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TOXICOLOGICAL CONFERENCE

TOXCON 2005

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2005

DEAR READER

This Supplement to the **BIOMEDICAL PAPERS, Volume 149** (Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub), is devoted to the 10th Interdisciplinary Czech-Slovak Toxicological Conference held at the Palacký University in Olomouc, September 14–16, 2005. It contains 74 abstracts of lectures and posters and 10 communications (papers, which do not quite fit into the regular Number 2 of this Journal). Daniela Walterová was the Guest Editor of this Supplement.

The BIOMEDICAL PAPERS (formerly ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS FACULTATIS MEDICAE, Volumes 1–144, Volume 1, first published in 1955) is a peer-reviewed international journal. Main features of the journal include:

- *Frequency:* 1 volume, 2 issues per year
- *ISSN:* 1213-8118
- Online version: http://biomed.papers.upol.cz
- *Indexed in:* MEDLINE, CHEMICAL ABSTRACTS, BIBLIOGRAPHIA MEDICA CECHOSLOVACA
- *Free full text articles* available via PubMed Linkout Service from Vol. 146 (2002).
- *Scope:*
  The journal is internationally oriented and the Editorial Board includes experts from Germany, United Kingdom and Lebanon. Its main priority is to publish key reviews from all biomedical fields with the emphasis on clinical-pathological correlations, and clinical and laboratory diagnostics. Thus, this Journal plays an important role in postgraduate medical education and also represents a valued publication forum for postgraduate students. Original papers on fundamental clinical and laboratory medical research as well as on physiological, epidemiological and toxicological studies are also accepted. Brief communications describing simple modifications of laboratory methods and clinical case reports are also welcome.
- *Peer-review process*
  Manuscripts are evaluated by the editorial board and by a panel of independent reviewers. The process lasts up to 4 weeks. The time period from the acceptance of a manuscript to its publication does not exceed 4 months.

I hope that the content of this Supplement will not only represent a basic brochure useful to all participants of the Conference but also provide a lot of valuable new information on recent results of leading laboratories working in all the fields of toxicology.

Vilím Šimánek
Co-Editor-in-Chief

10th Interdisciplinary Czech and Slovak Toxicological Conference

Olomouc, Czech Republic, September 14–16, 2005

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CONFERENCE PROGRAM

Lectures will be given in Czech, Slovak or English. Titles of all presentations listed in this program are solely in English according to the rules of Biomedical Papers

Wednesday, September 14

Main Lecture Hall

16,30 Conference opening (P. Anzenbacher, V. Šimánek, guests)
17,00–18,00 Šantavý Lecture of The Czech Chemical Society: J. Ulrichová (Olomouc) – Toxicology of quaternary benzo[c]phenanthridine alkaloids
19,00 Welcome Party (Hospital cafeteria)

Thursday, September 15

Main Lecture Hall

MORNING SESSION (EXPERIMENTAL TOXICOLOGY)

Chair: J. Ulrichová, J. Květina
8,30–9,00 M. Gómez-Lechón (Valencia) – New strategies and *in vitro* models to predict drug metabolism and hepatotoxicity
9,00–9,30 J. Vondráček, M. Machala (Brno) – Studies on mechanisms of toxicity of polycyclic aromatic hydrocarbons using *in vitro* models
9,30–9,50 Coffee Break

EXPERIMENTAL TOXICOLOGY, METHODS IN TOXICOLOGY I.

Chair: M. Gómez-Lechón, E. Ujházy
9,50–10,10 M. Kuneš, Z. Svoboda, J. Květina, V. Herout, J. Herink, J. Bajgar (Hradec Králové) – Intestinal single-pass *in situ* perfusion technique in rat: Influence of L-carnitine on absorption of 7-methoxytacrine
10,10–10,30 S. Kašparová, Z. Sumbalová, J. Horecký, P. Bystrický ,V. Mlynárik, A. Gvozdjáková, T. Liptaj (Bratislava) – New magnetic resonance spectroscopy biomarker for monitoring neurodegenerative diseases: Animal models
10,30–10,50 V. Marešová, J. Chadt (Praha) – Extraction methods for systematic toxicological analysis of basic, neutral and acidic drugs in biological material by GC-MS
10,50–11,10 J. Petrlová, O. Blaštík, R. Průša, J. Kukačka, D. Potěšil, R. Mikelová, V. Adam, J. Zehnálek, R. Kizek (Brno) – Use of electrochemical methods for studying of metallothionein content in the human blood serum of a patient poisoned by lead and treated by platinum
11,10–11,30 R. Mikelová, V. Adam, L. Trnková, P. Babula, A. Horna, R. Kizek (Brno) – Analysis of silver ions by stationary and flow electrochemical techniques on the surface of glassy carbon electrode
11,30–11,50 D. Kotyzová, V. Eybl, M. Mihaljevič, E. Glattre (Plzeň, Oslo) – Effect of long-term administration of arsenic (III) and bromine with and without selenium and iodine supplementation on the element level in the thyroid of rat
11,50–12,10 A. Kolman (Stockholm) – Implementation of *in vitro* tests for acute human toxicity assessment – The MEIC and ACuteTox projects
12,10–15,00 Lunch, Poster presentation (Posters will be on display both days.)
13,30–14,30 Experimental Toxicology – authors to be present at their posters (First authors: Ovádeková (2×), Theiszová, Repický, Kmoníčková, Anzenbacherová, Bartošová, Sadloňová, Hurbánková, Černá, Tulinská, Kováčiková, Tátrai, Štětinová, Račková, Mačičková, Nečas, Bartošíková, Nosáĺová, Ondrejíčková)

Thursday, September 15

Main Lecture Hall

AFTERNOON SESSION (EXPERIMENTAL TOXICOLOGY, NATURAL SUBSTANCES, DIETARY COMPONENTS)

Chair: J. Navarová, M. Tichý

15,00–15,30 J. Hajšlová (Praha) – Residua of pesticides and of other biologically active exogenous contaminants in human food chain

15,30–15,55 L. Hanuš (Jerusalem) – History of anandamide discovery

Program continues in two parallel sessions:

Main Lecture Hall
EXPERIMENTAL TOXICOLOGY II.

Chair: A. Kolman, J. Brtko

16,00–16,30 M. Tichý (Praha) – Experimental toxicology „in silico“

16,30–16,50 V. Dohnal, K. Kuča, D. Jun (Brno) – What are artificial neural networks and what they can do?

16,50–17,10 Coffee Break

17,10–17,40 J. Brtko (Bratislava) – Nuclear retinoic acid receptors and their role in therapy of selected malignancies

17,40–18,00 J. Navarová, E. Ujházy, R. Sotníková, M. Dubovický, M. Mach, V. NosáĺOvá (Bratislava) – Antioxidants and protection of organism against oxidative damage under experimental conditions

18,00–18,20 K. Bauerová, J. Kucharská, D. Mihalová, J. Navarová, A. Gvozdjáková, Sumbalová (Bratislava) – Effect of Coenzyme Q_{10} supplementation in the rat model of adjuvant arthritis

18,20–18,40 P. Babula, R. Mikelová, D. Potěšil, V. Adam, R. Kizek, L. Havel (Brno) – Simultaneous determination of 1,4-naphthoquinone, lawsone, juglone and plumbagin by liquid chromatography with UV detection

Lecture Hall A (“Levá posluchárna”)
NATURAL COMPOUNDS, DIETARY CONSTITUENTS

Chair: D. Slameňová, Z. Zídek

16,00–16,30 V. Šimánek (Olomouc) – Chemoprevention with nutraceuticals and phytochemicals. How to assess their effects on human organism?

16,30–16,50 D. Slameňová, J. Navarová (Bratislava) – Importance of nutrition in the etiology of human cancer

16,50–17,10 Coffee Break

17,10–17,40 M. Modrianský, Z. Dvořák (Olomouc) – Microtubule disruptors and their interaction with biotransformation enzymes

17,40–18,00 P. Hanuštiak, R. Mikelová, D. Potěšil, P. Hodek, M. Stiborová, R. Kizek (Brno) – Electrochemical behaviour of flavonoids on a surface of a carbon paste electrode

18,00–18,20 Z. Ovesná, K. Horváthová-Kozics (Bratislava) – Structure-activity relationship of *trans*-resveratrol and its analogues

18,20–18,40 K. Horváthová-Kozics, Z. Ovesná (Bratislava) – Electrochemical behaviour and determination of antitumour promoting activities of flavonoids

Friday, September 16

Main Lecture Hall

MORNING SESSION (FORENSIC AND APPLIED TOXICOLOGY)

Chair: K. Bauerová, R. Štětina

8,30–9,00 P. Souček, S. Šůsová, P. Vodička, J. Novotný (Praha) – Genetic polymorphisms of biotransformation enzymes and their role in carcinogenesis

9,00–9,30 M. Balíková (Praha) – Hair analysis for drugs of abuse. Plausibility of interpretation

9,30–9,50 Coffee Break

Program continues in two parallel sessions:

Main Lecture Hall
FORENSIC TOXICOLOGY

Chair: M. Balíková, P. Ondra

9,50–10,10 P. Ondra, K. Zedníková, R. Matlach (Olomouc) – Possibilities and problems with identification and determination of "new" hypnotics

10,10–10,30 M. Staňková, P. Kurka (Ostrava) – Toxicological analysis of tianeptine by LC/MS

10,30–10,50 M. Křenová, D. Pelclová, T. Navrátil, M. Merta (Praha) – Experience of the Czech Toxicological Information Centre with ethylene glycol poisoning

10,50–11,10 V. Habrdová, M. Balíková (Praha) – Simultaneous analysis of buprenorphine, norbuprenorphine and morphine-like opiates in human urine by GC/MS

11,10–11,30 R. Oros (Praha) – Possibilities of 2D-LCMS in determination of drug residues

11,30–11,50 L. Spáčilová, H. Klusoňová, P. Višňovský (Hradec Králové) – Medicaments abuse among drug addicts

Lecture Hall A ("Levá posluchárna")
APPLIED TOXICOLOGY

Chair: P. Višňovský, J. Kassa

9,50–10,10 Z. Zídek, E. Kmoníčková, A. Holý (Praha) – Cytotoxicity of pivoxil esters of antiviral acyclic nucleoside phosphonates: Adefovir dipivoxil *vs.* adefovir

10,10–10,30 J. Kassa, K. Kuča, J. Cabal (Hradec Králové) – Comparison of the potency of trimedoxime and other currently available oximes to reactivate tabun-inhibited acetylcholinesterase and eliminate toxic effects of tabun

10,30–10,50 K. Kuča, J. Cabal, D. Jun, J. Kassa, L. Bartošová, G. Kunešová, V. Dohnal (Hradec Králové) – Development of new acetylcholinesterase reactivators – antidotes used for treatment of nerve agent poisonings

10,50–11,10 L. Bartošová, K. Kuča, G. Kunešová (Hradec Králové) – Effectivity of new acetylcholinesterase reactivators in treatment of cyclosarin poisoning in mice and rats

11,10–11,30 H. Klusoňová, J. Vlková, P. Višňovský (Hradec Králové) – Natural opium as one of the possibilities for drug abusers

11,30–11,50 P. Višňovský, A. Kavalírová (Hradec Králové) – Use of marijuana in pharmacy students (2000–2005)

11,50–12,10 I. Brucknerová, M. Benedeková, K. Holomáň, E. Bieliková, A. Kostrová, E. Ujházy, M. Dubovický (Bratislava) – Delivery as "physiological stress" and its influence on liver enzymatic systems in asphyxial newborns

12,10–14,00 Lunch, Poster presentation

13,00–14,00 Natural Substances, Dietary Components, Forensic and Applied Toxicology, Clinical Toxicology and Drug Interactions, Carcinogenesis, Mutagenesis and Teratogenesis
– authors to be present at their posters (First authors: Paulíková (2×), Letašiová, Psotová, Slunská, Račková, Ondrušová, Vaľko, Svobodová; Zorec-Karlovšek, Beránková, Kresánek; Svoboda, Anzenbacherová; Rýdlová, Aimová, Poljaková, Birošová (3×), Miadoková, Molnárová).

Friday, September 16

AFTERNOON SESSION (CLINICAL TOXICOLOGY, DRUG INTERACTIONS; CARCINOGENESIS, MUTAGENESIS, TERATOGENESIS)

Main Lecture Hall
CLINICAL TOXICOLOGY, DRUG INTERACTIONS

Chair: M. Kriška, F. Perlík

14,00–14,20 P. Anzenbacher, J. Jezdinský (Olomouc) – „Top ten“ – Ten particularly dangerous drug interactions in long term care

14,20–14,40 M. Grundmann (Ostrava) – Therapeutic drug monitoring (TDM) and clinically important drug interactions

14,40–15,00 M. Kriška (Bratislava) – Clinical relevancy of drug interactions from the viewpoint of sources of information

15,00–15,20 F. Perlík (Praha) – Genetic polymorphism and possible adverse effects

15,20–15,40 J. Pivnička, H. Samková, A. Brzobohatá, M. Špačková (Brno) Determination of halothane using gas chromatography within quality control of patient malignant hyperthermia

15,40–15,50 **Closing session**

Lecture Hall A (“Levá posluchárna”)
CARCINOGENESIS, MUTAGENESIS, TERATOGENESIS

Chair: M. Stiborová, P. Souček

14,00–14,20 E. Ujházy, M. Mach, M. Dubovický, J. Navarová, I. Brucknerová (Bratislava) – Developmental toxicology – an integral part of safety evaluation of new drugs

14,20–14,40 M. Stiborová, V.M. Arlt, C.J. Henderson, C.R. Wolf, E. Frei, H.H. Schmeiser, D.H. Phillips (Praha) – Molecular mechanisms of genotoxicity of the environmental pollutant 3-nitrobenzanthrone

14,40–15,00 R. Štětina, P. Vodička, J. Varvařovská (Hradec Králové) – Changes in an individual capacity of DNA reparation induced by exposure to xenobiotics and by some pathological states (*Diabetes mellitus*)

15,00–15,20 M. Jágr, J. Mráz, K. Ctibor, V. Stránský, M. Pospíšil (Praha) – Preparation and characterization of styrene oxide adducts with amino acids in human globin

15,20–15,40 I. Juránek (Bratislava) – Reactive oxygen species – cause or consequence of tissue injury?

15,40–15,50 **Closing session (Main Lecture Hall)**

CONTENTS

EXPERIMENTAL TOXICOLOGY

TOXICOLOGY OF QUATERNARY BENZO[c]PHENANTHRIDINE ALKALOIDS*

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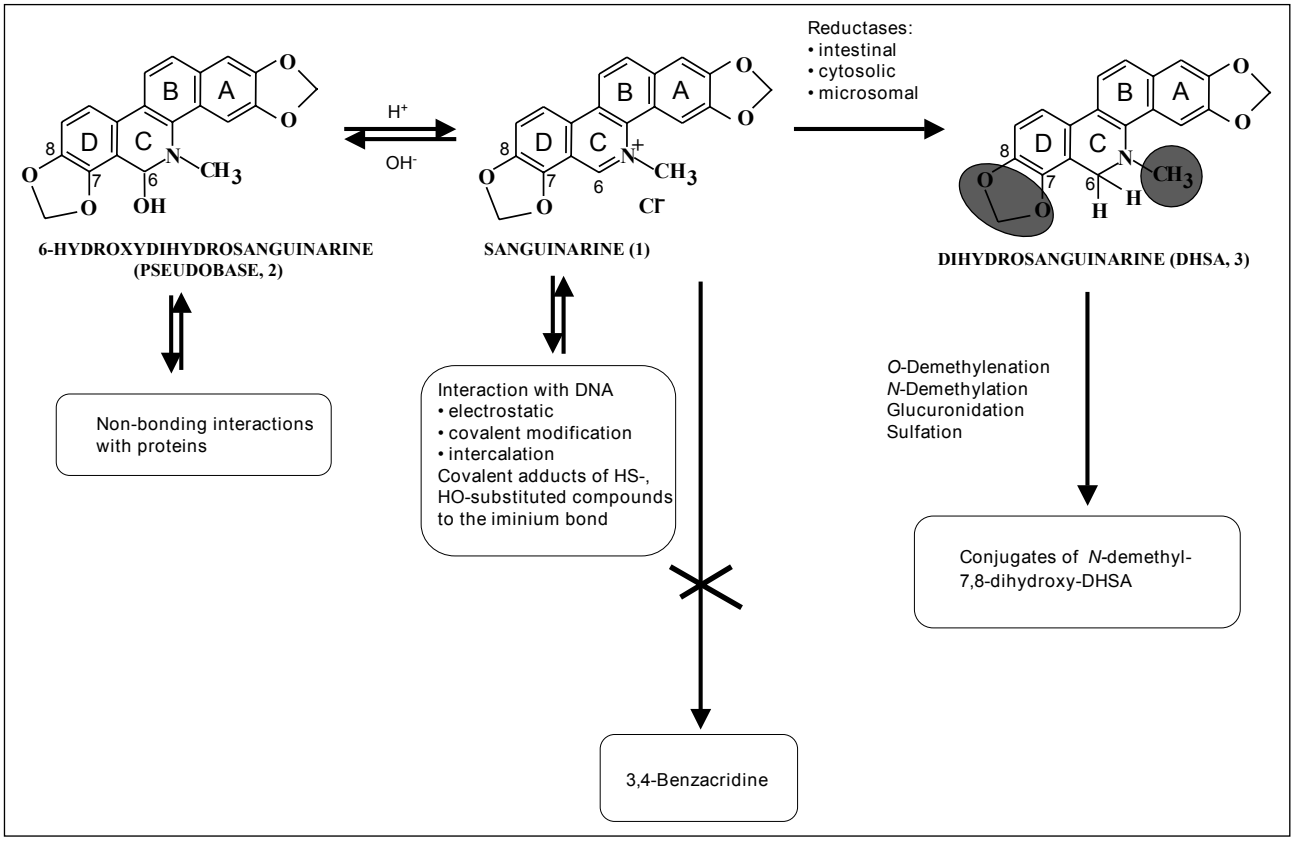
**** Šantavý Lecture of the Czech Chemical Society***

Key words: Sanguinarine/Chelerythrine/Biological activity/Pharmacokinetics/Metabolism/Adverse effect/ Safety/Medical application

Quaternary benzo[c]phenanthridine alkaloids (QBA) sanguinarine (SA) and chelerythrine (CHE) are found within the families Fumariaceae, Papaveraceae, Ranunculaceae and Rutaceae. These alkaloids originate from the aromatic amino acid tyrosine. An important intermediate of their biosynthesis are the protopines. For example, dihydrosanguinarine (DHSA) is formed after hydroxylation of protopine in a reaction that depends on NADPH as a reduction cofactor, and molecular oxygen. This reaction is catalyzed by a microsomal cytochrome P450-linked monooxygenase. Then, DHSA is readily converted to SA by an oxidase. The strong antimicrobial activity of QBA points to their function as plant defense secondary metabolites or phytoalexines. If these alkaloids are administrated to living organisms such as insects, fish, and mammalian species, they can be absorbed, distributed, retained and/or metabolized as quaternary cations, 6-hydroxydihydroderivatives (pseudobases) and/or dihydroderivatives (Scheme 1) (ref.[1]). In the blood and organs the equilibrium between these forms depends mainly upon the pH, ionic strength and oxidative capacity of milieu. Their resulting mode of action reflects the sum of reactions of all three chemical structures with cellular components. In consequence, plethora of biological effects of QBA may be due to their non-selectivity towards specific targets or to the toxicity resulting from metabolic activation to reactive intermediates that are capable of covalent or electrostatic binding or interaction/intercalation with cellular macromolecules.

The full explanation of QBA adverse/toxic effects is linked to some questions, to which the answers based on experimental evidence have still been lacking: (i) How are QBA absorbed and distributed in the mammalian organism after oral administration? (ii) How are QBA



Schema 1. The transformation of sanguinarine in the living organism. Dynamic equilibrium between sanguinarine (1) and its pseudobase (2) depends on pH value and cell milieu. Intestinal, cytosolic and microsomal reductases convert sanguinarine (1) to dihydrosanguinarine (3). Dihydrosanguinarine (3) is transformed in phase I and II reactions to polar conjugates.

metabolized by a normal and tumor cell and which of the QBA structures is biologically active in the cell? (iii) Is the reduction of the iminium bond the first step of QBA detoxification in mammalian species and what is the biological activity of the metabolites? (iv) Is benz[c]acridine really a QBA metabolite? (v) Has it been evidenced that QBA are toxic agents of argemone oil (*Epidemic dropsy syndrome*)? (vi) Are toxic effects described in mice after an *i.p.* administration of QBA (in a dose corresponding to 50% of acute *i.v.* LD_{50} in rats) justified to avert the use of QBA-containing plant extracts as anti-inflammatory and antimicrobial components in cosmetic/veterinary preparations? The aim of the lecture is to describe the *in vitro/in vivo* biological effects of QBA and to offer answers to some of the above-mentioned questions.

ACKNOWLEDGEMENT

Financial support by the Ministry of Education (grant MSM 6198959216) is gratefully acknowledged.

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NEW STRATEGIES AND *IN VITRO* MODELS TO PREDICT DRUG METABOLISM AND HEPATOTOXICITY IN MAN

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Key words: Cytochrome P450/CYP-engineered cells/ Hepatotoxicity/Human hepatocytes/Metabolism

Drug metabolism is the major determinant of drug clearance and the factor most frequently responsible for the interindividual differences in drug pharmacokinetics. The expression of drug metabolising CYPs is variable, many being polymorphic or inducible, which accounts for the human interindividual variability in drug metabolism. Due to the clinical implications of this variability, there is considerable interest in developing reliable methods to investigate human CYPs and their role in drug metabolism. Drug metabolism studies are routinely performed in laboratory animals, but due to significant metabolic interspecies differences when compared to man they are not accurate enough to anticipate the metabolic profile of a drug in humans. Human hepatocytes in primary culture provide the closest *in vitro* model to human liver and the only model that can produce a metabolic profile of a given drug that is very similar to that found *in vivo*[1]. Human hepatocytes can, therefore, be used to predict the metabolic profile of a new drug in man[2]. However their availability is seriously limited because of the restricted access to suitable tissue samples preventing their use in high throughput screening assays. To overcome this restriction the pharmaceutical industry has directed great efforts to develop fast and reliable *in vitro* methods for this purpose. Several hepatic systems which could give an indication of the metabolism of a drug are presently being proposed as alternative models to investigate human metabolism of drugs *in vitro:* liver microsomes, liver-derived cellular models (human CYP-engineered cells, hepatic derived cell lines) and tissue slices. Pharmaceutical companies are making use of *in vitro* models to speed up the selection of pharmacologically active but safe new drug candidates. In this context key issues, that should be implemented at the earliest stages of drug development for better candidate selection, are: 1) classification and labelling of the candidates on the basis of their increasing molar toxicity *in vitro* (toxic potential), 2) evaluation of hepatotoxicity risk assessment and the major metabolic alteration to hepatocytes, 3) comparative studies of the metabolic profile of a drug in hepatocytes or liver microsomes from different species to help the selection of the animal model closest to man for investigating drug effects at early preclinical stages, 4) identification of the CYP isozymes involved by using CYP-expressing cells, and finally, 5) analysis of the potential enzyme-inhibiting or enzyme-inducing properties on human CYPs of a compound evaluated in human hepatocytes[3].

The molecular mechanisms involved in the hepatotoxicity of xenobiotics are of major concern. Some xenobiotics are electrophilic in nature, and others are biotransformed by the liver to highly reactive metabolites generally more toxic than the parent compound. This activation process is the key to many hepatotoxic phenomena. Key points in the risk assessment of hepatotoxicity of drugs are the metabolic relevance of the observed alteration and its reversion upon withdrawal of the xenobiotic from incubation media. Certain cell functions can be transiently altered by a xenobiotic, yet this might lack *in vivo* significance if the cell rapidly recovers upon elimination of the xenobiotic. The ultimate goal of *in vitro* experiments is to generate the type of scientific information needed to identify compounds that are potentially toxic to man. For this purpose, not only the design of experiments but also the interpretation of results is essential.

ACKNOWLEDGEMENT

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STUDIES ON MECHANISMS OF TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS USING *IN VITRO* MODELS

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Key words: Polycyclic aromatic hydrocarbons/Toxicity/ Intercellular communication

Polycyclic aromatic hydrocarbons (PAHs) are a large group of diverse environmental organic pollutants formed mainly by incomplete combustion. Many of them are known or suspected carcinogens that have been reported to possess tumor-initiating and/or tumor-promoting properties. PAHs and/or their metabolites have been extensively studied as genotoxic, initiating agents. In contrast, their nongenotoxic effects that might be linked to carcinogenesis, reproductive or developmental impairment are poorly characterized. This presentation is intended to provide an overview of our recent experimental studies describing the impact of a wide array of PAHs and their derivatives on: i) activation of intracellular receptors, such as aryl hydrocarbon receptor (AhR) or estrogen receptor (ER); ii) disruption of gap junctional intercellular communication (GJIC); iii) expression of PAH-metabolizing enzymes; iv) deregulation of cell proliferation via ER- or AhR-dependent mechanisms; v) induction of programmed cell death; and vi) modulation of intracellular signaling pathways, such as the activation of mitogen-activated protein kinases (MAPK). These experiments have been carried out using two principal *in vitro* models, human breast carcinoma MCF-7 cells, which are widely employed for studies of effects of xenobiotics on ER activation and regulation; and in rat liver epithelial 'stem-like' WB-F344 cell line. This cell line, which is considered to represent an *in vitro* model of poorly differentiated liver progenitor cells, which might be an important target of carcinogens, is a suitable tool for studies on toxic effects of PAHs associated with carcinogenesis.

The studied PAHs elicited multiple toxic effects in these experimental models and our results allowed us to classify the studied compounds according to their prevalent mode of action. The low-molecular-weight PAHs, which are present at high levels in environmental samples or in cigarette smoke, such as anthracene, phenanthrene, fluoranthene or their methylated derivatives, were generally poor AhR ligands, weak activators of ER, efficient inhibitors of GJIC and they induced rapid effects, such as MAPK activation, when used at high concentrations ($> 10^{-5}$ M). These compounds did not induce cell death at micromolar concentrations and their effects on cell proliferation were only weak or negligible. These PAHs were also poor inducers of enzymes involved in metabolic activation of PAHs, such as cytochrome P450 1A1 (CYP1A1). In a marked contrast, the compounds such as benz[a]anthracene (BaA), benzo[a]pyrene (BaP), chrysene (Chr), benzofluoranthenes, dibenzo[a,h]anthracene or dibenzopyrenes were mostly strong inducers of AhR-mediated activity, as well as the expression of CYP1A1 and other metabolizing enzymes. Their impact on cell proliferation, apoptosis and cell signaling strongly depended on their genotoxicity. While the less genotoxic compounds stimulated cell proliferation in contact-inhibited WB-F344 cells (in the AhR-dependent manner) or in MCF-7 cells (via ER activation), the strong mutagens, such as BaP or DBalP, induced accumulation of cells in S-phase of cell cycle (probably partly mediated through S-phase arrest) and/or programmed cell death. The induction of apoptosis was associated with accumulation and phosphorylation of p53 tumor suppressor and simultaneous activation of kinases ERK1/2, p38, but not JNK/SAPK kinases. The introduction of methyl group further modified the impact of methylated derivatives of BaA or Chr on cell cycle, cell proliferation, programmed cell death, inhibition of GJIC or activation of intracellular receptors. Taken together, the impact of PAHs on cell proliferation, cell death and intercellular communication reflects both genotoxic and nongenotoxic events induced by these compounds. Our results demonstrated that PAHs (or their metabolites) can modify both intercellular communication and the balance between cell proliferation and cell death, and we identified some of the mechanisms responsible for these effects. Disruption of intercellular communication and cell proliferation control might significantly contribute to tumor promotion and carcinogenicity of individual PAHs.

ACKNOWLEDGEMENT

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INTESTINAL SINGLE-PASS IN SITU PERFUSION TECHNIQUE IN RAT: THE INFLUENCE OF L-CARNITINE ON ABSORPTION OF 7-METHOXYTACRINE

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Key words: Methoxytacrine/Carnitine/single-pass perfusion/Rat small intestine/intestinal absorption

7-Methoxytacrine (7-MEOTA) is an acetylcholine esterase inhibitor that is potentially useful in the therapy of some neurodegenerative disorders. L-carnitine (CRT) is an naturally occuring compound that is known among others to increase penetration of some compounds or chemical groups through biological barriers. Aim of this study was how CRT influenced transintestinal absorption transport of 7-MEOTA in rat using single-pass intestinal *in situ* perfusion method. It was compared the rate of absorption of 7-MEOTA during luminal perfusion with single 7-MEOTA with rate of absorption during simultaneous perfusion with 7-MEOTA and CRT and with absorption rate after the pre-medication with CRT for period of three days before beginning of perfusion. The methodical system was the perfusion of mesenterial bed (from arteria mesenterica superior to vena portae) and intestinal luminal perfusion (from duodenum to ileum). The lower transintestinal absorption in the course of simultaneous administration of CRT than just in case of perfusion with single 7-MEOTA has been found. On the contrary a significantly higher absorption of 7-MEOTA has been noted in group of rats pre-medicated with CRT for three consecutive days. The interpretation suggested that molecules of CRT incorporated into the metabolism of intestinal cells facilitated transport of 7-MEOTA (as a representative substance which is at least partly transferred by carrier mechanism). In case of simultaneous luminal perfusion with CRT and 7-MEOTA competitive over-saturation of carrier systems is probably concerned.

NEW MAGNETIC RESONANCE SPECTROSCOPY BIOMARKER FOR MONITORING NEURODEGENERATIVE DISEASES: ANIMAL MODELS

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Key words: Creatine kinase/Saturation transfer ^{31}P NMR/Chronic cerebral hypoperfusion/ Neurodegenerative diseases/Animal model

Creatine kinase (CK) plays a central role in energy transfer in cells with high-energy demands, and the enzyme is rather susceptible to oxidative inactivation. The aim of the present study was to investigate whether the rate constant of forward CK reaction (k_{for}) is a suitable indicator of alterations in cerebral energy metabolism. We monitored k_{for} in the rat brain non-invasively by *in vivo* phosphorus (^{31}P) magnetic resonance spectroscopy (MRS). To alter energy metabolism, we applied following experimental models: Huntington's disease, diabetes mellitus, chronic alcohol intoxication and chronic cerebral hypoperfusion (vascular dementia model). Results of our ^{31}P MRS experiment confirm importance of CK/PCr system in the regulation of brain energy metabolism *in vivo* because a kinetic parameter k_{for} was significantly changed in all above animal models that simulate neurodegenerative diseases or commonly during oxidative stress. Using this method we distinguished vascular dementia (VD) and Huntington disease (HD), because in VD model a kinetic parameter k_{for} decreased and in the case HD increased. Considering the importance of CK for the maintenance of energy homeostasis in the brain, it is conceivable that an alteration of this enzyme activity in the brain may be one of the mechanisms by which various neurodegenerative diseases might be monitored just by means saturation transfer method ^{31}P MRS.

ACKNOWLEDGEMENT

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EXTRACTION METHODS FOR SYSTEMATICAL TOXICOLOGICAL ANALYSIS OF BASIC, NEUTRAL AND ACIDIC DRUGS IN BIOLOGICAL MATERIAL BY GC-MS

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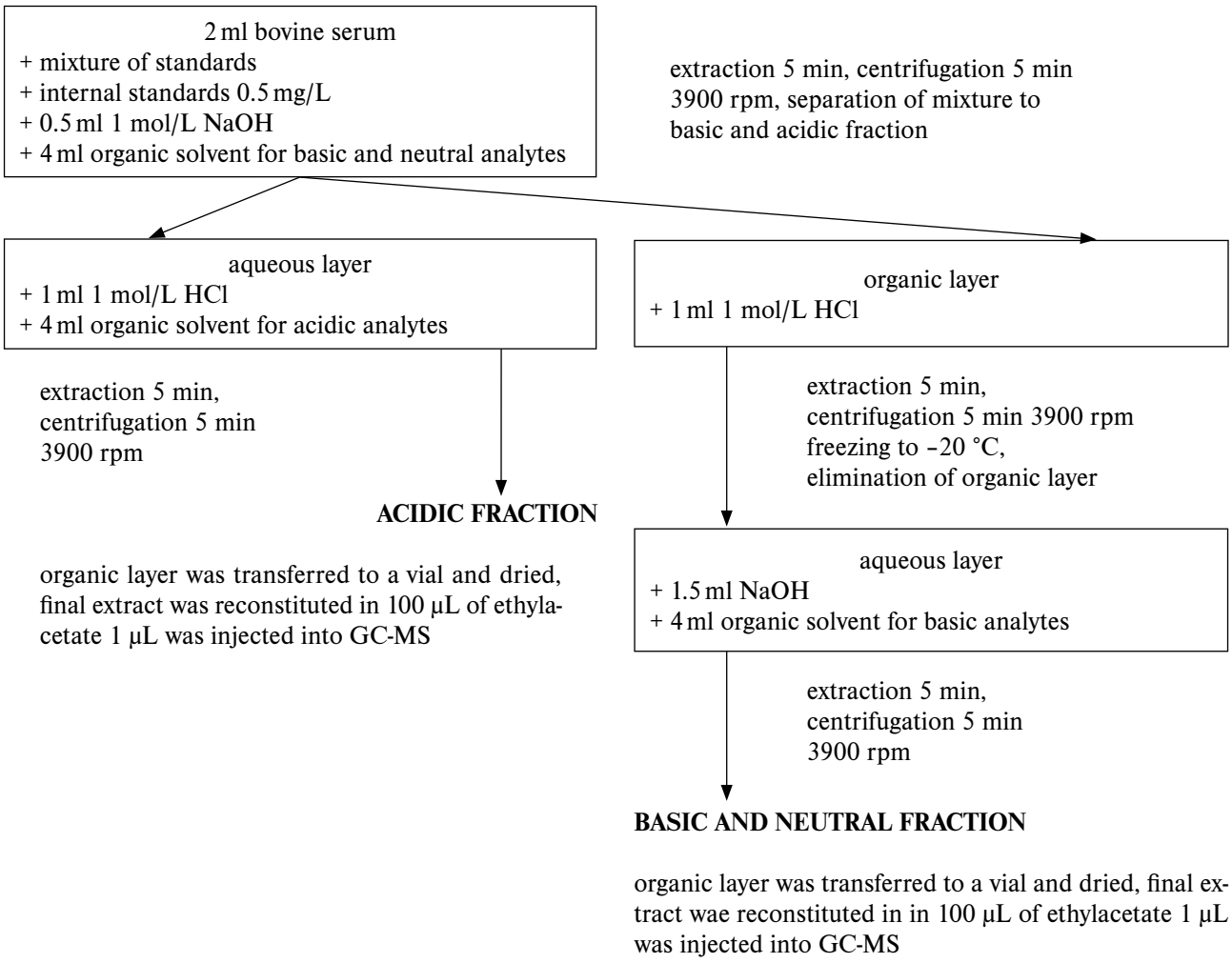
Key words: Drug/Screening/Liquid-liquid extraction/ Solid phase extraction

ABSTRACT

The aim of the study was to compare liquid-liquid (LLE) and solid phase (SPE) extraction techniques for simultaneous screening for basic, neutral and acidic drugs in spiked bovine serum according to the extraction recoveries of selected standards of drugs. A mixture of ten analytes with different acid-base character was selected for evaluation of extraction methods. The tested mixture of standards contained 5 mg/L phenobarbital, pentobarbital, methaqualone, mephentermine, benzocaine, codeine, quinine, haloperidol, strychnine and 25 mg/L morphine. The internal standard for acidic analytes was 0.5 mg/L hexobarbital and 0.5 mg/L trimipramine for basic analytes, respectively. After addition of internal standards, 2 ml of bovine serum were spiked with a tested mixture of standards at concentration levels: 0.5; 1.0; 2.0; 5.0 mg/L and extracted: LLE technique for acidic drugs with 4 ml acetone : chloroform (1 : 9 v/v) mixture and for basic and neutral drugs reextraction method with 4 ml ethylacetate:1-chlorbutane: cyclohexane (1 : 2 : 2 v/v/v) mixture (n = 6). SPE technique with mixed-mode columns for acidic and neutral drugs with acetone:chloroform (1 : 1 v/v) mixture and for basic drugs with dichlormethane : isopropanol : ammonia (8 : 2 : 0,2 v/v/v) mixture (n = 6). All extracts were analyzed by GC-MS in full scan mode. Morphine and quinine were analyzed after consequent silylation. The extraction efficiency of both extraction methods was evaluated on the base of the extraction recoveries of individual drugs of spiked bovine serum. LLE reached maximum of re-



Schema 1. Liquid-liquid extraction

covery of drugs at 0.5 and 1.0 mg/L concentration levels (53–88 %), SPE gave high recovery of drugs for all concentration levels (52–96 %). For these extraction conditions SPE reached higher extraction efficiency than LLE.

INTRODUCTION

Chromathographical and immunochemical methods are applied for comprehensive screening in clinical and forensic toxicology now. Immunochemical methods are not sufficient for extensive toxicological screening. These techniques are widely used for their rapidity, and absence of sample handling[1–6], but they have serious limitation in sensitivity and selectivity and may cause both false negative and false positive results. Therefore, chromatographic methods are more suitable for the screening of unknown drugs or poisons. Today, mass spectrometry coupled with gas chromatography (GC-MS) in the full scan mode is still the most often used method for screening of xenobiotics in biological samples[7–16]. Liquid chromatography-mass spectrometry (LC-MS) is now also applied for these purposes [8, 11, 12, 17–24].

The aim of the study was to compare liquid-liquid extraction (LLE) and solid phase extraction (SPE) techniques for simultaneous screening for basic, neutral and acidic drugs in spiked bovine serum according to the extraction recoveries of selected standards of drugs.

MATERIAL AND METHODS

A mixture of ten analytes with different acid-base character was selected for evaluation of extraction methods. The tested mixture of standards contained 5 mg/L phenobarbital, pentobarbital, methaqualone, mephentermine, benzocaine, codeine, quinine, haloperidol, strychnine and 25 mg/L morphine. The internal standard for acidic analytes was 0.5 mg/L hexobarbital and 0.5 mg/L trimipramine for basic analytes, respectively. After addition of internal standards, 2 ml of bovine serum were spiked with a tested mixture of standards at concentration lev-

Sample preparation:
2 ml bovine serum, mixtures of standards, internal standards 0.5 mg/L, 6 ml 0.1 mol/L phosphate buffer (pH=6.0), Bond Elute Certify

1) Column conditionation
2 ml CH_3OH
2 ml 0.1 mol/L phosphate buffer pH = 6, low vacuum

2) Specimen application
low vacuum

3) Column rinse
1 ml DI H_2O

4) pH adjustment
0.5 ml 0.01 mol/L acetic acid, pH = 3.4
low vacuum

5) Column dry
vacuum, 100 µL CH_3OH

6) Elute acidic and neutral drugs
3 × 1.5 ml acetone : chloroform (1 : 1 v/v)

extract of acidic and neutral drugs was reconstituted in 100 µl of ethylacetate, 1 µl was injected into GC-MS

7) Elute basic drugs
3 × 1.5 ml dichlormethane:isopropanol:ammonia (8 : 2 : 0.2 v/v/v)

extract of basic drugs was reconstituted in 100 µl of ethylacetate, 1 µl was injected into GC-MS

Schema 2. Solid phase extraction

els: 0.5; 1.0; 2.0; 5.0 mg/L and extracted: LLE technique for acidic drugs with 4 ml acetone:chloroform (1 : 9 v/v) mixture and for basic and neutral drugs reextraction method with 4 ml ethylacetate:1-chlorbutane:cyclohexane (1 : 2 : 2 v/v/v) mixture (n = 6). SPE technique with mixed-mode columns for acidic and neutral drugs with acetone:chloroform (1 : 1 v/v) mixture and for basic drugs with dichlormethane : isopropanol : ammonia (8 : 2 : 0,2 v/v/v) mixture (n = 6) was used. Morphine and quinine were analysed after consequent silylation. The content of a vial was evaporated to dryness. After addition 100 µL *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), ammonium iodide (NH_4I), 1,2-ethanedithiobis(trimethylsilane) (EDTBTS) (1000 : 2.5 : 3 (v/w/v)) derivatization was carried out at 80 °C for 20 min and 1 µL was injected into GC-MS. The samples were analyzed using a Finnigan MAT MAGNUM ion trap GC-MS with Varian 3400 GC fitted with SPI injector and A200S autosampler. The column Rtx-5ms 30 m x 0.25 mm i.d. with 0.25 µm film thickness was used. Column temperature conditions: 60 °C for 1 min increased to 280 °C at 10 °C/min and held at this temperature 20 min, for injector 60 °C for 6 s increased to 260 °C at 100 °C/min and held at this temperature 3 min. The carrier gas was helium 1 mL/min. The MS was operated in full scan mode. The transfer line heater 270 °C, electron ionization mode (EI), ionization energy 70 eV. The scan range was between 35 and 500 amu, scan speed 1 scan/s.

RESULTS

All extracts were analyzed by GC-MS in full scan mode. Morphine and quinine were analyzed after consequent silylation. The extraction efficiency of both extraction methods was evaluated on the base of the extraction recoveries of individual drugs of spiked bovine serum (Tables 1–4).

The extraction efficiency was evaluated on the basis of recoveries of all substances of the mixture taken as a unit. LLE reached maximum of recovery of drugs at 0.5 and 1.0 mg/L concentration levels (53–88 %), SPE gave high recovery of drugs for all concentration levels (52–96 %). For these extraction conditions SPE reached higher extraction efficiency than LLE.

Table 1. Extraction efficiency of basic and neutral standards – LLE

Concentration of analytes	Extraction efficiency
500 µg/L	52.6 – 84.0 %
1000 µg/L	54.7 – 88.4 %
2000 µg/L	53.6 – 73.1 %
5000 µg/L	55.1 – 64.8 %

Table 2. Extraction efficiency of acidic standards – LLE

Concentration of analytes	Extraction efficiency
500 µg/L	54.8 – 66.3 %
1000 µg/L	59.6 – 75.5 %
2000 µg/L	59.2 – 70.3 %
5000 µg/L	55.1 – 57.6 %

Table 3. Extraction efficiency of basic standards – SPE

Concentration of analytes	Extraction efficiency
500 µg/L	51.6 – 85.0 %
1000 µg/L	54.5 – 84.8 %
2000 µg/L	56.9 – 88,9 %
5000 µg/L	65.7 – 88.0 %

Table 4. Extraction efficiency of acidic and neutral standards – SPE

Concentration of analytes	Extraction efficiency
500 µg/L	54.8 – 70.7 %
1000 µg/L	59.1 – 71.3 %
2000 µg/L	52. 3 – 86.0 %
5000 µg/L	58.4 – 96.3 %

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USING OF ELECTROCHEMICAL METHODS FOR STUDYING OF METALLOTHIONEIN CONTENT IN THE HUMAN BLOOD SERUM OF A PATIENT POISONED BY LEAD AND TREATED BY PLATINUM

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Key words: Metallothionein/Brdicka reaction/ Peak H/Catalytic hydrogen reaction/Cancer/Lead/ Differential pulse voltammetry/Adsorptive stripping voltammetry

Metallothioneins belong to group of intracellular, high molecular and cysteine-rich proteins whose content increase with increasing concentration of a heavy metal. Here we applied the adsorptive transfer stripping differential pulse voltammetry Brdicka reaction for the determination of metallothionein in human blood serum of patient poisoned by lead and/or treated by platinum. The increased of metallothionein concentrations in both cases were observed.

ACKNOWLEDGEMENT

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ANALYSIS OF SILVER IONS BY STATIONARY AND FLOW ELECTROCHEMICAL TECHNIQUES ON THE SURFACE OF GLASSY CARBON ELECTRODE

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Key words: Silver/Toxicity/Glassy carbon electrode/ Ecology/Flow injection analysis/Electrochemistry

ABSTRACT

It is common knowledge that silver ions belongs to one of the most toxic elements causing acute toxicity of organisms e.g. fifty percent living organisms die after exposition of 10 μg/l Ag^+ per 96 hours. Here we applied two different electrochemical techniques (differential pulse voltammetry and flow injection analysis with electrochemical detection) for determination of silver ions. As we shown, not only the stationary electrochemical technique but also the flow injection analysis in connection with electrochemical detector are suitable for toxicological analysis of environmental samples.

INTRODUCTION

The effects of silver in the environment have been intensively studying. Existence of International researches organisation such as Silver Coalition (1991–1995) and The Silver Council (from 1996 up to present; http://www.silvercouncil.org/html/default.htm) proves this fact. These organisations also established International Conference on Transport, Fate and Effects of Silver in the Environment[1]. Silver ions come to the environment, first of all, from industry. The highest amounts of silver are used in photographical industry (more than 40 %), in electrotechnical industry (about 30 %), in electrochemical plating (production of jewellery, more than 22 %), in chemical industry (more than 6 %) and in health service (silver amalgam, about 2 %). In addition, amounts of silver ions in waste waters represents the greatest source of their entering to food chain[2]. Moreover, marked toxicity of silver ions has been described[2–10].

A quantification of silver ions in water is very difficult because there are formed numbers of silver complexes with inorganic and organic compounds, which marked decrease toxicity of silver[1, 11, 12]. A determination of silver ions is obviously performed by atomic absorption spectrometry[13, 14]. It is necessary to preconcentrate a real sample for sensitive analysis, but this process is time consuming and high cost procedure[14–16]. On the other hand electrochemical techniques are able to determine a silver on carbon electrodes at nanomolar concentrations without preconcentration[17–21].

The aim of this work was to analyse the silver ions by the flow and stationary electrochemical techniques on the surface of the glassy carbon electrode.

MATERIAL AND METHODS

Chemicals

Silver nitrate and sodium acetate was purchased from Sigma Aldrich (St. Louis, USA). Acetic acid was purchased from Fluka chemie AG (USA). All reagents used were ACS purity. Stock standard solutions were prepared by ACS water (Sigma-Aldrich, USA). Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45 μm teflon filter discs (MetaChem, Torrance, CA, USA) prior to HPLC analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by the personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix- H, pH 0–14/3M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

Electrochemical stationary analysis

Differential pulse voltammetric (DPV) measurements were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a glassy carbon electrode. The reference electrode was an Ag/AgCl/3M KCl electrode and the auxiliary electrode was a graphite electrode. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed. The supporting electrolyte (0.2 M acetate buffer, pH 5.0) was prepared by mixing buffer components. The DPV measurements were carried out at room temperature. DPV parameters were as follows: an initial potential of –1.2 V, an end potential 1.2 V, a modulation time 0.02 s, a time interval 0.2 s, a step potential of 5 mV/s, a modulation amplitude of 250 mV.

Electrochemical flow analysis

An flow injection analysis with electrochemical detection FIA ED system consisted of solvent delivery pump (Model 583 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m), and an electrochemical detector. The electrochemical detector (ED) includes one low volume flow-through amperometric analytical cells (Model 5040, ESA, USA), which is consisted of glassy carbon working electrode, palladium electrode as reference electrodes and auxiliary carbon electrode, and Coulochem III (ESA Inc. Chelmsford, MA, USA) as a control module. The working electrode was purified by setting of potentials of -1000/+1000 mV. The sample (5 µl) was injected manually. The obtained data were treated by CSW 32 software. The experiments were carried out at room temperature.

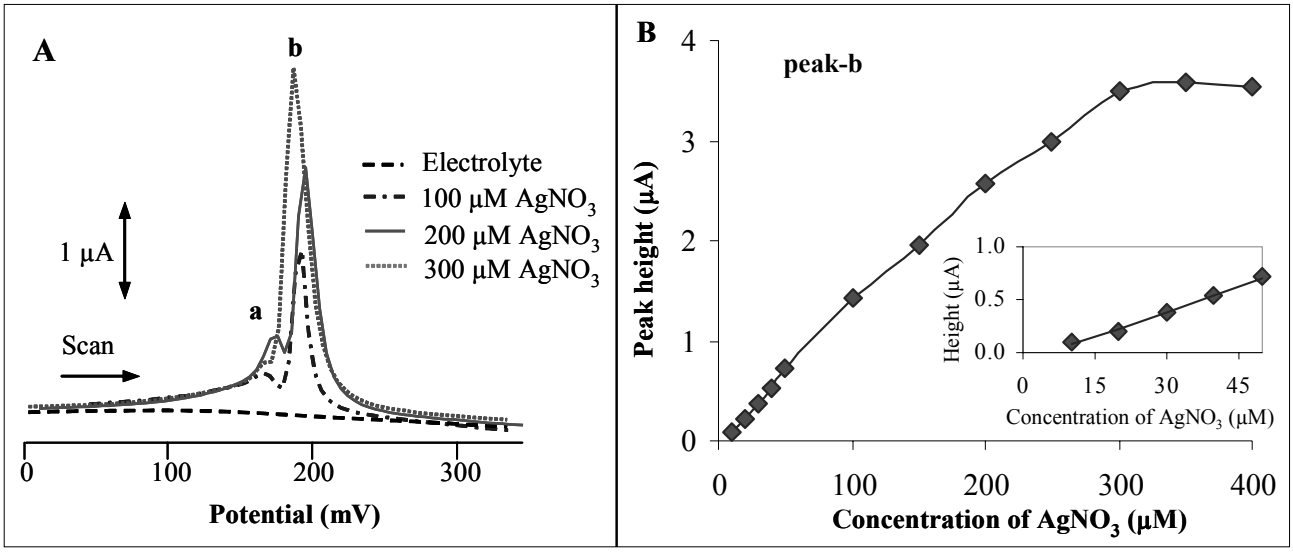

Fig. 1. Electrochemical stationary analysis. Typical DPV voltammograms of 100, 200 and 300 µM of $AgNO_3$ (**A**). Dependence of peak *b* height on concentration of silver ions in the range of 0–400 µM (**B**) and in the range of 0–50 µM (**inset in B**). The supporting electrolyte (0.2 M acetate buffer, pH 5.0) was prepared by mixing buffer components. DPV parameters were as follows: supporting electrolyte of 0.2 M acetate buffer (pH 5.0), an initial potential of -1.2 V, an end potential 1.2 V, a modulation time 0.02 s, a time interval 0.2 s, a step potential of 5 mV/s, a modulation amplitude of 250 mV.

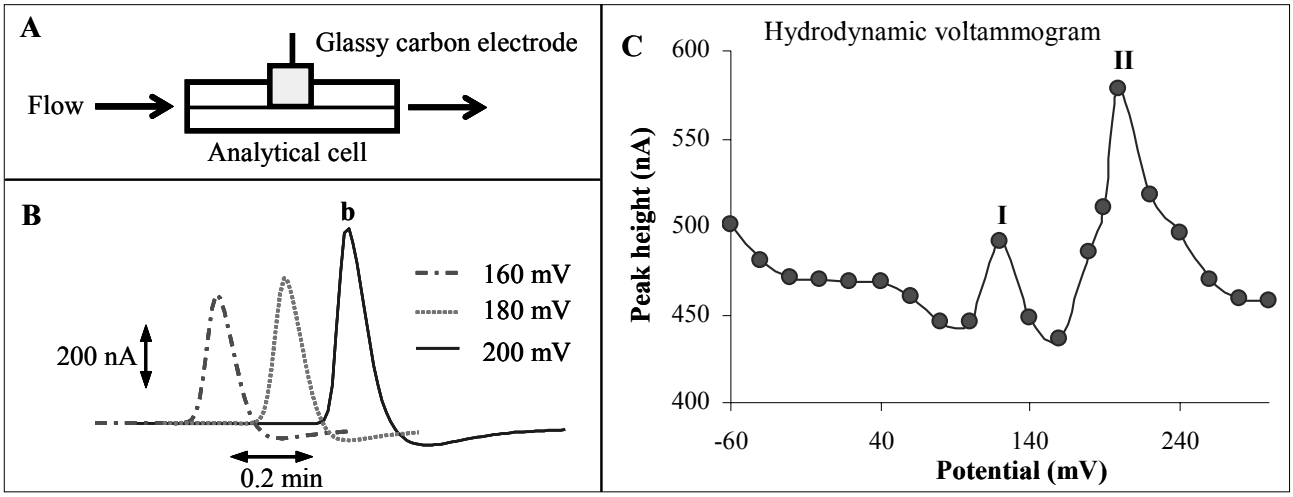

Fig. 2. Electrochemical flow analysis. The simplified scheme of flow-through analytical cell (**A**). Typical FIA-ED chromatograms of silver ions at three different potentials of working electrode (**B**). Dependence of the silver ions peak height on the potential of the glassy carbon electrode – hydrodynamic voltammogram (**C**). The FIA ED parameters were as follows: mobile phase of 0.2 M acetate buffer (pH 5.0), flow rate 0.8 $ml.min^{-1}$, potential guard cell 0 V, filter 2 sec, current 20 µA, sample injection 5 µl.

RESULTS AND DISCUSSION

Primarily the silver ions were analysed by differential pulse voltammetry (DPV) on the surface of glassy carbon electrode. We observed two signals: i) peak *a* at potential of 0.16 V and ii) peak *b* at potential of 0.19 V (Fig. 1A). The voltammetric signals *a* and *b* changed according to different concentration of silver ions, but we selected peak *b* for analytical purposes. Changes in peak *b* height with increasing concentration of silver ions are shown in Fig. 1B. If the concentration of silver ions exceeded 300 μM, the changes of peak *b* height was very low. This phenomenon relates with perfect coverage of the surface of the working electrode. In addition the concentration dependence of silver ions (0 – 50 μM) was strictly linear: $y = 0.0158x - 0.0873$; $R^2 = 0.9953$ (Fig. 1B). It is evident from the obtained results that it is possible to detect the silver ions on the surface of glassy carbon electrode.

A flow electrochemical determination (flow injection analysis; FIA) of silver ions was selected due to analysis of environmental samples. The simplified scheme of flow-through analytical cell used in our experiments is shown in Fig. 2A. We observed well separated signal of silver ions (Fig. 2B). On the base of the obtained results from stationary system, we selected the range of potentials used for plotting of hydrodynamic voltammogram. To be specific, we applied the potential ranged from –60 mV to +300 mV. We observed two maximums (I and II) on the hydrodynamic voltammogram (Fig. 2C). The first maximum (I) was obtained at potential of 120 mV and the second at potential of 200 mV (Fig. 2C). As we shown, the flow injection analysis in connection with electrochemical detector is suitable for toxicological analysis of environmental samples.

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EFFECT OF LONG-TERM ADMINISTRATION OF ARSENIC(III) AND BROMINE WITH AND WITHOUT SELENIUM AND IODINE SUPPLEMENTATION ON THE ELEMENT LEVEL IN THE THYROID OF RAT

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Key words: Iodine/Thyroid gland Selenium/Bromine/ Arsenic

The aim of this study was to evaluate the influence of arsenic and bromine exposure with or withour iodine and selenium supplementation on the element level in the thyroid of rats. Four major groups of Wistar female rats were fed with respective diets: group A – standard diet, group B – iodine rich diet (10 mg I/kg food), group C – selenium rich diet (1 mg Se/kg) and group D – iodine and selenium rich diet (as in group B and C). Each group was divided into four subgroups per 7 animals each receiving either $NaAsO_2$ ip (6.5 $mg.kg^{-1}$ twice a week for two weeks and 3.25 $mg.kg^{-1}$ for six weeks) or KBr in drinking water (58.8 $mg.l^{-1}$) for 8 weeks or combined administration of both substances. Remaining subgroup served as controls. After 8 weeks thyroid glands were analyzed by ICP-MS for As, Br, Se, and I content. The exposition of rat to arsenic or bromine causes the accumulation of these elements in the thyroid gland (~18 ppm of As, ~90 ppm of Br) and significantly affects iodine and selenium concentration in the thyroid. In iodine and/or selenium supplemented rats the bromine intake into the thyroid was lowered to ~50 % of the level in unsupplemented animals. Also selenium thyroid level elevated due to KBr administration was lowered by iodine supplementation in the diet. The accumulation of arsenic in the thyroid was not influenced by selenium or iodine supplementation; however, As(III) administration increased iodine thyroid level and suppressed selenium thyroid level in selenium or iodine supplemented group of animals.

IMPLEMENTATION OF *IN VITRO* TESTS FOR ACUTE HUMAN TOXICITY ASSESSMENT – THE MEIC AND ACUTETOX PROJECTS

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Key words: Human/Toxicity/Testing

In the last two decades new strategies in toxicology have been proposed in order to replace the animal lethal toxicity tests by alternative *in vitro* methods. Several studies have been conducted to develop reliable cellular assays and to evaluate their predictive value for acute human toxicity.

The most outstanding study in this field was the Multicentre Evaluation of In Vitro Cytotoxicity (MEIC) project (1989–1999), which was initiated and supervised by Dr. Björn Ekwall. The main goals of this international project were to evaluate the relevance of *in vitro* cytotoxicity tests for predicting acute human systemic toxicity and to select best combination of tests (test battery) for use in acute toxicity testing. Twenty nine laboratories in different countries around the world have tested 50 reference chemicals in 61 cytotoxicity assays. The MEIC project demonstrated a high relevance of human cell tests for the estimation of acute human toxicity *in vivo*. At the same time, the MEIC study outlined the need of organ-specific and mechanism-based *in vitro* assays, where biotransformation, toxicokinetics, and passage through biological barriers could be taken into account.

Recently, the European Commission (EC) proposed a new regulatory system called REACH (Registration, Evaluation and Authorisation of Chemicals). About 30,000 chemicals will be tested, which will cost billions of Euros, and require millions of experimental animals.

Two international projects have been granted by the EC, one of them is the ACuteTox (Acute Systemic Toxicity, with 35 partners from 13 countries), and the second one is ReProTect (Reproductive Toxicology, with 26 partners from 9 countries).

The European Centre for the Validation of Alternative Methods (ECVAM, Ispra, Italy) has elaborated a 10-year programme to support the implementation of the *in vitro* tests and their acceptance by the legislation.

The main aim of the ACuteTox project is to develop a forceful *in vitro* testing strategy for prediction of acute human systemic toxicity, and to replace the acute animal toxicity tests, used today for regulatory purposes, by *in vitro* and *in silico* alternatives. The project also includes validation of already existing and newly designed *in vitro* tests, e.g., analysis of their variability and reproducibility, analysis of outliers and comparison of human and *in vitro* data. A database with human, animal and *in vitro* acute toxicity data will be also created.

EXPERIMENTAL TOXICOLOGY *IN SILICO*

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Key words: Toxicity Testing/In Silico Methods/QSAR/ Validation of Computer Models

A review on applicability of *in silico* methods for toxicity testing by calculation for regulatory purposes, their survey and background of QSAR (Quantitative Structure-Activity Relationships) analysis are presented.

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WHAT ARE ARTIFICIAL NEURAL NETWORKS AND WHAT THEY CAN DO?

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Key words: Artificial neural networks/QSAR/ Expert systems

The artificial neural networks (ANN) are very often applied in many areas of toxicology for the solving of complex problems, such as the prediction of chemical compound properties and quantitative structure-activity relationship. The aim of this contribution is to give the basic knowledge about conception of ANN, theirs division and finally, the typical application of ANN will be discussed. Due to the diversity of architectures and adaptation algorithms, the ANNs are used in the broad spectrum of applications from the environmental processes modeling, through the optimization to quantitative structure-activity relationship (QSAR) methods. In addition, especially ANNs with Kohonen learning are very effective classification tool. The ANNs are mostly applied in cases, where the commonly used methods does not work.

NUCLEAR RETINOIC ACID RECEPTORS AND THEIR ROLE IN THERAPY OF SELECTED MALIGNANCIES

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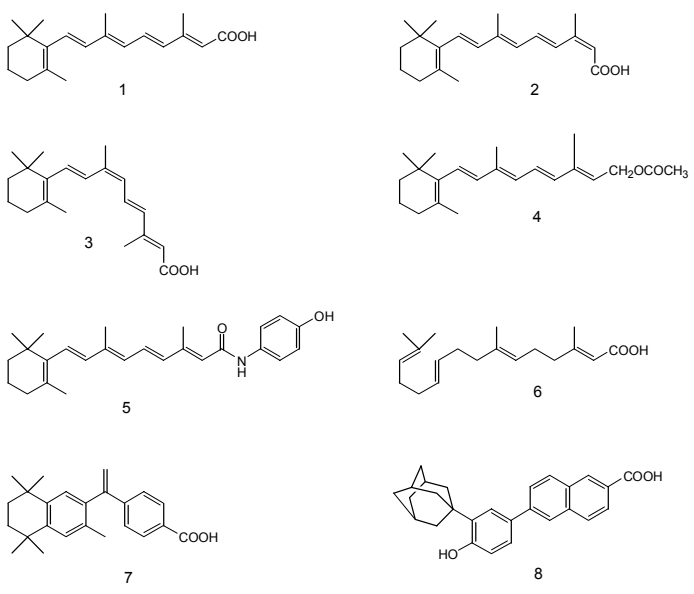
Key words: Retinoids/Rexinoids/Nuclear retinoid acid receptors/Retinoid inducible transcription factors/Cancer therapy/Regulation of transcription

ABSTRACT

Retinoids and rexinoids are natural or synthetic compounds related to retinoic acids that act through interaction with two basic types of nuclear receptors: retinoic acid receptors (RARα, RARβ and RARγ) and retinoid X receptors (RXRα, RXRβ and RXRγ) as ligand-activated, DNA-binding, trans-acting, transcription-modulating nuclear proteins. They are parts of molecular mechanisms responsible for transcriptional responses in target genes. Retinoids posses both beneficial and detrimental activity, they exert tumour-suppressive activity but on the other hand they are teratogenic. Retinoids also suppress tumour invasion in a variety of tissues. Natural and synthetic retinoids or rexinoids have therapeutical effects due to their antiproliferative and apoptosis-inducing effects. They cause redifferentiation or are able to prevent further dedifferentiation of various neoplastic cells. A number of nuclear receptor selective retinoids and rexinoids, have been successfully tested using animal models or different cancer cell lines. In spite of that rapid progress, novel synthetic retinoids and rexinoids with greater retinoid/ rexinoid receptor selectivity, therapeutic effects, reduced teratogenic and other side effects are highly required.

Retinoids and rexinoids are involved in the complex arrangements of physiological and developmental responses in many tissues of higher vertebrates that include embryonic development, vision, reproduction, bone formation, haematopoiesis, metabolism, growth and differentiation of a variety of cell types, apoptosis and processes of carcinogenesis[1,2,3]. Carboxy- group containing natural derivatives of vitamin A include all-trans –, 9-cis – and 13-cis retinoic acids (Fig. 1). In cells, all-trans retinoic acid formation, the main biologically active derivative of vitamin A, involves oxidation of all-trans retinol to all-trans retinaldehyde and subsequently its irreversible oxidation to all-trans retinoic acid by enzymes which are predominantly present in the intestine, liver and kidney. The all-trans retinaldehyde plays an important role for proper functioning of the eye, where it can be isomerised to 11-cis retinaldehyde, as a part of the visual cycle[4]. It is well known that retinoids are also teratogens and



1. All-trans retinoic acid
(TRETINOIN, VASANOID)
2. 13-cis retinoic acid
(ISOTRETINOIN, ROACCUTANE)
3. 9-cis retinoic acid
(ALITRETINOIN)
4. Retinyl acetate
5. N-(4hydroxyphenyl)retinamide
(FENRETIDINE)
6. All-trans-3,7,11,15-tetramethyl-2,4,6, 10,14-hexadecapentanoic acid
(NIK 333, Acyclic retinoid)
7. 4-[1-(5,6,7,8-tetrahydro-3,5, 5,8,8-pentamethyl-2-naphtalenyl)ethenyl] benzoic acid
(LGD1069, TARGRETIN, BEXAROTENE)
8. 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene carboxylic acid
(AHPN, CD437)

Fig. 1. Chemical structure of selected retinoids/rexinoids

the therapeutic doses of them are contraindicated during pregnancy[5].

A breakthrough in the field of nuclear receptors was discovery of the nuclear receptor superfamily that includes nuclear receptors for retinoids, steroid and thyroid hormones, vitamin D_3 and a number of "orphan" nuclear receptors for which the most of the ligands remains still unknown. It is generally accepted that nuclear receptors represent a superfamily of ligand-inducible transcription factors. The amino terminal A/B-domain of the nuclear receptor molecule contains a constitutive activation function (AF-1), the central C-domain is a cysteine-rich DNA-binding region consisting of two highly conserved zinc fingers. The D-domain represents a highly flexible structure, and it plays a role as a hinge of the receptor molecule. The carboxy-terminal E-domain is responsible for the ligand binding, dimerization, and it contains inducible transactivation function (AF-2) (ref.[6,7,8]) (Fig. 2). All-trans retinoic acid receptors (RARs) upon all-trans retinoic acid binding act as all-trans retinoic acid-inducible transcription factors by directly interacting as heterodimers with 9-cis retinoic acid receptor (retinoid X receptor, RXR). The RAR/RXR heterodimer interacts with specific DNA response elements of target genes and its effect on transcription is mediated also through recruitment of a number of coregulators (corepressors and coactivators)[9,10,11]. Ligand occupancy of both receptors in RAR/RXR heterodimes was found to synergistically increase transcriptional activity[12,13]. RARs bind both all-trans retinoic acid and 9-cis retinoic acid with similar affinities while RXRs bind only 9-cis retinoic acid. The diversity of retinoic acid-induced signalling pathway is associated with existence of at least three subtypes for RAR (α, β and γ) and three subtypes for RXR (α, β and γ) with distinct amino- and carboxy-terminal domains. Recently, two major isoforms for RARα (α1 and α2) and for RARγ (γ1 and γ2) and four major isoforms for RARβ (β1, β2, β3 and β4) have been found. Similarly, two major isoforms for RXRα (α1 and α2), RXRβ (β1 and β2), and RXRγ (γ1 and γ2) have been identified up to now[10,14]. RAR/RXR heterodimers bind to specific DNA sequence – retinoic acid response elements (RAREs), characterized by direct repeats of two hexamers (A/G)GGTCA separated predominantly by two nucleotides (DR+2) or five nucleotides (DR+5). In the absence of all-trans retinoic acid, the RAR/RXR heterodimer recruits nuclear receptor corepressor proteins, e.g. nuclear receptor corepressor (N-CoR) or silencing mediator of retinoid and thyroid hormone receptor (SMRT). All-trans retinoic acid binding leads to the dissociation of corepressor proteins and enables association of coactivator proteins with liganded receptor complex yielding in activation of gene transcription[15]. Thus, the retinoid receptors are considered to be ligand-activated, DNA-binding, trans-acting, transcription-modulating nuclear proteins involved in a general molecular mechanism responsible for transcriptional responses in target genes[11,14].

The retinoids selective for specific binding to RXRs are called rexinoids[16], and RXRs play an crucial role in the nuclear receptor mediated transcription processes for their ability to heterodimerize with many other members of nuclear receptor superfamily, including RARs, thyroid hormone receptors, vitamin D_3 receptor, peroxisome proliferator-activated receptor γ (PPARγ), liver X receptor (LXR) and farnesoid X-activated receptor (FXR) (ref.[17,18]). RXRs behave as promiscuous dimerization partners for a large number of nuclear receptors, and thus play an integrative and crucial role in nuclear receptor mediated pathways, suggesting that specific RXR ligands

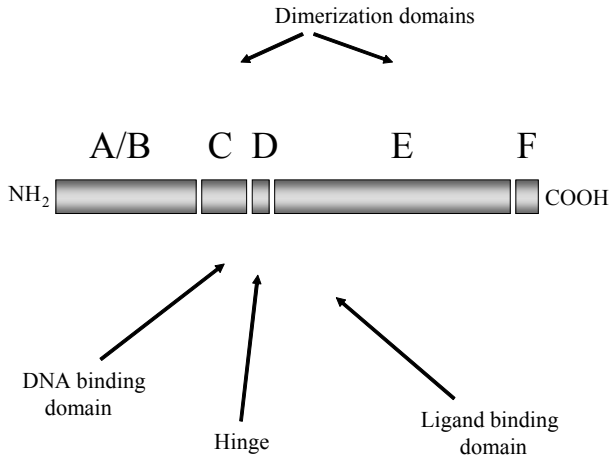


Fig. 2. Essential nuclear receptor domains

might modulate transcription processes of target genes independently of RARs (ref.[19,20]).

Acute promyelocytic leukaemia (APL) is characterized by selected expansion of immature myeloid precursors or malignant myeloid cells blocked at the promyelocytic stage of hemopoietic development[21]. RARα fuses to the PML gene on chromosome 15 yielding the PML – RARα chimeric gene, which is expressed in all APL patients with t(15;17) (ref.[22]). Thus, chimeric receptors are responsible for development of leukemias by interfering with the functions of RARs, RXRs and PML expressed by the normal alleles present in the same cell. The all-trans retinoic acid-induced differentiation of cells is based on important findings that it can activate degradation of PML – RARα, induce dissociation of corepressors from PML – RARα (ref.[23]).

Breast cancer development is associated with deregulation of cell growth and cell death. It has been shown that retinoids are also able to inhibit human breast cancer. They are effective inhibitors of breast cancer cells at the early stages of tumour progression and their effectiveness diminishes as tumours become more aggressive[24].

In follicular thyroid cancer cells, 13-cis retinoic acid has been found to induce radioiodine avidity of cells formerly unable to accumulate radioiodine[25]. Papillary and follicular thyroid carcinomas belong to so-called well-differentiated carcinomas with a good prognosis after treatment involving surgery, radioiodide therapy and thyrotropin-suppresive L-thyroxine application. However, approximately 30 % of the thyroid tumours may start to dedifferentiate and thus finally develop into highly malignant anaplastic thyroid carcinomas Non-operable thyroid follicular tumours, which due to loss of thyroid specific functions also fail to take up radioiodine, have been treated with 13-cis retinoic acid. The beneficial outcome of this treatment was interpreted as partial redifferentiation of thyroid cancer cells[26,27,28].

Abnormalities of RAR subtype expression in a high percentage of human lung cancer cell lines have been also reported. These abnormalities included predominantly failure to express both RARβ transcript isoforms with our without loss of normal induction of RARβ expression in response to all-trans retinoic acid[29].

Recent studies have also demonstrated a growth-retarding effect on prostate cancer cells and suppression of the development of prostate tumours by retinoids[30].

A variety of retinoids has been tested on different ovarian cancer lines in order to evaluate the mechanism of action. In ovarian carcinoma and number of other types of tumours (neuroblastoma, skin, bladder) retinoids induced both apoptosis and differentiation thus representing a potentially powerful alternative to present chemotherapeutic treatment of late stages of cancer[31,32].

In conclusion, a number of novel chemical compounds, receptor selective retinoids and rexinoids, have been synthesized up to now and tested both in vitro and in vivo. In spite of that rapid progress novel synthetic retinoids and rexinoids with greater retinoid receptor selectivity, reduced teratogenic and other side effects are highly required[32,33].

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ANTIOXIDANTS AND PROTECTION OF ORGANISM AGAINST OXIDATIVE DAMAGE UNDER EXPERIMENTAL CONDITIONS

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Key words: Melatonin/Vitamin E/Stobadine/Oxidative stress models

Subject of our study was focused on the effect of natural antioxidants (melatonin and vitamin E) and synthetic antioxidant stobadine on selected biochemical variables. We determined the activity of lysosomal enzymes and the glutathione level in rats tested in the model of cyclophosphamide and ^{60}Co irradiation induced oxidative stress. We applied the cyclophosphamide model on rats in the prenatal study as well. The antioxidative effect in rats was monitored in the model of experimental diabetes induced by streptozotocin, the mesenteric ischaemia/reperfusion model, the model of chronic intrauterine hypoxia induced by phenytoin and the model of neonatal anoxia in nitrogen chamber (100 % N_2). Obtained results demonstrate a partial preventive effect of the above studied antioxidants.

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EFFECT OF COENZYME Q_{10} SUPPLEMENTATION IN THE RAT MODEL OF ADJUVANT ARTHRITIS

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Key words: Coenzyme Q10/Supplementation/Adjuvant arthritis/Mitochondrial function/Antioxidants/Inflammation

Adjuvant arthritis (AA) is a model of chronic inflammation induced by *Mycobacterium butyricum* and

characterized by similar pathophysiological and pathobiochemical changes as rheumatoid arthritis (RA) in humans. In this study the antirheumatic activity of coenzyme Q_{10} supplementation was tested not only as to its capability to suppress the inflammation edema of the hind paw and to improve the body weight of the arthritic animals, but also to improve so important biochemical parameters as markers of inflammation and oxidative stress, and of mitochondrial bioenergetics. Despite the unfavorable effects on the rheumatic processes observed by monitoring biometric parameters (hind paw volume, relative body weight, relative weight of spleen), a significant protective effect was observed on the level of mitochondrial energetic and antioxidant disbalance. This finding speaks in favor of CoQ_{10} supplementation in rheumatic patients, presumably as combinatory therapy with classical antirheumatics, e.g. NSAIDs.

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SIMULTANEOUS DETERMINATION OF 1,4-NAPHTOQUINONE, LAWSONE, JUGLONE AND PLUMBAGIN BY LIQUID CHROMATOGRAPHY WITH UV DETECTION

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Key words: Naphtoquinones/High performance liquid chromatography/Diode array detector/Plants/1,4-Naphtoquinone/Lawsone/Juglone/Plumbagin

ABSTRACT

Naphthoquinones, compounds of natural origin, mostly appeared as chromatic pigments. They are deposited in cells vacuoles, where they are dissolved (in glycoside form). Naphthoquinones are very toxic, antimicrobial, antifungal, antiviral and antiparasitic effects were observed. The aim of our work was to optimize the high performance liquid chromatography coupled with diode array detector for the determination of naphtoquinones (1,4-naphtoquinone, lawsone; juglone and plumbagin). Detection limits (3 S/N) of naphtoquinones were – lawson 65 $ng.ml^{-1}$, 1,4-naftochinon 50 $ng.ml^{-1}$, juglon 75 $ng.ml^{-1}$ and plumbagin 39 $ng.ml^{-1}$. The optimized method was consequently used for the determination of the mentioned naphtoquinones in plant sample (*Dionaea muscipula*).

INTRODUCTION

Naphthoquinones, compounds of natural origin, mostly appeared as chromatic pigments. They are deposited in cell vacuoles, where are dissolved in form of glycosides[1]. Naphtoquinones as a group of secondary metabolites occur in number of plant families (*Plumbaginaceae, Juglandaceae, Ebenaceae, Boraginaceae, Dioncophyllaceae, Ancistrocladaceae, Iridaceae, Verbenaceae, Scrophulariaceae, Avicenniaceae, Balsaminceae, Bignoniaceae, Gentianaceae, Droseraceae, Nepenthaceae, Lythraceae, Euphorbiaceae*[2-5], fungi and microorganisms (*Streptomyces, Fusarium*)[6]. Biosynthesis of naphtoquinones proceed by way of six biosynthetic pathways[6-9]. It was found that the basic precursor of biosynthesis of majority part of naphtoquinones is shikimic acid.

An anticancer effect of naphthoquinones awakes an interest in determination and characterization of single derivates of 1,2 and 1,4 quinone in biological samples. High performance liquid chromatography with UV detection and electrochemical techniques are the most commonly used method for these purposes[10-13]. In addition de Paiva et. al. used mass spectrometry as a tool for study of plumbagin[14]. On the other hand a just a few scientists occupy themselves with the determination of naphthoquinones in the biological samples[10-14].

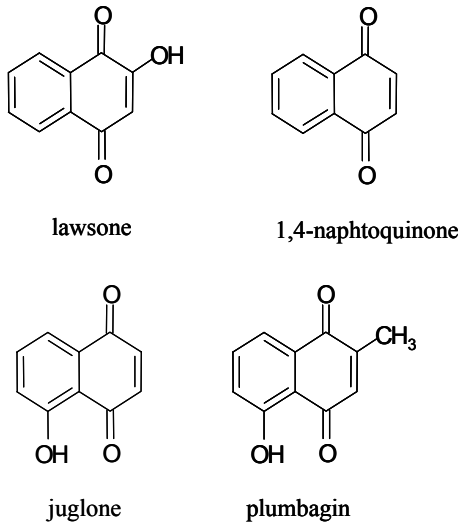

Fig. 1. Chemical structures of 1,4-naphtoquinone, lawsone; juglone and plumbagin.

The aim of this work was to optimize the simultaneous analysis of naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin; see in Fig. 1) by high performance liquid chromatography coupled with diode array detector (HPLC-DAD). The optimized technique was consequently used for detection and quantification of naphthoquinones in plant material (*Dionaea muscipula*).

MATERIAL AND METHODS

Chemicals

Naphtoquinones (1,4-naphtoquinone, lawsone, juglone and plumbagin) were purchased from Sigma Aldrich Chemical Corp. (St. Louis, USA). Methanol for HPLC and other analytical reagents of ACS purity were purchased from Sigma Aldrich too. Solutions were prepared using deionised ACS water (Sigma). The stock standard solutions of naphtoquinones at 100 μg.ml^{-1} concentration were prepared in ACS methanol and stored in the dark at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45 μm Teflon membrane filters (MetaChem, Torrance, USA) prior to HPLC separations.

Plant material

Dionaea muscipula Ell. plants were originally established at the Department of Natural Medicinals of the University of Veterinary and Pharmaceutical Sciences in Brno. The plants were cultivated on a 20 ml of Murashige Skoog Medium (MS)(ref.[15]) with addition of sucrose (30 g/l) and of activated carbon (1.5 g/l) in Erlenmayer flasks (100 ml).

The cultivation was performed in a cultivation boxes for 7 days, 14 hours long daylight per a day (maximal light intensity was about 100 μEm^{-2}s^{-1}), at a temperature 26.5 °C and humidity 60–70 %.

HPLC analysis

An HP 1100 liquid chromatographic system (Hewlett Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an auto sampler (G1313A), a column thermostat (G1316A), and a UV-VIS diode array detector (model G1315B) working at 190–690 nm. The ChemStation software (Rev. A 08.01) controlled the whole liquid chromatographic system. Spectra were registered in the range of 190-400 nm (SBW 100 nm). Chromatograms were registered at 280 nm. Naphtoquinones were separated on a reversed-phase Zorbax C18-AAA chromatographic column (150 mm × 4.6 mm, 3.5 μm particle size, Agilent Technologies, USA) in an isocratic mode. The most effective chromatographic

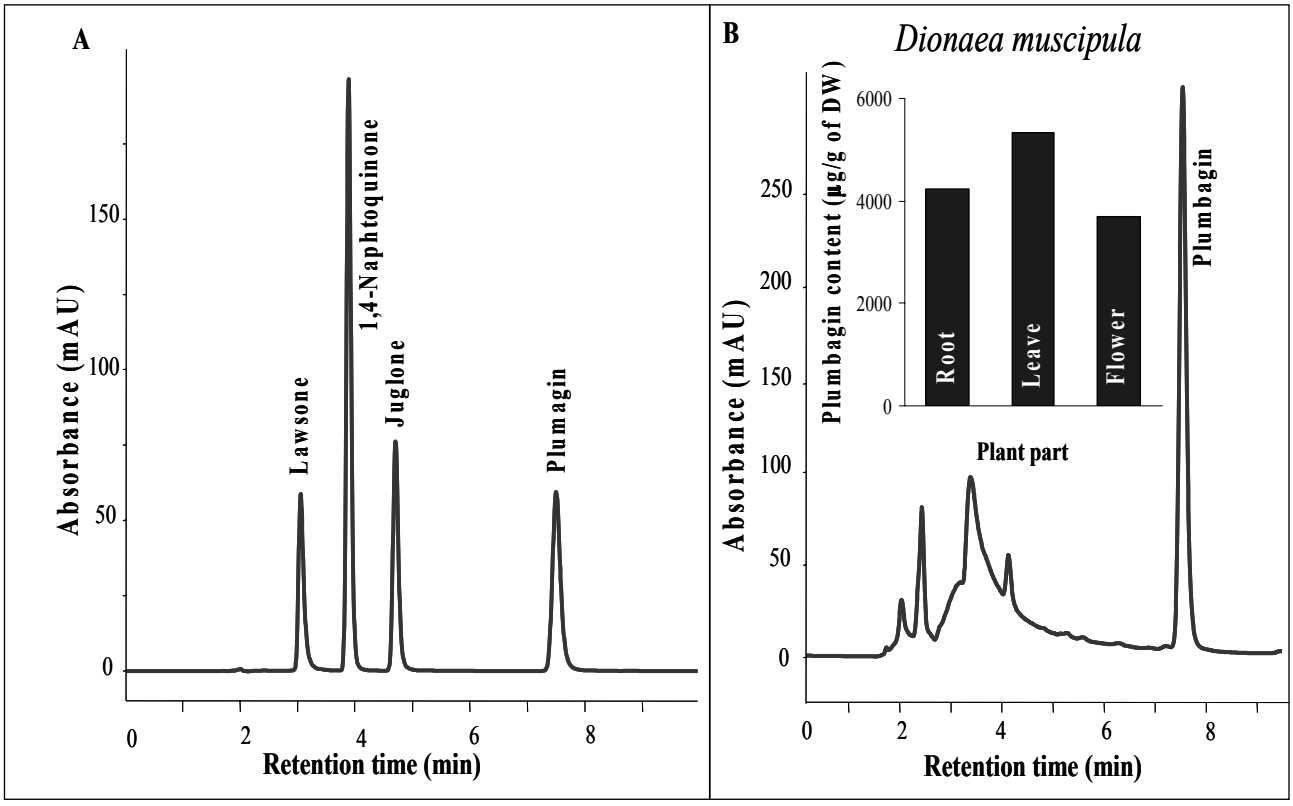

Fig. 2. Simultaneous determination of the naphthoquinones. HPLC-DAD chromatogram of 1,4-naphtoquinone, lawsone; juglone and plumbagin (**A**) and of *Dionaea muscipula* leave (**B**); in inset: content of plumbagin in leave, root and flower of *Dionaea muscipula*. HPLC-DAD conditions were as follows: mobile phase: 0.1 mol.l^{-1} acetic acid:methanol in ratio 35 : 65; flow rate: 0.8 ml.min^{-1}; column and detector temperature: 40 °C; auto sampler injection was 5 μl.

Tab. 1. HPLC-DAD characteristics of analysed naphthoquinones

Naphthoquinone	t_R (min)	Equation[a]	R^2	LOD ($ng.ml^{-1}$)	LOQ ($ng.ml^{-1}$)	R.S.D. (%)
1,4-naftochinone	3.8	y = 47.733x – 9.0435	0.9999	50.5	168.3	1.91
Juglone	4.7	y = 20.756x – 5.6828	0.9998	75.6	252.0	2.25
Lawsone	3.0	y = 15.951x – 5.2675	0.9997	65.3	217.7	2.86
Plumbagin	7.4	y = 24.416x – 6.0176	0.9998	38.5	128.3	1.11

[a] ... The concentration range was from 1.7 to 25.0 $\mu g.ml^{-1}$.

conditions were – mobile phase: 0.1 $mol.l^{-1}$ acetic acid: methanol in ratio 35 : 65; flow rate: 0.8 $ml.min^{-1}$; column and detector temperature: 40 °C. Auto sampler injection was 5 µl.

Sample preparation

Plant tissues were lyophilized at –51°C for 48 h (CHRIST-Alpha 1-2). Then the lyophilized samples were homogenized by an Ika A11 basic grinder (IKA Werke GmbH and Co., Staufen, KG, Germany). The homogenized samples (0.02–0.10 g) were dissolved in 99.999 % methanol and sonicated at the laboratory temperature for 30 min on K5 Sonicator (Czech Republic) at 150 W, 38 kHz. The samples were filtered through a 0.45 µm Teflon membrane filters (MetaChem, Torrance, CA, USA) prior to injection into the HPLC system.

RESULTS AND DISCUSSION

As we mentioned in the Introduction section, naphthoquinones have a broad range of biological action. That is why we aimed on simultaneous determination of naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin; see in Fig. 1). The chromatogram of the mentioned naphthoquinones obtained at the most effective chromatographic conditions (see Materials and Methods section) is shown in Fig. 2A. The dependence of height and area of DAD signal (254 nm) of naphthoquinones on their concentrations were strictly linear in the range from 1.7 to 25.0 $\mu g.ml^{-1}$, R^2 0.9995 – 0.9999 (Tab. 1). The limit of detection and quantification of naphthoquinones are shown in Tab. 1.

In addition we applied the optimised technique for the determination of naphthoquinones in biological sample – *Dionaea muscipula* (Fig. 2B). To be specific, we analysed the content of naphthoquinones in leaves, root and flower of the mentioned plant specie. We found out that the *Dionaea muscipula* contained only one naphthoquinone – plumbagin. The highest amounts of plumbagin were determined in leaves (5338 $\mu g.g^{-1}$), followed by root (4230 $\mu g.g^{-1}$) and flower (3698 $\mu g.g^{-1}$), see in inset in Fig. 2B. The HPLC-DAD chromatogram of the analysis of *Dionaea muscipula* leave is shown in Fig. 2B.

As we mentioned above, we did not determine any other naphthoquinone, which we studied. In addition the presence of other quinones derivates such as 3-chloroplumbagin, hydroplumbagin 4-O-β-glukopyranoside, diomuscipulone, diomuscinone and 8,8`-biplumbagin in *Dionaea muscipula* has been described[16–20]. The suggested technique could be used for studying of naphthoquinones not only in plant material but also in cell, embryos and food samples.

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COMPARISON OF "HIGH THROUGHPUT" MICROMETHODS FOR DETERMINATION OF CYTOCHROME P450 ACTIVITIES WITH CLASSICAL METHODS USING HPLC FOR PRODUCT IDENTIFICATION

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Key words: Cytochrome P450/CYP/Luminiscence/ Fluorescence/HPLC

Enzyme activities of the CYP enzymes (CYP3A4, CYP2C9 and CYP2A6) were determined using classical substrates (testosterone, diclofenac and coumarin, respectively) as well as with luminogenic or fluorogenic substrates in micromethod arrangement. The luciferin-based luminogenic substrates for CYP3A4 and CYP2C9 as well as coumarin in micromethod for assay of CYP2A6 activity gave results well comparable with the classical methods with determination of reaction products by the HPLC.

ACKNOWLEDGEMENT

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EFFECT OF ACRIDINE ORANGE ON EUGLENA GRACILIS AND DETECTION OF DAMAGE TO DNA BY USING SCREEN-PRINTED ELECTRODE

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Key words: Euglena gracilis/Acridine orange/ Mutagenicity/DNA damage/DNA biosensor

ABSTRACT

Genotoxic properties of chemical compounds have been mostly described using bacterial system[1]. Among the unicellular eukaryotic systems, different strains of yeast have been used[2]. A less known model of detection of genetically active substances is unicellular flagellate *Euglena gracilis*. Many genotoxic agents exert a mutagenic effect on the unicellular flagellate *E. gracilis*[3]. Green autotrophic cells of *E. gracilis* affected by mutagens are converted to heterotrophic cells due to their irreversible loss of chloroplasts. This cytoplasmatic mutation, "permanent bleaching", is induced, among others, by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-methyl-*N*-nitrosourea[4] and acridine orange.

INTRODUCTION

Interaction of DNA with other molecules represents a fundamental issue in life sciences. They are, therefore, also a subject of investigations done with the DNA-based biosensors. Comparing to biocomponents used typically as sensing parts of the biosensors, the DNA biopolymer is of interest for the study of DNA itself including DNA association with low-molecular-weight compounds, DNA hybridization and DNA damage[5]. An evaluation of DNA interactions using the biosensors helps to understand action mechanisms of drugs as well as health risk chemicals, to develop compounds of desired activity and, on the other hand, to predict unwanted toxic effects and damage to DNA. Different particularly electrochemical DNA sensors have been used advantageously in recent years for rapid screening of various compounds[6]. DNA biosensors are integrated receptor-transducer devices that use DNA as biomolecular recognition element to measure specific binding processes with DNA, usually by electrical, thermal, or optical signal transduction.

MATERIAL AND METHODS

Chemicals

Acridine orange (AO, Fig. 1 and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemie, Steinheim, Germany. Acridine orange was dissolved immediately before use in DMSO, its final concentration in the medium never exceeded 0.4 % (v/v) in either control and treated cells.

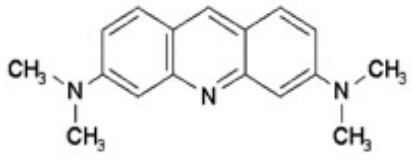

Fig. 1. Acridine orange

Organism

Euglena gracilis strain Z was originally obtained from S. H. Hutner, Haskins Laboratories, Pace University, NY, USA. The cells were cultivated statistically on a modified Cramer-Myers medium supplemented with 0.5 % sodium acetate at pH 6.8 at 26±2 °C under permanent illumination[7].

Cell viability assay

The viability of euglena cells was estimated by counting the total colony number affected by mutagens compared to the total colony number without any treatment.

Euglena gracilis assay

The cells taken from exponential growth phase and diluted to final concentration 10^5 cells/ml were used. Euglena cells were treated with 5.5 µmol/l, 11 µmol/l, 22 µmol/l AO by using temperature 17, 22, 38 °C. Following a 24-h treatment, the cells were washed out, diluted and spread on agar plates with CM medium (1.2 % agar). Green and white (mutant) colonies were analyzed after 10-14 days of cultivation in the light at 26 °C. The experiments were repeated in three independent series. The number of bleached and green colonies was counted and thein percentage were calculated.

Measurement of the interaction of acridine orange with dsDNA by using DNA - modified screen - printed electrode[8]

In our work, a screen-printed electrode based on a carbon paste with the surface modified by double stranded (ds) calf thymus DNA has been prepared and applied to the study this AO. Interactions of AO with DNA have been detected voltammetrically using the tris(1,10-phen anthroline)cobalt(III) complex $[Co(phen)_3]^{3+}$ as a DNA redox indicator.

The screen-printed three-electrode assembly (Fig. 2) includes a working carbon electrode (SPE, 25 mm^2 geometric surface area and about 35 mm^2 effective area) together with silver/silver chloride reference electrode (Ag/AgCl/SPE with potencial of 0.284 V vs conventional Ag/AgCl/3 mol/l KCl electrode) and counter electrode. A working electrode was chemically modified by covering with 5 µl 0.1 mg/l DNA stock solution and leaving to dry overnight. After 2 min pretreatment in blank (pH = 7.0) solution before use, the final dsDNA layer fixed by adsorption is rather stable and can be exploit analytically with sufficient repeatability (RSD of 12 %, n = 15).

To detect the damage of DNA, the same DNA sensor was first incubated for 10 min with AO (5.5 µmol/l, 11 µmol/l, 22 µmol/l) in 5 mmol/l phosphate buffer solution (PBS) by using temperature 17, 22, 38 °C, pH 7.0, under string, and then rinsed with water. Then, the marker peak current (I) was obtained using differential pulse voltammetric (DPV) measurement/biosensor regeneration scheme and the normalized (relative) indicator signal I/I_o was calculated (I_o is the signal obtained before the incubation).

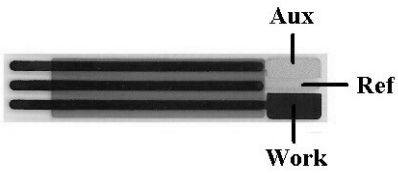

Fig. 2. Screen-printed three-electrode assembly

The electrochemical procedure for the detection of adenine and guanine oxidation signals from dsDNA involved two steps: DNA immobilization and voltammetric transduction. The oxidation signals of adenine and guanine were measured by using differential pulse voltammograms (DPV) in the blank 5 mmol/l PBS.

RESULTS AND DISCUSSION

The hereditary bleaching test on *Euglena gracilis* used for detecting extranuclear mutation showed positive results for AO (5.5 µmol/l, 11 µmol/l, 22 µmol/l). The presence of DMSO in separate control incubations with the euglena cells did not demonstrated the mutagenic activity. We tested effect of AO in temperature 17, 22, 38 °C (Fig. 3). The highest concentration of AO (22 µmol/l) by temperature 22 and 38 °C induced a high number of irreversible white mutants of *E. gracilis*, their percentage reached 100. With increasing concentration of AO and with increasing temperature of cultivation *E. gracilis* with AO, increased a number of bleached colonies.

The redox marker can be accumulated effectively within the DNA double helix from the $[Co(phen)_3]^{3+}$ solution at both a polarization of the modified electrode by a positive potential as well as open circuit. Depending on an ionic strength of the medium, intercalation (predominantly at high ionic strength) and electrostatic forces (predominantly at low ionic strength) take part in binding of the marker particles. The interaction as well as electrostatic binding are equilibrium processes which can be utilized for a removal of $[Co(phen)_3]^{3+}$ from the DNA layer in solution without $[Co(phen)_3]^{3+}$.

Damage to DNA was expressed by the relative signal I/I_o, where I and I_o are the corrected marker DPV peak currents in experiments with and without AO. Calf thymus DNA on biosensor was exposed to different concentrations (5.5 µmol/l, 11 µmol/l, 22 µmol/l) of AO for 17, 22 and 38 °C for 10 minutes. The treatment of the sensor in solution of AO lead to a change in the signal. We can see on Fig. 4, that AO caused change in calf thymus dsDNA. The highest damage was by using 22 µM AO and temperature 38 °C (I/Io = 0.06), so the damage to DNA was 94 %. AO was investigated in 0.4 % DMSO solution (5 mmol/l PBS, pH = 7.0). A potential shift of the guest molecule is usually taken as a confirmation of its binding mode.

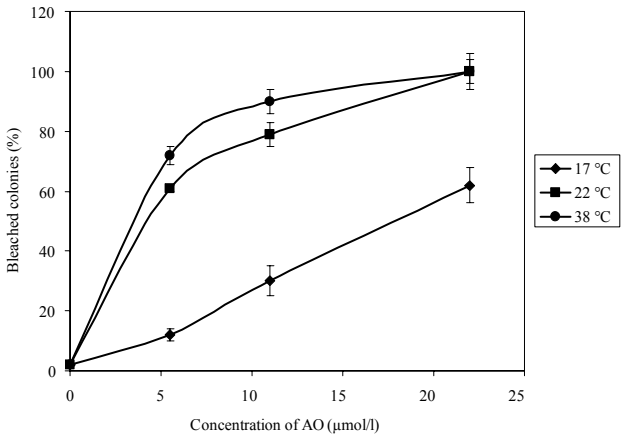

Fig. 3. Effect of AO at different concentrations on the frequency of white mutants (% of bleached colonies) in *Euglena gracilis* after 24 h incubation at 17, 22 and 38 °C.

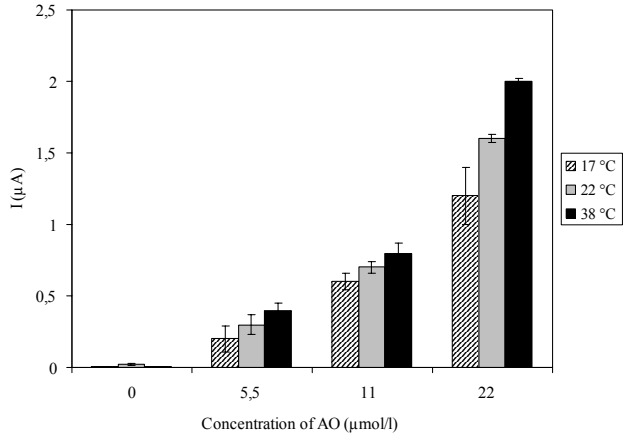

Fig. 4. Effect of AO on DNA at 10 min incubation of DNA/SPE sensor in AO solution for temperature 17, 22 and 38 °C. Conditions: 5 mmol/l phosphate buffer solution pH 7.0 under stirring.

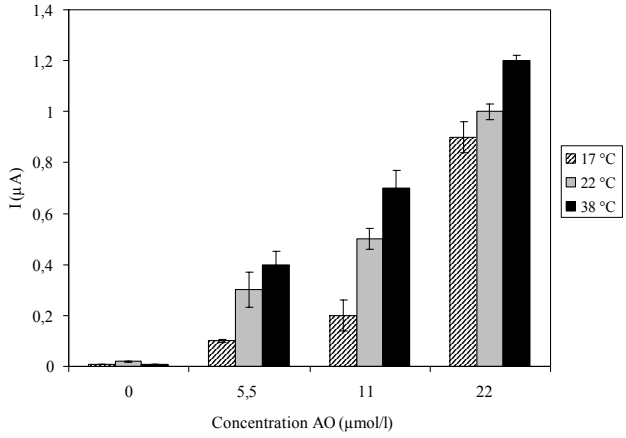

Fig. 5. Voltammetric peak currents of guanine for the interaction of AO with dsDNA for various temperature. Conditions: 5 mmol/l phosphate buffer solution pH 7.0 under stirring.

We can see on the Fig. 5, that the oxidation signals of guanine obtained with the dsDNA modified SPE without the interaction of AO was very small than signals of guanine obtained after the interaction dsDNA with AO for various temperature (17, 22 a 38 °C). Voltammetric peak currents (VPC) increased by using higher concentration of AO and by using the higher temperature (AO is 22 μmol/l, temperature is 38 °C and VPC is 2 μA).

Fig. 6 displays the triplicate results from the oxidation signals of adenine before and after the interaction of AO with dsDNA in solution. The signal of adenine obtained before interaction with AO was smaller than the signal obtained after interaction with AO. The highest VPC (1.2 μA) was measured by temperature 38 °C and concentration of AO 22 μmol/l. No signal of 5 mmol/l PBS at SPE and no signal of AO at SPE were also found. The signals of guanine was higher than signals of adenine.

Yamabe (1967) tested by using spectrofotometric *in vitro* interaction of AO with calf thymus DNA. From spectral change concluded, that AO interact with deoxyadenosine a deoxyguanosine. AO was not effective on *E. gracilis* which was cultivated in dark.

The interaction of electroactive and non-electroactive agents with DNA can be monitored by using the procedures reported here. The DPV detection system provides lower detection limits than the other voltammetric systems, because trace amount of agent could be easily detected. The oxidation signals of guanine and adenine were used for detecting the interaction mechanism of AO with DNA at the electrode surface. Detecting the voltammetric behavior of several drugs that interact with DNA would be valuable in the design of sequence-specific DNA binding molecules for application in chemotherapy and in the development of biotechnological tools for the point-of-care tests based on DNA.

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CYTOTOXICITY AND DETECTION OF DAMAGE TO DNA BY 3-(5-NITRO-2-THIENYL)–9–CHLORO-5-MORPHOLIN-4-YL [1,2,4] TRIAZOLO [4,3-*c*] QUINAZOLINE ON HUMAN CANCER CELL LINE HELA

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Key words: Quinazoline derivative/HeLa cells/ Cytotoxicity/DNA damage/DNA biosensor

Quinazolines – 1,3-benzodiazines are biological active compounds, which are used in the phamaceutical industry, in agriculture and in medicine. As documented in the literature, many derivatives demonstrated anticancer activity and they act as multitarget agents. 3-(5-Nitro-2-thienyl)-9-chloro-5-morpholin-4-yl [1,2,4] triazolo [4,3-*c*] quinazoline (NTCHMTQ) – a new synthetically prepared quinazoline derivative was the most effective derivative in our primary cytotoxic screening. In this study, we evaluated cytotoxic/antiproliferative activity of NTCHMTQ using human tumor cell line HeLa. Possible interaction of 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl [1,2,4] triazolo[4,3-c] quinazoline with calf thymus DNA was tested by the DNA – modified screen – printed electrode. Quinazoline derivative acted cytotoxically on tumor cell line HeLa. The IC_{100} value was 10 μg/ml. The IC_{50} values was found to be less than 4 μg/ml, a limit put forward by the National Cancer Institute (NCI) for classiffication of he compound as a potential anticancer drug. Quinazoline at micromolar concetrations induced morphological changes and necrosis of HeLa cells. Using the DNA based electrochemical biosensor, we have not found damage to DNA under *in vitro* conditions at an incubation of the biosensor in mixture with quinazoline.

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COMPARISON THE CYTOTOXICITY OF HYDROXYAPATITE MEASURED BY DIRECT CELL COUNTING AND MTT TEST IN MURINE FIBROBLAST NIH-3T3 CELLS

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Key words: Hydroxyapatite/Cytotoxicity/Indirect test/ Cell proliferation/MTT test/NIH-3T3 Cells

The worldwide growing interest to biomaterials over the last years results from their irreplaceable role in medical clinic. Hydroxyapatite is used in bone reconstruction because of its similar chemical structure compared to the inorganic composition of human bone and it is basic building component of many new prepared biomaterials. In this study, we evaluated cytotoxic/antiproliferative activity of hydroxyapatite extract using murine fibroblast cell line NIH-3T3 and two *in vitro* different cytotoxic assays: growth inhibition assay and MTT assay. Hydroxyapatite extract after 72 h of incubation manifested the significant *in vitro* cytotoxic/antiproliferative effect only at the highest tested concentration (100 %). The antiproliferative effect of hydroxyapatite extract at other concentrations tested (75 %, 50 %, 25 %, 10 %, 5 % and 1 %) was directly proportional to the concentration and the time of influence. The inhibition of cell proliferation was 86.8–0 %. The sensitivity of cell growth inhibition assay (direct counting of viable cells) to the extract influence was higher than that of MTT test.

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CYTOTOXICITY AND INDUCTION OF APOPTOSIS BY 4-AMINO-3-ACETYLQUINOLINE IN MURINE LEUKEMIA CELL LINE L1210

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Key words: 4-Amino-3-acetylquinazoline/L1210 cells/ Cytotoxic activity/Apoptosis

Nitrogen heterocyclic compounds are used in the pharmaceutical industry, in medicine and in agriculture for their biological activity. 4-Amino-3-acetylquinazoline a new synthetically prepared quinoline derivative was the most effective compound in our primary cytotoxic screening. In this study, we evaluated cytotoxic/antiproliferative activity of quinoline using murine leukemia cell line L1210. Its ability to induce of apoptosis was studied, too. Quinoline derivative acted cytotoxically on tumor cell line L1210, the IC_{100} value were 50 μg/ml (for 24 h), 25 μg/ml (for 48 h) and 10 μg/ml (for 72 h). The IC_{50} values was found to be less than 4 μg/ml, a limit put forward by the National Cancer Institute (NCI) for classiffication of he compound as a potential anticancer drug. The cytotoxic concentrations of quinoline induced morphological changes of L1210 cells and the apoptotic DNA fragmentation.

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MODULATOR OF INTRACELLULAR Ca^{2+}, THAPSIGARGIN, INTERFERES WITH *IN VITRO* SECRETION OF CYTOKINES AND NITRIC OXIDE

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Key words: Thapsigargin/TNF-α/IL-10/RANTES/MIP-1α

Interference of thapsigargin (TG), an inhibitor of endoplasmic reticulum Ca^{2+} ATPase, with immune reactivity of murine macrophages was investigated under conditions *in vitro*. The activation of cells with lipopolysaccharide (LPS), interferon-γ (IFN-γ), and with acyclic nucleoside phosphonate N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (N^6-isobutyl-PMEDAP) resulted in enhanced production of cytokines TNF-α, IL-10, chemokines RANTES/CCL5 and MIP-1α/CCL3, as well as in substantially augmented production of nitric oxide (NO) triggered by IFN-γ. The effects were in a dual mode of action influenced by TG (1 μM). While TG upregulated secretion of TNF-α, it inhibited secretion of IL-10 and RANTES. The immune-stimulated secretion of MIP-1α remained virtually unaffected, though TG on its own activated expression of MIP-1α in macrophages. The high-output NO production induced by IFN-γ, high concentrations of LPS, or by combination of IFN-γ plus LPS or N^6-isobutyl-PMEDAP was inhibited by TG. On the other hand, production of NO which was marginally activated by low concentration of LPS was upregulated by TG.

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EFFECT OF A NEW ULTRASHORT BETALYTIC AGENT ON ACONITINE-INDUCED ARRHYTHMIA

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Key words: Rats/Aconitine/Ventricular fibrillations/ 2-Hydroxy-3-(alkylamino)propyl-4-[(alkoxycarbonyl)amino]benzoate

The anti-arrhythmic effect was tested on the model of aconitine-induced arrhythmia. The experiment was performed *in vivo* with 31 male Wistar laboratory rats. Group A was first administered aconitine and, after the onset of the first sinus rhythm disorders, the 44Bu compound was administered. Group B was first administered the 44Bu compound and only after that aconitine. The control group was administered aconitine and saline as a replacement of the tested compound. In the group A, there was a decrease in the ventricular fibrillation occurrence from 100 % to 8 % ($p < 0.001$) after the administration of the 44Bu compound. In the B group, the onsets of all monitored arrhythmia types were delayed by an average of 15.6 minutes. Ventricular rhythm occurrence was decreased from 100 to 20 %, as well as ventricular fibrillations, from 100 to 0 % ($p < 0.001$).

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COMPARISON OF BIOCHEMICAL AND HISTOPATHOLOGICAL RESULTS IN 90 DAYS' TOXICITY IN DOGS TREATED WITH THE 2,4-D HERBICIDE

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The study was performed to investigate the toxicity of 2,4-dichlorophenoxyacetic acid (2,4-D). Groups of dogs of both sexes received 2,4-D in daily doses of 0, 0.5, 5 and 7.5 mg/kg per os during 90 days. Blood samples for hematology and clinical chemistry analysis were taken on days 0, 30, 60 and 90, and in animals of the satellite groups 28 days after the last application of the test substance. The animals were sacrificied and necropsied and principal organs were weigthed and processed for microscopic examination. No death occurred among the dogs in the course of the study. Mean body weights of dosage groups and control dogs of either sex were comparable. After 30 days AST and ALT increased, ALP decreased in all animals of dosage groups versus controls and elevation of urea and creatinine was observed. The trend continued until the end of application of 2,4-D. A dose-dependent response was evident for ALT, creatinine and urea. No dose dependence was observed for the mean levels of AST and ALP. An increase in thyroid relative weight in animals of the 7.5 mg/kg dose group was observed (males +33 %, females +14 % versus control). Focal hepatocellular necrosis, moderate inflammatory reaction in the portal tract, mononuclear nodules, diffuse steatosis were observed predominantly in all treated animals. There were no histopathologic changes observed in the kidney. Occurrence of inflammatory and dystrophic changes in the liver and the results of biochemical evaluation indicate a moderate adverse effect of 2,4-D.

INFLUENCE OF REFRACTORY CERAMIC FIBRES – ASBESTOS SUBSTITUTE – ON THE SELECTED PARAMETERS OF BRONCHOALVEOLAR LAVAGE 6 MONTHS AFTER INTRATRACHEAL INSTILLATION TO W-RATS

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Key words: Refractory ceramic fibers/Subchronic effect/ Bronchoalveolar lavage/Inflammation/Cytotoxicity/ Intratracheal instilation

Industrial fibrous dusts are applied in many industrial branches and represent adverse factors in occupational and enviromental area. Refractory ceramic fibers (RCFs) – amorphous alumina silicates – are used as one kind of asbestos substitutes. Because RCFs are relatively durable and some RCFs are respirable, they may present a potential health hazard by inhalation. The aim of the present work was to find out the subchronic effect of RCFs on selected parameters of bronchoalveolar lavage (BAL) in W-rats, confirm the biopersistence of RCFs after 6 month instillation and contribute to the understanding of the pathomechanism of lung injury after fibrous dust

exposure. Wistar rats were intratracheally instilled with 4 mg/animal of RCFs – exposed group and with 0.4 ml saline solution/animal – control group. Animals were sacrificed after 6 month exposure. Bronchoalveolar lavage (BAL) was performed and selected BAL parameters (mainly inflammatory and cytotoxic) were examined. Following changes were observed after treatment with RCFs: statistically significant increase of proportion of lymphocytes and polymorphonuclears as well as % of immature alveolar macrophages (AM) and phagocytic activity of AM; statistically significant decrease of viability of AM and proportion of AM (from the differential cell count) in comparison with the control group. The results of this study indicated that RCFs even 6 months after intratracheal instillation very significantly changed the majority of examined BAL parameters. The presence of inflammatory and cytotoxic response in lung may signalize beginning or developing disease process.

LUNG CYTOTOXICITY OF COMBINED EXPOSURE TO REFRACTORY CERAMIC FIBRES AND CIGARETTE SMOKE

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Key words: Lungs/Bronchoalveolar lavage/Refractory ceramic fibres/Smoking/Cytotoxicity/ Lysosomal enzymes

Changes in some lung cytotoxic parameters after exposure to refractory ceramic fibres (RCF) or to cigarette smoke (S) and after combined exposure to RCF+S were studied in male Wistar rats in order to evaluate their potential adverse health effects. Four group of rats were treated as follows: 1) intratracheally instilled by saline solution (0.4 ml); 2) intratracheally instilled by 4 mg of RCF; 3) exposed only to S (85 mg of total particulate matter/m^3 air) for two hours daily; 4) exposed to RCF+S. After 6 months the animals were exsanguinated and the bronchoalveolar lavage (BAL) was perfomed. Viability and phagocytic activity of alveolar macrophages (AM), activity of lactate dehydrogenase (LDH) in cell-free BAL fluid (cfBALF), acid phosphatase (ACP) and cathepsin D (CATD) in cfBALF, in BALF cells and in the lung tissue were estimated. Viability of AM was depressed by every type of exposure with RCF+S effect being at least additive. Phagocytic activity of AM increased in the presence of RCF. No significant changes in LDH activity were found. Activities of lysosomal enzymes measured in the lung tissue homogenates were not significantly changed, but those in the cfBALF increased especially after exposure to S with most expressive increase in BALF cells after exposure to S and RCF+S. In the case of CATD the effect of RCF+S was more than additive. The results point out to the presistence of the RCF exposure cytotoxic effects and their amplification by cigarette smoke.

THE EFFECT OF CERAMIC FIBERS ON THE IMMUNE SYSTEM

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Key words: Ceramic fibers/Immune system/ Immunotoxicity/Lymhpocytes/Phagocytosis/ Immunosuppression

Male Sprague-Dawley rats were treated by intratracheal instillation with 1 mg/animal of refractory ceramic fibers. Intratracheal exposure to ceramic fibers led to significant changes of immune response. Results of proliferative activity of spleen lymphocytes showed significantly decreased proliferative activity of T-cells in response to mitogens phytohemagglutinin and concanavalin A in animals given ceramic fibers in comparison with control rats. Similarly, T-dependent B-cell response to pokeweed mitogen was significantly suppressed. Spontaneous proliferative activity of lymphocytes in non-stimulated spleen cell cultures did not differ in exposed and control rats. No significant changes were found among groups in percentage of phagocytic blood polymorphonuclear leukocytes and percentage of cells with respiratory burst.

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EFFECT OF INTRATRACHEAL FIBRES EXPOSURE ON THE RAT LUNG

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Key words: Lung/Bronchoalveolar lavage/Antioxidant status/Stone wool/Glass fibres

The changes in antioxidant status of rat lung after intratracheal instillation of stone-wool and glass fibres were studied. The animals were exposed to 2 or 8 mg of fibres for 4 or 16 weeks, the bronchoalveolar lavage was performed and the activity of superoxide dismutase, glutathione peroxidase and the total amount of glutathione was estimated both in tissue and in cell free fraction of bronchoalveolar lavage and the ascorbic acid in lung tissue. The results showed the higher burden by stone-wool. Most changes were detected in groups exposed to higher dose of fibres for shorter time period, the most sensitive parameter was superoxide dismutase. The lung tissue was studied also by light microscopy and transmission electron microscopy.

THE EFFECT OF ASBESTOS AND STONE-WOOL FIBRES ON SOME CHEMOKINES AND REDOX SYSTEM OF PULMONARY ALVEOLAR MACROPHAGES AND PNEUMOCYTES TYPE II

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Key words: Stone-wool/Alveolar macrophages/ Pneumocytes type II/Antioxidant system/ Chemokines

The *in vivo* effect of stone-wool has been studied in primary cultures of pulmonary alveolar macrophages (AM) and type II pneumocytes (T2) by morphological, biochemical and immunological methods. UICC crocidolite was applied as positive control. Although stone-wool brought about frustrated phagocytosis, it did not induce serious membrane damage, whereas crocidolite gave rise to very severe membrane alterations. Stone-wool significantly reduced the activity of Cu,Zn/superoxide dismutase (SOD) in alveolar macrophages and significantly decreased the activity of γ-glutamyl transpeptidase (GGT) in pneumocytes type II. Crocidolite, on the other hand, decreased the activity of all enzymes (glutathione peroxidase – GSH-Px, glutathione reductase – GSH-Rd) of glutathione metabolism in alveolar macrophages. It decreased the activity of all enzymes in pneumocytes type II except for Cu,Zn/SOD. On exposure to stone-wool, the production of inflammatory proteins, macrophage chemoattractant protein-1 (MCP-1) and macrophage inhibitory protein-1α (MIP-1α) increased in both cultured cells but did not reach the level induced by crocidolite. Although this study provided a useful insight into the toxicity of the stone-wool, we can not draw the conclusions how the intact pulmonary tissue may respond on the exposure to these fibres, exclusively based on the *in vitro* tests.

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RABBIT TESTING FOR BIOEQUIVALENT PREDICTION OF ORALLY ADMINISTRATED XENOBIOTIC (MOLSIDOMINE)

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A selection of suitable experimental species for the interspecific comparison (with a tendency towards human applications) of hardly soluble substances (and other solid peroral medication forms) has been quite a serious question. In that sense, we aimed our work to determine the basic pharmacokinetic parameters of three pharmaceutical charges of molsidomine (pills with controlled release of a' 8 mg) currently under development, compare to a reference drug (Corvaton). Molsiton (medication form of molsidomine pro-drug, the vasodilatation effect of which is induced by biotransformation metabolites) was applied under total anaesthesia (ketamine 60 mg/kg + xylazine 40 mg/kg i.m.) to 'Chinchilla' rabbits intraduodenally after performed laparotomy. Blood samples for the detection of hydrolysed molsidomine metabolite (SIN 1 C) by HPLC (ref.[1]) method were taken from cannulated a. auricularis after 15, 30, 45, 60, 120 and 180 minutes. According to calculated kinetic parameters, the charge closest to Corvaton was the Molsiton, values of which

were: $AUC_{0\text{-}3h}$ = 530 + 227 ng/ml.h (103 % AUC Corvaton), c_{max} = 230 + 90 ng/ml (99 % c_{max} Corvaton), t_{max} = 0.7 + 0.3 h (equal to t_{max} of Corvaton). Despite severe interindividual differences of AUC and c_{max}, the design of this study brought certain reference preclinical predictive validity.

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FLUORESCENT LABELING OF MEMBRANES OF THE HUMAN NEONATAL FIBROBLASTS (HNF-1) CELL LINE

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Key words: cis-Parinaric acid/Fluorescent probe/ Fluorescent labeling/Fibroblasts/Cell membrane/HNF-1 cell line

The aim of the study was to optimize the method of fluorescent labeling of the cell membranes, which is used for the purposes of measurement of the membrane oxidative damage. We used the sensitive fluorescent probe, *cis*-parinaric acid, and human neonatal fibroblasts (HNF-1) cell line in our labeling experiments. The cells were incubated in the phosphate buffered solution containing the probe (c = 5 μmol/l) at 37 °C in dark. The amount of the incorporated probe was determined spectrofluorimetrically in the isopropanol extracts of the cells. Membrane labeling using the suspension culture was shown to be more effective and exact method, than labeling of the cells on the Petri dishes. We observed only slight increase of amount of the incorporated probe with the time within 170 minutes-time period for the suspension method. The efficiency of the probe incorporation showed remarkable dependence on the heat exchange between the incubation system and the cells. The method showed good reproducibility for the time intervals 50, 60 and 70 min and we observed 95–100 % cell viability for the time interval up to 80 minutes. Our results indicate that the suspension cultivation of fibroblasts with the *cis*-parinaric acid within the time interval of 1 hour could be an effective, sensitive and non-toxic method for fluorescent labeling of the cell membrane.

INFLUENCE OF CARVEDILOL ON SUPEROXIDE GENERATION AND ENZYME RELEASE FROM STIMULATED HUMAN NEUTROPHILS

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Key words: Carvedilol/Human neutrophils/Superoxide generation/Myeloperoxidase

Activation of neutrophils induces generation of reactive oxygen species and release of granule enzymes, which not only participate in the bactericidal mechanisms of these cells, but also in possible tissue damage. We studied the effect of carvedilol (CARV) [0.1–100 μmol/l], an antihypertensive and cardiovascular drug with antioxidative properties, on superoxide generation (SO) and myeloperoxidase (MPO) release from isolated human neutrophils stimulated with fMLP, a specific receptor activator, or with PMA, a receptor bypassing stimulus. Unstimulated cells showed neither SO formation nor MPO release after preincubation with drug. CARV decreased fMLP and PMA stimulated MPO release and SO generation dose dependently. The inhibitory effect of CARV may attributed to non-specific action since its effect was not influenced by the type of stimulation. It might inhibit SO generation as well as MPO release either by membrane-operating stimulus (fMLP) or membrane bypassing activator (PMA).

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INFLUENCE OF ANTIOXIDANT EFFECT OF STOBADINE DERIVATIVE IN CONDITION OF KIDNEY ISCHEMIA-REPERFUSION IN A PRE-CLINICAL EXPERIMENT (EFFECT IN PROPHYLAXIS)

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Key words: Stobadine derivative/Ischemia-reperfusion/ Antioxidative enzymes/Laboratory rat

The goal of the study was to monitor the antioxidative effect of stobadine derivative in the conditions of ischemia-reperfusion of laboratory rat kidney tissue. The animals were divided by random selection into 5 groups (n = 10). The treated groups were given stobadine derivative in peroral doses of 5, 10 and 20 mg/kg in 0.5% solution of Avicel once a day; the placebo group was given only the solution of Avicel. The last group was an intact group (without ischemia-reperfusion and without treatment). After conclusion of medication on the 15th day all animals were subject to kidney tissue ischemia (60 min) followed by reperfusion (10 min). All animals were subsequently exsanguined and single identification of superoxide dismutase, glutathione peroxidase, total antioxidative capacity and malondialdehyde level in the blood were determined. Kidneys were recovered for histopathological examination. It was discovered a statistically significant decrease of the superoxide dismutase and statistically significant increase of the glutathione peroxidase catalytic activity in the treated groups compared to the groups of placebo and intact. There was also a statistically highly significant increase of total antioxidative capacity in the treated groups compared to the groups of placebo and intact. A statistically significant decrease of malondialdehyde level was identified in the treated groups compared to the groups of placebo and intact. The results of biochemical examination show a protective antioxidative effect of stobadine derivate. The results of histopathological examination support this assumption.

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INFLUENCE OF ANTIOXIDANT EFFECT OF STOBADINE DERIVATIVE IN CONDITION OF KIDNEY ISCHEMIA-REPERFUSION IN A PRE-CLINICAL EXPERIMENT (EFFECT IN THERAPY)

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Key words: Stobadine derivative/Ischemia-reperfusion/ Antioxidative enzymes/Laboratory rat

The goal of the study was to monitor the antioxidative effect of stobadine derivative in the conditions of ischemia-reperfusion of laboratory rat kidney tissue. 40 animals were subject to kidney tissue ischemia (60 min) followed by reperfusion (10 min). After that, the animals were divided by random selection into 4 groups (n = 10). The treated groups were given stobadine derivative in peroral doses of 5, 10 and 20 mg/kg in 0.5 % solution of Avicel once a day, the placebo group was given only the solution of Avicel. One group (n = 10) was an intact group (without ischemia-reperfusion and without treatment), for comparison. Once a week, selected laboratory parameters were determined in all animals. On the 15th day the animals were exsanguined and organs were recovered for histopathological examination. We discovered a statistically significant changes of the superoxide dismutase and glutathione peroxidase catalytic activity; changes of total antioxidative capacity and malondialdehyde in the treated groups compared to the groups of placebo and intact. Other examined laboratory parameters (creatinine, urea and uric acid in blood; creatinine, urea, total protein in urine; diuresis) exhibit the significant changes too. The results of biochemical examination show a protective antioxidative effect of stobadine derivative. The results of histopathological examination support this assumption.

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PROTECTION BY STOBADINE OF THE INTESTINAL AND VASCULAR TISSUE AGAINST TOXIC INFLUENCE OF REACTIVE OXYGEN SPECIES BY MESENTERIC ISCHAEMIA/REPERFUSION

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Reactive oxygen species (ROS) have been implicated in the injury associated with reperfusion of ischaemic intestine. The objective of this study was to determine the effects of the antioxidant, pyridoindole compound stobadine, on intestinal and vascular injury following mesenteric ischaemia/reperfusion (I/R) in rats. Ischaemia was induced by occlusion of the superior mesenteric artery (SMA) for 60 min, followed by 30 min reperfusion. Gut impairment was characterised, vascular permeability was measured and SMA functional studies were performed. A method of amplified chemiluminescence was used to detect ROS production by SMA specimens.

Following I/R, pronounced intestinal damage was observed, with increased vascular permeability and subsequent haemorrhagic injury of the intestine. Vessel dysfunction was manifested by a decrease of the maximal relaxation response to acetylcholine. Stobadine was found to reduce the extent of small intestine injury and to protect endothelial-dependent relaxation of the mesenteric artery. The chemiluminescence response was suppressed by stobadine more sufficiently than by tempol, a superoxide dismutase agonist. The effect of stobadine was more pronounced also in isometric tension studies of SMA rings.

The observed beneficial effect of stobadine indicates its possible application in the preventive and/or therapeutic approach to I/R induced pathologies.

PROTECTIVE EFFECT OF 2,3-DIHYDROMELATONIN ON ISCHEMIC BRAIN INJURY

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Key words: Locomotor activity/Oxidative stress/ Antioxidant drug/Protein carbonyl groups/ GSSG/GSH

The neuroprotective effect melatonin was demonstrated in numerous *in vivo* studies. A new melatonin derivative (2,3-dihydromelatonin, DHM) with enhanced antioxidant activity was prepared by our team. To examine putative neuroprotective effect of DHM on locomotor hyperactivity and brain content of lactate, malondialdehyde, glutathione, and protein carbonyl groups (PCG) after short-lasting brain ischemia. Brain ischemia (I) was induced by 12 min bilateral carotid occlusion in adult male gerbils (60–70 g b.wt.). DHM (10 mg/kg) was administered i.p. 20 min before the operation, at the beginning of reperfusion (R), and 2 and 6 hours later. Locomotor activity was recorded during the first 24 hours by open field. After 48-hour R, brain lactate, malondialdehyde, and PCG were determined by spectrophotometric methods and oxidized (GSSG) and reduced glutathione (GSH) by microplate reader spectrophotometry. DHM returned hyperactivity induced by I/R almost to control value in an interval of up to 6 hours of R (from 13000 to 5000 cm distance traveled, $p < 0.05$). The increased level of lactate in the hippocampus and cortex reflected sustained perturbance in aerobic metabolism. DHM diminished the increase in the I/R-induced brain PCG by 30.01 % ($p < 0.05$). The GSSG/GSH ratio increased by I/R ($p < 0.05$) was non-significantly reduced by DHM. No significant effect of DHM on brain lactate and MDA changes occurred. The results may indicate a protective effect of DHM against functional injury induced by brain I/R in gerbils along with improvement in some biochemical indicators of oxidative stress in the brain. More detailed biochemical and functional analyses are needed.

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NATURAL SUBSTANCES, DIETARY COMPONENTS

CHEMOPREVENTION WITH NUTRACEUTICALS AND PHYTOCHEMICALS. HOW TO ASSESS THEIR EFFECTS ON HUMAN ORGANISM?

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Key words: Life styl/Diet/Chemoprotective compounds/ Biomarkers/In vitro assays/Clinical trials

One of the imperatives of a modern community is to increase the quality of life and to decrease the health care expenditures for all age groups of the population[1,2]. Dietary supplements (including vitamins and minerals) play an important role in maintaining the good quality of life and in the prevention of some diet-related diseases – cancer, coronary heart diseases and osteoporosis[3,4]. The dietary supplements contain: (i) parts of intact plants (ii) enriched extracts, (iii) fractions of extracts supplemented by e.g. vitamins/minerals and/or (iv) a chemically defined substance – a nutraceutical/phytochemical. Within all dietary supplements that exhibit a demonstrable positive physiological effect, there is a group of substances, nutraceuticals, and/or phytochemicals responsible for that effect. Examples of such phytochemicals are polyphenolic compounds, phytosterols, β-oligofructanes, glykosinolates, ω-3/ω-6 polyunsaturated fatty acids, β-glucans, and others. The biological effect of an extract/fraction is not triggered by one chemically defined and pharmacologically characterized substance acting on a single target (protein, glycoprotein, lipoprotein, DNA) in the cell, but it is caused by tens of substances of the plant affecting in a complex way the metabolic processes. The lecture offers a critical review of: (i) chemoprotective effects of selected natural substances, (ii) biomarkers used to assess the effects of natural substances in *vitro/in vivo*[5] and (iii) evidences from controlled clinical trials/epidemiological studies performed with some nutraceuticals/phytochemicals/dietary supplements.

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IMPORTANCE OF NUTRITION IN THE ETIOLOGY OF HUMAN CANCER

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Key words: Case-Control Epidemiological Studies/ Potential Risk and Protective Factors/Micronutrients/ Inhibition of Carcinogenesis/Chemoprotective Agents

Diet belongs among the most important factors in the etiology of human cancer and is responsible for around one third of all cases. It is presumed that dietary effects are responsible for the large international differences in incidence seen for most cancers.

EPIDEMIOLOGICAL STUDIES

Epidemiological studies performed during the last 20 years support an inverse relationshi p between the individual intake of fruits and vegetables and the risk of cancer[1,2]. Epidemiological studies are usually performed by two ways, i.e. as 1. Case-control studies to assess the associations between serum or plasma micronutrient and antioxidant levels and the risk of cancer or 2. Population-based case-control studies to assess the associations of cancer risk with vegetables, fruits and related micronutrient intake, where the usual dietary habits are assessed by in-person interviews or questionnaires. Epidemiological studies have shown that low plasma levels of antioxidant micronutrients, which are commonly found in fruit and vegetables, are associated with increased risk for cancer,

heart disease, metabolic disorders, and the like[3,4]. Significant increases in blood nutrient levels after active supplementation were observed for beta-carotene, vitamin C, vitamin E, selenium and folate[5]. Ranges measured, after supplementation, often fell into those associated with a reduced risk for disease. Fruits and vegetables provide numerous phytochemicals which, in part, may explain their beneficial effect. More investigation in the identification of the biologically active constituents, in the knowledge of their availability and the mechanism by which they contribute to lower the risk of cancer, will increase the scientific support of a public health policy. Dietary studies are limited in their conclusions because the protection afforded by the consumption of a particular nutrient may be multifactorial, with different components of the food exerting potential chemopreventive effects[6].

POTENTIAL RISK AND PROTECTIVE FACTORS

Despite strenuous attempts in identification, no single causal factor stands out. Obesity, nutrient spars food such as concentrated sugars and refined flour products that contribute to impaired glucose metabolism, low fiber intake, and imbalance of omega 3 and omega 6 fats all contribute to excess cancer risk[7]. Meat protein and fat were reported as potential risk factors, particularly for colon, breast, and prostate cancer, however, meat, including liver, is not only composed of fat and protein but contains also essential nutrients and micronutrients (vitamin A, vitamin B12, folate, selenium, zinc). On balance, meat consists of a few, not clearly defined cancer-promoting factors and a lot of cancer-protecting factors[8]. It was also found that carotenoid cleavage products, including highly reactive aldehydes and epoxides, which are formed during oxidative attacks in the course of antioxidative action may increase oxidative stress by impairing mitochondrial function. The inhibition of mitochondrial respiration, accompanied by changes in mitochondrial membrane and deterioration of the adenine nucleotide translocator may reflect a basic mechanism of increasing the risk of cancer induced by carotenoid cleavage products[9]. Deficiency of micronutrients is a further potential risk factor throughout the world and even marginal deficiency of some of the micronutrients might be involved in the etiology of many of the so-called lifestyle diseases, including cancer. On the other side, there is strong evidence that the intake of vegetables and fruits reduces the risk of cardiovascular disease and is inversely associated with several forms of cancer. The sources of well-known micronutrients, such as minerals and vitamins and also of microconstituents, are contained in the Mediterranean and Japanese diet[10]. Hydrosoluble vitamins (e.g. folic acid and vitamin C), which play a protective role in chronic degenerative diseases, are well represented in these diets, more so than vitamin A, a liposoluble vitamin obtained primarily from animal foods. Vitamin E is important for antioxidant and cellular functions. The Mediterranean diet is also rich in provitamins A, such as alpha- and beta-carotene and beta-cryptoxanthine. Microconstituents, non – nutritional compounds known to protect plants and more recently suggested to have a protective effect in humans, play a role in the antioxidant defense of the organism. Their effect on various enzyme activities appears even more promising and is still under investigation. It is nevertheless difficult to isolate the effect of the numerous biofactors present in the Mediterranean diet from the foods themselves, especially because of the possible synergy between the various biofactors. Abundant micronutrients in a human diet are polyphenols. Evidence for their role in the prevention of degenerative diseases is emerging. The positive health effects of polyphenols depend on the amount consumed and on their bioavailability. The bioavailability of polyphenols depends on intestinal absorption and the influence of chemical structure (eg, glycosylation, esterification, and polymerization), food matrix, and excretion back into the intestinal lumen.

INHIBITION OF CARCINOGENESIS BY CHEMOPREVENTIVE AGENTS

Different micronutrients may prevent cancer development by interfering with detrimental actions of mutagens, carcinogens, and tumor promoters[11,12]. The mechanism of action is related evidently to their abilities to prevent critical carcinogen metabolism and to increase detoxification of carcinogens and tumor promoters, however, only a deeper understanding of the mechanisms by which micronutrients exert their chemopreventive benefits may provide an improved strategy in designing therapeutic regimens. The selection of micronutrients, defined as essential and nonessential dietary components consumed in minute quantities, for testing in clinical chemoprevention trials is based on integrated evidence arising from epidemiologic, *in vitro*, animal, and clinical studies. The goal of current studies in cancer prevention is to determine the mechanisms of synergistic action of the natural source compounds known to inhibit one or more stages of carcinogenesis, that is, initiation and promotion/progression. Those micronutrients that surface with chemopreventive potential, in terms of high efficacy and low toxicity in early-phase clinical studies, are then candidates for large-scale, randomized clinical chemoprevention trials with cancer endpoints.

CONCLUSIONS

With the exception of tobacco consumption, diet is probably the most important factor in the etiology of human cancer. There is strong evidence that the intake of vegetables and fruits is inversely associated with several forms of cancer. The beneficial role of vegetable and fruit consumption can also be inferred by considering the health effects of two dietary patterns, the Mediterranean and Japanese ones, in both of which the consumption of plant foods holds a prominent position. Different vitamins

and other micronutrients in vegetables, fruits, and other natural plant products may prevent cancer development by interfering with detrimental actions of mutagens, carcinogens, and tumor promoters. The mechanism of action is related to their abilities to prevent critical carcinogen metabolism and to increase detoxification of carcinogens and tumor promoters.

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MICROTUBULE DISRUPTORS AND THEIR INTERACTION WITH BIOTRANSFORMATION ENZYMES

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Key words: Antimitotic drugs/Drug metabolism/ Cytochromes P450/Nuclear receptors/Gene expression

Microtubule disruptors, widely known as antimitotics, have broad applications in human medicine, especially as anti-neoplastic agents. They are subject to biotransformation within human body frequently involving cytochromes P450. Therefore antimitotics are potential culprits of drug-drug interactions on the level of activity as well as expression of cytochromes P450. This review discusses the effects of four well-known natural antimitotics: colchicine, taxol (paclitaxel), vincristine, and vinblastine, and a synthetic microtubule disruptor nocodazole on transcriptional activity of glucocorticoid and aryl hydrocarbon receptors. It appears that microtubules disarray restricts the signaling by these two nuclear receptors regardless of cell cycle phase. Consequently, intact microtubules play an important role in the regulation of expression of cytochromes P450, which are under direct or indirect control of the two nuclear receptors.

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ELECTROCHEMICAL BEHAVIOUR OF FLAVONOIDS ON A SURFACE OF A CARBON PASTE ELECTRODE

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Key words: Flavonoids/Antioxidant/Rutin/Quercetin/ Quercitrin/Diosmin/Chrysin/Carbon paste electrode/ Square wave voltammetry

ABSTRACT

Recent papers discuss relation of flavonoid compounds and tumour diseases. In addition it is impossible to say that effect of flavonoids is positive or negative without epidemiological studies. That is why it is necessary to suggest a simple, low cost and sensitive technique for determination of these compounds in biological material. The electrochemical techniques are very suitable for these purposes. Here we suggested the electrochemical technique for the determination of rutin, quercetin, quercitrin, chrysin and diosmin by square wave voltammetry using carbon paste electrode as a working electrode. The limits of detection of the studied flavonoids were about tens of nM, except diosmin (tens of μM).

INTRODUCTION

It is common knowledge that compounds contained in food considerably affect our health. People living in developed country have not usually problems with insufficient food and nutrient intake, contrarywise, they consume more than need[1]. Therefore there is needed to take care of compounds which are present in minor amount in food as well. For example vitamins or mineral elements belong to the minor food component, but there are many other constituents which have both positive and negative affect on health of the consumer. In relation to this, some authors speak about foodstuffs called nutraceutics[2]. Nutraceutics represent foodstuffs that could positively affect physiological functions of a human organism. To be specific, it was proved that they influence general state of health and could significantly support a physiological capacity or decrease a possibility of a disease appearing[3]. Different groups of metabolites such as phenolic acids, lignans, phytosterols, carotenoids, glucosinolates and also flavonoids influence human health as described above[2]. Flavonoids comprise a wide-ranging group of plant phenols. Up to now, more than 4,000 of flavonoid compounds are known and new ones have been still discovering. Flavonoids are derived from heterocyclic oxygen compound, flavan, which is formed by two benzene rings linked by heterocyclic pyran (Fig. 1). All three rings could be substituted by hydroxy- or methoxy-groups and particular derivates differs only in degree of substitution and oxidation. We distinguish following basic structures of flavonoids: catechins, leukoanthocyanidins, flavanons, flavononols, flavons and anthocyanides. Natural flavonoids occur mostly in form of *O*-glycoside. Existence of free aglycones is rare, but it was observed that concentration of free aglycones could increase in specific conditions (during technological processing at high temperatures and also in acidic medium) when hydrolysis of glycosides may proceed[4,5].

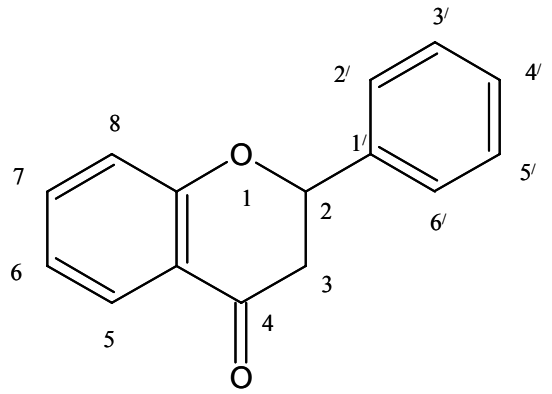

Fig. 1. Flavonoid structure made of two benzene rings linked by heterocyclic pyran.

Antioxidative activity of flavonoids, which means e.g. capability to scavenging of reactive oxygen species or protection of LDL fractions of lipids against their oxidative modification (crucial during atherosclerosis progress), has been studying. Moreover, flavonoids have other effects – anticoagulant, antiestrogenic, antiinflammatory (digestive tract), antimicrobial, or spasmolytic (Fig. 2). In addition opinions of the scientists markedly vary in case of the relation of flavonoids with tumour diseases. Number of authors assign to them anticancer effect which is ascertained in *in vitro* tests and on animal models. On the other hand, this effect has not been proved in epidemiological studies[2,6–8]. The same scientists allege that an anticancer activity of flavonoids could be deduced from theirs chemical structure and that the activity appear in both initiatory and promotional phase of carcinogenesis. In connection with this activity, the following mechanisms are discussed: **i)** inhibition of enzymes of phase I (PIE) encouraging oxidation, **ii)** induction of conjugating and detoxificating enzymes of phase II, **iii)** direct interaction

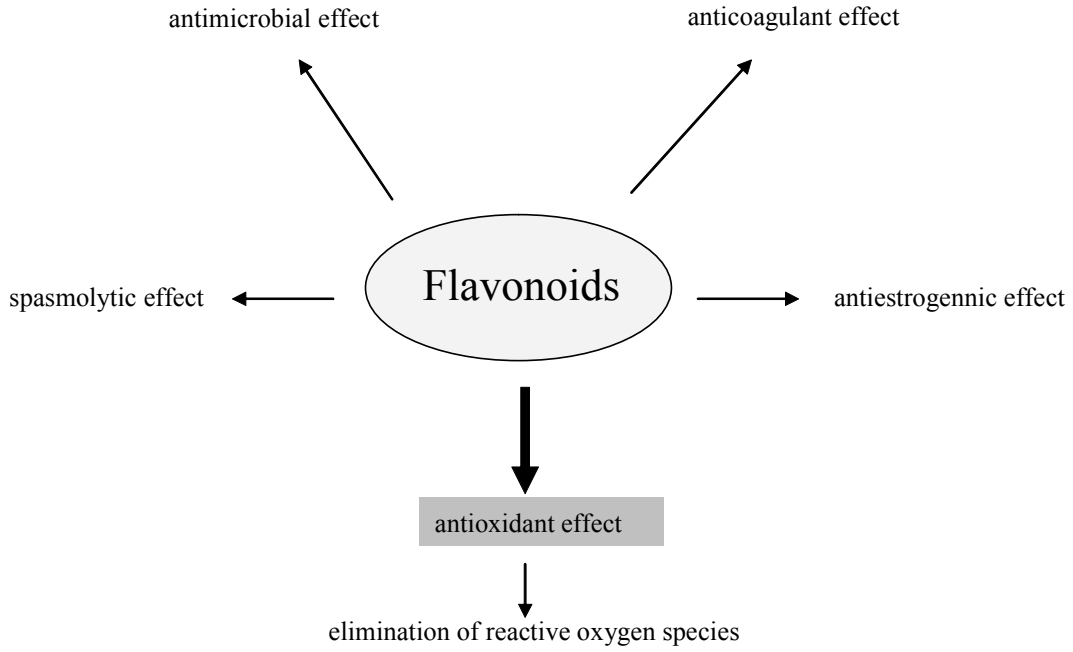

Fig. 2. Scheme of possible effects of flavonoids.

with DNA, **iv)** induction of apoptosis (programmed cell death), **v)** inhibition of cell proliferation, **vi)** antioxidant action, **vii)** modulation of immune system.

On the contrary, there are also authors which are convinced of carcinogen affecting of some flavonoids[9, 10]. They assert that applying of high dose of flavonoids, for example diosmin, on the one hand effectively protects formation of certain adducts with DNA but on the second hand has simultaneously genotoxic influence. It could be taken an exception that as high amount of flavonoid is not able to achieve without food supplements. But it was found out that flavonoids concentration can increase to mmol.l^{-1} due to limited resorption of them in small intestine and water resorption in large intestine[6, 7].

In this work we were concerned with suggestion of electroanalytical technique for determination of quercetin, quercitrin, rutin, diosmin and chrysin on the surface of a carbon paste electrode.

MATERIAL AND METHODS

Chemicals

Methanol, NaH_2PO_4, diosmin, chrysin, quercetin dihydrate, carbon powder, and mineral oil were purchased from Sigma Aldrich Chemical Corp. (St. Louis, MO, USA). Rutin trihydrate and quercitrin dihydrate were from Roth GmbH (Karlsruhe, Germany). Na_2HPO_4 was purchased from Merck (Darmstadt, Germany). Solutions were prepared using ACS water from Sigma Aldrich.

Electrochemical measurements

Electrochemical measurements were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a carbon paste electrode. The reference electrode was an Ag/AgCl/3M KCl electrode and the auxiliary electrode was a graphite electrode. The supporting electrolyte was prepared by mixing buffer components. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed. SWV experiments were performed at room temperature. The measurements were performed using the following parameters: initial potential = 0.1 V, end potential = 1.2 V, pulse amplitude = 49.85 mV, step potential = 1.95 mV, and frequency = 180 Hz. Phosphate buffer (0.1 M NaH_2PO_4 + 0.1 M Na_2HPO_4, pH 7.0) was used as a supporting electrolyte. The carbon paste (about 0.5 g) was made of graphite powder (Aldrich) and mineral oil (Sigma; free of DNase, RNase, and protease). The ratio of the graphite powder and mineral oil was 70/30 (*w*/*w*). This paste was housed in a Teflon body having a 2.5-mm-diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing it with soft filter paper.

RESULTS AND DISCUSSION

Using of an electroanalytical approach for detection of flavonoids is rare[11, 12]. Analytical determination of flavonoids is currently maintained mainly by chromatographic procedures[13–17] and capillary electrophoresis[15, 18, 19]. Electrochemical procedures represent advantageous possibility of miniaturisation and simultaneous analysis *in vivo* without disruption of living organism in comparison with these commonly used methods.

Recently, method for electrochemical determination of quercetin using glassy carbon electrode was published[12]. A renewing of the electrode surface (polishing by alumi-

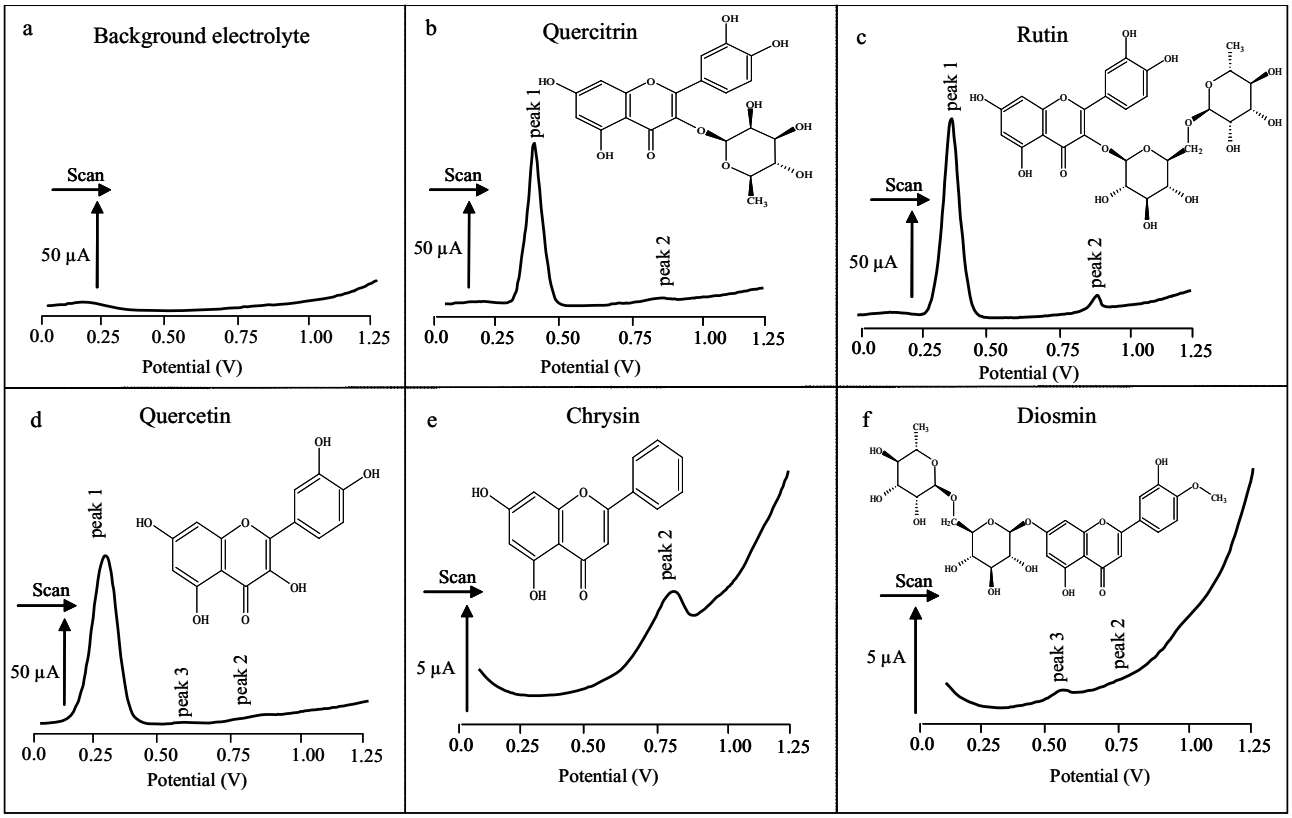

Fig. 3. Square-wave voltammograms of the studied flavonoids at 10 µM concentration – a) background electrolyte, b) quercitrin, c) rutin, d) quercetin, e) chrysin and e) 100 µM of diosmin. The SWV parameters were as follows: initial potential = 0.1 V, end potential = 1.2 V, pulse amplitude = 49.85 mV, step potential = 1.95 mV, and frequency = 180 Hz. Phosphate buffer (0.1 M NaH_2PO_4 + 0.1 M Na_2HPO_4, pH 7.0) was used as a supporting electrolyte. For other details see Materials and Methods section.

na, ultrasound) is the main disadvantage of this approach. Therefore we suggested the method utilising carbon paste electrode (CPE) for determination of quercetin, rutin, quercitrin, chrysin, and diosmin by square wave voltammetry (SWV) in the presence of phosphate buffer. The surface of the CPE is renewed by polishing using filtration paper prior to this purpose, which is much easier in comparison with glassy carbon electrode.

Four oxidative signals were observed on the obtained voltammograms. The signals were called as peak 1, peak 2, peak 3 and peak 4 (Fig. 3b, c, d, e, f). Brett[12] described two of these signals – peak 1 and peak 2 as electrochemical responses of oxidation of hydroxyl group in position 7 (Fig. 1). We assume that two other signals (peak 3 and peak 4) probably correspond to oxidation of hydroxyl group in position 5 according to Janeiro and Brett[20] (Fig. 1). Moreover, the influence of glycoside part of the flavonoids on the resulted electrochemical signal was studied. We observed the peak 1 potential of quercetin at potential of 0.15 V and signals of glycosided quercetin (rutin and quercitrin) at potential 0.25 V (Fig. 3b, c, d). The potential shifting (more than 100 mV to positive potential) could be caused by presence of glycoside part covalently bound on the quercetin (see in Fig. 3). Electrochemical signal of chrysin and diosmin were lower in comparison with signal of quercetin and its glycosylated derivatives (Fig. 3d, e). The observed decrease of electrochemical response is probably caused by different chemical structure of the studied compounds. The highest decrease of peak height was noticed in case of diosmin, which contain two glycoside molecules in its structure. In addition the detection limits (3 S/N) of studied analytes were calculated, see Table 1.

Table. 1. Limits of detection (LOD, 3S/N) of the studied flavonoids

Flavonoids	LOD (nM)
Quercetin	1.04
Quercitrin	0.73
Rutin	0.85
Chrysin	56
Diosmin	2663

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STRUCTURE-ACTIVITY RELATIONSHIP OF *TRANS*-RESVERATROL AND ITS ANALOGUES

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Key words: trans-Resveratrol/Piceatannol/Structure-activity relationship/Polyphenols/Chemopreventive activity/ Antioxidant activity

ABSTRACT

Cancer is one of the main reasons of death in both men and women, claiming over 6 million people each year worldwide. Chemoprevention in combination with anticancer treatment is therefore important to reduce morbidity and mortality. *trans*-Resveratrol is a naturally occurring phytoalexin present in grapes and many other plants. It was found that this compound possesses a variety of biological activities. One of the most striking biological activities of *trans*-resveratrol soundly investigated during the late years has been its cancer-chemopreventive potential. To improve the beneficial effects of *trans*-resveratrol it is necessary to know the structure-activity relationship of *trans*-resveratrol and its analogues. This gives us useful information for further chemopreventive drug design.

trans-Resveratrol (3,4',5-*trans*-trihydroxystilbene; t-RES) is a polyphenolic compound accounting to the stilbene class (Fig. 1). It has been found in high concentrations in a wide variety of plants, including grapes, peanuts, berries, pines and traditional oriental medicine plants[1]. Thus, relatively high concentrations of this compound are present in grape juice and, especially, in red wine[2,3]. In plants t-RES is synthesized in response to stress conditions such as trauma, UV irradiation, exposure to ozone and fungal infection, and thus it can be considered to be a phytoalexin, a class of antibiotics of plant origin[4,5]. t-RES has been reported to be a phytoestrogen due to its structural similarity to the estrogenic agent diethylstilbestrol[6]. In recent years, it has been shown to exhibit estrogenic activity in mammals[7,8]. t-RES has been reported to have both anti-carcinogenic and cardioprotective activities, which could be attributed to its antioxidant and anti-coagulant properties[9,10]. Besides these effects, t-RES has been reported to be effective in inhibiting platelet aggregation and lipid peroxidation, altering eicosanoid synthesis, modulating lipoprotein metabolism[11–13], and exhibiting vasorelaxing and anti-inflammatory activities[3,5]. In different rodent species as well as in humans, t-RES is well absorbed, distributed to various organs, and metabolized to *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate[14–16].

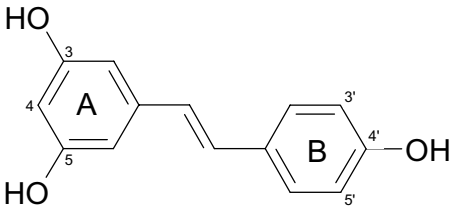

Fig. 1. Structure of *trans*-resveratrol

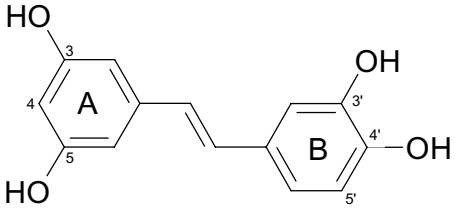

Fig. 2. Structure of piceatannol

The anti-cancer activity of t-RES was first revealed by its ability to reduce incidence of carcinogen-induced development of cancers in experimental animals[17,18]. It has since been demonstrated that it possesses chemopreventive and cytostatic properties via the inhibition of tumor initiation, promotion and progression[17]. It causes cell arrest in the S and G_2 phases of the cell cycle[19] and is capable of inducing differentiation and apoptosis in a multitude of tumor cell lines, such as human leukemia, breast cancer and esophageal cells via CD95-dependent or independent mechanisms or through activation of caspase 3 or cleavage of poly(ADP-ribose) polymerase[20–22]. It has also been demonstrated that t-RES inhibits the ribonucleotide reductase catalyzing the rate limiting step of de novo DNA synthesis[23].

In order to established the influence of the spatial position of the hydroxyl groups on the activity of t-RES, several writers have compared the activity of t-RES with activities of its analogues[10,24–27]. They found that the activity of t-RES depends on the position of the hydroxyl groups. *para*-Hydroxyl group of t-RES dominates in the radical-scavenging efficiency whereas its *meta*-hydroxyl groups show only minor reactivity[10]. t-RES and its analogues with *ortho*-dihydroxyl and/or *para*-hydroxyl functionalities have been shown to possess highest antioxidative activity[25]. This can be understood because the *ortho*-hydroxyl phenoxyl radical, the oxidation intermediate, is more stable due to the intramolecular hydrogen bonding interaction, as evidenced from both experiments[28] and theoretical calculations[29]. Their results suggest, that the 4'-hydroxyl group in *trans*-conformation (hydroxystyryl moiety) is not the sole determinant for antioxidant properties, while it is absolutely required for antiproliferative activity[24].

In contrast to the detailed knowledge of t-RES activities in biological systems much less is known about the effects of higher hydroxylated stilbens. t-RES undergoes cytochrome P450 catalyzed hydroxylation to piceatannol (3,3',4',5-*trans*-tetrahydroxystilbene; PCA) (Fig. 2) and to two other unidentified mono- and dihydroxy-t-RES analogues. PCA differs from t-RES by having an additional hydroxyl group. It demonstrates that a natural dietary cancer preventative agent can be converted to a compound with known anticancer activity by an enzyme that is found in human tumors[30].

PCA has been shown to exert a more than 6600-fold higher anti-radical activity than t-RES and its two other analogues. Furthermore, in HL-60 leukemic cells hydroxystilbens with *ortho*-hydroxyl groups exhibited a more than three-fold higher cytostatic activity compared to hydroxystilbenes with other substitution patterns[31]. These data were supported by other investigators who also found free radical scavenging activity that was several times better, along with a higher growth-inhibitory activity of PCA compared to t-RES in tumor cells[32]. PCA was about 160 fold more potent than t-RES in superoxide anion scavenging. These results showed the possible structural criteria important for the antioxidant activities of these polyphenolic compounds. Deletion of the hydroxyl group at the B-4 of t-RES reduces its antioxidant activity. In contrast, the presence of *ortho*-dihydroxy structure in ring B (PCA) enhanced its activity to inhibit LDL peroxidation and free radical trapping, especially superoxide anion[33].

CONCLUSION

It has been found that the biological activity of t-RES and its analogues depends significantly on the position of the hydroxyl groups. The observation that *trans*-stilbene compounds having 4' hydroxyl group and bearing *ortho*-diphenoxyl or *para*-diphenoxyl functionalities possess remarkably higher chemopreventive activity than t-RES gives us useful information for anti-cancer drug design.

ACKNOWLEDGEMENT

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Full text submitted into Neoplasma

ELECTROCHEMICAL BEHAVIOUR AND DETERMINATION OF ANTI-TUMOUR PROMOTING ACTIVITIES OF FLAVONOIDS

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Key words: DC polarography/Anhydrous conditions/ Anti-tumour promoting activity/Flavonoids/ 7,12-Dimethylbenz(a)anthracene/ 12-O-teradecanoylphorbol-13-acetate

ABSTRACT

This paper deals with determination of anti-tumour promoting activity of 11 flavonoids performed by DC polarography. The anti-tumour promoting activity of flavonoids was studied in the presence of polyaromatic carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) or by combination of DMBA and 12-O-tetradecanoylphorbol-

13-acetate (TPA), known as a specific tumour promoter for an epidermal carcinogenesis.

We found that in this experimental system the promotory or inhibitory activity of studied flavonoids depends on the number and position of hydroxyl groups in the chemical structure and is related to the polarographic behaviour of these compounds.

The acquired results demonstrated that the electrochemical method – DC polarography is very sensitive, simple technique for determining of anti-tumour promoting activities of studied compounds.

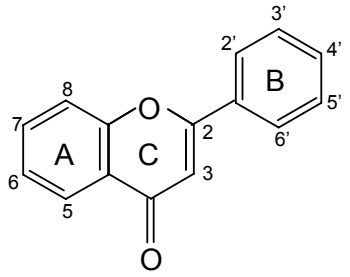
INTRODUCTION

Several polyphenolic compounds are known as cancer chemopreventive agents. In particular, flavonoids are a class of natural polyphenolic compounds, widely distributed in the plant kingdom, that display a variety of biological activities, including tumour growth inhibition and chemoprevention[1].

Structurally diverse flavonoids were reported to inhibit tumourigenesis induced by polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines[2]. DMBA, a more potent skin tumour initiator, binds to a greater extent to epidermal DNA (ref.[3]). Balasubramanian and Govindasamy[4] investigated the inhibitory effect of quercetin on DMBA-induced hamster buccal pouch carcinogenesis. Dietary quercetin inhibited the incidence of both papillomas and tumours induced by DMBA (ref.[4]).

12-O-Tetradecanoylphorbol-13-acetate (TPA) is known to act as a specific promoter of epidermal tumourigenesis iniciated by DMBA, originally detected in oil prepared from seeds of Croton tiglium L. TPA has highly pleiotropic effects on cells in culture and on tissues in vivo[5]. Fisetin and kaempferol potently inhibited TPA-caused epidermal ornitine decarboxylase induction and epidermal lipoxygenase activity in CD-1 mice[6]. Quercetin markedly suppressed the effect of TPA on skin tumour formation in the CD-1 mice initiated by DMBA (ref.[7]).

In the present study we have examined the anti-tumour promoting activity of flavonoids (Structure 1) – galangin (GA), chrysin (CH), baicalein (BA), 7-hydroxyflavone (7-OH-FL), 7,8-dihydroxyflavone (7,8-OH-FL), quercetin (QU), rutin (RU), luteolin (LU), apigenin (AP), fisetin (FI) and kaempferol (KA) performed by electrochemical method DC polarography. The use of DC polarography as a pre-screening method for identifying potential inhibitors of chemical carcinogens and tumour promoters is convenient as compared to the conventional *in vivo* tests[8], which are all expensive and time consuming.



Flavonoids	***3***	***5***	***6***	***7***	***8***	***3'***	***4'***
Baicalein		OH	OH	OH			
Galangin	OH	OH		OH			
Chrysin		OH		OH			
7-Hydroxyflavone				OH			
7,8-Dihydroxyflavone				OH	OH		
Quercetin	OH	OH		OH		OH	OH
Luteolin		OH		OH		OH	OH
Kaempferol	OH	OH		OH			OH
Fisetin	OH			OH		OH	OH
Apigenin		OH		OH			OH
Rutin	Rham*	OH		OH		OH	OH

* Rham – rhamnosyl-glucosyl

Structure 1

MATERIAL AND METODS

Chemicals and reagents

Studied flavonoids and TPA were obtained from Sigma-Aldrich Chemie, Steinheim, Germany. N,N-dimethylformamide (DMF) and DMBA were a commercial origin from Fluka Chemie AG, Switzerland. DMF was additionally purified prior to all electrochemical measurements by double vacuum distillation in a dry nitrogen atmosphere according to Riddick and Bunger[9]. DMF water content did not exceed 0.01 wt %. Terabutylammonium perchlorate (TBAP), used as the supporting electrolyte at a concentration of 0.15 mol l^{-1}, was product of Fluka Chemie AG, Switzerland.

Polarographic conditions

All compounds were investigated using the polarographic analyzer PA4 equipped with the two-line recorder XY 4106 from Laboratorní přístroje, Prague, Czech Republic. Polarographic experiments were performed in a polarographic cell adapted for the work in anhydrous system. As the indicating electrode, a mercury dropping electrode was used with a drop time of 3 s and mercury flow rate of 2.27 mg s^{-1} at a mercury column high 81 cm. As the reference electrode, a saturated calomel electrode (SCE) modified for anhydrous conditions was used. As the auxiliary electrode, a platinum electrode (Radelkis, Budapest, Hungary) was used.

Anti-tumour promoting activity of compounds was determined based on the decrease or increase of current values observed with the chemical carcinogen DMBA ($3.98 .10^{-5}$–$4.94.10^{-4}$ mol l^{-1}) in the absence or presence of the proved tumour promoter TPA ($5.4.10^{-4}$ mol l^{-1}). This activity is expressed in percents as the difference of the I_d values measured for the carcinogen alone or in the presence of tumour promoter and of the I_d values measured after addition of the substance under evaluation.

The concentration of all compounds tested during their polarographic reduction was 5.10^{-4} mol l^{-1}.

RESULTS AND DISCUSSION

Polarographic reduction of studied flavonoids in anhydrous conditions

All the flavonoids investigated are stable in anhydrous DMF at room temperature. The values of their reduction half-wave potentials ($E_{1/2}$) are presented in Table 1. Generally, all flavonoids tested are reduced in anhydrous DMF in the presence of 0.15 mol l^{-1} TBAP on a mercury dropping electrode in two or three well defined steps.

Compounds, which are not hydroxylated at the ring B (GA, CH, BA, 7-OH-FL, 7,8-OH-FL) are reduced in anhydrous DMF on a mercury dropping electrode in three diffuse one-electron steps, the first and the second step being reversible and the third irreversible. QU, LU, FI, KA and AP are reduced in strictly anhydrous DMF on a mercury dropping electrode in two diffuse well defined steps. The first step is one-electron reversible reduction, the second step is one-electron irreversible reduction. The $E_{1/2}$ values of these compounds are demonstred in Table 1.

Table 1. Values of half wave potentials ($E_{1/2}$) of tested flavonoids

Compound	$E_{1/2\,I.}$/V	$E_{1/2\,II.}$/V	$E_{1/2\,III.}$/V
GA	−1.550	−1.950	−2.420
7,8-OH-FL	−1.540	−1.900	−2.340
CH	−1.460	−1.880	−2.400
7-OH-FL	−1.510	−1.920	−2.290
BA	−1.410	−1.810	−2.270
QU	−1.680	–	−2.390
LU	−1.590	–	−2.380
KA	−1.600	–	−2.380
FI	−1.610	–	−2.390
AP	−1.560	–	−2.360
RU	−1.560	–	−2.160

$E_{1/2\,I.}$ – the half wave potential of the first polarographic wave

$E_{1/2\,II.}$ – the half wave potential of the second polarographic wave

$E_{1/2\,III.}$ – the half wave potential of the third polarographic wave

Effects of flavonoids on the activity of DMBA

The goal of our work was to evaluate the effects of studied flavonoids on the activity of DMBA. We found that in this experimental system the promotory or inhibitory activity of studied flavonoids depends on the number and position of hydroxyl groups in the chemical structure and is related to the polarographic behaviour of these compounds.

Flavonoids, which are not hydroxylated at the ring B and are reduced in three one-electron steps (GA, CH, BA, 7-OH-FL, 7,8-OH-FL) have been shown to enhance the activity of DMBA (Fig. 1A, Table 2). The highest promotory activity against DMBA was found for BA (66.21 %). Promotory activity of flavonoids is probably related to hydroxylation of the ring A in the positions 5, 6 and 7. Increase of the number of hydroxyl groups in these positions enhanced the promotory activity of flavonoids against DMBA (Fig. 1A, Table 2).

Flavonoids, which are hydroxylated at the ring B and are reduced in anhydrous DMF on a mercury dropping electrode in two one-electron steps (QU, RU, LU, AP, FI, KA) have been shown to inhibit the activity of DMBA (Fig. 1A, Table 2). Decrease of the number of hydroxyl groups at the rings A and B caused enhancement of inhibitory activity of studied flavonoids.

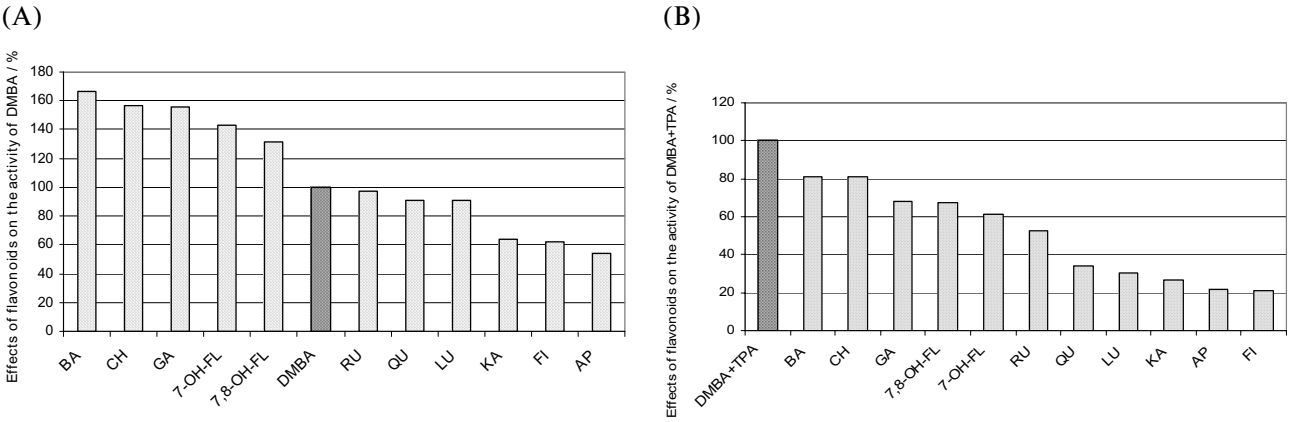


Fig. 1. Determination of anti-tumour promoting effect of tested flavonoids against the chemical carcinogen DMBA (A) and against the combination of tumour promoter TPA and DMBA (B) by the DC polarographic method.

Table 2. Determination of anti-tumour promoting effect of tested flavonoids against the chemical carcinogen DMBA and against the combination of tumour promoter TPA and DMBA by the DC polarographic method.

Flavonoids	Activity DMBA/ %[a]	Activity DMBA+TPA/%[b]
BA	+ 66.21	- 19.01
CH	+ 56.80	- 18.90
GA	+ 55.40	- 31.70
7-OH-FL	+ 43.24	- 38.74
7,8-OH-FL	+ 31.08	- 32.80
RU	- 2.70	- 47.20
QU	- 9.50	- 66.20
LU	- 9.50	- 69.70
KA	- 36.51	- 73.30
FI	- 37.84	- 78.87
AP	- 45.90	- 78.17

[a] Activity DMBA is expressed in percents for the figure (A).

[b] Activity DMBA+TPA is expressed in percents for the figure (B).

Effects of flavonoids on the simultaneous reduction of DMBA and TPA

The polarographic behaviour of TPA which is a specific promoter of epidermal tumourigenesis iniciated by DMBA, has been studied by several writers[10].

It has been shown that in experimens with DMBA+TPA all investigated flavonoids have inhibitory activity. The value of inhibitory activity depends on the number and position of hydroxyl groups at the ring B.

Similary to experiments with DMBA flavonoids QU, RU, LU, AP, FI and KA, which are hydroxylated at the ring B, have been shown to have high inhibitory activity against DMBA+TPA (Fig.1B, Table 2). Another important factor was the number of hydroxyl groups at the rings A and B. FI, KA and AP have been shown to be the most potent compounds (Fig.1B, Table 2).

7-FL, 7,8-FL, GA, BA and CH, which are not hydroxylated at the ring B have been shown to inhibit the activity of DMBA+TPA (Fig.1B, Table 2). Their inhibitory activity is much more lower than of flavonoids, which possess hydroxyl groups at the ring B (Fig.1B, Table 2).

CONCLUSION

The presented data demonstrated that electrochemical method - DC polarography is very sensitive, simple technique for determining of anti-tumour promoting activities of studied compounds. Use of electrochemical methods could therefore contribute much to our understanding of anticancer agents. The obtained results are correlated to their structural arrangement and organization of the hydroxyl groups. Flavonoids might thus warrant further study as sources for possible cancer chemopreventive agents.

ACKNOWLEDGEMENT

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THE EFFECT OF QUERCETIN AND GALANGIN ON GLUTATHIONE REDUCTASE

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Quercetin and galangin can change the activity of glutathione reductase. Quercetin (a catechol structure in the B-ring) and galangin (any hydroxyl group in the B-ring) have different biological activities but, both possess high antioxidant abilities. Quercetin during the antioxidative action, is converted into an oxidized products (*o*-semiquinone and *o*-quinone), and subsequently glutathionyl adducts may be formed or SH-enzyme can be inhibited. We have tried to see whether inhibition of glutathione reductase (GR) can be influenced by preincubation of enzyme with NADPH (a creation of reduced form of enzyme, GRH_2) and whether diaphorase activity of the enzyme is decreased by these flavonoids. The results confirmed that quercetin inhibits GRH_2 and inhibition is reduced by addition of EDTA or N-acetylcysteine. Both of flavonoids have no effect on diaphorase activity of glutathione reductase and this enzyme could increase the production of free radicals by catalysis of reduction of *o*-quinone during action of quercetin *in vivo*.

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INVOLVEMENT OF GLUTATHIONE IN THE CYTOTOXICITY OF 9-ISOTHIOCYANATOACRIDINE

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Isothiocyanates (ITCs) are phytochemicals with promising cancer-preventive potential. To elucidate the mechanism of cytotoxicity of ITCs, their accumulation by cells and the role of intracellular glutathione, fluorescent 9-isothiocyanatoacridine (AcITC) was synthesized. The kinetic parameters for the reactions of AcITC with thiols were estimated and the influence of AcITC on human chronic myeloid leukemia cell line (K562) in regard to intracellular glutathione was studied. Cytotoxicity was evaluated by MTT assay, IC_{50}= 29.2 ± 2.5 μM (48 h incubation). This acridine derivative was able to induce apoptosis of cells (morphological changes of cells and DNA fragmentation were observed) at least within certain dose that only decreased the level of intracellular glutathione, excessive doses (completely depleted intracellular pool of glutathione) induced necrosis rather than apoptosis. Our results indicated that apoptosis of leukemia cells induced by ITC is possible only if intracellular glutathione is not entirely depleted.

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ANTIPROLIFERATIVE ACTIVITY OF BERBERINE *IN VITRO* AND *IN VIVO*

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Key words: Berberine/B16 cells C57BL/6 mice/ Antiproliferative activity/In vitro and in vivo interaction

Berberine, an isoquinoline plant alkaloid acted cytotoxically *in vitro* on tumour cell lines B16. Its anticancer activity *in vivo* was studied in mice with the transplanted B16 line in the range of doses from 1 mg/kg to 10 mg/kg. The significant reduction of tumor volume was observed on day 16 at doses of 5 and 10 mg/kg. The dose of 1 mg/kg stimulated the tumor mass, but other tested concentration, 5 and 10 mg/kg, reduced the tumor weight.

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REDUCTION OF IMINIUM BOND – THE FIRST STEP OF SANGUINARINE TRANSFORMATION IN RAT

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Key words: Quaternary benzo[c]phenanthridine alkaloid/Rat/Hepatocyte/Metabolism/LC-ESI-MS/ Dihydrosanguinarine

Sanguinarine (SA) is a quaternary benzo[c]phenanthridine alkaloid (QBA) that has been found in Papaveraceae, Fumariaceae, Ranunculaceae and Rutaceae families. Cellular studies have shown that SA affects nonspecifically a great number of molecular targets at the level of the membrane, organelles, and in the nucleus[1]. Studies on living organisms have demonstrated that SA has therapeutically interesting anti-inflammatory and antimicrobial effects. SA at physiological pH interconverts between the cationic (iminium) and neutral (6-hydroxydihydrosanguinarine or pseudobase) forms. *In vitro*, the iminium bond in SA is susceptible to nucleophilic addition (of mainly SH-compounds) and is reduced by NADH/NADPH[1,2]. A fraction of QBA, containing SA and its congener chelerythrine, is an active component in oral hygiene preparations and in additives for farm animal feed[1]. On the other hand, SA is considered to be linked to the formation of premalignant lesions on the oral mucous membrane[3] and to the intoxication by the oil from *Argemone mexicana* seeds – the "epidemic dropsy" syndrome[4]. It is surprising that SA metabolism in animals has been discussed in two papers only, both of them giving benz[c]acridine as a SA metabolite[5,6]. Despite obvious absurdity of the suggested rearrangement of the benzo[c]phenanthridine skeleton into the benz[c]acridine structure, this deduction has been quoted in the literature ever since.

This study deals with the transformation of orally administered SA in rat. Sanguinarine was isolated from *Macleaya cordata* (Papaveraceae). Dihydrosanguinarine (DHSA) was prepared from SA by reaction with $NaBH_4$. Male Wistar rats (n = 4) were administered by gavage, after 10 h fasting, a single oral dose of SA (10 mg SA/kg body weight) in 1.0 ml water. Urine was collected. Animals were terminally narcotized (*i. m.* anesthesia by fentanyl (4 μg/100 g b.w.), medetomidin (20 μg/100 g b.w.) and diazepam (0.5 mg/100 g b.w.) after 3 h. After opening the abdominal cavity, a macroscopic examination of the main organs, GI-tract, heart, kidney, liver, lungs, was performed. Urine was diluted 1 : 1 withy mobile phase, vortexed vigorously for 1 min, sonicated in an ultrasonic bath for 2 min, and centrifuged at 2500 g for 1 min at room temperature. Supernatant (50 μl) was applied onto an HPLC column. The blood sample was collected from the aortic bifurcation into a tube containing Na_2EDTA (1 mg/ml) and NaN_3 (0.1 mg/ml). The blood sample was centrifuged at 4 °C and 2500g for 10 min to obtain the plasma. The plasma aliquots and the phosphate buffer-washed liver were stored for the determination of the alkaloid content at –80 °C. For LC-MS analysis, standard stock solutions of SA and DHSA in methanol or plasma specimens (0.5 ml) were diluted by 1 ml of 0.01 M 1-heptanesulfonic acid in water, added to the conditioned (4 ml of methanol, 2 ml of water, and 1 ml of 0.01 M 1-heptanesulfonic acid in water) C_{18} SPE cartridges, washed by 2 ml of 0.01 M 1-heptanesulfonic acid in water, and QBA were eluted by 2 ml of 0.01 M 1-heptanesulfonic acid in 95% methanol. After evaporation of the eluate, dissolution in the mobile phase and filtration through a 0.45 μm filter, the samples (50 μl) were applied onto an HPLC column. Liver (1 g) was homogenized with a mechanical homogenizer in 4 ml of a 0.01 M 1-heptanesulfonic acid in 95% acetonitrile. The homogenate was vortexed vigorously for 1 min, sonicated in an ultrasonic bath for 2 min, and centrifuged at room temperature and 2500g for 1 min. The supernatant (3 ml) was evaporated under N_2 at 50 °C and dissolved in methanol. For a separation and identification of metabolites, a liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) method had to be optimized. The best separation of SA and its biotransformation products was achieved within 20 min in a linear gradient mode elution (0.01 M ammonium formate/methanol) employing a CN reversed phase column. A sensitive and selective detection was possible due to the SIM of molecular and/or main fragment ions based on specific collision-induced dissociation (CID). The electrospray spectra were acquired for SA and DHSA. The full scan mass spectra of SA and DHSA showed an abundant $[M+H]^+$ ions at *m/z* 332 and 334 for SA and DHSA, respectively. A higher fragmentation voltage (100–200 V) at the skimmer was used to increase the fragmentation of the original protonated molecules. In these experiments, *m/z* 332→274 for SA and *m/z* 334→318 for DHSA were used as qualifier ions.

In the plasma and the liver, DHSA was unambiguously identified as a SA metabolite and determined. SA and DHSA were not detected in the urine. In both the plasma and the liver we found a significantly higher level of DHSA in comparison with that of SA. In a parallel experiment, we evidenced the conversion of SA into DHSA in primary cultures of rat hepatocytes after their 3 h incubation with SA. Benz[c]acridine was found neither in urine nor in plasma and liver. The formation of DHSA might be the first step of SA detoxification in the organism and its subsequent elimination in phase II reactions. The reduction of SA to DHSA may be started in the

GI-tract by intestinal reductases[7]. After the absorption of both alkaloids SA can be further reduced by nonspecific cytosolic and microsomal reductases. Unlike in plants, where oxidation of DHSA is elicited by a defense reaction triggered by an external stimulus (the strong antimicrobial activity of SA points to a function as a defense compound and/or a phytoalexin) (ref.[8]), the reduction of iminium bond is a detoxification reaction for mammalian species. In contrast to SA, DHSA displayed *in vitro* low biological activities[9]. To date, we have putatively identified SA metabolites in rat liver as 7,8-demethylene-DHSA and/or *N*-demethyl-7,8-demethylene-DHSA conjugates with sulfuric/glucuronic acid. Their definitive identification as well as the identification of the CYP 450 isoforms involved in *O*-demethylenation at C_7 and C_8 and/or *N*-demethylation as possible metabolic pathways of DHSA is the subject of our further research. Although the structures of the conjugates have not been fully elucidated, it has been evidenced that they are formed from their precursor dihydrosanguinarine, which is the first metabolite in sanguinarine transformation *in vivo/in vitro*.

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ANTIPROLIFERATIVE ACTIVITIES OF SELECTED BENZO[c]PHENANTHRIDINE ALKALOIDS

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Quaternary benzo[c]phenanthridine alkaloids (QBA) fall into a group of isochinoline alkaloids that have phenylalanin as their precursor. They are isolated from many plant species for instance from Papaveraceae, Fumariaceae and Rutaceae families. Sanguinarin and chelerytrin are the two best known QBA and their biological activities have been widely studied[1–3]. In addition to them about 10 other QBA differing only by substitution on the aromatic rings have been isolated as minor components in several species. The informations about biological effects of this minor QBA are rare.

In this study the biological effect of sanguinarine (SA), chelerythrine (CHE) and their congeners sanguirubine (SR), chelirubine (CHR) and macarpine (MA) were tested. One normal (skin fibroblasts) and 3 tumour cell lines (HeLa; A 431; HL-60) were used as a model object. Cytotoxicity (IC_{50}) and apoptosis on living cells were studied. After 72 hours, MTT assay was performed to evaluate the *in vitro* cytotoxicity induced by tested alkaloids at concentration 0.01-5μg/ml. Promyelocytic leukemia cell line HL-60 has been proved the most sensitive to the alkaloid treatment followed by skin fibroblasts, while the carcinoma cell lines HeLa and A-431 appeared much more resistant. Cytotoxicity of individual alkaloids descended (the value of IC_{50} increased) in the order CHE > MA > SA > SR > CHR. For apoptosis detection morphology of nuclei of alkaloid treated HL-60 cells after DAPI staining was studied and flow cytometric analysis using Annexin V-FITC and PI was applied. SA, CHE, MA and SR appeared to be inductors of apoptosis.

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MECHANISM OF ANTI-INFLAMMATORY ACTION OF LICORICE EXTRACTS AND THEIR MAJOR CONSTITUENT, GLYCYRRHIZIN

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Key words: Licorice/Glycyrrhiza glabra/Glycyrrhizine/ DPPH/Liposomes/Neutrophils/ Reactive oxygen species

The antiradical, antioxidant activity and inhibitory effect on generation of reactive oxygen species (ROS) by whole blood of two extracts isolated from *Glycyrrhiza glabra* and its major constituent, glycyrrhizin, were compared. Both licorice extracts showed significant activity in all the systems used in a dose dependent manner. They exerted remarkable reactivity with stable α,α'-diphenyl-β-picrylhydrazyl (DPPH) radical, inhibitory efficacy in oxidatively damaged unilamellar dioleoylphosphatidylcholine (DOPC) liposomes induced by 2,2'-azobis-(2-amidinopro pane)hydrochloride (AAPH) azoiniciator and inhibition of chemiluminiscence of ROS generated by whole blood induced by both receptor-bypassing stimuli (PMA) and receptor-operating stimuli (OpZ) in the ranking order of stimuli PMA > OpZ. These activities well correlated with the content of polyphenols and flavonoids determined in the extracts tested. On the other hand, triterpenoic derivative, glycyrrhizin, showed no efficiency in the system of reaction with free stable DPPH radical, ·OH radical determined by EPR spectroscopy and the system of peroxidation of DOPC liposomes. However, this compound slightly decreased luminol-enhanced chemiluminiscence of whole blood-generated ROS. The present study indicates that anti-inflammatory efficiency of licorice extracts could be predominantly ensured by their polyphenolic constituents, which are able to reduce reactive oxygen species as the most potent inflammatory mediators at the site of inflammation. However, the anti-inflammatory action of glycyrrhizin is most probably due to ability to decrease the generation of ROS by neutrophils.

ANTIPROLIFERATIVE ACTIVITY, CELL CYCLE ANALYSIS AND INDUCTION OF APOPTOSIS BY ROSEMARY EXTRACT IN MURINE LEUKEMIA CELL LINE L1210

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Key words: Rosemary extract/Leukemia L1210 cells/ Cytotoxicity/Cell cycle/Apoptosis

Rosemary is a widely distributed compound in various Labiatae herbs. It contains flavonoids, phenols, volatile oil and terpenoids. Rosemary is stated to act as a biological active agents. It was reported that an extract of rosemary leaves contained high antioxidant activity, anticancer and chemopreventive effects, and inhibited the process of carcinogenesis. Rosemary is commonly used as a spice and flavoring agent in food processing. In this study, we evaluated cytotoxic/antiproliferative activity of rosemary extract using murine leukemia cell line L1210. The cell cycle analysis and the ability of this extract to induce apoptosis were studied, too. Rosemary extract acted cytotoxically on tumor cell line L1210, the IC_{100} values were 1 μg/ml (for 24 h) and 10 μg/ml (for 48 h and 72 h). The IC_{50} values were found to be less than 4 μg/ml, a limit put forward by the National Cancer Institute (NCI) for classiffication of he compound as a potential anticancer drug. After 24 h of influence, rosemary extract concentrations of 50 and 10 μg/ml induced morphological changes of L1210 cells and the apoptotic DNA fragmentation.

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CYTOTOXICITY OF WATER EXTRACTS FROM LEAVES AND BRANCHES OF *PHILADELPHUS CORONARIUS* L

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Key words: Philadelphus coronarius L/Plant water extracts/A431 cells/MCF-7 cells/MTT cytotoxicity

Philadelphus coronarius L. is big, leggy and deciduous old-fashioned shrub known for its fragrant white flowers in the late spring. Some members of genus *Philadelphus* L. are known for their antibacterial, antiradical and immunomodulatory effects. Therefore, these herbs represent prospective sources for the isolation of active substances with desired effects.

We have investigated the cytotoxicity effects of water extracts from leaves and branches of *Philadelphus coronarius* L.(Hydrangeaceae). A431 cells (human skin carcinoma cell line) and the human breast adenocarcinoma cell line (MCF-7) were treated with various doses of individual extracts (0.1–100 μg dry matter/ml) for 24h and 72 h. MTT cytotoxicity assay (cell proliferation test) measures metabolic activity of the cells, e.g. active mitochondrial dehydrogenases of living cells. The results expressed as EC_{50} values display the dose and time dependent cytotoxic effect of analyzed samples.

The highest toxic effects of both plant parts extracts were observed on MCF-7 cells regardless the time of treatment.Cells A431 were less sensitive to toxic effects of leaves and branches extracts but the time dependence was present with the tendency of increased toxicity after chronic treatment. There were no differences in the extend of toxic effects between branches and leaves extracts. The results obtained so far will provide the basis for the future studies with isolated active substances from these extracts.

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MODULATION OF NICOTINIC RECEPTORS BY ANTICHOLINESTERASES PHYSOSTIGMINE AND GALANTHAMINE

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Physostigmine (Phy) and galanthamine (Gal) are known as reversible inhibitors of acetylcholinesterase. Besides, the different direct effects of these drugs were also observed on the nicotinic receptor. We studied the direct influence of these drugs on mouse muscle nicotinic acetylcholine receptor expressed in COS–7 cells, by whole-cell patch-clamp method.

Currents were recorded in the whole-cell mode 3-5 days after cell transfection by plasmids coding αβγδ combination of receptor subunits. As expected, transfected cells responded to application of acetylcholine (ACh) by desensitizing inward currents. However, no reliable specific whole-cell membrane responses could be induced by Phy and Gal when applied up to 1 mM. Phy (1.10^{-6}–1.10^{-3} M) and Gal (1.10^{-5}–1.10^{-3} M) decrease response amplitude and accelerate receptor desensitization when applied together with ACh. Combined with ACh, they also increase the final level of desensitization in concentration dependent manner. These findings on COS-7 cells are in contrast to the suppression and slowing down of desensitization by Phy and 1-methyl-galanthamine observed in *Torpedo* and PC 12 nicotinic receptors[1]. The effect of Phy on ACh response amplitude is partially voltage dependent. These drugs apparently bind to different binding sites other than nicotinic agonists and competitive antagonists. Our results also indicate that the blockade of ionic channel in open state could take place beside allosteric modulation.

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FORENSIC AND APPLIED TOXICOLOGY

HAIR ANALYSIS FOR DRUGS OF ABUSE. PLAUSIBILITY OF INTERPRETATION

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Key words: Hair/Drugs of abuse/Toxicology/Detection

Over more than 20 years hair analysis for drugs has been gaining increasing attention and recognition in various toxicological fields as preemployment and employment screening, forensic sciences, doping control of banned substances, clinical diagnostics in health problems. Hair analysis for drugs can expand the toxicological examination of conventional materials and thus contribute with additional important information to the complex evaluation of a certain case. Hair is a unique material for the retrospective investigation of chronic drug consumption, intentional or unintentional chronic poisoning in criminal cases, gestational drug exposure or environmental exposure to pollutants and adulterants and with specific ultrasensitive procedures allow to demonstrate even a previous single dose administration in a very low amount. Assuming the ideal hair steady and uniform growth, segmental hair analysis can provide the information about the time course of the substance use or exposure. However, the physiological background of hair growth, mechanisms of drug incorporation are not simple, not yet understood in full details and need not be evaluated exactly in all cases. The hair sampling, storage, sample preparation, analytical performance themselves are also very important for final results. Different laboratory attitudes can produce different results. The full information on circumstances of the case examined must be taken into account during interpretation. The pitfalls in hair analysis should be known and avoided to assure the responsible and correct interpretation of laboratory results adequate to an individual case.

POSSIBILITIES AND PROBLEMS WITH IDENTIFICATION AND DETERMINATION OF "NEW" HYPNOTICS

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Key words: Hypnotics/Flunitrazepam/Zolpidem/SPE/ TLC/GC-MS/GC-ECD/Identification/ Determination/ Forensic toxicology

Authors discuss problems with identification and determination of flunitrazepam and zolpidem in biological material. Over the recent years, these two structurally different substances have become the most frequently used as well as abused hypnotic drugs. This study presents applicability of immunochemical methods in the screening of flunitrazepam, one of the most commonly prescribed drugs among the benzodiazepines. Herein described techniques, a liquid-liquid (L-L) extraction, solid phase extraction (SPE) and the so-called "freeze out" method are used for isolation of the above mentioned compounds from biological material. Besides the thin layer chromatography (TLC) and gas chromatography – mass spectrometry (GC-MS) applied in qualitative analysis, the study also describes a gas chromatography with electron capture detector (GC-ECD) and gas chromatography with nitrogen phosphorus detector (GC-NPD) optimized for the determination of flunitrazepam and zolpidem in blood (serum). Successful analyses of these two substances are of major importance, especially in interpreting the results of forensic toxicological examinations.

TOXICOLOGICAL ANALYSIS OF TIANEPTINE BY LC-MS

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Key words: Tianeptine/LC-MS analysis/Pretreatment column/Antidepressant/Toxicological analysis

ABSTRACT

Tianeptine is a widely used antidepressant. Tianeptine is relatively safe, but hight doses and combination with other drugs can cause serious problems when taken in

overdose situations. In this case a toxicological investigation of tianeptine in biological materials must be provided. A LC-MS method with using pretreatment column was optimized for determination of tianeptine and its metabolites in urine and serum. In this method, a Simpack MAYI-ODS column as a pretreatment column, and a Gemini C18 110, 150 × 2 mm as an analytical column were used. This method was applied to the determination of tianeptine in serum and urine samples obtained after a single oral dose of tianeptine and after a lethal overdose with mixture of drugs including tianeptine.

INTRODUCTION

Tianeptine is a dibenzothiazepine tricyclic antidepressant agent. In fact, tianeptine, in contrast to typical tricyclic antidepressants or selective serotonin uptake inhibitors (SSRI), stimulates presynaptic serotonin uptake in the brain and platelets after short and long term administration. On the other hand, tianeptine does not directly affect the uptake or release of dopamine. It appears to have a more favourable tolerability profile than amitriptyline in terms of anticholinergic and cardiovascular averse effects.

Pharmacokinetic studies showed that tianeptine is mainly metabolised by the extrarenal route. β-Oxidation is the major metabolic pathway and the pentanoic and propionic acid side-chain derivatives are the major metabolites in urine and plasma, further displaying therapeutic activity. Tianeptine is relatively safe, but hight doses and/or combination with other drugs it can cause serious problems when taken in overdose situations. In this cases a toxicological investigation of tianeptine in biological materials must be provided. LCMS method for determination of tianeptine in urine and quantification in serum is reported.

MATERIALS AND METHODS

Biological specimens were obtained from patients with tianeptin therapy (urines), from a volunteer after using 25 mg of tianeptine (urine and blood two hours after using) and from one fatal overdose case (stomach content, blood).

Urine samples (2–5 ml) were extracted with diethylether at pH 3–4, evaporated under nitrogen streem and reconstituted in mobile phase. Serum was precipitated with acetonitrile (1 : 1) and centrifuged. Supernatant was used for analysis by LC-MS. The calibration samples were prepared from negative blood serum spiked with tianeptine to concentrations 50, 75, 125 and 250 ng/ml.

LC-MS was performed on Shimadzu system consisting of binary pumps LC-10AC, degaser DGU-14A, autosampler SIL-HT, column oven CTO-10AC and MS-2010A detector, APCI. The pretreatment column, 10 × 4.6 mm (i.d.) Sim-pack MAYI-ODS (50 μm particle size), was from Shimadzu. The analytical column, Gemini 5 μm C18 110A, 150 × 2 mm(i.d.), was from Phenomenex. An injection volume of 10 μl was used in all samples. The mobile phase for pretreatment column: water-acetonitile (90 : 10) acidified with acetic acid (0.2 ml to 100 ml of solution), the mobile phase for isocratic analysis: 65 % of 0,01 M ammonium acetate with 0.1 % of formic acid and 35 % of acetonitrile. Total flow was 0.55 ml/min, pretreatment column flow was 35 % from total flow. The programm of switching pretreatment and analytical columns: 0.01–0.3 min – sample goes on the pretratment column; 0.31–30 min. – removal analytes from the pretreatment column and go to the analytical column.

MS conditions: the MS (with APCI interface) operated in the positive mode; interface temperature was 400 °C, CDL temperature was 250 °C, heat block temperature was 200 °C, nebulizing gas (nitrogen) flow was 2.5 l/min, drying gas flow was 10 l/min.

RESULTS

The structure of tianeptine is shown in Fig. 1. Tianeptine gives ions of M/Z = 436 (a molecular ion) and M/Z = 291 as a major fragment (this fragment conforms to tricyclic part of a molecule of tianeptine). Peak of tianeptine is symetric and in this system no interference of other molecules could be detected. The calibration curve of tianeptine in the range 50 to 250 ng/ml was created. An external calibration was used. The limit of detection for tianeptine is about 5 ng/ml, the limit of quantification is about 10 ng/ml.

Urine was analyzed both without extraction (direct injection of urine) and after extraction from acidic milieu with diethylether. We obtained better results after extraction procedures.

Analysis of urine samples are shown in Fig. 2. We detected a modicum of parent form of tianeptine (RT about 3.5 min), pentanoic acid side-chain derivate (RT about 2.8 min) with M/Z = 408 and 291, propionic acid side-chain derivate (RT about 2.9 min) with M/Z = 380 and 291 and a major metabolite with M/Z = 291 with relatively long retention time (about 23 min). It could be a dealkylated metabolite of tianeptine (Fig. 3).

Qualitative analysis of serum (2 hours after application of 25 mg of tianeptine) identified tianeptine and one

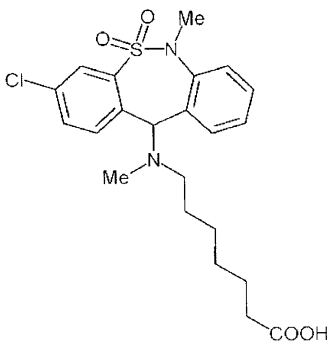

Fig. 1. The structure of tianeptine

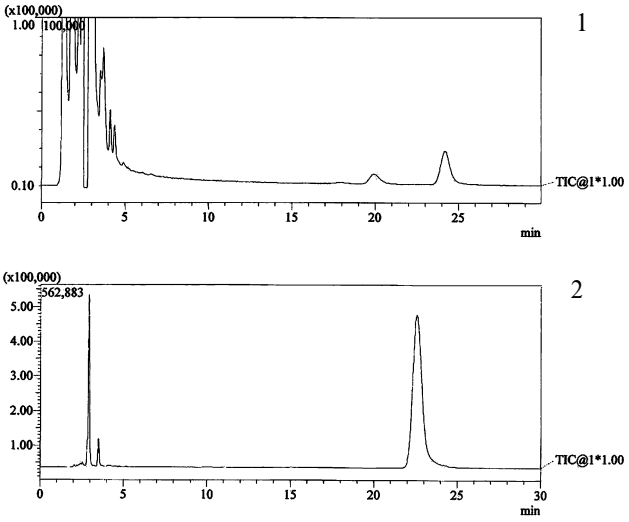

Fig. 2. The analysis of urine
1 – direct analysis of urine (without extraction)
2 – analysis of urine after extraction with diethyl-ether

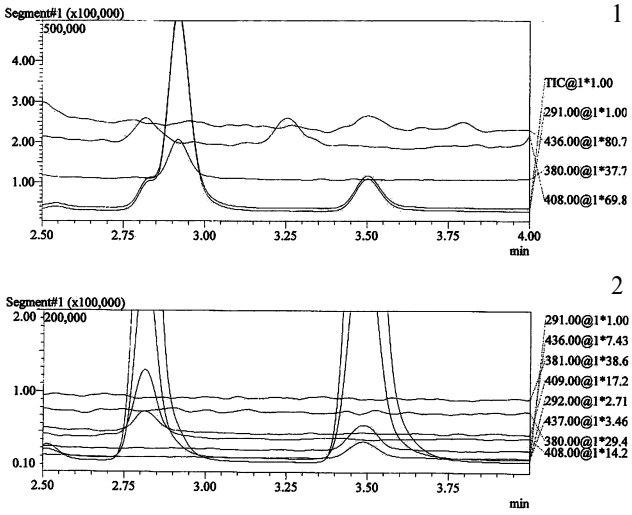

Fig. 3. Tianeptin and its metabolites
1 – analysis of urine: parent form of tianeptine (RT about 3.5 min), pentanoic acid side-chain derivate (RT about 2.8 min) with M/Z = 408 and 291, propionic acid side-chain derivate (RT about 2.9 min) with M/Z = 380 and 291
2 – analysis of serum: parent form of tianeptine (RT about 3.5 min), pentanoic acid side-chain derivative with M/Z = 408 and 291 (RT about 2.8 min)

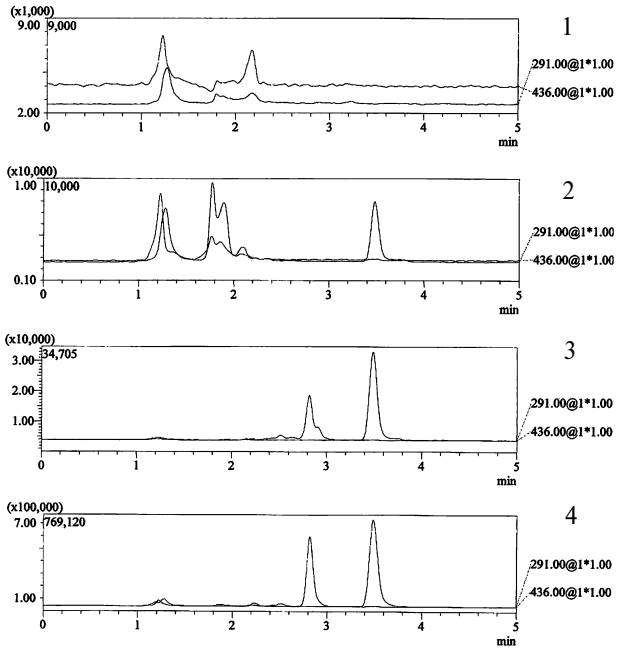

Fig. 4. The analysis of serum
1 – negative serum
2 – spiked serum with 75 ng/ml of tianeptine
3 – serum 2 hours after application of 25 mg of tianeptine
4 – serum of lethal overdosed patient

of the major metabolites of the parent form (pentanoic acid side-chain derivative) with M/Z = 408 and 291 (RT about 2.8 min). The concentration of tianeptine in serum was 595 ng/ml (Fig. 3). Analysis in serum obtained during autopsy from a 32-year-old man who overdosed with mixture of drugs identified tianeptine, promethazine and ibuprofen. The concentration of tianeptine in serum was 5.87 mg/l. The analysis of negative serum, spiked serum and serum of patients are shown in Fig. 4.

DISCUSSION

Tianeptine is widely used nowadays in the treatment of depressive disorders. Even if it is well tolerated by the organism and cause few side effects, an overdose in combination with other drugs can be fatal.

It was created a sensitive and reliable method for determination of tianeptine and its metabolites in urine and quantification of tianeptine in blood by LC-MS method. This method is suitable for detection and quantification of tianeptine.

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EXPERIENCES OF THE CZECH TOXICOLOGICAL INFORMATION CENTRE WITH ETHYLENE GLYCOL POISONING

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Key words: Ethylene glycol/Intoxication/Haemodialysis/Acute renal failure/Nephrotoxicity/Czech Republic

The objective was to evaluate the severity of ethylene glycol (EG) intoxications in a 3-year retrospective study of the calls to the Toxicological Information Centre (TIC). Data about clinical course of patients with EG poisoning reported to the TIC in the years 2000–2002 were analysed. They were completed by the data from discharge records from the hospitals and by toxicological analyses. The χ-square test, Student's t-test, Fisher's test and the calculation of linear correlation coefficient were used for statistical analysis. The significance level was set at 0.05. TIC received total 188 calls concerning EG, from which 33 discharge reports were gained. There were 30 males (age 5–74 years) and 3 females (age 10–54 years). The patients ingested 252 ml on average (30–1000 ml); lethal dose (100 ml) was exceeded in 14 patients. Mean time interval from ingestion to admission was 3 hours (3–24 hours), mean length of hospitalisation 6 days (1–76 days). Fourteen patients developed metabolic acidosis, nine unconsciousness, thirteen signs of nephrotoxicity and nine signs of hepatotoxicity. Three patients died. Antidote ethanol was given in 30 patients. Other treatment included haemodialysis (20 cases) and B vitamins (23 cases). Ingested dose and the time interval between ingestion and admission correlated with severity of kidney damage. These data confirm that EG poisoning could seriously threaten the life. Renal parameters were abnormal in 30 % of patients who were discharged from the hospital. Those patients will be followed to evaluate the reversibility of EG toxic kidney damage.

ACKNOWLEDGEMENT

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SIMULTANEOUS ANALYSIS OF BUPRENORPHINE, NORBUPRENORPHINE AND MORPHINE-LIKE OPIATES IN HUMAN URINE BY GC/MS

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Key words: Buprenorphin/Norbuprenorphine/Morphine-like opiates/Human urine/GC/MS

INTRODUCTION

Buprenorphine has been used to treat opiate addiction in the Czech Republic since 2000. As the availability of buprenorphine increases, so does the risk for its abuse and criminal misuse. These facts establish the need for an appropriate technique to facilitate the monitoring of the population of buprenorphine users and abusers. As buprenorphine can be abused in combination with other drugs, a specific GC/MS method is needed to distinguish buprenorphine and other opiates present in urine (e.g. heroin metabolites). Buprenorphine and its active metabolite norbuprenorphine are excreted in urine, almost exclusively as glucuronides. It has been found that acidic and basic conditions of hydrolysis can destroy buprenorphine; therefore, the enzymatic hydrolysis is necessary in sample preparation for GC/MS analysis. The aim of the study was to develop and evaluate the GC/MS method for the rapid screening and quantification of buprenorphine, norbuprenorphine and other morphine-like opiates in human urine.

METHODS

In order to find the optimal conditions of hydrolysis, the aliquots of urine samples of individuals treated with buprenorphine were used. The optimal incubation conditions of the enzymatic hydrolysis (β-glucuronidase of *Helix pomatia*) of 5 ml urine were as follows: 250 µl of β-glucuronidase, incubation 1.5 h at 55 °C. The hydrolyzed samples were then extracted using Bond Elut Certify SPE cartridges and derivatized. Various types of derivatization (silylation and trifluoroacetylation) were evaluated and the optimal one for the purpose of our study selected.

RESULTS

Silylation was selected as the appropriate derivatization for the opioids analyzed. The method was validated according to international guidelines. It was linear from 1 to 500 ng/ml for all 11 analytes. The detection lim-

its for all analytes were 0.5 ng/ml using the SIM mode method.

CONCLUSIONS

This method was applied to the routine analysis of urine specimens collected from subjects treated with buprenorphine as well as to analysis of post-mortem samples.

MEDICAMENTS ABUSE AMONG DRUG ADDICTS

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ABSTRACT

The aim of our survey is to assess medicaments abuse among clients of contact centres in the Region of Zlín via questionnaires. We have evaluated the influence of various factors on abuse and have analyzed abuse details. Data from 147 questionnaires are presented. 44 % of respondents abused medicaments more than six times.We have created a profile of frequent medicament user who abused medicaments more than 20 times. Some statistical discrepances via ψ^2 (chi square) test between frequent medicament users and medicament non-users in several socioeconomic factors have been found. Collection of data will continue.

INTRODUCTION

Abuse of medicaments is connected with an addictive action of the medicaments[1]. We have premised that drug users (who the clients of the contact centres are) take medicamets because of their psychotomimetic potention rahter than because of the curative action. We have decided to evaluate the influence of socioeconomic factors on abuse of medicaments and analyze abuse in details.

There are 3 main groups of abused medicaments: analgetics (opioids, non-steroid analgetics and combinated analgetics), hypnotics (benzodiazepines and barbiturates) and anxiolytics (propandiols, benzodiazepins and others)[2,3]. The main common effect of these substances is a depression of the central nervous system[3].

MATERIALS AND METHODS

Our sample includes clients of two contact centres in the Region of Zlín: the Contact Centre Haus in Zlín and the Contact Centre Klíč in Vsetín. The contact centres are institutions of terciary drug prevention focusing on drug addicts who can not or don't want to quit drug use. The contact centres provide social and sanitary service, the most demanded service is syringe and needles exchange.

The clients have filled in our questionnaires focusing on general characteristics of the respondents, socio-economic data, drug use, risk behaviour and occurence of infection disease. 147 respondents underwent this questionnaire during years 2002–2005. We plan to continue the data collection.

RESULTS AND DISCUSSION

Our respondents stated number of medicaments abuses. They could choose from these posibilities: never; once; from two to five times; from six to twenty times; more than twenty times. We have dealt with clients who took medicaments more than 20 times, we inscribe them as frequent users of medicaments (MU). There were 40 (27 %) persons from all 147 who abused medicaments more than 20 times. We created a profile of a frequent medicament user (Table 1).

Are there any differences between group of all clients and group of MU? We confirmed some statistical discrep-

Table 1. Profile of a frequent medicament user

value	***typical MU***	***%***
gender	male	55
age	average	23.8 years
domicil	>10 000 inhabitans	57
cohabitation	with drug user	62
education	skilled without graduation	33
	advanced vocational training	33
employment	unemployed without soc. support	40
father education	graduation	30
mother education	skilled without graduation	40
relationship with parents	bad/not in touch	75
smoking	regulary	38
alcohol	several times a month	33
harmfullness of medicaments	little	35
	when using for a long time	30
legalization of "soft drugs"	free using and distribution	53
	free using, controlled distribution	35
basic drug	pervitin	83

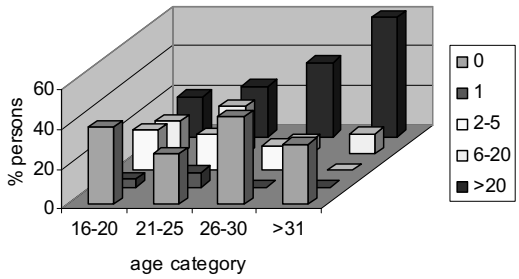

Fig. 1. Dependance on age

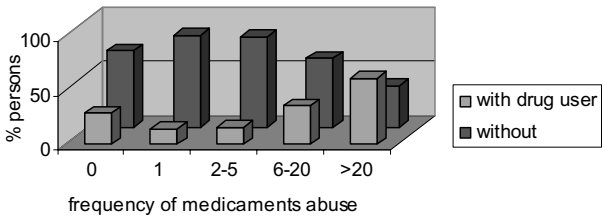

Fig. 2. Dependance on cohabitation with drug user

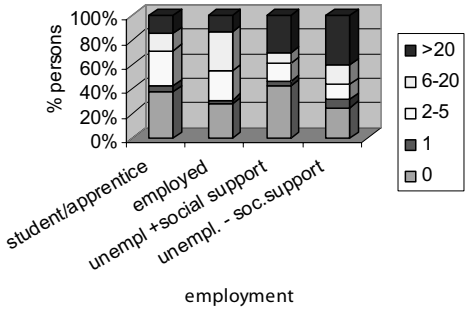

Fig. 3. Dependance on employment

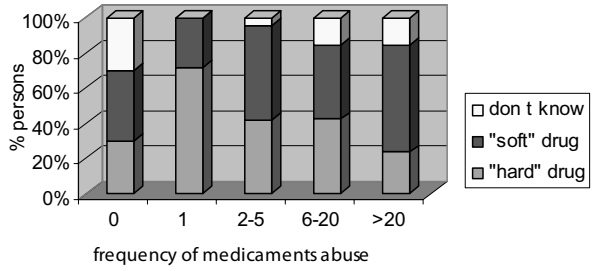

Fig. 4. Appreciation of medicaments danger

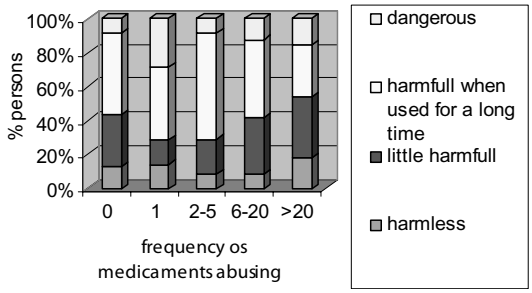

Fig. 5. Appreciation of medicaments harmfullness

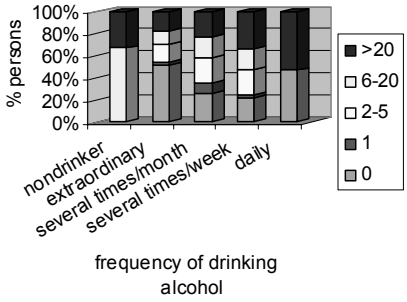

Fig. 6. Dependance on drinking alcohol

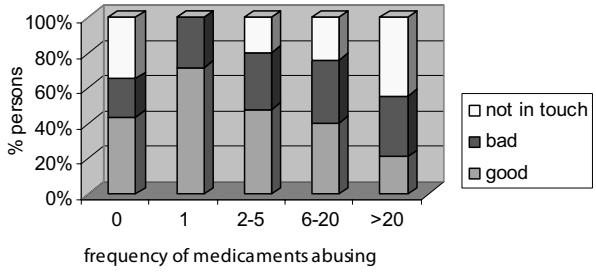

Fig. 7. Dependance on relationship with parents

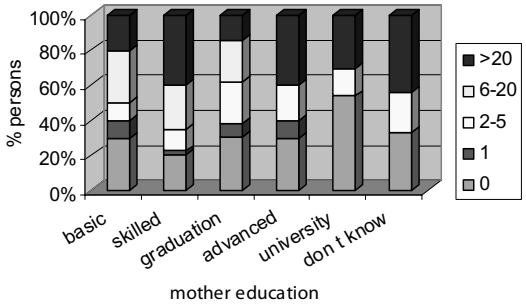

Fig. 8. Dependance on mother education

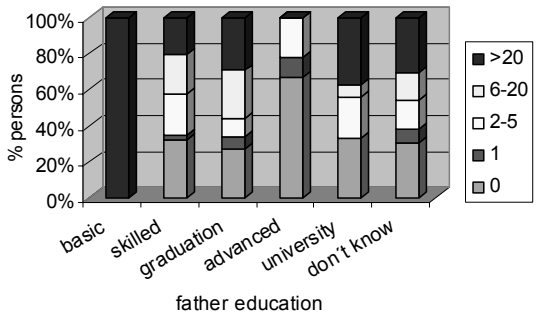

Fig. 9. Dependance on father education

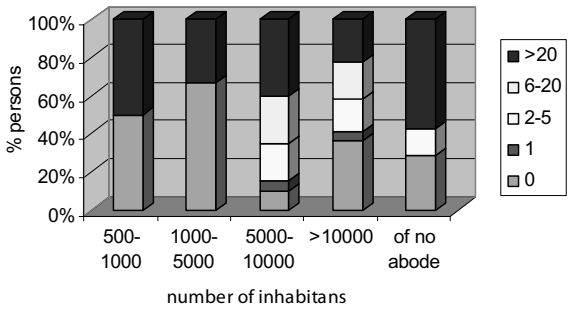

Fig. 10. Dependance on domicil

ances via ψ^2 test. Statistically more MU live together with another drug user than it is in the population of all clients. Statistically less mothers of MU underwent graduation than mothers of all clients. MU stated a good relationship with their parents statistically less times than it is in the population of all clients. More MU are unemployed without social support, also total unemployment (with or without social support) is bigger among MU and there are more students and employed among all clients.

We have also statistically compared a group without medicament experiences (stated "never" or "once") – the medicament non-users (MNU) with MU. Following differences were found:

- more MU live in a village with 5–10 thousands inhabitans (than MNU) and more MNU live in a village with more than 10 thousands inhabitans;
- more MU live with another drug user; less fathers of MU achieved advanced vocational training; more mothers of MNU achieved graduation exam;
- more MNU stated good relationships with parents.
- what is not suprising, less MU are extraordinary drinkers, more MU consider medicaments as a "soft drug" and according to our expectations there are more MU with alcohol as a basic drug;
- there are more unemployed persons among MU (with or without social support) than among MNU.

We bring ten figures (Fig. 1–10) summarizing characteristics of all clients who are arranged to groups according to their experince with abusing medicaments (0; 1; 2–5; 6–20; >20). These Figures show dependance of medicaments abusing on various factors.

CONCLUSIONS

We confirmed that abuse of medicaments is a concomitant phenomenon of intravenous drug using. Reccurence of us, pharmaceutical experts, should be to reduce this phenomenon not only among drug users but also in our whole population.

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NATURAL OPIUM AS ONE OF THE POSSIBILITIES FOR DRUG ABUSERS

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Natural opium is a popular seasonal alternation for substance dependent people. Its processing, way of using, experience and change of habits was the aim of our study carried out via anonymous questionnaire and directed interview. 47 questionnaires were obtained. According to our results natural opium has been abused by 19–42 years old people, more than a half of them were male. Basic drug were pervitin in 57 %, marihuana in 33 %, a considerable part (30 %) was natural opium. Each grower produced approximately 35 g of raw opium in one season. The most frequent way of application was smoking, injecting and "opium tea" drinking. Almost a half of the clients (40 %) felt attenuation after application; relaxation, hallucination and central stimulation admitted 1/4 of respondents. Health troubles had majority of the users. Combinations of natural opium with other drugs (alcohol, marihuana, pervitin) declared 45 % respondents, the effects of the combinations admitted 1/3 users. The examination for viral hepatitis confirmed more than a half of the clients; the major part of users from Olomouc, but only 25 % of users from Prostějov were tested for HIV. Opium consumption influenced habits of personal hygiene of more than a half of the users (57 %). Routine servicing of the spot of the needle insertion practiced a half of the responders. Majority of the abusers (79 %) obtained and closed out paraphernalia throw to the "harm reduction" program.

USE OF MARIJUANA IN PHARMACY STUDENTS (2000–2005)

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In 2000–2005, a survey on the consumption of legal and illegal drugs of abuse was conducted in 1.571 students of Faculty of Pharmacy in Hradec Králové, Charles University in Prague. The availability of cannabis and the prevalence rates of its use in university students were investigated. A standardized anonymous questionnaire was employed for the survey.

The average age of respondents was 20 years. The number of females was higher (82.8 %) than that of males. Marijuana was the most available and the most commonly used illegal drug of abuse in the group of pharmacy students. Its offer and the life-time prevalence increased over the 5 year period of the survey from 55.8 % to 72.9 % and from 30.3 % to 48.4 %, respectively. There was a predominance of the male users over the female ones, mainly in a category of high frequency of marijuana consumption ("used more than five times"). Almost three quarters of marijuana consumers admitted more than one experience with marijuana. Our data provide worrying statistics and support the need of continuous education also in university students to advise them on the risks of drug misuse.

CYTOTOXICITY OF PIVOXIL ESTERS OF ANTIVIRAL ACYCLIC NUCLEOSIDE PHOSPHONATES: ADEFOVIR DIPIVOXIL VERSUS ADEFOVIR

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Key words: Adefovir/Adefovir dipivoxil/Pivoxil/Formaldehyde/Cytostatic activity/Cytocidal effects/Nitric oxide

Biological effectiveness of antiviral acyclic nucleoside phosphonate adefovir, 9-[2-(phosphonomethoxy) ethy]adenine (PMEA) and its more lipophilic (bis)pivaloyloxymethyl ester prodrug adefovir dipivoxil (bis-POM-PMEA) were compared under *in vitro* conditions in mammalian cell systems. Proliferation of murine splenocytes was inhibited in a concentration-dependent manner, the bis-POM-PMEA being more effective than PMEA. In contrast to PMEA, bis-POM-PMEA inhibited production of nitric oxide (NO) in macrophages activated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS). Viability of both splenocytes and macrophages remained uninfluenced by PMEA, whereas pronounced cytocidal effects were exhibited by bis-POM-PMEA. The IC_{50}s reached the values of 15 μM and 30 μM in cultures of macrophages and splenocytes, respectively (assayed at the interval of 24 hrs). The effects could partly be mimicked by formaldehyde, a decomposition product of the pivoxil moiety of bis-POM-PMEA. The other possible product, pivalic acid, was ineffective in this respect. The present data are consistent with the view that pivoxil prodrug of PMEA, bis-POM-PMEA possesses enhanced but also broader spectrum of biological effects than the parent compound.

ACKNOWLEDGEMENT

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A COMPARISON OF THE POTENCY OF TRIMEDOXIME AND OTHER CURRENTLY AVAILABLE OXIMES TO REACTIVATE TABUN-INHIBITED ACETYLCHOLINESTERASE AND ELIMINATE ACUTE TOXIC EFFECTS OF TABUN

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Key words: Tabun/Acetylcholinesterase/Trimedoxime/Pralidoxime/Obidoxime/HI-6/Rats/Mice

Tabun (O-ethyl-N,N-dimethyl phosphoramidocyanidate) belongs to highly toxic organophosphorus compounds misused as chemical warfare agents for military as well as terroristic purposes. It differs from other highly toxic organophosphates by its chemical structure and by the fact that tabun-inhibited acetylcholinesterase is extraordinarily difficult to reactivate.

The potency of trimedoxime and other commonly used oximes (pralidoxime, obidoxime, the oxime HI-6) to reactivate tabun-inhibited acetylcholinesterase and to eliminate tabun-induced acute effects was evaluated using *in vitro* and *in vivo* methods. *In vitro* calculated kinetic parameters of reactivation of tabun-inhibited acetylcholinesterase from rat brain homogenate and *in vivo* deter-

mined percentage of reactivation of tabun-inhibited blood and tissue acetylcholinesterase in poisoned rats show that trimedoxime seems to be the most efficacious reactivator in the case of tabun poisonings. Trimedoxime was also found to be the most efficacious oxime in the elimination of acute lethal toxic effecs in tabun-poisoned rats and mice. The oxime HI-6, so efficacious against soman, does not seem to be sufficiently effective oxime to reactivate tabun-inhibited acetylcholinesterase and to counteract acute lethal effects of tabun.

ACKNOWLEDGEMENT

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DEVELOPMENT OF NEW ACETYLCHOLINESTERASE REACTIVATORS – ANTIDOTES USED FOR TREATMENT OF NERVE AGENT POISONINGS

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Key words: Acetylcholinesterase/Nerve agent/Reactivator/ Molecular design/Artificial neural networks/Synthesis/ In vitro/In vivo/Neurotoxicity

The mechanism of intoxication with organophosphorus compounds, including highly toxic nerve agents, is based on the formation of irreversibly inhibited acetylcholinesterase (AChE; EC 3.1.1.7) that could be followed by a generalized cholinergic crisis. Nerve agent poisoning is conventionally treated using a combination of a cholinolytic drug (atropine mostly) to counteract the accumulation of acetylcholine at muscarinic receptors and AChE reactivators (pralidoxime or obidoxime) to reactivate inhibited AChE.

At our department, we are interested in searching for new more potent AChE reactivators consisting of several steps: description of the nerve agent intoxication mechanism on the molecular basis (molecular design), prediction of the biological active structure of AChE reactivators (artificial neural networks), their synthesis, *in vitro* evaluation of their potencies (potenciometric titration and Elman method), *in vivo* studies (therapeutic index, LD_{50} of newly synthesized reactivators, reactivation in different tissues, neuroprotective efficacy). In this contribution, we would like to show the whole process of AChE reactivators development in Czech Republic.

ACKNOWLEDGEMENT

This work was supported by the grants of Ministry of Defense No. OBVLAJEP20032 and ONVLAJEP20031.

EFFECTIVITY OF NEW ACETYLCHOLINESTERASE REACTIVATORS IN TREATMENT OF CYCLOSARIN POISONING IN MICE AND RATS

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Key words: Cyclosarin/Oximes/Atropine/Acute toxicity/ Therapeutic ratio/Mice/Rats

The present study was performed to assess and compare a therapeutic efficacy of obidoxime, HI-6, BI-6 and HS-6 administered in equimolar doses and combined with atropine in cyclosarin-poisoned mice and rats. It was demonstrated that all the therapeutic regimens tested, were able to decrease the cyclosarin -induced toxicity significantly – at least 1.5 times. Higher therapeutic ratios, almost three times, were achieved in rats in comparison with mice. The highest therapeutic ratio was achieved for therapeutic regimen consisting of HI-6 and atropine in both mice and rats. Obidoxime was the least effective oxime in the treatment of cyclosarin intoxication. The BI-6 oxime was significantly more efficacious than obidoxime (in both mice and rats) and HS-6 (in rats) but its effectiveness did not reach the efficacy of HI-6.

ACKNOWLEDGEMENT

The study was supported by the Grant of Ministry of Defense of Czech Republic No. ONVLAJEP20031.

DELIVERY AS "PHYSIOLOGICAL STRESS" AND ITS INFLUENCE ON LIVER ENZYMATIC SYSTEMS IN ASPHYXIAL NEWBORNS

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Key words: Newborn/Oxidative stress/Aspartate aminotransferase/Alanine aminotransferase/ Quotient DeRitis

A new aspect on delivery as "physiological stress" provides the possibility for assessing its influence in asphyxial newborns. Asphyxia is characterized by hypoxic ischemic damage of organs with subsequent damage due to reoxygenation and reperfusion.

In the case of healthy term newborns (HTN) (Department of Neonatology, 2nd Dept. of Gynecology – Obstetrics) to determine on the 1st and 5th day of life the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), as well as to determine the quotient of DeRitis (ratio AST/ALT) and to compare them with asphyxial term newborns (ATN) (1st Department of Pediatrics, Department of Pathological Newborns), as well as to find the enzymatic answer of the liver on the delivery in healthy newborns.

The series consisted of 15 HTN and 24 ATN. In both groups AST, ALT and AST/ALT were investigated. Reference values of AST on the 1st day of life 0.80 ± 0.12 μkat/l and on the 5th day of life 0.78 ± 0.11 μkat/l, of ALT on the 1st day of life 0.29 ± 0.04 μkat/l and on the 5th day of life 0.54 ± 0.21 μkat/l and of the quotient of DeRitis on the 1st day of life 3.35 ± 0.6 and on the 5th day of life 2.14 ± 0.22 were established.

The quotient of DeRitis expresses better the influence of stress on HTN since isolated evaluation of liver enzymes and their dynamics, which is decreased on the 5th day of life shows stabilization of the baby and improvement of liver cell function in comparison with the 1st day of life. The authors found that delivery is considered to be a certain form of physiological stress.

ACKNOWLEDGEMENT

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AN EXTREMELY HIGH CONCENTRATION OF BREATH METHANOL

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Key words: Breath methanol/Blood methanol/Formate/ Ethanol/Dräger Alcotest 7410 Plus

Head-space gas-chromatographic analyses of a blood sample from the driver confirmed that the reason for the high Dräger Alcotest 7410 response (averaging 3.75 pro mille) was mainly methanol and partly ethanol in the breath, corresponding to a blood methanol concentration of 2.8 g/kg and a blood ethanol concentration of 0.46 g/kg. With the help of a wet-bath simulator and the standard methanol and ethanol solutions, the influence of breath methanol on the Dräger Alcotest 7410 response was evaluated. The case report described highlights the important role of ethanol as an antidote in cases of methanol intoxication.

STUDY ON METABOLITES OF 2,5-DIMETHOXY-4-BROMAMPHETAMINE (DOB) IN HUMAN URINE USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Key words: 2,5-Dimethoxy-4-bromamphetamine/DOB/ Biotransformation/Metabolites

The study of the biotransformation of a new synthetic drug 2,5-dimethoxy-4-bromamphetamine (DOB) and identification of its metabolites in urine of a poisoned person is described using gas chromatography mass spectrometry (GC-MS) with various ways of derivatization. It has been confirmed that one of its metabolic pathways leads to the corresponding 2-*O*-desmethyl and 5-*O*-desmethyl metabolites when the latter is prevailing. It is important to know the metabolism of this neurotoxic and hallucinogenic substance as it is a prerequisite for developing reliable toxicological diagnostic procedures and for assessment of toxicological risks.

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DRUG ABUSE IN SLOVAK REPUBLIC IN THE YEARS 1992–2004

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Key words: Drugs of abuse/Acute intoxication

ABSTRACT

The drug abusing structure has dramatically changed since 1989. While in 1989 the sniffing of the fluid drugs represented 98 % of the global drug abuse, the most abused drugs were: heroin, marihuana, cocaine, amphetamine and its derivates. During last 10 years situation with drug abuse has changed. Currently the most abused drugs: cannabinoides, amphetamines.

The plant drugs (*Datura stramonium*, hallucinogenic mushrooms–*Psilocybe semilanceata*, nutmeg–the seed of *Myristica fragrans*) combined with the alcohol are popular among the young abusers. According to an analysis of the phone consultations in our Toxicological Information Centre (TIC) we found out, that the number of intoxications with the plant drugs has increased five times during the last year (comparing with the yr 2000), because of their easy availibility, low price and quick spreading of information.

INTRODUCTION

Authors inform about the drug scene in Slovak Republic using own results from the Toxicological Information Centre (TIC).

The drug abusing structure has dramatically changed since 1989. While in 1989 the sniffing of the fluid drugs represented 98 % of the global drug abuse, the most abused drugs were: heroin, marijuana, cocaine, amphetamine and its derivates. During last 10 years situation with drug abuse has changed. Currently the most abused drugs: cannabinoides, amphetamines, plant drugs[1].

The plant drugs (*Datura stramonium*, hallucinogenic mushrooms-*Psilocybe semilanceata*, nutmeg-the seed of *Myristica fragrans*) combined with the alcohol are popular among young abusers. According to an analysis of the phone consultations in our TIC we found out, that the number of intoxications with the plant drugs has increased five times during the last year (comparing with the yr 2000), because of their easy availibility, low price and quick spreading of information[1,2].

The sources of intoxication in TIC are following: drugs 2.2 %, plant and mushrooms 7.7 %, chemical products 32.1 %, pharmaceuticals 43.9 % (ref.[2,6]) (Fig. 1). Suicidal intoxications have risen in the last 10 years with most

occurences among girls between the ages of 11 and 19, which is consistent to a certain extent with drug abuse in this age group. We have recorded 3 suicidal intoxications in the age group under 10 (Fig. 2). Within the last year for the first time we have recorded most intoxications with analgesics mostly containing Paracetamol and Ibuprofen[1,3] (Fig. 3).

Until 1989 the most abused substances were alcohol and siccatives. Abuse of other drugs was rare. Slovakia was mostly a transit zone for drugs heading to Western Europe. Heroin became the number 1 drug after the borders were open (number of users, addicted, being treated). This situation lasted until 1999 when plant drugs occured on the drug scene and gradually multiplied. The situation is currently comparable with other countries within the E.U. Most abused are cannabinoids and amphetamines (number of users, addicted, being treated). The use of heroin has declined considerably. However, the use of plant drugs is on the rise (Fig. 4, 5, 6).

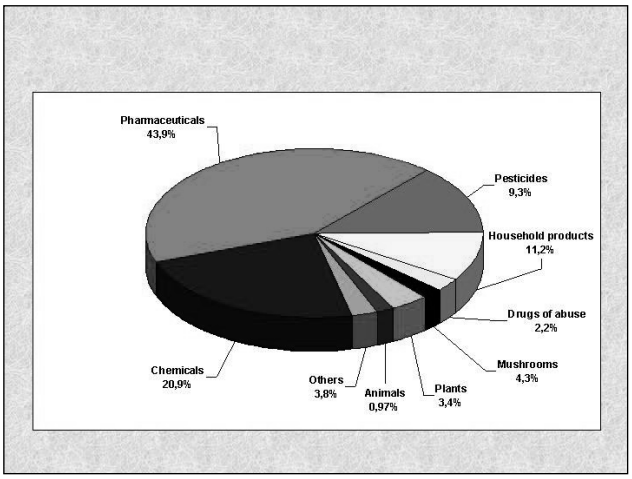

Fig. 1. Substances involved in poisoning – 2004

60 % of adult population of both genders have problems with alcohol abuse. The current situation is alarming and the most problematic issue is the continual shift of the alcohol abuse to the younger and younger population groups (12–13 year girls)[1,4] (Fig. 7, 8).

The situation with drug abusing in Slovakia is lowly transparent, even though the Slovak government is putting a stress on this issue. There is no global knowledge about the number of drug abusing and drug addicted individuals in Slovakia neither about the volume of drugs circulating on the Slovak territory. There is 47 specialized agencies focusing on the treatment of drug addicted individuals in the Slovak Republic[5].

A study was conducted in Bratislava High schools using a questionnaire. The results are following: 38.5 % of students admitted to having used drugs, predominantly alcohol and marijuana (Fig. 9). Somewhat smaller percentage of the students admitted to having smoked cigarettes at least once. The reasons why students have used drugs varied as well. More than one half claimed they would never use drugs. Almost one third would try drugs out of curiosity. And the remainder of the students would use drugs for various other reasons. Very few stated that they would use drugs if others were using them. Students demonstrated high tolerance towards so called legal drugs. More than a half does not consider alcohol, coffee and cigarettes to be drugs. Alcohol use is more common for students who have been intoxicated at least once. This represents almost a half of the students surveyed. Most students were aware of substances or medications which enhance intoxication by alcohol. However many students indicated only general interaction between alcohol and medication without knowing whether it enhances intoxication or not. Most occurring were analgesics, hypnotics-sedatives, energy drink, amphetamines and marijuana. About a third would welcome legalization of marijuana. Most students were aware of plants with intoxicative effects. Widely mentioned were cannabis, poppy seed, mushrooms, tobacco leaves, jimson, and sage.

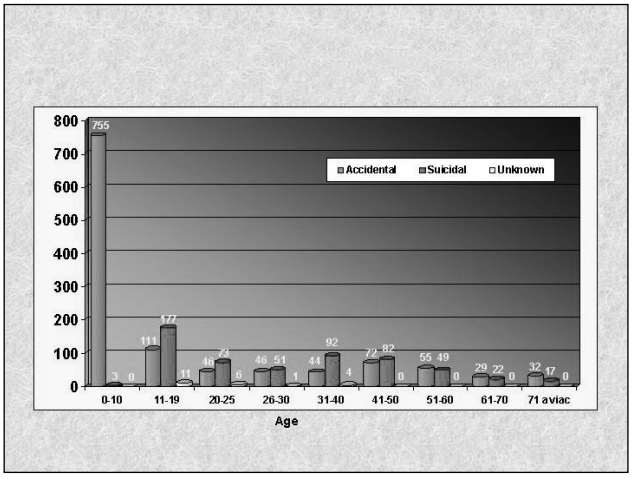

Fig. 2. Acute poisonings – 2004. Age distribution, intent of exposure

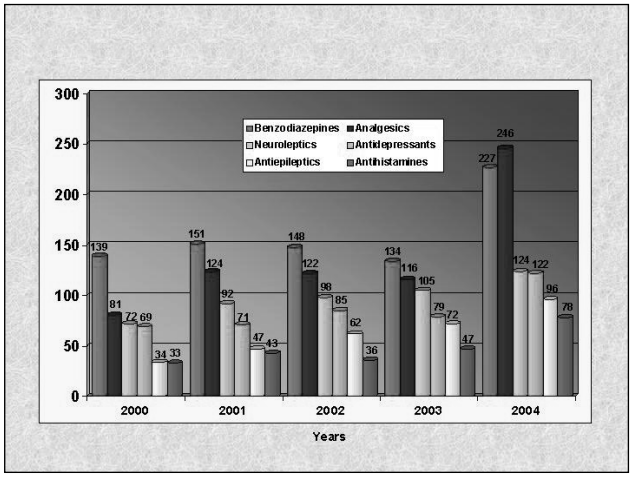

Fig. 3. Distribution og drug acute poisoning cases (2000–2004)

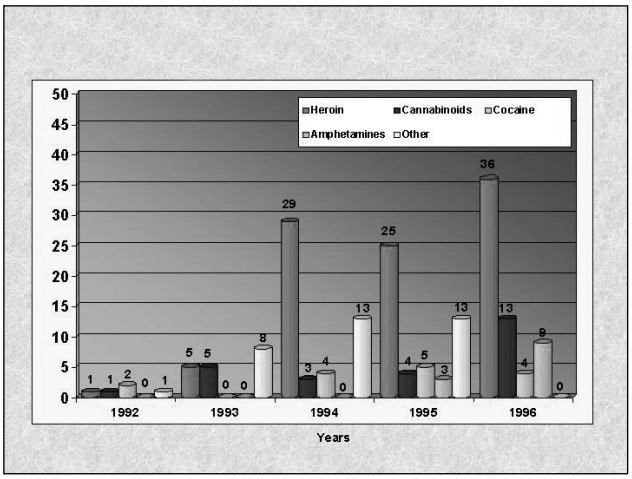

Fig. 4. Intoxication by drugs of abuse in the years 1992–1996

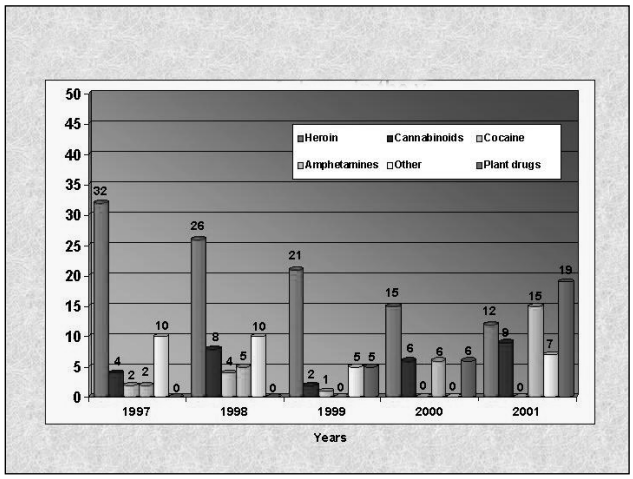

Fig. 5. Intoxication by drugs of abuse in the years 1997–2001

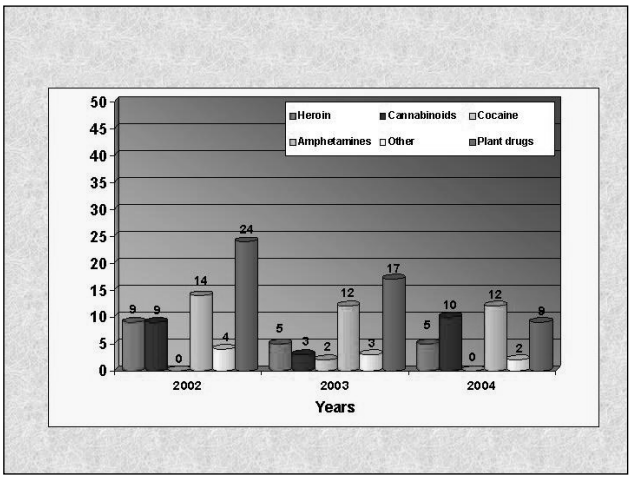

Fig. 6. Intoxication by drugs of abuse in the years 2002–2004

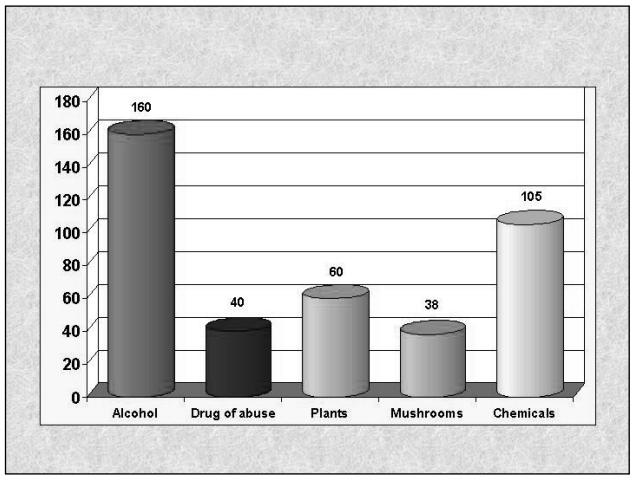

Fig. 7. Non-drug poisoning in children – 2004

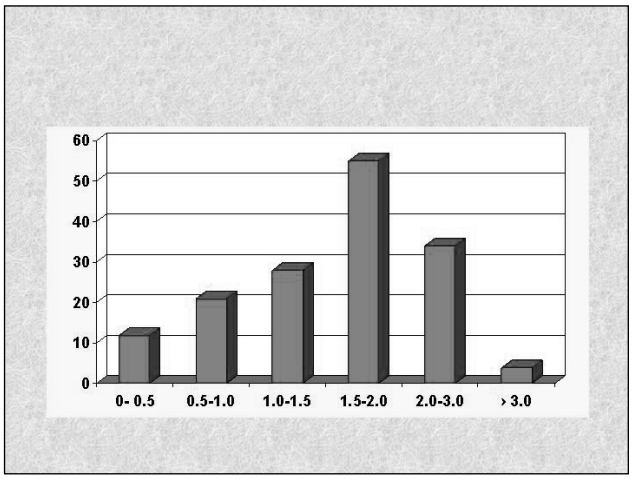

Fig. 8. Children's blood ethanol concentration (‰) – 2004

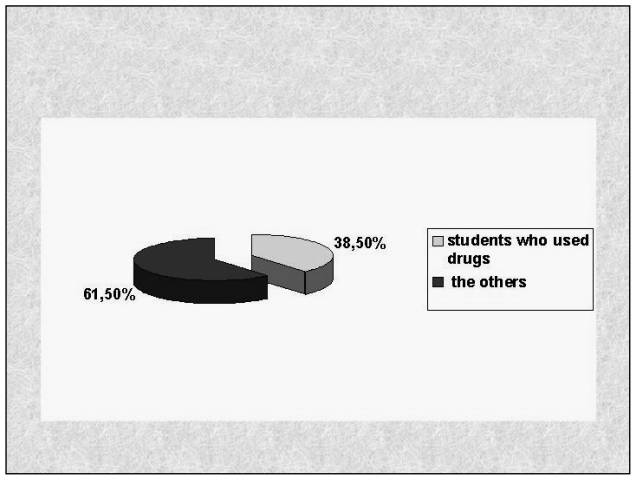

Fig. 9. Drug abuse among the students

CONCLUSIONS

More than 60 % of all intoxications consulting with our Toxicological Information centre (TIC) concern the 0–21 year old population. The sources of intoxication in TIC are following: pharmaceuticals 43.9 %, chemical products 32.1 %, plant/mushrooms 7.7 % and drugs 2.2 %. The drug abusing structure has dramatically changed since 1989. While in 1989 the sniffing of the fluid drugs represented 98 % of the global drug abuse, currently the most abused drugs become cannabinoides and amphetamine and its derivates. The plant drugs combined with the alcohol are popular among the young abusers. 60 % of adult population of both genders have problems with alcohol abuse. Incidence of drug abuse among 0–4 year olds was 6 %, 15–19 year olds 60 %, 20–24 year olds 30 %, 25–29 year olds 7 % , 30–40 year 2 % and older group less than 1 %. The most frequented start of drug abuse is among the 12–13 year old individuals. The TIC is closely cooperating with Toxicological Laboratory. The number of drug related laboratory tests have increased 10 times since 1995 and this trend is still growing. The number of drug addicted individuals increased from 2000 in 1994 to 50 000 at present.

The Slovak population has aged. The average age increased, which makes the situation appear relatively stabilized. We must note that the population is more informed and more alert about drugs, especially children and young adults.

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CLINICAL TOXICOLOGY, DRUG INTERACTIONS

"TOP TEN" – TEN PARTICULARLY DANGEROUS DRUG INTERACTIONS IN LONG TERM CARE

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Key words: Drug interactions/Warfarin/CYP2C9/ Cytochromes P450

Drug interactions, mainly these joined with severe adverse effects, are often the cause of patient hospitalization and, if unrecognized, may lead to patient death. The current studies (e.g. this performed by American Association of Medical Directors, AMDA[1]) have pinpointed ten most dangerous drug interactions which have been observed in patients taking prescription drugs for long time, as e.g. in nursing homes or in hospitals providing long term care. The importance of the adverse drug reactions has been judged on the basis of the clinical significance of the interaction and by the potential to cause harm, by the frequency with which the interaction occurs and by the frequency with which the drugs are prescribed.

The first five most important drug interactions involve warfarin. Next two cases are based on interactions of ACE inhibitors (interactions with drugs and preparations leading to hyperkalemia), two are of pharmacodynamic basis involving digoxin and the last, tenth most important drug interaction is this of theophylline with quinolones based on their metabolism.

Drug interactions of warfarin are quite frequent due to its narrow therapeutic window and because of relatively complicated metabolism of this compound. Among the five most dangerous interactions of warfarin, these with (i) non-steroidal anti-inflammatory drugs, (ii) sulfonamides, (iii) macrolides, (iv) quinolones and (v) with phenytoin are involved. NSAID are listed here because of a known increase of gastric irritation and formation of gastrointestinal bleeding caused by these compounds; a concomitant treatment of warfarin is then extremely dangerous. However, also another mechanism may be involved, namely, an interaction at the level of metabolism mediated by cytochrome P450 form 2C9 (CYPC9). With antibiotics, the situation is rather similar. Primarily, it is believed that the interactions are due to diminished synthesis of vitamin K by intestinal microflora (suppressed by antibiotics or sulfonamides). However, at least in the case of certain macrolides and quinolones, an interaction at the level of cytochromes P450 (mostly the CYP3A4 form) may take place. Phenytoin and warfarin (its *S*-enantiomer) are typical substrates of CYP2C9 and the interaction here is clearly at the pharmacokinetic basis.

The danger of warfarin toxicity is even increased in patients with certain mutant alleles of CYP2C9 (CYP2C9*2 and CYP2C9*3). These subjects are known to metabolize warfarin more slowly and hence therapeutic level of this drug may be easily exceeded. Here, recent pharmacogenetic approaches unequivocally show the usefulness of phenotyping and genotyping procedures as e.g. patients with mutant CYP2C9 alleles need in average three months to find an optimal warfarin dosage[2]. Geno- or phenotyping procedures may shorten the time considerably. Drug interactions may even complicate this situation considerably. Hence, both in vitro as well as clinical approaches are needed to understand and, moreover, to avoid dangerous drug interactions.

ACKNOWLEDGEMENT

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DETERMINATION OF HALOTHANE USING GAS CHROMATOGRAPHY WITHIN QUALITY CONTROL OF PATIENT MALIGNANT HYPERTHERMIA SUSCEPTIBILITY DIAGNOSTICS

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Key words: Halothane/Gas chromatography/Malignant hyperthermia/Contracture test/European malignant hyperthermia group

ABSTRACT

The aim of this work was to develop the method of determination of halothane in a liquid (Krebs-Ringer solution) for the quality control performance of preoperational diagnostics of malignant hyperthermia patient's suscepti-

bility on base of so-called *in vitro* contracture test (IVCT) according to protocol of the European Malignant Hyperthermia Group (EMHG). The gas chromatography with head-space sampling technique coupled to flame ionization detector (FID) have been chosen for this purpose. Quantification was performed by method of internal standard using 2-propanol (IS). Within the validation of method its linearity in concentration range 0.038–3.788 mmol of halothane/l was verified ($R > 0.9999$), limits of detection (LOD) and quantification (LOQ) were 2.33 and 7.76 μmol of halothane/l, respectively. The precission expressed by RSD was better than 6 % in whole dynamic range of the method. The accuracy and the confidence interval of obtained results were verified as well. Proposed method has been found suitable for intended purpose.

INTRODUCTION

The malignant hyperthermia (MH) is latent genetically conditioned diathesis in man that doesn't manifest itself within common life.The manifests of MH may arise as the consequence of excessive stress, temperature or within the induction and maintaining of general preoperational anaesthesia using certain so-called MH-triggers. The fundamentals of this disease are regulation disorders of calcium metabolism on sarcoplasmatic reticulum receptor level in muscle cells[1]. The state, when the symptoms of this diathesis are well developed, is called MH-crisis. It manifests through muscle rigidity, ventilation disorders and heart dysfunctions, changes of blood pressure and fast increase of body temperature up to 42°C. The last mentioned fact that mustn't necessarily arise in all cases, gave paradoxically the name to whole this phenomenon. The MH-crisis may be fatal without early intensive medical care.

The substances that may trigger MH-crisis belong to group of depolarization muscle relaxants such as succinylcholine, caffeine or suxamethonium, furthermore ryanodine or 4-chloro-*m*-cresol were detected as potential MH-triggers, and the group of gaseous general anaesthetics as well, that includes: Halothane (1,1,1-trifluoro-2,2-chlorobromoethane), Enflurane (2-chloro-1,1,2-trifluoroethyl difluoromethylether), Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethylether), Sevoflurane (fluoromethyl-1,1,1,3,3,3-hexafluoroisopropylether), Desflurane (1,2,2,2-tetrafluoroethyl difluoromethylether), Methoxyflurane (1,1-difluoro-2,2-dichloroethyl methylether). This group of halogenderivatives of aliphatic alkanes and ethers is used for anaesthetical purposes from the World War 2nd up to now in spite of adverse effect mentioned above. The reason is fast onset and deletion of anaesthetic effect and easy regulation and maintaining of anaesthesia, respectively.

From the geneticist's point of view the cause of MH-susceptibility is aberration on 19th chromosome, however this aberration was proved at about 50 % of families only where MH occured. The most recent research in this field led to discovery already more than 20 different kinds of this genetic aberration that may be the cause of MH-susceptibility and even another different kinds of muscular disease. Therefore it is not possible to use the molecular genetics as stand-alone method for reliable diagnostics of MH-susceptibility at present.

Statistical mortality in cases of MH-crisis development (progress) was 70–80 %. The MH represented the most frequent cause of death during anaesthesia in the USA in the early 1970s, for example[2]. In 1975 G. G. Harisson discovered dantrolene (hydantoin derivative) – the MH-remedy – within the testing on MH-susceptible swines[3]. Introduction of this substance has led to significant decrease of mortality to 10–20 %. The mortality associated with MH has decreased to 5–10 % at present. In order to minimize the risk associated with anaesthesia using volatile general anaesthetics, specialized laboratories dealing with preoperational diagnostics of MH-susceptibility in patients has started arise all over the world. There are 22 diagnostic centres engaging in this issue situated in 15 countries of the Europe at present. One such laboratory, the only one in Czech Republic, was established in 2003 at the Department of Anaesthesiology and Intensive Care of the St. Anne's University Hospital in Brno.

The diagnostic examination itself is performed on muscular tissue from patient's quadriceps isolated at the biopsy. Immediately after excision the tissue is dipped to bath with perfusion of Krebs-Ringer solution kept at 37°C which carbogen (95% O_2 + 5% CO_2) bubbles through in order to maintain vital reaction of the tissue. The muscle is situated in bath vertically in such way to be tightly fixed to anchoring wire with lower end and with its upper end is tied to sensitive transducer of contraction intensity. Simultaneously the muscle touches with its ends to electrodes which stimulate it. In accordance to EMHG protocol the muscle is exposed to gradually increasing concentrations of halothane that is introduced to bath in gaseous phase with carbogen blowing through vaporizer, and caffeine which is added to bath in bolus-form. From parallel recording of muscular contraction changes versus changing concentrations of both testing substances the evaluation is performed and patient is sorted to one of three groups: MHS (MH susceptible) = MH diathesis, MHN (MH non-susceptible) = MH diathesis excluded, MHE (MH equivocal) = MH diathesis ambiguous (case of incidence of pathological contracture at one of testing substances only). The MH Emergency card is issued to MHS-patients afterwards. This card exercises within acute states of patient requiring emergency surgery.

Within diagnostics of MH-susceptibility in accordance to EMHG protocol it was necessary to develop the method for determination of halothane in liquid (Krebs-Ringer solution) for quality control purposes concerning to performance *in vitro* contract tests. In accordance to protocol mentioned above the quality control testing is performed every 3 months.

There are many different reports engaging to determination of volatile anaesthetics in liquid (buffer, urine, blood) where distinguish approaches utilising UV-spectroscopic method or liquid chromatography were applied. However, the most suitable method of analysis is gas chromatography (GC) for its availability and simplicity of use. For its sufficient sensitivity, wide linear dynamic range together with sampling of reasonable sample volume the

flame-ionization detector (FID), besides mass (MS) and electron-capture detectors (ECD), is the most frequently used for this type of analysis[4]. The sample may be injected either directly (this type of sampling isn't recommended for contamination and humidity introduction to the system), or after sample pretreatment as it is mentioned by Farthing *at al.*[5] who injected 1 µl of trichloromethane extract of Krebs buffer within the parallel determination of halothane, enflurane and isoflurane by GC coupled with splitter sampler. Another method of introduction of sample to the GC system mentioned in report by Snoeck *at al.*[6] is head-space method (HS) of sample injection. The authors presented the use of the system for control analysis of sevoflurane concentration in testing bath within the research of occurance of abnormal contractures of skeletal muscles in patients exposed to this gas anaesthetic. This sampling technique provides within the determination of volatile substances in a liquid the advantage of elimination of sometimes laborious sample pretreatment [e.g. L-L extraction or solid phase microextraction (SPME) (ref.[7])], which leads to time savings. So that was one of the reasons why we have chosen the adapted method of HS-GC used for evidence and determination of the volatile substances in biological samples in our toxicological laboratory.

MATERIAL AND METHODS

Instrumental equipment. The gas chromatograph Perkin-Elmer SIGMA 2000 with head-space autosampler HS-100 and flame-ionization detector (FID) was used for determination of halothane.

Chemicals. The pharmaceutical NARCOTAN™ (Léčiva Praha, a.s., Czech Republic) containing 0.01% of thymole as stabiliser was used as halothane standard. Trichloromethane stabilised by ethanol (~ 1 %) and 2-propanol, both p.a. grade, were delivered by Lach-Ner, s.r.o. and Lachema, a.s. (both Neratovice, Czech Republic), respectively. Krebs-Ringer solution, pH 7.40, containing: Natrium chloride (118.1 mmol/l), Kalium chloride (3.4 mmol/l), Magnesium sulphate (0.8 mmol/l), Kalium dihydrogenphosphate (1.2 mmol/l), Glucose (11.1 mmol/l), Natrium hydrogencarbonate (25.0 mmol/l), Calcium chloride (2.5 mmol/l) was prepared by hospital pharmacy, St. Anne's University Hospital in Brno.

Standard stock solutions of halothane were prepared by diluting of primary standard with trichloromethane to required concentration as follows: Into the 25 ml volumetric flasks it was pipeted 10, 5, 2, 1, 0.5, 0.2 and 0.1 ml of halothane and particular flasks were filled up to mark of flask with trichloromethane. To the vial, volume 20 ml, 1 ml of Krebs-Ringer solution was pipeted and immediately after addition of 1 µl of 2-propanol (IS) the vial was closed using hand crimper. It was injected 1 µl of respective stock standard solutions through the septum to minimize the loss of volatile standard.

Sample preparation. Sampling was performed by intake of 1 ml Krebs-Ringer solution from testing bath maintained at 37°C and previously saturated by gas mixture of carbogen (medicinal gas of 95% O_2 and 5% CO_2) and halothane in concentration of 2 vol.% as required by EMHG protocol. Sampling was performed using so-called insuline-syringe, 1 ml volume, to the vials, 20 ml volume, through rubber septum in which 1 µl of 2-propanol and 1 µl of trichloromethane had been previously pipeted in order to maintain the same conditions of analysis both the samples and the standards.

Analysis conditions. For GC determination the chromatographic column packed with 15 % Carbowax 1500 on Chrom WNAW 80/100, 2 m length and 1/8'' ID, was used. The mobile phase was nitrogen 5.0 at 160 kPa. The flame-ionisation detector was used for detection with hydrogen 5.0 and air at 160, resp. 110 kPa. Samples were incubated at 60°C for 12 min and subsequently analysed at 90 °C (isothermal analysis). The temperature of injector and detector were the same, 140°C. Injected sample volume of gaseous phase was 1 ml, time of analysis 5min. This time may be shortened within the further optimization of method.

RESULTS AND DISCUSSION

Using the developed method it was achieved the sufficient separation of individual components, i.e. halothane, 2-propanol (IS) and trichloromethane to baseline (see chromatogram below, Fig. 1). Individual components eluated with values of retention time are mentioned in Table 1.

Table 1. Retention time values of separated components

substance	retention time Rt [min]
halothane	1.75
2-propanol (IS)	2.13
trichloromethane	3.10

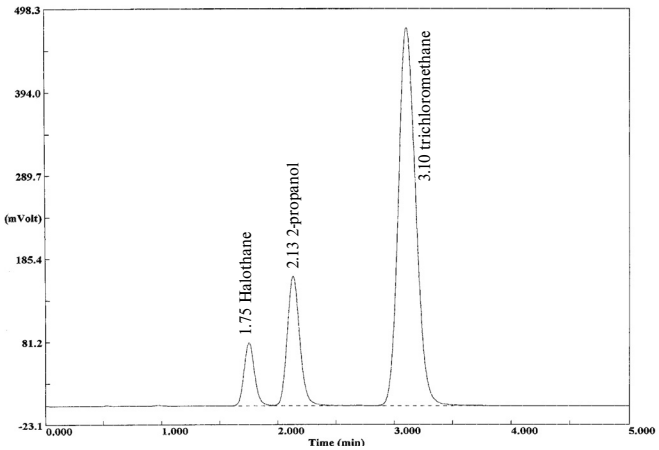

Fig. 1. Chromatogram of sample analysed within quality control of MH-diagnostics

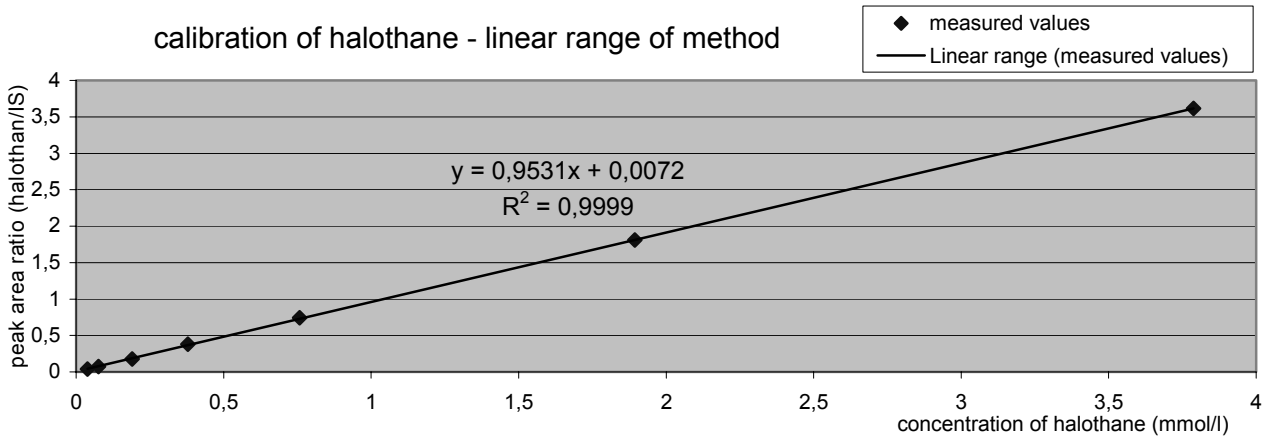

Fig. 2. Calibration plot of halothane – linear range verification

Validation of method. Linear range of method was verified by analysing of batch of standard solutions and plotting measured chromatographic data versus concentration (see Fig. 2). The method showed linear response in the range of 0.038 – 3.788 mmol of halothane/l with regression coefficient R better than 0.9999 and the equation of linear plot was y = 0.9531x + 0.0072.

Limit of detection (LOD) and limit of quantification (LOQ) were determined as 3-, resp. 10-multiple of baseline noise of blank chromatogram (N = 10) in range of R_t = 1.50–2.00 min (R_t of halothane was 1.75 min). The LOD and LOQ were 2.33, resp. 7.76 μmol of halothane/l.

Precision of method expressed by reproducibility of results was examined at limit values of linear range, i.e. at concentration of 0.038 and 3.788 mmol of halothane/l, inside the interval at concentration of 0.379 mmol of halothane/l and the concentration which was controled within quality control measurements, i.e. 0.44 mmol of halothane/l. For concentrations mentioned above we calculated RSD values (N = 8) as they are summarised in Table 2:

Table 2. RSD values for halothane analysis

conc. of halothane [mmol/l]	RSD [%]
0.038	5.81
0.379	2.02
0.440	4.88
3.788	3.67

For verification of accuracy of method by means of Student's t-test the same data files identical with those used for verification of reproducibility were used. The confidence interval of measured data obtained from control measurement of halothane is 0.435 ± 0.008 at statistical significance level of α = 0.05, i.e. rate of statistical probability P = 95 %.

CONCLUSION

Despite problematical handling with analyzed substance especially caused by high volatility (boiling point of halothane is 50.2 °C) the developed GC method for determination of halothane in Krebs-Ringer solution may be accepted as suitable for intended purpose, particularly when we take account of measuring error of ± 10 % allowed by EMHG protocol within the quality control of IVCT performance.

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GALANTAMINE ANTIACETYLCHOLINESTERASE ACTIVITY IN RAT BRAIN INFLUENCED BY L-CARNITINE

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Key words: Galantamine/Acetylcholinesterase/L-carnitine/ Brain/Rat/Alzheimer's disease

Galantamine (GAL) is a selective, competitive and reversible acetylcholinesterase (AChE) inhibitor, which increases the activity of the cholinergic system and hence gives rise to an improvement of cognitive functions in patients suffering from dementia of Alzheimer type. L-carnitine (CAR) is a natural component of the mammalian tissue and is known to increase penetration of some chemical compounds/groups across biological membranes. The aim of this study was to evaluate the influence of pretreatment with CAR on AChE inhibition caused by GAL in selected brain parts in rat (basal ganglia, septum, frontal cortex, hippocampus) and in hypophysis, which does not lay beyond the blood-brain-barrier. During the first stage of the study, GAL was administered i.m. in different doses ranging from 2.5 to 10 mg/kg. The highest degree of AChE dose dependent inhibition was observed in hypophysis, while that in CNS was lower and became apparent in frontal cortex and hippocampus only after the administration of the dose of 10 mg/kg i.m. In the second stage, CAR was administered daily during 3 consecutive days at a dose of 250 mg/kg p.o. prior to the administration of GAL (10 mg/kg i.m.). Pretreatment with CAR enhanced trend of AChE inhibition in all selected brain parts comparing with single GAL administration, however, significant difference was not observed. Comparing this results with control group, statistical significance was found in frontal cortex, hippocampus and hypophysis.

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INTERACTION OF AROMATIC CYTOKININS WITH HUMAN LIVER MICROSOMAL CYTOCHROMES P450

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Key words: Cytokinins/Cyclin dependent kinase inhibitor/ Cytochrome P450/CYP/Human microsomes

Aromatic cytokinins (*o*-topolin riboside, benzylaminopurine riboside and 2-hydroxy-3-methoxybenzylaminopurine riboside) were tested for their possible interaction with human liver microsomal cytochromes P450 by absorption difference spectroscopy. All three compounds were shown to bind to the CYP enzymes producing a high to low spin shift of the heme iron yielding a Soret absorption band shift to approximately 425 nm. As this type of spectral change means that the substance is able to bind directly to the heme iron, the results obtained open the possibility of an interaction of these compounds with metabolism of other drugs or, in general, with other substrates of cytochromes P450.

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CARCINOGENESIS, MUTAGENESIS, TERATOGENESIS

GENETIC POLYMORPHISMS OF BIOTRANSFORMATION ENZYMES AND THEIR ROLE IN CARCINOGENESIS

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During the process of carcinogenesis many changes arise, however very little is known about their order and time periods between individual occurrences. Presumed cause for the genesis of tumorous disease is the interaction between genetic (sensitivity) and environmental factors, or to put it differently the factors of our lifestyle and natural environment. Lately, these interactions have been monitored by means of research focusing on genetic polymorphisms in genes with low penetration. Hereditary variations of these genes are probably responsible for the origin of higher percentage of carcinoma cases than in genes with high penetration, as for instance tumour suppressors or oncogenes.

During our presentation, we shall introduce results of the pilot study aimed at determination of relations between genetic polymorphisms of biotransformation enzymes and colorectal carcinoma (CRC). Within the study frequency and distribution of polymorphisms in genes of cytochrome P450 1B1, epoxide hydrolase, NAD(P)H: quinone oxidoreductase and glutathione S-transferases were monitored in the CRC patients and the control subjects of the Czech nationality. The statistical analysis demonstrated that i) individual polymorphisms have no statistically significant effect on the risk of CRC genesis in general population. ii) In the group of women quinone oxidoreductase polymorphism was determined to be the risk factor. The female carriers of the variant genotype had more than threefold higher risk of CRC genesis compare to women with normal genotype ($P = 0.034$). This polymorphism played no significant role in male individuals. Nevertheless, its function in breast carcinoma in women was published lately not only in the Czech but also Austrian population. iii) As for the gene combinations, the most notable were the glutathione S-transferases combinations. In all tested combinations, the recorded relations were just at the significance level ($P < 0.1$). iv) Age had no influence on the statistical analyses results.

The very first research of this kind performed within the Czech population demonstrated that polymorphisms of some biotransformation enzymes may participate in the CRC genesis. Further research should be targeted particularly at the analysis of differences in exposition between the two genders, at the importance of polymorphisms combinations and also the broader spectra of genes with low penetration.

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DEVELOPMENTAL TOXICOLOGY – AN INTEGRAL PART OF SAFETY EVALUATION OF NEW DRUGS

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Key words: Developmental toxicology/Teratology/Screening tests/Biomodels

The thalidomide tragedy stimulated an intense research in the etiology, prevention and treatment of congenital malformations. The Government requires that drugs and food additives be evaluated pre-clinically for toxicity, including developmental toxicity, before being marketed. The aim of developmental toxicology is to detect any adverse effects of xenobiotics on the pregnant female and on the development of the embryo and foetus as a consequence to exposure from starting with implantation through the entire period of gestation, parturition and maturation. The number of compounds which must be tested has increased dramatically with the continuous development of therapeutic, cosmetic and food additive chemicals. Such tests include: *in vitro* studies which can serve as efficient pre-screens to rank chemicals for further batteries of *in vivo* tests on pregnant animals. The reliability and extent of testing depends on the methods and ex-

periences of the laboratory where the tests are performed. The current methodology derived from basic developmental toxicology research is able to detect the detrimental potential of the majority of substances, to identify the dose range of embryotoxicity and to determine the relationship between the dose and the effect. However, precise extrapolation will require not only knowledge of the specificity of the human xenobiotic metabolites but also the identification of the sensibility of the morphogenetic systems and their distribution in the time horizon of the development of the human embryo. Finaly, the safety of any drug would be determined only by a post-marketing epidemiological survey. Taking into account the altered susceptibility to different drugs in a pregnant individual, it could be said that administration of any drug during the first trimester is an experiment in human teratology.

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MOLECULAR MECHANISM OF GENOTOXICITY OF THE ENVIRONMENTAL POLLUTANT 3-NITROBENZANTHRONE

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Key words: 3-Nitrobenzanthrone/3-Aminobenzanthrone/ Cytochrome P450/NAD(P)H:quinone oxidoreductase/ Peroxidase/DNA adducts/^{32}P-postlabelling/Diesel/ Air pollution

3-Nitrobenzanthrone (3-NBA) is a suspected human carcinogen identified in diesel exhaust and air pollution. The article reviews the results of our laboratories showing which of the phase I and II enzymes are responsible for 3-NBA genotoxicity, participating in activation of 3-NBA and its human metabolite, 3-aminobenzanthrone (3-ABA), to species generating DNA adducts. Among the phase I enzymes, the most of the activation of 3-NBA *in vitro* is attributable to cytosolic NAD(P)H:quinone oxidoreductase (NQO1), while *N*,*O*-acetyltransferase (NAT), NAT2, followed by NAT1, sulfotransferase (SULT), SULT1A1 and, to a lesser extent, SULT1A2 are the major phase II enzymes activating 3-NBA. To evaluate the importance of hepatic cytosolic enzymes in relation to microsomal NADPH:cytochrome P450 (CYP) reductase (CPR) in the activation of 3-NBA *in vivo*, we treated hepatic CPR-null and wild-type C57BL/6 mice with 3-NBA. The results indicate that 3-NBA is predominately activated by cytosolic nitroreductases such as NQO1 rather than microsomal CPR. In the case of 3-ABA, CYP1A1/2 enzymes are essential for the oxidative activation of 3-ABA in livers. However, cells in the extrahepatic organs have the metabolic capacity to activate 3-ABA to form DNA adducts, independently from the CYP-mediated oxidation in the liver. Peroxidases such as prostaglandin H synthase, lactoperoxidase, myeloperoxidase, abundant in several extrahepatic tissues, generate DNA adducts, which are formed *in vivo* by 3-ABA or 3-NBA. The results suggest that both CYPs and peroxidases may play an important role in metabolism of 3-ABA to reactive species forming DNA adducts, participating in genotoxicity of this compounds and its parental counterpart, 3-NBA.

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PREPARATION AND CHARACTERIZATION OF STYRENE OXIDE ADDUCTS WITH AMINO ACIDS IN HUMAN GLOBIN

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Key words: Styrene oxide/Globin/Adducts/Pronase Cysteine/Histidine/Lysine

ABSTRACT

Styrene, an important monomer used in plastics industry, is metabolized to a genotoxic intermediate styrene-7,8-oxide (SO). Besides extensive detoxification through the enzyme-mediated hydrolysis, a minor part of SO can produce stable covalent adducts at various nucleophilic sites in the body, e.g. with DNA or proteins. In blood protein globin, the amino acids reported to form adducts with SO are Cys, His, Asp, Glu, N-terminal Val, Lys, and Tyr. However, several SO adduct species are commonly detected for each amino acid because of simultanous for-

mation of both 2-hydroxy-1-phenylethyl (21HPE) and 2-hydroxy-2-phenylethyl (22HPE) regioisomers, combined with the diastereomerism due to the chiral properties of SO. Additionally, binding of SO to His can occur via N_π or N_τ in the imidazol ring of His.

The main aim of our study was to identify major isomers of the SO adducts with Cys, Lys, and His in human globin. For reference purposes, the synthetic standards were prepared. The (*S*)- and (*R*)- SO enantiomers were reacted with Cys or N_α-protected Lys and His. Following deprotection and purification on a silica gel column, the individual isomers were separated by semi-preparative HPLC and characterized using GC/MS, HPLC/MS, and ^{1}H-NMR. Human globin incubated with a 100-fold molar excess of SO was hydrolyzed with pronase. Released amino acids and adducts were then derivatized by a terc-butyldimethylsilyl reagent, and analyzed by GC-MS. The most abundant regioisomers were 21HPE in Cys-SO and Lys-SO, and N_τ-22HPE in His-SO. All remaining regioisomers were also detected. The diastereomers were not resolved by GC, however, some pairs could be separated by HPLC. In the future, we wish to develop a sensitive method allowing determination of the amino acid-SO adducts in subjects occupationally exposed to styrene.

INTRODUCTION

Styrene, one of the most important monomers used in the manufacture of plastics and resins, is metabolized in rats and humans via hepatic cytochrome P-450 isozymes to the reactive metabolite styrene-7,8-oxide (SO)[1–3]. SO is mutagenic and genotoxic in various biological systems. The main metabolic route of SO involves its enzymatic hydrolysis to phenylethylene glycol and further oxidation to mandelic and phenylglyoxylic acids. These represent about 90 % of the absorbed styrene dose in man, and are rapidly excreted in urine[2]. Both acids are commonly determined as biomarkers of short-term exposures to styrene. Another metabolic pathway of SO, important in rodents but very minor in humans, is initiated by the conjugation of SO with glutathione, and finally results in the formation of N-acetyl-S-hydroxyphenylethyl cysteines (mercapturic acids) which are excreted in urine[1–3]. SO can also react with nucleophilic amino acid residues in plasma proteins and globin. If produced *in vivo*, such products (adducts) persist in the body for a long time, and may be used for biomonitoring. The most abundant covalent SO adducts with globin are formed at cysteine (Cys) and histidine (His), followed by carboxylic residues of aspartic and glutamic acids (Asp, Glu), lysine (Lys), N-terminal valine (Val), and tyrosine (Tyr)[4–7]. Several specific procedures have been developed to determine the adducts at selected amino acids, e.g., the N-terminal Val, Cys, or carboxylic residues. In contrast, the non-specific procedures permit to determine the complete adduct profile simultaneously. In our preliminary study[7] we systematically investigated the adducts in human globin (HG) treated with a 100-fold molar excess of SO. Globin was subjected to enzymatic hydrolysis with pronase which cleaved peptidic bonds between amino acids but not the bonds between the amino acids and SO. The hydrolysate was analyzed by GC/MS. For each reactive amino acid, several peaks corresponding to the isomers of amino acid-SO adducts were detected. This observation, which is in agreement with the reported analyses of various SO conjugates, is due to the presence of regio- and stereoisomers. Both carbon atoms in the oxirane ring of SO are reactive. Therefore, two positional isomers (regioisomers), namely, the 2-hydroxy-1-phenylethyl (21HPE) or 2-hydroxy-2-phenylethyl (22HPE) products from SO can be formed at each binding site. Further, SO is a chiral compound, existing in the form of (*R*)- and (*S*)-SO enantiomers. This is also the case of metabolically produced SO. The chiral centre is retained during the SO reactions, and is transferred on the adducts[2]. Doubled number of adduct species is expected in His-SO adducts, due to the possibility of adduct formation at N_π (N1) or N_τ (N3) of the His imidazole ring.

The main aim of this study was to identify the major regio- and stereoisomers of the SO adducts with Cys, His, and Lys in human globin treated in vitro with racemic SO. For this purpose, standards of the above compounds with pure SO enantiomers were prepared, characterized using GC/MS, HPLC/MS, and NMR spectrometry, and compared with the adducts present in biological material. The most abundant adducts will be further evaluated as potential biomarkers of the long-term cumulative exposures to styrene.

MATERIAL AND METHODS

Chemicals

(R)-Styrene-7,8-oxide (> 98%), L-cysteine, N_α-terc. butyloxycarbonyl-L-histidine (N_α-BOC-L-His), N_α-BOC-L-lysine, and *N*-tert-butyldimethylsilyl-*N*-methyl-trifluoroacetamide (MTBSTFA) were purchased from Fluka. (*S*)-Styrene-7,8-oxide (98 %) was from Aldrich. Pronase® (mixture of proteolytic enzymes from *Streptomyces griseus*) was bought from Calbiochem. All other chemical and solvents were of analytical grade and were purchased from various sources.

Preparation of synthetic standards

The Cys-SO adducts were prepared by direct reaction of L-Cys with a two-fold excess of either (R)- or (S)-SO in the presence of triethylamine. A mixture of the Cys-monoSO adducts free of parent Cys and Cys-bisSO adducts was obtained. The Lys-SO and His-SO adducts were prepared from the respective N_α-BOC-protected Lys and His, which were reacted with slight excess of SO enantiomers in the presence of triethylamine. The BOC-groups were then deprotected by trifluoroacetic acid. The crude reaction mixtures were purified by silica gel column chromatography to separate the mixture of the requested Lys-monoSO adducts and His-monoSO adducts from the unreacted amino acids and bisSO by-products. The progress of the reactions and purification procedures was monitored by thin-layer chromatography (TLC) on silica gel plates, using ninhydrin as a visualizing reagent.

The isomers of Cys-, Lys-, and His-monoSO adducts were then separated by semipreparative HPLC on a Synergi Polar RP column. Eluate fractions containing single components were collected and the solvent was evaporated to afford pure compounds. These were then characterized by HPLC/MS, GC/MS, and NMR spectrometry (data not shown here). The ratio of the regio- and stereoisomers in a mixture was assessed by HPLC using the ratio of peak areas at 220 nm. No difference in mass spectra was observed for the matching adducts prepared from (*R*)- and (*S*)-SO (below).

Cys-SO adducts (1, 2)

ESI-MS: m/z 242 $(M+H)^+$, 224 $(M+H-H_2O)^+$; specific for 1: 162, 121 $(CHPhCH_2OH)^+$, 103 $(PhCH=CH)^+$; specific for 2: 207, 135 $(M+H-CHOHPh)^+$ 120; GC/MS: (trisTBDMS derivatives): m/z 568 $(M-CH_3)^+$, 526 $[M-C(CH_3)_3]^+$, 498 $[M-C(CH_3)_3-(C=O)]^+$, 424 $(M-COOTBDMS)^+$, 316, 302, 262; specific for 1: 235 $(CHPhCH_2OTBDMS)^+$.

Lys-SO adducts (3, 4)

ESI-MS: m/z 267 $(M+H)^+$, 249 $(M+H-H_2O)^+$; specific for 3: 147; 130 $(M-NHCHPhCH_2OH)^+$, 121 $(CHPhCH_2OH)^+$, 103 $(PhCH=CH)^+$; GC/MS: (trisTBDMS derivatives): m/z 593 $(M-CH_3)^+$, 551 $[M-C(CH_3)_3]^+$, 419, 356, 300; specific for 3: 463 $(M-CH_2OTBDMS)^+$; specific for 4: 387 $(M-CHPhOTBDMS)^+$, 256.

His-SO adducts (5, 6, 7, 8)

ESI-MS: m/z 276 $(M+H)^+$, 258 $(M+H-H_2O)^+$, 156 $(M+H-CH_2CHOHPh)^+$ or $(M+H-CHPhCH_2OH)^+$; specific for 5: 110; specific for 6: 232, 110; specific for 7: 230, 110; specific for 8: 230, 215; GC/MS (trisTBDMS derivatives): 602 $(M-CH_3)^+$, 560 $[M-C(CH_3)_3]^+$, 532 $[M-C(CH_3)_3-(C=O)]^+$, 458 $(M-COOTBDMS)^+$, 400, 316, 302; specific for 5: 234 $(CHPhCH_2OTBDMS-H)^+$; specific for 7: 298, 235 $(CHPhCH_2OTBDMS)^+$.

List of the synthetic standards and their structures are presented in Table 1 and Fig. 1. Note that the configuration at the chiral carbon coming from SO is retained in the 22HPE products and inverted in the 21HPE products.

Table 1. The adducts of SO with cysteine, lysine and histidine. Structures of the compounds are shown in Fig. 1.

Amino acid	(R,S)-SO	(R)-SO	(S)-SO
	Regioisomers*	Diastereomers	
L-cysteine	S-(21HPE)-L-Cys: 1 S-(22HPE)-L-Cys: 2	1-S 2-R	1-R 2-S
L-lysine	N_ω-(21HPE)-L-Lys: 3 N_ω-(22HPE)-L-Lys: 4	3-S 4-R	3-R 4-S
L-histidine	N_π-(21HPE)-L-His: 5 N_π-(22HPE)-L-His: 6 N_τ-(21HPE)-L-His: 7 N_τ-(22HPE)-L-His: 8	5-S 6-R 7-S 8-R	5-R 6-S 7-R 8-S

* 21HPE: 2-hydroxy-1-phenylethyl

* 22HPE: 2-hydroxy-2-phenylethyl

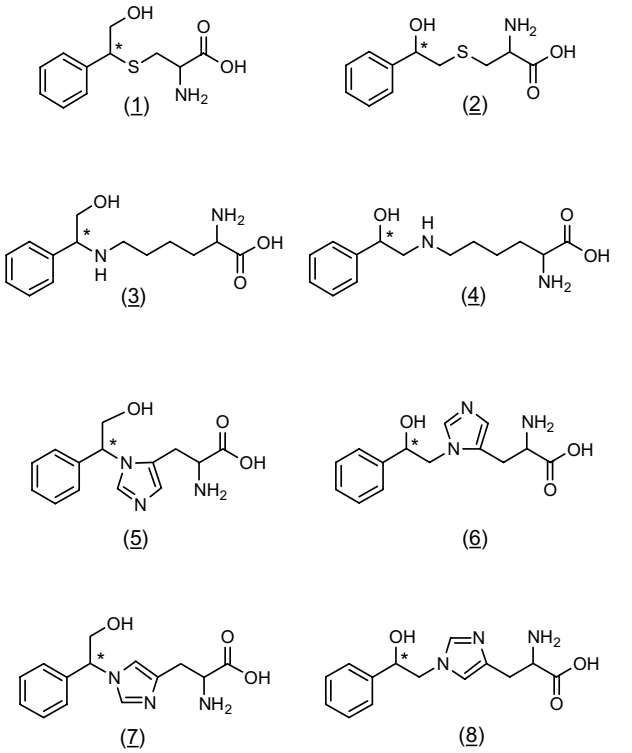

Fig. 1. Structure of SO adducts with cysteine, lysine and histidine. The compounds are marked in accordance with Table 1. The asterisk denotes the chiral centre in the SO moiety.

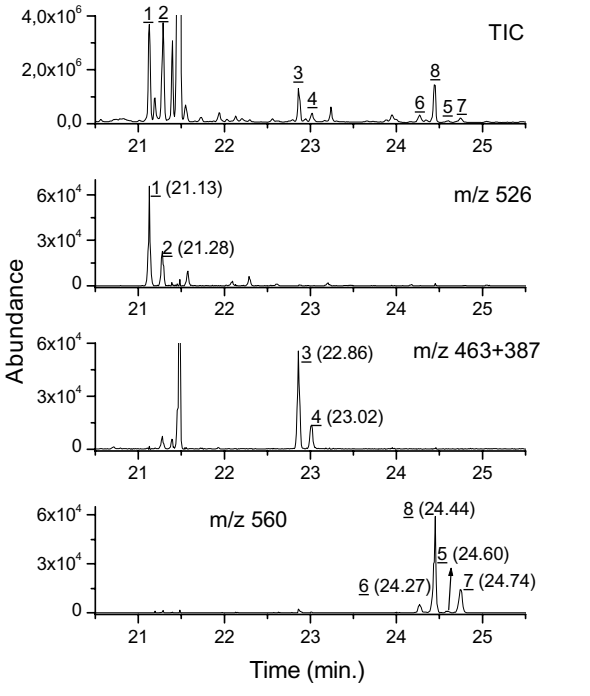

Fig. 2. GC-MS analysis of pronase hydrolysate of human globin treated with 100-fold molar excess of SO and derivatized by MTBSTFA. TIC: total ion chromatogram; m/z 526: detection of the adducts Cys-SO; m/z (463+387): detection of Lys-SO; m/z 560: detection of His-SO. No peaks at the retention times of these adducts were detected in pronase hydrolysate of control human globin.

Preparation of human globin modified with SO (HG-SO) in vitro

Full human blood was centrifuged to separate the erythrocytes. These were washed, hemolyzed, and diluted with phosphate buffer, pH 7.4, to a 5-fold volume of the blood sample. Racemic SO was added to a final concentration of 0.15 mmol/ml, which corresponded to the molar ratio SO/globin of ca. 100 : 1. After a 24 h incubation at 37 °C, globin was precipitated by a standard procedure using cold acetone with 2 % hydrochloric acid, washed, and dried.

Enzymatic hydrolysis

Globin sample (2 mg) was dissolved in water (0.25 ml), then 0.1 M phosphate buffer pH 7.4 (0.25 ml) and 1% suspension of pronase in buffer (50 µl) were added. The sample was incubated at 37°C for 24 hours, filtered on a 0.45 µm filter, and dried in vacuum. The residuum was derivatized for GC/MS analysis.

Derivatization procedure

The standards or dried globin hydrolyzates were dissolved in N,N-dimethylformamide (100 µl) and a silylating reagent (MTBSTFA, 50 µl) was added. The solution was heated in sealed vials for 1 hour at 110°C. The derivatized compounds were extracted into 200 µl of dry heptane and 1 µl of this solution was analyzed by GC/MS.

Instrumentation

Semi-preparative HPLC was performed on a Waters HPLC instrument which consisted from 606 pump and 490E UV detector. The Synergi Polar RP 80 Å, 4 µm column (250 × 10 mm) from Phenomenex was used. The compounds were eluted with water/acetonitrile mobile phase with 0.1% addition of TFA, and were monitored at 220 nm.

ESI-MS spectra were measured on a Bruker Daltonics Esquire 2000 MS spectrometer, equipped with the electrospray ionization (ESI) probe.

^{1}H-NMR spectra were measured on a Oxford Varian 300 NMR spectrometer, using d_6-dimethylsulfoxide as a solvent. The spectra were obtained in the Fourier transform mode.

GC/MS was performed on a Finnigan GCQ ion trap GC/MS system. Analysis was carried out on a DB-5ms fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm phase thickness) (J&W Scientific). The mass spectrometer was operated in electron impact (EI) ionization mode. Ion source temperature was held at 200 °C.

RESULTS AND DISCUSSION

Synthetic standards

The SO adducts of the selected amino acids (Cys, Lys, His) were prepared by direct reaction of SO with the reactive amino acid residues (Table 1, Fig. 1). However, only Cys could be used for the synthesis in a free form since the adduct formation at the SH group at ambient temperature greatly predominated over that at α-NH_2 group (no adducts at N_α were observed). The requested adducts with Lys and His had to be prepared from the intermediates with protected α-amino group at elevated temperature (80°C) so that SO could only react with the N_ω in Lys, and N_π or N_τ in His.

Reaction of L-Cys with the (*R*) or (*S*) enantiomers of SO afforded in each case both regioisomers, 21HPE and 22HPE, at a ratio 3:1. These were clearly distinguished by the characteristic ions in spectra of the 21HPE isomer, m/z 121 $(CHPhCH_2OH)^+$ in ESI-MS spectrum, and m/z 235 $(CHPhCH_2OTBDMS)^+$ in GC/MS spectrum. No difference was observed in ESI-MS, GC/MS and NMR spectra of the pairs of diastereomers i.e., 1-*S*/1-*R* and 2-*S*/2-*R*. All four Cys-SO diastereomers were separated well on the HPLC column. Somewhat surprisingly, the elution order was 1-*R*, 2-*R*, 2-*S*, 1-*S*, which means an inverse elution order of the matching regioisomers derived from the opposite SO enantiomers.

In Lys-SO adducts prepared from both SO enantiomers, the elution order of the regioisomers on HPLC column was 21HPE and 22HPE and their ratio was 1.3 : 1. The regioisomers were distinguished using characteristic ions in the spectra of the 21HPE adducts, e.g., m/z 121 $(PhCHCH_2OH)^+$ or m/z 463 $(M\text{-}CH_2OTBDMS)^+$. Moreover, a characteristic ion m/z 387 $(M\text{-}CHPhOTBDMS)^+$ was present in GC/MS spectrum of the 22HPE isomer. Unlike with the Cys-SO adducts, the matching regioisomers produced from the opposite SO enantiomers exhibited not only identical spectral data but also identical retention times in HPLC.

With regard to His-SO adducts, four well resolved products in the ratio of ca. 5:1:28:18 were detected for each SO enantiomer. This corresponds to regioisomerism on both SO and His moieties (21HPE vs. 22HPE; N_π-His vs. N_τ-His). Of the matching four diastereomeric pairs, only one pair (5-R /5-S) was resolved by HPLC. Among the His-SO adducts, 21HPE and 22HPE were distinguished by characteristic ions in spectra of the 21HPE adducts. On the other hand, we were not confident which of the adducts were N_π and N_τ isomers, as we were unable to interpret the observed differences in the NMR spectra. In previous studies on His adducts with propylene oxide[8] and sulphur mustard[9], the N_τ isomers were found to be less polar and, therefore, faster eluting on a silica gel column. In agreement with this, elution on a reversed-phase column should be in the order N_π, N_τ. By combining all data together, we hypothesize that the elution of the His-SO adducts in HPLC was in the order: N_π-22HPE, N_π-21HPE, N_τ-22HPE, N_τ-21HPE.

SO adducts in globin

The enzymatic hydrolysate of HG-SO (prepared with racemic SO) was silylated and analyzed by GC/MS together with the synthetic standards. All adducts 1 to 8 were detected, the major species being 1, 3, and 8 (Fig. 2). Thus, the major regioisomers in Cys-SO and Lys-SO were 21HPE, and N_τ-22HPE in His-SO. None of these products was detected in control globin. The current GC/MS analy-

sis does not allow to separate the diastereomers. Thus, in its present form it would not permit to study the stereoselectivity of SO formation and binding to amino acid residues following an in vivo exposure to styrene. This can be partially achived using HPLC (for Cys-SO adducts), or perhaps on a more suitable GC column. Nevertheless, knowledge on the diastereomeric composition of the identified regioisomers is not necessary if the measurement is used for biomonitoring purposes. In future stage of the project, highly sensitive method allowing determination of the major SO-amino acid adducts in subjects exposed to styrene will be developed.

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REACTIVE OXYGEN SPECIES – CAUSE OR CONSEQUENCE OF TISSUE INJURY?

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Key words: Reactive oxygen species/Antioxidants/ Oxidative stress/Tissue injury

Reactive oxygen species (ROS)-mediated harmful actions towards essential constituents of the cell are considered to be essential events in the etiopathogenesis of those diseases where ROS have been implicated. Since ROS easily react *in vitro* with most biological molecules, causing their degradation and destruction, for some investigators this implicitly suggests that ROS, when excessively produced, are deleterious to integral cellular components *in vivo* and may cause their dysfunctions. Some experimental data indicate that ROS-mediated lipid peroxidation, protein oxidation and oxidative alterations to nucleic acids are crucial events of unfavorable actions of ROS. Yet the most convincing evidence, i.e. unambiguous inhibition of tissue injury by pretreatment with antioxidants, has not been provided. On the contrary, there are quite a few papers reporting failure in applying antioxidants to heal those pathologies where the causal role of ROS was supposed. Other papers reported serious complications arising from antioxidant therapy, which is quite in contradiction to its expected effect. Moreover, an increasing number of recent findings have provided evidence of a key role of ROS in both intracellular signaling and intercellular communication, processes involved in maintaining homeostasis. Hence, other investigators consider excessive production of ROS to be rather a smoke after the fire than a deleterious fire itself, suggesting the occurrence of overproduced ROS as being the consequence of tissue damage.

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CARCINOGENIC POLLUTANTS *o*-NITROANISOLE AND *o*-ANISIDINE ARE SUBSTRATES AND INDUCERS OF CYTOCHROMES P450

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Key words: o-Nitroanisole/o-Anisidine/Carcinogen/ Cytochromes P450/Induction

2-Methoxyaniline (*o*-anisidine) and 2-methoxynitrobenzene (*o*-nitroanisole) are important pollutants and potent carcinogens for rodents. *o*-Anisidine is oxidized by microsomes of rats and rabbits to *N*-(2-methoxyphenyl)hydroxylamine that is also formed as the reduction metabolite of *o*-nitroanisole. *o*-Anisidine is a promiscuity substrate of rat and rabbit cytochromes P450 (CYP), because cytochromes P450 of 1A, 2B, 2E and 3A subfamilies oxidize *o*-anisidine. Using purified cytochrome P450 enzymes, reconstituted with NADPH:cytochrome P450 reductase, rabbit cytochrome P450 2E1 was the most efficient enzyme oxidizing *o*-anisidine, but the ability of cytochromes P450 1A1, 1A2, 2B2, 2B4 and 3A6 to participate in *o*-anisidine oxidation was also proved. Utilizing Western blotting and consecutive immunoquantification employing chicken polyclonal antibodies raised against various CYPs, the effect of *o*-anisidine and *o*-nitroanisole on the expression of the CYP enzymes was investigated. The expression of CYP1A1/2 was found to be strongly induced in rats treated with either compounds. In addition, 7-ethoxyresorufin *O*-deethylation, a marker activity for both CYP1A1 and 1A2, was significantly increased in rats treated with either carcinogen. The data demonstrate the participation of different rat and rabbit cytochromes P450 in *o*-anisidine oxidation and indicate that both experimental animal species might serve as suitable models to mimic the *o*-anisidine oxidation in human. Furthermore, by induction of rat hepatic and renal CYP1A1/2, both *o*-nitroanisole and *o*-anisidine influence their carcinogenic effects, modifying their detoxification and/or activation pathways.

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ANTITUMOR DRUG ELLIPTICINE INHIBITS THE ACTIVITIES OF RAT HEPATIC CYTOCHROMES P450

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Key words: Ellipticine/Anticancer drug/Cytochrome P450/ Inhibition/Binding

Ellipticine is a potent antineoplastic agent, whose mode of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II. Recently, we found that ellipticine also forms the cytochrome P450 (CYP)-mediated covalent DNA adducts. Here, we study the effect of ellipticine on CYP enzymes in rat hepatic microsomes, studying its binding to the enzymes and its potential to inhibit the CYP activities measured with their selective substrates. Although ellipticine was reported to be a selective and strong inhibitor of CYP1A1/2, we found that its inhibitory potential is non-specific. Ellipticine is the most potent inhibitor for CYP3A-dependent 6β-hydroxylation of progesterone, followed by CYP1A1/2-dependent ethoxyresorufin O-deethylation and CYP2B-mediated pentoxyresorufin O-dependentylation. Lower inhibition was detected for 1'-hydroxylation of bufurarol, 21-hydroxylation of progesterone and 6-hydroxylation of chlorzoxazone catalyzed by CYP2D, CYP2C and CYP2E1, respectively. Ellipticine binds to several CYPs of rat hepatic microsomes. The binding titration of ellipticine typically give reverse type I spectrum with CYPs in rat hepatic microsomes. The results indicate that inhibition of CYPs by ellipticine cannot be explained only by its differential potency to bind to individual CYPs.

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OXIDATION OF AN ANTITUMOR DRUG ELLIPTICINE BY PEROXIDASES

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Key words: Ellipticine/Anticancer drug/Peroxidase/ Cytochrome P450/Oxidation

Ellipticine is a potent antineoplastic agent, whose mode of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II. Since we found that ellipticine also forms the cytochrome P450 (CYP)-mediated covalent DNA adducts, this anticancer drug is considered to function as a pro-drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues. Here, we demonstrate that ellipticine is also oxidized by peroxidases, which are abundantly expressed in several target tumor tissues. Lactoperoxidase, myeloperoxidase and horseradish peroxidase were used as models. Peroxidases in the presence of hydrogen peroxide oxidize ellipticine to an ellipticine dimer and N^2-oxide of ellipticine as the major and minor metabolite, respectively. Inhibition of the peroxidase-mediated ellipticine oxidation by radical scavengers ascorbate, glutathione and NADH suggests a one-electron mechanism of the oxidation. The implication of the oxidation of ellipticine by peroxidases in its mechanism of action is discussed.

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ANTIMUTAGENIC EFFECT OF PHENOLIC ACIDS

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Key words: Phenolic acid/Antimutagenity/Salmonella typhimurium

In the present study, the *Salmonella typhimurium* tester strain TA 100 was used in the plate-incorporation test to examine the antimutagenic potential of caffeic, ferulic and cichoric acids extracted from plant species of genera *Echinacea* (L) Moench, as well as of another phenolic acids, on 3-(5-nitro-2-furyl)acrylic acid (5NFAA) and sodium azide mutagenicity. All tested compounds possess antimutagenic activity. In the case of 5NFAA, the antimutagenic potency of tested compounds was in the order of gallic acid>ferulic acid>caffeic acid>syringic acid>vanillic acid. The mutagenic effect of sodium azide was inhibited by tested phenolic acids by about 20–35 %. The most effective compound, gallic acid inhibits this effect by 82 % in the concentration of 500 μg/plate. The only exception from favourable properties of tested phenolic acids is cichoric acid, which in the contrary significantly increased the mutagenic effect of 5NFAA.

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THE EFFECT OF PHENOLIC AND POLYPHENOLIC COMPOUNDS ON THE DEVELOPMENT OF DRUG RESISTANCE

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Key words: Antibiotic resistance/Ciprofloxacin/ Gentamicin/Antimutagen

The effect of two phenolic compounds vanillin (4-hydroxy-3-methoxybenzaldehyde) and lignin on the development of drug/antibiotic resistance in *Salmonella*

typhimurium was studied. Using the modified Ames test we have shown that vanillin alone has negligible effect on spontaneous mutability to ciprofloxacin and gentamicin resistance. At the tested concentrations vanillin reduces the toxicity of 4-nitroquinoline-N-oxide (4NQO) and reduces the ability of this compound to induce mutations leading to ciprofloxacin but not to gentamicin resistance. Lignin at higher concentrations increases mutagenicity to ciprofloxacin resistance and possess considerable inhibition effect on the spontaneous and 4NQO induced mutability to gentamicin resistance.

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DEVELOPMENT OF CIPROFLOXACIN RESISTANCE DUE TO CHROMOSOMAL MUTATIONS INDUCED BY 2-NITROFLUORENE

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Key words: 2-Nitrofluoren/Antibiotic resistance/ Ciprofloxacin/Salmonella typhimurium

In this study we investigated the ability of 2-nitrofluorene to induce mutations leading to antibiotic resistance in quinolone sensitive strain *Salmonela typhimurium*. After preincubation of bacteria with 2-nitrofluorene, the frequency of mutation to ciprofloxacin resistance was 57 fold higher than in the case of spontaneous mutability. Some of resultant resistant colonies showed a great increase of ciprofloxacin MIC.

ACKNOWLEDGEMENT

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THE ROLE OF NATURAL BIOPOLYMERS IN GENOTOXICITY OF MUTAGENS/ CARCINOGENS ELIMINATION

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Key words: Antigenotoxicity/Carboxymethyl glucan/ Biological response modifiers/Cancer prevention and therapy/Natural biopolymer

Nowadays naturally occuring compounds with the potential antimutagenic and anticarcinogenic effects are of great importance for their prospective use in cancer chemoprevention and treatment. The new water soluble derivative of microbial polysaccharide β-D-glucan – carboxymethyl glucan (CMG) belongs to such a category of natural substances. CMG isolated from the cell wall of baker's yeast *Saccharomyces cerevisiae* is included into the class of biopolymers known as biological response modifiers (BRMs) with a broad range of activities, above all ones interfering with cancer therapy. It was demonstrated on four experimental model systems that biological and consequential medicinal importance of CMG is based on the combined application with another active compound. In the *Saccharomyces cerevisiae* antimutagenicity assay CMG significantly reduced ofloxacin-induced mutagenicity in the yeast strain D7. CMG exerted bioprotective (anti-toxic and antimutagenic) effect after its simultaneos application with methyl methanesulphonate on the repair-deficient strain *uvs10* of the unicellular green alga *Chlamydomonas reinhardtii.* In the *Vicia sativa* simultaneous phytotoxicity and anti clastogenicity assay CMG exerted statistically significant anticlastogenic efect against maleic hydrazide-induced clastogenicity in *Vicia sativa* L. Only in the Salmonella/microsome assay CMG did not exert statistically significant antigenotoxic effect, despite of the fact that it reduced 9-aminoacridine-induced mutagenicity in *S. typhimurium* TA97, but his^+ revertants decreasing was statistically significant only at the highest CMG concentration used. The data presented unambiguously documented that even biopolysaccharides (e.g., derivatives of β-glucan) belonging to the most abundant class of natural biopolymers may contribute to cancer prevention and therapy.

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THE PRENATAL CHLAMYDIAL AND MYCOPLASMAL INFECTIONS AND THEIR RELATIONSHIP TO OROFACIAL CLEFTS

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Chlamydia and *Mycoplasma* are microorganisms widespread in nature and have many common features, namely similar clinical symptoms in urogenital and respiratory tracts. Some results referred about of possibility to cause congenital defects.

In our work we determined whether specific IgG and IgA antibodies in sera of mothers and their newborns with orofacial cleft (OC) are formed in response to *Mycoplasma pneumoniae* and *Chlamydia trachomatis* infections as compared to mothers and their healthy newborns; sera were tested by ELISA method (r ELISA medac, Germany).

Of 90 sera tested for *M. pneumoniae* antibodies, we found IgG antibodies in 3 (3.3 %) samples of newborns with OC and 25 (27.8 %) of mothers, respectively. Ten (11.1 %) and 3 (3.3 %) of them had also IgA antibodies. In healthy mothers and newborns (41 samples each) IgG antibodies were found in 7 (17.0 %) and 4 (9.8 %) sera, respectively. IgA was detected only in two (4.9 %) and one (2.4 %) serum sample, respectively.

At the same time, IgG antibodies to *C. trachomatis* were found in 3 (3.3 %) and one (1.1 %) respectively. IgA antibodies were not detected. In healthy mothers and newborns IgG antibodies were detected in two (4.9 %) newborns and mothers samples each. IgA antibodies were found in 3 (7.3 %) mother samples only.

Our findings do not exclude the possibility of contracting the *M. pneumoniae* infection from infected mothers and influencing on intrauterine developing of fetus. The occurrence of specific antibodies to *C. trachomatis* is low and similar to that in the control groups, so that in our study this microorganism could not have any effect on developing the OC.

The risk of occurrence of congenital defects associated with intrauterine exposure to *M. pneumoniae* and *C. trachomatis* remains unclear and more studies are needed to ascertain the possible role of these microorganisms also in creation of OC.

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AUTHOR INDEX

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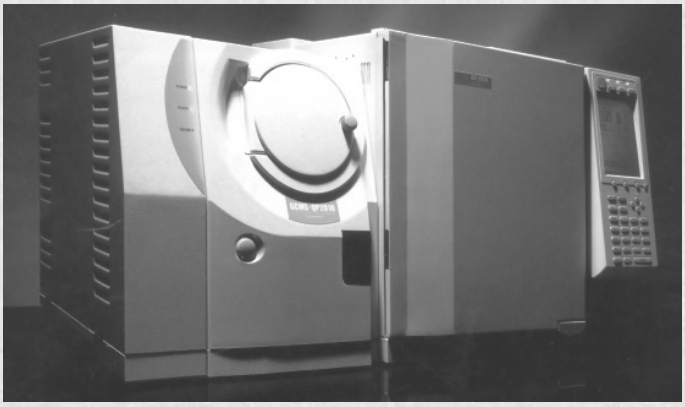

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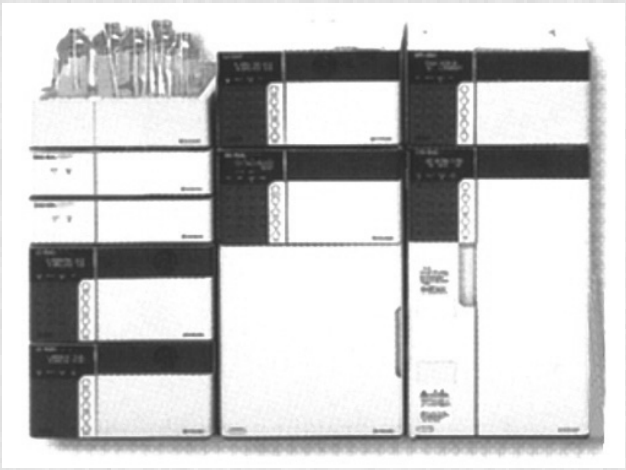

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extrakce sorbenty, kolony
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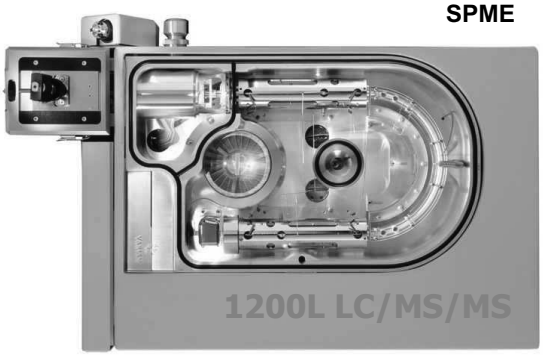
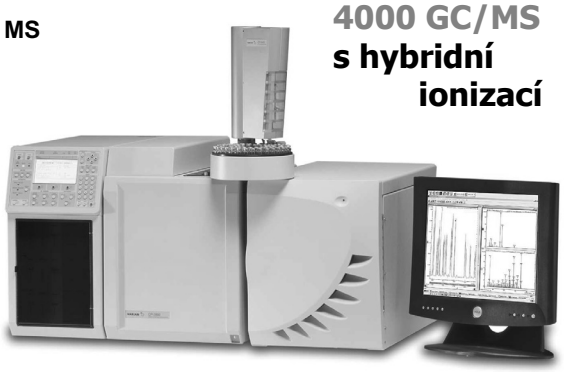


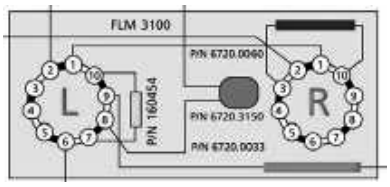
disoluční systémy pro testování tablet
fyzikální testy tablet (VanKel)

4000 GC/MS
s hybridní ionizací



AAS, ICP-MS, ICP-OES, UV/VIS/NIR
fluorescenční spektrometrie
FT-IR, analyzátory olejů
FT-IR mikroskopie
Ramanova spektrometrie

DIONEX



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