POSSIBILITIES AND PROBLEMS WITH IDENTIFICATION AND DETERMINATION OF “NEW” HYPNOTICS

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Authors discuss problems with identification and determination of flunitrazepam and zolpidem in biological material (BM). 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 BM. 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.

INTRODUCTION

In the 1990s a significant increase in findings of benzodiazepine drugs occurred in forensic and clinical toxicological examinations of biological material (BM). In this period, benzodiazepine hypnotic drugs largely began to replace the at-that-time commonly used barbiturate hypnotics, mainly because of their lower toxicity and adverse side effects. Within the wide range of all used benzodiazepines, it was flunitrazepam, the active substance of Rohypnol™, that became the most frequently abused drug, particularly for a deliberate induction of sleep because of crime. Its identification and determination in BM was at first associated with various analytical problems¹.

By the end of the 20th century, another change in the field of hypnotics abuse occurred, specifically in connection with the use of new non-benzodiazepine hypnotic drugs such as zopiclon and zolpidem. Above all, zolpidem became the substance that replaced flunitrazepam not only in the treatment of insomnia, but in commission of criminal acts as well. The presented study deals with the problems of identification and determination of flunitrazepam and zolpidem in BM, especially in toxicological forensic cases. The similar problems have recently been solved by many toxicological laboratories^{2–4}.

MATERIAL AND METHODS

For the objective assessment of the degree of flunitrazepam and zolpidem intoxication blood and urine are the most frequently analysed BM in forensic toxicological cases. The blood analysis serves for determination of pertinent substance quantity, while the urine analysis assesses the substance quality.

Liquid-liquid (L-L) extraction was used to extract zolpidem and flunitrazepam metabolite (7-aminoflunitrazepam). These were extracted by diethylether from 50 ml of urine under i) acidic conditions (pH 1, M_A fraction obtained) and ii) alkaline conditions (pH 10, M_B fraction obtained).

Solid Phase Extraction (SPE – Bond Elut Certify™ columns – Varian) was used to extract zolpidem from BM under alkaline conditions. 1 ml of urine, blood or plasma was processed according to Rokus de Zeeuw^{6–7} (fraction M_B was obtained).

„Freeze out“ methods was used for isolation of flunitrazepam from blood or serum: 200 µl of serum + 20 µl of 2M TRIS buffer of pH 9 + 50 µl of butylacetate were mixed and shaken for 5 min. After centrifugation at 5000 × g for 10 min, the serum with a separated organic phase was kept at – 20 °C for 30 min. The upper layer was removed and 1 µl of it was applied to GC-MS.

Thin Layer Chromatography is suitable for zolpidem and the flunitrazepam metabolite (7-aminoflunitrazepam) identification in the M_B fraction after their L-L extraction. The modified procedure according to Večerková

was employed with Bratton-Marshall⁸ location reagent for 7-aminoflunitrazepam, and sulphuric acid in ethanol as the location reagent for zolpidem (ultraviolet light at 366 nm).

Gas Chromatography with Mass Spectrometry was used for zolpidem identification in the M_B fraction after its isolation by SPE method (GC TRACE 2000, MD – PolarisQ, Thermo Finnigan), equipped with the capil-

lary column ZB 5 MS (length 15 m $\times$ I.D. 0.25 mm $\times$ film thickness 0.25 μ m), injector temperature 230 °C, column temperature 70 °C, detector temperature 230 °C, temperature gradient 70 °C (1 min), programming 15 °C/min to final temperature 260 °C (held for 10 min), TIC mode – m/z range 40-450 AMU, or SIR mode – m/z 235 AMU for trace analysis.

Fig. 1. Total ion current and selected ion monitoring chromatograms of zolpidem in a patient's blood (t_R 15.04 min. – zolpidem).

Fig. 2. Mass spectrum of zolpidem in a patient's blood.

Fig. 3. Gas chromatogram of flunitrazepam in a patient's blood ($t_R = 5.31$ min. – lorazepam as IS, $t_R = 6.51$ min. – flunitrazepam).

Fig. 4. Gas chromatogram of zolpidem in a patient's blood ($t_R = 15.91$ min. – amitriptyline as IS, $t_R = 21.30$ min. – zolpidem).

Table 1. Concentration range of flunitrazepam and zolpidem, correlation coefficient R^2 , LOD and methods of determination

Analytes	Concentrat. Range $\mu\text{g/ml}$	R^2	LOD ng/ml	Methods
Flunitrazepam	0.001 – 1.0	0.9987	0.5	GC-ECD
Zolpidem	0.01 – 10.0	0.9971	3	GC-NPD

Gas Chromatography with electron capture detector (GC-ECD) was applied to determine flunitrazepam in blood (serum) after the extraction by the „freeze out“ method done with PerkinElmer device (AutoSystem XL), equipped with capillary column ZB 5 (length $15 \text{ m} \times \text{I.D. } 0.25 \text{ mm} \times \text{film thickness } 0.25 \text{ mm}$), injector temperature 160°C , column temperature 180°C , detector temperature 300°C , temperature gradient 180°C , programming 20°C/min to temperature 250°C , then programming 10°C/min to final temperature 280°C (held for 5 min).

Gas Chromatography with nitrogen-phosphorus detector (GC-NPD) was applied to determine zolpidem in blood (serum) after its extraction by the SPE method (AutoSystem XL, PerkinElmer), capillary column ZB 5 (length $15 \text{ m} \times \text{I.D. } 0.25 \text{ mm} \times \text{film thickness } 0.25 \text{ mm}$), injector temperature 70°C , column temperature 250°C , detector temperature 280°C , temperature gradient 70°C , programming 10°C/min to temperature 280°C (held for 10 min).

For the assessment of zolpidem quantity in blood or serum also the GC-MS method (under the conditions mentioned above) was applied, nevertheless the GC-NPD technique proved to be more suitable due to a non-linear response of the MS detector.

RESULTS AND DISCUSSION

Urine is the most commonly used BM, analysed for the presence of toxicologically important substances including flunitrazepam and zolpidem. Frequent procedures employed for the screening of benzodiazepines are the immunochemical methods, whose main advantage lies in a direct urine analysis without any sample preparation.

The immunochemical method FPIA (Fluorescence Polarization Immunoassay) was performed in our lab with ADx or AxSYM analysers (Abbott) on urine samples for the screening of benzodiazepines. However, in the case of flunitrazepam or its metabolite, the applied systems demonstrate false negativity, thus being unsuitable for the screening of this metabolite in urine⁵. Instead, the thin layer chromatography (TLC) has to be used. The TLC can also be applied for the screening of zolpidem, yet it is not always sensitive sufficiently.

This method is sensitive enough after ingestion of a larger amount of tablets together with collection of the urine sample in an appropriate time relation. When the concentrations of zolpidem in urine are low, the application of SPE followed by GC-MS in SIR mode is required. The retention time (Fig. 1) and the mass spectrum were used for its identification (Fig. 2).

To assess the quantitative representation of flunitrazepam and zolpidem in real samples of blood or serum, which is of major importance for objective evaluation of the influence of the substances on the user, the application of sensitive methods GC-ECD and GC-NPD is required, respectively (Fig. 3, Fig 4). The linear response of ECD and NPD was verified using the standard solutions of flunitrazepam and zolpidem in the concentration range of $0.001 - 100 \text{ mg/l}$ and $0.01 - 10.0 \text{ mg/l}$, respectively (injection volume $1 \mu\text{l}$). The concentration ranges were chosen with respect to the method sensitivity and the actual concentration of measured substances in real blood samples. To evaluate the regression curve slope, section on y axes

as well as correlation coefficient R^2 , the method of linear regression was employed. The calibration curves parameters including LOD (Limits of Detection) are shown in Table 1. Since the response of MS detection is non-linear, the GC-MS method remains unsuitable for the assessment of zolpidem in blood or serum.

The identification and possibly also determination of hypnotic drugs (flunitrazepam and zolpidem) is crucial for the objective assessment of the actual impact onto the drug user. Thus such findings contribute to solving severe criminal cases.

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