ANALYSIS OF ESTAZOLAM IN POST-MORTEM MATERIAL

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ABSTRACT: In this study, an evaluation of suitable extraction and clean up techniques for Estazolam in whole blood and organ material was done. Solvent extraction methods were compared with solid-phase extraction using Extrelut® or modified silica adsorbents. For end-step detection method TLC, UV-spectrophotometry, high pressure liquid chromatography (HPLC) with UV detector and gas chromatography-mass spectrometry (GC/MSD) were evaluated.

KEY WORDS: Estazolam; Post-mortem material; Solid-phase extraction; GC/MS.

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INTRODUCTION

Estazolam (Estazolam, Nuctalon, ProSom, Eurodin) is triazolobenzodiazepine derivate first obtained by K. Megura i Y. Kuwada in 1969 [10]. Estazolam is an effective, low dosed new generation hypnotic introduced to the Polish market in 1988.

Estazolam exerts sedative, hypnotic, anti-anxiety, anti-convulsive and central muscle relaxant activity. It effects the central nervous system, especially the limbic system and hypothalamus. Like other benzodiazepines it increases the inhibition of GABA-dependent neuronal transmission [10].

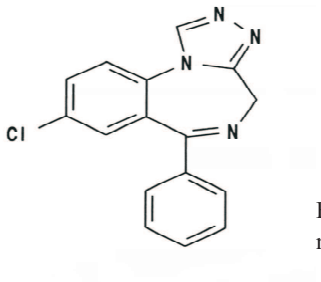

Fig. 1. Structure of estazolam (8-chloro-6-phenyl-4H-[1,2,4]-triazolo [4,3-a] [1,4] benzodiazepine).

Estazolam is easily resorbed from the gastrointestinal tract reaching its maximal concentration in plasma after about 2 hours. The protein binding rate in plasma is 93%. Estazolam is metabolised in the liver to 1-oxo-estazolam and 4-hydroxy-estazolam. The metabolites are mainly eliminated *via* urine [11]. The biological half-life ($T_{0.5}$) of estazolam is about 17.0 hours (8.3–31.2 h) [1]. Estazolam penetrates the placental barrier. It is excreted in small amounts with the milk. Typical therapeutical doses range from 2–6 mg/day, and therapeutic plasma concentrations range from 0.055–0.1 mg/l [16]. There are no data concerning toxic or fatal doses.

Several other analytical methods are described in the literature for screening and quantitation of estazolam such as spectrophotometric methods [6], immunoassays [8, 13], GC [4, 5, 9], capillary electrophoresis [15] and mostly HPLC [2, 3, 7, 12, 14]. All of these methods have generally been developed for the analysis of estazolam in plasma and urine.

This paper describes the identification of estazolam in postmortem material (blood, urine, liver, kidney, bile). The efficiency of the isolation of estazolam from biological material by the use of 3 methods was compared. The methods were: conventional liquid-liquid extraction, Extrelut® extraction and solid-phase extraction using narc-2 columns. Qualitative and quantitative analyses were performed using of thin-layer chromatography (TLC), UV-spectrophotometry, high pressure liquid chromatography (HPLC) and gas chromatography with mass-selective detector (GC/MS).

CASES

The following three cases, in which estazolam was identified during postmortem toxicological analysis, were investigated by the Institute of Forensic Medicine, Medical Academy in Bydgoszcz, Poland. Complete autopsies were performed in each case including routine toxicological screening. A brief history of each case is given in Table I.

TABLE I. HISTORIES OF THE PRESENTED CASES

Case	History
1	39-year-old woman found dead in her bedroom, history of depression
2	15-year-old woman, suspicion of suicidal poisoning
3	25-year-old man, known history of heart disease

EXPERIMENTAL

Materials and reagents

Estazolam and IS (diazepam) were purchased from Polfa (Poland) and dissolved in distilled water to give a concentration of 1 mg/ml each. All other chemicals used were analytical grade and obtained from Merck (Darmstadt, Germany). Solid-phase extraction columns used were 3 ml Bakerbond disposable extraction columns packed with 200 mg narc-2 (Baker, Holland). Extrelut® 20 was purchased from Merck (Darmstadt, Germany).

The evaluate the method by stocking experiments biological fluids, e.g. urine, blood, bile and organ samples (liver, kidney) were obtained from cases in which a toxicological investigation should reveal the cause of death and which were tested negative for estazolam and diazepam.

Sample preparation

100 µl of estazolam (1 µg/ml) and 100 µl of diazepam (1 µg/ml) as internal standard and were added to 1 ml or 1 g of each specimen in 5 ml glass tubes. The tubes were mixed for 5 min and centrifuged at 3000 rpm for 10 min.

Extraction procedure

Isolation of estazolam from biological material was performed using conventional liquid-liquid extraction, Extrelut® extraction and solid-phase extraction with Bakerbond narc-columns in accordance with the procedures presented in Figures 2, 3, 4.

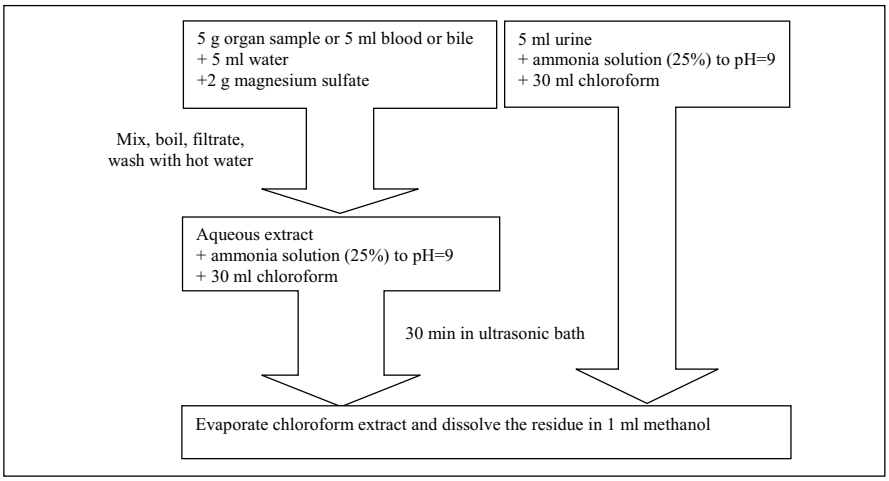

Fig. 2. Procedure for liquid-liquid extraction.

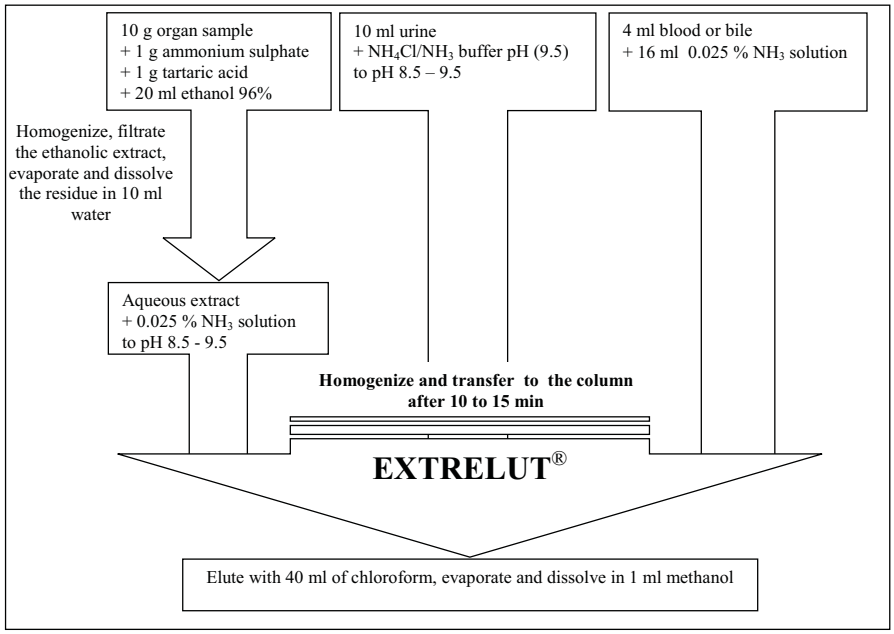

Fig. 3. Procedure for extraction with the use Extrelut®.

Instrumentation

TLC

Estazolam was determined using Merck Silica gel GF_{254} plates (Darmstadt, Germany) under the conditions presented in Table II.

UV measurement

Ultraviolet spectra of the extracted samples were determined between 200 and 500 nm on a Pharmacia LKB spectrometer (Ultrospec III, Pharmacia, Sweden) after dissolving the residues in 0.1 M HCl (absorbance maximum 267 nm). In 0.1 M sodium hydroxide and in methanol estazolam does not give a absorbance maximum.

HPLC

HPLC analyses were performed on a Pharmacia LKB liquid chromatograph (Pharmacia, Sweden) with a pump type 2248, a UV-detector type VWM 2141 and a Supelcosil LC-18DB column (15 cm x 4.6 mm, 5 μm, Supelco, USA). Samples were eluted at a flow-rate of 1.2 ml/min using methanol/water (65:35; v/v) as an eluent. The UV-detector was set to a wavelength of 230 nm, diazepam was used as internal standard.

The retention times of estazolam and diazepam (IS) were 4.0 min and 5.6 min, respectively.

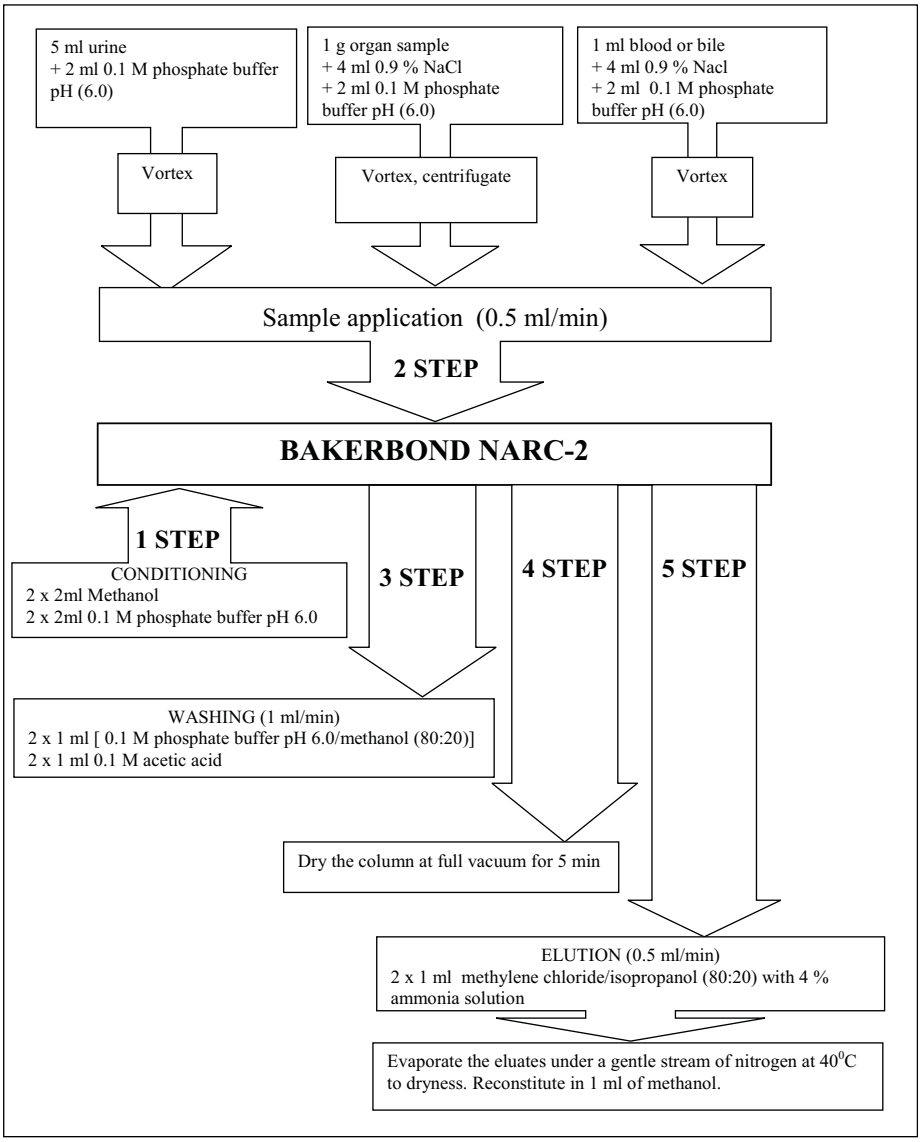

Fig. 4. Procedure for solid-phase extraction using narc-2 column.

TABLE II. CONDITIONS FOR ESTAZOLAM ANALYSIS USING THIN-LAYER CHROMATOGRAPHY

Mobilep Phase	Rf	
1. Methanol/ammonia 25% solution (100:1,5)	71	
2. Cyclohexane/toluene/diethylamine (75:15:10)	00	
3. Chloroform/methanol (90:10)	50	
4. Chloroform/acetone (90:10)	01	
5. Ethyl acetate/methanol/ammonia 25% solution (85:10:5)	45	
6. Ethyl acetate	08	
Detection	Colour	Limit of detection
1. Dragendorff spray reagent	Orange	500 ng
2. Acidified iodoplatinate solution	Blue-violet	600 ng
3. UV	–	100 ng
Marquis reagent, Mandelin's reagent, Forrest reagent, acidified potassium permanganate give negative results for estazolam.		

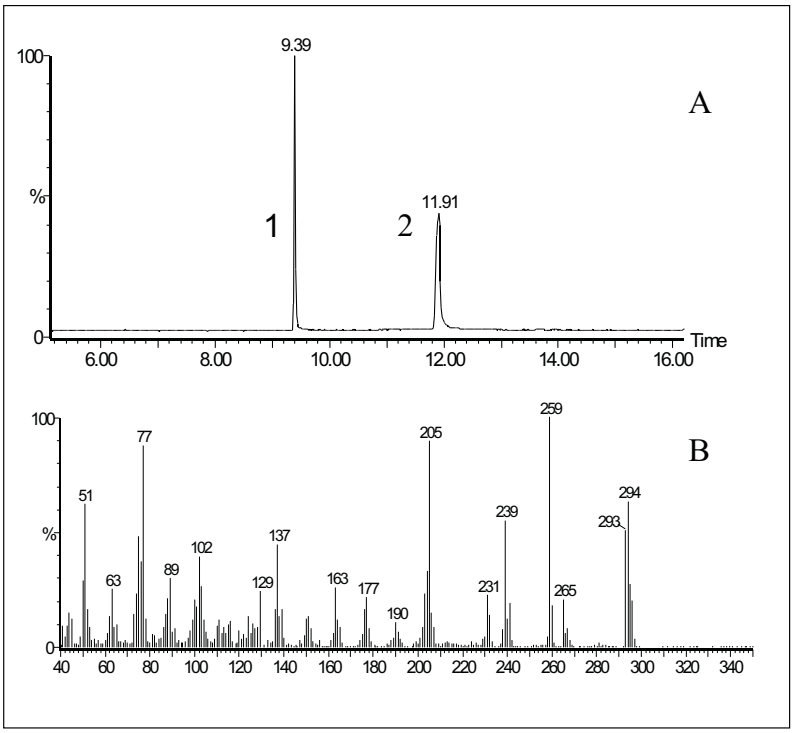

Fig. 5. a) Gas chromatogram of an extracted blood specimen using narc-2 columns. Peak identification: 1. internal standard-diazepam (t_R = 9.39); 2. estazolam (t_R = 11.91); b) full scan mass spectrum of estazolam.

GC/MS

The samples were analysed using a Perkin-Elmer AutoSystem XL with a turbo mass-detector. The analytes were separated on a Perkin Elmer PE-5ms capillary column (30 m x 0.32 mm; 0.25 μm film thickness). Helium was used as a carrier gas with a flow rate of 50 cm/s. The splitless injection mode was used with 1 μl samples being injected. The injection port and the transfer line were set at 210°C and 275°C, respectively. The oven temperature started at 150°C and was hold for 3 min, increased first with a rate of 30°C/min to 200°C, then with a rate of 15°C/min to 300°C. The final temperature was hold for 5 min. Data were automatically processed with so called "macros" using the Willey's database for reference mass spectra and unequivocal identification.

RESULTS AND DISCUSSION

Quantitative analysis of estazolam was performed by GC/MS. The mass spectrum of estazolam is presented in Figure 5. The base peak for estazolam was m/z = 259 and the molecular ion was m/z = 294. Ions with smaller abundance at m/z = 205, 239 and 77 were also seen.

Table III presents the recovery rates of estazolam isolated from biological material using three methods: conventional liquid-liquid extraction, Extrelut® extraction and solid-phase extraction with Bakerbond narc-2 columns. Solid-phase extraction using of Bakerbond narc-2 turned out to be most effective (mean 88.1%). When Extrelut® was used the recovery rate reached 68.8% (mean), in the case of liquid-liquid extraction it was 64.4% (mean). Apart from the fact that the use of the Bakerbond narc-2 method is effective, it is also simple and rapid. Therefore this method was employed to analyse the autopsy cases mentioned above.

TABLE III. RECOVERY RATES OF ESTAZOLAM ISOLATED FROM BIOLOGICAL MATERIAL

Sample	Recovery and coefficients of variation [%] Concentration of estazolam = 100 ng/ml; (n = 3)		
	Liquid-liquid	Narc-2	Extrelut®
Water	72.0 ± 2.8	92.5 ± 3.5	75.0 ± 1.4
Blood	67.0 ± 4.2	90.0 ± 2.8	69.0 ± 2.8
Urine	70.5 ± 2.1	90.5 ± 0.7	75.0 ± 1.4
Bile	62.5 ± 3.5	89.5 ± 3.5	67.5 ± 3.5
Kidney	57.0 ± 4.2	82.5 ± 3.5	67.5 ± 3.5
Liver	57.5 ± 6.4	84.0 ± 5.7	59.0 ± 2.8

The results of the postmortem material analysis are summarized in Table IV. The concentration of estazolam in each blood sample significantly exceeded the therapeutic level.

TABLE IV. CONCENTRATIONS OF ESTAZOLAM IN POSTMORTEM MATERIAL IN AUTOPSY CASES

Sample	Concentration		
	Case 1	Case 2	Case 3
Blood	139.28 μg/ml	142 μg/ml	44 μg/ml
Brain	–	21 μg/g	8 μg/g
Kidney	–	73 μg/g	11 μg/g
Liver	–	47 μg/g	20 μg/g
Gastric contents	–	1.68 mg/45 g	183 μg/14 g

In case 1 and 2 alcohol or other drugs could not be detected. In case 3 apart from estazolam, the antiarrhythmic propafenon was found in the following quantitaties: blood = 7.39 μg/ml, liver = 360 μg/g, kidney = 189 μg/g, brain = 143 μg/g, gastric contens (14 g) = 3.19 mg (therapeutic level of propafenon in plasma ranges from 0.4 to 1.6 μg/ml [16]).

CONCLUSION

Solid-phase extraction using Bakerbond narc-2 columns turned out to be the most effective method when there is a need to isolate estazolam from autopsy material. Gas chromatography with mass-selective detector (GC/MS) carried out according to the conditions above described in 3.4.4. is simple, sensitive and selective. If GC/MS in not available, HPLC, UV and TLC methods are also effective.

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