

A COMPARATIVE STUDY ON FLUNITRAZEPAM AND 7-AMINOFLUNITRAZEPAM ISOLATION FROM HUMAN PLASMA BY LIQUID-LIQUID EXTRACTION AND SOLID PHASE EXTRACTION

CĂTĂLINA BOGDAN*, BÉLA KISS, ANCA POP, FELICIA
LOGHIN

*Department of Toxicology, Faculty of Pharmacy, University of
Medicine and Pharmacy "Iuliu Hațieganu" Cluj-Napoca*

**corresponding author: catalinab2202@yahoo.com*

Abstract

The aim of this work was to establish the best experimental conditions for the isolation of flunitrazepam and its main metabolite 7-aminoflunitrazepam from human plasma. Several mixtures of solvents (ethyl acetate, *tert*-butylmethyl ether, hexane, chloroform/ ethyl ether (20/80), dichloromethane / ethyl ether (20/80) and extraction cartridges (Oasis[®] MCX, SepPak[®] C18, Chromafix[®] C18, Oasis[®] HLB) were tested at different pH values on the samples. In order to assay flunitrazepam and its major metabolite, an ultra performance liquid chromatography with UV detection (UPLC-UV) method was applied. The best results for liquid-liquid extraction (LLE) of flunitrazepam and 7-aminoflunitrazepam from human plasma were obtained using as extraction solvent ethyl acetate after a previous sample alkalization with ammonia hydroxide. Recovery rates were 82.37% for flunitrazepam and 63.70% for 7-aminoflunitrazepam and the interferences at the retention time of these two compounds were minimal. In the case of solid phase extraction (SPE), the best recovery rates were obtained using SepPak[®] and HLB[®] cartridges (108.38% and 81.46% for flunitrazepam and 82.53% and 76.81% for 7-amino flunitrazepam, respectively), these results being correlated with minimal interferences at the retention time of flunitrazepam and 7-aminoflunitrazepam. LLE proved to be as efficient as SPE for the isolation of flunitrazepam and aminoflunitrazepam from human plasma. Liquid phase extraction using ethyl acetate as solvent in basic conditions and solid phase extraction using SepPak[®] and HLB[®] cartridges can be used in toxicological studies in order to efficiently isolate flunitrazepam and 7-aminoflunitrazepam from human plasma.

Rezumat

Scopul acestei lucrări a fost de a stabili condițiile experimentale optime pentru izolarea flunitrazepamului și a metabolitului său principal 7-aminoflunitrazepam, din plasma umană. S-au testat mai multe amestecuri de solvenți organici (acetat de etil, *terț*butilmetileter, hexan, cloroform/eter etilic (20/80), diclorometan/eter etilic (20/80) și cartușe de extracție pe fază solidă (Oasis[®] MCX, SepPak[®] C18, Chromafix[®] C18, Oasis[®] HLB) la diferite valori de pH, iar determinările cantitative au fost realizate printr-o metodă UPLC-UV. Cele mai bune rezultate pentru extracția în fază lichidă a flunitrazepamului și a 7-aminoflunitrazepamului din plasma umană au fost obținute utilizând ca solvent acetatul

de etil, după o prealabilă alcalinizare a probei cu hidroxid de amoniu 10%. Randamentele obținute au fost 82,37% pentru flunitrazepam și 63,70% pentru 7-aminoflunitrazepam, iar interferențele prezente la timpii de retenție ai celor 2 compuși au fost minime. Pentru extracția în fază solidă (EFS) cele mai bune randamente au fost obținute utilizând cartușele SepPak® și HLB® (108,38% și 81,46% pentru flunitrazepam, respectiv 82,53% și 76,81% pentru 7-aminoflunitrazepam), aceste rezultate fiind corelate cu interferențe minime la timpii de retenție ai celor 2 compuși. Extracția în fază lichidă (ELL) s-a dovedit a fi la fel de eficientă ca EFS în izolarea flunitrazepamului și a 7-aminoflunitrazepamului din plasma umană. Extracția în fază lichidă, utilizând ca solvent de extracție acetatul de etil în mediu bazic, și extracția în fază solidă, utilizând cartușe SepPak® și HLB®, pot fi aplicate în studii toxicologice pentru izolarea eficientă a flunitrazepamului și 7-aminoflunitrazepamului din plasma umană.

Keywords: flunitrazepam, 7-aminoflunitrazepam, plasma, liquid-liquid extraction, solid phase extraction

Introduction

Flunitrazepam is a long acting benzodiazepine, with a hypnotic effect overcoming the sedative, anxiolytic, muscle relaxant and anticonvulsivant effects characteristic for other benzodiazepines. It is prescribed in the symptomatic treatment of sleep disorders and as premedication before anaesthesia. Flunitrazepam is also involved in an important number of sexual aggressions, being used to sedate the victim in combination with alcohol [10].

After therapeutic administration, the plasma levels of flunitrazepam and its metabolites are low, due to the low dosage, the intensive biotransformation and the large volume of distribution [11]. In flunitrazepam-associated fatal accidents, its metabolite 7-amino-flunitrazepam is usually present in high plasma concentrations, while flunitrazepam is undetectable in most cases [8]. Due to the very low plasma levels (ng/mL) of flunitrazepam and 7-aminoflunitrazepam, very efficient isolation methods are needed (with high extraction yields and clean extracts), which, coupled with a sensitive and selective chromatographic method, will allow an accurate and precise quantification of flunitrazepam and its main metabolite [1].

Chromatographic separation of flunitrazepam from human plasma involves an isolation step using an extraction method. Several HPLC or GC methods are available for flunitrazepam quantification in biological samples after liquid-liquid extraction (LLE) [2,3,6,9,12] or solid-phase extraction (SPE) [4,13,14,15].

The aim of this study was to compare the efficiency of LLE and SPE extractions of flunitrazepam and its metabolite 7-aminoflunitrazepam from

human plasma in order to develop an extraction method with maximum yield that can be used in quantitative toxicological studies.

Materials and Methods

Reagents and materials

Flunitrazepam, 7-aminoflunitrazepam and chlordiazepoxide standard references were purchased from Lipomed AG (Arlesheim, Switzerland). All reagents were of analytical grade. HPLC-grade acetonitrile, methanol, hexane, chloroform, diethyl ether were purchased from Sigma-Aldrich (Steinheim, Germany). Ethyl acetate and dichloromethane (HPLC-grade) were purchased from Merck (Darmstadt, Germany). HPLC-grade *tert*-butylmethyl ether was provided by Riedel-de Haën (Seelze-Hanover, Germany). Ammonia hydroxide 25% (p.a.) was provided by Fluka (Buchs SG, Switzerland), p.a. grade phosphoric acid 85% and p.a. grade potassium hydroxide were purchased from Merck (Darmstadt, Germany). Dipotassium phosphate (p.a.) was purchased from Chimopar (Bucharest, Romania).

Deionised water obtained from a Millipore Milli-Q (Milford, MA, SUA) system was used throughout the experiments. The human blank plasma was supplied by the Blood Donation Centre Cluj-Napoca, Romania. For SPE the following cartridge types were used: Oasis[®] MCX, 3 cc (Waters Corporation, Massachusetts, USA), SepPak[®] C18, 3cc (Lida Manufacturing Corporation, Kenosha, WI, USA), Chromafix[®] C18 (Macherey-Nagel, Düren, Germany), Oasis[®] HLB 3cc (Waters Corporation, Massachusetts, USA).

Apparatus and chromatographic conditions

An UPLC Waters Acquity (Waters, Millford, MA, USA) equipped with a binary pump, an autosampler, a degasser, a column thermostat and a diode array UV detector was used in this experiment. Chromatographic separation was performed at 30°C on a C18 BEH chromatographic column (50 mm x 2.1 mm i.d., 1.7 µm). The mobile phase consisted of a mixture of 10 mM dipotassium phosphate 10 mM/acetonitrile with gradient elution (Table I). The flow-rate was 0.275 mL/min and the injection volume was 5 µL.

Table I
The gradient system of the mobile phase

Time (min)	10 mM Dipotassium phosphate (%)	Acetonitrile (%)
0	90	10
3	50	50
4	50	50
4.2	90	10
6	90	10

Chromatographic data acquisition and processing were performed using Empower 2 software.

The following apparatus were used in this study: 2-16 Sigma Centrifuge (Sigma Laborzentrifugen GmbH, Germania); Discovery Analytical Balance (Ohaus, Pine Brook, NJ, SUA); Heidolph vortex mixer (Heidolph Instruments, Schwabach, Germany) and Glas-Col Multipulse vortex (Glas-Col, Terre Haute, IN, USA); Elma Transsonic 700/H ultrasonic bath (Singen, Germany); solid phase extraction Chromabond® Vacuum manifolds (Macherey-Nagel, Düren, Germany).

Standard solutions

Stock solutions of flunitrazepam, 7-aminoflunitrazepam (1 mg/mL) and chlordiazepoxide (internal standard) (1 mg/mL) were prepared by dissolving the drugs in methanol and were stored at 4°C. Working standard solutions were prepared by dilution of stock solutions with deionised water. Due to the fact that the toxic effects of flunitrazepam occur at plasmatic levels of 50 ng/mL or higher, we prepared spiked plasma samples with a concentration of 40.16 ng/mL flunitrazepam and 41.36 ng/mL 7-aminoflunitrazepam by diluting specific volumes of flunitrazepam and 7-aminoflunitrazepam working solutions with blank plasma.

Sample preparation

a. Liquid-liquid extraction procedure

1 mL plasma samples were spiked with 50 µL of the internal standard (I.S.) solution (1.3 µg/mL chlordiazepoxide in MilliQ water) and vortexed for 20 seconds at low speed (55.10 ng/mL I.S. in plasma). The plasma samples were mixed with 3.5 mL organic solvent, with or without previous alkalisation of the sample using 10% ammonia hydroxide (50 µL) and vortexed at 3000 rpm in a multi-pulse vortex-mixer for 5 min. The organic phase was evaporated to dryness under a nitrogen stream at 45°C. The dry extract was reconstituted in 150 µL of a mixture of 10mM dipotassium phosphate (pH=8.5)/acetonitrile (50/50, v/v), vortexed at high speed for 20 sec. and centrifuged at 8000 rpm for 5 min. The supernatant was transferred to an autosampler vial and 5 µL were injected into the chromatographic system.

The organic solvents used were: ethyl acetate, *tert*-butylmethyl ether, hexane, chloroform/ ethyl ether (20/80), dichloromethane / ethyl ether (20/80). In the case of ethyl acetate, the extraction was performed with or without alkalisation with 50 µL 10% ammonia hydroxide.

b. Solid phase extraction procedure

For SPE, SepPak[®] C18, Chromafix[®] C18 and Oasis[®] HLB cartridges were conditioned with methanol, deionised water and 2% ammonia hydroxide. A mixture of 1 mL plasma sample, 1 mL water and 40 μ L 2% ammonia hydroxide was passed through the cartridges, followed by a washing step with methanol: 20% ammonia hydroxide (5:95, v/v). Analytes were eluted with acetonitrile containing 2% acetic acid and the eluate was evaporated to dryness at 45°C under a nitrogen stream. The residues were reconstituted in 150 μ L mixture of 10mM dipotassium phosphate (pH=8.5)/acetonitrile (50/50, v/v) and 5 μ L were injected into the chromatographic system.

The Oasis[®] MCX cartridges were conditioned with methanol and deionised water. Then, a mixture of 1mL plasma samples, 1 mL water and 40 μ L phosphoric acid was passed through the cartridges which was afterwards washed with 2 mL 2% formic acid. The eluate was evaporated to dryness at 45°C under a nitrogen stream and residues were reconstituted in 150 μ L of a mixture of 10 mM dipotassium phosphate (pH=8.5)/acetonitrile (50/50, v/v). An aliquot of 5 μ L was injected into the chromatographic column.

Each extraction method was applied on blank plasma and on plasma spiked with 7-aminoflunitrazepam and flunitrazepam. In each case samples were analyzed in triplicate.

Results and Discussion

In order to quantitate flunitrazepam and its major metabolite we applied an UPLC-UV method previously developed and validated in our laboratory [6,7]. The UV detection was performed at two wavelengths, 242 nm for 7-aminoflunitrazepam and 314 nm for flunitrazepam, respectively.

Taking into account that the current study aims to optimize the extraction methods for flunitrazepam and its major metabolite, using an already validated method for the quantification of the two substances, the recovery of analytes and the precision were calculated for each extraction method based on three determinations (samples)/method.

Ethyl acetate can quantitatively extract flunitrazepam from human plasma with or without pH adjustment, but for 7-aminoflunitrazepam the recovery was superior after extraction in alkaline medium. The best results with good recovery and insignificant endogenous interferences were obtained with ethyl acetate after addition of ammonia hydroxide.

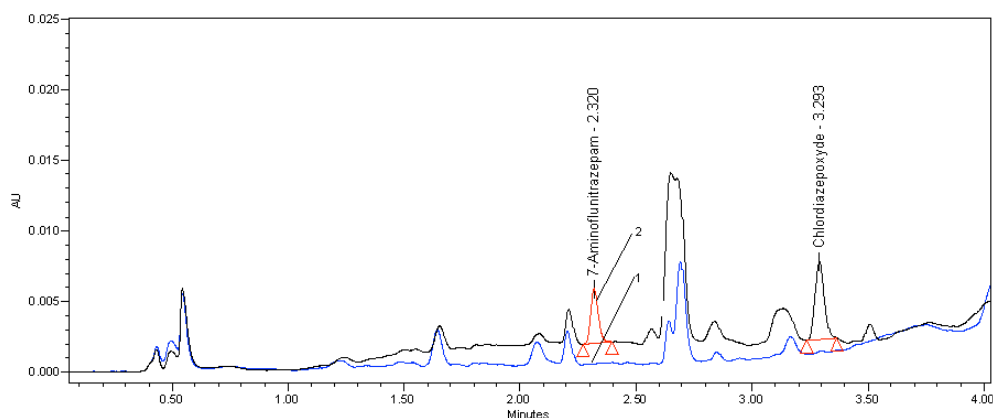


Figure 1

Chromatograms obtained for drug free, blank plasma (1) and plasma sample spiked with 7-aminoflunitrazepam (41.36 ng/mL), chlordiazepoxide (55.10 ng/mL) and flunitrazepam (40.16 ng/mL)- UV detection at 242 nm (2), extraction solvent: ethyl acetate + 50 μ L ammonia 10%

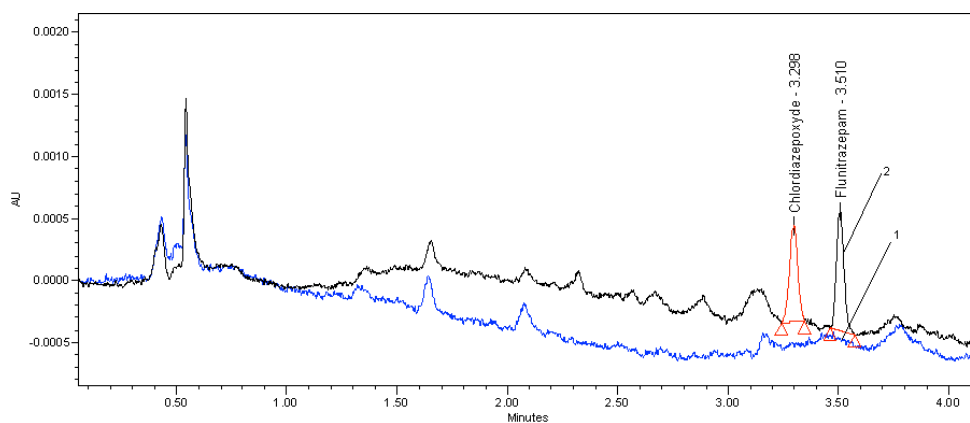


Figure 2

Chromatograms obtained for drug free, blank plasma (1) and plasma sample spiked with 7-aminoflunitrazepam (41.36 ng/mL), chlordiazepoxide (55.10 ng/mL) and flunitrazepam (40.16 ng/mL)- UV detection at 314 nm (2), extraction solvent: ethyl acetate + 50 μ L ammonia 10%

Extraction procedure using chloroform: ethyl ether and *tert*-butylmethyl ether as extraction solvents revealed a good recovery for

flunitrazepam, but significant interferences from the endogenous compounds were observed. For dichloromethane: ethyl ether extraction, a major drawback was the formation of an emulsion, which influenced the extraction efficiency and made impossible the separation of the organic phase.

Table II

Extraction efficiency of flunitrazepam by LLE using different solvents and pH conditions

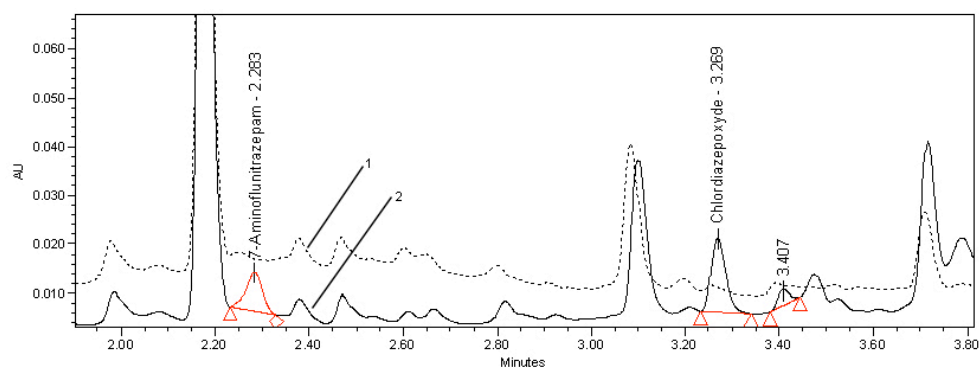
Solvent	Nominal conc. (ng/mL)	Mean of measured conc. (ng/mL) $\pm$ SD	RSD (%)	Recovery (%)
Ethyl acetate	41.36	39.45 $\pm$ 5.53	14.03	95.38
Ethyl acetate + 50 μ L ammonia 10%	41.36	34.07 $\pm$ 4.20	12.35	82.37
Tert-butylmethyl ether	41.36	35.71 $\pm$ 3.54	9.93	86.34
Hexane	41.36	0	0	0
Chloroform: ethyl ether 20/80	41.36	34.09 $\pm$ 8.48	24.89	82.42
Dichloromethane: ethyl ether 20/80	41.36	34.59 $\pm$ 6.72	19.42	83.63

Table III

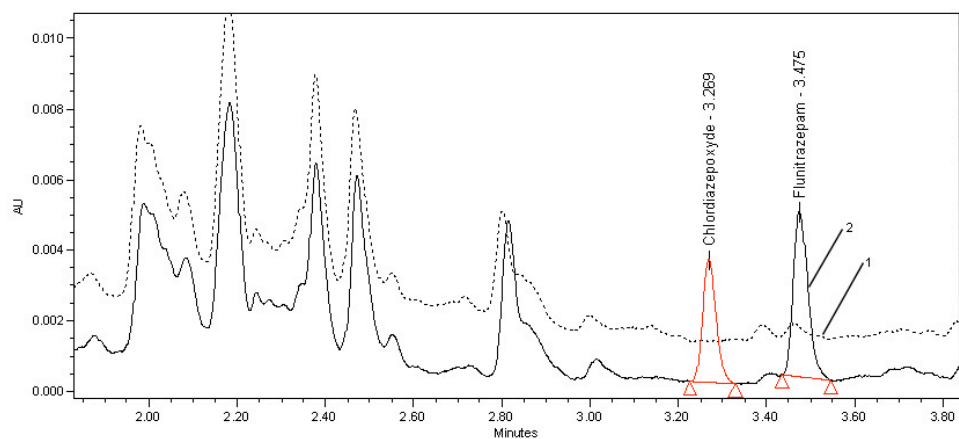
Extraction efficiency of 7-aminoflunitrazepam by LLE using different solvents and pH conditions

Solvent	Nominal conc. (ng/mL)	Mean of measured conc.(ng/mL) $\pm$ SD	RSD (%)	Recovery (%)
Ethyl acetate	40.16	23.7 $\pm$ 5.05	23.23	59.01
Ethyl acetate + 50 μ L ammonia 10%	40.16	25.58 $\pm$ 3.30	12.90	63.70
Tert-butylmethyl ether	40.16	16.85 $\pm$ 3.67	21.78	41.96
Hexane	40.16	2.91 $\pm$ 0.63	21.92	7.25
Chloroform: ethyl ether 20/80	40.16	13.59 $\pm$ 5.39	39.67	33.84
Dichloromethane: ethyl ether 20/80	40.16	14.23 $\pm$ 5.34	37.58	35.43

The best results for SPE were obtained using SepPak® and Oasis® HLB cartridges. The washing step with methanol: 2% ammonia hydroxide guaranteed a selective extraction of the analytes from the matrix without significant interferences from endogenous plasma compounds.

**Figure 3**

Chromatograms obtained for drug free, blank plasma (1) and plasma sample spiked with 7-aminoflunitrazepam (35.72 ng/mL), chlordiazepoxide (58.12 ng/mL) and flunitrazepam (40.32 ng/mL)- UV detection at 242 nm (2), solid phase extraction cartridge: SepPak®

**Figure 4**

Chromatograms obtained for drug free, blank plasma (1) and plasma sample spiked with 7-aminoflunitrazepam (35.72 ng/mL), chlordiazepoxide (58.12 ng/mL) and flunitrazepam (40.32 ng/mL)- UV detection at 242 nm (2), solid phase extraction cartridge: SepPak®

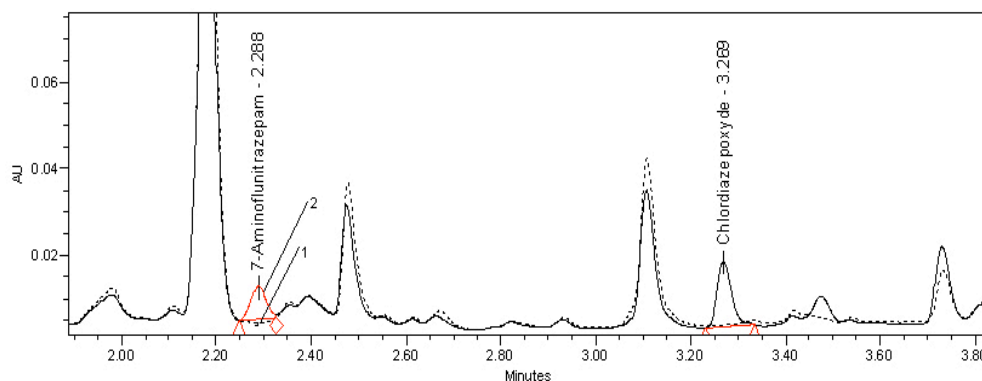


Figure 5

Chromatograms obtained for drug free, blank plasma (1) and plasma sample spiked with 7-aminoflunitrazepam (35.72 ng/mL), chlordiazepoxide (58.12 ng/mL) and flunitrazepam (40.32 ng/mL)- UV detection at 242 nm (2), solid phase extraction cartridge: Oasis[®] HLB

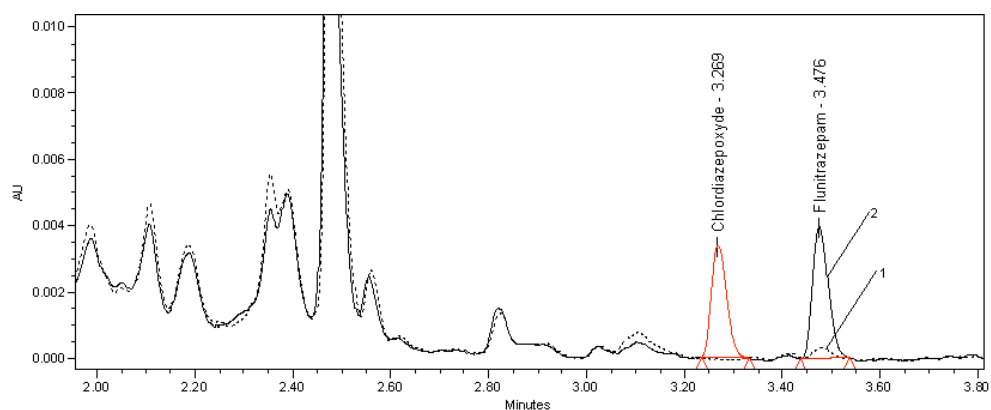


Figure 6

Chromatograms obtained for drug free, blank plasma (1) and plasma sample spiked with 7-aminoflunitrazepam (35.72 ng/mL), chlordiazepoxide (58.12 ng/mL) and flunitrazepam (40.32 ng/mL)- UV detection at 242 nm (2), solid phase extraction cartridge: Oasis[®] HLB

Table IV

Extraction efficiency of flunitrazepam by SPE using different cartridges

Eluate	Cartridge	Nominal conc. (ng/mL)	Mean of measured conc. (ng/mL) $\pm$ SD	RSD (%)	Recovery (%)
Methanol: ammonia hydroxide 20% (5:95)	SepPak®	40.32	43.70 $\pm$ 1.36	3.11	108.38
	Chromafix®	40.32	2.31*	NA**	5.72
	HLB®	40.32	32.85 $\pm$ 4.58	13.93	81.46
Formic acid 2%	MCX®	40.32	14.19 $\pm$ 11.42	80.50	35.18

* quantification of flunitrazepam was possible for only one of the three samples

** NA – not applicable

Table V

Extraction efficiency of 7-aminoflunitrazepam by SPE using different cartridges

Eluate	Cartridge	Nominal conc. (ng/mL)	Mean of measured conc. (ng/mL) $\pm$ SD	RSD (%)	Recovery (%)
Methanol: ammonia hydroxide 20% (5:95)	SepPak®	35.72	29.48 $\pm$ 2.15	7.29	82.53
	Chromafix®	35.72	42.08 $\pm$ 6.38	15.16	117.81
	HLB®	35.72	27.44 $\pm$ 3.35	12.22	76.81
Formic acid 2%	MCX®	35.72	0	0	0

When solid phase extraction was performed using Chromafix® cartridges, flunitrazepam extraction was inefficient and at the retention time of 7-aminoflunitrazepam, interferences from endogenous plasma components were observed, explaining the high recovery value of 117.81%.

The recoveries obtained for flunitrazepam extraction with MCX® cartridges were poor (35.17%) and the 7-aminoflunitrazepam separation was inefficient.

The best results for the isolation of both analytes from human plasma were obtained using ethyl acetate after adding ammonia hydroxide (LLE) and SepPak® and Oasis® HLB cartridges (SPE). These extraction methods showed very good precision with RSD values in the range recommended by the bioanalytical method validation guide ($RSD \leq 15\%$) [16]. The comparison of the two extraction techniques (LLE and SPE) shows that SPE offers several advantages over LLE: higher selectivity, cleaner extracts and the avoidance of emulsion formation.

Previous studies revealed that flunitrazepam is difficult to analyze due to the low concentration present in blood/urine samples and because the parent drug can break down to form the metabolite even after the sample has been withdrawn from the body and stored appropriately. In this situation, the concentrations of both flunitrazepam and 7-aminoflunitrazepam are reduced even further. The ability to extract and analyze both compounds simply and efficiently is the goal of many laboratories working in this field. The presented assay proposes some SPE and LLE procedures which provide a highly efficient sample clean up with excellent recoveries. The optimized extraction method together with the Ultra performance liquid chromatography (UPLC/PDA) method developed earlier in our laboratory [7] represent a good alternative to the existing methods for the quantification of flunitrazepam (FNZ) and 7-amino flunitrazepam (AFNZ) in human plasma, without laborious sample treatment, with lower solvent consumption and superior sensitivity, when compared to published HPLC/UV assays.

Conclusions

Ethyl acetate (for LLE) and SepPak® and Oasis® HLB cartridges (for SPE) proved good efficiencies for flunitrazepam and 7-aminoflunitrazepam extraction in terms of extraction yields and elimination of endogenous interferences. These optimum experimental conditions could be used in different pharmacokinetic and toxicological studies of drugs and their metabolites such flunitrazepam and 7-aminoflunitrazepam.

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