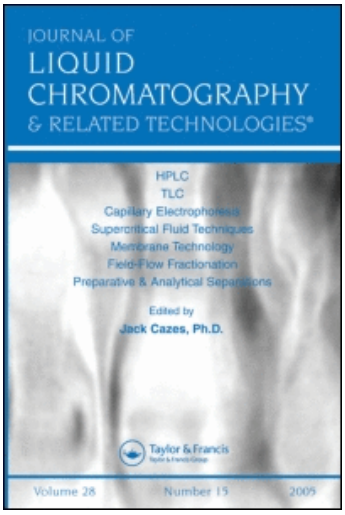

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ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF FLUNITRAZEPAM AND 7-AMINOFLUNITRAZEPAM IN HUMAN PLASMA

Béla Kiss[a]; Catalina Bogdan[a]; Anca Pop[a]; Felicia Loghin[a]
[a] Department of Toxicology, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania

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Béla Kiss, Catalina Bogdan, Anca Pop, and Felicia Loghin

Department of Toxicology, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania

□ *A high-throughput ultra-performance liquid chromatography (UPLC) method with UV detection was elaborated and validated for the simultaneous quantification of flunitrazepam and its major metabolite, 7-aminoflunitrazepam, in human plasma. After a simple liquid-liquid extraction with ethyl acetate, chromatographic separation was performed on a BEH C18 chromatographic column with a binary mixture of acetonitrile/10 mM potassium monohydrogen phosphate (pH = 8.5) as the mobile phase, under gradient elution (flow rate = 0.275 mL/min). The total run time of the chromatographic analysis was 4.2 min. UV detection was performed at 314 nm for flunitrazepam and at 242 nm for 7-aminoflunitrazepam. To our knowledge, this is the first reported UPLC-PDA method for the quantification of flunitrazepam and 7-aminoflunitrazepam in human plasma. The assay was applied successfully for the analysis of the selected two analytes in real human plasma samples obtained from a healthy volunteer, after ingestion of a single dose of flunitrazepam.*

Keywords 7-aminoflunitrazepam, flunitrazepam, plasma, ultra-performance liquid chromatography, validation

INTRODUCTION

Flunitrazepam (5-(2-Fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, Rohypnol) (Figure 1) is a short acting benzodiazepine recommended especially as a hypnotic agent but also as premedication before anaesthesia. While this drug is no longer approved for medical use in the United States due to its increasing abuse, it is still legally sold in Japan, Europe, and Latin America.[1–3]

Flunitrazepam has become increasingly popular among teenagers and young adults because it causes a drunken-like high and heightens the effect

Address correspondence to Béla Kiss, Department of Toxicology, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, no. 6 Pasteur, RO-400349, Cluj-Napoca, Romania. E-mail: kbela@umfcluj.ro

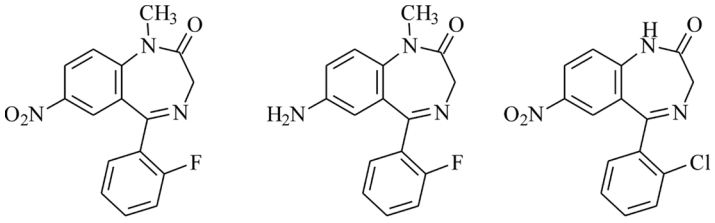

FIGURE 1 Chemical structures of flunitrazepam, 7-aminoflunitrazepam, and chlordiazepoxide.

of alcohol and other drugs. It is the most popular benzodiazepine among heroin addicts, probably due to the attenuation of some unpleasant symptoms of opiate withdrawal, but also due to its capacity to induce euphoria in methadone maintained patients.[1,4–8]

Several reports point to flunitrazepam (alone or with concomitant alcohol consumption) as a date rape drug. The significant sedative effect of the drug, which is enhanced by alcohol, increases the vulnerability to sexual assault. Another effect that makes this compound a "perfect" agent for drug facilitated sexual assault is the anterograde amnesia, reported after exposure to flunitrazepam.[1,2,9]

In order to confirm cases of drug abuse or drug-facilitated sexual assaults, it is important to elaborate high-throughput and sensitive methods for the analysis of flunitrazepam in biological samples. The most frequently analyzed samples are human whole blood and plasma.

Several chromatographic methods, such as gas-chromatography with mass spectrometry or tandem mass spectrometry (GC/MS or GC/MS/MS), and high-performance liquid chromatography with ultraviolet or MS detection (HPLC/UV, HPLC/MS or HPLC/MS/MS) for the quantification of flunitrazepam and its metabolites in human whole blood and plasma were published. Extraction of the analytes from these matrices is performed either by classic liquid-liquid extraction (LLE), liquid-phase microextraction, or solid-phase extraction (SPE). GC/MS and GC/MS/MS methods presented generally good sensitivity, with the lower limit of detection between 0.025–1 ng/mL.[3,10–21] Terada et al.[11] reported a GC/MS/MS method for the quantification of flunitrazepam (FNZ) and its major metabolite, 7-aminoflunitrazepam (AFNZ) from human serum with a limit of detection (LOD) of 15 ng/mL and 1 ng/mL, respectively. The main problem of GC/MS and MS/MS methods is the need for a derivatization step to increase the volatility of the analytes. This step means a prolonged, laborious sample preparation and a supplementary source of errors. Smink et al.[13] quantified 33 benzodiazepines, metabolites, and benzodiazepine-like substances by HPLC/MS/MS with good sensitivity for FNZ and AFNZ (LLOQ = 0.7 and 2.6 ng/mL, respectively). The chromatographic run time

was 45 min. Bugey et al.[14] separated 8 benzodiazepines from whole blood in 5 min, by using a monolithic column and MS detection (LOQ = 2.5 ng/mL). Marchi et al.[15] reported a HPLC/MS method for the quantification of flunitrazepam, alprazolam, and their main metabolites from haemolysed blood, with an analysis run time of 10 min and a LLOQ of 1 ng/mL. A common drawback of GC and HPLC methods with MS or MS/MS detection is the high cost, which makes it less accessible, especially for smaller laboratories or in developing countries.

The HPLC/UV methods (with detection at 220, 230, 240, or 242 nm) described in the literature present poor sensitivity, with a lower limit of quantitation (LLOQ) between 10–50 ng/mL.[16–20] Hackett et al.[3] reported a HPLC/UV method with LLOQ of 5 ng/mL for both, FNZ and AFNZ. Benhamou and co-workers[21] achieved the same sensitivity in a HPLC/UV assay for FNZ in plasma. In addition to the sensitivity problem, most of these HPLC/UV methods are also time consuming, with a chromatographic analysis run time of 8 to 50 min and involve high solvent consumption. Bugey et al.[19] reported the separation and quantification of flunitrazepam and seven other benzodiazepines by HPLC/UV, using a monolithic column, with a run time of 4 min. The use of a monolithic column allowed the reduction of analysis time by increasing flow rate (2 mL/min), which means that the solvent consumption is not diminished. However, HPLC/UV remains the most used method for the analysis of FNZ and its metabolites from human plasma, probably due to the widespread/accessibility of this technique.

We previously reported a HPLC/UV[22] and a HPLC/MS/MS[23] method for the quantification of flunitrazepam in human plasma (and in urine, in case of MS/MS detection) with a LLOQ of 5 ng/mL and 0.77 ng/mL, respectively.

The aim of this study was to elaborate and validate an ultra-performance liquid chromatography (UPLC) method coupled with photodiode array (PDA) detection for the quantification of FNZ and AFNZ in human plasma, which could represent a high-throughput and sensitive alternative to other methods described in the literature.

EXPERIMENTAL

Materials and Reagents

Flunitrazepam, 7-aminoflunitrazepam, chlordiazepoxide (internal standard, I.S.), bromazepam, temazepam, alprazolam, oxazepam, nitrazepam, and diazepam standards were purchased from Lipomed (Arlesheim, Switzerland). HPLC gradient grade acetonitrile and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Ethyl acetate

(HPLC grade), potassium monohydrogen phosphate, and potassium hydroxide (analytical grade) were obtained from Merck KgaA (Darmstadt, Germany), whereas, analytical grade 25% ammonium hydroxide was obtained from Fluka (Buchs, Switzerland). Distilled, deionized water was produced by a Direct Q-3 Millipore (Millipore SA, Molsheim, France) water purification system. Drug free human plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

Preparation of Standard and Quality Control Solutions

Stock standard solutions of FNZ, AFNZ, and chlordiazepoxide were prepared by dissolution of each compound (accurately weighed on a Discovery analytical balance (Ohaus Corp., Pine Brook, USA)) in methanol to obtain a final concentration of 1 mg/mL. These solutions were stored at 4°C, protected from light.

Less concentrated working solutions, containing both FNZ and AFNZ, were prepared daily by serial dilution of stock solutions with deionized water. These working solutions were used to spike drug free human plasma, finally providing seven calibration standards (ranging from 5.02 to 251 ng/mL in case of FNZ, and from 5.17 to 258.5 ng/mL in case of AFNZ).

Quality control (OQ) samples at 5.02, 80.32, and 251.00 ng/mL for FNZ and at 5.17, 82.72, and 258.50 ng/mL for AFNZ were prepared by spiking drug free human plasma with working standard solutions containing both FNZ and AFNZ.

In case of IS a working solution with a concentration of 1.32 μg/mL was prepared on a daily basis by dilution of stock solution with deionized water.

Sample Preparation

Aliquots of 1 mL human plasma were spiked with 50 μL of I.S. working solution (corresponding to a final concentration in plasma of 66.12 ng/mL chlordiazepoxide). After a gentle shaking for 10 sec, 50 μL of 10% ammonium hydroxide solution was added, followed by 3.5 mL ethyl acetate. In case of blank samples, 50 μL deionized water was added instead of I.S. working solution. Samples were submitted to liquid-liquid extraction by vortexing thoroughly for 5 min with a Multi Pulse vortexer (Glas Col, Terre Haute, USA). In order to facilitate separation of the two liquid phases, samples were centrifuged for 5 min at 3000 rpm (Sigma 2–16, Sigma Laborzentrifugen GmbH, Osterode, Germany). The organic phases were transferred to other glass tubes and evaporated at 40°C under nitrogen flow. The residues were dissolved in 150 μL of a mixture of acetonitrile/10 mM potassium monohydrogen phosphate (pH = 8.5) (50/50, v/v), transferred

to 1.5 mL Eppendorf tubes and centrifuged at 8000 rpm. The clear supernatants were transferred to autosampler vials and 5 µL were injected into the chromatographic column.

Instrumentation and Chromatographic Conditions

The analyses were carried out using a Waters Acquity UPLC (Waters, Millford, MA, USA) system. Chromatographic separation was achieved on an Acquity BEH C18 (Waters, Milford, MA, USA) column (50 mm × 2.1 mm I.D., 1.7 µm particle size), maintained at 30°C.

The mobile phase was a mixture of acetonitrile (solvent A)/10 mM potassium monohydrogen phosphate (pH = 8.5, adjusted with 5 N KOH) (solvent B), with gradient elution. The elution started with a linear gradient from 10% to 50% from start to 3 min, it was held at 50% for 1 min, and returned to 10% A in 0.2 min. After each injection the column was re-equilibrated for 1 min, at 10% A. A flow rate of 0.275 mL/min was maintained throughout the analysis. Each component of the mobile phase was filtered through 0.45 µm filters (Millipore SA, Molsheim, France) and degassed in an Elmasonic S 100 H (Elma Hans Schmidbauer GmbH, Singen, Germany) ultrasonic bath before use for UPLC. The temperature in the sample compartment was held at 4°C.

Empower 2 software was used for instrument control, data acquisition, and data handling.

Method Validation

The selectivity of the method was assessed by analyzing six different blank plasma extracts and comparing them with the corresponding spiked plasma samples.

The linearity of the analytical method was evaluated in the concentration range of 5.02–251 ng/mL and 5.17–258.5 ng/mL plasma for FNZ and AFNZ, respectively. Calibration standards were prepared and analyzed according to the sample preparation procedure. Singlicate calibration standards at each concentration were analyzed on three consecutive days and curves for FNZ and AFNZ were constructed using peak area ratio of drug to I.S. versus nominal concentration of the analytes. The calibration model was accepted if the residuals were within ±20% at the LLOQ and within ±15% at all other calibrator levels.

The quantification limit (LLOQ) of the method was also evaluated, and it was defined as the lowest concentration with a precision and accuracy below 20%.

The extraction recoveries of FNZ and AFNZ from plasma were determined by comparing the analytical results for extracted samples at all seven

calibration levels with the response of unextracted standards in a mixture of acetonitrile/10 mM potassium monohydrogen phosphate (pH = 8.5) (50/50, v/v), with the same concentration of FNZ and AFNZ as the plasma calibrators.

The precision and accuracy of the method were evaluated by analyzing QC samples at three concentrations (low, medium, and high), representing the entire calibration range. In order to determine intra-day and inter-day accuracy and precision, each QC sample was extracted and analyzed five times/run in three consecutive days. Accuracy was calculated as the percentage difference between the concentration of drug calculated from the calibration curve and the concentration of drug added to the blank plasma. Precision was expressed as coefficient of variation (CV %).

In addition, a stability study of FNZ and AFNZ in human plasma was performed as part of the validation process. This study included the evaluation of room temperature stability, post preparative stability (stability of the analytes in the final extract) in the autosampler, and freeze-thaw stability after 3 cycles.

Application to Real Biological Samples

The applicability of the developed method was assessed by the analysis of two real human plasma samples. Whole blood samples were obtained from a healthy 49-year-old woman, with a body weight of 50 kg, after a single dose of flunitrazepam (1 mg). Written consent of the volunteer was obtained for this study. Samples were drawn at 1.2 hr and 5 hr after ingestion of the drug. In order to obtain plasma samples, the whole blood was centrifuged at 5000 rpm for 5 min.

RESULTS AND DISCUSSION

The aim of this study was to develop and validate a UPLC-PDA method for the analysis of FNZ and its major metabolite, AFNZ in human plasma. HPLC with UV detection, with previous sample preparation by LLE or SPE are the most employed techniques used in routine analysis of FNZ. To our knowledge, this is the first UPLC/PDA method for the quantification of FNZ and AFNZ from human plasma.

Based on the pK_a values reported in the literature for FNZ and chlordiazepoxide ($pK_a = 1.8$ and 4.8, respectively)[24] a simple, inexpensive LLE in the presence of ammonium hydroxide was chosen for sample preparation, which guaranteed the presence of analytes in non-ionized form, easily extractible in the hydrophobic organic solvent. Information relating the pK_a of AFNZ was not available.

After extraction, chromatographic separation of the analytes was performed with PDA detection at 242 nm and 314 nm, for AFNZ and FNZ, respectively. Figure 2 shows the UV spectra of FNZ, AFNZ, and I.S. Regarding the UV detection, selectivity, and not sensitivity, was the main criteria for wavelength selection. These wavelengths guaranteed increased selectivity, but also provided good sensitivity for both compounds. Under the described chromatographic conditions, FNZ and AFNZ were well separated, with retention times of 3.47 min (Figure 3) and 2.29 min (Figure 4), respectively. The internal standard eluted at 3.27 min (Figure 3).

The method was validated according to the industrial guidance for the bioanalytical method validation.[25,26] The selectivity study showed no interfering peaks at the retention time and specific detection wavelength of the analytes (Figures 3 and 4).

Regarding the calibration curves, response ratios of peak areas between the corresponding analyte and the I.S. versus theoretical concentration was fitted by a least-square linear regression to the equation $y = bx + a$, where y is the response ratio, b the slope, a the intercept, and x represents the concentration. The calibration curves showed good linearity over the studied concentration range for both analytes, with correlation coefficients (r^2) of 0.9994 ± 0.0004 and 0.9989 ± 0.0010 (mean $\pm$ S.D., n = 3) for FNZ and AFNZ, respectively.

The mean absolute recoveries ($\pm$S.D.) were $80.44\% \pm 11.56$, $77.24\% \pm 9.68$, and $85.17\% \pm 8.88$ for FNZ, AFNZ, and I.S., respectively.

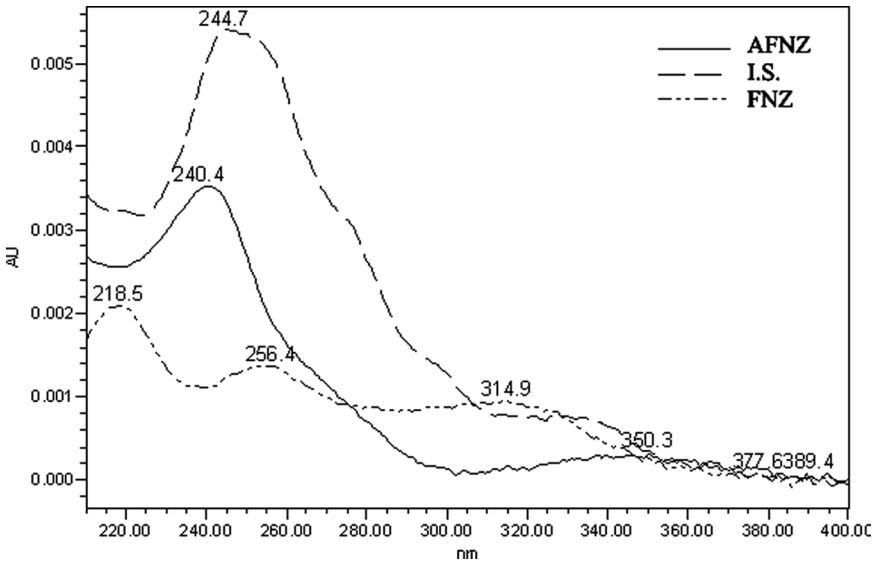

FIGURE 2 Ultraviolet spectra of FNZ, AFNZ, and chlordiazepoxide.

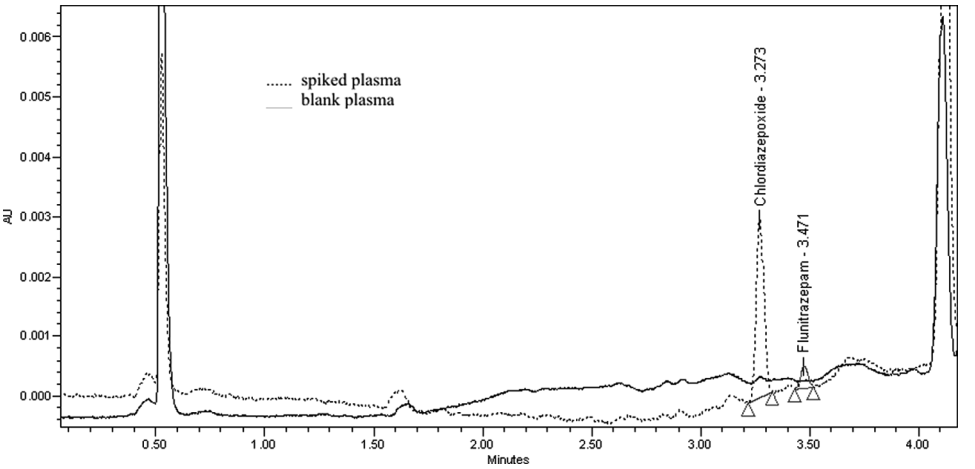

FIGURE 3 Chromatograms of blank plasma and spiked plasma at LLOQ (5.02 ng/mL flunitrazepam and 66.12 ng/mL I.S.) with UV detection at 314 nm (wavelength used for detection/quantification of FNZ).

The mean relative recovery of FNZ and AFNZ was 95.55% ± 11.46 and 89.74% ± 4.30, respectively.

The within- and between-run accuracy and precision data are summarized in Table 1. These results confirmed that the assay is accurate and precise over the studied concentration range.

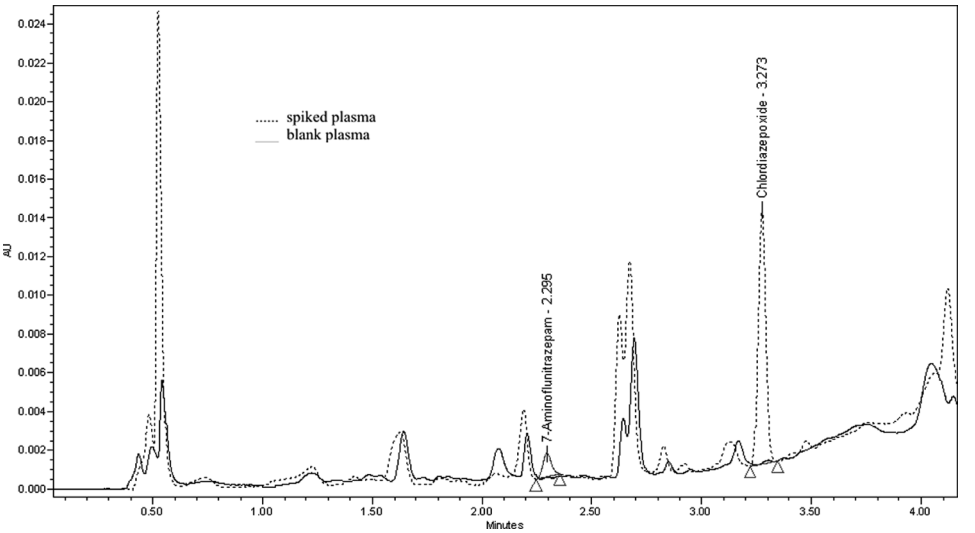

FIGURE 4 Chromatograms of blank plasma and spiked plasma at LLOQ (5.17 ng/mL 7-aminoflunitrazepam and 66.12 ng/mL I.S.) with UV detection at 242 nm (wavelength used for detection/quantification of AFNZ).

TABLE 1 Within-run and Between-run Precision and Accuracy for Flunitrazepam and 7-aminoflunitrazepam

		Within-Run			Between-Run		
Analyte	$c_{nominal}$ (ng/mL)	Mean c_{found} (ng/mL) ± S.D.	CV (%)	Inaccuracy (%)	Mean c_{found} (ng/mL) ± S.D.	CV (%)	Inaccuracy (%)
FNZ	5.02	5.32 ± 0.21	3.97	5.95	5.37 ± 0.18	3.42	6.97
	80.32	86.58 ± 1.70	1.96	7.79	83.43 ± 3.28	3.93	3.87
	251.00	277.51 ± 9.91	3.57	10.56	264.26 ± 11.17	4.23	5.28
AFNZ	5.17	5.82 ± 0.13	2.16	12.55	5.34 ± 0.54	10.14	3.35
	82.72	87.00 ± 1.92	2.21	5.17	87.68 ± 5.27	6.01	5.99
	258.50	263.46 ± 15.28	5.80	1.92	266.93 ± 14.10	5.28	3.26

Regarding the sensitivity of the method, based on the accuracy and precision values obtained for the low QC samples, the LLOQ was set at the level of the lowest calibrator in the case of both analytes (5.02 ng/mL for FNZ and 5.17 ng/mL for AFNZ).

The results of the stability study revealed no significant changes in FNZ and AFNZ concentration under the tested conditions. In the case of short-term stability at room temperature (protected from light), both analytes proved to be stable for at least 40 hr (inaccuracy <15%). In the case of final extracts maintained in the autosampler at 4°C, both analytes proved to be stable for at least 24 hr, with inaccuracy values between −3.96%–9.41% and −5.31–5.10% for FNZ and AFNZ, respectively. The mean changes in FNZ and AFNZ concentration after three freeze-thaw cycles indicated no stability problems under these conditions.

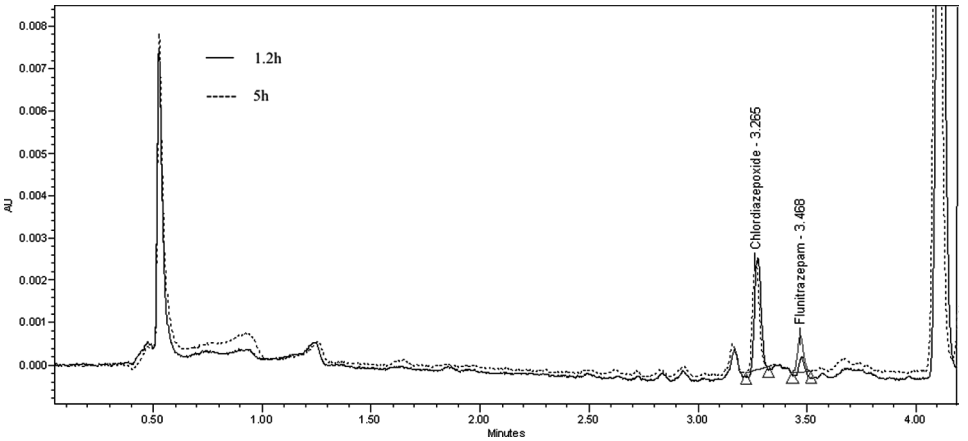

FIGURE 5 Chromatograms of real plasma samples, collected at 1.2 h and 5 h after a single oral dose of 1 mg flunitrazepam, spiked with 66.12 ng/mL I.S.; UV detection performed at 314 nm (for FNZ).

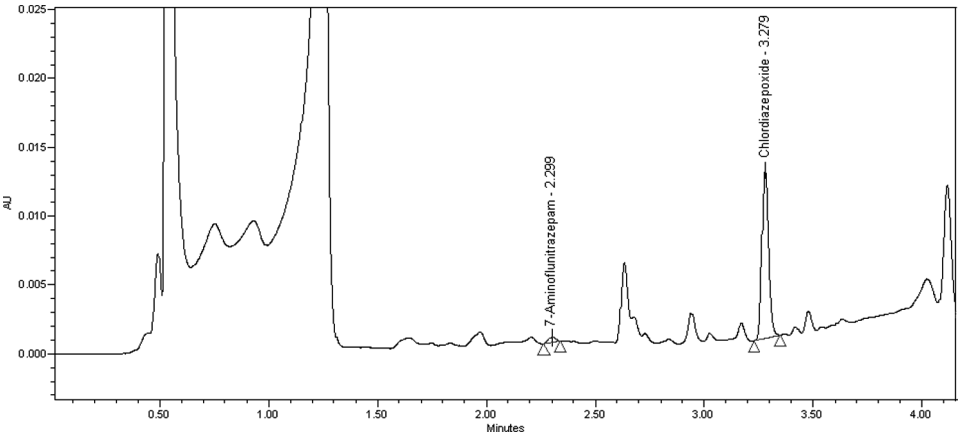

FIGURE 6 Chromatogram of real plasma samples, collected at 5 h after a single oral dose of 1 mg flunitrazepam, spiked with 66.12 ng/mL I.S.; UV detection performed at 242 nm (for AFNZ).

The validated method was applied to analyze real human plasma samples obtained from one healthy volunteer after a single dose of 1 mg flunitrazepam. At each time point, samples were analyzed in triplicate. In the case of FNZ, the mean concentrations found (mean ± SD) were of 12.82 ± 1.16 ng/mL and 6.71 ± 0.70 ng/mL, at 1.2 hr and 5 hr after ingestion (Figure 5). AFNZ could be detected only in the plasma sample collected at 5 hr after ingestion, but only qualitative analysis was possible (confirmation of the presence of this metabolite) since the concentration found was under the LLOQ value of the method (Figure 6). In cases of real forensic or clinical samples, higher concentrations of FNZ and AFNZ are expected than those observed in the case of the one healthy volunteer, since these cases involve chronic administration or higher doses.

CONCLUSIONS

The assay presented in this paper is the first UPLC/PDA method reported in the literature for the simultaneous quantification of FNZ and AFNZ in human plasma. The sample preparation consisted in a fast and easy LLE with very good recoveries. The method was fully validated in terms of selectivity, linearity, accuracy, and precision. The range of linearity and the LLOQ of 5.02 ng/mL FNZ and 5.17 ng/mL AFNZ make this method suitable for forensic and clinical purposes. This UPLC method could represent a good alternative to existing methods for quantification of FNZ and AFNZ in human plasma, being less expensive then GC/MS/MS or LC/MS/MS methods, without laborious sample treatment (there is no need for derivatization as in case of GC methods), and with shorter analysis

time, lower solvent consumption, and superior sensitivity when compared to published HPLC/UV assays.

The assay was applied successfully for the analysis of FNZ and AFNZ in real human plasma samples obtained from a healthy volunteer. The results obtained proved that the method described in this paper is suitable for the analysis of FNZ and AFNZ from real plasma samples for forensic and clinical purposes.

REFERENCES

1. Druid, H.; Holmgren, P.; Ahlner, J. Flunitrazepam: An Evaluation of Use, Abuse and Toxicity. *Forensic Sci. Int.* **2001**, *122*, 136–141.
2. Ohshima, T. A Case of Drug-Facilitated Sexual Assault by the Use of Flunitrazepam. *J. Clin. Forensic Med.* **2006**, *13*, 44–45.
3. Hackett, J.; Elian, A. A. Extraction and Analysis of Flunitrazepam/7-aminoflunitrazepam in Blood and Urine by LC-PDA and GC-MS Using Butyl SPE Columns. *Forensic Sci. Int.* **2006**, *157*, 156–162.
4. McCabe, S. E. Correlates of Nonmedical Use of Prescription Benzodiazepine Anxiolytics: Results from a National Survey of US. *Drug Alcohol Depend.* **2005**, *79*, 53–62.
5. San, L.; Tato, J.; Torrens, M.; Castillo, C.; Farre, M.; Cami, J. Flunitrazepam Consumption Among Heroin Addicts Admitted for In-Patient Detoxification. *Drug Alcohol Depend.* **1993**, *32*, 281–286.
6. Gelkopf, M.; Bleich, A.; Hayward, R.; Bodner, G.; Adelson, M. Characteristics of Benzodiazepine Abuse in Methadone Maintenance Treatment Patients: A 1 Year Prospective Study in an Israeli Clinic. *Drug Alcohol Depend.* **1999**, *55*, 63–68.
7. De Wet, C.; Reed, L.; Glasper, A.; Moran, M.; Bearn, J.; Gossop, M. Benzodiazepine Co-Dependence Exacerbates the Opiate Withdrawal Syndrome. *Drug Alcohol Depend.* **2004**, *76*, 31–35.
8. Rickert, V. I.; Wiemann, C. M.; Berenson, A. B. Prevalence, Patterns, and Correlates of Voluntary Flunitrazepam Use. *Pediatrics.* **1999**, *103* (1), E6.
9. Scott-Ham, M.; Burton, F. C. Toxicological Findings of Alleged Drug-Facilitated Sexual Assault in the United Kingdom Over a 3-year Period. *J. Clin. Forensic Med.* **2005**, *12*, 175–186.
10. Elian, A. A. Detection of Low Levels of Flunitrazepam and Its Metabolites in Blood and Bloodstains. *Forensic Sci. Int.* **1999**, *101*, 107–111.
11. Terada, M.; Masui, S.; Hayashi, T.; Watanabe, R.; Inoue, H.; Iino, M.; Nakatome, M.; Matoba, R.; Shinozuka, T.; Murai, T.; Tanak, E.; Honda, K. Simultaneous Determination of Flunitrazepam and 7-aminoflunitrazepam in Human Serum by Ion Trap Gas Chromatography-Tandem Mass Spectrometry. *Leg. Med.* **2003**, *5*, S96–S100.
12. Cui, S.; Tan, S.; Ouyang, G.; Pawliszyn, J. Automated Polyvinylidene Difluoride Hollow Fiber Liquid-Phase Microextraction of Flunitrazepam in Plasma and Urine Samples for Gas Chromatography/Tandem Mass Spectrometry. *J. Chromatogr. A* **2009**, *1216*, 2241–2247.
13. Smink, B. E.; Brandsma, J. E.; Dijkhuizen, A.; Lusthof, K. J.; de Gier, J. J.; Egberts, A. C. G.; Uges, D. R. A. Quantitative Analysis of 33 Benzodiazepines, Metabolites and Benzodiazepine-Like Substances in Whole Blood by Liquid Chromatography-(Tandem) Mass Spectrometry. *J. Chromatogr. B* **2004**, *811*, 13–20.
14. Bugey, A.; Rudaz, S.; Staub, C. A Fast LC-APCI/MS Method for Analyzing Benzodiazepines in Whole Blood Using Monolithic Support. *J. Chromatogr. B* **2006**, *832*, 249–255.
15. Marchi, I.; Schappler, J.; Veuthey, J. L.; Rudaz, S. Development and Validation of a Liquid Chromatography-Atmospheric Pressure Photoionization-Mass Spectrometry Method for the Quantification of Alprazolam, Flunitrazepam, and Their Main Metabolites in Haemolysed Blood. *J. Chromatogr. B* **2009**, *877*, 2275–2283.
16. Berthault, F.; Kintz, P.; Mangin, P. Simultaneous High-Performance Liquid Chromatographic Analysis of Flunitrazepam and Four Metabolites in Serum. *J. Chromatogr. B* **1996**, *685* (2), 383–387.
17. He, W.; Parissis, N. Simultaneous Determination of Flunitrazepam and Its Metabolites in Plasma and Urine by HPLC/DAD After Solid Phase Extraction. *J. Pharm. Biomed. Anal.* **1997**, *16*, 707–715.

18. El Mahjoub, A.; Staub, C. Simultaneous Determination of Benzodiazepines in Whole Blood or Serum by HPLC/DAD with a Semi-Micro Column. *J. Pharm. Biomed. Anal.* **2000**, *23*, 447–458.
19. Bugey, A.; Staub, C. Rapid Analysis of Benzodiazepines in Whole Blood by High-Performance Liquid Chromatography: Use of a Monolithic Column. *J. Pharm. Biomed. Anal.* **2004**, *35*, 555–562.
20. Bastos Borges, K.; Freire, E. F.; Martins, I.; Bastos De Siqueira, M. E. P. Simultaneous Determination of Multibenzodiazepines by HPLC/UV: Investigation of Liquid-Liquid and Solid-Phase Extractions in Human Plasma. *Talanta* **2009**, *78*, 233–241.
21. Benhamou-Batut, F.; Demotes-Mainard, F.; Labat, L.; Vincon, G.; Bannwarth, B. Determination of Flunitrazepam in Plasma by Liquid Chromatography. *J. Pharm. Biomed. Anal.* **1994**, *12* (7), 931–936.
22. Kiss, B.; Popa, D. S.; Bojita, M.; Loghin, F. Development and Validation of a High-Performance Liquid Chromatography Method with Ultraviolet Detection for the Determination of Flunitrazepam in Human Plasma. *Rev. Chim.-Bucharest* **2008**, *59* (12), 1301–1304.
23. Vlase, L.; Kiss, B.; Loghin, F.; Leucuta, S. E. Determination of Flunitrazepam in Human Plasma and Urine by HPLC with Mass Spectrometry Detection. *J. Liq. Chromatogr. Rel. Technol.* **2008**, *31* (16), 2442–2454.
24. Moffat, A. C.; Osselton, M. D.; Widdop, B. (Eds.) *Clarke's Analysis of Drugs and Poisons.* 3rd ed.; Pharmaceutical Press: London, UK, 2004; Vols.775–776; pp 1044–1045.
25. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. *Guidance for Industry. Bioavailability and Bioequivalence Studies for Orally Administered Drug Products-General Considerations*; Rockville, MD, USA, March 2003, http://www.fda.gov/cder/guidance/index.htm
26. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. *Guidance for Industry-Bioanalytical Method Validation*; Rockville, MD, USA, May 2001.