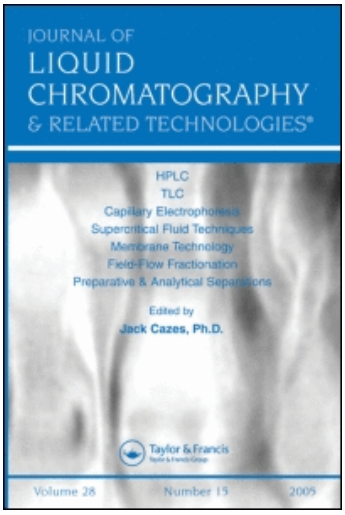

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Determination of Flunitrazepam in Human Plasma and Urine by HPLC with Mass Spectrometry Detection

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Abstract: A new, sensitive, and selective liquid chromatography coupled with mass spectrometry (LC/MS/MS) method for quantification of flunitrazepam in human plasma and urine was validated. The detection of flunitrazepam was in multiple reaction monitoring mode using an ion trap mass spectrometer equipped with an atmospheric pressure chemical ionisation ion source. The method was validated and proved to be linear, accurate, and precise over the range of 0.7–49.4 ng/mL in plasma samples and 0.5–33 ng/mL in urine. This is the first reported method for analysis of flunitrazepam in human plasma and urine that uses protein precipitation for plasma/direct injection for urine as a sample processing procedure. The total run time of the analytical method is less than 2 minutes. Another advantage of the method, besides its simplicity, is the very good recovery of the analyte. The validated LC/MS/MS method has been successfully applied to a pharmacotoxicological study of flunitrazepam.

Keywords: Flunitrazepam, Liquid chromatography, Mass spectrometry, Plasma, Urine, Validation

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INTRODUCTION

The abusive use of prescription drugs has been known for decades and is reaching now the level of an epidemic not only in the United States and in Western Europe, but also in developing countries. A national survey conducted by Substance Abuse and Mental Health Services Administration (SAMHSA), showed that, in 2003, approximately 15 million Americans, ages 12 and older used a psychotherapeutic for a condition other than medical use; the drugs most frequently involved being opioid analgesics, sedative/tranquilizers, and stimulants.[1] Studies conducted by SAMHSA also provide evidence that nonmedical use of prescription benzodiazepine anxiolytics is increasing among adolescents and young adults, and that the prevalence is correlated to a high degree with the nonmedical use of other drugs, such as prescription opiates.[2]

Since the first benzodiazepines were introduced on the market in 1960, there has been an evolution in the development of these drugs that have a broad range of therapeutic uses and are widely prescribed for their anxiolytic, hypnotic, anticonvulsive, and muscle relaxing properties. Newer derivatives have lower therapeutic doses, shorter action, and sometimes a more specific effect. A particular place is reserved to flunitrazepam (Rohypnol®), a fast acting hypnotic benzodiazepine recommended primary for insomnia and as premedication before anaesthesia in doses of 0.5–1 mg, with a maximum dose of 2 mg.[3,4] Due to its increasing abuse, the drug is no longer approved in US, but it is still used in Europe and Japan. It has a major toxicological interest due to its increased abuse among alcohol and drug addicts, to the fact that it may precipitate violent behaviour in predisposed subjects and as it is frequently involved in cases of drug facilitated sexual assaults.[3,4] In all these cases, a quick analysis, with a minimum sample pretreatment, but sensitive and selective, is essential from a legal and toxicological point of view.

To date, some high performance liquid chromatography (HPLC) methods with UV or MS/MS detection and gas chromatography methods with MS/MS detection have been published for the quantitative analysis of flunitrazepam in biological samples.[5–15] The major disadvantages of UV detection based assays are the low sensitivity (LLOQ $\geq$ 10 ng/mL) and lack of specificity. Another disadvantage of these methods (with UV or MS/MS detection) is the laborious sample preparation (consisting in liquid-liquid or solid phase extraction) that precedes the chromatographic analysis.[5–12] Fuh et al. elaborated recently an on-line solid phase extraction coupled with a HPLC/MS/MS method for quantification of flunitrazepam and 7-aminoflunitrazepam in human urine, but the total run time for one sample is very long (22 min) and the method's sensitivity is poor in the case of flunitrazepam (LLOQ = 3 ng/mL).[13] Macek et al. elaborated a HPLC/MS/MS

method for determination of omeprazole in plasma, using flunitrazepam as the internal standard. In this case, the sample treatment consisted in a simple protein precipitation, but since flunitrazepam is the internal standard, there is no information in the paper concerning the sensitivity of the method in the case of flunitrazepam.[14] In the GC analysis method a supplementary derivatization step is needed before the chromatographic analysis, which complicates sample treatment.

The aim of this study was to elaborate and validate a new liquid chromatography method coupled with mass spectrometry detection for the quantification of flunitrazepam in human plasma and urine, which can be successfully applied to toxicological studies.

EXPERIMENTAL

Materials and Reagents

Flunitrazepam standard (Figure 1) was obtained from Sigma (Steinheim, Germany). Acetonitrile, methanol (HPLC gradient grade), formic acid, ammonium acetate, and perchloric acid (analytical grade) were purchased from Merck KgaA (Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. Drug free human plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania. The human blank urine was obtained from healthy volunteers.

Preparation of Standard and Quality Control Solutions

The primary stock solution of flunitrazepam was prepared by dissolving an accurately weighed quantity of flunitrazepam in methanol (weighed on an Analytical Plus balance from Ohaus, USA).

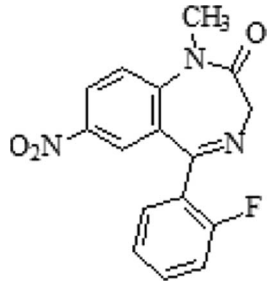

Figure 1. Chemical structure of flunitrazepam.

Working solutions of flunitrazepam were obtained by diluting specific volumes of stock solution with blank plasma or urine. (0.15 and 10.3 μg/mL flunitrazepam for plasma; 0.10 and 10.3 μg/mL flunitrazepam for urine).

These working solutions were used to spike different volumes of human plasma and urine blank, finally providing seven standards with the concentrations ranged between 0.77–49.44 ng/mL (plasma) and 0.52–32.96 ng/mL (urine), respectively.

Quality control (QC) samples at 2.32, 15.45, and 30.90 ng/mL for plasma and at 1.55, 5.15, and 10.30 ng/mL for urine were prepared by diluting specific volumes of flunitrazepam working standard with blank human plasma or urine, respectively.

Sample Preparation

Plasma Samples

Blank plasma (200 μL), calibration standards, and QC samples were vortex mixed (Vortex Genie 2, Scientific Industries) for 10 sec with 100 μL 7% perchloric acid in 1.5 mL polypropylene tubes. The samples were then centrifuged at 5000 rpm for 6 min (204 Sigma centrifuge, Osterode am Harz, Germany). Of the supernatant, 150 μL was transferred to an autosampler vial and 20 μL were injected into the HPLC system.

Urine Samples

A 0.4 mL aliquot urine sample was transferred to a 1.5 mL polypropylene tube and centrifuged at 5000 rpm for 6 min. Of the supernatant, 200 μL was transferred to an autosampler vial and a 20 μL aliquot was then injected for HPLC-MS/MS analysis.

LC-MS/MS Analysis

LC-MS analysis was performed using an Agilent Technologies Series 1100 LC (Agilent Technologies, Palo Alto, CA)/MSD VL (Brucker Daltonics GmbH, Germany) system with a Zorbax SB-C18 column, 3.5 μm, 100 mm × 3 mm i.d. (Agilent Technologies). The mobile phase consisted of a mixture of water and acetonitrile (55:45, v/v), each component being degassed before elution for 10 min in an Elma Transsonic 700/H (Singen, Germany) ultrasonic bath. The flow rate

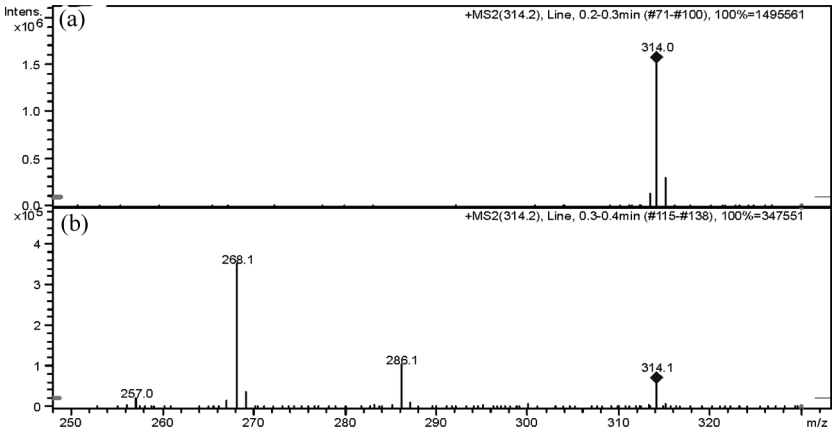

Figure 2. Full scan mass spectra of flunitrazepam (a) and mass spectra of the pseudo-molecular ion $[M + H]^+$ at $m/z = 314.1$ (b).

was set at 1 mL/min. The column temperature was maintained at 45°C. Chromatograms were processed using QuantAnalysis software.

The MS detection was in MRM mode using an ion trap mass spectrometer, with electrospray positive ionisation and atmospheric pressure chemical ionisation being tested.

The ion sources parameters were as follows: for ESI – capillary 4500 V, nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 325°C; for APCI – capillary 3500 V, nebulizer 60 psi (nitrogen), vaporiser 450°C, dry gas nitrogen at 5 L/min, dry gas temperature 350°C.

The monitored ion transitions were m/z 314 → (268 + 286).

Figure 2 shows a typical full scan EIC mass spectrum of flunitrazepam and a MS/MS spectra (indicating the 314 → (268 + 286) transition) obtained through the fragmentation of the protonated molecular ion $m/z = 314.1$.

Method Validation

The assay was validated in accordance with the industrial guidance for the bioanalytical method validation.[16–18]

The selectivity was checked by comparing six different plasma/urine blanks with the corresponding spiked plasma/urine samples.

Linearity was studied by analyzing singlicate calibration standards at concentration levels of 0.77, 1.55, 3.09, 6.18, 12.36, 24.72, and 49.44 ng/mL. Samples were prepared and analyzed as described

in sections below. The concentration of analyte was determined automatically by the instrument data system using the external standard method. The calibration curve model was determined by the least squares analysis. The applied calibration model was $y = c + bx + ax^2$, weight $1/y$ $(1/y)$ quadratic response, where $y =$ area and $x =$ concentration. Linearity was determined by checking five calibration curves on five different working days. The calibration model was accepted if the residuals were within $\pm 20\%$ at the lower limit of quantification (LLOQ) and within $\pm 15\%$ at all other calibration levels, and at least two thirds of the calibration standards meet this criterion, including highest and lowest calibration levels.

Regarding the sensitivity of the assay, the LLOQ was set at the lowest calibration level with an accuracy and precision less than 20%.

Precision is defined as coefficient of variation (CV%) and accuracy as relative deviation expressed as percentage error of the calculated value as compared with target added concentrations (true value).

The accuracy and intra-run precision were determined by analysis on the same day of five different samples at 2.32, 15.45, and 30.90 ng/mL of flunitrazepam for urine. The inter-run accuracy and precision were determined at the same concentrations of flunitrazepam, but on five different experimental days.

The relative recoveries were analyzed at each of the three QC levels and also at the LLOQ, by comparing the response of treated plasma/urine samples with the response of untreated standards in solvent with the same concentration of flunitrazepam as the plasma/urine QC sample.

The stability study of flunitrazepam in human plasma included the evaluation of room temperature stability (RTS) and post preparative stability (PPS) in the autosampler. In the case of urine samples only the RTS was evaluated, since the biological matrix is not physically (chemically) pre-treated. The evaluation of stability was performed at all three QC levels.

Clinical Application

The method described above has been applied to a pharmacotoxicological study of flunitrazepam.

RESULTS AND DISCUSSION

It's well known that in LC/MS, the MS signal can be greatly improved by changing in a proper way the ionisation source and especially the

mobile phase. For testing method sensitivity, two ionisation techniques were involved: ESI (electrospray) and APCI (atmospheric pressure chemical ionisation). ESI and APCI are the most currently used atmospheric pressure ionisation sources. For each of these, several mobile phases were tested. However, besides the absolute signal intensity, another important factor in LC/MS analysis of compounds in biological matrixes is signal suppression (ion suppression). This ion suppression is due to a phenomenon termed matrix effect. Different mechanisms have been proposed to explain the matrix effect. One possible explanation is the ionisation competition between the different species present in the effluent. The data published regarding the matrix effect shows clearly that APCI is less susceptible to matrix effect than ESI, because in case of APCI ionisation takes place in the gas phase.[19,20]

In order to find the best MS interface and mobile phase composition, for each MS interface/mobile phase tested, we recorded both the peak area (and signal to noise ratio) and ion suppression percent. These tests were conducted using as biological matrix human plasma and urine, respectively. The ion suppression test was done as follows: a sample of 4 ng/mL flunitrazepam in either plasma or urine was prepared and analysed according to procedures described in the Experimental sections (50 pg flunitrazepam injected on the column). Another sample with the same concentration but prepared in water was treated as the biological sample and also injected in the LC/MS system. The ionisation suppression ratio was calculated as follows:

$$\text{Ionisation suppression } \% = 100 \times (1 - \text{area in case of biological sample/area in case of standard solution})$$

Table 1 presents the results obtained during these tests for both ESI and APCI ionisation sources and for the different mobile phases tested in the case of human plasma and urine, respectively.

Based on results obtained during the optimisation process with the two ionisation sources and different mobile phases, the final LC/MS/MS conditions chosen consisted of a mixture of acetonitrile/water (45:55, v/v) as mobile phase and an APCI ionisation source.

Usually, the recommended strategies to diminish signal suppression effect (matrix effect) are an improved sample preparation (a more extensive sample clean-up) or improved chromatographic separation.

The assay described in this paper is the first LC-MS method for the quantification of flunitrazepam that involves only a simple protein precipitation with 7% perchloric acid in the case of plasma and a direct injection into the column, after centrifugation, in the case of human urine. Despite the very simple and rapid sample preparation,

Table 1. Results obtained during method optimization (signal intensity, ion suppression)

Ionisation source	Mobile phase (v/v)	Signal intensity (Arbitrary units)	Ion suppression (%)	
			Plasma	Urine
ESI	Acetonitrile/0.1% Formic acid (45/55)	1.2×10^5	62.0	80–90
	Acetonitrile/0.2% Formic acid (45/55)	1.3×10^5	64.2	
	Acetonitrile/1 mM Ammonium acetate (45/55)	2.2×10^5	59.4	
	Acetonitrile/3 mM Ammonium acetate (45/55)	2.4×10^5	61.9	
	Methanol/3 mM Ammonium acetate (60/40)	0.8×10^5	55.7	
	Acetonitrile/Water (45/55)	0.2×10^5	78.1	
APCI	Methanol/3 mM Ammonium acetate (55/45)	1.25×10^5	6.0	5.3
	Methanol/0.1% Formic acid (55/45)	1.25×10^5	6.8	9.0
	Methanol/Water (55/45)	1.2×10^5	5.6	7.5
	Acetonitrile/Water (45/55)	1.3×10^5	5.2	7.2

the APCI ionisation source and the final mobile phase guaranteed an ion suppression of only 5.2% and 7.2% for plasma and urine samples, respectively. The results obtained during the optimisation process (Table 1) were in accordance with the information already published regarding the influence of matrix effect in ESI and APCI.

Using the final chromatographic conditions and the APCI source, the method was validated in accordance with the industrial guidance for the bioanalytical method validation.

Regarding the selectivity, no significant interferences or ion suppression from endogenous compounds was observed at the retention time of the analyte in the case of both biological matrixes. Figure 3 shows the typical chromatograms of blank plasma, spiked plasma with flunitrazepam at LLOQ level (0.77 ng/mL flunitrazepam), blank urine and spiked urine with flunitrazepam at LLOQ level (0.5 ng/mL flunitrazepam). The retention time for flunitrazepam was 1.4 min.

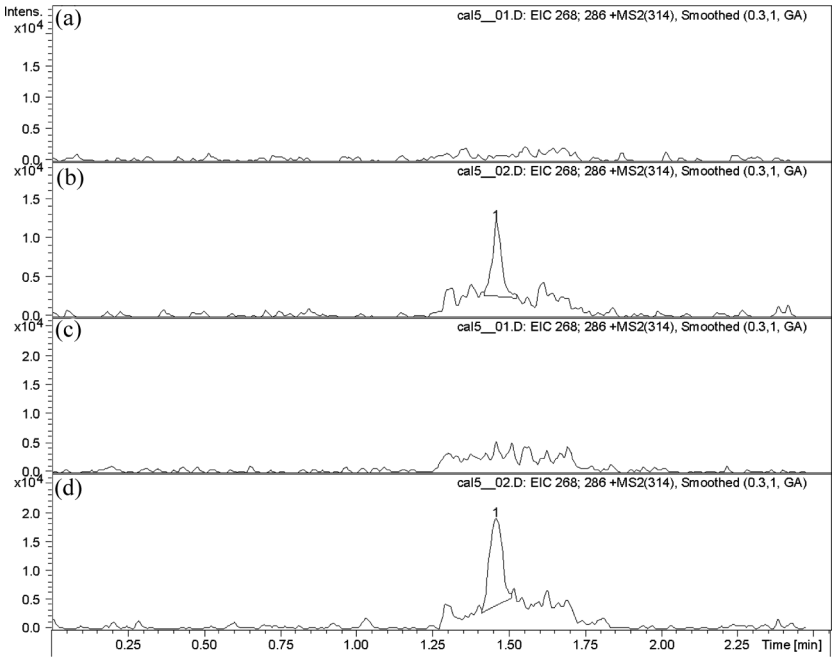

Figure 3. (a) Chromatograms of plasma blank; (b) spiked plasma at LLOQ (0.77 ng/mL flunitrazepam); (c) urine blank; and (d) spiked urine at LLOQ (0.5 ng/mL flunitrazepam).

The calibration curves showed good linearity over the studied concentration range (0.77–49.44 ng/mL for plasma and 0.52–32.96 ng/mL for urine), with correlation coefficients (r) 0.9988 ± 0.000614 and 0.9978 ± 0.001058 (mean $\pm$ S.D., $n = 5$) for human plasma and urine, respectively.

Having the advantage of simple and rapid sample preparation and short chromatographic run time, the method showed similar or even superior sensitivity to methods described in other scientific papers, based on LC-MS, GC-MS or LC-PDA analysis and a more laborious sample preparation.[5–15]

Due to the simple sample preparation, involving only protein precipitation and centrifugation, without any extraction process, the recovery of flunitrazepam was very good (superior to 80%). The LLOQ was established at 0.77 ng/mL and 0.5 ng/mL flunitrazepam for plasma and urine, respectively, with accuracy and precision less than 20% (Tables 2 and 3).

Table 2. Within-run precision, accuracy and recovery for flunitrazepam ($n = 5$)

Sample	$c_{nominal}$ (ng/mL)	Mean c_{found} (ng/mL) ± S.D.	CV (%)	Inaccuracy (%)	Recovery (%) ± S.D.
Plasma	0.77	0.72 ± 0.05	7.0	−6.7	115.7 ± 7.9
	2.32	2.21 ± 0.15	6.6	−4.8	101.6 ± 6.6
	15.45	15.09 ± 0.60	4.0	−2.4	109.0 ± 4.3
	30.90	32.39 ± 1.49	4.6	4.8	105.4 ± 4.8
Urine	0.52	0.58 ± 0.04	7.0	12.9	90.1 ± 8.2
	1.55	1.63 ± 0.13	7.8	5.8	106.0 ± 9.0
	5.15	5.08 ± 0.23	4.4	−1.4	90.7 ± 4.1
	10.30	10.72 ± 0.52	4.9	4.0	101.1 ± 5.0

The within- and between-run precision and accuracy data are summarized in Tables 2 and 3. According to these results the assay is accurate and precise in the studied concentration range.

The results of the stability study showed that no significant degradation of flunitrazepam occurred under the tested conditions in the two biological matrixes. In the case of storage at room temperature, flunitrazepam proved to be stable in plasma and urine samples for at least 4 h, the mean change in analyte content being −4.8% and −1.3% in the case of plasma, and +12.3% and +11.1% in urine at the two concentration levels tested. In the plasma samples the post-preparative stability study showed the stability of flunitrazepam after sample preparation for at least 3 h (inaccuracy <15%).

The validated method was applied in a pharmacotoxicological study of flunitrazepam, in order to quantify the analyte in human plasma and urine in two healthy volunteers after a single dose of flunitrazepam of 1 or 2 mg. The results are summarized in Table 4.

Table 3. Between-run precision and accuracy for flunitrazepam ($n = 5$)

Sample	$c_{nominal}$ (ng/mL)	Mean c_{found} (ng/mL) ± S.D.	CV (%)	Inaccuracy (%)	Recovery (%) ± S.D.
Plasma	0.77	0.86 ± 0.06	7.4	11.8	104.8 ± 18.4
	2.32	2.32 ± 0.33	14.1	0.3	98.6 ± 8.6
	15.45	14.72 ± 0.73	5.0	−4.7	102.3 ± 9.3
	30.90	30.79 ± 2.23	7.2	−0.3	103.3 ± 4.6
Urine	0.52	0.49 ± 0.09	18.9	−4.1	83.4 ± 9.4
	1.55	1.65 ± 0.04	2.7	6.5	103.1 ± 11.8
	5.15	5.32 ± 0.24	4.4	3.3	99.9 ± 6.6
	10.30	10.94 ± 0.26	2.4	6.2	100.9 ± 6.3

Table 4. The results obtained in case of single dose ingestion by healthy volunteers

Healthy volunteers	Gender	Body weight (kg)	Flunitrazepam dose (mg)	c_{found} (ng/mL)			
				Plasma		Urine	
				3 h*	6 h*	5 h*	9 h*
Subject 1	F	47	1	3.492	1.560	1.268	0.784
Subject 2	M	102	2	3.122	1.544	1.598	0.553

*Time after ingestion of flunitrazepam (h).

CONCLUSIONS

The method described in this paper is the first reported in the literature for the quantification of flunitrazepam after a very simple and rapid sample preparation consisting of protein precipitation in the case of human plasma or by direct biological matrix injection in the case of urine samples. The method was validated in accordance with the bioanalytical methods validation guidelines and showed good linearity, accuracy, and precision in the studied concentration range. No interferences due to endogenous compounds were observed. The use of the APCI ionisation source permitted the minimization of matrix effect, so that despite the very simple sample preparation the method showed very high sensitivity. Another advantage of the method is the short chromatographic run time of only 2 min. The method was successfully used in for flunitrazepam quantification during a pharmacotoxicological study on healthy human volunteers.

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