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Automated solid-phase extraction and liquid chromatography–electrospray ionization-mass spectrometry for the determination of flunitrazepam and its metabolites in human urine and plasma samples

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Abstract

A sensitive and specific method using reversed-phase liquid chromatography coupled with electrospray ionization-mass spectrometry (LC–ESI-MS) has been developed for the quantitative determination of flunitrazepam (F) and its metabolites 7-aminoflunitrazepam (7-AF), *N*-desmethylflunitrazepam (*N*-DMF) and 3-hydroxyflunitrazepam (3-OHF) in biological fluids. After the addition of deuterium labelled standards of F,7-AF and *N*-DMF, the drugs were isolated from urine or plasma by automated solid-phase extraction, then chromatographed in an isocratic elution mode with a salt-free eluent. The quantification was performed using selected ion monitoring of protonated molecular ions ($M+H^+$). Experiments were carried out to improve the extraction recovery (81–100%) and the sensitivity (limit of detection 0.025 ng/ml for F and 7-AF, 0.040 ng/ml for *N*-DMF and 0.200 ng/ml for 3-OHF). The method was applied to the determination of F and metabolites in drug addicts including withdrawal urine samples and in one date-rape plasma and urine sample.
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Keywords: Flunitrazepam; 7-Aminoflunitrazepam; *N*-Desmethylflunitrazepam; 3-Hydroxyflunitrazepam

1. Introduction

Flunitrazepam (F) is a pronounced hypnotic effect benzodiazepine used in the treatment of insomnia and as preanesthetic medication [1]. However, clinical observations have shown that F is frequently misused by polydrug users and is often involved in date-rape cases [2,3,1].

Because of the small dosage of the drug administered (orally or intravenously in 1- to 2-mg doses) and extensive metabolism through several pathways [4], identification of F and its metabolites is especially difficult. Only a small amount is excreted as unchanged drug. The principal metabolites are the 7-aminoflunitrazepam (7-AF), the 3-hydroxyflunitrazepam (3-OHF) and the *N*-desmethylflunitrazepam (*N*-DMF) with at least one of these compounds (the 7-amino metabolite) with anaesthetic activity (Fig. 1) [5]. Furthermore, the decomposition of F has been reported previously in biological fluids like for the other nitrobenzodiazepines (clonazepam, nitrazepam, loprazolam) [6–8]. The parent drug and metabolites *N*-DMF and

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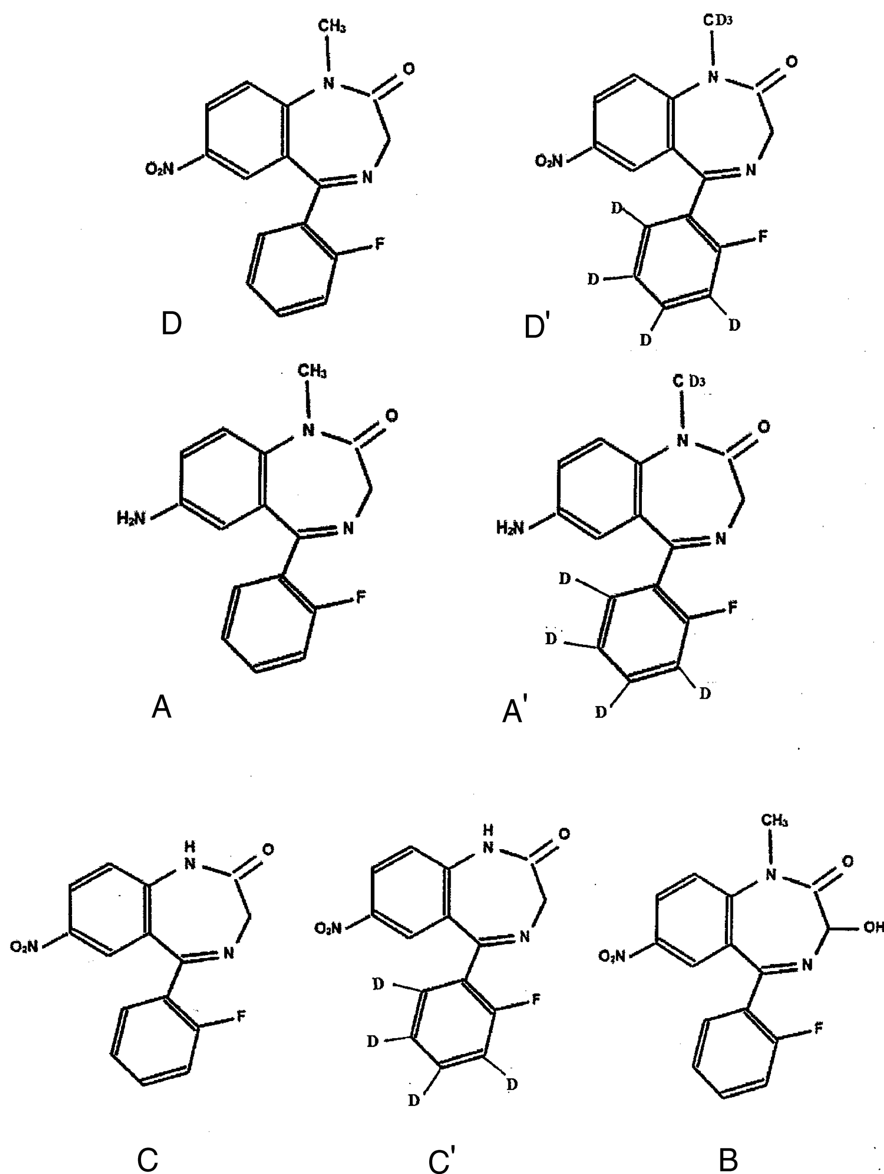

Fig. 1. Chemical structures of flunitrazepam $C_{16}H_{12}FN_3O_3$ (D) and flunitrazepam-d$_7$ $C_{16}H_5D_7FN_3O_3$ (D′), 7-aminoflunitrazepam $C_{16}H_{14}FN_3O$ (A) and 7-aminoflunitrazepam-d$_7$ $C_{16}H_7D_7FN_3O$ (A′), *N*-desmethylflunitrazepam $C_{15}H_{10}FN_3O_3$ (C) and *N*-desmethylflunitrazepam-d$_4$ $C_{15}H_6D_4FN_3O_3$ (C′), 3-hydroxyflunitrazepam $C_{16}H_{12}FN_3O_4$ (B).

3-OHF disappeared from biological fluids within a few days at room temperature and in solutions exposed to light. The amino metabolite is the predominant compound in urine and its concentration can greatly exceed the concentration of unchanged F in stored blood samples as a consequence of an in vitro reduction of the 7-nitro group to the 7-amino group [9].

Several analytical methods for the determination of F in biological fluids have been published previously [1–4,9–19]. Benzodiazepine immunoassays generally used as screening tests (radioimmunoassays, fluorescence polarisation immunoassays, enzyme immunoassays), identify the presence of benzodiazepine compounds with good specificity. However, this monitoring could be of questionable significance for its lack of sensitivity for some benzodiazepines, especially for low dose benzodiazepines like F [1,20–23]. The development of radioreceptor assays with a lower limit of detection (LOD) allows measurement of pharmacologically active concentrations of F, but does not allow its identification [24,25].

Gas chromatography (GC) using electron-capture detection (ECD) has been used with great sensitivity, however this methodology lacks specificity and does not detect 7-AF and 3-OHF [15]. Reported GC methods with mass-spectrometric (MS) detection using electron impact (EI) or chemical ionisation (CI) are sensitive and specific, but do not detect the polar metabolites without derivatization [9]. Furthermore, commonly used silylation procedures are not appropriate for 7-amino-groups and do not allow 7-amino benzodiazepine determination [18].

In contrast, liquid chromatography coupled with MS detection (LC–MS) when using electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) leads to highly sensitive methods without a derivatization step [9,13–15,26]. Bogusz et al. [15] stated that APCI leads to a seven-fold higher sensitivity for F (0.2 ng/ml) when compared to an ESI method. The same APCI detection mode enabled a limit of detection of 0.19 ng/ml for F by Darius et al. [26].

In the present work we developed a very sensitive ESI procedure (LOD 0.025 ng/ml for F and 7-AF) using an eluent without any electrolyte, allowing both identification and quantification of F and metabolites in clinical or forensic samples, after automated solid-phase extraction (SPE). Only a few methods include the optimised simultaneous determination of F, 7-AF, *N*-DMF and 3-OHF. The determination of 3-OHF needs a previous 3-OHF glucuronide enzyme-based hydrolysis step yielding 3-OHF, which is then extracted and determined.

2. Experimental

2.1. Reagents

Dichloromethane (for trace analysis), methanol (for pesticide analysis) and isopropanol (HPLC grade) were purchased from SDS (Valdonne-Peypin, France). Acetonitrile (HPLC grade), potassium dihydrogenorthophosphate, potassium hydroxide, orthophosphoric acid 85% (v/v) (analytical grade) and ammonia solution 25% (v/v) (analytical grade) were provided by Merck (Nogent sur Marne, France). Deionised water was generated in the laboratory with an Elgastat UHQ PS filtration system (Elga Labwater, Le Plessis Robinson, France). F, flunitrazepam-d_7 (F-d_7, the internal standard for F and 3-OHF), 7-AF, 7-aminoflunitrazepam-d_7 (7-AF-d_7), *N*-DMF and *N*-desmethylflunitrazepam-d_4 (*N*-DMF-d_4) were purchased from Promochem (Molsheim, France). 3-OHF was obtained from Lipomed (Arlesheim, Switzerland). A 0.1-*M* phosphate buffer was prepared by dissolving 13.61 g potassium dihydrogenorthophosphate in water (900 ml), adjusting the pH to 6.0 with 1.0 *M* potassium hydroxide and diluting to 1 l with water. A 0.33-*M* orthophosphoric acid was prepared by mixing 9.5 ml of a solution of orthophosphoric acid 85% (v/v) with 50 ml of water, and diluting to 500 ml with water. The SPE elution mixture was prepared daily by adding 2 ml ammonia solution 25% (v/v) to 98.0 ml of dichloromethane–isopropanol (70:30, v/v). β-Glucuronidase 374 000 U/g from the *Helix pomatia* was obtained from Sigma (St. Quentin, Fallavier, France).

Isolation of drugs was performed by SPE using Isolute HCX mixed-mode cartridges, 3 ml/130 mg (Touzart et Matignon, Courtaboeuf, France) positioned on an Aspec XL automated SPE-device (Gilson, Villiers le Bel, France) working with positive air pressure pushes.

2.2. Stock solutions and calibration samples

Stock solutions (1 g/l or 100 mg/l in methanol or acetonitrile) were stored at−28 °C. Spiking solutions containing F and its three metabolites were prepared by combining aliquots of the stock solutions and diluting to 100, 10 and 1 mg/l with the appropriate

solvent. The internal standard solutions were prepared at final concentrations of 1 mg/l by diluting the stock solutions with methanol or acetonitrile. The spiking solutions were stored at −28 °C and found to be stable for at least 1 month.

For preparation of the urine or plasma calibrators an appropriate amount of the spiking solutions were added to blank, drug-free urine or plasma, to yield the mentioned range of calibration concentrations (0.5, 1.0, 2.5, 5.0 10.0 and 20.0 ng/ml). Quality control (QC) samples were similarly prepared from independant methanolic stock solutions of standards at concentrations of 0.8, 3.0 and 12 ng/ml. Internal standards were added at concentration of 10 ng/ml. A Medidrug® benzodiazepine S level 1 control (Medichem, Molsheim, France) containing F and 7-AF, 10 ng/ml each, was also incorporated into the run for quality control purposes. Calibration curves were constructed by linear least squares regression using analyte:deuterated standard peak area ratio versus concentration.

Stability of flunitrazepam and its metabolites in urine was assessed by five replicate analyses of the three in-house control samples. The samples were initially analysed within 2 h of preparation to obtain reference concentrations. The samples were then used to assess the stability of analytes during storage under refrigeration (4 °C) after 24 h and 12 days, respectively. Aliquots of the three samples were also stored deep frozen (−28 °C), then thawed at ambient temperature and reassayed. The three aliquots were refrozen and thawed twice more to provide data from three freeze–thaw cycles for a period of 3 weeks.

2.3. Biological samples

Four urine samples taken from four drug addicts (male subjects, 26–37 years of age) showed positive results for 7-AF in LC–photo-diode array detection screening and were subjected to LC–ESI-MS. One urine and one plasma sample, obtained from a victim of drug-facilitated rape (male subject, 22 years of age), were collected within 4–6 h of the alleged event and were also analysed. Finally, eleven urine specimens taken at 24-h intervals were collected from a polydrug addict (male subject, 30 years of age, hospitalised for a F withdrawal treatment after an official chronic daily ingestion of 2 mg F) and were analysed.

2.4. Sample preparation

Urine or plasma samples were extracted directly as follows: after priming the SPE column with 2 ml of methanol and 2 ml of 0.1 *M* phosphate buffer, samples (1 ml previously diluted in 3 ml of 0.1 *M* phosphate buffer, spiked with 10 ng of the internal standards and equilibrated for 15 min) were allowed to pass through the column under positive air pressure. The cartridge was rinsed successively with 4 ml of methanol–water (20:80, v/v), 4 ml of 0.33 *M* orthophosphoric acid, 4 ml of a methanol–water (20:80, v/v) and 4 ml of dichloromethane. The analytes were eluted with 2 ml of the elution solvent and the eluant was dried under a stream of nitrogen. The residue was dissolved in 30 μl LC mobile phase, transferred to autosampler vials fitted with 200-μl inserts and 5 μl of the solution were injected.

Urine samples for 3-OHF specific determination were hydrolysed prior to extraction. The specimens were buffered to pH 4.5 with acetic acid and incubated for 1 h at 56 °C with 125 μl of β-glucuronidase. The internal standards were added after hydrolysis.

2.5. Instrumentation

2.5.1. HPLC

The following HPLC system (Perkin-Elmer, Courtaboeuf, France) was used: two series 200 LC pumps, a series 200 cooled autosampler (4 °C), a column oven maintained at 30 °C, a Nova-Pak C_{18} column, 2.1 mm I.D.×150 mm, 4 μm particle size, (Waters, St. Quentin, France), eluted with a water–acetonitrile–methanol (49.5:5.5:45.0, v/v/v) for the mobile phase at a flow-rate of 150 μl/min.

2.5.2. Mass spectrometry

A TurboIonSpray® source equipped API 150 EX mass spectrometer (Applied Biosystems, Courtaboeuf, France) was used in ESI positive ion mode. The mass spectrometer was operated with the following specifications: Turbo heater temperature, 200 °C; air (ultrahigh purity) at 7 1/min as Turbo heater gas. The ionspray voltage was adjusted to 5500 V. Orifice

Table 1
Selected ions, voltage adjustments and retention times for flunitrazepam, metabolites and deuterated standards

Compound		t_R [a] (min)	Quantification ions		Identification ions	
			m/z	OR[b]/RNG[c]	*m/z*	OR[b]/RNG[c]
A′	7-AF-d_7	3.65	291.3	16/100	244.1	60/150
A	7-AF	3.72	284.1	16/100	227.1	60/150
B	3-OHF	7.03	330.2	6/110	284.1	40/110
C′	*N*-DMF-d_4	7.37	304.3	21/120	257.9	60/120
C	*N*-DMF	7.52	300.2	21/120	253.9	60/120
D′	F-d_7 [d]	9.46	321.2	6/130	275.2	40/130
D	F	9.70	314.2	6/130	268.2	40/130

[a] Retention time.
[b] Orifice voltage (V).
[c] Ring voltage (V).
[d] Internal standard for F and 3-OHF.

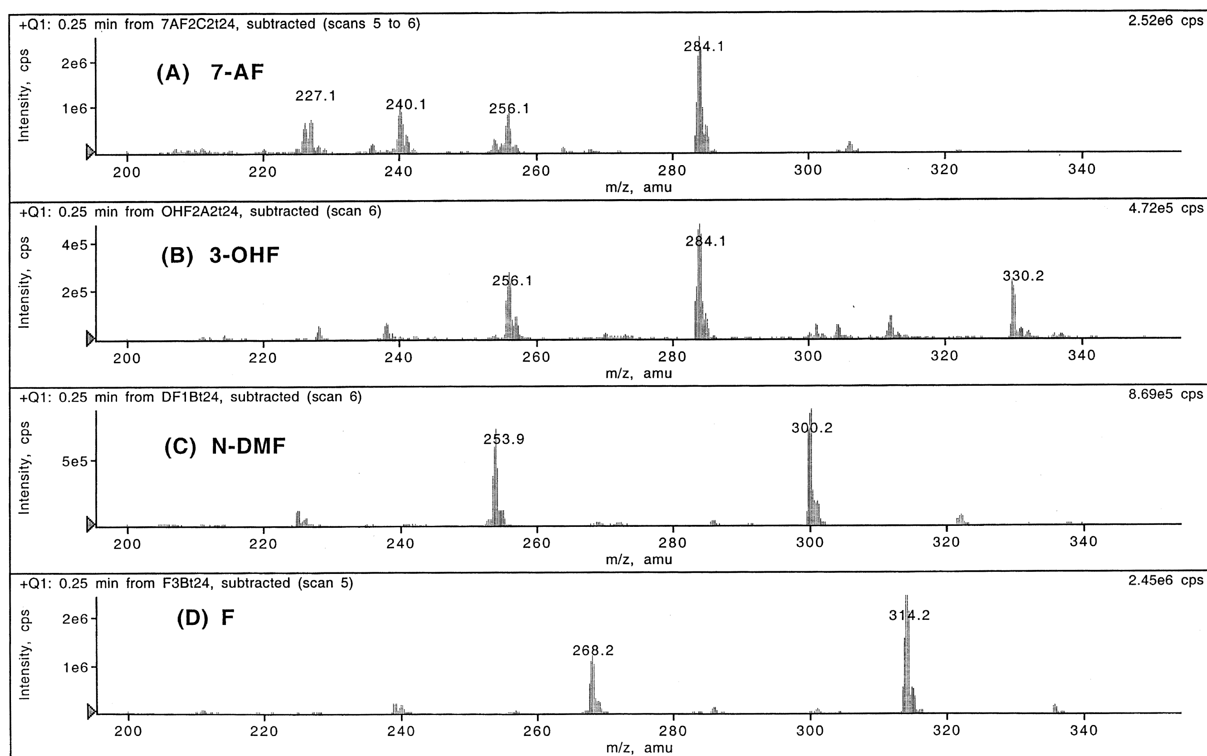

Fig. 2. Positive ion mass spectra registered at orifice and ring voltages (OR, RNG) allowing fragmentation: (A) 7-AF, (B) 3-OHF, (C) *N*-DMF and (D) F. OR/RNG (V): (A) 60/150, (B) 40/110, (C) 60/120, (D) 40/130.

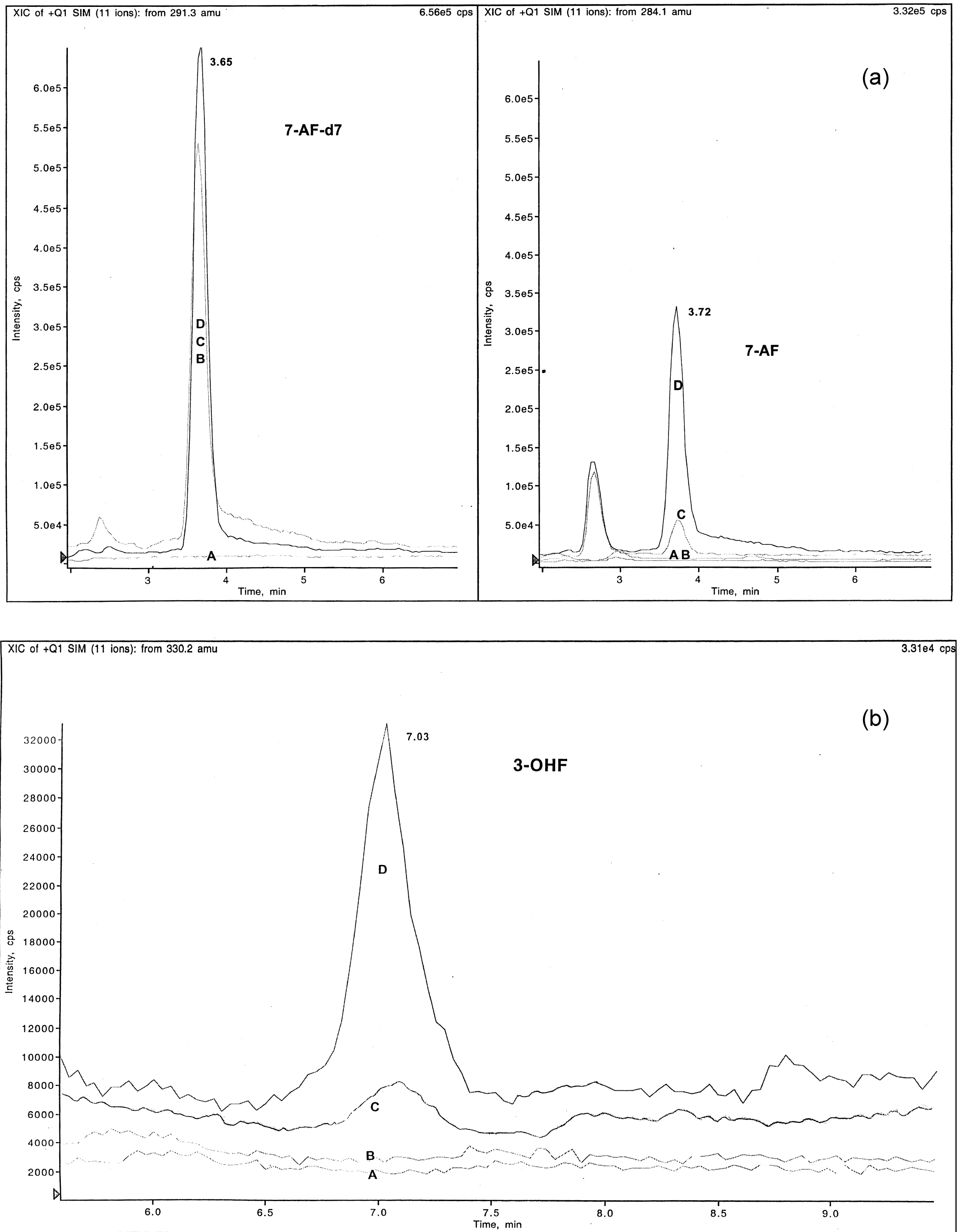

Fig. 3. Ion chromatograms registered from (A) double blank healthy human urine; (B) blank urine spiked with deuterated internal standards; (C) blank urine spiked with Flunitrazepam and metabolites at the concentration of 0.5 ng/ml; (D) blank urine spiked with flunitrazepam and metabolites at the concentration of 5 ng/ml.

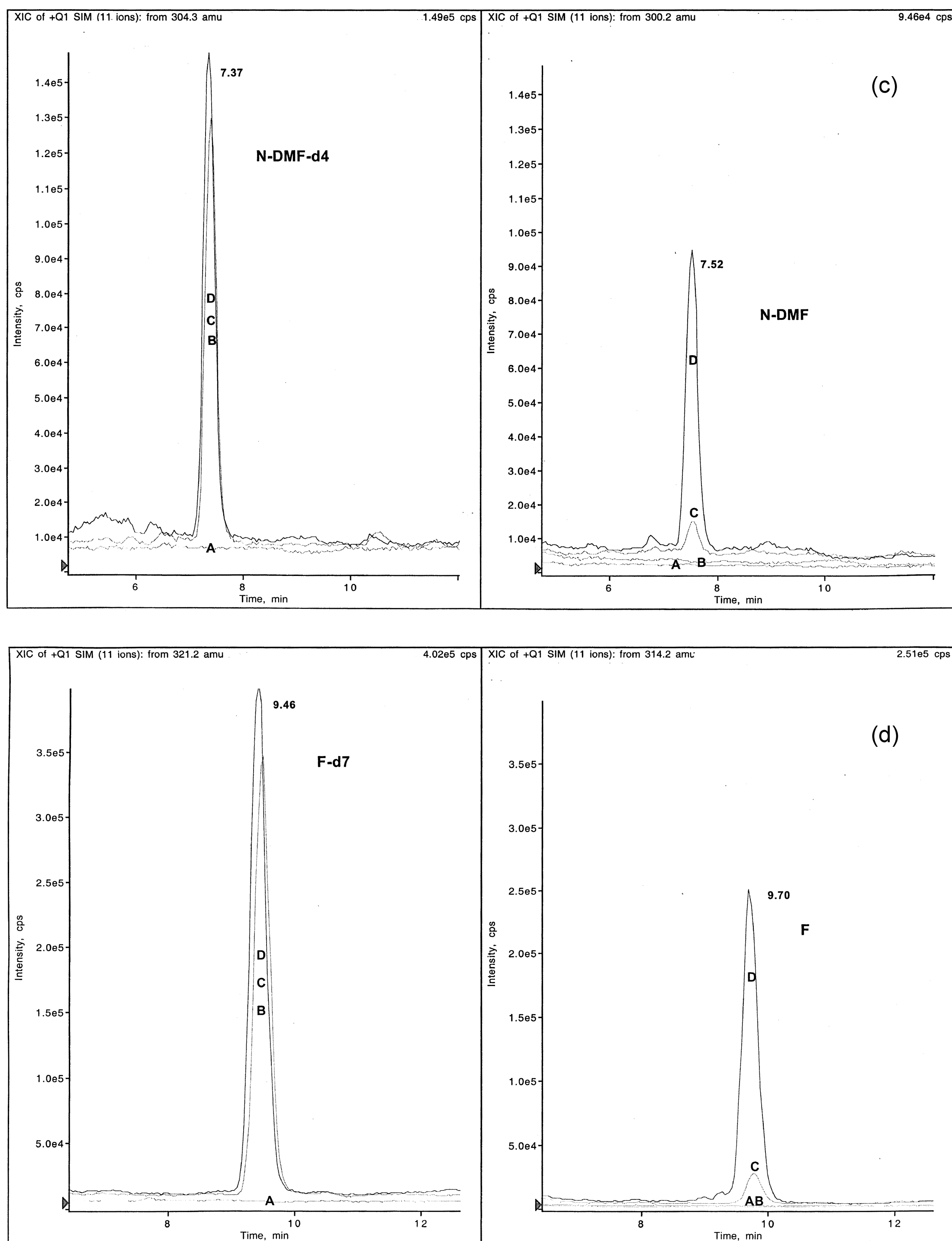

Fig. 3. (*continued*)

and Ring voltages were optimised for each ion. Detection was performed using selected ion monitoring (SIM).

3. Results

3.1. Selected ions, mass spectra and chromatograms

Table 1 shows the selected ions for each compound, the different voltage adjustments used and their retention times. ESI positive ion mass spectra of F and metabolites are presented in Fig. 2: protonated molecular ions ($M+H^+$) were selected for quantification. Ions m/z 268.2, 253.9 and 284.1 (M−46), corresponding to loss of NO_2 from the molecular ions, were selected for the F, *N*-DMF and 3-OHF confirmation, respectively. Ion m/z 227.1 (M−57), perhaps loss of $CH_3N{=}C{=}O$ from the molecular ion, was selected for 7-AF confirmation. The retention times of F and its metabolites were between 3.72 and 9.70 min. Fig. 3 shows selected ion chromatograms registered from (A) double blank healthy human urine; (B) blank urine—the same human urine sample spiked with deuterated internal standards; (C) blank urine spiked with F and metabolites at the concentration of 0.5 ng/ml (LLOQ); (D) blank urine spiked with F and metabolites at the concentration of 5 ng/ml. No interfering peaks of endogenous compounds from urine or plasma samples were found. A run of 20 min was selected to secure against late eluting peaks.

3.2. Investigation of mobile-phase composition

Different mobile-phase compositions were investigated to improve the sensitivity (Fig. 4). Methanol and/or acetonitrile were used as the mobile-phase organic solvent; water, 5 m*M* ammonium formate buffer or 0.1% v/v formic acid solution for the mobile-phase aqueous solvent. Ion current was registered after injection of 50 ng 7-AF under each condition. The water–acetonitrile–methanol (49.5:5.5:45.0, v/v/v) mobile-phase composition resulted in the greatest sensitivity.

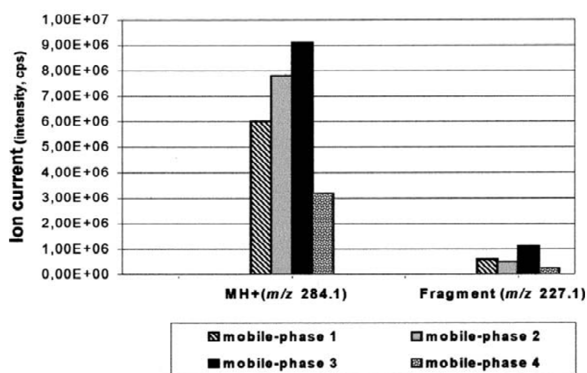

Fig. 4. Effect of mobile-phase composition on the sensitivity of the determination of 7-aminoflunutrazepam (50 ng injected). Mobile phases: 1=0.1% formic acid–methanol (40:60, v/v); 2=0.1% formic acid–acetonitrile (40:60, v/v); 3=water–acetonitrile–methanol (49.5:5.5:45.0, v/v/v); 4=5 m*M* ammonium formate buffer, pH 3.0–(acetonitrile–5 m*M* ammonium formate buffer, pH 3.0, 90:10, v/v) (65:35, v/v).

3.3. Effects of turboionspray probe temperature on sensitivity

The rate of solvent evaporation and consequent droplet diameter reduction is enhanced significantly, leading to improvements in sensitivity, with the use of heated gas (200 °C) as shown in Fig. 5. Under these conditions, the experiment leads to 4×, 3.5×, 2× and 1.3× higher sensitivity for the determination of *N*-DMF, 3-OHF, F and 7-AF, respectively compared with gas at room temperature (24 °C). No

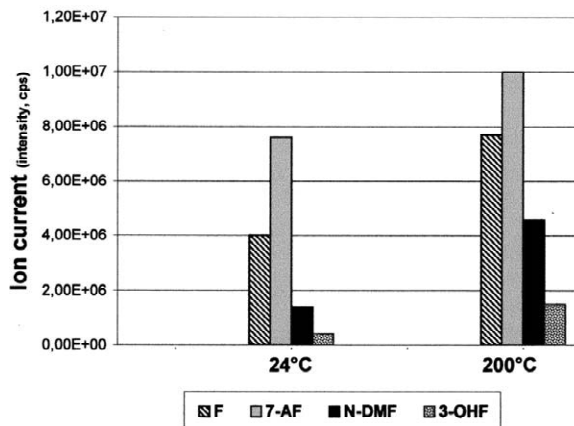

Fig. 5. Ion current after injection of 50 ng F and its metabolites. Turbospray heater gas temperature: 24 °C or 200 °C.

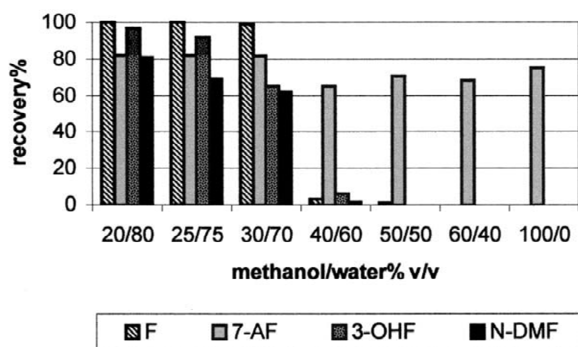

Fig. 6. Extraction recoveries from spiked urine samples for flunitrazepam and its metabolites as a function of methanol concentration in the washing solution (methanol–water).

improvement has been observed under higher temperature conditions. Despite the use of heat, no evidence of thermal degradation has been observed.

3.4. Extraction

We slightly modified the extraction method described by Deinl et al. [9]. Our extraction conditions (composition of washing and elution solvents) provide clean extracts for later chromatographic analysis and high extraction recoveries (81%). Recoveries from spiked urine samples are a function of the methanol concentration in the washing solution (methanol–water). Optimal conditions for the extraction of F and its main metabolites are obtained with methanol–water washing solvent (20:80, v/v). The results shown in Fig. 6 indicate a general decrease in F, *N*-DMF and 3-OHF recoveries with a concentration of methanol higher than 20%. Only the 7-AF recovery remained constant under these conditions.

3.5. Validation

The validation data on urine and plasma samples are presented in Tables 2 and 3. Linear correlation was verified over the range 0.5–100 ng/ml for all compounds. The LODs calculated for a signal-to-noise ratio of 3 were 0.025 ng/ml for F and 7-AF, 0.040 ng/ml for *N*-DMF and 0.200 ng/ml for 3-OHF. The lower limit of quantification (LLOQ) was set at 0.5 ng/ml, the value of the lowest calibrator. The upper limit of quantification (ULOQ) was set at 20 ng/ml, the value of the highest calibrator. Ex-

Table 2
Validation data in urine samples

	F			7-AF			*N*-DMF			3-OHF		
Limit of detection (LOD) (ng/ml)	0.025			0.025			0.040			0.200		
Lower limit of quantification (LLOQ) (ng/ml)	0.5			0.5			0.5			0.5		
Upper limit of quantification (ULOQ) (ng/ml)	20.0			20.0			20.0			20.0		
Regression analysis	$y=0.170\ (\pm 0.002)x + 0.030\ (\pm 0.008)$ $r=0.9991$			$y=1.112\ (\pm 0.044)x + 0.014\ (\pm 0.005)$ $r=0.9993$			$y=1.015\ (\pm 0.019)x + 0.012\ (\pm 0.004)$ $r=0.9991$			$y=0.012\ (\pm 0.001)x + 0.006\ (\pm 0.03)$ $r=0.9983$		
Extraction recovery (%)	100.2			82.4			81.3			97.5		
Within-day[a]												
Nominal concentrations (ng/ml)	0.5	5.0	20.0	0.5	5.0	20.0	0.5	5.0	20.0	0.5	5.0	20.0
Precision (RSD[b], %)	4.5	1.1	1.6	4.6	1.6	0.9	4.0	3.2	0.8	13.8	10.1	5.0
Accuracy[c] (%)	98.4	101.2	98.3	97.0	98.2	97.6	98.2	96.2	97.0	93.4	104.1	95.2
Between-day[a]												
Nominal concentrations (ng/ml)	0.5	5.0	20.0	0.5	5.0	20.0	0.5	5.0	20.0	0.5	5.0	20.0
Precision (RSD[b], %)	4.7	3.5	1.3	7.8	2.4	1.2	7.1	1.8	1.4	13.1	7.5	5.0
Accuracy[c] (%)	97.3	99.7	99.3	96.0	95.2	96.3	96.7	95.4	95.3	92.8	106.2	104.0

[a] Calculated in eight series.
[b] Relative standard deviation.
[c] Found/nominal × 100.

Table 3
Accuracy and precision for the analysis of fluitrazepam and its metabolites in spiked plasma samples

	F			7-AF			*N*-DMF			3-OHF		
Nominal concentrations (ng/ml)	0.5	2.5	20	0.5	2.5	20	0.5	2.5	20	0.5	2.5	20
Within-day[a]												
Precision (RSD[b], %)	10.5	4.6	2.5	10.7	2.6	2.8	14.2	3.6	2.6	10.4	3.8	3.5
Accuracy[c] (%)	96.9	102.2	101.5	94.3	96.4	96.3	94.2	95.3	96.2	107.9	98.3	102.6
Between-day[a]												
Precision (RSD[b], %)	11.7	7.1	8.0	6.6	6.1	3.0	14.7	6.6	6.1	8.7	9.5	4.0
Accuracy[c] (%)	95.7	103.4	102.5	96.0	97.8	94.2	95.8	93.4	95.3	93.4	106.3	105.8

[a] Calculated in five series.
[b] Relative standard deviation.
[c] Found/nominal × 100.

traction recovery, expressed as a percentage, was defined as the ratio of calibration curve slope of extracted analytes to calibration curve slope of nonextracted analytes. All extraction recoveries were between 81 and 100%. Within-run precision and accuracy were established from repeated analysis of spiked runs with F, 7-AF, *N*-DMF and 3-OHF at three different concentrations during 1 working day. Similarly between-day precision and accuracy were established for the same concentrations and analysed

Table 4
Stability of flunitrazepam and metabolites in spiked human urine samples ($n=5$)

Storage conditions	Conc. (ng/ml)	F		7AF		*N*-DMF		3-OHF	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
24 h at 4 °C	0.8	96.2	4.2	95.6	4.8	98.2	4.9	104.2	11.6
	3	103.8	3.8	102.4	1.4	95.6	6.8	96.3	8.2
	12	102.2	3.8	102.6	1.2	97.6	3.4	98.7	4.8
12 days at 4 °C	0.8	103.4	5.6	102.3	5.2	103.4	7.0	103.1	10.4
	3	97.0	3.9	100.9	4.9	96.1	7.2	93.7	8.2
	12	102.7	4.7	98.7	2.8	95.6	7.2	101.6	4.4
Three freeze–thaw cycles	0.8	95.9	4.9	106.3	6.3	103.2	5.6	95.2	12.8
	3	104.8	4.0	99.1	4.1	104.9	4.8	100.2	9.5
	12	100.2	5.2	97.9	3.7	99.1	4.8	96.8	6.9

Table 5
Concentrations of flunitrazepam and its metabolites in biological samples

Case[a]	Body fluid[b]	Concentration (ng/ml)			
		F	7-AF	*N*-DMF	3-OHF[c]
1	U	4.8	445	2.9	114
2	U	1.6	494	2.7	120
3	U	3.4	522	4.4	77
4	U	3.7	844	6.2	379
5	U	<LLOQ	11.9	<LLOQ	<LLOQ
5	P	1.0	0.9	<LLOQ	<LLOQ

[a] Cases 1–4: drug addicts, case 5: date-rape victim.
[b] U, urine; P, plasma.
[c] After hydrolysis step.

for a period of 2 weeks. Stability studies show no degradation of flunitrazepam and metabolites (RSD recoveries between 1.2 and 12.8%) for up to 3 weeks when stored at −28 °C (Table 4).

3.6. Biological samples

Table 5 shows the results of the LC–ESI-MS determination of F and its metabolites in biological samples. The main metabolite found in urine after volunteer intake or date-rape was 7-AF. The results of drug addict urine show high concentrations of 7-AF ranging from 445 to 844 ng/ml (with urine dilution prior to quantification), unchanged F and *N*-DMF in low concentrations (<5 and<7 ng/ml, respectively) and 3-OHF concentrations between 77 and 379 ng/ml. Fig. 7 shows the necessity of a hydrolysis step before determination of the 3-OHF conjugated compound. The results from the date-rape victim showed concentrations of F and 7-AF = 1 ng/ml in a plasma sample; only the 7-AF was detected in urine (11.9 ng/ml). Fig. 8 presents urinary excretion profiles of all substances determined during F withdrawal period from a polydrug addict. The different curves show that urinary concentrations reach maximal concentrations at day 5 or 6 (F 5.1 ng/ml, *N*-DMF 10.3 ng/ml, 7-AF 2626 ng/ml and 3-OHF 506 ng/ml). All measured concentrations were minimal at day 11 of the withdrawal period. Normalising the values with creatinine concentrations does not change the excretion profiles considerably.

4. Discussion

This optimised procedure allowed us to reach detection limits (LODs) lower than those observed in APCI [15,26]. The previous F ingestion by the date-rape victim, undetected by LC–photo-diode array detection, showed positive results for 7-AF and F by LC–ESI-MS. GC with sensitive, but unspecific ECD would have detected F concentrations as low as 0.3–1.0 ng/ml too [27,28], but not 7-AF [18].

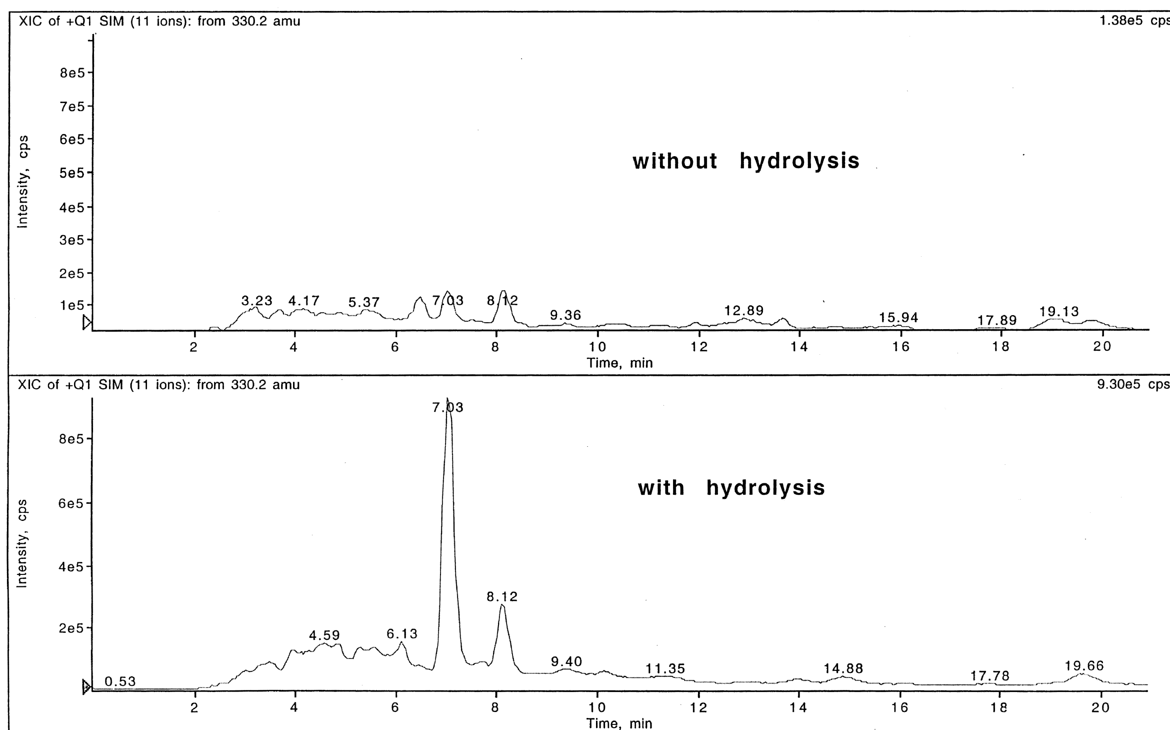

Fig. 7. LC–ESI-MS determination of 3-OHF (t_R 7.03 min) in the same urine sample without and with hydrolysis step.

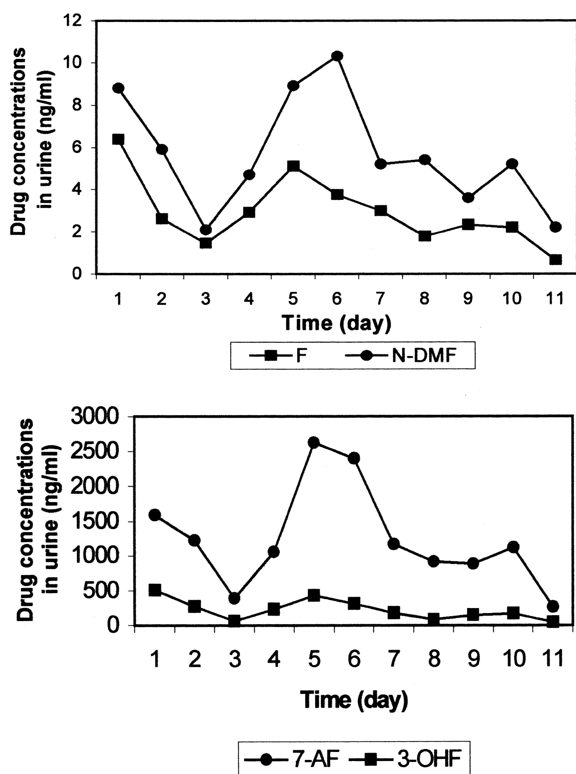

Fig. 8. Urinary excretion profiles of F, *N*-DMF, 7-AF and 3-OHF using LC–ESI-MS from a drug addict in the F withdrawal period.

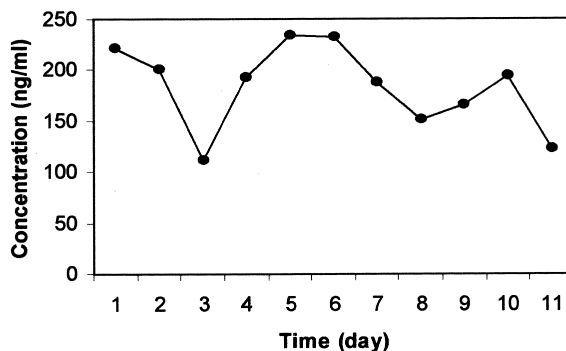

Fig. 9. Total urinary excretion profile (nordiazepam equivalents) using AxSYM benzodiazepines Abbott immunoassays from a drug addict during the F withdrawal period.

According to previous studies, doses higher than 2 mg would be expected to produce concentrations between 445 and 844 ng/ml of 7-AF in urine [19]. The 7-AF and 3-OHF urinary concentrations as high as 2626 and 506 ng/ml measured during the drug addict withdrawal period actually corresponded to confessed 7–8 mg F daily doses. Maximal concentrations observed at days 5 and 6 for the four analytes (especially 7-AF urinary levels increasing from 392 to 2626 ng/ml in 48 h), suggested the evidence of F reintake, confirmed after interrogation. The results of immunochemical tests (AxSYM benzodiazepines Abbott immunoassay reagents) are shown in Fig. 9. The different curves show unexpectedly similar profiles. Owing to low cross-reactivity of F in fluorescence polarisation immunoassays, the immunochemical results reflected in this case especially high concentrations of the analytes. Only a few methods included detection of the 3-OHF metabolite [4,14,15,21]. Salamone et al. [21] detected the 3-OHF metabolite at concentrations of 60–93 ng/ml between 8 and 24 h after ingestion of 4 mg. Parissis and Kirartzidis [14] found the 3-OHF at a concentration of 720 ng/ml in an urine sample of a patient who had taken probably four Rohypnol®. Bogusz et al. [15] studied serum concentrations of 3-OHF in five forensic cases and two clinical cases after ingestion of 1 mg F. The 3-OHF metabolite was detected only in three forensic serum samples with concentrations as low as 1.2–12 ng/ml.

Our results showed that the 3-OHF metabolite is the most important metabolite after 7-AF in F chronic user urine and can reach concentrations as high as 500 ng/ml after high F doses. Conditions for the enzymatic hydrolysis of benzodiazepines were examined by Meatherall [29]. Optimal recovery of the free drug occurs when 1 ml of urine buffered to pH 4.5 is incubated with 5000 U of *Helix pomatia* β-glucuronidase at 56 °C for 2 h. Our assays under the same conditions showed complete hydrolysis of 3-OHF at 56 °C after 1 h. Nevertheless, important losses of F, 7-AF and *N*-DMF occurred. Heating for 2 h at a lower temperature (37 °C) did not affect these compounds, but led to incomplete hydrolysis of 3-OHF. So heating at 56 °C for optimal recovery of 3-OHF required that the internal standard be added after the hydrolysis step but before SPE extraction. Consequently, two extraction procedures, with and without the hydrolysis step, were necessary to quantify all the compounds.

5. Conclusion

The optimisation of ion current with orifice and ring voltages, turbospray heater gas temperature, composition of mobile-phase solvent and the optimisation of the mixed-mode SPE procedure with appropriate washing solutions provided a particularly high sensitivity useful to detect F and its main metabolites in body fluids. This procedure is particularly helpful in alleged date-rape situations, overcoming the problem of very low concentrations. Optimal recovery of 3-OHF in urine occurs after an enzyme-based hydrolysis step requiring a second extraction procedure. Performing systematic 3-OHF determination is currently under investigation.

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