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Determination of flunitrazepam and its metabolites in blood by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

A selective assay of flunitrazepam (F) and its metabolites 7-aminoflunitrazepam (7-AF), N-desmethylflunitrazepam (N-DF) and 3-hydroxyflunitrazepam (3-OHF) with liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS, positive ions) is described. The drugs were isolated from serum, blood or urine using a solid-phase extraction procedure previously applied to various drugs of abuse. F-d_3 and 7-AF-d_3 were used as internal standards. The drugs were separated on ODS column in acetonitrile–50 m*M* ammonium formate buffer, pH 3.0 (45:55, v/v). After analysis of mass spectra taken in full scan mode, a selected-ion monitoring detection was applied with following ions: *m/z* 284 (7-AF and F), 287 (7-AF-d_3 and F-d_3), 314 (F), 300 (N-DF and 3-OHF), 317 (F-d_3), 330 (3-OHF). The limits of detection were: 0.2 μg/l for F and 7-AF, 1 μg/l for N-DF and 3-OHF. The method was linear in the range 1–500 μg/l, the recoveries ranged from 92 to 99%. The method was applied for determination of F and metabolites in clinical and forensic samples. LC–APCI-MS seems to be a method of choice for these compounds.

Keywords: Flunitrazepam; 7-Aminoflunitrazepam; N-Desmethylflunitrazepam; 3-Hydroxyflunitrazepam

1. Introduction

Flunitrazepam belongs to most potent hypnotic benzodiazepines and is available on prescription in most European countries, in Australia, South Africa and Latin America. In the US flunitrazepam is not approved. Nevertheless, the drug is smuggled into the US and its metabolite has been identified several times in clinical samples during drug screening [1–3].

Flunitrazepam is usually administered orally in doses of 0.5–2 mg. The therapeutic concentration ranges from 5 to 15 μg/l. Flunitrazepam undergoes reduction to 7-aminoflunitrazepam (7-AF), hydroxylation to 3-hydroxyflunitrazepam (3-OHF) and N-demethylation to N-desmethylflunitrazepam (N-DF) (Fig. 1). After acetylation or glucuronidation the metabolites are excreted in urine [4]. 7-AF was the most abundant metabolite and could be found up to 72 h after ingestion of 1 mg of drug, whereas the parent drug was not detectable in urine [5].

The assay of flunitrazepam and its metabolites is of forensic importance for several reasons. The drug shows toxicity higher than any other benzodiazepine

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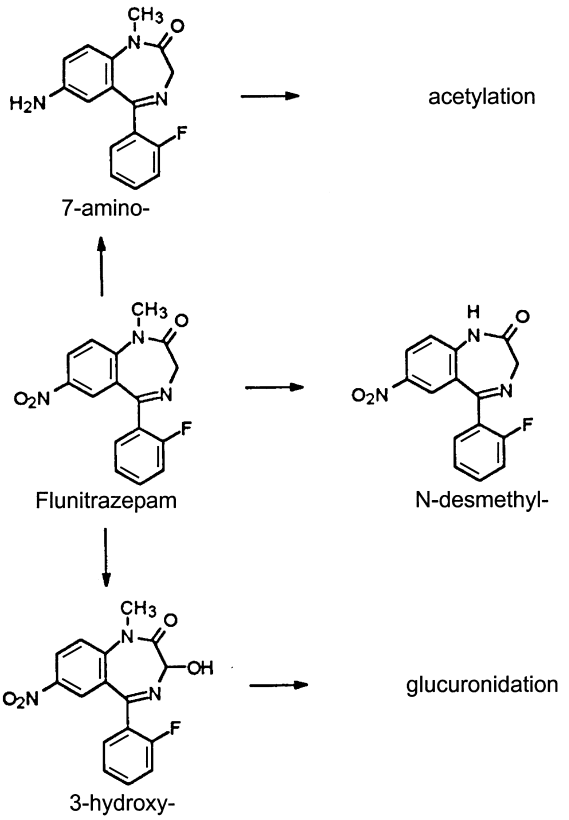

Fig. 1. Metabolic scheme of flunitrazepam, showing the substances determined.

derivative [6,7], and primary addiction potential which may precipitate a withdrawal syndrome [8] or paradoxical agitation among addicts [9]. Heroin addicts consume flunitrazepam more often than other drugs [10–12].

Flunitrazepam solution is odorless, tasteless and colorless. These features, together with its potential use with ethyl alcohol have often caused the drug to be used as an incapacitating agent in rape or robbery [13–15].

There are difficulties in determining flunitrazepam and its metabolites. The parent drug is sensitive to light [16] and is also rapidly converted to 7-AF by bacteria [17–20]. Therefore, in flunitrazepam-associated deaths high concentrations of 7-aminoflunitrazepam are usually present and the parent substance is not found [6,7].

All assay methods for flunitrazepam and its metabolites show some limitations. Benzodiazepine immunoassays often lack the sensitivity required to detect flunitrazepam metabolites in urine [21–23]. The on-line enzyme hydrolysis may improve the detectability of 7-AF with immunoassay [6,24]. FPIA immunoassay applied to blood samples also showed questionable sensitivity [25]. Whole blood radioimmunoasay, specific for flunitrazepam, showed low crossreactivity for 3-OHF and DMF and no crossreactivity for 7-AF [26]. Gas chromatography (GC) with sensitive, but unspecific electron capture detection (ECD) allows detection of flunitrazepam, but not 7-AF or 3-OHF. GC–MS is sufficiently specific and sensitive, but metabolites must be derivatized [5,27,28]. In contrast to GC, all substances involved may be separated by high-performance liquid chromatography (HPLC) without any derivatization. The weak points of HPLC with UV detection were: questionable specificity and low sensitivity (the limit of quantitation was 10 μg/l [29–32].

The purpose of the present study was to develop a sensitive and specific method of determination of flunitrazepam and its metabolites in biological samples without any derivatization, using solid-phase extraction (SPE) and liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS).

2. Experimental

2.1. Reagents and materials

Flunitrazepam, flunitrazepam-d_3 (purity 99.89%), 7-aminoflunitrazepam, 7-aminoflunitrazepam-d_3 (purity 99.79%), N-desmethylflunitrazepam and 3-hydroxyflunitrazepam were supplied by Lipomed (Arlesheim, Switzerland). SPE cartridges Bond Elut C_{18} 200 mg were purchased from ICT (Bad Homburg, Germany). The cartridges were rinsed with 1 ml of methanol, 1 ml of water and 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) before use. The preparation of 0.01 *M* ammonium carbonate buffer (pH 9.3) and 0.05 *M* ammonium formate buffer (pH 3.0 is described elsewhere [33,34].

2.2. Validation standards

Serum samples used for validation were obtained from a local blood bank and were preliminarily screened for the absence of drugs using immunochemical (CEDIA®, Boehringer Mannheim, Mannheim, Germany) and GC–MS procedures. The samples were spiked with flunitrazepam, 7-AF, N-DF and 3-OHF in the concentration range of 1 to 500 μg/l. Medidrug Benzodiazepine Level 1 control serum (Medichem, Stuttgart, Germany), containing flunitrazepam and 7-AF, 10 μg/l each, was used for precision control.

2.3. Biological samples

Five blood samples taken from subjects suspected of driving under the influence of drugs showed positive results for flunitrazepam in GC–ECD examination and were subjected to LC–APCI-MS assay. Urine samples from two subjects taken 0, 12, 18, 24 and 48 h after ingestion of 1 mg flunitrazepam (Rohypnol®) were analyzed with immunoassay (CEDIA® DAU, Boehringer Mannheim) and with LC–APCI-MS. From one of these subjects also blood samples were taken 2 and 12 h after ingestion and analyzed.

2.4. Sample preparation

The rationale behind the applied procedure was to use the same isolation method, which already gave very good results for various drugs of abuse, like opiates and their glucuronides, synthetic opiate agonists, cocaine and its metabolites and LSD [33–35]. A 1–1.5-ml volume of sample was centrifuged for 5 min at 14 000 *g*, to remove cell debris. A 0.5–1-ml volume of supernatant was vortex-mixed with 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) and with internal standard mixture (flunitrazepam-d_3 and 7-aminoflunitrazepam-d_3, 100 ng each). After 10 min centrifugation at 5000 *g*, 2 ml of clear supernatant were applied on SPE cartridge and slowly passed through it (~5 min). The SPE cartridge was rinsed with 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) and vacuum dried for 5 min. The retained drugs were eluted under gravity force with 2×0.5 ml of methanol–0.5 *M* acetic acid to 1-ml Eppendorf tubes containing 5 μl 0.001 *M* HCl and dried under nitrogen at 37°C. The residue was reconstituted in 100 μl of HPLC mobile phase and centrifuged 4 min at 14 000 *g*. A 5–20-μl volume of clear supernatant was injected into LC–MS.

2.5. HPLC

The separation was performed using Superspher RP 18 columns (Merck, Darmstadt, Germany), 125× 3 mm I.D., 4 μm particle size. Acetonitrile–50 m*M* ammonium formate buffer, pH 3.0 (45:55, v/v) was used as mobile phase at flow-rate of 0.3 ml/min.

2.6. APCI-MS

A SSQ 7000 single quadrupole instrument (Finnigan MAT, San Jose, CA, USA) was used. In preliminary experiments electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) were used. APCI was used in positive and negative ionization modes. All further experiments were done with APCI and positive ionization. The following APCI inlet conditions were applied: sheath gas (nitrogen) pressure 70 p.s.i., (1 p.s.i.=6894.76 Pa) auxiliary gas (nitrogen) 20 ml/min, heated vaporizer temperature 450°C, heated capillary temperature 190°C, corona current 5 μA. In order to establish the appropriate selected ion monitoring (SIM) conditions, the full scan mass spectra of substances involved were taken in the range of 100–350 a.m.u. at octapole offset values of 10, 20, 30 and 40 V. The increase of octapole offset voltage was associated with higher acceleration of ions and consecutive distinct collision-induced dissociation (CID). For these examinations direct injections of single pure drugs (10 ng each in 5 μl mobile phase), without HPLC separation, were applied.

On the base of observed fragmentation the procedures were written for SIM detection of particular substances. The octapole offset voltage was set at 10 V. Following ions were monitored: *m/z* 284 (for 7-AF and flunitrazepam)), 287 (for 7-AF-d_3 and flunitrazepam-d_3), 300 (for N-DF and 3-OHF), 314 (for flunitrazepam), 317 (for flunitrazepam-d_3) and 330 (for 3-OHF). The quantitative determinations of flunitrazepam, N-DF and 3-OHF) were performed against flunitrazepam-d_3 as internal standard. For

7-AF assay, 7-AF-d_3 was used. Blank sera spiked with flunitrazepam, 7-AF, N-DF and 3-OHF to the concentrations of 5 and 20 μg/l each were used as calibration standards. The concentration of internal standards was always 100 μg/l.

3. Results and discussion

3.1. APCI mass spectra

Full-scan LC–MS spectra of all substances involved, taken at octapole offset values of 10 and 40 V in positive ionization, are shown in Figs. 2–4. As a rule, at octapole offset voltage of 10 and 20 V only slight fragmentation of flunitrazepam and 3-OHF was observed. 7-AF showed only a protonated molecular peak, in the case of N-DF adduct ions were seen. At 40 V a distinct fragmentation, caused by CID, was noted. The fragmentation patterns of deuterated analogs of flunitrazepam and 7-AF were identical with nondeuterated drugs.

Mass spectra, taken in negative ionization mode, showed very low signals for flunitrazepam, N-DF and 3-OHF. The mass spectrum of 7-AF was not detectable.

3.2. LC–APCI-MS results

In the preliminary study a comparison was made between the results obtained with ESI and APCI, both in positive ionization mode, using SIM described above. This comparison showed that APCI was seven times more sensitive for flunitrazepam,

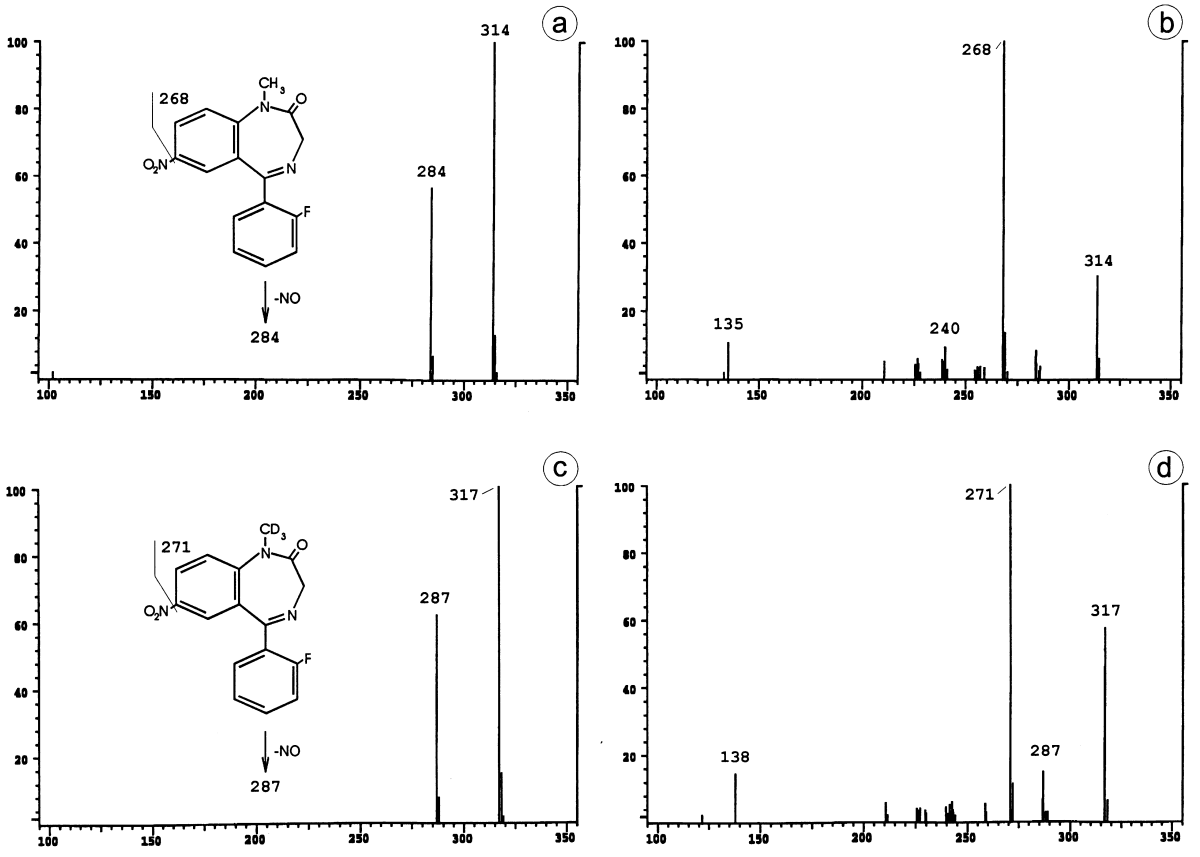

Fig. 2. Mass spectra of flunitrazepam taken at octapole offset voltage 10V (a) and 40 V (b) and of flunitrazepam-d_3 taken at octapole offset voltage 10 V (c) and 40 V (d).

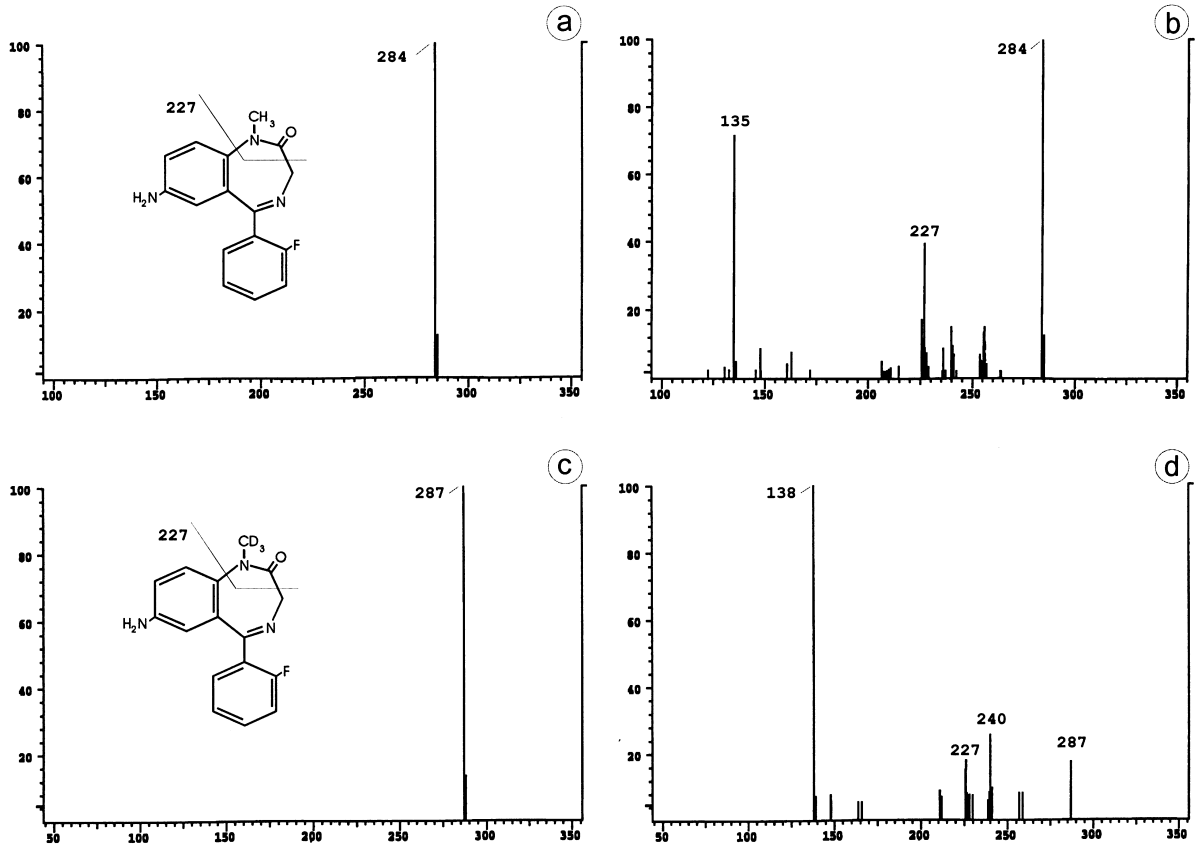

Fig. 3. Mass spectra of 7-aminoflunitrazepam taken at octapole offset voltage 10 V (a) and 40 V (b) and of 7-aminoflunitrazepam-d_3 taken at octapole offset voltage 10 V (c) and 40 V (d).

~20 times more sensitive for N-DF and ~40 times more sensitive for 3-OHF than ESI. The sensitivity of both APCI and ESI for 7-AF was almost identical. Therefore, for the main study an APCI ionization source was chosen.

In all the examined extracts no interfering matrix peaks of similar retention time or mass were observed when the developed SIM procedure was applied. The declared very high purity of deuterated internal standards was confirmed in practice. Fig. 5 shows mass chromatograms of blank serum extract, spiked with flunitrazepam-d_3 and 7-AF-d_3. In the case of 7-AF, no peak corresponding to nondeuterated 7-AF was observed. The peak area corresponding to nondeuterated flunitrazepam amounted to 0.2%.

All drugs were fully separated in ~3–7 min. This was true also for closely eluting N-DF and 3-OHF. This is of practical importance because both compounds produce a signal at m/z 300. The separation of all examined compounds in spiked serum extract is depicted in Fig. 6.

3.2.1. Validation

The validation was done in five series of experiments with serum samples, spiked with flunitrazepam, 7-AF, N-DF and 3-OHF to the concentrations of 1, 5, 10, 50, 100 and 500 μg/l each, and with commercially available control serum Medidrug Benzodiazepine Level 1, containing flunitrazepam and 7-AF, 10 μg/l each. The precision in the lower concentration range was additionally tested for flunitrazepam using authentic urine sample taken 12 h

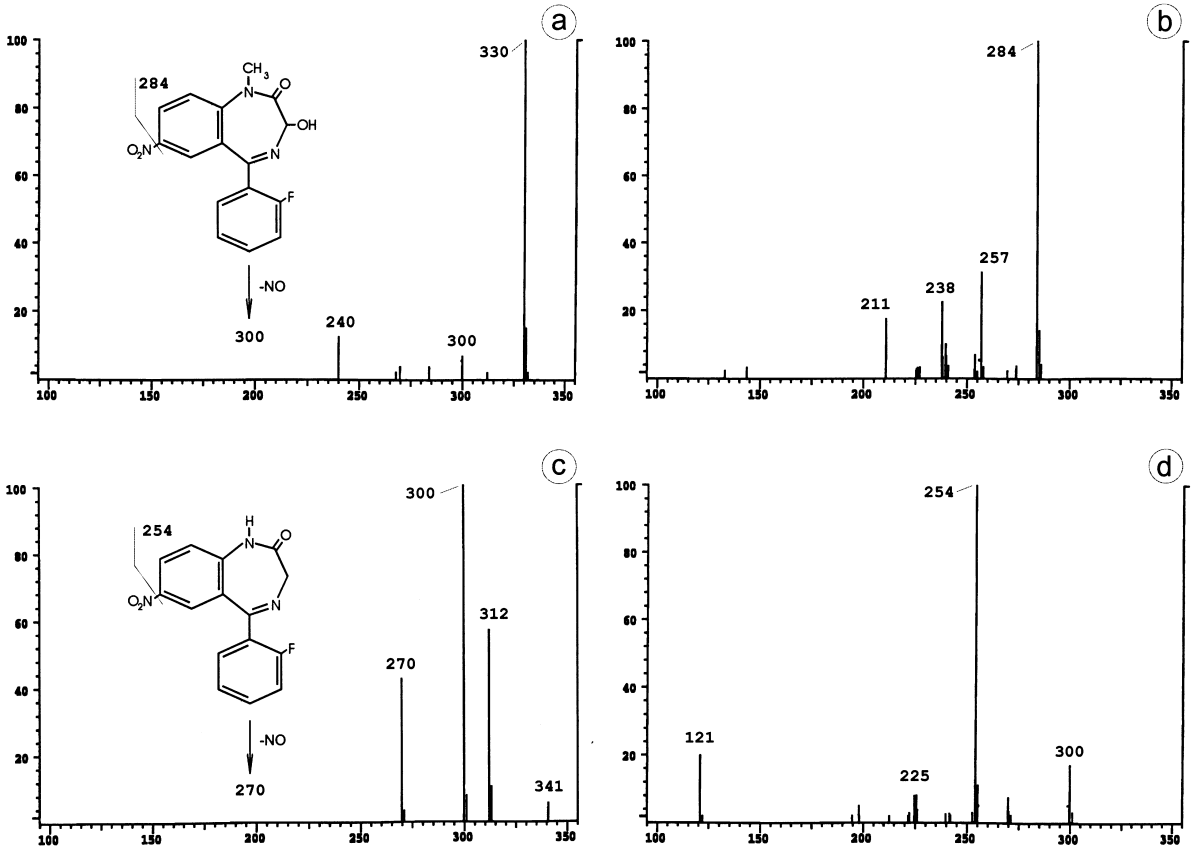

Fig. 4. Mass spectra of 3-hydroxyflunitrazepam taken at octapole offset voltage 10 V (a) and 40 V (b) and of N-desmethylflunitrazepam taken at octapole offset voltage 10 V (c) and 40 V (d).

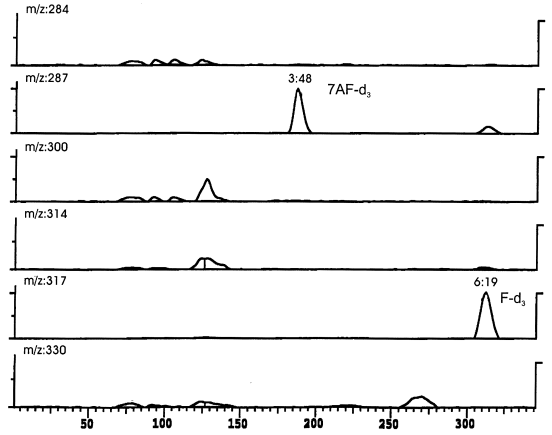

Fig. 5. Chromatogram of blank serum spiked with I.S. mixture. The ion intensities were: for m/z 284: 2.182[3], for m/z 287: 1.392[6], for m/z 300: 9.896[3], for m/z 314: 7.597[3], for m/z 317: 3.565[5], for m/z 330 4.807[3].

after Rohypnol® intake (see Table 3). This sample was measured five times on different days, showing variation coefficient of 17% at the level of about 1 μg/l.

The results of validation are summarized in Table 1. The extraction recovery was consistently high and reproducible, irrespective of concentration, for all examined substances. This confirms our previous experience concerning application of the same extraction procedure for other groups of basic drugs [33–35]. APCI-MS method applied showed sufficient sensitivity, with limit of detection ca. one-tenth of the lower therapeutic concentration of flunitrazepam.

All substances were stable in extracts when stored at −20°C, showing no significant losses during up to 2 months of storage.

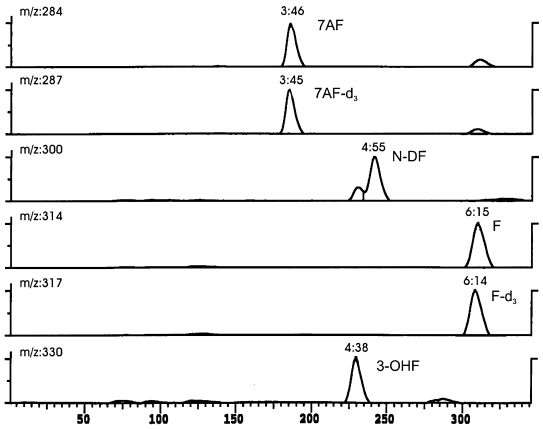

Fig. 6. Chromatogram of blank serum spiked with flunitrazepam (F), 7-aminoflunitrazepam (7-AF), 3-hydroxyflunitrazepam (3-OHF), N-desmethylflunitrazepam (N-DF) (50 ng/ml each) and with I.S. mixture (flunitrazepam-d_3 and aminoflunitrazepam-d_3). The ion intensities were: for m/z 284: 6.332[5], for m/z 287: 1.014[6], for m/z 300: 8.493[4], for m/z 314: 1.742[5], for m/z 317: 1.723[5], for m/z 330 3.170[4].

3.2.2. Clinical and forensic samples

Table 2 shows the results of LC–APCI-MS determination of flunitrazepam and its metabolites in five forensic and three clinical blood samples. In these cases GC–ECD determination was also done. The comparison of the methods shows that the LC–APCI-MS is both more selective (see case 3) and more sensitive, enabling detection of flunitrazepam and/or 7-AF after intake of low dose of the drug. Fig. 7 shows the chromatogram from case 4.

The main metabolite found in urine taken after volunteer intake of 1 mg flunitrazepam (Rohypnol®) was 7-AF. Unchanged flunitrazepam was detected only in two urine samples, taken from one person (MB), 12 and 18 h after intake. N-DF and 3-OH were not detected. The quantitative results observed were in agreement with the data published recently [5,27]. The results of LC–APCI-MS determinations, compared with the results of immunochemical examination (CEDIA® DAU), are shown in Table 3.

Table 1
Validation data

Compound	LOD[a] (μg/l)	Linearity	r^2 (%)	Recovery[b] (R.S.D., %)	Precision[c] (R.S.D., %)
Flunitrazepam	0.2	$y=0.0100x+0.03$	1.000	93±3	3
7-AF	0.2	$y=0.0093x-0.07$	0.9995	92±2	3
N-DF	1	$y=0.0062x+0.01$	0.9998	99±4	6
3-OHF	1	$y=0.0024x-0.001$	0.9993	96±6	5

[a] Defined as 3×signal-to-noise ratio of 3; as the limit of quantitation a double LOD was used.
[b] Defined as percent peak area of corresponding amounts of nonextracted drugs injected into LC–MS.
[c] Calculated in five series (day-to-day) at the concentrations of 10 and 50 μg/l for all substances. In authentic urine sample after Rohypnol intake a concentration of 1.4±0.24 μg/l flunitrazepam was found (mean from five determinations on different days).

Table 2
Concentration of flunitrazepam (F) and its metabolites in serum by LC–APCI-MS in forensic cases (1–5) and in clinical samples (6–8)

Case	Concentration (μg/l)				
	F (GC–ECD[a])	F	7-AF	N-DF	3-OHF
1	40	55	32	36	12
2	33	27	32	3.1	3.1
3	20	0.0	0.0	0.0	0.0
4	8	5.0	26	5.2	0.0
5	31	48	36	0	1.2
6[b]	N.D.	3	Trace (0.1)	0	0
7[c]	N.D.	0	1.8	0	0
8[d]	N.D.	0	3.0	0	0

[a] GC–ECD: concentrations of flunitrazepam measured with gas chromatography–electron capture detector.
[b] Serum taken from volunteer (WF) 2 h after ingestion of 1 mg flunitrazepam.
[c] serum taken from volunteer (WF) 12 h after ingestion of 1 mg flunitrazepam.
[d] Serum taken from patient 12 h after ingestion of 1 mg flunitrazepam.

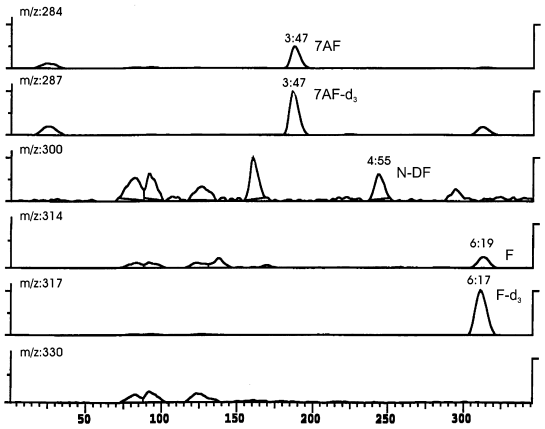

Fig. 7. Chromatogram of serum extract from the case 4 in Table 2. The ion intensities were: for m/z 284: 1.428^5, for m/z 287: 8.406^5, for m/z 300: 6.987^3, for m/z 314: 1.482^4, for m/z 317: 2.976^5, for m/z 330: 3.004^4.

This comparison showed surprisingly good agreement between the methods. The quantitative results of benzodiazepine immunoassay were expressed as concentration of nitrazepam-equivalent (calibrator used in CEDIA DAU benzodiazepine assay). Cross-reactivity of flunitrazepam, according to manufacturer, was 135%, the crossreactivity of 7-AF was not stated. According to the recommended cut-off value of 200 μg/l, all immunochemical results should be regarded as negative.

The practical conclusion from the comparison of the immunoassay, GC–ECD and LC–APCI-MS methods is that the two former methods may be used as screening presumptive tests for the latter.

To date, we have had no opportunity to examine flunitrazepam and its metabolites in authentic post-mortem material. The isolation method applied was, however, used successfully in many cases of heroin, cocaine or methadone intoxication [33–35].

4. Conclusion

The solid-phase extraction method gave excellent recoveries of all substances involved and provided clean extracts. LC–APCI-MS appeared to be a very sensitive and selective method for determination of flunitrazepam and its metabolites in body fluids. This technique combines the universality of liquid chromatographic separation with the sensitivity and selectivity of mass spectrometric detection.

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Table 3
Urinary excretion profiles of flunitrazepam (F) and 7-aminoflunitrazepam (7-AF) (measured by LC–APCI-MS) and benzodiazepines (nitrazepam equivalents measured by CEDIA DAU) in two volunteers after a 1-mg oral dose of flunitrazepam (Rohypnol)

Subject	Time after ingestion (h)	F (μg/l)	7-AF (μg/l)	Benzodiazepines[a] (μg/l)
MB	0	0	0	27
WF	0	0	0	8
MB	12	1.4	27	22
WF	12	0	54	57
MB	18	1.2	27	35
WF	18	0	143	156
MB	24	0	84	101
WF	24	0	28	18
MB	48	0	27	43
WF	48	0	61	92
MB	60	0	20	31
WF	60	0	64	61

[a] Expressed as concentration of nitrazepam-equivalent (calibrator used in CEDIA immunoassay). Crossreactivity of flunitrazepam, according to the manufacturer, was 135%, the crossreactivity of 7-aminoflunitrazepam was not stated.

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