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# Simultaneous analysis of flunitrazepam and its major metabolites in human plasma by high performance liquid chromatography tandem mass spectrometry

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**Abstract**

A sensitive and specific high performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry (HPLC–APCI–MS–MS) method has been developed for the simultaneous determination of flunitrazepam and its major metabolites, 7-aminoflunitrazepam and *N*-desmethylflunitrazepam, in human plasma. After the addition of a deuterium labelled internal standard of flunitrazepam, plasma samples were extracted using Oasis® MCX solid phase extraction cartridges. The compounds were separated on a 5 μm Symmetry C18 (Waters) column (3.0 × 150 mm, i.d.) with a step gradient of acetonitrile-0.1% formic acid at a flow rate of 0.6 ml/min. The overall extraction efficiency was more than 89% for all three compounds. The limits of detection were 0.25 g/l for flunitrazepam, 0.5 μg/l for 7-aminoflunitrazepam, and 2.0 μg/l for *N*-desmethylflunitrazepam. Within-run accuracies for quality-control samples were between 92.5 and 101.3% of the target concentration, with coefficients of variation < 8%. The proposed method enables the unambiguous identification and quantitation of flunitrazepam and its major metabolites in both clinical and forensic specimens. 

*Keywords:* Flunitrazepam; 7-Aminoflunitrazepam; High performance liquid chromatography; Mass spectrometry; Solid phase extraction

## 1. Introduction

Flunitrazepam (Rohypnol®), a 1,4-Benzodiazepine, is a psychotrope substance whose hypnotic effect predominates over the anxiolytic, sedative, and muscle relaxant properites characteristic for benzodiazepines. Flunitrazepam is indicated for the symptomatic treatment of clinically relevant sleep disorders as well as for premedication prior to anaesthesia and intensive care. The non-prescription abuse of flunitrazepam has greatly increased compared with other benzodiazepines over the last decades [1–3]. Moreover,

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flunitrazepam has been misused to incapacitate victims of rape and robbery since solutions of flunitrazepam are tasteless and odorless [4–6].

Due to low dosage, intensive biotransformation, and the high volume of distribution, flunitrazepam and its metabolites occur at low blood levels after therapeutic administration [7]. The therapeutic concentration of flunitrazepam ranges from 5 to 15 μg/l and toxic symptoms have been observed at plasma levels above 50 μg/l [8]. There are two major metabolites of flunitrazepam, 7-aminoflunitrazepam and *N*-desmethylflunitrazepam [9]. The concentration of 7-aminoflunitrazepam can greatly exceed the concentration of the unchanged parent drug in stored blood or plasma samples owing to the in vitro reduction of the 7-nitro group [10,11]. In flunitrazepam-associated fatalities, high 7-aminoflunitrazepam levels are usually found, while the parent drug is often undetectable [12–14]. Hence, the unambiguous identification and quantitation of flunitrazepam requires a specific and sensitive method that includes the detection of its main metabolites.

Several analytical methods have been developed for the determination of flunitrazepam and its metabolites. Immunoassays for the detection of benzodiazepine intake often lack the required sensitivity [15–17]. Although GC–MS is a sufficiently sensitive and specific method for the detection and quantitation of flunitrazepam, the major disadvantage is the need of time-consuming derivatization of the metabolites prior to analysis [18–20]. In comparison to GC, HPLC allows the simultaneous separation of flunitrazepam and its metabolites without any prior derivatization [21–24]. However, the shortcoming of HPLC with UV detection is the unreliability in regard to specificity. The coupling of HPLC to MS with atmospheric pressure ionization (API) leads to a much more sensitive and specific analytical technique. A second stage of mass analysis (MS–MS) further enhances specificity and provides an improved signal to noise ratio compared with single stage MS. Two HPLC–MS–MS assays for the determination of flunitrazepam [25,26], as well as two HPLC–MS applications for the analysis of flunitrazepam and its metabolites [27,28], have been described so far. However, no HPLC–MS–MS method was described for the simultaneous determination of flunitrazepam and its major metabolites.

The purpose of this work was to develop and validate an HPLC–MS–MS method for the simultaneous analysis of flunitrazepam and its major metabolites in human plasma that is suitable for routine analysis. An ion trap detector operated in tandem MS mode was used to provide the specificity and sensitivity necessary for the unambiguous identification and quantitation of these analytes, which is crucial for both clinical and forensic applications.

## 2. Experimental

### 2.1. Reagents

Flunitrazepam, flunitrazepam-$d_7$, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam were supplied by Promochem (Herts, UK). HPLC grade acetonitrile and analytical grade concentrated formic acid were obtained from Promochem (Wesel, Germany). Isopropanol, ammonia (33%), methylene chloride, and orthophosphoric acid (85%) were of analytical grade and were purchased from Merck (Darmstadt, Germany). A Milli-Q® Plus water purification system was used to obtain purified water for the HPLC solvent (Millipore Corp., Vienna, Austria). Oasis® MCX solid phase extraction cartridges were supplied by Waters (Vienna, Austria).

### 2.2. LC–APCI–MS–MS

The LC–MS–MS analyses were performed using a TSP LC system consisting of a vacuum degasser, a P4000 quaternary pump, an AS3000 autosampler, a UV6000LP diode array UV detector, and a Finnigan LCQ$^{DUO}$ ion trap mass spectrometer equipped with an APCI source (Finnigan MAT, USA) run by XCALIBUR 1.2 software.

HPLC separations were performed on a Symmetry C18 (Waters, USA), 5 μm, 3.0 × 150 mm internal diameter (i.d.) HPLC column, operated

at ambient temperature and protected by a Sentry guard column Symmetry C18 (Waters), 5 μm, 3.9 × 20 mm i.d. Each 12 min chromatographic run was carried out at a flow rate of 0.6 ml/min with a binary mobile phase consisting of acetonitrile (A) and 0.1% formic acid (B) using a step gradient profile of 25% A for 3 min, increase up to 50% A in 1 min, isocratic at 50% for 4 min, down to 25% A in 1 min. After re-equilibration at 25% A for 3 min, the next sample was injected.

Operating conditions for the APCI source used in the positive ionization mode were optimized by repeatedly adding a mixture of 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, flunitrazepam-$d_7$, and flunitrazepam, each at a concentration of 1 mg/l in methanol to the HPLC flow by a syringe pump via a T connector in the infusion mode. The signal was optimized on the total ion current in MS mode, resulting in a vaporizer temperature of 450 °C, a heated transfer capillary temperature of 200 °C, a corona discharge intensity of 5 μA, and a sheath gas flow of 60 units (units refer to arbitrary values set by the LCQ software). At the same time, the selection of ions and the collision voltages were optimized using LCQ software.

In the MS–MS experiments, the protonated precursor molecular ions $[M + H]^+$ of 7-aminoflunitrazepam ($m/z$ 284), *N*-desmethylflunitrazepam ($m/z$ 300), flunitrazepam-$d_7$ ($m/z$ 321), and flunitrazepam ($m/z$ 314) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 36%. The mass spectra resulting from these fragmentations were acquired in the full scan mode from $m/z$ 100 to 400. Several product ions were observed for each compound. The most abundant product ions, $m/z$ 264 for 7-aminoflunitrazepam, $m/z$ 254 for *N*-desmethylflunitrazepam, $m/z$ 275 for flunitrazepam-$d_7$, and $m/z$ 268 for flunitrazepam, were extracted and chosen for quantitation.

### 2.3. Calibration standards and quality control samples

Stock solutions of 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, flunitrazepam-$d_7$ (internal standard, IS), and flunitrazepam were prepared by dissolving 1 mg of the respective analyte in 1 ml of methanol. The stock solutions of 7-aminoflunitrazepam, *N*-desmethylflunitrazepam and flunitrazepam were combined and diluted with methanol to obtain working solutions containing 0.1, 1, and 10 mg/l of each of the three analytes. The stock solution of the IS was also diluted in methanol to a final concentration of 1 mg/l. To prepare calibration samples, drug free plasma was spiked with the appropriate volume of working solution to contain 7-aminoflunitrazepam and flunitrazepam at concentrations of 1, 2, 5, 10, 20, 30, 40, 50, and 100 μg/l. For *N*-desmethyl-flunitrazepam, calibration samples at concentrations of 5, 10, 20, 30, 40, 50, and 100 μg/l were prepared accordingly. Three replicate analyses were performed for each calibrator to evaluate linearity. The calibration curves were constructed by linear regression using the peak area ratios of 7-aminoflunitrazepam, *N*-desmethylflunitrazepam and flunitrazepam, respectively, to IS, plotted against the corresponding concentrations.

A similar dilution procedure was used to make separate working solutions containing 1 and 10 mg/l of 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, and flunitrazepam. These solutions were used to prepare quality-control samples in drug free plasma at concentrations of 8, 15, and 50 μg/l.

### 2.4. Solid phase extraction procedure

To one milliliter of each calibrator, quality-control plasma sample, or patient sample 20 μl of orthophosphoric acid and 40 μl of the diluted internal standard solution (1 mg/l) were added and these mixtures were vortex mixed.

Solid phase extraction was performed using Oasis® MCX cartridges (1cc/30 mg), a vacuum manifold device, and a vacuum source. SPE cartridges were conditioned and equilibrated with 1 ml of methanol and 1 ml of water. Each spiked and acidified specimen was applied to the cartridge and passed through the bed at a constant flow rate (1 ml/min). Cartridges were washed sequentially with 1 ml of 0.1 N hydrochloric acid and 1 ml of methanol. Analytes were eluted with

1 ml of methylene chloride: isopropanol: ammonia (78:20:2), collected into glass tubes and evaporated to dryness under a stream of nitrogen at 35 °C. The residues were reconstituted in 100 μl of mobile phase and were transferred to autosampler vials. 20 μl of the reconstituted extracts were injected.

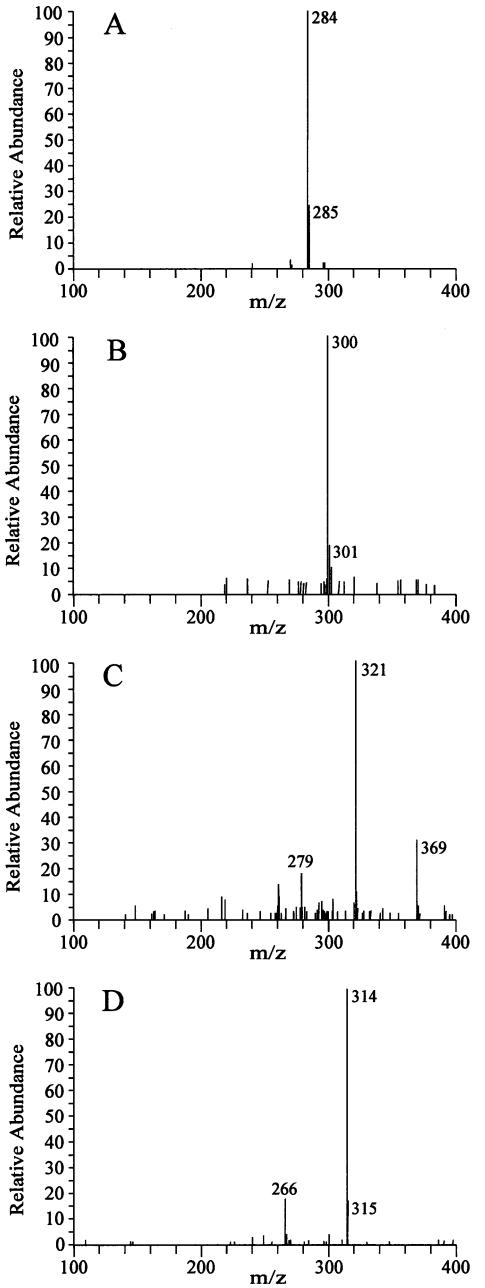


Fig. 1. Atmospheric pressure chemical ionization mass spectra of (A) 7-aminoflunitrazepam, (B) *N*-desmethylflunitrazepam, (C) flunitrazepam-$d_7$, and (D) flunitrazepam.

### 2.5. Extraction efficiency

The extraction efficiencies for 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, and flunitrazepam were experimentally determined at concentrations of 5, 15, and 50 μg/l. The absolute extraction recoveries were evaluated by comparing the analyte peak areas obtained from plasma samples spiked pre-extraction ($n = 5$) to those obtained from plasma samples spiked with equal amounts of the analytes post-extraction.

### 2.6. Matrix effect

The degree of ion suppression or enhancement that could be attributable to the sample matrix was estimated in a separate set of experiments. Blank plasma samples were extracted as described above and the derived eluants were spiked to contain 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, and flunitrazepam each at a concentration of 15 μg/l. Additionally, pure eluant was spiked to contain the analytes at the same concentration. The matrix effect was calculated by comparing analyte peak areas obtained from unextracted reference standards with those obtained from plasma samples spiked post-extraction ($n = 5$).

## 3. Results and discussion

### 3.1. Chromatography and mass spectra

All of the compounds investigated gave protonated precursor molecular ions $[M + H]^+$ in the MS mode. Fig. 1 shows the full scan mass spectra ($m/z$ 100–400) of the compounds investigated. The major ions observed were $m/z$ 284 for 7-aminoflunitrazepam, $m/z$ 300 for *N*-desmethylflunitrazepam, $m/z$ 321 for flunitrazepam-$d_7$, and $m/z$ 314 for flunitrazepam. The product ion chromatograms and the corresponding full scan

Table 1
Extraction efficiencies ($n = 5$) of 7-aminoflunitrazepam (7-AF), *N*-desmethylflunitrazepam (N-DF), and flunitrazepam (F)

| Compound | 8 μg/l Recovery (%) | S.D. | 15 μg/l Recovery (%) | S.D. | 50 μg/l Recovery (%) | S.D. |
|---|---|---|---|---|---|---|
| 7-AF | 95.1 | ±4.8 | 98.6 | ±5.3 | 101.8 | ±5.6 |
| N-DF | 93.7 | ±4.5 | 100.8 | ±4.9 | 103.1 | ±4.8 |
| *F* | 94.1 | ±4.9 | 103.8 | ±4.7 | 105.4 | ±4.9 |

Table 2
Inter- and intraday accuracy and precision data for F and its major metabolites

| Intra-assay ($n = 5$) | 8 μg/l | | | 15 μg/l | | | 50 μg/l | | |
|---|---|---|---|---|---|---|---|---|---|
| | 7-AF | N-DF | *F* | 7-AF | N-DF | *F* | 7-AF | N-DF | *F* |
| Mean | 7.4 | 7.7 | 8.1 | 14.7 | 14.3 | 14.8 | 46.9 | 47.7 | 49.4 |
| S.D. | 0.27 | 0.38 | 0.44 | 0.41 | 0.73 | 0.72 | 2.50 | 3.39 | 2.67 |
| Percent CV | 3.6 | 4.9 | 5.4 | 2.8 | 5.1 | 4.9 | 5.3 | 7.1 | 5.4 |
| Percent of Target | 92.5 | 96.2 | 101.3 | 98.0 | 95.3 | 98.7 | 93.8 | 95.4 | 98.8 |
| *Inter-assay* ($n = 5$) | | | | | | | | | |
| Mean | 8.3 | 7.7 | 7.8 | 15.7 | 14.2 | 13.9 | 51.0 | 51.9 | 49.2 |
| S.D. | 0.75 | 0.66 | 0.59 | 1.27 | 1.08 | 1.25 | 3.36 | 4.17 | 2.75 |
| Percent CV | 9.0 | 8.6 | 7.6 | 8.1 | 7.6 | 9.0 | 6.6 | 8.0 | 5.6 |
| Percent of target | 103.8 | 96.3 | 97.5 | 104.7 | 94.7 | 92.7 | 102.0 | 103.8 | 98.4 |

product ion spectra of 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, flunitrazepam-$d_7$, and flunitrazepam, extracted from spiked plasma are depicted in Fig. 2. The most intense product ions observed in MS–MS spectra were, $m/z$ 264 for 7-aminoflunitrazepam, $m/z$ 254 for *N*-desmethylflunitrazepam, $m/z$ 268 for flunitrazepam, and $m/z$ 275 for IS. The retention times of 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, flunitrazepam-$d_7$, and flunitrazepam were 2.76, 6.28, 7.17, and 7.25 min, respectively. The total HPLC–MS–MS analysis time was 12 min per sample.

The specificity of the method was evaluated by analyzing blank plasma samples from six different plasma pools. No interferences from endogenous plasma components at the retention times corresponding to the analytes of interest were observed. The processes that occur in the ion trap detector, operated in MS–MS full scan mode, can be broken down into the following steps: ionization of the molecules, storage of the ions formed in the ion source, selection of ions of a single mass-to-charge ratio (precursor ions) and ejection of all other ions, collision induced dissociation of the precursor ions, and detection of the product ions formed. In comparison to LC–MS, this technique produces a higher signal to noise ratio, which is hardly affected by the matrix. Hence, analytical background noise has significantly less influence on product ion chromatograms and mass spectra obtained by the LC–MS–MS technique than on those generated by LC–MS. The main advantage of the LC–MS–MS technique compared with the so-called collision induced dissociation LC–MS interface, where analyte fragments are formed from any substance eluting from the HPLC column, is that there are no uncertainties as to the origin of the fragments observed in the product ion spectra.

### 3.2. Method validation

The extraction recoveries are presented in Table 1. The recoveries for all compounds were greater than 89% at all three concentrations tested.

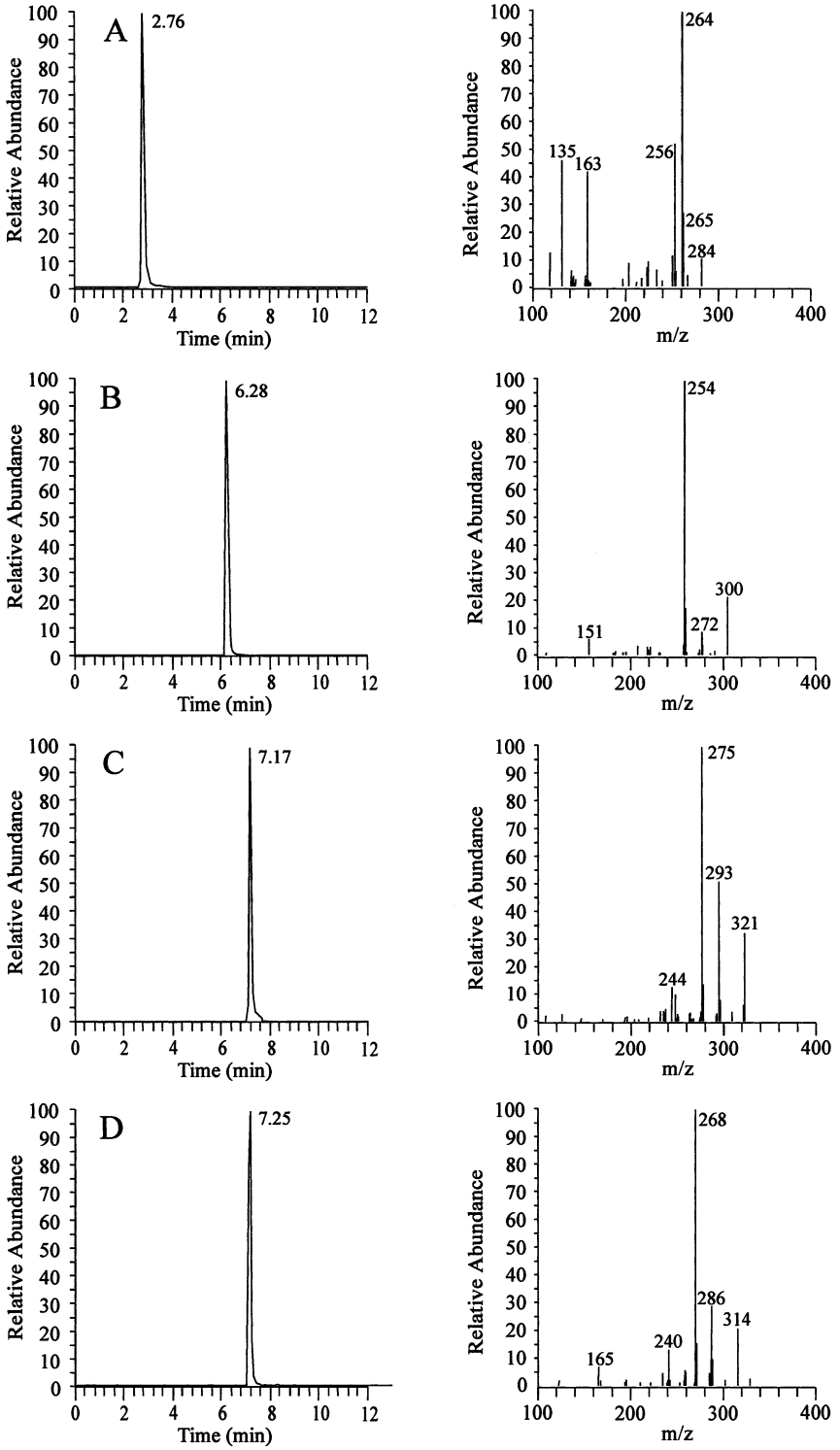


Fig. 2. Product ion chromatograms obtained from an extract of human plasma spiked with 15 ng/ml of (A) 7-aminoflunitrazepam, (B) *N*-desmethylflunitrazepam, (C) flunitrazepam-$d_7$, and (D) flunitrazepam. The product ion traces were $m/z$ 264 for 7-aminoflunitrazepam, $m/z$ 254 for *N*-desmethylflunitrazepam, $m/z$ 275 for flunitrazepam-$d_7$, and $m/z$ 268 for flunitrazepam. On the right of each chromatogramm the corresponding full scan product ion spectra are shown.

Calibration lines for all compounds were linear in the concentration range investigated with coefficients of determination ($r^2$ values) $\geq 0.997$. Slopes and intercepts, the standard deviations of the slope and at the intercept, and the coefficients of determination, evaluated by triplicate analysis of the calibration samples, were as follows: for 7-aminoflunitrazepam, $y = (0.1259 \pm 0.0019)\ x - (0.0531 \pm 0.0903)$, $r^2 = 0.997$; for *N*-desmethylflunitrazepam, $y = (0.0179 \pm 0.0002)\ x + (0.0623 \pm 0.0112)$, $r^2 = 0.998$; for flunitrazepam, $y = (0.1001 \pm 0.0013)\ x - (0.0330 \pm 0.0605)$, $r^2 = 0.998$.

The limit of detection (LOD), defined as the lowest concentration of the analyte which can be detected with a signal-to-noise ratio greater than 7, was established by serial extraction of plasma samples spiked with decreasing concentrations of

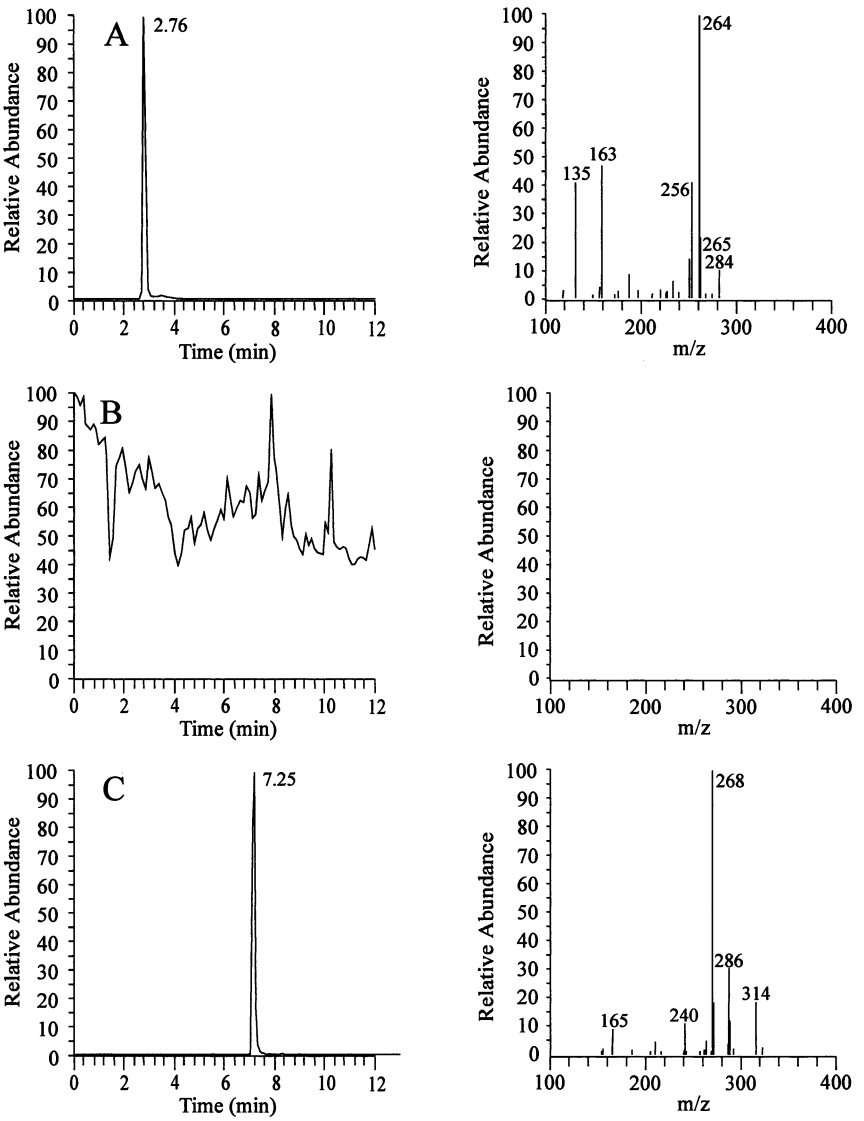


Fig. 3. Product ion chromatograms and spectra obtained from an extracted patient plasma sample. (A) 7-aminoflunitrazepam (5.6 μg/l), (B) *N*-desmethylflunitrazepam was not detected, (C) and flunitrazepam (9.9 μg/l).

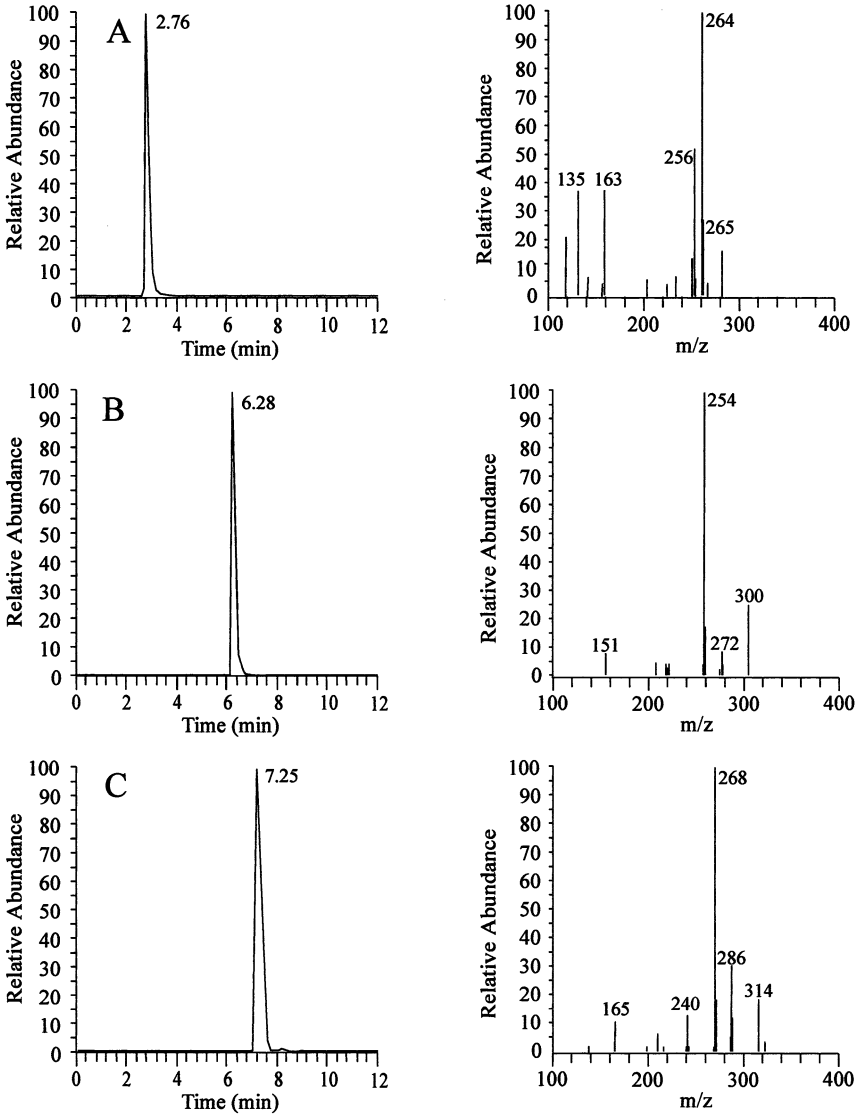


Fig. 4. Product ion chromatograms and spectra obtained from an extracted plasma sample of victim of flunitrazepam-facilitated sexual assault. (A) 7-aminoflunitrazepam (23.1 μg/l), (B) *N*-desmethylflunitrazepam (11.7 μg/l), (C) and flunitrazepam (48.3 μg/l).

each analyte. The LOD was found to be 0.5 μg/l for 7-aminoflunitrazepam, 2.0 μg/l for *N*-desmethylflunitrazepam, and 0.25 μg/l for flunitrazepam. The limit of quantitation (LOQ) was defined as the lowest concentration of the analyte that can be analyzed with both an accuracy of $\pm 20\%$ of the true value and a coefficient of variation (CV) $\leq 20\%$. The limits of quantitation were 1 μg/l for 7-aminoflunitrazepam and flunitrazepam, and 5 μg/l for *N*-desmethylflunitrazepam.

In order to evaluate the accuracy and precision of the assay, analyses of the prepared quality control samples containing 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, and flunitrazepam at concentrations of 8, 15, and 50 μg/l were carried out. Results are summarized in Table 2. To determine intra-assay accuracy and precision, five

replicate analyses were performed at each of the three concentrations. Inter-assay accuracy and precision were determined at the same concentrations over a period of 10 days, by establishing calibration curves for the analytes on five different days. The mean, standard deviation, and percent coefficient of variation (percent CV) for 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, and flunitrazepam were calculated at each concentration. The intra-assay percent CV for 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, and flunitrazepam were $\leq 7.1\%$. All inter-assay percent CV were below 9.2%. The accuracies, referred to as percent of Target in Table 2, were determined by comparing the mean calculated concentration with the spiked target concentration of the QC samples. The intra- and inter-assay accuracies for all analytes were found to be within 1.0 and 7.0%, respectively, of the target values.

As a part of the method validation, the change in the efficiency of ionization that could be attributable to components of the sample matrix was estimated by comparing analyte peak areas ($n = 5$) obtained from unextracted preparations of the analytes with those obtained from plasma samples spiked with an equivalent amount of each analyte post-extraction. The average signal suppression was 1.5% for 7-aminoflunitrazepam, 1.3% for *N*-desmethylflunitrazepam, and 1.2% for flunitrazepam.

### 3.3. Application of the method

The described method was used to identify and quantitate flunitrazepam in plasma samples of both patients who were victims of rape or robbery and drivers suspected of driving under the influence of drugs.

The product ion chromatograms and corresponding spectra obtained from an extracted plasma sample of a patient who repeatedly denied taking flunitrazepam are depicted in Fig. 3. Measured plasma concentrations were 5.6 μg/l of 7-aminoflunitrazepam and 9.9 μg/l of flunitrazepam. *N*-desmethylflunitrazepam was not detected.

The HPLC–MS–MS analysis of an extracted plasma sample of a patient who had been a victim of rape is depicted in Fig. 4. Measured plasma concentrations were 23.1 μg/l of 7-aminoflunitrazepam, 11.7 μg/l of *N*-desmethylflunitrazepam and 48.3 μg/l of flunitrazepam.

## 4. Conclusion

An analytical procedure combining mixed mode SPE and LC–MS–MS was developed for the simultaneous determination and quantitation of flunitrazepam and its major metabolites in human plasma. The new simple mixed mode SPE procedure provides a highly efficient sample clean up with excellent recoveries. The combination of HPLC and MS–MS with an APCI interface leads to specificity and sensitivity of drug identification, which is crucial in both clinical and forensic applications.

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