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Short communication

Simultaneous high-performance liquid chromatographic analysis of flunitrazepam and four metabolites in serum

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Abstract

A high-performance liquid chromatographic method for the simultaneous determination of flunitrazepam and four metabolites, desmethylflunitrazepam (DMF), 7-aminodesmethylflunitrazepam (7-NH₂DMF), 7-aminoflunitrazepam (7-NH₂F) and 3-hydroxyflunitrazepam (3-OHF), in serum is described. The method involves a simple extraction from alkalized plasma (pH 9.5) into diethyl ether–chloroform (80:20, v/v). Prazepam was used as an internal standard for the quantification of the five compounds. Separation was achieved with a 10 μ m RSil CN column (300 \times 3.9 mm I.D.). The detection wavelength was set at 242 nm. The limits of detection ranged from 2.5 to 5 μ g/l with a limit of quantification of 10 μ g/l for all analytes.

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

1. Introduction

Flunitrazepam (Rohypnol) is the N-methyl-2'-fluoro analogue of nitrazepam. It is available in a number of Western European countries for use as a hypnotic and anesthetic induction agent and is administered orally or by intravenous injection in doses of 2 mg.

Flunitrazepam undergoes biotransformation via N-demethylation, 3-hydroxylation and glucuronidation, and reduction of the nitro group to an amine with subsequent acetylation (Fig. 1) [1].

At least eleven metabolites are present in urine; the major ones include 7-aminoflunitrazepam (7-

NH₂F, 10% of a dose), 3-hydroxyflunitrazepam (3-OHF, 3.5%), 7-acetamidodesmethylflunitrazepam (7-NH₂DMF, 2.6%) and 3-hydroxy-7-acetamidoflunitrazepam (2.0%).

Desmethylflunitrazepam (DMF) and 7-acetamidodesmethylflunitrazepam are active metabolites. Less than 0.2% is excreted unchanged.

Numerous methods are available for flunitrazepam using various techniques: Gas chromatographic methods [2–4] are sensitive but often require a derivatization or hydrolysis procedure prior analysis. High-performance liquid chromatographic (HPLC) methods [5,6] have often been proposed for the determination of flunitrazepam, but few present the simultaneous determination of the drug with metabolites; determination of flunitrazepam and its N-demethyl metabolites [7,8] has been accomplished by some authors.

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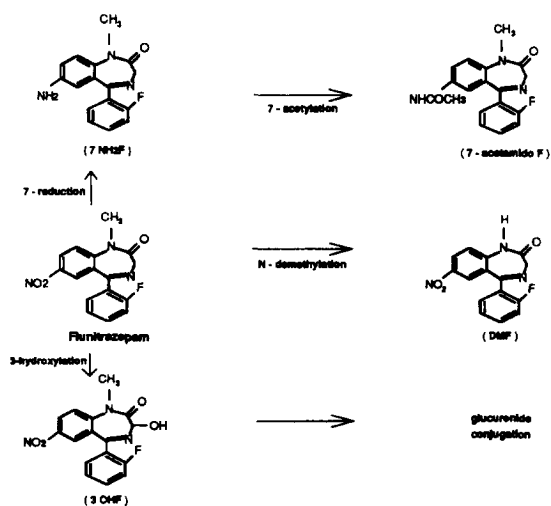


Fig. 1. Flunitrazepam metabolism [1].

Therefore, in this paper, we present the simultaneous determination and quantification of this drug and DMF, 7-NH₂DMF, 7-NH₂F and 3-OHF.

2. Experimental

2.1. Chemicals and reagents

Methanol, chloroform and diethyl ether were HPLC grade (Merck, Darmstadt, Germany). All other chemicals and reagents were analytical grade (Merck and Prolabo, France).

Flunitrazepam and metabolites (7-NH₂F, 7-NH₂DMF, 3-OHF and DMF) were obtained from Roche (Paris, France). Prazepam (internal standard, IS) was obtained from Substantia Division Parke-Davis (Paris, France). Stock solutions (100 mg/l free base) of drugs and prazepam were prepared in methanol and stored at 4°C.

2.2. Apparatus

The HPLC system consisted of a pump (Waters 590) and an automatic sample injection module (Waters Wisp 710B), which were coupled to a multiwavelength detector (Waters 490) operated at 242 nm. The flow-rate was set at 1.6 ml/min.

The separation of flunitrazepam and metabolites

was performed on a 10 μm RSil CN column (300 × 3.9 mm I.D.).

The mobile phase was methanol–30 mM phosphate buffer (KH₂PO₄) (17:83, v/v), and was adjusted to pH 4.0 with phosphoric acid.

2.3. Extraction procedure

To 2 ml of plasma, 20 μl of internal standard solution (prazepam, 100 mg/l), 500 μl NH₄Cl saturated buffer (pH 9.5) and 5 ml of diethyl ether–chloroform (80:20, v/v) were added in glass centrifuge tubes. After horizontal agitation (20 min) and centrifugation (15 min at 2800 g), the organic phase was removed and evaporated to dryness at 45°C in a Speed Vac concentrator. The residue was dissolved in 100 μl of the eluent, and 80 μl was injected into the chromatographic system.

3. Results and discussion

The simultaneous separation of flunitrazepam and four metabolites in serum was achieved by HPLC in this paper.

Fig. 2 shows chromatograms from (A) blank human plasma, and (B) human plasma spiked with a mixture of 7-NH₂DMF, 7-NH₂F, 3-OHF, DMF and flunitrazepam (each compound containing 250 μg/l), and the internal standard (20 μl, 100 mg/l).

The retention times were 4.43, 5.68, 7.23, 8.15, 10.67 and 19.38 min, respectively.

As an example of application, Fig. 3 represents the identification of 7-NH₂F in a post-mortem blood sample. Concentration was 35 μg/l. Flunitrazepam, as well as 7-NH₂DMF, 3-OHF and DMF were not detected.

The limits of detection were determined by spiking plasma with decreasing concentrations of the five compounds until a response equivalent to three times the background noise was observed. They were in the range 2.5 to 5.0 μg/l.

Extraction recoveries were determined for each compound by comparing the representative peak areas of extracted plasma with the peak areas of methanolic standards at the same concentration.

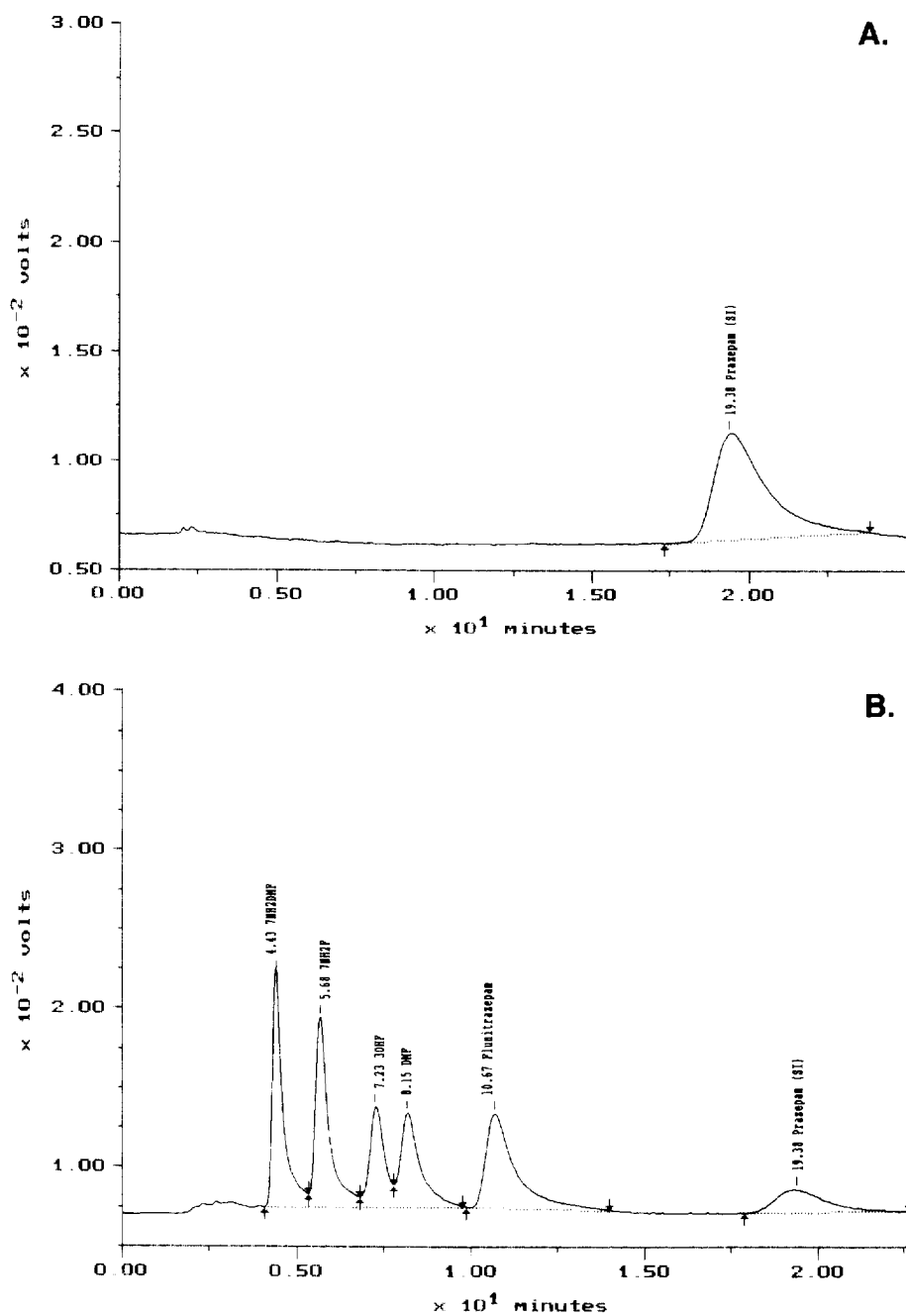


Fig. 2. Chromatograms of extracts from (A) blank human plasma, (B) human serum spiked with a mixture (each component containing 250 $\mu\text{g/l}$ 7-NH₂DMF, 7-NH₂F, 3-OHF, DMF, flunitrazepam) and the internal standard (20 μl , 100 mg/l). t_R (7-NH₂DMF)=4.43 min, t_R (7-NH₂F)=5.68 min, t_R (3-OHF)=7.23 min, t_R (DMF)=8.15 min, t_R (flunitrazepam)=10.67 min and t_R (prazepam)=19.38 min.

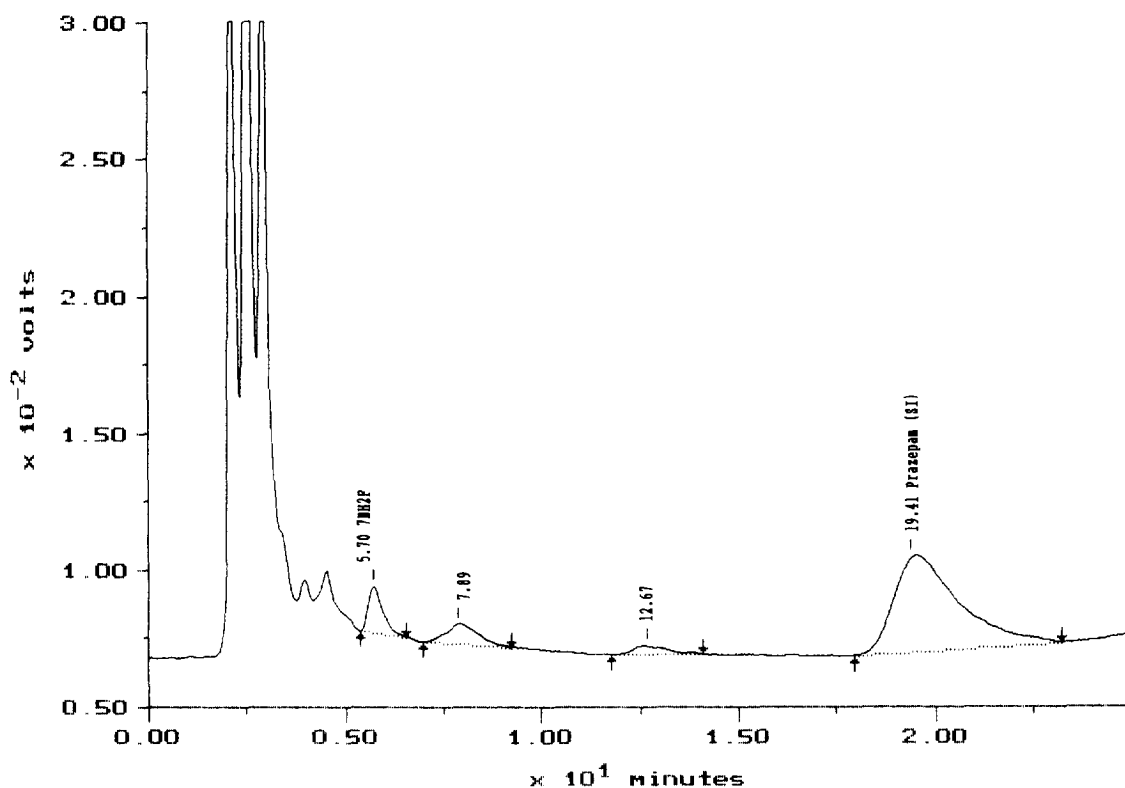


Fig. 3. Blood post-mortem sample containing 35 $\mu\text{g/l}$ of 7-NH₂F. t_R (7-NH₂F)=5.70 min and t_R (prazepam)=19.41 min.

The values of these two parameters are given in Table 1. They were in the range 30 to 83%.

Intra-assay reproducibility was assessed using five replicates of three standards 0.05, 0.1 and 0.5 mg/l for each compound. Coefficients of variation (C.V.) for intra-assay variability are shown in Table 2.

Calibration curves for 7-NH₂DMF, 7-NH₂F, 3-OHF, DMF and flunitrazepam were linear over the concentrations tested.

The mathematical expressions and the correlation

Table 1
Recovery data and detection limits of flunitrazepam and metabolites

Compound	Recovery (%)	Detection limit ($\mu\text{g/l}$)
Flunitrazepam	83	5
7-NH ₂ F	30	5
3-OHF	80.5	2.5
DMF	57	2.5
7-NH ₂ DMF	83	2.5

Table 2
Coefficients of variation (C.V.) (intra-assay)

Compound	Concentration added ($\mu\text{g/l}$)	C.V. (%) (n=5)
Flunitrazepam	50	17.7
	10	21.7
	500	23.8
7-NH ₂ F	50	24.2
	10	25.9
	500	22.9
3-OHF	50	22.0
	10	11.6
	500	20.3
DMF	50	23.5
	10	26.8
	500	21.9
7-NH ₂ DMF	50	28.7
	10	22.6
	500	21.4

Table 3
Regression equations and correlation coefficients (*r*)

Compound	Range of concentrations tested (mg/l)	Regression equation	<i>r</i>
Flunitrazepam	0.01–5	$y=0.097x+0.032$	0.999
7-NH ₂ MF	0.01–1	$y=0.13x-0.059$	0.979
7-NH ₂ F	0.01–1	$y=0.147x+0.021$	0.998
3-OHF	0.01–1	$y=0.088x+0.039$	0.993
DMF	0.01–1	$y=0.142x-0.017$	0.999

y: peak areas, *x*: concentrations (mg/l).

coefficients are listed in Table 3, indicating good linearity between the detector response and the concentrations tested.

4. Conclusion

The HPLC procedure described here for the simultaneous determination and quantification of flunitrazepam, N-methyl, N-desmethyl and 3-hy-

droxy metabolites appears rapid, simple, and suitable for therapeutic drug monitoring.

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