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Determination of flunitrazepam in plasma by liquid chromatography

F. BENHAMOU-BATUT, F. DEMOTES-MAINARD,* L. LABAT, G. VINÇON✝ and B. BANNWARTH

Department of Clinical Pharmacology and Toxicology, CHU and Université de Bordeaux II, 33076 Bordeaux Cedex, France

Abstract: A reversed-phase liquid chromatographic method has been used to determine flunitrazepam in plasma. Extraction was simple and there was no need to hydrolyse the drug. Separation was achieved on a 150 × 3.9 mm i.d. column packed with 4-μm Nova Pack C_{18} using a mobile phase of water–acetonitrile–triethylamine (700:300:4, v/v/v) (adjusted to pH 7.5 with orthophosphoric acid). The method was shown to be rapid and reliable with a lower limit of detection of 5 ng ml^{-1}. Results are reported of simple experiments on the effects of temperature and light on the stability of flunitrazepam in plasma kept on the laboratory bench.

Keywords: *Flunitrazepam; reversed-phase liquid chromatography; stability; toxicology.*

Introduction

Flunitrazepam (Fig. 1), 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepine 2-one, is administered orally for the treatment of sleep disorders and parenterally for premedication and induction of anaesthesia.

Although flunitrazepam is a relatively safe drug as a hypnotic, its use for a prolonged time can lead to dependence [1]. During acute overdose, observed with relatively low doses (20 mg), this drug may induce a deeper coma than other benzodiazepines with oligopnea and bradycardia [2]. Therefore, it seems necessary to determine flunitrazepam in plasma samples, especially in cases of polymedication poisoning.

Several chromatographic methods have been described for the determination of flunitrazepam in biological fluids, such as gas–liquid chromatography [3–5] and high-performance

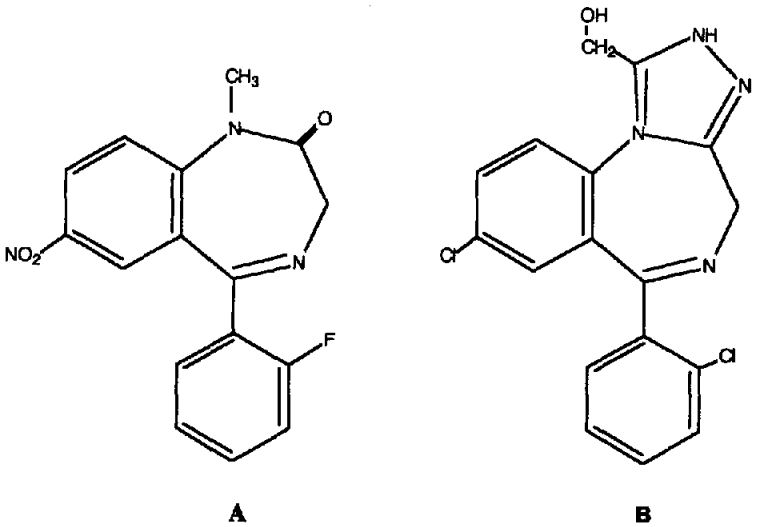

Figure 1
Chemical structures of (A) flunitrazepam and (B) internal standard (α-hydroxytriazolam).

* Author to whom correspondence should be addressed.
✝ Deceased.

liquid chromatography (HPLC) [6–9]. Among the previously reported HPLC assays, some involved hydrolysis of the drug before extraction from plasma or urine samples [7] or were time-consuming [6, 8, 9]. Moreover, none of these methods had been used to study the stability of flunitrazepam in biological samples although significant degradation by light has been demonstrated for clonazepam and nitrazepam, structurally related benzodiazepines [9, 10].

The method described is rapid since it involves a simple extraction of flunitrazepam from plasma in the presence of an appropriate internal standard. This specific assay is particularly adapted to analytical toxicology in cases of acute poisoning.

Experimental

Chemicals

Flunitrazepam was supplied by Roche (Neuilly sur Seine, France) and the internal standard (α-hydroxytriazolam) by Upjohn (Paris, France). Diethyl ether and acetonitrile were supplied by Info-Labo (Sainte-Foy-la Grande, France) and were of HPLC grade. Methanol (HPLC grade) was supplied by Prolabo (Paris, France). Water was de-ionized and double-distilled in glass. Orthophosphoric acid was supplied by Farmitalia Carlo Erba (Milan, Italy), triethylamine by Rathburn (Walkerburn, Scotland, UK) and sodium hydroxide by Prolabo (Paris, France); these were all of analytical grade. Human heparinized plasma was supplied by the Regional Centre of Blood Transfusion (Bordeaux, France).

HPLC conditions

The Waters Assoc. (Milford, MA, USA) chromatographic system used comprised a Model 45 constant-flow pump, a Wisp Model 710 B automatic injector and a Model 490 ultraviolet detector operated at 230 nm. The detector was interfaced with an integrator D-2000 (Merck-Hitachi, Nogent sur Marne, France). Separation of compounds was carried on a 150 × 3.9 mm column packed with 4-μm Nova Pack C_{18}. The mobile phase was water–acetonitrile–triethylamine (700:300:4, v/v/v) (pH adjusted to 7.5 with orthophosphoric acid). Before use, the mobile phase was filtered through a 0.45-μm filter (Sartorius, Göttingen, Germany). The flow-rate was maintained at 2 ml min^{-1}. The chromatograms were recorded at a chart speed of 0.5 cm min^{-1} and peak-height ratios of flunitrazepam to internal standard were measured.

Standard solutions

Stock solutions of flunitrazepam and internal standard (1 mg ml^{-1}) were prepared in methanol and stored at −20°C for 12 months without degradation. Dilutions of flunitrazepam were made in drug-free human plasma to provide concentrations of 20, 50, 100 and 200 ng ml^{-1}. The internal standard dilution (1 μg ml^{-1}) was made in double-distilled water.

Sample preparation

In a 10-ml polypropylene tube, 1 ml of plasma was added to 50 μl of the internal standard solution, 200 μl of 0.1 M sodium hydroxide and 7 ml of diethyl ether. The mixture was rotary mixed for 10 min and centrifuged at 3000*g* for 5 min. The organic layer was then transferred to a small glass tube and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The residue was dissolved in 100 μl of the mobile phase. After centrifugation, 40 μl was injected into the liquid chromatograph.

Results and Discussion

Under the described chromatographic conditions, flunitrazepam and the internal standard were well resolved from endogenous plasma compounds (Fig. 2). The retention times of flunitrazepam and α-hydroxytriazolam were 5.2 and 2.8 min, respectively.

Retention times and capacity factors of other benzodiazepines extracted under the assay conditions described and studied as potential sources of interference are shown in Table 1. The limit of detection for flunitrazepam was 5 ng ml^{-1} (signal-to-noise ratio = 3).

From 20 different calibration curves (n = 100) ranging from 0 to 200 ng ml^{-1}, there was a high correlation coefficient (r = 0.993). The equation for the relationship between the peak-height ratio (y) and the concentration (x) was: $y = 0.012x - 0.017$; the SE of the slope was 0.0001 and the SE of the intercept was 0.0017.

The reproducibility (intra-day assay, n = 10) and the repeatability (inter-day assay, n = 10) of the HPLC procedure were determined by replicate analysis of three different

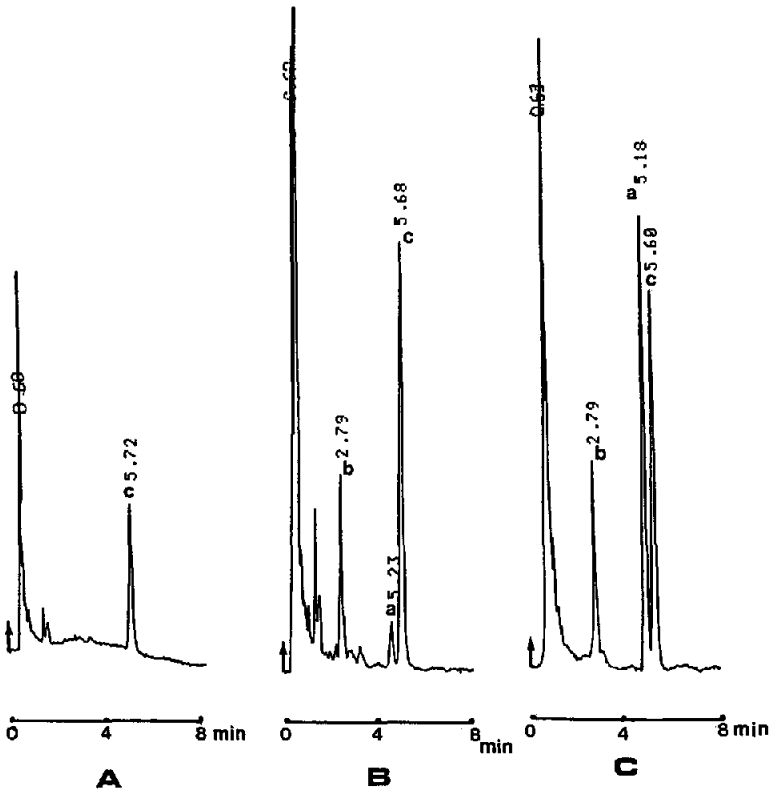

Figure 2
Chromatograms of (A) human drug-free plasma; (B) extracted human plasma spiked with 20 ng ml^{-1} of flunitrazepam and (C) plasma extract from a poisoned patient (180 ng ml^{-1}). Peak a corresponds to flunitrazepam and peak b to the internal standard. Peak c is an unknown plasma compound.

Table 1
Retention times (t_r) and capacity factors (k') of some benzodiazepines

Compounds	t_r (min)	k'
Bromazepam	1.06	0.76
Internal standard	2.79	3.50
Lorazepam	3.16	4.26
Oxazepam	3.18	4.30
Nitrazepam	3.23	4.38
Clonazepam	3.99	5.65
Alprazolam	4.39	6.39
Triazolam	4.80	7.00
Flunitrazepam	5.18	7.58
Temazepam	5.41	8.01
Nordiazepam	6.53	9.88
Clobazam	6.88	10.46
Diazepam	10.70	16.83

control plasmas containing 35, 70 and 140 ng ml^{-1} of flunitrazepam. The results are presented in Table 2. The within-day precision data had a relative standard deviation (RSD) of 3.6–9.1% and an accuracy of 106.4–117.1%; between-day data gave a RSD of 7.6–9.3% and an accuracy of 103.1–110.3%, respectively.

To study the influence of temperature on the stability of flunitrazepam, plasma solutions spiked with 140 ng ml^{-1} of the drug were stored under three different conditions: −20°C, +4°C and room temperature. No deterioration was observed in the quick frozen sample during 3 weeks. Storage for as long as 1

Table 2
Accuracy and precision of results for plasma spiked with flunitrazepam

Spiked concentration (ng ml^{-1})	Repeatability (n = 10)			Reproducibility (n = 10)		
	Mean (ng ml^{-1})	RSD (%)	Accuracy* (%)	Mean (ng ml^{-1})	RSD (%)	Accuracy* (%)
35	41.0	3.6	117.1	38.6	9.3	110.3
70	75.1	4.6	107.3	76.2	9.0	108.8
140	149.0	9.1	106.4	144.4	7.6	103.1

*Accuracy = (amount found/amount added) × 100%.

Table 3
Stability of flunitrazepam in plasma after storage under different conditions of temperature and light*

	−20°C, dark	+4°C, dark	Room temperature, dark	Room temperature, light
Replicates (n)	33	24	33	33
Mean concentration found (ng ml^{-1})	136.7	132.9	128.3	56.03
SD (ng ml^{-1})	11.3	9.0	11.2	42.4
Statistical tests				
F-value	1.07	1.68	1.04	13.2
Theoretical F-values	2.27	2.38	2.27	2.71
t-value	1.90	0.22	3.97	10.70
Theoretical t-values	2.021	2.042	2.021	2.294

*Statistical analyses by the Fisher–Snedecor test for the variances (F-value) and the Fisher–Behrens test or the Fisher–Student test (t-value) for the means, ($P < 0.05$ for all tests) [13].

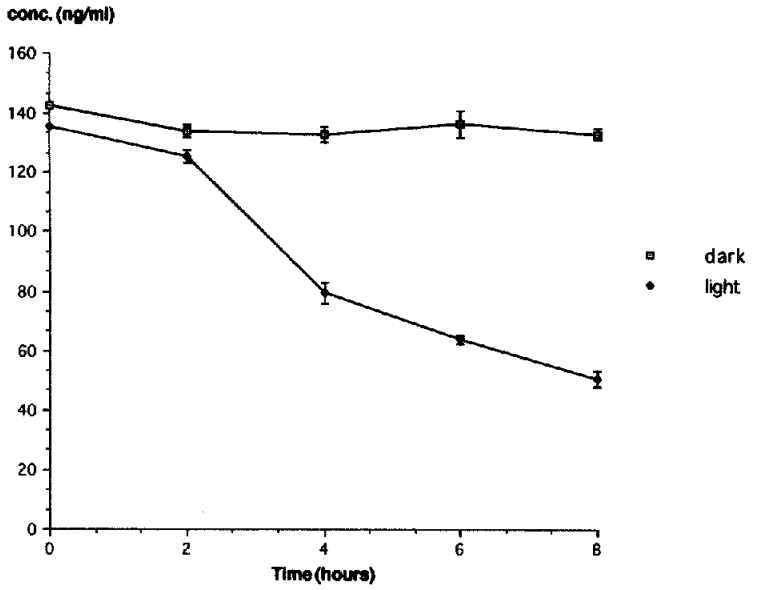

Figure 3
Degradation of flunitrazepam at room temperature (20°C). The plasma sample containing 140 ng ml^{-1} of benzodiazepine was kept in a dark place or exposed to light. Each point is the mean of three analyses (mean ± SD).

week at +4°C produced no significant decrease of flunitrazepam concentration. But a slight degradation of this benzodiazepine occurred when plasma was stored at room temperature for 2 days in a dark place (Table 3, Fig. 3). This moderate degradation was a first-order rate process with a half-life of about 6 days. These observations are in agreement with those of other workers observed for other nitrobenzodiazepines [10–11].

The potential influence of light was studied on a plasma sample kept at ambient temperature (+20°C). This experiment was done by exposing glass tubes containing 140 ng ml^{-1} of flunitrazepam to sunlight passed through a double-glass window. Highly significant degradation of this benzodiazepine by light during 1 day was observed (Table 3, Fig. 3).

A similar influence of light on two other nitrobenzodiazepines (clonazepam and nitrazepam) has previously been reported [10–12], leading to the protection of plasma samples from light during analysis.

Conclusions

The described HPLC method is sensitive, reliable and allows rapid quantification of flunitrazepam in human plasma. It presents some advantages over previously described techniques: extraction is simple and there is no need for the drug to be hydrolysed. The relatively low limit of detection (5 ng ml^{-1}) and the short total time of the assay are particularly adapted to emergency use in toxicological analyses. This method is now routinely used for the determination of the plasma levels of flunitrazepam in poisoned patients.

A remarkable decrease of flunitrazepam concentration was observed in plasma samples stored on the laboratory bench and exposed to light during working hours. As a consequence of this finding, it is recommended that samples be protected from light during analysis by wrapping the tubes in aluminium foil.

References

[1] M.B. Scharf, E.O. Bixler, A. Kales and C.R. Soldatos, *Pharmacology* **19**, 173–181 (1979).
[2] C. Mignée, R. Garnier, F. Conso, M.L. Efthymiou and E. Fournier, *Thérapie* **35**, 581–589 (1980).
[3] R. Jochemsen and D.D. Breimer, *J. Chromatogr.* **227**, 199–206 (1982).
[4] D.J. Greenblatt, H.R. Ochs, A. Locniskar and P.M. Lauven, *Pharmacology* **24**, 82–87 (1982).
[5] Y.C. Surmirtapura, C. Aubert and J.P. Cano, *Arzneim.-Forsch./Drug Res.* **32**, 252–257 (1982).
[6] T.B. Vree, B. Lenselink and E. Van Der Kleijn, *J. Chromatogr.* **143**, 530–534 (1977).
[7] C. Violon, L. Pessemier and A. Vercruysse, *J. Chromatogr.* **14**, 370–384 (1982).
[8] A. Boukhabza, A.A.J. Lugnier, P. Kintz and P. Mangin, *J. Anal. Toxicol.* **15**, 319–322 (1991).
[9] J. Guichard, G. Panteix, J. Dubost, P. Baltassat and C. Roche, *J. Chromatogr.* **612**, 269–274 (1993).
[10] N. Wad, *Ther. Drug Monit.* **8**, 358–360 (1986).

[11] H. Kelly, A. Huggett and S. Dawling, *Clin. Chem.* **28**, 1478–1481 (1982).
[12] P.J.G. Cornelissen, G.M.J. Beijersbergen Van Henegouwen and G.R. Mohn, *Photochem. Photobiol.* **34**, 345–350 (1981).
[13] M. Gaultier, *Analyse, Probabilités et Méthode statistique*, part 2, pp. 107–111. Vuibert Université, Paris (1984).

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