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The determination of bromazepam in plasma by reversed-phase high-performance liquid chromatography

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Abstract: A reversed-phase high-performance liquid chromatographic method has been developed for the determination of bromazepam, an anxiolytic benzodiazepine, in plasma. After a single-step extraction from alkalinized plasma with diethyl-ether in the presence of an internal standard (α-hydroxy-triazolam), the residues were chromatographed on a reversed-phase Nova Pak 5 μm C_{18} column, with a mobile phase of acetonitrile–water–triethylamine (700:300:4, v/v/v) adjusted to pH 7.4 with orthophosphoric acid. The limit of detection was 50 ng ml^{-1}, using a 20 μl injection with UV detection at 240 nm. Between-day and within-day relative standard deviations were lower than 6%. Studies of drug stability during sample storage at −20°C and at +4°C showed no degradation of bromazepam. However, bromazepam seemed to be degraded at ambient temperature, without any influence of light. This method is applied to the determination of bromazepam plasma levels in analytical toxicology.

Keywords: *Bromazepam; reversed-phase chromatography; toxicology.*

Introduction

Bromazepam, belonging to the 1,4-benzodiazepine class of anxiolytic compounds, was first synthesized by Fryer [1] in 1964. Since its introduction in clinical practice, it has been increasingly used in anxiolytic therapeutics [2]. This compound possesses two chemical substituents which are unusual for benzodiazepines, namely the 7-bromo and the 5-(2-pyridyl) groups (Fig. 1).

Although benzodiazepines, including bromazepam, are relatively safe drugs in current therapeutic use, high doses for a prolonged period of time can lead to dependence [3]. Furthermore, acute overdose, alone or in combination with alcohol or other CNS depressants, may result in coma [4]. Because of the wide use of bromazepam in self-poisoning, it seemed necessary to evaluate this drug in plasma samples.

Several methods have been described for the determination of bromazepam in plasma, such as thin-layer chromatography (TLC) [5], gas–liquid chromatography (GLC) [6–8] and high-performance liquid chromatography (HPLC) [9–12]. Quantification of bromazepam in body fluids by TLC and GLC did not give satisfactory results, because of lack of specificity and reproducibility. Amongst the HPLC procedures reported for determination of bromazepam, only one is adapted to emergency toxicology [12]. However, this method is time- and plasma-consuming.

The method described below is rapid, specific and adapted to routine and analytical toxicology in case of acute poisoning.

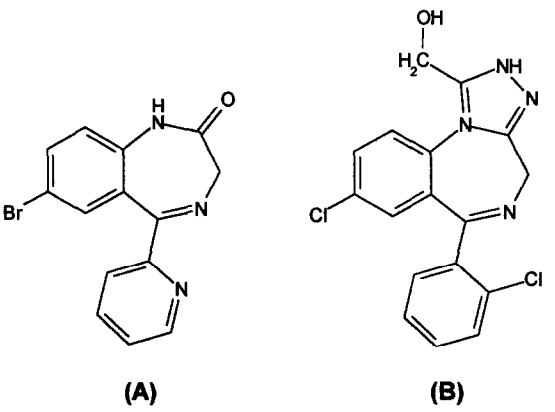

Figure 1
Chemical structures of (A) bromazepam and of (B) internal standard (α-hydroxy-triazolam).

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Experimental

Chemicals

Bromazepam was supplied by Roche (Neuilly sur Seine, France), and the internal standard (α-hydroxy-triazolam) by Upjohn (Paris, France). Acetonitrile and diethyl-ether were supplied by Info-Labo (Sainte-Foy-la-Grande, France) and were of HPLC reagent grade. Methanol (HPLC grade) was supplied by Prolabo (Paris, France). Water was de-ionized and doubly-glass distilled. Triethyl-amine was supplied by Rathburn (Walkerburn, Scotland, UK), orthophosphoric acid by Farmitalia Carlo Erba (Milan, Italy), sodium carbonate by Prolabo (Paris, France). They were all of analytical grade. Human heparin-ized plasma was supplied by the Regional Centre of Blood Transfusion (Bordeaux, France).

HPLC conditions

The analysis were performed on a Waters Assoc. (Milford, MA, USA) chromatographic system, including a Model 45 constant-flow pump, a Wisp Model 710 B automatic injector and a Lambda Max Model 480 ultraviolet detector operated at 240 nm. Samples were chromatographed on a Nova Pak C_{18} 5 μm (3.9 mm × 15 mm i.d.) stainless steel column (Waters). Chromatograms were recorded on an integrator D-2000 (Merck-Hitachi, Nogent sur Marne, France). Chart-speed was 0.5 cm min^{-1}.

The composition of the mobile phase was water–acetonitrile–triethylamine (700:300:4, v/v/v), the pH of the aqueous phase being adjusted to 7.4 with orthophosphoric acid. Before use, the mobile phase was filtered through a 0.45 μm filter (Sartorius, Göttingen, Germany). The flow rate was 2 ml min^{-1} and the peak-height ratios of bromazepam to internal standard were measured.

Standard solutions

Stock solutions of bromazepam and internal standard (1 mg ml^{-1}) were prepared in double distilled water. These solutions were frozen and stored at −20°C without degradation for 12 months.

Appropriate dilutions of solutions of bromazepam were made in drug-free human plasma to provide concentrations of 0.5, 1, 2 and 5 μg ml^{-1}.

Extraction procedure

A 100 μl volume of plasma, 50 μl of internal standard solution (10 μg ml^{-1} in double distilled water), 50 μl of sodium carbonate (0.1 M in double distilled water) and 1 ml of diethyl-ether were combined in a 1.5 ml conical centrifuge tube. The tube was then shaken for 1 min with a vortex mixer and centrifuged for 2 min at 8700*g* with a Microfuge (Beckman, Palo Alto, CA, USA). The organic layer was transferred to another conical centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The residues were dissolved in 100 μl of mobile phase and 20 μl were injected into the chromatographic system after centrifugation.

Results and Discussion

Figure 2 shows representative chromatograms of human plasma prepared and analysed as described in the procedure above.

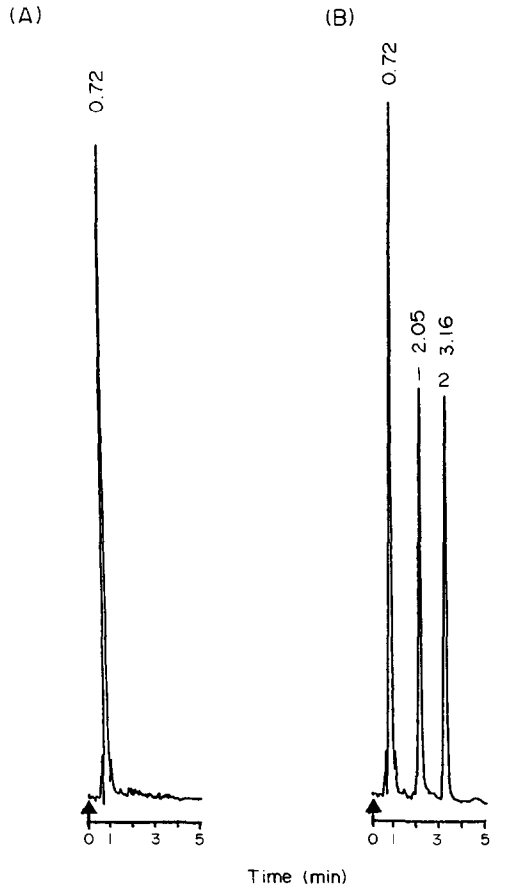

Figure 2
Typical chromatograms of (A) human drug-free plasma; (B) human plasma, after extraction, containing 2 μg ml^{-1} of bromazepam (peak 1 corresponds to bromazepam and peak 2 to internal standard).

The retention times were 2.05 min for bromazepam and 3.16 min for α-hydroxy-triazolam. The capacity factors (k') were 1.9 and 3.4, respectively.

The ability of some benzodiazepines or other structural analogues to interfere with bromazepam or the internal standard was investigated. The retention times and capacity factors of the compounds assayed are shown in Table 1. Under experimental conditions, no interference could be observed with these substances. Furthermore, no interferences were found with the main benzodiazepine metabolites. From our clinical experience, using the extraction procedure and the chromatographic conditions described herein, no interference was observed with the drugs usually associated with bromazepam, such as antidepressive, antipsychotic or barbiturate derivatives.

A 250 μl volume of internal standard was added to 5 ml of plasma containing either 1, 2 or 5 μg ml^{-1} bromazepam; 10 replicate analyses were then performed. Comparable concentrations prepared in mobile phase were used to determine absolute recovery. The peak-height measured in this latter experiment gave the 100% values destined to be compared to the peak-height of bromazepam and internal standard obtained after extraction. The percentage recoveries were between 97.2 and 104.1% for bromazepam and between 85.2 and 92.3% for α-hydroxy-triazolam (Table 2).

From a combination of 19 different assays (n = 95), a five-point calibration graph was calculated in the range 0–5 μg ml^{-1}. A strong correlation coefficient (r = 0.982) was obtained. The relation between the peak-height ratio (y) and the concentration (x) was: $y = 0.546x - 0.033$ with standard deviations being equal to 0.011 for the slope and 0.188 for the intercept.

The limit of detection (LOD, signal to noise ratio = 3) and the limit of quantitation (LOQ, signal to noise ratio = 10) were calculated and found to be 50 and 167 ng ml^{-1}, respectively.

The repeatability (n = 10) and the reproducibility (n = 10) of the HPLC procedure were tested on plasma spiked with bromazepam (0.75, 1.5 and 4 μg ml^{-1}). Accuracy was defined as (concentration found/concentration added) × 100.

The intra-assay precision had a relative standard deviation (RSD) between 1.8 and 3.1% (Table 3). The inter-assay provided RSD from 4.6 to 5.9% (Table 3). The accuracy was between 93.5 and 102.5% for the within-day assay and between 91.0 and 101.7% for the between-day assay (Table 3).

The stability of bromazepam in plasma at three different temperatures (−20, +4 and +25°C) was studied and also the potential influence of light on the solution kept at ambient temperature.

No degradation of the drug was noted at −20°C during a storage of 1 month.

One plasma sample containing 1.5 μg ml^{-1} was kept at +4°C for 8 days or at ambient temperature for 53 h either exposed to the light or kept in a dark place. These results are summarized in Table 4.

Interestingly, at +4°C, the apparent difference with the reference (between-day experiment) was not statistically significant. Con-

Table 1
Retention times (t_r) and capacity factors (k') of different benzodiazepines or structural analogues

Compounds	t_r (min)	k'
Bromazepam	2.05	1.9
Internal standard	3.16	3.4
Oxazepam	3.63	4.0
Lorazepam	3.65	4.1
Nitrazepam	3.72	4.2
Estazolam	3.90	4.4
Zolpidem	4.20	4.8
Clonazepam	4.44	5.2
Triazolam	5.62	6.8
Flunitrazepam	5.90	7.2
Temazepam	6.33	7.8
Nor-diazepam	6.83	8.5
Clobazam	9.77	12.6
Alprazolam	10.03	12.9
Diazepam	12.35	16.1

Table 2
Analytical recovery of bromazepam and the internal standard

	Bromazepam conc.		
	1 μg ml^{-1}	2 μg ml^{-1}	5 μ ml^{-1}
Replicates (n)	5	5	5
Bromazepam recovery	104.1 ± 9.5%	102.4 ± 3.6%	97.2 ± 3.0%
Internal standard recovery	85.2 ± 1.7%	86.4 ± 1.9%	92.3 ± 1.7%

Table 3
Intra-day and inter-day assays. Relative standard deviation (RSD) and accuracy were obtained for each assay using three standards (0.75, 1.5 and 4 μg ml^{-1})

Spiked conc. (μg ml^{-1})	Replicates (n)	Mean conc. found (μg ml^{-1})	RSD (%)	Accuracy (%)
Intra-day assay				
0.75	10	0.70	3.1	93.5
1.50	10	1.52	2.6	101.2
4.00	10	4.10	1.8	102.5
Inter-day assay				
0.75	10	0.68	4.6	91.0
1.50	10	1.50	4.2	99.9
4.00	10	4.07	5.9	101.7

Table 4
Statistical analysis of bromazepam stability in plasma, using the Fisher–Snedecor test for the variances (F-value) and the Fisher–Behrens test [13] for the means (t-value). Values given between brackets are theoretical values. ($P < 0.05$ for all tests)

Assay	Replicates (n)	Mean conc. found (μg ml^{-1})	SD (μg ml^{-1})	F-value*	t-value*
Stability at −20°C	30	1.49	0.12	3.41 (2.71)	0.193 (2.129)
Stability at +4°C	21	1.45	0.12	3.27 (2.90)	1.403 (2.176)
Stability at +25°C (exposure to light)	33	1.36	0.15	5.04 (2.71)	4.225 (2.129)
Stability at +25°C (stored in dark)	33	1.35	0.17	6.24 (2.71)	4.177 (2.045)

* References for the statistical comparison are between-days values.

sequently, bromazepam seemed to stay unchanged at this temperature, during the time of the assay. But, if the F-values were comparable between the −20°C and the +4°C assays, the comparison between the respective t-values showed a strong discrepancy, indicating that at +4°C, a slight degradation process could occur, but too slowly to pass beyond the statistical level.

On the other hand, a more pronounced degradation was observed during storage at +25°C, this phenomenon being more marked after exposure to light. This moderate degradation was shown to be a first-order rate process, with a degradation half-life of about 122 h (light) and 165 h (dark). However, the difference between these two processes of degradation did not reach statistical significance (the Gauss reduced variable $t = 0.21$) and is lower than the theoretical value ($t_0 = 1.96$; $P = 0.05$).

Conclusions

The HPLC method described for the determination of bromazepam in human plasma was specific, reliable and rapid. The limit of detection (50 ng ml^{-1}) was sufficient and the total time of the assay was short. Thus, this method is particularly adapted to emergency use in toxicological conditions. Moreover, it required a small volume of plasma, allowing possible further determinations on the same samples, in case of poly-intoxications.

This method is now routinely used for the determination of the plasma levels of bromazepam in intoxicated people.

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