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Determination of bentazepam in plasma by high-performance liquid chromatography

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Introduction

Bentazepam (2,3-tetramethylene-4-phenyl-7-oxo-thiene), Fig. 1, is a derivative of thienodiazepine showing anxiolytic, tranquillizing, anticonvulsant, miorelaxing and sleep-inducing properties [1]. Pre-clinical trials in volunteers have shown psychotropic activity for this drug such that the preparation may be considered as a tranquillizer with slight psychostimulatory properties. The drug also shows activity in combating symptoms of anxiety, restlessness, depression, difficulties in touch, sleep disorders [2] and in treatment of childhood anxiety [3].

Acute and chronic intolerance to the drug is rare at the clinical doses of 30–150 mg and is generally accompanied by side-effects similar to those displayed by several benzodiazepines. However these side-effects appear at doses greater than those normally used in clinical practice [4].

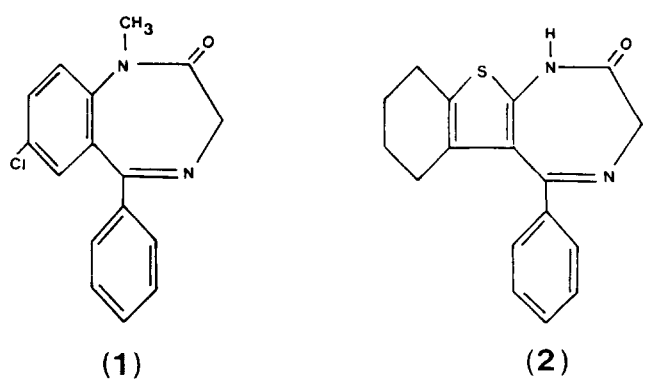

Figure 1
Chemical structures of diazepam (1) and bentazepam (2).

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This paper describes a simple and sensitive reversed-phase high-performance liquid chromatography method for the measurement of bentazepam in plasma.

Experimental

Apparatus

The liquid chromatograph used consisted of a solvent delivery pump (Varian 5000), a fixed-wavelength 254 mn UV detector model Varichrom (Varian, Palo Alto, CA), a fixed-volume loop injector (40 μl) (Valco) and an electronic integrator model CDS 111 (Varian, Palo Alto, CA). A Waters 3.9 mm × 30 cm μ-Bondapack C-18 column (Waters Associates, Milford, Massachusetts) was used.

Reagents

Reagent grade sodium hydroxide, glycine, HPLC grade benzene and methanol (E. Merck, Darmstadt, West Germany), diazepam (USP reference standard) and bentazepam (Laboratorios MADE S.A. Spain) were used as received. The pH 10.2 buffer used consisted of 0.1 M glycine adjusted with sodium hydroxide solution.

Analytical conditions

For the analysis of bentazepam in plasma a μ-Bondapack C-18 column was employed with methanol–water (65:35, v/v) as mobile phase at a flow rate of 1 ml/min. Chromatography was performed at room temperature and quantification achieved by U.V. absorption measurements at 254 nm with an attenuation of 0.04–0.02 AUFS. Diazepam was used as the internal standard.

Preparation of standards

A set of standard samples was prepared by adding 100, 80, 40, 20, 10 and 5 μl of a 5 μg/ml solution of bentazepam in water to 1 ml of plasma blank by means of a 40 microlitre syringe (Hamilton Co., Reno, Nevada). The internal standard was prepared by adding 100 ml of methanol to 5 mg of diazepam in a 100 ml volumetric flask and shaking manually for 5 min. This solution was later diluted 1:200 with the eluting solvent, employing 0.5 ml of solution and bringing up to a volume of 100 ml. The concentration of the resulting solution was 250 ng/ml and 10 μl of this solution were added to each plasma sample.

Subjects

After an overnight fast, 10 healthy male volunteers (23–30 years old, 50–66 kg) were administered a single 25 mg bentazepam tablet. Blood samples of sufficient volume to obtain 5 ml of plasma were drawn prior to administration and at regular intervals after administration. Samples were collected in vacuum heparinized containers (Vacutainer, Becton, Dickinson and Co., Oxnard, CA) 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 and 12.0 h, respectively after administration of the drug, and immediately centrifuged at 750 **g**. The separated plasma was stored at −20°C until required for analysis.

Extraction procedure and analytical conditions

Plasma (1 ml) was pipetted into a 20 ml screw-cap test tube (Teflon-line screw cap, No. 9826; Pirex), and 5 ml of benzene, 100 ml of glycine–NaOH buffer and 100 μl of

internal standard were added. The tubes were placed on a rocking shaker (Heidolph; Typ Reax 2) at a slow speed for 15 min and then centrifuged at 250 **g**. Any emulsion formed was broken by lightly tapping the tubes which were recentrifuged. The organic layer was transferred to a 12 ml glass-stoppered conical-tip tube using a Pasteur pipette. The extraction of the aqueous layer was repeated with an additional 5 ml of benzene. This was centrifuged, the organic layer was combined with the first extract in the conical-tip tube, and the tubes were placed in a water bath maintained at 40°C. The combined extracts were evaporated under a stream of nitrogen. The dry residue was redissolved in 100 μl of the mobile phase prior to injection into the chromatograph.

Retention times for bentazepam and diazepam were 10.8 and 8.7 min, respectively. The peak height ratios of bentazepam to the internal standard in the sample plasma were measured and compared with those for the calibration standards.

Calibration curve

The linearity of the calibration was determined by adding 25–500 ng of drug to 1 ml plasma blanks. The coefficient of variation was determined using replicate samples of plasma spiked with bentazepam.

Recovery

The recovery of bentazepam from plasma was determined by analysing spiked samples containing 500, 100 and 25 ng/ml bentazepam, respectively.

Results and Discussion

The method was evaluated in terms of extraction efficiency, selectivity, linearity, and precision. The peaks observed for bentazepam and diazepam were well separated from any naturally occurring plasma constituents. With blank plasma, containing no bentazepam, no peaks with the same retention as bentazepam were observed (Fig. 2). A typical chromatogram of plasma from a typical subject 2 h after the administration of 25 mg of bentazepam is shown in Fig. 3. Between 0.5 and 16 h after drug administration, the concentration of bentazepam in plasma was generally in the range of 5–400 ng/ml (Fig. 4). The peaks obtained for bentazepam and internal standard were symmetrical and well separated.

In order to determine the precision and accuracy of the analytical methods, replicate samples ($n = 4$) were analysed for seven concentrations of bentazepam in plasma. The results of these analyses are summarized in Table 1. The mean predicted plasma concentrations range from 97 to 105% of the calculated concentrations. The relative standard deviation (RSD) of the peak height ratios ranged from 2.6 to 6.2% (mean = 4.1%). The data from Table 1 were subjected to linear regression analysis and standard curves constructed which were linear from 25 to 500 ng of bentazepam per ml of plasma ($r = 0.9989$).

Mean recoveries of bentazepam from the extraction procedure were 95.4, 98.9 and 97.5 at 25, 100 and 500 ng/ml, respectively.

The limit of detection defined with respect to double that of the background noise with 1 ml of plasma and 40 μl sample is 5 ng/ml of plasma. If a 100-μl loop and a greater volume of plasma are used the limit of detection can be reduced to <3 ng/ml of plasma.

The choice of a pharmacokinetic model to describe the evolution of bentazepam plasma levels with the time elapsed after oral administration of the drug was carried out with the aid of the programme STRIP [5].

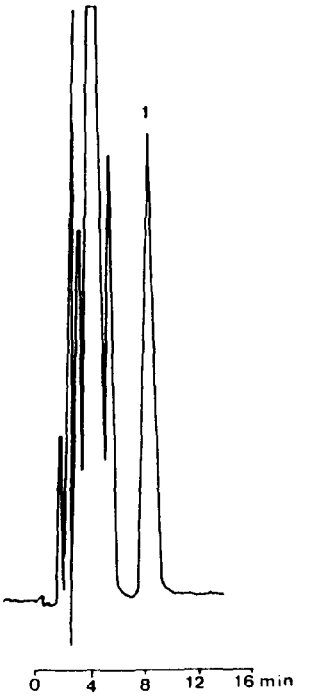

Figure 2
Chromatogram of blank plasma spiked with diazepam (1).

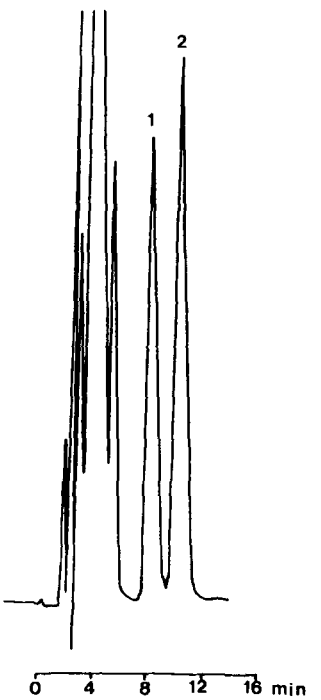

Figure 3
Chromatogram of a plasma sample taken from a healthy volunteer 2 h after the oral administration of a 25 mg bentazepam tablet. Key: (1) diazepam, (2) bentazepam (252.9 ng/ml).

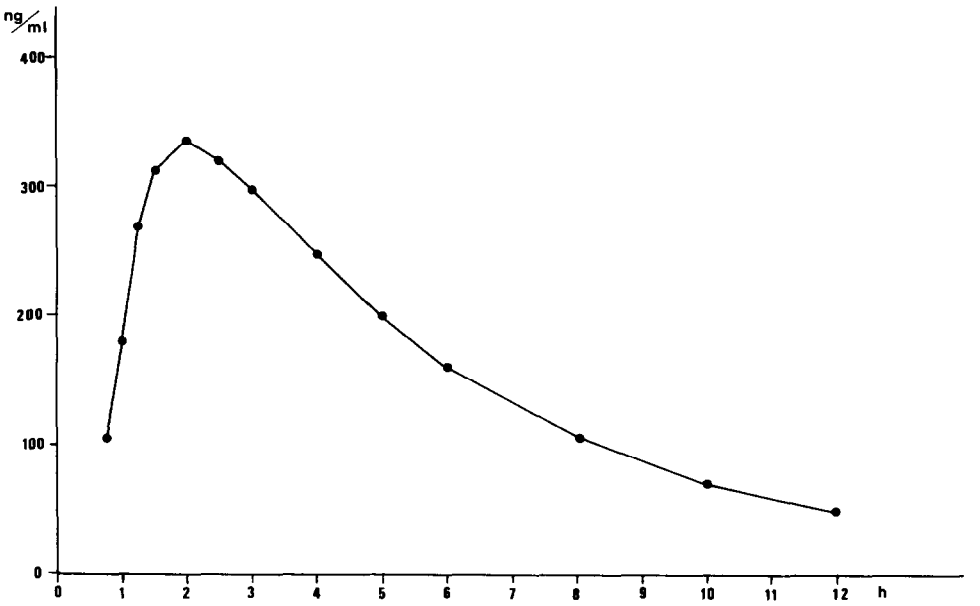

Figure 4
Mean plasma levels of bentazepam, for the volunteers included in the study, as a function of the time elapsed after administration of the drug.

Table 1
Precision and accuracy data for the determination of bentazepam in plasma

Calculated concentration of bentazepam (ng/ml)	Observed mean peak area ratio	Predicted mean concentration of bentazepam (% of theoretical) (ng/ml)	Relative standard deviation (%)*
25	0.16	25.3 (101%)	6.2
50	0.25	49.2 (98%)	5.7
100	0.51	104.7 (105%)	4.2
200	1.05	193.8 (97%)	3.6
400	2.08	410.5 (103%)	3.0
500	2.55	482.9 (97%)	2.6
			Mean = 4.1

*n = 4 at each concentration studied.

The plasma concentration data for bentazepam was analysed in accordance with a single compartment open kinetic model, according to the following equation:

$$C = B_0 \, e^{-K_e(t-t_0)} - A_0 \, e^{-K_a(t-t_0)}.$$

The fundamental parameters for this model; the exponentials K_a and K_e, the absorption and elimination constants, respectively, and the coefficients A_0 and B_0 were calculated by the iterative calculus program using minimum expanded squares

ELSFIT (B.L. Sheiner, University of California — private communication). The two calculus programs employed were used on a Hewlett–Packard 85 computer. The remaining pharmacokinetic parameters, lag time (t_o), absorption half-life ($t_{1/2a}$), the time at which the maximum plasma levels were reached (t_{max}), the maximum plasma concentration (C_{max}), and the elimination half-life ($t_{1/2e}$) were determined according to the criteria of Wagner [6]. The mean and standard deviation of the pharmacokinetic parameters established for the experimental data in accordance with the single compartment model proposed are shown in Table 2.

Further experimental work should permit the establishment of the bioavailability kinetics of bentazepam in the formulation employed and the determination of other pharmacokinetic properties of the drug.

Table 2
Pharmacokinetic parameters for bentazepam determined using LC analytical data

Parameters	Units	Mean	Standard deviation
K_a	(h^{-1})	3.1	1.8
$t_{1/2a}$	(h)	0.3	0.2
t_o	(h)	0.7	0.3
C_{max}	(ng/ml)	424.6	40.6
t_{max}	(h)	1.1	0.4
K_e	(h^{-1})	0.2	0.1
$t_{1/2e}$	(h)	3.3	1.1

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