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Note

Quantitative determination of premazepam in human plasma by high-performance liquid chromatography

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Premazepam*, 1,7-dihydro-6,7-dimethyl-5-phenylpyrrolo-[3,4-*e*][1,4]-diazepin-2(3H)one (Fig. 1), is a pyrrolodiazepine which originates from the search for drugs with a clear-cut dissociation between the desired pure anti-anxiety activity and undesired sedative effects.

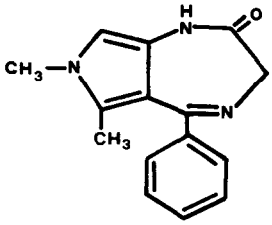

Fig. 1. Chemical structure of premazepam.

Premazepam shows benzodiazepine-like activity in the pharmacological tests considered to be predictive of anti-anxiety activity in man [1, 2]. Unlike benzodiazepines, premazepam does not induce ataxia or sedation at comparable or much higher doses than those effective in the above-mentioned tests.

Premazepam binds selectively to brain benzodiazepine receptors in vitro and in vivo [1, 2], acting also as a benzodiazepine antagonist at these receptors, i.e. it reduces or abolishes the effects of diazepam on motor coordination or muscular tone [1].

In a pilot study on tolerance and efficacy of premazepam, the drug was

*International non-proprietary name (World Health Organization).

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well tolerated by fifteen anxious patients up to 75 mg twice daily, and as judged by many rating scales for the evaluation of anxiety caused an improvement in clinical symptoms [3].

The aim of this work was to develop a sensitive, selective and reproducible method for assaying premazepam in human plasma.

Several analytical methods have been published for the determination of benzodiazepines in biological fluids by thin-layer chromatography (TLC), gas chromatography (GC) with sensitive and specific detection, and high-performance liquid chromatography (HPLC) [4, 5].

GC analysis is often unsuitable for the assay of some benzodiazepines because of their thermal instability or the need for derivatization. Some trials conducted on premazepam using this technique were unsuccessful.

A TLC method, validated for the concentration range 0.3—3.0 μg/ml, was previously developed [6] for assaying premazepam in the plasma of laboratory animals.

HPLC followed by UV detection was also found to be suitable for the determination of premazepam [7] and it was used in developing the present method for assaying the drug in human plasma. This method proved to have a higher sensitivity than the TLC method [6] since the application range tested was 20—200 ng/ml. Moreover, the HPLC method was particularly suitable for routine analysis using an automatic sampler.

Polarography was also used for the determination of some benzodiazepines in biological fluids [8], because of the presence of the reducible groups >C=N—, =N<O, and —NO_2 in the molecules [9]. The polarogram of premazepam displays a reduction wave with $E_{1/2}$ = —0.9 V under the conditions reported elsewhere [7]. This property of premazepam suggested the application of an electrochemical detector, a system more sensitive than ultraviolet (UV) detection, for the quantitative determination of premazepam in plasma after HPLC. Trials are in progress.

EXPERIMENTAL

Materials

Premazepam (Fig. 1) was Lepetit analytical standard with a chemical purity of 99.9%. Betamethasone (Fig. 2), crystalline, from Sigma (St. Louis, MO, U.S.A.), was used as the internal standard. Solvent and reagents were high purity grade from Merck (Darmstadt, F.R.G.). Distilled water was filtered through a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Plasma was from healthy volunteers.

The Bransonic 12 ultrasonic equipment was supplied by Smithkline (Soest,

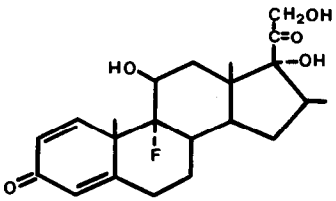

Fig. 2. Chemical structure of betamethasone, internal standard.

The Netherlands). The Continental Alter 2864 shaker was from Passoni (Milan, Italy).

A Supelcosil LC-18 DB column, 15 cm × 4.6 mm, particle size 5 μm, from Supelco (Bellefonte, PA, U.S.A.), was used as the stationary phase.

Instrumentation

A Waters Assoc. liquid chromatograph equipped with a Waters Model 620 solvent programmer, a Waters Model 440 absorbance detector, a WISP Model 710A automatic sampler, and a Tarkan 600 W+W recorder connected to an HP 3357 data system, or a Hewlett-Packard Model 3380A integrator, was used for the quantitative determination of premazepam with reference to the internal standard.

Standard solutions

For premazepam, 10 mg of premazepam were dissolved in 100 ml of acetonitrile—2-propanol (1:1). For internal standard, 10 mg of betamethasone were dissolved in 10 ml of acetonitrile—2-propanol (1:1).

Extraction procedure

A 1-ml volume of human plasma was pipetted into a screw-cap tube containing 1 ml of 0.2 *M* carbonate/bicarbonate pH 10 buffer solution and 0.1 ml of 0.05% ascorbic acid solution. The internal standard, betamethasone (500 ng), was added and then solid sodium chloride was dissolved until saturation by vortex mixing. The pH was adjusted to 10—10.5 with 0.5 *M* sodium hydroxide and the sample was extracted with 15 ml of chloroform by shaking for 10 min at 300 inversions per min. After centrifuging at 3000 *g* for 10 min (ambient temperature), the organic phase was transferred to another screw-cap tube containing 1 ml of ammonium hydroxide.

The tube was shaken for 5 min at 300 inversions per min and was centrifuged at 3000 *g* for 10 min (ambient temperature). The aqueous phase was aspirated; 12 ml of the organic phase were poured into a conical tube and evaporated to dryness in a water bath (37°C) under a stream of nitrogen.

The residue was concentrated at the bottom of the tube by washing the walls with chloroform saturated with ammonia and by evaporating the solvent in a water bath (37°C) under nitrogen.

Chromatography

The residue was redissolved in 20—25 μl of acetonitrile—2-propanol (1:1) and transferred to the microvial of the HPLC automatic sampler.

A 10-μl aliquot of the extracted sample was injected onto a Supelcosil LC-18 DB column (stationary phase). Isocratic elution was made using a mixture of 22% B in A as mobile phase, where A is 0.1 *M* ammonium hydrogen phosphate—ammonium dihydrogen phosphate solution pH 7.5, and B is acetonitrile. The flow-rate was 2 ml/min, and UV detection was at 254 nm. The retention times of premazepam and betamethasone were 6.5 and 20 min, respectively.

RESULTS

Plasma samples from untreated subjects, processed by the method described, gave tracings without any peaks that could interfere with the determination of premazepam or the internal standard (Figs. 3 and 4).

Plasma samples of untreated healthy subjects were spiked with premazepam and betamethasone (internal standard) in order to determine the recovery, precision (repeatability), accuracy and linearity of the method. Five samples were prepared for each of the following concentrations of premazepam: 20, 50, 100, 200 ng/ml of plasma. The samples were extracted and analysed as described.

The results of the recovery trials are given in Table I. The mean recovery of premazepam relative to betamethasone internal standardization from human plasma was 63—82% in the concentration range tested, indicating that premazepam is extracted less than the internal standard used. The lowest recovery (63%) was for the lowest concentration (20 ng/ml). The true value of the

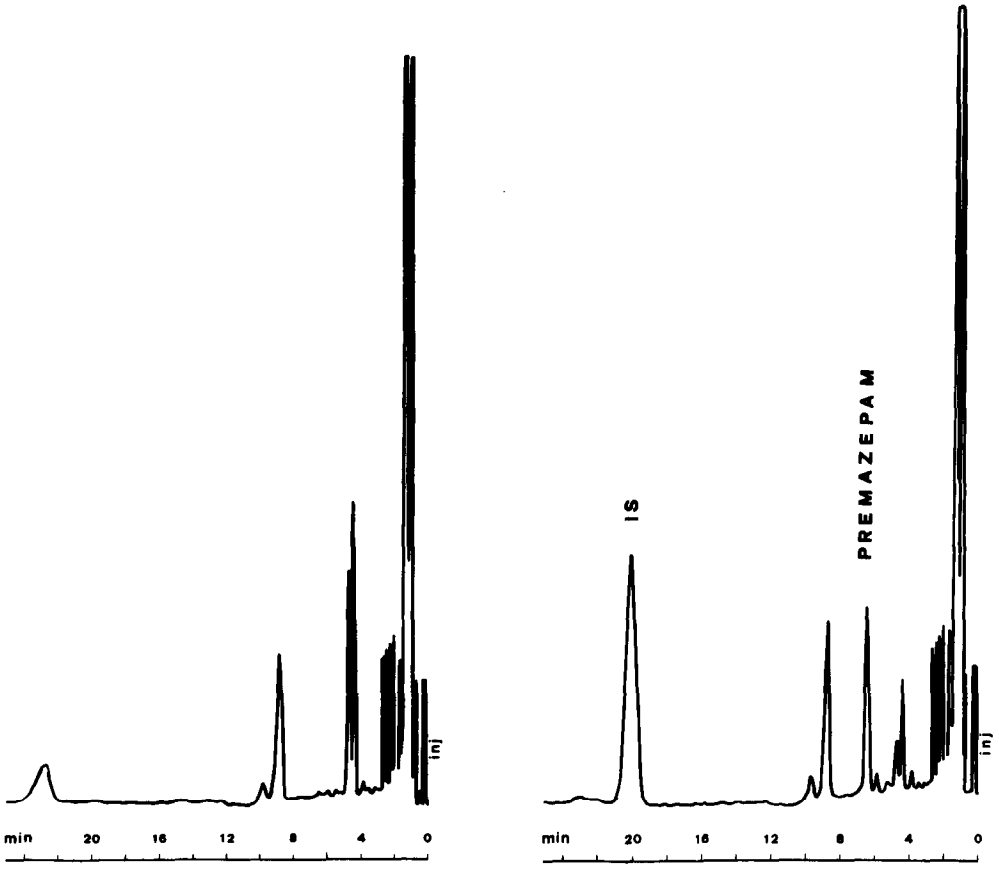

Fig. 3. Chromatogram of a plasma sample of an untreated subject. The retention times of premazepam and betamethasone (internal standard) are 6.5 and 20 min, respectively.

Fig. 4. Chromatogram of a plasma sample of an untreated subject, spiked with premazepam (100 ng/ml) and the internal standard (IS, 500 ng/ml).

TABLE I

RECOVERY OF PREMAZEPAM FROM HUMAN PLASMA SAMPLES (RELATIVE TO BETAMETHASONE)

Added (ng/ml)	Found (ng/ml)					Mean ± S.D. (ng/ml)	R.S.D.	Mean recovery (%)
20.5	11.9	12.6	14.5	13.8	12.0	13.0 ± 1.1	8.5	63.2
50.5	38.1	36.4	40.9	34.7	39.8	38.0 ± 2.5	6.6	75.2
100.8	74.4	76.8	76.5	75.0	77.2	76.0 ± 1.2	1.6	75.4
205.9	165.5	164.7	171.0	174.2	164.4	168.0 ± 4.4	2.6	81.6

plasma concentration in a plasma sample can be found by the regression analysis equation given below.

The precision of the method was established by the value of the relative standard deviation (R.S.D.) and ranged from 1.6% to 8.5% (Table I) in the concentration range analysed.

The accuracy of the method was calculated using the twenty determinations reported in Table I, with a mean recovery of 73.8% and an R.S.D. of 10.4% over the concentration range analysed.

The linear-regression analysis made with twenty values of X (premazepam added, ng/ml) and twenty values of Y (premazepam found, ng/ml) (Table I) provided the equation $Y = 0.8348X - 5.1102$ (correlation coefficient, $r = 0.9987$).

DISCUSSION

In setting up the extraction procedure, a simple extraction with chloroform of premazepam and the internal standard, followed by evaporation under nitrogen, gave varying recoveries of premazepam from spiked plasma samples particularly at the lowest concentrations.

Initial trials indicated that the critical step was the evaporation of the organic phase under nitrogen, after which the recovery of premazepam was found to be non-reproducible, probably due to the adsorption of premazepam on the glass. In our efforts to improve the precision of the method, several trials were conducted and the use of ammonia gave the best results. The organic phase has to be saturated with ammonia before evaporating it under nitrogen and chloroform saturated with ammonia has to be used to collect the residue at the bottom of the conical tube.

It is well known that the pyrrole ring is sensitive to oxidation [10]. Thus, ascorbic acid was added in the tube as anti-oxidizing agent before extracting the sample.

The validation range was established on the basis of the plasma concentration of premazepam expected in humans after the administration of the drug. The method was applied to observe the time course (Fig. 5) of premazepam in the plasma of subjects ($n = 4$) treated with a single 30-mg oral dose [3]. Premazepam was rapidly absorbed by the gastrointestinal tract, reaching plateau plasma concentrations of 330—370 ng/ml 1.5—6 h after administration. The concentration of premazepam decreased thereafter with a mono-

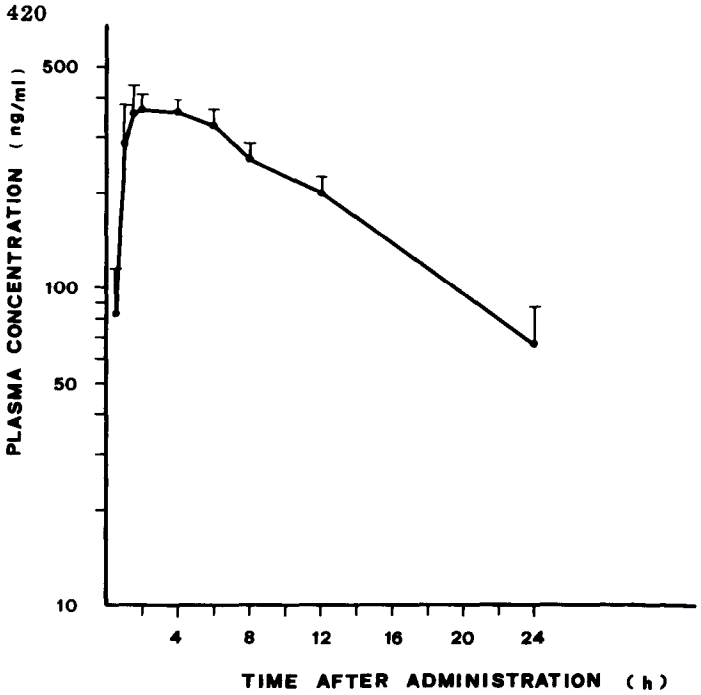

Fig. 5. Mean plasma levels ± S.E. of premazepam in man (n = 4) after a 30-mg oral dose.

exponential slope characterized by an elimination half-life of about 8 h. Twenty-four h after administration, the plasma concentration declined to about 70 ng/ml.

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