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## Short Communication

# Determination of a potential anxiolytic drug (CGS 20625) in human plasma by high-performance liquid chromatography

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ABSTRACT

An analytical method employing reversed-phase high-performance liquid chromatography is described for the determination of a potential anxiolytic agent in human plasma. This experimental drug candidate has potent and selective affinity for the central benzodiazepine receptor complex. The compound and internal standard are extracted from buffered plasma (pH 9.0) into ethyl acetate. The solvent is evaporated and the residue is reconstituted in chromatographic mobile phase. Separation is achieved on a 5-$\mu$m phenyl column with ultraviolet absorbance detection of the drug and internal standard at 270 nm. Recovery and reproducibility assessments indicate good accuracy (overall relative recovery of 101%) and precision (coefficients of variation from 2.0 to 11%) over the concentration range 10–1000 ng/ml. The limit of quantification for the method is 10 ng/ml. The method is suitable for pharmacokinetic analysis following the administration of 80 mg of drug to normal volunteers.

## INTRODUCTION

The compound (CGS 20625), 2-(4-methoxyphenyl)-5,6,7,8,9,10-hexahydrocyclohepta[*b*]pyrazolo[3,4-*d*]pyridin-3(2H)-one (I, Fig. 1), has potent and selective affinity for the central benzodiazepine receptor complex [1,2]. This experimental drug candidate is being studied in clinical trials as a potential anxiolytic agent. It is appropriate, therefore, to have analytical methodology available for assessing the pharmacokinetics and bioavailability of the compound.

In this paper we describe a sensitive and selective high-performance liquid chromatographic (HPLC) method for the analysis of this drug candidate in human plasma and its application to clinical studies.

## EXPERIMENTAL

### *Chemicals and reagents*

The drug (I) and internal standard (CGS 17867) (II, Fig. 1) were synthesized

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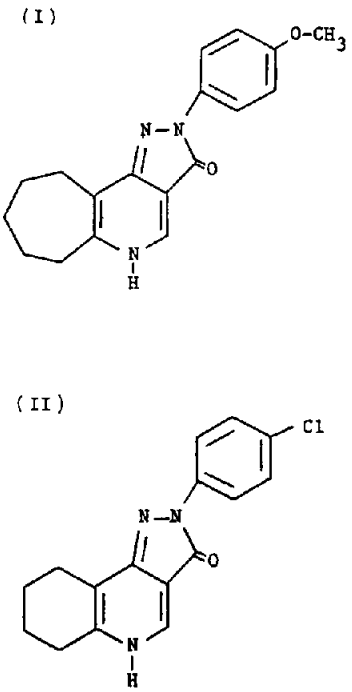


Fig. 1. Structures of I and II.

and supplied by Naokata Yokoyama, Ciba-Geigy (Summit, NJ, USA). The HPLC-grade ethyl acetate, methanol, tetrahydrofuran (THF) and water were purchased from Burdick & Jackson (Muskegon, MI, USA). Boric acid, potassium chloride and sodium hydroxide pellets, all reagent grade, were purchased from J. T. Baker (Phillipsburg, NJ, USA). Human plasma from heparinized blood was obtained from Biological Specialty (Landsdale, PA, USA) and Ciba-Geigy volunteers (Ardsley, NY, USA). All procedures were performed using Pyrex containers, disposable borosilicate glass containers, PTFE-lined caps and plastic pipet tips, purchased from Baxter Healthcare (Edison, NJ, USA).

*Preparation of reagents, stock and standard solutions*

A 0.1 *M* boric acid–potassium chloride–sodium hydroxide buffer (pH 9.0) was prepared from boric acid–potassium chloride and sodium hydroxide solutions. This buffer solution was refrigerated and brought to room temperature prior to use.

A stock solution of I (1.0 mg/ml) was prepared by dissolving the compound in methanol. Additional solutions at 10 and 20 μg/ml were prepared by further diluting this solution with methanol. These solutions were used to prepare methanolic spiking solutions at concentrations of 0.2, 0.4, 1.0, 2.0 and 5.0 μg/ml.

A 1.0 mg/ml stock solution of II (internal standard) was prepared in methanol and was further diluted with methanol to give the internal standard spiking solution (5.0 μg/ml).

The solutions were stored at about 4°C. Under these conditions both compounds remained stable for more than two months.

Plasma (calibration) standards were obtained by spiking 1.0-ml portions of control human plasma with 50-μl aliquots of the appropriate solution of I to give final concentrations of 10, 20, 50, 100, 250, 500 and 1000 ng/ml. Calibration standards were prepared on a daily basis.

Quality control samples (50-ml pools) used for method validation were prepared at drug (I) concentrations of 10, 50, 500 and 1000 ng/ml. The quality control samples were prepared in advance of method validation and stored as 1-ml aliquots at about −20°C until analyzed.

The HPLC mobile phase consisted of methanol–water–THF (45:40:15, v/v). The mobile phase was filtered through a 0.45-μm Millipore GVWP membrane filter (Millipore, Bedford, MA, USA) and degassed under vacuum prior to use.

A chromatographic standard solution was prepared in HPLC mobile phase at concentrations equivalent to final plasma concentrations of 100 ng/ml I and 250 ng/ml II. This solution was stored at about 4°C until analysis.

*Sample preparation procedure*

A 1.0-ml plasma sample was added to a screw-cap culture tube followed by the addition of 50 μl of the internal standard spiking solution (5.0 μg/ml II). A 1-ml volume of 0.1 *M* boric acid–potassium chloride–sodium hydroxide buffer (pH 9.0) was added and the sample was vortex-mixed. Ethyl acetate (5 ml) was added and the sample tube was capped and placed on a rotator at medium speed for 5 min, then centrifuged at 1250 *g* for 5 min. The organic phase was transferred to a culture tube and the contents were evaporated until dry under a stream of nitrogen at 40°C. The residue was reconstituted in 200 μl of mobile phase solution, vortex-mixed for 0.5 min and then transferred to an autosampler micro-vial for injection into the HPLC system.

*Chromatographic conditions*

The chromatographic system consisted of a Waters 590 programmable solvent delivery module (Waters Assoc., Milford, MA, USA), a WISP 710B autosampler (Waters Assoc.), a Kratos 783 variable-wavelength UV–VIS detector (ABI Analytical, Ramsey, NJ, USA) and a PE Nelson 970 (256-kB memory) dual-channel interface and TurboChrom chromatography workstation (PE Nelson Systems, Cupertino, CA, USA).

Analyses were performed on a Zorbax 5-μm, 150 mm × 4.6 mm I.D., phenyl column (Mac-Mod Analytical, Chadds Ford, PA, USA) in series with a 10-μm Guard-PAK ODS cartridge module (Waters Assoc.). The mobile phase was passed through the system at a flow-rate of 1.0 ml/min. The UV detector was set

to 270 nm and maximum sensitivity output to the computer/data system. The chromatographic system was operated at ambient temperature under a pressure of 80–90 bar. A 25-μl aliquot was used for the chromatographic analysis.

Small changes (1–2%) in the organic modifiers of the mobile phase achieved reproducible retention characteristics (±0.5 min) of the analytes from one analytical column to another.

*Data acquisition and quantitation*

Peak areas for the drug (I) and internal standard (II) were measured using a PC-based, TurboChrom chromatography workstation (PE Nelson systems). The chromatographic data were processed for peak-area ratios of I to II using TurboChrom 2700 (version 1.04) software (PE Nelson Systems). Calibration curves were generated from the daily calibration standards using weighted (1/peak-area response ratio) linear least-squares regression of the amount of drug added *versus* the measured peak-area ratio of I to II. Quantification of unknown and quality control samples was obtained by interpolation from the regression equations of the respective daily calibration curves.

## RESULTS AND DISCUSSION

*Chromatographic selectivity and sensitivity*

Typical chromatograms of blank human plasma and plasma spiked with II (250 ng/ml) and I at 10 or 100 ng/ml are shown in Fig. 2. Drug (I) and internal standard (II) were eluted with retention times of about 4.7 and 7.5 min, respectively. No interferences were observed at the retention times of these compounds in any of the blank plasma samples tested.

Using a signal-to-noise ratio of 3:1 (statistically different from an analytical blank), the minimal detectable amount of drug is equivalent to a drug plasma concentration of 2.5 ng/ml. The limit of quantification (LOQ), defined as the lowest drug concentration where acceptable accuracy (100 ± 15%) and precision (coefficient of variation, C.V. ≤ 15%) are obtained, for this method is 10 ng/ml.

*Recovery from plasma*

Absolute recoveries (extraction efficiencies) of I and II were determined by comparing peak areas of the analytes from extracted plasma standards to those from a chromatographic standard solution prepared in mobile phase at the equivalent concentrations and chromatographed directly. Mean (±S.D.) recoveries of 108 ± 8.1 and 99.1 ± 4.5% were obtained for I (100 ng/ml) and II (250 ng/ml), respectively.

*Linearity of calibration curves*

Daily calibrators (10–1000 ng/ml) were prepared and used to generate linear regression curves. These calibration curves were represented by a plot of peak-area ratios of I to II *versus* concentration of the calibrator.

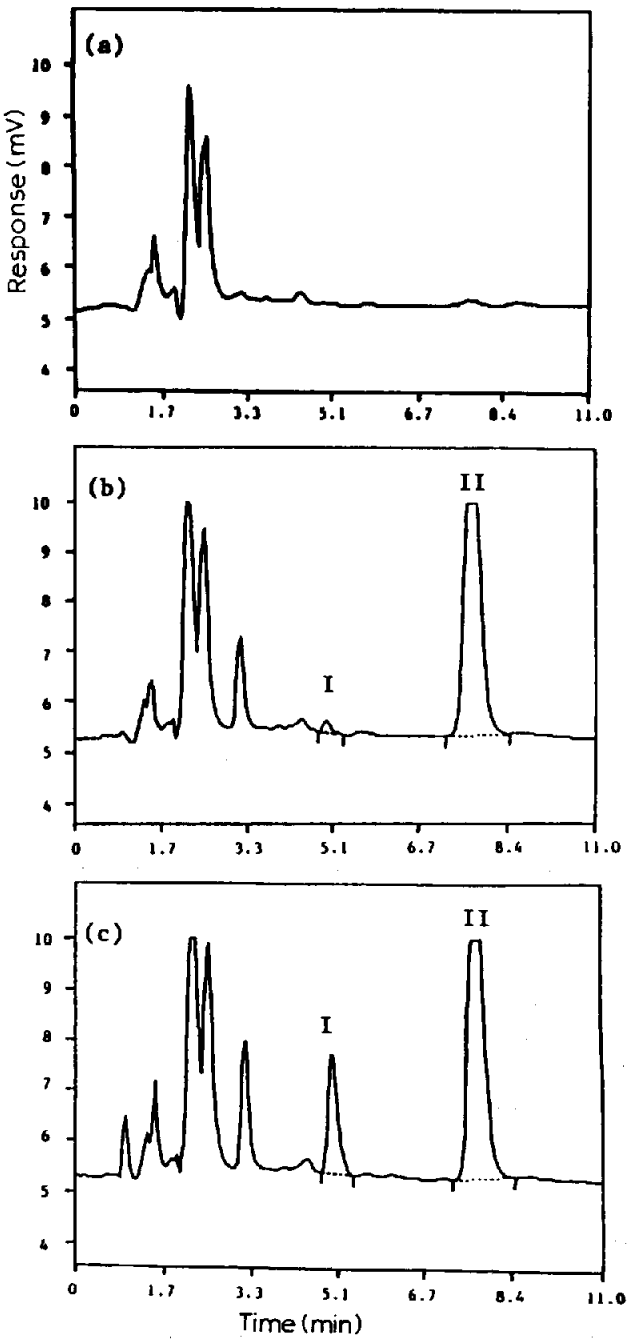


Fig. 2. Sample chromatogram of (a) blank human plasma, (b) plasma spiked with 10 ng/ml I and 250 ng/ml II and (c) plasma spiked with 100 ng/ml I and 250 ng/ml II.

TABLE I

INTRA-DAY ($n$ = 4–5) AND INTER-DAY ($n$ = 19–20) ACCURACY AND PRECISION DATA FOR QUALITY CONTROL SAMPLES

Values in parentheses are coefficients of variation (%).

| Added concentration (ng/ml) | Mean relative recovery (%) | | | | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Inter-day |
| 10 | 116 (7.6) | 103 (8.4) | 98.9 (3.1) | 106 (4.4) | 120 (3.1) | 106 (8.4) |
| 50 | 102 (0.5) | 90.0 (1.2) | 91.6 (0.9) | 105 (1.2) | 91.4 (4.5) | 96.0 (6.9) |
| 500 | 90.3 (0.8) | 104 (1.2) | 98.6 (0.6) | 101 (1.8) | 98.4 (1.4) | 98.5 (4.8) |
| 1000 | 106 (1.6) | 102 (0.6) | 100 (0.6) | 104 (0.8) | 103 (1.6) | 103 (2.0) |

Overall mean recovery = 101%

The calibration curves were found to be linear, with a mean correlation coefficient ($r$) of 0.9980, indicating a good fit to the linear regression model used. The mean slope data, associated with a C.V. of 5.9%, indicated good inter-day reproducibility.

*Accuracy and precision*

Results obtained from the quality control samples were used to assess the accuracy of the method. The reproducibility of the data was used to determine the precision of the method.

The intra-day ($n$ = 4–5) and inter-day accuracy and precision results for I are presented in Table I. The data are expressed as mean percentage found and percentage C.V. The inter-day values were calculated using all the determinations ($n$ = 19–20) at each of the indicated concentrations.

Mean relative recoveries ranged from 90.3 to 120% (intra-day) and 96.0 to 106% (inter-day). The overall accuracy was determined to be 101%. The data showed good inter-day precision with C.V. values of 8.4% at the LOQ (10 ng/ml) and 2.0–8.4% over the entire concentration range (10–1000 ng/ml). Intra-day precision was also good, with C.V.s ranging from 3.1 to 8.4% at the LOQ, as compared to 0.5–8.4% over the entire concentration range.

*Stability*

Plasma spiked with I at concentrations of 15–750 ng/ml and stored in both glass and plastic containers was found to be stable, with mean relative recoveries ranging from 88 to 111%, after storage under various conditions (room temperature, refrigerated and frozen) for periods of up to one month. Additional samples taken through several freeze–thaw cycles were also found to be stable (mean relative recoveries of 86–108%).

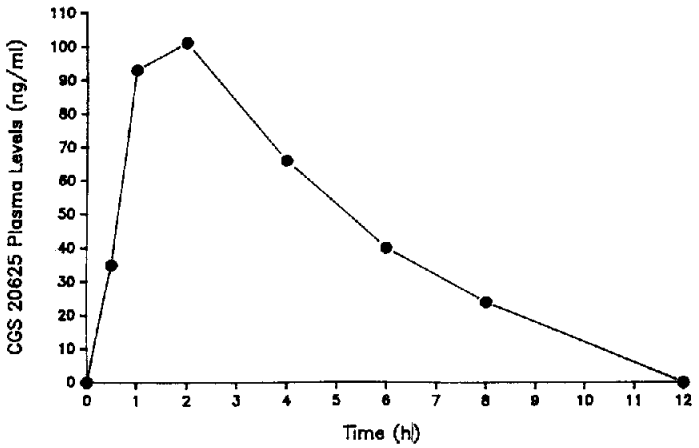


Fig. 3. Plasma level profile from a normal volunteer who received a single 80-mg oral dose of I.

*Application*

The applicability of the method was demonstrated by analyzing plasma samples from a clinical study in which normal volunteers received 80-mg single oral doses of I. The plasma concentration *versus* time profile for one of the participants of the trial is shown in Fig. 3. Approximately fifty samples can be analyzed daily on a routine basis.

## CONCLUSIONS

An HPLC method has been developed and validated for quantifying concentrations of a new anxiolytic drug candidate (I) in human plasma. The method has been shown to be rugged and reproducible. The overall accuracy of the method was determined to be 101%. The precision of the method was 2.0–8.4% over the concentration range 10–1000 ng/ml, with a limit of quantification of 10 ng/ml. The method has been successfully applied to the analysis of plasma samples originating from human drug disposition studies.

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