Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 4, pp. 351–355, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

Short Communication

Determination of CGS 15040A in human plasma by liquid chromatography

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Keywords: *CGS 15040A; liquid chromatography; plasma; human pharmacokinetics.*

Introduction

1,3,4,16b-Tetrahydro-2-methyl-2-h,1-OH-indolo-2(2,1c) pyrazino(1,2a)(1,4)benzodiazepine-16-carboxylic acid methyl ester hydrochloride (CGS 15040A; Fig. 1), is a new drug candidate being studied in clinical trials as a potential anxiolytic agent. Analytical methodology is therefore required for studying the pharmacokinetics of the compound. In this paper a sensitive method is described for the analysis of CGS 15040A in human plasma and its application to clinical studies.

Experimental

Materials

CGS 15040A was synthesized and supplied by J.W. Wasley and co-workers, Chemistry Research, CIBA-GEIGY Corporation (Summit, NJ, USA). The *N*-propyl ester analogue of CGS 15040A was kindly synthesized by H.P. Egger and R. Ianucci, Clinical Pharmacokinetics and Disposition, CIBA-GEIGY Corporation (Ardsley, NY, USA). The HPLC-grade acetonitrile, methanol, methyl t-butyl ether and water were purchased from Burdick & Jackson (Muskegon, MI, USA). Human plasma from heparinized blood was obtained from Biological Specialty Corporation (Landspale, PA, USA) and CIBA-GEIGY Corporation volunteers (Ardsley, NY, USA). Sodium bicarbonate, anhydrous sodium carbonate and phosphoric acid (85%), all reagent grade, were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Diisopropylamine (DPA), 99% reagent grade, was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Preparation of reagents and solutions

A 1.0 mg ml^{-1} stock solution of CGS

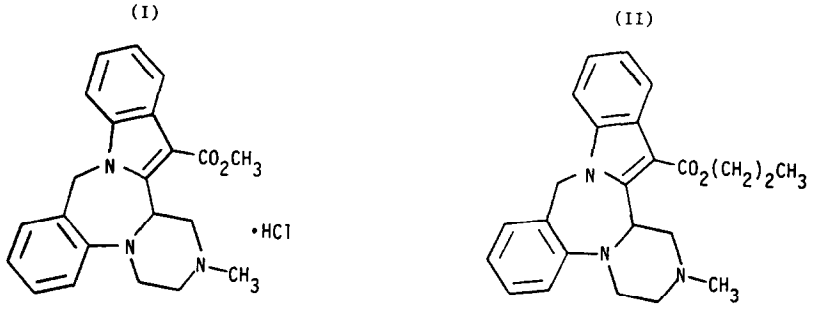

Figure 1
Structural formulae of CGS 15040A (I) and the internal standard, its *N*-propyl ester analogue (II).

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15040A was prepared in methanol. This solution was diluted with methanol to give spiking solutions of 1.0, 10 and 100 μg ml^{-1}. A 1.0 mg ml^{-1} internal standard (*N*-propyl ester analogue of CGS 15040A) stock solution was prepared in methanol. This stock solution was further diluted with methanol to give the internal standard spiking solution (10 μg ml^{-1}). Several chromatographic standard solutions were prepared in mobile phase to represent equivalent final plasma concentrations of 25, 100 and 500 ng ml^{-1} CGS 15040A, and 500 ng ml^{-1} of its *N*-propyl ester analogue. These solutions were used for the extraction efficiency studies. All solutions were stored at about 4°C and brought to room temperature prior to use.

Calibration standards for establishing the standard curves and quality control samples for evaluating the accuracy and precision of the method were prepared by adding the appropriate aliquots (10–50 μl) of the CGS 15040A stock or spiking solutions to 1.0 ml of blank human plasma to give concentrations of 10, 25, 50, 100, 200, 350, 500 and 1000 ng ml^{-1}. The calibration standards were prepared on a daily basis and the quality control samples were stored at about −20°C until analysed.

The mobile phase was prepared by combining acetonitrile and 0.2% aqueous DPA solution (90:10, v/v), and adjusting to pH* 6.5 with 10% H_3PO_4 solution. The mobile phase was filtered through a 0.45-μm GVWP membrane filter (Millipore Corp., Milford, MA, USA) and degassed under vacuum prior to use.

Chromatographic equipment and conditions

The HPLC system consisted of a Waters 590 programmable solvent delivery module (Waters Assoc., Milford, MA, USA) with flow rate at 1.0 ml min^{-1}; a WISP 710B autosampler (Waters Assoc.); an Ultrasphere ODS analytical column (5 μm, 250 × 4.6 mm, I.D.) (Beckman Instruments, Inc., San Ramon, CA, USA) in series with a Waters Guard-PAK ODS (10 μm) cartridge module (Waters Assoc.); and an SPD-6A variable wavelength UV detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) set at a wavelength of 290 nm. The chromatographic separation was performed at room temperature under a pressure of 350–500 psi.

Data acquisition

Peak areas for the drug and internal standard were measured using a Model 970 (256KB memory) interface and a TurboChrom workstation (PE Nelson Systems, Inc., Cupertino, CA, USA). The chromatographic data were processed for peak area ratios of drug to internal standard using TurboChrom 2700 (version 1.04) software (PE Nelson Systems, Inc.).

Sample preparation and analysis

A 1.0-ml plasma sample was added to a screw-cap culture tube followed by addition of 50 μl of the internal standard spiking solution (10 μg ml^{-1} *N*-propyl ester analogue of CGS 15040A). Two ml of a carbonate buffer (pH 10; 0.05 M) were added and the sample was vortexed for 15 s followed by the addition of 5 ml of methyl t-butyl ether. The sample tube was capped and placed on the rotator for 10 min (60–80 rpm) and then centrifuged for 5 min at 2500 rpm. The organic phase was transferred to a culture tube and the contents were evaporated to dryness under a stream of nitrogen at 45°C. The residue was reconstituted in 200 μl of mobile phase, vortexed for 30 s and then transferred to a WISP vial. A 50-μl aliquot was used for the chromatographic analysis.

Calibration and sample quantification

Calibration curves were generated using weighted (1/peak area response ratio) linear least-squares regression and were represented by a plot of peak area ratios of CGS 15040A to internal standard versus the concentration of the standards (10–1000 ng ml^{-1}). Quantification of quality control sample concentrations was obtained by interpolation from the equations of the regression lines of the respective calibration curves.

Results and Discussion

Chromatographic specificity and sensitivity

Typical chromatograms of blank human plasma, and plasma spiked with drug at the quantification limit (10 ng ml^{-1}) and at 1000 ng ml^{-1} in the presence of the internal standard are shown in Fig. 2. Drug and internal standard eluted with retention times of about 6.9 and 9.1 min, respectively. No interferences were observed at the retention time of the drug from any of the plasma samples tested. However, a small interference inherent in the human plasma tested was found at/near the

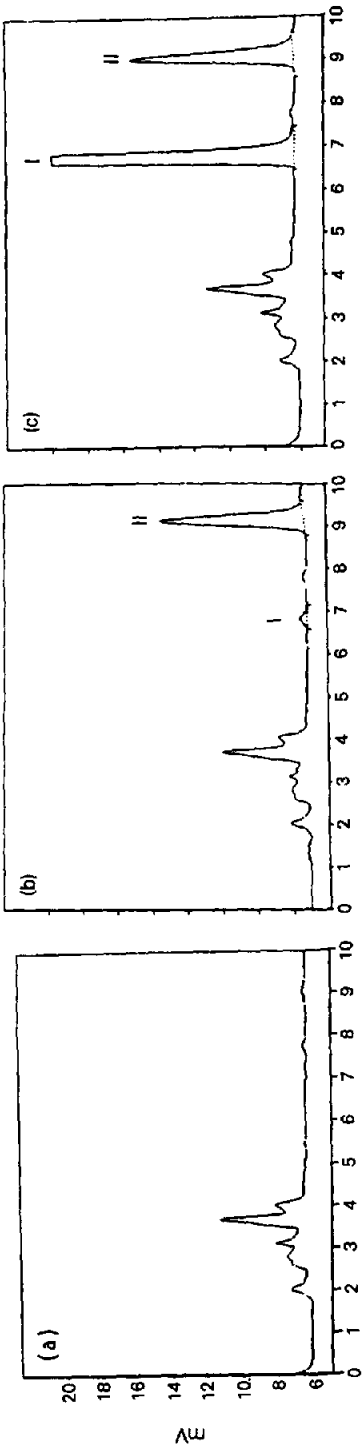

Figure 2
Typical chromatogram of (a) blank plasma extract, (b) plasma spiked with CGS 15040A (I) at 10 ng ml^{-1} and the internal standard (II) at 500 ng ml^{-1}, and (c) plasma spiked with CGS 15040A at 1000 ng ml^{-1}, and 500 ng ml^{-1} internal standard.

retention time of the internal standard, but this peak consistently contributed ≤3% to the total area. The limit of quantification (LOQ) for CGS 15040A is 10 ng ml^{-1}, and is defined as the lowest drug concentration where acceptable accuracy (100 ± 15%) and precision (RSD ≤15%) were obtained. The limit of detection for CGS 15040A was 2 ng ml^{-1}, at a S/N ratio of 4:1. Over 500 injections could be made before necessitating a change of the analytical column.

Linearity of calibration curves

The statistical data for the calibration curves are shown in Table 1. All of the calibration curves showed a good fit to the weighted linear regression model, with an average correlation coefficient (r) of 0.999. The mean slope data were associated with a relative standard deviation (RSD) of 7.9%, indicating good inter-day reproducibility.

Accuracy and precision

Relative recovery data were used to assess the accuracy of the method. The reproducibility of the recovery data was used to determine the precision of the method. The intra- and inter-day data, expressed as mean per cent found and RSD, respectively, are shown in Table 2. The inter-day values were calculated using all the determinations at the indicated concentrations.

The overall mean recovery (representing method accuracy) for CGS 15040A was 104%. The inter-day RSD was 6.9% at the LOQ and 3.5–9.2% for the entire concentration range (10–1000 ng ml^{-1}).

Extraction efficiency

The extraction efficiency (absolute recovery) was expressed as the ratio of the peak areas of the analytes from extracted plasma standards to those obtained from chromatographic standard solutions prepared in mobile phase at equivalent concentrations and chromatographed directly. The extraction efficiencies of CGS 15040A (25–500 ng ml^{-1}) and the internal standard (500 ng ml^{-1}) from plasma were 94–112% and 94%, respectively (Table 3).

Stability

Plasma spiked with CGS 15040A at various concentrations (12, 80 and 600 ng ml^{-1}), and stored in both glass and plastic containers, was found to be stable (relative recoveries of 86.5–

Table 1
Reproducibility of daily (N = 3) calibration curves

Analysis day	Slope* (m)	Intercept* (b)	Correlation coefficient (r)
1	0.0016	0.0027	0.9983
2	0.0014	0.0029	0.9992
3	0.0014	0.0008	0.9995
Mean	0.0015	0.0021	0.9990
SD (±)	0.0001	0.0012	0.0006
RSD (%)	7.9	54	0.06

*Slope (m) and intercept (b) coefficients of the calibration curves relating peak area ratio to concentration (in ng ml^{-1}).

Table 2
Intra- (N = 3) and inter-day accuracy and precision data for CGS 15040A quality control samples

Added concentration (ng ml^{-1})	Mean relative recovery (RSD, %) Day 1	Day 2	Day 3	Inter-day
10	104 (6.4)	102 (5.1)	110 (8.8)	105 (6.9)
25	103 (2.4)	103 (1.1)	108 (3.7)	105 (3.5)
50	106 (4.4)	107 (6.5)	119 (5.3)	111 (8.2)
100	91.8 (3.3)	100 (1.5)	113 (2.8)	102 (9.2)
200	98.3 (3.3)	104 (1.5)	109 (5.3)	104 (5.4)
350	92.9 (3.3)	100 (0.8)	104 (2.5)	99.0 (5.4)
500	97.5 (3.7)	106 (1.9)	110 (7.7)	105 (7.0)
1000	97.9 (7.2)	99.0 (1.1)	102 (3.6)	99.5 (4.3)

Overall mean recovery = 104%

Table 3
Extraction efficiency (absolute recovery, %) from plasma (N = 4)

	CGS 15040A (25 ng ml^{-1})	CGS 15040A (100 ng ml^{-1})	CGS 15040A (500 ng ml^{-1})	N-propyl ester analogue (500 ng ml^{-1})
Mean	94.5	112	96.2	93.5
SD (±)	3.4	13.2	1.3	4.4
RSD (%)	3.6	11.8	1.4	4.8

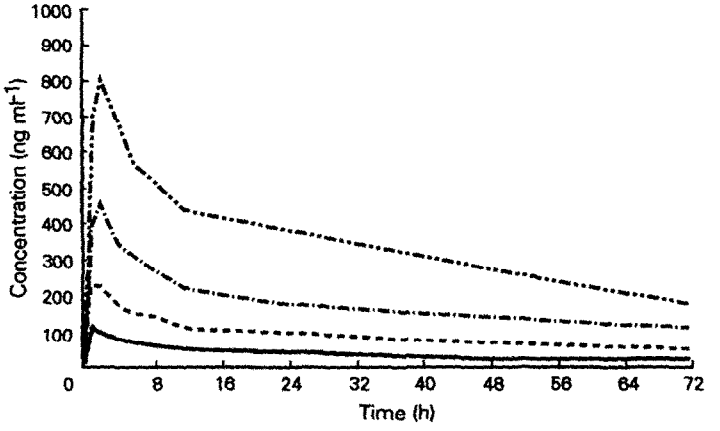

Figure 3
Mean (N = 10) plasma level profiles from healthy normal volunteers receiving single ascending oral doses of 5–40 mg CGS 15040A. ——, 5 mg; --, 10 mg; - · -, 20 mg; — -- —, 40 mg.

103%) for periods up to 2 months when stored at about −20°C. Additional samples, thawed and brought to room temperature for 1 h on each of 4 consecutive days and subsequently analysed, were also stable (relative recoveries of 90.4–98.8%).

Application of the method

The method was used in the analysis of samples from a clinical study in which normal volunteers received single ascending oral doses of 5–40 mg CGS 15040A. The mean (N = 10) drug concentration versus time profiles resulting from the analyses are depicted in Fig. 3.

Conclusions

A sensitive HPLC method has been developed for quantifying CGS 15040A concentrations in human plasma. Validation of the method over the concentration range 10–1000 ng ml^{-1} resulted in an overall mean accuracy of 104% and precision (RSD) of 3.5–9.2%. The limit of quantification was 10 ng ml^{-1}. Approximately 50 samples could be analysed daily on a routine basis. The stability of the compound in plasma under the tested conditions allows for intermediate storage prior to analysis of samples from clinical trials. The method has been successfully applied to the analysis of plasma samples from healthy volunteers' participation in a rising dose tolerance clinical trial.

[*Manuscript received for review 18 September 1990; revised version received 14 December 1990*]