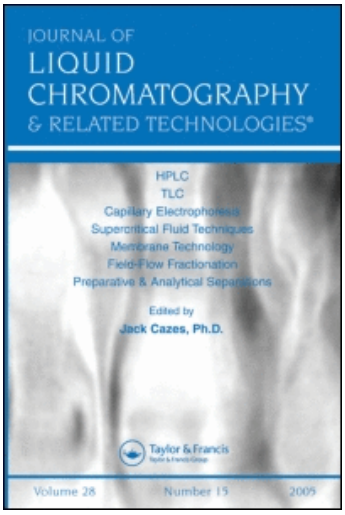

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ASSAY OF THE ANTI-INFLAMMATORY COMPOUND CGS 5391B IN BLOOD PLASMA BY AUTOMATED HPLC

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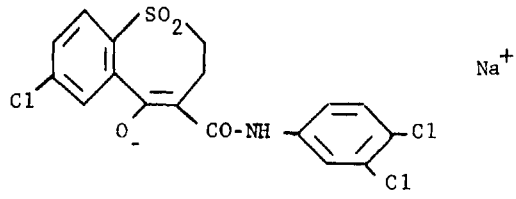
ABSTRACT

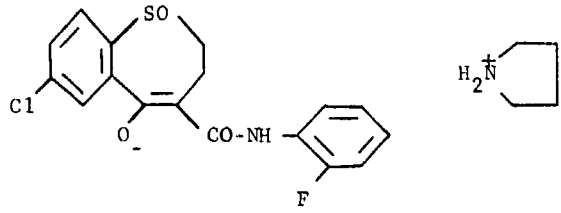
An automated HPLC method for the quantitative determination of the anti-inflammatory compound CGS 5391B in blood plasma was devised and tested. The method provides quantitation in the concentration range of 1 to 200 μg/ml of drug in plasma, with an average recovery of 96.9 ± 6.0%.

INTRODUCTION

CGS 5391B is the sodium salt of 7-chloro-N-(3,4-dichlorophenyl)-2,3,4,5-tetrahydro-5-oxo-1-benzothiepin-4-carboxamide-1,1-dioxide. The structural formula is shown in Figure 1. CGS 5391B is presently being tested for the treatment of arthritis. Reversed phase HPLC proved to be an effective means for quantitative determination of the compound in blood plasma.



CGS 5391B



CGS 5089A (Internal Standard)

FIGURE 1: Structural formulas.

MATERIALS

Chemicals and Reagents

Solutions of CGS 5391B (0.2 and 2.0 mg/ml) and of the internal standard CGS 5089A (0.25 mg/ml) were prepared in absolute ethanol and stored in a refrigerator. The pH 5 buffer used for extractions was freshly prepared each week by mixing 0.1 M citric acid and 0.1 M Na_2HPO_4 solutions in the volume ratio of 1.00 to 1.06. CGS 5089A solution was added to the extraction buffer to provide a concentration of 10 µg/ml of the internal standard. The buffer used for chromatography was 7.0 g of KH_2PO_4 per liter of water, adjusted to pH 2.9 with 85% H_3PO_4. This buffer was

mixed with glass distilled acetonitrile (Burdick and Jackson Laboratories Inc.) in the volume ratio of 35:65 and passed through an 0.5 μ filter (Millipore FH) which was prewetted with acetonitrile. Glass distilled chloroform (Burdick and Jackson Laboratories Inc.), HPLC grade methanol (Fisher Scientific Co.) and house distilled water were used.

Chromatograph

The chromatography system consisted of a Waters Associates Model 710A Intelligent Sample Processor, Model 6000A Solvent Delivery System, Model 440, 254 mμ Absorbance Detector, and a Hewlett-Packard Model 3385A Automation System for plotting and integration. A Whatman pre-column filled with Du Pont Permaphase packing and a Du Pont 250 x 4.6 mm Zorbax 10 μ C8 column were used. Instrument paramenters were: sample processor - 15 μl injection volume, run time 6 minutes; pump - flow rate 2.0 ml/min; detector - sensitivity 0.005 AUFS; integrator - chart speed 1.00 cm/min, zero 10% of scale, attenuation 2 to the third power, slope sensitivity zero, area reject 99980 changed to 10 at 1.8 min, stop at 7.0 min.

METHODS

An aliquot (0.5 ml) of each plasma for analysis and six to eight aliquots of control plasma for calibration standards were pipetted into 40 ml screw-cap centrifuge tubes. CGS 5391B stock solutions were added to the calibration standard tubes. One ml of extraction buffer containing internal standard was added to each plasma, followed by 10 ml of chloroform. The tubes were

closed with teflon-lined screw caps and rotated 15 minutes at 15 RPM. The phases were separated by centrifugation for 10 minutes at 1300-1400 RPM.

The upper, aqueous layer and associated solids were removed by aspiration. The chloroform extracts (ca 9 ml) were transferred into 12 ml centrifuge tubes and evaporated to dryness under nitrogen, with a bath temperature of 45-55° C. To each tube was added 2.0 ml of acetonitrile and the tubes were vortexed for 5 seconds. To allow complete dissolution of the drug, residues from human and monkey plasma required storage for 1.5 hours at room temperature, or overnight in a freezer before further processing. A different procedure was necessary for rat plasma. Residues from rat plasma dissolved readily but the accompanying CGS 5391B was subject to decomposition. The reasons for the observed differences are not known. Reconstituted residues from rat plasma were either chromatographed immediately or stored in a freezer and then chromatographed with a maximum interval of two hours at room temperature.

The ratio of peak areas for CGS 5391B to internal standard were calculated and linear least squares curve fitting was carried out with the calibration data. Concentration values for the unknowns were then calculated from the slope of the regression line.

The composition of the HPLC peak corresponding to CGS 5391B in the plasma of dosed patients was examined. A Waters Associates C-18 Sep-Pak Cartridge was washed by passing through in succession 3 x 5 ml of acetonitrile, 3 x 5 ml of methanol and 3 x 5 ml of water. The cartridge was allowed to drain between portions. Approximately 100 ml of chromatograph eluate containing about 10 µg

of apparent CGS 5391B was obtained by repeated collections. The eluate was diluted three fold with water and passed through the washed Sep-Pak cartridge, which was then rinsed with 3 x 5 ml of water. Retained materials were then eluted with 2 ml of methanol. The Sep-Pak eluate was compared with authentic drug by HPLC in three chromatographic systems: (1) methanol/water (60:40) on Applied Science Laboratories Inc. Lichrosorb C-18; (2) methanol/water/acetic acid (80:20:1) on Waters Associates μ Bondapak Phenyl; and (3) methylene chloride on E. Merck Lichrosorb Si 60.

RESULTS AND DISCUSSION

CGS 5391B and the internal standard CGS 5089A are chemically related (Figure 1) but are sufficiently different in structure to permit good resolution by reversed phase HPLC. A representative chromatogram showing distinct, well-resolved peaks for the two compounds is shown in Figure 2. Extracts of blank plasma showed no significant interference, and chromatograms of samples from dosed humans and animals were similar to that shown in Figure 2.

Six to eight calibration standards were prepared on each analysis day to bracket and thoroughly cover the expected range of unknowns (Figure 3). The slopes of regression lines obtained on different occasions were in good agreement, intercept values were insignificant and the correlation coefficients demonstrated good definition of the calibration curves (Table 1).

The assay procedure was further tested by blind analysis of human plasma samples to which 1.2 to 75.0 μg/ml of CGS 5391B had been added. Spiked plasma of each concentration was divided into

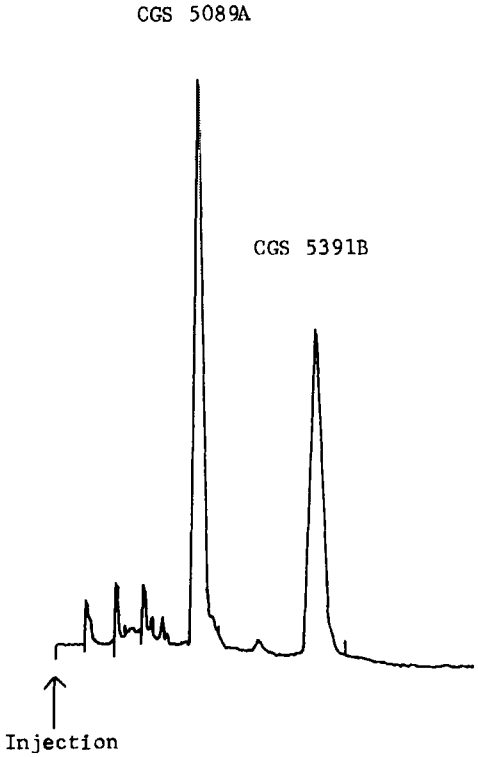


FIGURE 2: Chromatogram of an extract of monkey plasma containing 10 μg/ml of CGS 5391B.

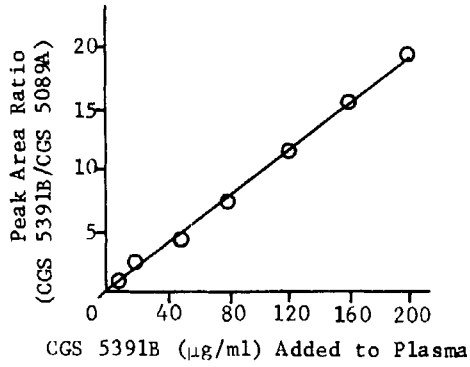


FIGURE 3: A typical standard curve for the assay of CGS 5391B in human plasma.

TABLE 1

CGS 5391B Assay Calibration Data

Plasma	Number of Analysis Occasions	Average ± Relative Standard Deviation		
		Slope	Intercept	Correlation Coefficient
Human	13	0.0946 ± 9	-0.08 ± 86	0.9988 ± 0.1
Rat	10	0.0934 ± 4	-0.12 ± 69	0.9986 ± 0.1
Cynomolgus monkey	4	0.0817 ± 6	0.06 ± 41	0.9994 ± 0.0

aliquots for replicate analyses to allow assessment of precision as well as accuracy. The resulting samples were number coded in random sequence and frozen to await analysis. The concentrations were made known to the analyst only after the assay results were recorded. Similar experiments were done on three occasions and the results demonstrated good accuracy and good precision (Table 2).

The identity and homogeneity of the CGS 5391B HPLC peak from plasma of dosed patients was established by comparing the collected peak material to authentic compound by HPLC in three systems of diverse nature, as described previously. The recovered material gave a single peak corresponding in retention to authentic CGS 5391B in all systems.

The validation studies showed that the assay provides sufficient accuracy and precision for pharmacokinetic studies of CGS 5391B in plasma. The blind validation data indicated satisfactory performance in the 1 to 75 μg/ml concentration range, while the

TABLE 2

Percent Recoveries for Blind Analysis of Human Plasma Spiked with CGS 5391B

CGS 5391B Added (μg/ml)	Number of Analyses	Average Percent Recovery ± Relative Standard Deviation
1.2	3	102.7 ± 4.5
1.8	3	100.0 ± 6.0
3.0	3	99.0 ± 5.6
4.4	3	95.0 ± 3.8
5.0	3	94.7 ± 3.2
6.0	9	92.6 ± 5.1
17.5	3	87.3 ± 2.4
18.0	3	99.7 ± 6.4
55.0	3	91.3 ± 1.3
75.0	5	107.0 ± 1.9
	38	96.9 ± 6.0

linearity and precision of calibration curves showed the method performed well up to 200 μg/ml. The use of an automatic sample injector and electronic integrator proved very convenient because the chromatography required only infrequent attention, and the preparation of additional samples could be done concurrently with operation of the chromatograph.