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Note

High-performance liquid chromatographic method for a clozapine analogue, CGS 13429, and its N-oxide and desmethyl metabolites

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CGS 13429 (compound I, Fig 1, 2-methyl-5-(4-methyl-1-piperazinyl)-11H-[1,2,4]-triazolo-[1,5,-C][1,3]-benzodiazepine) is a clozapine analogue currently under development as an antipsychotic drug [1] A recent manuscript [2] presenting a gas chromatographic method for clozapine indicated the limited number of published analytical methods for this compound Moreover, this paper described a method unlikely to detect metabolism of compound I Therefore, in anticipation of potential demethylation or N-oxidation of piperazine (compounds II and III, respectively, Fig 1), a high-performance liquid chromatographic (HPLC)method was developed for preclinical pharmacokinetic and bioavailability studies to quantify all three analogues in rat and dog plasma

EXPERIMENTAL

Chemicals

Methanol and distilled water were of High Purity Solvent brand (HPLC grade) manufactured by American Burdock and Jackson (Muskegon, MI, U S A) Triethylamine (HPLC grade) and glacial acetic acid were obtained from Fisher Scientific (Springfield, NJ, U S A) 1-Heptanesulfonic acid, sodium salt, was purchased from Sigma (St Louis, MO, U S A) All other reagents were of Baker Analyzed Reagent grade and were purchased from American Scientific Products (McGaw Park, IL, U S A)

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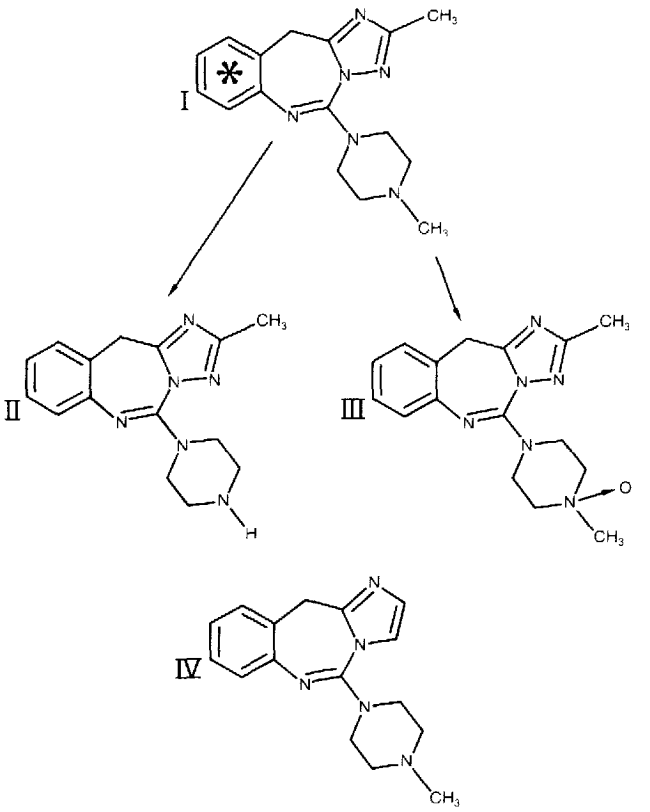

Fig 1 Structures of compounds I (CGS 13429), II (desmethyl metabolite), III (N-oxide metabolite) and IV (internal standard) Compounds I and II were provided as maleate salts The asterisk indicates the ring where hydroxylation is thought to occur

Standards and solutions

The structures of CGS 13429 (I), two metabolites (II and III) and the internal standard, CGS 11760 (IV), are shown in Fig. 1 The maleate salt of both I and the desmethyl compound ('A' salts) were used for all studies

Stock solutions of I and its desmethyl (CGS 19649A, II) and N-oxide (CGS 22996, III) metabolites were prepared in a mixture of methanol–50 m*M* phosphate (pH 7 0) (50 50, v/v) Specific volumes of the stocks were combined in such a way that only 10 μl of each dilution were added to prepare each plasma standard.

A stock solution of the internal standard, prepared at 1 mg per 10 ml water, was diluted ten-fold to obtain a 10 ng/μl working solution

Quality control samples containing all three compounds were prepared at 150 and 2500 ng/ml of dog plasma Portions of 200 μl were pipetted into 1 5-ml polypropylene tubes and frozen pending analysis.

High-performance liquid chromatography

Chromatography was controlled through a Waters Assoc (Milford, MA, U S A) 840 data station, components included a Waters Model 710B WISP automated injector and two Model 590 pumps Effluent was monitored at 280 nm with a Kratos (American Bioanalytical, Ramsey, NJ, U S A) Model 783 variable-wavelength detector Separations were performed at ambient temperature on a Supelcosil (Supelco, Bellefonte, PA, U S A) LC-8-DB column (15 cm×4 6 mm I D , 5 μm particle size) preceded by an on-line 0 5-μm particle filter and a Waters Guardpak module with a C_{18} insert

Mobile phase solvents were water and methanol, each containing 5 m*M* sodium heptanesulfonate, 0 5% glacial acetic acid and 0 5% triethylamine (pH 4) Both solutions were filtered through a 0 02-μm Anodisc (Alltech Assoc , Deerfield, IL, U S A) Each was then degassed by stirring under house vacuum Two pumps were programmed to deliver a total of 1 0 ml/min of mobile phase After 5 min at 30% methanol, a linear gradient increased the proportion of methanol to 55% over the next 15 min This was held for 2 min, then returned to 30% with a re-equilibration period of 8 min

Assay procedure

Standard curves were prepared each analysis day by addition of 10 μl of the appropriate mixture of I, II and III to 200 μl blank dog plasma such that the concentrations ranged from 100 to 5000 ng/ml All standards and test samples received 10 μl of a solution of IV (10 ng/μl) as the internal standard

Solid-phase extraction was performed with 500-mg C_{18} Bond Elut columns (Analytichem International, Harbor City, CA, U S A) pre-rinsed with methanol, water and buffer (0 2 *M* sodium dibasic phosphate, pH 9 5) Plasma samples were placed on the column followed by addition of 2 5 ml of the buffer The columns were washed with 2×2 5 ml water followed by 2 5 ml of 20% methanol–water Analytes were eluted with 2×1 0 ml methanol The methanol was evaporated with a gentle stream of nitrogen in a water bath at 37°C The dry residues were reconstituted in 100 μl of a 50 50 mixture of mobile phase components All samples were filtered through mobile phase-washed 0 2-μm PTFE syringe filters (ACRO LC-13, Gelman Sciences, Ann Arbor, MI, U S A) and placed in 1 0-dram vials fitted with limited-volume inserts for chromatography Usually 75 μl were injected onto the column

Data manipulation

Raw data from chromatograms were tabulated by keyboard entry onto a VAX computer Calculations and preparation of tables and graphs were performed using the Research System/1 (RS/1, Bolt, Beranek and Newman, Cambridge, MA, U S A) software package on a VAX 8800 computer with a VMS operating system (Digital Equipment Corporation, Merrimack, NH, U S A)

The peak-height ratios of analytes to internal standard were plotted versus their known concentrations Linear regression analyses were performed on the calibration standards, the resulting slopes and y-intercepts were used to obtain concentration values for that day's validation samples

RESULTS

The HPLC method described above was found to give a linear response for peak-height ratio of drug or metabolite to internal standard in the 100–5000 ng/ml concentration range Chromatograms of pre- and post-dose (50 mg/kg orally) dog plasma extracts are shown in Fig 2

Standard curves from extracted dog plasma (200 μl), with I, II and III concentrations ranging from 100 to 5000 ng/ml, had slopes with coefficients of variation of 1 8, 2 4 and 8 6%, respectively, during a two-week test (Table I) Intra-day and inter-day accuracy and precision of quality control samples (Table II) were acceptable, but decreased recovery was observed for the 2500 ng/ml samples and there was greater variation in the recovery of II Limit of de-

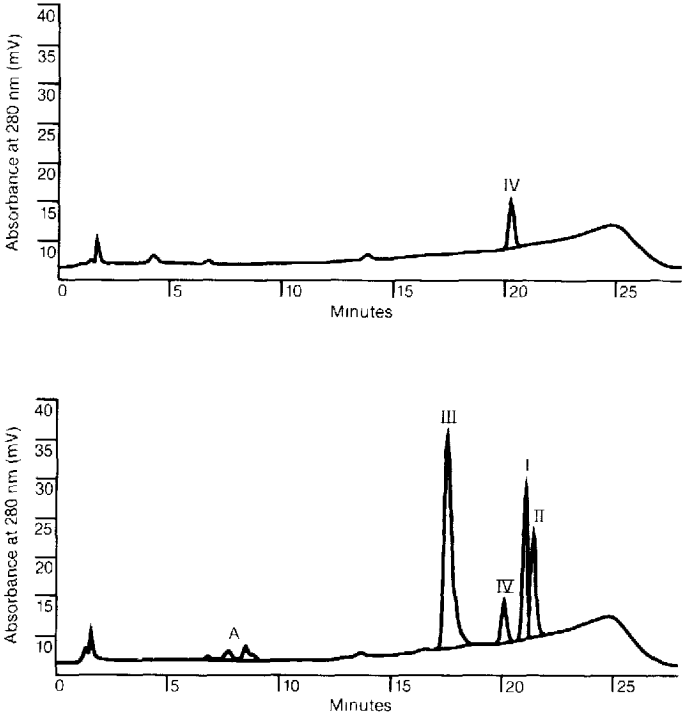

Fig 2 Chromatograms of dog plasma extracts (Top) Pre-dose with added internal standard, (bottom) 5 h after 50 mg/kg oral dose of compound I Peak A = unidentified metabolites (hydroxylated forms?), remaining compounds are identified by reference to Fig 1

TABLE I

SLOPES ($\times 10^3$) AND COEFFICIENTS OF DETERMINATION OF STANDARD CURVES USED FOR METHOD VALIDATION

Analysis day	I		II		III	
	Slope	r^2	Slope	r^2	Slope	r^2
1	1 812	0 999	1 483	0 995	1 140	0 999
2	1 771	0 999	1 734	0 999	1 132	0 999
3	1 828	0 999	1 526	0 999	1 175	0 999
4	1 808	0 999	1 770	0 998	1 145	0 999
7	1 865	0 999	1 821	0 998	1 207	0 997
8	1 849	0 999	1 794	0 999	1 153	0 999
Mean	1 822	0 999	1 688	0 998	1 159	0 999
S D	0 033	0 000	0 145	0 002	0 028	0 001
Coefficient of variation (%)	1 8		8 6		2 4	

TABLE II

SUMMARY OF QUALITY CONTROL DATA FOR ANALYSIS OF I, II AND III IN DOG PLASMA

Concentration added[a] (ng/ml)	n	I		II		III	
		C V[b] (%)	Accuracy[c] (%)	C V (%)	Accuracy (%)	C V (%)	Accuracy (%)
150	3	1 0	97	4 0	123	3 6	109
150	3	0 8	91	5 7	113	1 7	108
150	3	2 6	86	12 8	75	2 0	119
150	3	1 2	106	0 1	134	4 3	117
150	3	3 8	112	2 1	125	3 1	138
150	3	2 9	90	1 8	109	0 8	106
Inter-day	18	9 7	97	17 6	113	11 7	114
2500	3	2 6	93	4 9	84	3 1	93
2500	3	1 0	94	2 6	92	2 5	96
2500	3	3 4	90	1 6	82	3 6	85
2500	3	2 8	92	2 7	91	3 4	92
2500	3	1 7	87	2 0	85	0 7	86
2500	3	1 5	88	1 5	87	2 4	90
Inter-day	18	3 6	91	4 7	87	5 0	90

[a]Quality control samples containing compounds I, II and III analyzed over a two-week period
[b]Percentage coefficient of variation of each analysis
[c]Percentage accuracy for the specific compound (found/added $\times$ 100%)

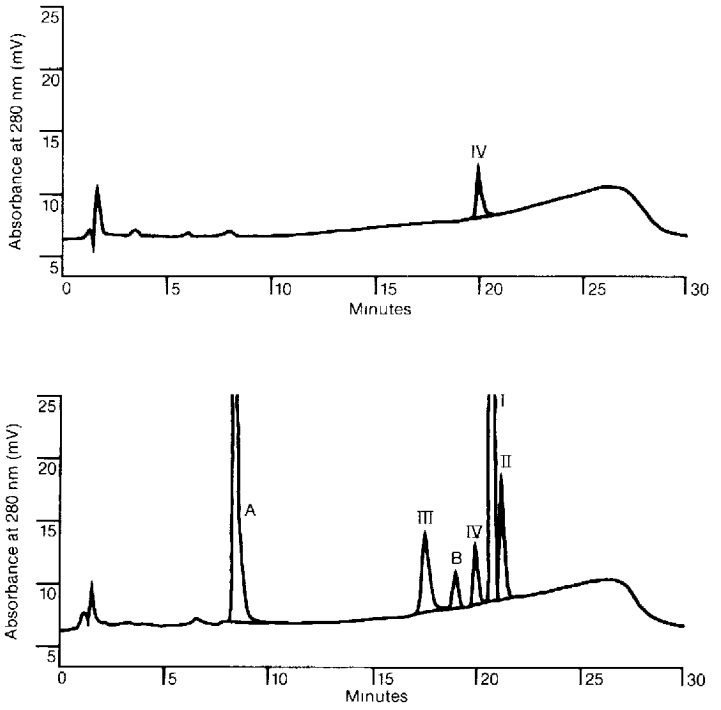

Fig 3 Chromatograms of rat plasma extracts (Top) Pre-dose with added internal standard, (bottom) 2 h after a 50 mg/kg oral dose of compound I Peak A = unidentified metabolites (hydroxylated forms?), peak B = unidentified metabolite, remaining compounds are identified by reference to Fig 1

tection (three times the signal-to-noise ratio) was approximately 40 ng/ml using 200-μl extracts

Dogs and rats were given an oral dose of 50 mg/kg I Blood samples were obtained at various times to determine applicability of the method In addition to the parent compound peak, post-dose chromatograms from dog (Fig 2) and rat (Fig 3) plasma extracts showed at least four more peaks not observed in control plasma Retention times of two of the peaks corresponded to those of analogues II and III Another peak of similar polarity was observed in rat plasma only, but levels appeared to be very low Additionally, more polar material, comprised of at least two components, was observed in relatively larger amounts in the rat than the dog

DISCUSSION

The HPLC method described herein had a limit of detection of 40 ng/ml for the determination of compounds I, II and III in 200 μl rat or dog plasma Inter-

and intra-day analytical variability was acceptable for pharmacokinetic analyses (Tables I and II) Extraction of all three components was found to be extremely pH-sensitive, since plasma pH below 8 led to no recovery of II and reduced recovery of the other two analytes

The observed metabolism of I in the rat was rather extensive Three drug-related peaks eluted near the parent compound on HPLC analysis Two of these coeluted with analogues II and III, while the third was unidentified More polar material eluting as a heterogeneous peak (Fig 3, bottom, peak A) was also not identified However, preliminary studies suggest that this peak may represent one or more ring-hydroxylated forms of I, II or III (see legend to Fig 1) Published results [3] on a structurally similar compound is consistent with this hypothesis

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