Journal of Chromatography, 578 (1992) 77–83
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6364

Automated-extraction, high-performance liquid chromatographic method and pharmacokinetics in rats of a highly A_2-selective adenosine agonist, CGS 21680

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(First received January 2nd, 1992; revised manuscript received March 3rd, 1992)

ABSTRACT

CGS 21680 (2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine, I) is a highly A_2-selective (A_2/A_1 = 140), high-affinity adenosine agonist. A method has been devised to extract the compound from biological matrices with automated solid-phase extraction using C_{18} bonded silica columns. This is followed by reversed-phase, paired-ion chromatography on a Supelco LC-18-S column with fluorescence detection. The limit of quantitation is 5 ng/ml, but 1 ng/ml (five times the signal-to-noise ratio) can readily be detected. Tritium-labeled compound was used to study the pharmacokinetics in rats. After an intravenous dose of 0.3 mg/kg, biphasic elimination kinetics were observed for parent I, characterized by half-lives of 1.8 min (distribution) and 15 min (elimination). The volume of distribution in the terminal phase (V_β) was low (0.27 l/kg) and plasma clearance was moderate (0.83 l/kg/h). Although the compound was rapidly absorbed (mean T_{max} = 13 min), low concentrations (mean C_{max} = 94 ng/ml) were observed after an oral dose of 3.0 mg/kg, and bioavailability was only approximately 1.4%. Radioactivity persisted in plasma longer than parent compound after either dose, but levels were too low for isolation and structure identification of drug-derived compounds.

INTRODUCTION

CGS 21680 (2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine, I, Fig. 1) possesses high affinity (K_b = 22 n*M*) for brain adenosine A_2 receptors. Selectivity for A_2 *versus* A_1 binding is excellent (A_2/A_1 = 140) [1]. The compound has been observed to have potent hypotensive activity when administered to spontaneously hypertensive rats [2]. Tritium-labeled I has been shown to directly label rat brain A_2 receptors [3] and has been used to determine their localization [4]. Additional studies have indicated

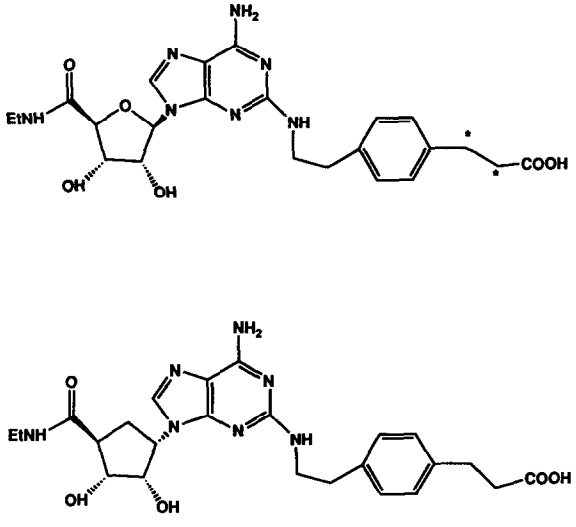

Fig. 1. Structures of I (top) and II (bottom). The asterisks show the position of the tritium label in compound I.

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0378-4347/92/$05.00

that the drug binds preferentially to the A_{2a} subclass of receptors in rat striatum [5]. A sensitive analytical method has been developed to assay I at levels in the range of its receptor affinity (22 n*M* = 11 ng/ml), and this method was utilized to determine intravenous pharmacokinetics and oral bioavailability in rats. Preliminary results of this work have been presented in abstract form [6].

EXPERIMENTAL

Materials

Methanol was of high-purity solvent brand (HPLC grade) and was obtained from American Burdock and Jackson (Muskegon, MI, USA). Ultrapure water was drawn from a Milli-Q system (Millipore, Bedford, MA, USA). Triethylamine (HPLC grade), glacial acetic acid and 1-pentanesulfonic acid sodium salt were purchased from Fisher Scientific (Springfield, NJ, USA). All other reagents were of Baker analyzed reagent grade and were purchased form Baxter Healthcare (Edison, NJ, USA). Unlabeled I and CGS 22484 (HPLC internal standard, compound II, Fig. 1) were obtained from the Chemistry Department, Pharmaceuticals Division, CIBA-GEIGY (Summit, NJ, USA). [^{3}H]I was purchased from NEN Research Products (Boston, MA, USA) and had a specific activity of 48.1 Ci/mmol.

High-performance liquid chromatography

The analytical column used was obtained from Supelco (Bellefonte, PA, USA) and was an LC-18-S, 5 μm particle size, 150 mm × 4.6 mm I.D. column. The chromatography system consisted of Waters Assoc. (Milford, MA, USA) components (two Model 590 pumps, a WISP 710B autosampler and a temperature control module, with control and data analysis with an 840 chromatography station). A Kratos (ABI, Foster City, CA, USA) Model 980 fluorescence detector was utilized for detection with excitation set at 222 nm and emission monitored at >345 nm. The flow-rate was maintained at 1.0 ml/min at a column temperature of 35°C.

Mobile phase solvents were separate solutions of water and methanol, each containing 5 m*M* pentanesulfonic acid sodium salt, 0.2% glacial acetic acid and 0.5% triethylamine (pH 5.6). Each solution was filtered through a 0.02-μm Anodisc 47 filter (Alltech Assoc., Deerfield, IL, USA) and vacuum-degassed each day of analysis. A linear 10-min gradient was used for compound analysis, beginning with 25% methanol and ending at 50% methanol. Return to starting conditions and re-equilibration was accomplished over an additional 7 min.

Standards and quality control samples

A stock solution of CGS 21680C (I, sodium salt) was prepared at a concentration of 0.1 mg/ml in water, then diluted with water to solutions in the concentration range 0.1–10 μg/ml. A stock solution of II was prepared at 0.1 mg/ml in methanol, then diluted with water to a concentration of 200 ng/ml for use as the HPLC internal standard. Kept refrigerated, these solutions remained stable for more than one month.

Quality control samples were prepared in plasma at 15 ng/ml by the addition of 60 μl of the 1.0 μg/ml stock of I to 4 ml of plasma and at 300 ng/ml by addition of 120 μl of a 10 μg/ml stock solution of I to 4 ml of plasma. Samples of 200 μl were pipetted into 75 mm × 10 mm borosilicate glass tubes and stored frozen at −20°C pending analysis.

Sample preparation and extraction

A calibration curve was prepared for each day of analysis by the addition of 10 μl of the appropriate stock solutions of I to 200-μl portions of rat control plasma in 75 mm × 10 mm glass tubes. Using 200 μl of plasma, the linear range of the calibration curve was from 5 to 400 ng/ml. Test samples of 200 μl were likewise added to glass tubes. The standards, quality control samples and test samples were then put onto a 96-place rack for automated solid-phase extraction using a Waters MilliLab workstation.

Briefly, Waters Sep-Pak Light C_{18} cartridges (120 mg solid-phase material) were chosen for the extraction. These were preconditioned with 3.0

ml of methanol, followed by 5.0 ml of water. All samples had 1.0 ml of water added which contained 200 ng of II. The plasma samples were loaded onto the cartridges, which were then washed consecutively with 3.0 ml of water and 3.0 ml of 10% methanol–water. Air was blown through the cartridges for approximately 15 s. I and II were then eluted into glass tubes with 2.0 ml of methanol. The eluates were filtered through 0.22-μm GV13 PVDF filters (Millipore) into separate glass tubes, which were then manually removed from the apparatus and dried on an N-EVAP (Organomation Assoc., South Berlin, MA, USA) with a gently stream of dry nitrogen at 40°C. All samples were resuspended in 100 μl of the aqueous portion of the mobile phase, and 75 μl were injected for analysis.

Animal studies

Male Sprague–Dawley (CRL, CDBR) rats (240–330 g) were obtained from Charles River Labs. (Wilmington, MA, USA). The animals were anesthetized with a 0.1 mg/kg intraperitoneal injection of ketamine–acepromazine (10:1, v/v). The carotid artery was cannulated for oral studies and both the carotid artery and jugular vein were cannulated for the intravenous studies. Cannulas were externalized to the back of the neck between the scapulae and were secured with an anchor button attached to a spring tether and swivel (Instech Labs., Horsham, PA, USA). After the rats were fully recovered, a pre-dose blood sample was obtained.

Three rats received an intravenous dose of 0.3 mg/kg [^{3}H]I (specific activity reduced to 6.4 μCi/mmol with unlabeled drug) given through the jugular cannula at a concentration of 0.3 mg/ml (1.0 ml/kg) in phosphate-buffered saline, pH 7.4 (PBS). Oral doses of 3.0 mg/kg (specific activity 24 μCi/mmol) were administered by gavage to three additional rats at a concentration of 1.0 mg/ml (3.0 ml/kg) in PBS. Blood samples (0.5 ml) were obtained through the carotid artery cannulas at appropriate times after each dose. An equal volume of whole blood from a littermate, diluted 1:1 (v/v) with sterile 0.9% sodium chloride, was infused through the cannula after each sample during the first day of testing. Cannulas were cleared with approximately 100 μl of heparinized saline.

Blood was pipetted into 1.5-ml lithium–heparin pre-coated polypropylene centrifuge tubes (Brinkman Instruments, Westbury, NY, USA), which were immediately centrifuged at 2000 *g*. Plasma (200 μl) was transferred to 75 mm × 10 mm borosilicate glass tubes and frozen at −20°C pending extraction and analysis as above. A 50-μl sample of plasma was also added to 10 ml of Ultima Gold scintillation cocktail (Packard Instrument, Downers Grove, IL, USA) for liquid scintillation counting on a Packard Tri-Carb 4530.

Data analysis

Concentrations of I in test samples were determined automatically by the Waters 840 chromatography workstation, using the calibration line generated on the day of analysis from the peak-height ratios of I/II. Pharmacokinetic parameters were calculated from the data as follows: the apparent rate constants (α and β) for elimination of I from plasma after the intravenous dose were determined for individual rats by iterative computer fit to a two-compartment model described by an equation of the form $F(t) = Ae^{-\alpha t} + Be^{-\beta t}$ where A and B are the extrapolated initial concentrations in the two compartments. The distribution and elimination half-lives are equal to 0.693 divided by α or β, the respective rate constants. The area under the plasma concentration *versus* time curve (AUC) after the intravenous dose was calculated using the expression AUC = $A/\alpha + B/\beta$ and was estimated for the oral doses utilizing the trapezoidal rule as follows:

$$\mathrm{AUC} = \sum_{0}^{i} [0.5(C_i + C_{i+1})(t_{i+1} - t_i)] + C_{i+1}/\beta$$

This is the sum of the areas of successive trapezoids from t_i to t_{i+1}, with the final time $i + 1$ set to the last detectable plasma concentration of I and the terminal portion estimated using the mean terminal elimination rate determined in the intravenous studies. The area under the moment curve (AUMC) was calculated for the intravenous dose with a similar procedure, substituting

the time-weighted concentration in the above formula. The mean residence time (MRT) was calculated by dividing the AUMC by the AUC. Total plasma clearance (Cl_p) was determined by dividing the intravenous dose by the AUC. The volume of distribution during the terminal phase (V_β) was calculated by dividing the Cl_p by β.

C_{max} is the maximum concentration observed after an oral dose, and this occurs at time T_{max}. The percent bioavailability (BA) after an oral dose is the ratio of the AUCs of equivalent oral (p.o.) and intravenous (i.v.) doses times 100%, or

$$BA = \frac{AUC\ [p.o.\ dose]}{AUC\ [i.v.\ dose]} \times \frac{[i.v.\ dose]}{[p.o.\ dose]} \times 100\%$$

The mean AUC of the intravenous dose was used in these calculations.

RESULTS

Method validation

Method validation commenced prior to analysis of study samples and was continued through study sample analysis to confirm extended reproducibility of the techniques. The results of the analyses of each individual point on the calibration curve are shown in Table I, with values expressed as the mean ± S.D. peak-height ratios of I/II. The coefficients of variation (C.V.) were all below 5.6% in the range 5–400 ng/ml, and as little as 1.0 ng/ml (five times the signal-to-noise ratio) could be detected using 200 μl of plasma. Studies using the radioactive compound showed >90% recovery throughout the range of this analysis. Using as little as 10 μl or as much as 1.0 ml of plasma, the calibration curve could be expanded to a range of 0.5 ng/ml to 8 μg/ml with-

TABLE I

MEAN PEAK-HEIGHT RATIOS FOR THE STANDARDS USED TO GENERATE THE DAILY CALIBRATION CURVE FOR THE ANALYSIS OF COMPOUND I IN RAT PLASMA

Calibration curves were conducted on six separate days during a 25-day period.

Concentration of I (ng/ml)	Response[a] (mean ± S.D.)	C.V. (%)
5	0.065 ± 0.004	5.6
25	0.256 ± 0.008	3.3
50	0.493 ± 0.010	1.9
125	1.197 ± 0.024	2.0
250	2.354 ± 0.073	3.1
400	3.999 ± 0.042	1.1
Slope (× 100) (mean ± S.D.)	0.986 ± 0.010	1.0
r^2 (mean ± S.D.)	0.9985 ± 0.0019	0.2

[a] Response is the peak-height ratio of compound I/II.

TABLE II

ANALYSIS OF QUALITY CONTROL SAMPLES OF COMPOUND I

Analysis day	Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	C.V.[a] (%)	Accuracy (%)	Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	C.V.[a] (%)	Accuracy (%)
1	15	15.8 ± 0.4	2.5	105.3	300	285.9 ± 8.6	3.0	95.3
3	15	17.9 ± 1.5	8.4	119.3	300	288.0 ± 10.7	3.7	96.0
4	15	16.8 ± 1.9	11.3	112.0	300	272.1 ± 4.6	1.7	90.7
5	15	18.2 ± 0.8	4.4	121.3	300	277.9 ± 9.1	3.3	92.6
14	15	16.2 ± 1.7	10.5	108.0	300	281.1 ± 2.3	0.8	93.7
25[b]	15	13.2	–	87.4	300	280.4	–	93.5
Interday (n = 17)	15	16.5 ± 1.9	11.4	110.2	300	280.9 ± 8.6	3.1	93.6

[a] Coefficient of variation (n = 3, except where indicated).

[b] Mean of two samples.

out a substantial increase in method variability. Table I also shows the mean slope and correlation coefficient of the calibration curves generated from the data and indicates the high degree of reproducibility and linearity of the method.

Table II shows the results from analysis of quality control samples prepared as a pool on the first day of analysis. Intra-day and inter-day coefficients of variation and accuracy were well within acceptable limits. Repeat analysis of plasma standards and test samples after up to four months of freezer storage were determined to be within 10% of the original values.

The method could also be used for the analysis of I in tissues (*e.g.*, brain, lung, heart and liver) by simply prefiltering a 1:8 (w/v) homogenate through a 5-μm filter prior to the extraction described above. Although not fully validated, standard curve slopes were similar, no substantial interference was observed, and [^{3}H]I added to control tissue was quantitatively recovered.

Pharmacokinetics and bioavailability in rats

Fig. 2 shows representative chromatograms from a 5 ng/ml standard (top), a pre-dose plasma sample (middle) and a sample obtained at 60 min after an oral dose of 3.0 mg/kg [^{3}H]I with a calculated concentration of 128 ng/ml. Standard retention times in this system were 11.3 and 11.8 min for compounds I and II, respectively. The lack of interference in the pre-dose sample and the ready detectability of 5 ng/ml (0.75 ng on column) are clearly evident.

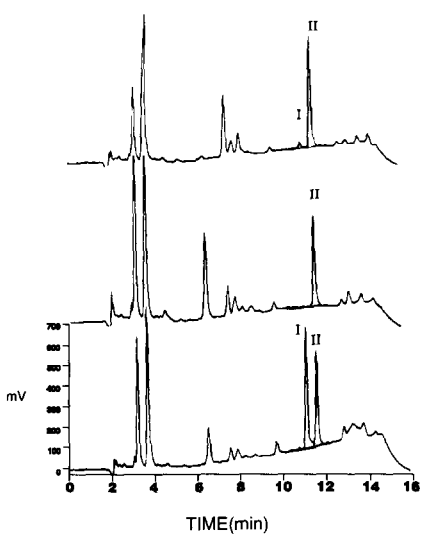

Fig. 2. Representative chromatograms showing a plasma standard (top) with a concentration of 5 ng/ml I, a pre-dose plasma extract (middle) and an extract of plasma obtained 60 min after an oral dose of 3.0 mg/kg [^{3}H]I, with a calculated concentration of 128 ng/ml.

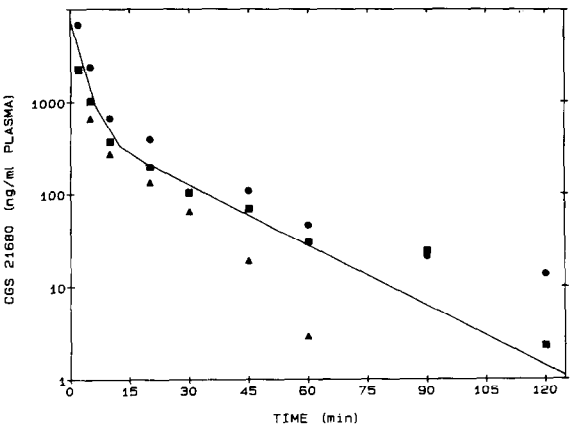

Fig. 3. Plasma concentrations of I in three rats administered an intravenous dose of 0.3 mg/kg [^{3}H]I. The curve shown is the biexponential fit derived from the mean data determined for individual animals (Table III).

Fig. 3 shows the individual data for rats administered an intravenous dose of 0.3 mg/kg [^{3}H]I and the biexponential curve fit derived from the mean values calculated for individual rats, as summarized in Table III. Distribution occurred

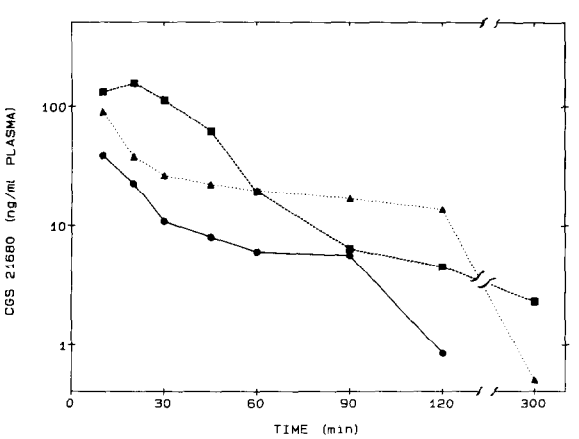

Fig. 4. Plasma concentrations of I in three rats administered an oral dose of 3.0 mg/kg [^{3}H]I.

TABLE III

PHARMACOKINETIC PARAMETERS CALCULATED FOR A 0.3 mg/kg INTRAVENOUS DOSE AND FOR A 3.0 mg/kg ORAL DOSE OF [^{3}H]I

Rat No.	A (ng/ml)	$k(\alpha)$ (min^{-1})	$t_{\frac{1}{2}\alpha}$ (min)	B (ng/ml)	$k(\beta)$ (min^{-1})	$t_{\frac{1}{2}\beta}$ (min)	AUC (h · ng/ml)	MRT (min)	Cl_p (l/kg/h)	V_β (l/kg)
0.3 mg/kg intravenous										
1	3000	0.500	2.1	574	0.073	9	231	7	1.30	0.30
2	3767	0.334	1.7	371	0.038	18	350	14	0.86	0.37
3	13713	0.410	1.7	729	0.039	18	873	20	0.34	0.15
Mean	6827	0.415	1.8	558	0.050	15	485	11	0.83	0.27
S.D.	5976	0.083	0.2	180	0.020	5	342	4	0.48	0.11

Rat No.	T_{max} (min)	C_{max} (ng/ml)	AUC (h · ng/ml)	BA (%)
3.0 mg/kg oral				
4	10	39	20	0.4
5	20	154	108	2.2
6	10	89	73	1.5
Mean	13	94	67	1.4
S.D.	6	58	44	0.9

rapidly and was characterized by a half-life of 1.8 min. Elimination was also rapid, with a half-life of 15 min. Total plasma clearance was moderate (0.83 l/kg/h) and MRT was only 11 min. V_β was 0.27 l/kg, suggesting little distribution out of extracellular water. Parent compound was not detected in plasma after 120 min. Total drug-derived material (radioactivity) in plasma was slightly higher than concentrations of I and generally paralleled that of parent compound through 20 min post-dose. Thereafter, concentrations of total radioactivity and parent compound diverged. While parent compound was not detected after 120 min post-dose, radioactivity at 3–40 times background levels persisted for up to 24 h.

Fig. 4 shows the plasma concentration–time profile for three rats administered an oral dose of 3.0 mg/kg [^{3}H]I. Although absorption was rapid (T_{max} by 10–20 min), C_{max} averaged less than 100 ng/ml (Table III). BA was low and variable (1.4 ± 0.9%). As was observed after the intravenous dose, total drug-derived material in plasma at early times post-dose approximated the concentrations of I, but radioactivity at 3–10 times background persisted for up to 48 h, while parent compound was not detected at 10 h after administration.

DISCUSSION

Compound I was chosen from over 200 adenosine derivatives because of its high affinity and selectivity for the adenosine A_2 receptor. It is a potent hypotensive [1,2] and vasorelaxing [7] agent. The extraction and HPLC method described in this report is useful for the analysis of I at concentrations approximating the binding constant (22 nM or 11 ng/ml) of the compound. Using 200 μl of rat plasma, the linear range of quantitation of the method was 5–400 ng/ml; however, this could easily be expanded to 0.5–8000 ng/ml by increasing or decreasing sample size. In addition, the method could readily be extended to the study of parent drug levels in tissues.

The compound has been shown herein to have a short elimination half-life after an intravenous dose; parent drug elimination from plasma paralleled the duration of its hypotensive activity reported in previous studies [2]. Although this activity persisted longer after relatively high oral doses (≥ 10 mg/kg), these experiments and others showed an oral BA of $<2\%$, even at higher doses. Analysis of total radioactivity and studies using isolated rat gut loops *in situ* (not reported herein) suggested that up to 14% of the drug was absorbed, but most parent compound was rapidly cleared from plasma by an as yet undetermined mechanism.

Although compound I has been removed from further development due to its low and variable BA, I remains one of the most useful tools for studying comparative binding to and localization of the adenosine A_2 receptor subtype. The analytical method described in these studies should prove useful for determining purity of the compound, as well as for the analysis of free and bound fraction of drug in comparative binding studies.

ACKNOWLEDGEMENT

The authors whish to thank Mrs. Elizabeth Gleich for expert help in the preparation of this manuscript.

REFERENCES

1 A. J. Hutchison, R. L. Webb, H. H. Oei, G. R. Ghai, M. B. Zimmerman and M. Williams, *J. Pharmacol. Exp. Ther.*, 251 (1989) 47.
2 R. L. Webb, R. B. McNeal, B. W. Barclay and G. D. Yasay, *FASEB J.*, 3 (1989) A1047.
3 M. F. Jarvis, R. Schulz, A. J. Hutchison, U. H. Do, M. A. Sills and M. Williams, *J. Pharmacol. Exp. Ther.*, 251 (1989) 888.
4 M. F. Jarvis and M. Williams, *Eur. J. Pharmacol.*, 168 (1989) 243.
5 C. R. Lupica, W. A. Cass, N. R. Zahniser and T. V. Dunwiddie, *J. Pharmacol. Exp. Ther.*, 252 (1990) 1134.
6 J. Chovan, P. A. Zane, S. L. Tripp, P. Robertson and F. L. Douglas, *FASEB J.*, 4 (1990) A462.
7 J. L. Balwierczak, M. J. S. Miller, C. Krulan, R. Sharif, F. P. Field and G. B. Weiss, *FASEB J.*, 3 (1989) A1047.