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RAPID, HIGH-SENSITIVITY REVERSED-PHASE LIQUID CHROMATOGRAPHIC ASSAY FOR 9-CHLORO-2-(2-FURYL)[1,2,4]TRIAZOLO[1,5-*c*]QUINAZOLIN-5-IMINE AND ITS OXO METABOLITE IN PLASMA USING FLUORESCENCE DETECTION

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SUMMARY

A rapid, sensitive and specific assay for 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-imine (I) and its oxo metabolite (II) in plasma was developed and validated employing reversed-phase high-performance liquid chromatography with fluorescence detection Sample preparation was achieved by a simple ethyl acetate extraction from plasma buffered at pH 10 (0 1 *M* boric acid–0 1 *M* potassium chloride) Chromatographic analyses were performed isocratically on a C_{18} column, with a mobile phase consisting of methanol–0 2 *M* sodium acetate buffer, pH 5 0 (67 33, v/v) Chromatographic run time was less than 8 min The assay was linear ($r > 0$ 9998) over the concentration range 1 50–10 000 ng/ml for both I and II, for individual studies, curves covering a range of two orders of magnitude were generally employed Limits of detection for I and II were 0 5 and 1 0 ng/ml, respectively A preliminary investigation of the plasma concentrations of I and II in the rat following a single 30 mg/kg oral dose demonstrated the applicability of the method for pharmacokinetic studies

INTRODUCTION

Historically, antagonists for adenosine receptors have been primarily of the xanthine structural class. Theophylline, caffeine and other xanthines have been exhaustively investigated due to a myriad of pharmacological effects, including central stimulation [1], antiasthmatic properties [2] and cardiotonic effects [3]. Non-specific effects such as phosphodiesterase inhibition, calcium mobi-

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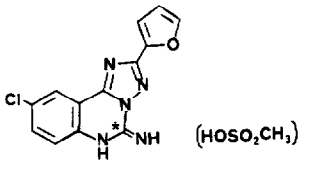
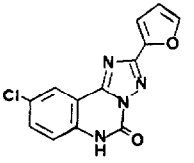
lizing effects [4,5] and non-selectivity for A_1 and A_2 adenosine receptors have limited the use of xanthine-type adenosine antagonists as pharmacological tools. Thus, considerable interest and effort has been directed toward identifying alternative structural types which exhibit adenosine antagonist activity without the concomitant phosphodiesterase inhibitory properties

IA [methane sulfonate salt of 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-imine (I), Fig. 1] is a potent adenosine antagonist with a novel non-xanthine heterocyclic ring structure. IA was selected for preclinical development based upon the results of biochemical [6] and pharmacological [7] screening of a newly synthesized series of compounds of the triazoquinoline type. It demonstrated selectivity for the A_2 receptor, exhibited no intrinsic phosphodiesterase activity, and was over 500-fold more potent than theophylline as an adenosine antagonist.

This report describes development, validation and application of a high-performance liquid chromatographic (HPLC) method for analyses of I and its oxo metabolite II in preclinical pharmacokinetic studies. The analytes were separated from plasma by a simple ethyl acetate extraction at pH 10, followed by isocratic HPLC. Baseline resolution of I, II and the internal standard, III, was obtained with run times of less than 8 min, permitting a high throughput of study samples. The method was subsequently used to determine the plasma concentration versus time profile of unchanged drug and metabolite in the rat following a single 30 mg/kg oral dose of IA.



I/IA



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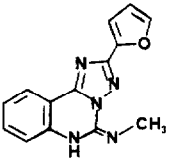

III

Fig 1 Structures of I (I is the free base, IA is the methane sulfonate salt), its oxo metabolite II and the internal standard III * indicates the position of the ^{14}C label, if present

EXPERIMENTAL

Preparation and source of reagents

HPLC-grade ethyl acetate, tetrahydrofuran and water were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) Dimethylacetamide (EM Science, Gibbstown, NJ, U.S.A.) and methanol (Mallinckrodt, Paris, KY, U.S.A.) were also HPLC grade Extraction buffers of pH 10 and 8 were prepared by adjusting the pH of a 0 1 *M* boric acid–0.1 *M* potassium chloride solution with 0.1 *M* sodium hydroxide Extraction buffers of pH 6 and 4 were prepared by adjusting the pH of a 0.2 *M* Na_2HPO_4 solution with a 0.1 *M* citric acid solution. 9-Chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5(6H)one (II, CGS 15696), 5-methylamino-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazoline (III, CGS 16936), 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-imine (I, CGS 15943) and 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-imine methane sulfonate (IA, CGS 15943A) were supplied by Chemistry Research, Ciba-Geigy [8] [^{14}C]IA was synthesized in the Preclinical Drug Metabolism Laboratories of Ciba-Geigy The specific activity of the compound was 306 kBq/mg; chemical and radiochemical purity by HPLC and thin-layer chromatography (TLC) was 99 7%. Structures of IA and its analogues are presented in Fig 1

Measurement of extraction efficiency

Solvent and pH dependence of recovery of I from plasma and urine was investigated by measuring recovery from plasma or urine spiked with [^{14}C]IA. Measured portions of a 0.6 mg/ml stock solution of [^{14}C]IA in methanol–tetrahydrofuran (2 1, v/v) were added to 40-ml centrifuge tubes and evaporated Subsequently, 500 μl of plasma or urine were added to each The resulting solutions were mixed with equal volumes of pH 10, 8, 6 or 4 buffer and extracted with 5.0 ml of ethyl acetate, cyclohexane or dichloromethane by horizontal shaking on an Eberbach shaking apparatus (Ann Arbor, MI, U.S A) for 15 min Following a 10-min centrifugation at 600 *g* (Centra-7 centrifuge, International Equipment, Needham Heights, MA, U.S.A) the organic layers were separated, and a 100-μl aliquot of each was mixed with 10 ml of Scintisol (Isolab, Akron, OH, U S.A) liquid scintillation fluid. Radioactivity was measured on a Packard Tri-Carb 4530 counter The ratios of radioactivity found to radioactivity added were multiplied by 100 and defined as percentage recovery of I The remaining organic layer in each case was evaporated to dryness under nitrogen on an N-EVAP apparatus (Organomation Assoc., South Berlin, MA, U S A). The residue was reconstituted in 0 2 ml of methanol and analyzed for interferences by endogenous substances by the chromatographic system described below.

High-performance liquid chromatography

The HPLC system (Waters Assoc., Milford, MA, U.S A.) included a Waters 840 data and chromatography station, a WISP 710B automatic sample processor, a Model 590 pump and a Kratos Model 983 fluorometric detector. Chromatography was performed at ambient temperature on a Rainin Microsorb (Rainin Instrument, Woburn, MA, U.S A.) 10 0 cm×4.6 mm I.D. octadecyl column (3 μm particle size) The mobile phase was methanol–0.2 *M* sodium acetate buffer pH 5.0 (67 33, v/v) and was degassed and filtered prior to use. The buffer was made by mixing a 0.2 *M* solution of sodium acetate and 0.2 *M* acetic acid solution The flow-rate was 1.0 ml/min. An Upchurch guard column packed with 30–40 μm octadecyl Perisorb and a 0 2-μm filter preceded the analytical column. Under these conditions, retention times were 4.4, 5.7 and 7 0 min for II, III and I, respectively. Fluorescence detection was based upon wavelengths of 260 nm (excitation) and 340 nm (emission). The detector was interfaced to the 840 computer system for data acquisition, integration and storage Hardcopies of chromatographic runs were printed on an LA75 printer

Preparation of standards

Standard solutions of I, II (oxo metabolite) and III (internal standard) were prepared from 200 μg/ml stock solutions (I and III, tetrahydrofuran; II, dimethylacetamide). The stock solutions were diluted to a convenient range for spiking (typically 100–500 ng/ml) with methanol–water (2 1, v/v) For standard curves, measured volumes of these working standards were added to 40-ml centrifuge tubes, the solvent was evaporated under nitrogen, and a volume of plasma equal to the analysis sample size for the experiment was added.

Preparation of doses

The dosage form of IA was prepared by suspending appropriate amounts of drug to yield a 7 5 mg/ml suspension in 3% aqueous cornstarch. The material was mixed in a Corning tissue homogenizer and briefly sonicated to achieve a uniform suspension. IA was administered orally at 30 mg/kg animal weight

Animal studies

Following an overnight fast, nine adult male Sprague–Dawley (Charles River) rats weighing 200–230 g were administered the 7 5 mg/ml IA–cornstarch suspension by oral gavage (4.0 ml/kg), to afford a dose of 30 mg/kg. At 0 5, 0.75, 1.0, 2.0, 3.0, 5 0 and 24 h following drug administration, blood was collected directly into 1.5-ml polypropylene vials from the orbital sinus of each of three animals, using heparinized microhematocrit capillary tubes (American Scientific, McGaw Park, IL, U.S.A.). Plasma was separated by centrifugation for 10 min at 600 *g*, and a 200-μl portion was added to a 40-ml centrifuge tube containing 5 ng of internal standard

Sample analysis

Standard curves for I and II were prepared daily. Internal standard (5 ng) and known amounts of II and I standard solutions were combined with 200 μl of control plasma in 40-ml glass centrifuge tubes to produce seven to ten non-zero concentrations in the range of interest An equivalent volume of pH 10 buffer was added to standards and samples, followed by 3 ml of ethyl acetate. After capping with a PTFE-lined cap, the layers were mixed by shaking 15 min on a horizontal shaker. After centrifugation for 10 min at 600 *g*, the organic layer was transferred to a clean tube and evaporated to dryness under nitrogen. The interior walls or each tube were rinsed with 0 50 ml of HPLC-grade methanol, followed by a second evaporation The residue was reconstituted with mobile phase to a volume one half of the original plasma aliquot and was vortexed for 20 s The reconstituted sample was transferred to a WISP injection vial, from which 50 μl were injected. Standard curves were constructed by plotting the peak-height ratios of I and II to the internal standard versus the analyte concentration (expressed as ng/ml or ng per sample with subsequent reconversion to ng/ml), with the equation of best fit obtained by linear regression analysis.

RESULTS AND DISCUSSION

Chromatographic separation

Chromatograms obtained from control and spiked rat plasma, using the HPLC procedure described above, are shown in Fig. 2 Baseline resolution of the compounds of interest was obtained and peak shapes were excellent, with asymmetry factors in the range 1.0–1 5 as measured at 10% above baseline. Under these conditions, II, III and I had retention times of 4.4, 5.8 and 7.0 min, respectively Blank control plasma exhibited no interferences relative to any of the compounds of interest.

Solvent and pH extraction dependence

Solvent and pH dependence of extraction efficiency of I from plasma and urine spiked with [^{14}C]IA was evaluated; the results are shown in Table I. Buffering at pH 10 afforded the highest yield of I from both fluids, as expected for a weakly basic molecule. Additionally, at pH 10 the extraction of early-eluting endogenous material was minimized, as demonstrated by subsequent HPLC analysis of the extracts. HPLC analysis of ethyl acetate, cyclohexane or dichloromethane extracts of blank plasma demonstrated no chromatographic interferences. Thereafter, ethyl acetate was employed due to its greater extraction efficiency from both biological fluids.

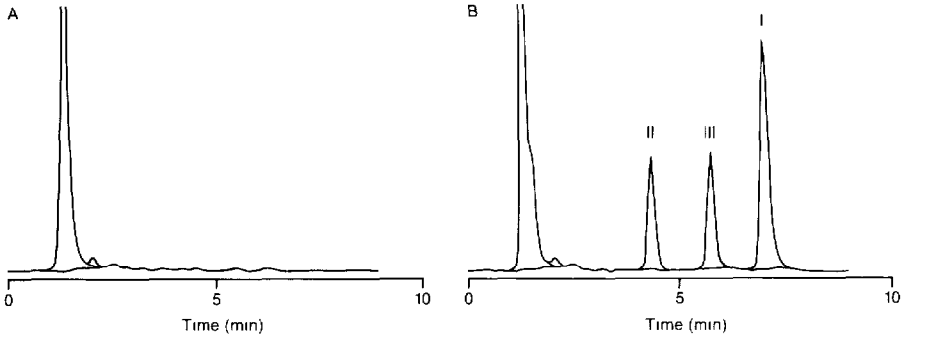

Fig 2 HPLC patterns of (A) control rat plasma and (B) spiked plasma Chromatography was performed on a Rainin C_{18} Microsorb Short-One, with methanol–0 2 *M* sodium acetate buffer pH 5 0 (67 33, v/v) as mobile phase, flow-rate, 1 0 ml/min Fluorescence detection was used, with an excitation wavelength of 260 nm and a 320-nm emission filter (A) Injection of 50 μl of a mobile phase reconstituted extract of 300 μl control rat plasma (B) Injection of 50 μl of reconstituted extract of 300 μl rat plasma previously spiked at 16 7 ng/ml III and 167 ng/ml I and II

TABLE I

EXTRACTION RECOVERY OF RADIOLABELED I FROM PLASMA AND URINE

n a = not assayed

pH	Recovery (%)					
	Ethyl acetate		Cyclohexane		Dichloromethane	
	Plasma	Urine	Plasma	Urine	Plasma	Urine
10	99 3	95 7	73 0	87 9	104 0	n a
8	83 0	94 1	76 7	88 8	89 2	n a
6	99 0	94 6	73 4	88 3	67 7	n a
4	97 2	64 9	79 6	87 4	54 0	n a

Assay linearity

Linear calibration curves were obtained throughout the range 1.5–10 000 ng/ml for both I and II. For individual studies, calibration samples typically spanned two orders of magnitude in the range of interest. Calibration curve linearity and reproducibility for both compounds were routinely excellent. Table II shows a compilation of standard curve results from five analysis dates. The inter-day mean of the correlation coefficients (r) was found to be greater than 0.9998 for both compounds. The high degree of reproducibility of the

TABLE II

INTER-DAY VARIABILITY OF STANDARD CURVES

Least-squares linear regression equations have the form $y=ax+b$, where a=slope and b=y-intercept, r=linear correlation coefficient Values are means ($n=5$)

Compound	a	b	r	R S D of a (%)
I	0 03793	−0 00274	0 9998	2 57
II	0 01905	−0 00078	0 9998	2 83

method was demonstrated by the low inter-day variability in the slope of the standard curves, with a relative standard deviation (R S D) of 2.57% for I and 2.83% for II

Intra-assay accuracy and precision

Intra-assay accuracy and precision were determined by analysis of replicate plasma samples ($n=4$) spiked to yield six non-zero concentrations within the typical working range of the assay. The results are presented in Table III The precision (R.S.D) of the method for both analytes was within 1 68–6 17%, indicating good reproducibility within the entire range of concentrations A consistently high degree of accuracy was observed at all concentrations, with mean absolute errors (M A E) in the range 2.62–4.16% for I and 2 31–10.00% for II

TABLE III

INTRA-ASSAY ACCURACY AND PRECISION

Compound	n	Concentration (ng/ml)		M A E (%)	R S D (%)
		Nominal	Found		
I	4	8 33	8 19	3 30	3 92
	3	16 67	16 63	3 62	5 15
	4	33 33	34 53	3 59	4 88
	4	83 33	86 20	3 44	3 40
	4	167 67	173 60	4 16	3 05
	4	333 33	342 06	2 62	4 40
II	4	8 33	8 61	5 42	5 00
	4	16 67	15 00	10 00	6 17
	3	33 33	34 81	4 43	1 68
	4	83 33	84 15	3 86	5 54
	4	167 67	170 51	2 31	2 13
	4	333 33	344 90	3 47	2 31

Inter-assay accuracy and precision

Inter-assay accuracy and precision were assessed by the analysis of replicate plasma samples spiked at a low (14.7–15.0 ng/ml) and a high (150.3–152.1 ng/ml) concentration in the typical working range of the assay. On multiple analysis dates, one or more samples from the low and high level were analyzed versus a standard curve. The results are tabulated in Table IV. For both analytes, extremely low inter-assay variability was observed. At both the high and low level for both compounds, the M.A.E was less than 2.0%, demonstrating excellent inter-assay accuracy. Good inter-assay precision was also observed, with an inter-assay R.S.D of 1.41–2.37% for II and 1.21–1.98% for I.

Assay limits

The limits of quantification (LOQ, defined as both an R S.D. and M A E $\leq 10\%$) for both compounds were evaluated Replicate plasma samples ($n=6$) containing known amounts of both analytes were analyzed versus a standard curve. The results are shown in Table V. Both accuracy (M A.E.) and precision (R.S.D.) for both compounds were well within the 10% criterion at 3 1 ng/ml. Accuracy, even at this low concentration, was not limiting, with M A.E. values of 3.40 and 9.65% for I and II, respectively. Precision was also good, with an R.S D of 4.02% for I and 9.02% for II. The limit of detection (LOD; signal-to-noise ratio ≥ 2) was estimated to be 0.5 ng/ml for I and 1.0 ng/ml for II

TABLE IV

INTER-ASSAY ACCURACY AND PRECISION

Compound	*n*	Nominal concentration (ng/ml)	M A E (%)	R S D (%)
I	10	14 80- 15 02	1 54	1 98
	8	151 72-152 12	1 77	1 21
II	10	14 74- 14 83	1 73	2 37
	8	150 29-151 97	1 25	1 41

TABLE V

LIMIT OF QUANTITATION

Compound	*n*	Concentration (ng/ml)		M A E (%)	R S D (%)
		Nominal	Found		
I	6	3 12	3 13	3 40	4 02
II	6	3 06	3 32	9 65	9 02

Animal studies

The described method was applied to the determination of unchanged I and its metabolite II in plasma of rats orally dosed (30 mg/kg) with IA. Mean plasma concentrations ($n=3$) of both analytes are presented graphically in Fig. 3.

The absorption of IA following oral administration was characterized by the rapid appearance of I and II in plasma. The peak concentration of I in plasma (C_{max}) of 208 ng/ml was attained by 1.0 h post-dose. Substantial variability was noted in the plasma drug levels at each timepoint and was a function of inter-animal variability Elimination of both compounds from plasma was also rapid, with neither compound detected (detection limit ~ 1 ng/ml) after 3 h For II, the C_{max} (93 8 ng/ml) was approximately one half that for parent drug, indicating it to be a major metabolite. These data indicated rapid conversion of I to II and rapid elimination of that metabolite from plasma. No other metabolites were apparent. As with all methods employing extractions, the possible existence of other highly polar metabolites cannot be eliminated.

There was an apparent correlation of the plasma pharmacokinetic profile of I with the observed pharmacodynamics of the in vivo inhibition [7] of the 2-chloroadenosine-induced depressor response in normotensive rats Inhibition (50–60%) by IA was maximal at 1 h and by 2 h was rapidly decreasing, suggesting that the observed biological effect was directly related to the plasma levels of I. The biological response profile did not correspond to the plasma concentration profile of the oxo metabolite II, for which peak plasma levels were observed at 2 h post-dose, indicating that II is not a significantly active metabolite

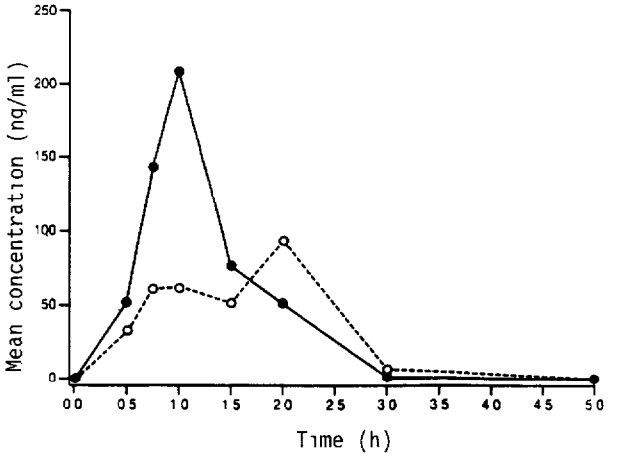

Fig 3 Mean plasma concentrations of I (●) and II (○) in the rat after oral administration of IA at 30 mg/kg as a suspension in cornstarch Each data point represents the mean of three samples drawn from a group of nine dosed rats

CONCLUSIONS

The analytical method developed has been shown to be of adequate sensitivity and selectivity to quantify I and II in rat plasma following oral administration of the methane sulfonate salt of I, IA. The method exhibited high accuracy and precision, both in intra- and in inter-assay tests, and is currently validated to 3.1 ng/ml for both I and its metabolite II. The R S D and M A E were not limiting factors even at 3 1 ng/ml; it is anticipated that the method could be extended to lower levels, if necessary.

When applied to its intended use, the analysis of animal pharmacokinetic samples, the short isocratic run time combined with the simple sample preparation allowed a high throughput of study samples If necessary, as many as 100 samples could be prepared and analyzed within a 24-h period.

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