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Simultaneous determination of L-693,612, a topical carbonic anhydrase inhibitor, and two potential metabolites in human whole blood by ion-pair high-performance liquid chromatography

E.J. Woolf*, T. Au, M. Kasper, M. Constanzer and B. Matuszewski
Merck Research Laboratories, Department of Drug Metabolism, West Point, PA 19486 (USA)

ABSTRACT

A method for the simultaneous determination of a topical carbonic anhydrase inhibitor, L-693,612, and two of its potential metabolites in human whole blood is described. The analytes are isolated from the matrix via liquid–liquid extraction with a mixture of toluene, ethyl acetate and isopropanol (49:50:1, v/v/v). The analytes are then back extracted into dilute phosphoric acid prior to injection into the HPLC system. A cyano column (Zorbax SB-CN, 150 × 4.6 mm) with a mobile phase of phosphoric acid(0.085%)–acetonitrile (73.5:26.5) containing 10 m*M* sodium decane sulfonate and adjusted to pH 3 is used for the analysis. Detection is based on UV absorbance at 252 nm. The assay was found to be linear in the concentration range of 5–500 ng/ml for each analyte when 1-ml aliquots of whole blood were extracted.

INTRODUCTION

Trans-(−)-5,6-dihydro-6-(3-methoxypropyl)-4-propylamino - 4H - thieno[2,3 - B]thiopyran - 2-sulfon-amide-7,7-dioxide (Compound **I**, L-693,612) is a member of a group of compounds that have been found to be potent inhibitors of the enzyme carbonic anhydrase. The ocular hypotensive activity that these compounds exhibit when applied topically has been demonstrated in various animal pharmacological models, as well as humans. During pre-clinical studies in rats, compounds **II** (O-desmethyl L-693612) and **III** (N-despropyl L-693612) were identified as active metabolites of **I** [1]. In order to support a clinical pharmacokinetic program, an assay of **I**, and its potential metabolites **II** and **III**, in human whole blood was required. An assay of **I**, **II**, and **III** in human whole blood using liquid–liquid extraction for analyte isolation followed by reversed-phase ion-pair high-performance liquid chromatography with UV detection is presented here.

* Corresponding author.

MATERIALS AND METHODS

Materials

The hydrochloride salts of compounds **I**, **II**, and **III** (Fig. 1) were obtained from the Medicinal Chemistry Department of Merck Research Laboratories (West Point, PA, USA). Acetonitrile and hexane (Omnisolve grade) were from EM Science (Gibbstown, NJ, USA). Ethyl acetate and toluene were obtained from Burdick and Jackson (Muskegon, MI, USA). Isopropanol (Optima grade) was from Fisher Scientific (Springfield, NJ, USA). The sodium salts of C5–C8 alkane sulfonates were from Regis (Morton Grove, IL, USA). Sodium 1-nonane

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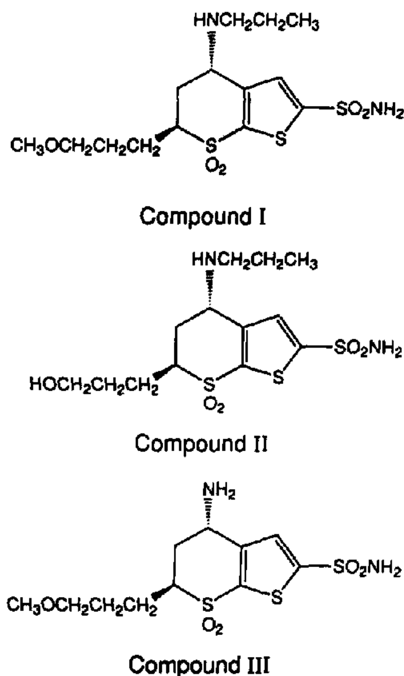

Fig. 1. Structures of analytes

and sodium 1-decane sulfonates were purchased from Lancaster Synthesis (Windham, NH, USA). Drug-free heparinized human whole blood was supplied by Biological Specialties, Inc. (Lansdale, PA, USA). All other reagents were of ACS grade and were used as received.

Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 410 pump, a Waters (Milford, MA, USA) WISP 715 automatic injector and an Applied Biosystems (Foster City, CA, USA) Model 785 absorbance detector. The analog output from the detector (1 V/AU) was connected to a PE-Nelson (Cupertino, CA, USA) Access-Chrom data system via a PE-Nelson 941 analog-to-digital interface.

Chromatography conditions

The mobile phase consisted of 2.4 g of sodium 1-decane sulfonate dissolved in a mixture of 735 ml of 0.085% *o*-phosphoric acid and 265 ml of acetonitrile. The final pH of the mobile phase was adjusted to 3.0 with 10 *M* sodium hydroxide. Prior to use, the mobile phase was passed through a Rainin (Woburn, MA, USA) 0.20-μm nylon membrane filter.

A Zorbax SB-CN column (5-μm particles, 80 Å pore size, 150 × 4.6 mm, Mac-Mod Analytical, Chadds Ford, PA, USA) was used for the separation. The mobile phase flow rate was 1.2 ml/min. The sample injection volume was 200 μl. Ultraviolet absorbance at 252 nm was used for detection.

Preparation of standards

A methanolic stock solution containing 20 μg (free base)/ml of each analyte was prepared by weighing 1.09 mg of the hydrochloride salts of **I** and **II** and 1.10 mg of the hydrochloride salt of **III** into a 50-ml volumetric flask and filling the flask to volume with methanol. A 2.0 μg/ml stock solution was prepared by diluting 5 ml of the 20 μg/ml solution to 50 ml with methanol.

Working standards of 10, 8, 4, and 2 μg of each analyte/ml were prepared by dilution of the 20 μg/ml stock solution with methanol. Working standards of 1, 0.4, 0.2 and 0.1 μg of each analyte/ml were prepared by dilution of the 2.0 μg/ml stock with methanol. Working standard solutions were found to be stable for at least 2 weeks when stored at room temperature.

Whole blood standards were prepared by adding 50 μl of each working standard to 1 ml of drug free blood. The resulting standards ranged in concentration from 5 to 500 ng/ml of each analyte.

Sample preparation

A 1-ml aliquot of blood (sample or standard) was pipetted, using a large orifice polypropylene pipet tip (PGC Scientifics, Gaithersburg, MD, USA), into a 50-ml disposable screw cap glass conical centrifuge tube (Kimble, Vineland, NJ, USA). Following the addition of 1 ml of water, the tube was vortexed vigorously for 60 s to lyse any intact cells. 600 μl of a 10% (w/v) aqueous solution of trichloroacetic acid was added to the tube, after which it was vortexed for 60 s to denature proteins in the sample. Buffer (0.2 *M* phosphate, pH = 8.0, 3.0 ml) was added followed by 8.0 ml of extraction solvent mixture (ethyl acetate–toluene–isopropanol; 50:49:1, v/v/v). The tubes were capped with teflon lined

closures and shaken for 10 min. Following centrifugation (5 min, 1500 *g*), 7.0 ml of the upper organic layer was transferred to a clean 50-ml glass centrifuge tube. The aqueous layer was then re-extracted with an additional 8.0 ml of extraction solvent. The second 8.0 ml of extraction solvent was transferred to the tube containing the first portion of solvent, and the combined extracts were back-extracted into 0.5 ml of 0.085% *o*-phosphoric acid. The acid extract was washed with 2.0 ml of hexane in order to remove most of the residual toluene from the extraction solvent. Following aspiration of the hexane to waste, the acid extract was transferred to an autosampler vial prior to injection into the HPLC system.

RESULTS

Assay specificity

Fig. 2 shows chromatograms of extracted drug-free whole blood, a whole blood standard containing each analyte at a concentration of 200 ng/ml and a whole blood sample taken from a subject after receiving an ocular dose of a 2% solution of **I**. A comparison of Fig. 2A with Fig. 2B illustrates that no endogenous peaks co-elute with any of the analytes. The specificity of the method is further illustrated by the fact that all pre-dose blood samples from subjects involved in clinical trials were free of interfering peaks.

Linearity

Weighted (weighting factor = $1/y$ where y = peak height) least-squares regression calibration curves, constructed by plotting the standard concentration of each analyte *vs.* peak height, yielded coefficients of regression typically greater than 0.999 over the concentration range, for each analyte, of 5–500 ng/ml of blood. The use of the weighted least-squares regression resulted in less than a 10% deviation between the nominal standard concentrations and the experimentally determined standard concentrations calculated from the regression equations.

Extraction recovery

The recovery of the extraction procedure was determined by comparing the responses of the

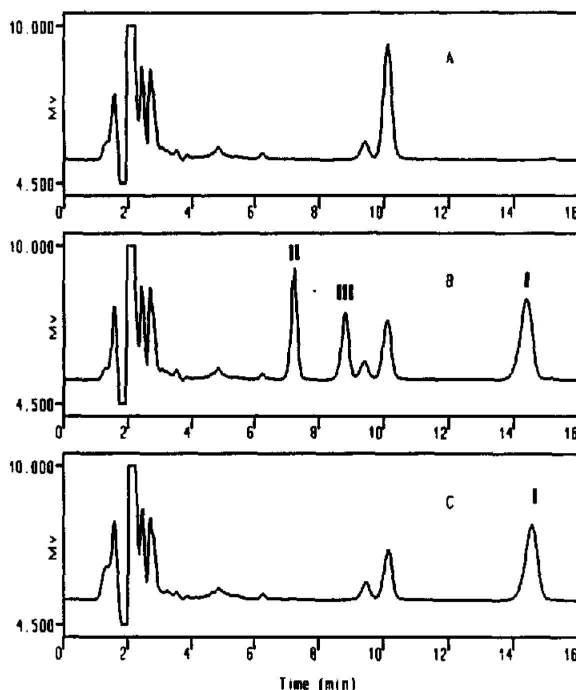

Fig. 2. Representative chromatograms of (A) control human whole blood, (B) whole blood spiked with 200 ng/ml of each analyte and (C) blood sample from human volunteer obtained after ocular administration of **I**; the concentration of **I** is equivalent to 184 ng/ml. Analytes **II** and **III** were not detectable. (*Y*-axis scale: 1 mV = 1 mAu).

working standards containing **I**, **II**, and **III**, with those of extracted whole blood standards. The results (Table I) indicate that the mean recoveries of **I**, **II**, and **III**, over the concentration range of 5–500 ng/ml blood, are 84.3, 62.1, and 41.5%, respectively. Although the extraction efficiency of **III** was under 50%, it was constant over the concentration range studied, and the precision of the extraction of **III** at each concentration, as measured by the relative standard deviation, was better than 6.0% (Table I).

Assay precision and accuracy

Replicate standards ($n = 5$) were analyzed to assess the within-day variability of the assay. The mean accuracy of the assayed concentration of each analyte as well as the relative standard deviation (R.S.D.) of the whole blood replicate standards are shown in Table I.

Standard curves over the assay range of 5–500

TABLE I

RECOVERY OF ANALYTES FROM HUMAN WHOLE BLOOD AND WITHIN-DAY VARIABILITY OF THE ASSAY

Nominal conc. of analyte (ng/ml)	Analyte I			Analyte II			Analyte III		
	Accuracy[a]	Precision[b]	Recovery (%)	Accuracy	Precision	Recovery (%)	Accuracy	Precision	Recovery (%)
5.0	98.6	5.9	72.9	97.0	3.9	65.2	98.0	5.7	41.3
10.0	97.3	2.0	84.3	97.6	2.4	62.7	99.5	1.7	41.1
20.0	103.9	1.9	85.8	99.0	1.9	60.8	100.4	3.1	40.9
50.0	96.1	6.5	87.8	98.4	3.7	64.5	98.1	3.9	43.5
100.0	98.8	5.8	85.5	100.3	2.0	58.7	99.9	1.8	39.6
200.0	102.9	2.8	88.0	103.9	2.1	63.6	104.8	2.0	43.1
400.0	99.7	2.4	84.9	98.1	1.5	60.4	98.5	1.5	41.0
500.0	95.4	6.5	85.6	98.6	2.6	62.1	98.2	4.3	41.3
Mean	99.1	4.2	84.3	99.1	2.6	62.1	99.7	3.0	41.5

[a] Calculated as [mean ($n = 5$) observed concentration/nominal concentration] × 100.
[b] Expressed as relative standard deviation.

ng/ml of each analyte per ml of blood were analyzed on each of 3 days to determine the inter-day variability of the assay. The inter-day mean accuracy and precision data is presented in Table II. The R.S.D.s of the slopes of the regression lines over the 3 runs were 4.5, 1.7, and 2.6% for analytes **I**, **II**, and **III**, respectively.

Analyte stability

Whole blood quality control samples were prepared at concentrations of 15.0 and 350 ng of each analyte per ml and stored frozen at −20°C. Analysis of these samples over a one-month period showed no degradation of any of the analytes.

Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded an within-day R.S.D. of less than 10% and an within-day accuracy between 90 and 110% of nominal concentration, was 5 ng/ml for each analyte.

DISCUSSION

The red blood cell (RBC) to plasma ratio of **I** in rats has been found to be concentration-dependent, presumably due to saturable binding of **I** to carbonic anhydrase in RBCs [2]. To eliminate the contribution of this effect, an assay of **I**,

TABLE II

INTER-DAY VARIABILITY OF THE ASSAY AS ASSESSED BY THE PRECISION AND ACCURACY OF THE CALCULATED STANDARD CONCENTRATIONS FROM THE REGRESSION LINES ON EACH OF 3 DAYS

Nominal conc. of analyte (ng/ml)	Analyte I		Analyte II		Analyte III	
	Accuracy[a]	Precision[b]	Accuracy	Precision	Accuracy	Precision
5.0	101.7	5.4	98.9	6.7	101.1	9.4
10.0	97.9	1.0	98.7	3.8	99.0	1.1
20.0	100.9	2.2	101.1	3.6	101.2	2.9
50.0	101.1	2.9	102.1	3.3	100.9	3.2
100.0	99.6	4.3	101.3	6.4	99.4	5.8
200.0	98.8	3.0	98.4	2.4	98.5	1.8
400.0	99.0	1.9	99.1	1.8	99.1	1.5
500.0	101.3	0.2	101.0	0.4	101.5	0.6

[a] Calculated as [mean ($n = 3$) observed concentration/nominal concentration] × 100.
[b] Expressed as relative standard deviation.

II, and III in whole blood rather than plasma was required.

In order to develop a simultaneous assay for I, II, and III, two problems had to be addressed. First, a method to simultaneously extract the analytes from the blood matrix was required. Second, a chromatographic system capable of not only separating the analytes from each other, but also, resolving them from co-extracted endogenous substances had to be developed.

We have previously reported that another carbonic anhydrase inhibitor, possessing structural similarities to I, was effectively extracted from buffered, deproteinized whole blood with a mixture of toluene, ethyl acetate and isopropanol [3]. Such an approach proved to be successful for the isolation of I, II, and III. Although the extraction recoveries of II and III were significantly less than that of I, the recovery of all analytes appeared to be constant and reproducible over the concentration range studied (Table I).

Development of a chromatographic system that could be used to analyze whole blood extracts proved to be more difficult. Initial attempts to chromatograph the extracts under isocratic reverse phase conditions using a base deactivated C-18 column (Zorbax Rx-C18) with mobile phases at pH = 3 and pH = 6.5 resulted in wide capacity factor differences between the possible metabolites (II and III) and the parent compound, I. Such chromatography led to poor assay sensitivity and long run times. The use of gradient conditions was not thought to be a viable option, as it was felt that the time saved by running a gradient would be lost in column re-equilibration time.

The use of a "stable bond" cyano column (Zorbax SB-CN) with a mobile phase at pH = 3 containing 9% acetonitrile allowed all analytes to be chromatographed with capacity factors ranging from 2 to 6. Unfortunately, interferences were present in whole blood extracts. The majority of the interferences could be moved to the solvent front by increasing the strength of the mobile phase to 27% acetonitrile; however, as expected, the retention of the analytes was also decreased. Adding alkane sulfonate ion-pairing agents to a mobile phase containing 27% acetonitrile was found to dramatically affect the retention of the analytes; retention was found to increase as the length of the carbon chain of the ion-pairing agent increased (Fig. 3). The behavior of the endogeneous species was unaffected by the ion-pairing agents; the majority continued to elute near the solvent front. Apparently,the endogenous material did not interact with any of the ion-pair reagents, whereas the retention of the analytes was found to be highly dependent on the ion-pair reagent used in the mobile phase. The retention of two remaining endogenous peaks, eluting between 9 and 11 min, was also found to be unaffected by any of the ion-pair reagents. These peaks could, how-

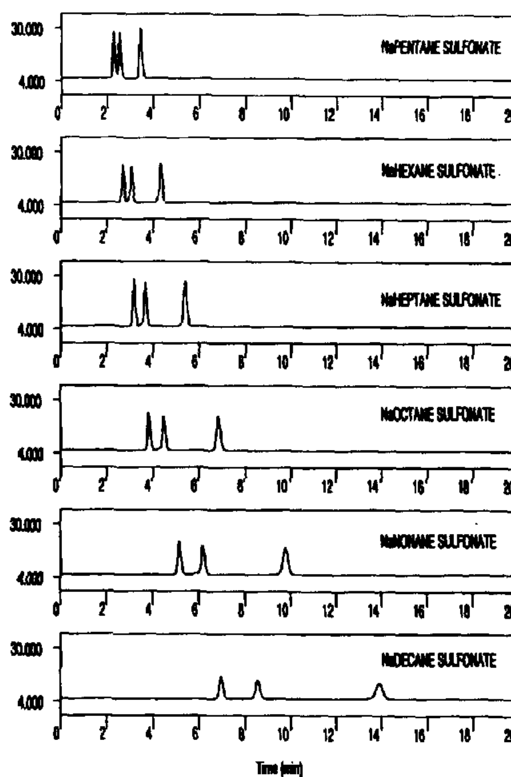

Fig. 3. Chromatography of analytes I, II, and III with mobile phases containing different ion-pairing reagents. The mobile phase in each case consists of acetonitrile–water (27:73, v/v) containing 1.4 ml/l *o*-phosphoric acid and 10 m*M* of the indicated ion-pair reagent. The final pH of the mobile phase was adjusted to 3.0 with 10 *M* NaOH. Column, Zorbax SB-CN (150 × 4.6 mm); flow rate, 1.2 ml/min; detection, UV absorbance at 252 nm, 1 mV = 1 mAu. Elution order: II, III, I. Each peak corresponds to approximately 200 ng of analyte injected on column.

ever, be resolved from the analytes by slightly adjusting the percentage of acetonitrile in the mobile phase. The optimum ion-pair mobile phase consisted of approximately 10 m*M* sodium decane sulfonate dissolved in a 26.5:73.5 (v/v) mixture of acetonitrile and phosphate buffer at pH = 3. Using this mobile phase, all analytes were resolved from endogenous substances in under 16 min (Fig. 2).

The described assay has been used to analyze clinical samples obtained after ocular administration of I. Twice a day, study subjects received 1 drop of a 2% solution of I in each eye. This dose was administered for seven days. Blood samples were collected pre-dose, and on days 2 and 7. Additional samples were collected at one week, three weeks, and 3 months post administration of the last dose. The resulting levels of I for two subjects are presented in Table III. As is the case with other members of this class of compounds [3], I has an extremely long terminal half-life; significant levels are still observed 3 months after drug administration. Metabolites **II** and **III**, previously identified in rat blood [1], were not detectable in any of the clinical samples.

TABLE III

WHOLE BLOOD LEVELS OF I AFTER OCULAR ADMINISTRATION OF A 2% SOLUTION

Time	Concentration (ng/ml)	
	Subject 1	Subject 2
Pre-dose	0.0	0.0
Day 2	32.1	36.9
Day 7	254.6	222.4
1 Week p.a.[a]	214.6	206.2
3 Weeks p.a.	188.1	170.5
3 Months p.a.	123.4	139.1

[a] Post administration.

CONCLUSIONS

An ion-pair HPLC assay for the simultaneous determination of I and its potential metabolites, **II** and **III**, in human whole blood has been developed. The method has been found to be precise, accurate, and suitable for the analysis of whole blood samples collected during clinical pharmacokinetic studies. Analytes **II** and **III** were not detected in human whole blood samples obtained after 7 days of ocular dosing of **I**.

ACKNOWLEDGEMENTS

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