Journal of Chromatography, 579 (1992) 307–317
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6452

High-performance liquid chromatographic determination of N-[2(*S*)-(mercaptomethyl)-3-(2-methylphenyl)-1-oxopropyl]-L-methionine, the active plasma metabolite of a prodrug atriopeptidase inhibitor (SCH 42495), using a thiol selective (Au/Hg) amperometric detector

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(First received February 26th, 1992; revised manuscript received April 30th, 1992)

ABSTRACT

A high-performance liquid chromatographic assay for the determination of N-[2(*S*)-(mercaptomethyl)-3-(2-methylphenyl)-1-oxopropyl]-L-methionine (SCH 42354; **II**), the active metabolite of the atriopeptidase inhibitor prodrug, N-[2(*S*)-(acetylthiomethyl)-3-(2-methylphenyl)-1-oxopropyl]-L-methionine ethyl ester (SCH 42495; **I**), in human plasma was validated for use in clinical pharmacokinetic studies. Plasma (200 μl) was processed by protein precipitation with acetone containing the internal standard, N-[2(*S*)-(mercaptomethyl)-3-(2-methylphenyl)-1-oxopropyl]-L-ethionine (**III**). Compound **II** was recovered (*ca.* 90%) in the supernatant after centrifugation and prepared for injection by the addition of 0.15 *M* monochloroacetic acid containing 0.2 m*M* EDTA. Separation of **II** and **III** was accomplished on commercially available reversed-phase C_8 columns designed for the separation of basic compounds. Both compounds were detected using amperometric detection (+0.125 V *versus* Ag/AgCl) on a thin-layer Au/Hg amalgam electrode. The lower limit of quantitation was 10 ng/ml, where the inter-assay precision (coefficient of variation) was ±11.4% and the inter-assay accuracy (bias) was +1.0%. No endogenous interferences were observed in the extracts obtained from drug-free plasma. The detector response (using either peak area or height ratios of **II** to **III**) was linear from 0.01 to 1.0 μg/ml. Compound **II** was stable in plasma supplemented with EDTA and sodium hydrogensulfite for at least 3 months when stored frozen at −78°C; no significant decomposition of **II** was observed following three freeze–thaw cycles. The feasibility of this liquid chromatographic assay with electrochemical detection was demonstrated with plasma samples from hypertensive subjects administered 100 mg of compound **I**.

INTRODUCTION

Atrial natriuretic factors (ANFs) are potent diuretic, natriuretic and vasorelaxant hormones secreted by the heart and are presumed to be involved in the regulation of blood pressure, fluid and electrolyte balance [1]. The half-life of ANFs is very short (1–2 min); therefore, inhibition of ANF metabolism should prolong the half-life of ANFs and enhance their biological effects [2,3]. Several workers have reported that neutral metalloendopeptidase (NEP; atriopeptidase) hydrolyzes ANFs to inactive products [4–7]. Inhibition of NEP [8–11] potentiated the renal and hypotensive responses to exogenous ANFs in spontaneously hypertensive rats and lowered blood pressure in desoxycorticosterone acetate salt hypertensive rats. N-[2(*S*)-(Mercaptomethyl)-3-(2-

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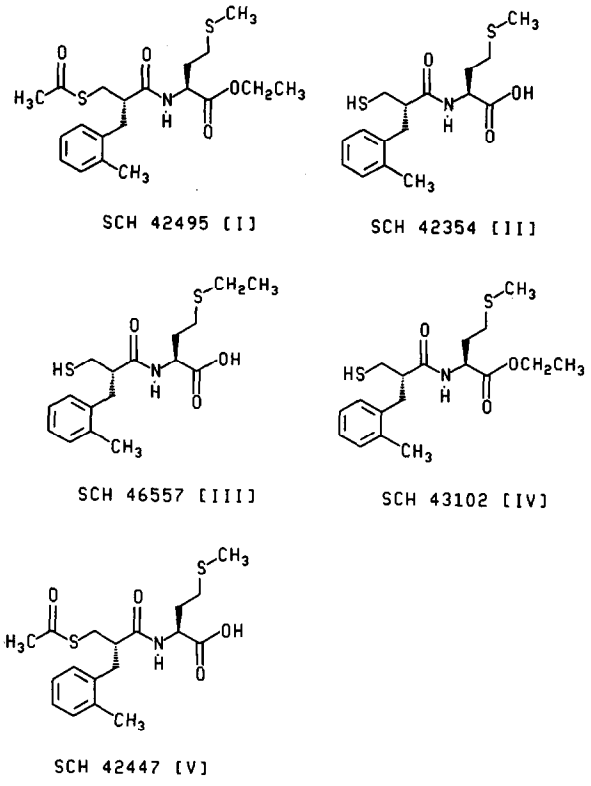

Fig. 1. Chemical structures of the prodrug (**I**), the active metabolite (**II**), the internal standard (**III**) and other potential metabolites (**IV** and **V**).

methylphenyl)-1-oxopropyl]-L-methionine (SCH 42354; **II**), the deacylated free acid form of the orally active prodrug N-[2(*S*)-(acetylthiomethyl)-3-(2-methylphenyl)-1-oxopropyl]-L-methionine ethyl ester (SCH 42495; **I**) has been characterized in biochemical tests as a potent and selective inhibitor of NEP (Fig. 1) [12,13].

Preliminary metabolism studies indicated that **I** is rapidly metabolized *ex vivo* in plasma to **II** and/or related hydrolysis products. In early exploratory studies with animals, a high-performance liquid chromatographic (HPLC) assay with fluorescent detection had been used to quantitate drug-related analyte in plasma [14]. This method required several reaction steps to ultimately produce a common ammonium-4-chloro-7-sulfobenzofuran (SBL-Cl) sulfoxide derivative. However, this relatively insensitive [limit of quantitation (LOQ) $\approx$ 0.5 μg/ml] and non-specific assay did not discriminate among the parent drug or its putative free thiol, disulfide or sulfoxide metabolites. A sensitive and selective quantitative procedure for **II**, the major active metabolite, was needed to support clinical pharmacokinetic as well as animal toxicokinetic studies. Amperometric detection methods which use thin-layer Au/Hg amalgam electrodes for the direct determination of thiols have been reported [15–19] for a number of drugs and endogenous substances. The electrochemical reaction on the detector surface is based on a highly selective oxidation of ground-state mercury in the presence of thiol analyte. The development and validation of a novel liquid chromatography (LC)–electrochemical detection (ED) method which exploits this highly selective electrochemical response to **II** in human plasma is decribed herein.

EXPERIMENTAL

Apparatus

Analyses were performed with an HPLC system composed of a Model 712 WISP automatic injector (Waters Assoc., Milford, MA, USA), a Waters M6000A pump, a Waters Model 481 UV spectrophotometer and an LC-4B amperometric detector equipped with a dual thin-layer Au/Hg amalgam working electrode housed in a CC-4 transducer kit (BAS, West Lafayette, IN, USA). The drug was detected at an oxidation potential of +0.125 V relative to an Ag/AgCl reference electrode; the electrometer sensitivity was adjusted to 2 nA/V. The amplified signal (10 mV) from the detector was connected to a strip-chart recorder (Model SE120, BBC Goerz/Metrawatt) to monitor real-time chromatographic tracings and a computer (HP3357 Lab Automation System, Hewlett-Packard, Palo Alto, CA, USA) to integrate peak signals. Polyetheretherketone (PEEK) tubing was purchased from Rainin Instrument, Woburn, MA, USA.

Reagents and solvents

The parent drug (**I**), **II** and the internal standard, N-[2(*S*)-(mercaptomethyl)-3-(2-methylphenyl)-1-oxopropyl]-L-ethionine (SCH 46557; **III**), were used as received from the Schering-Plough Research Institute Chemical Distribution Center (Bloomfield, NJ, USA). The chemical structures of these compounds along with other potential metabolites are shown in Fig. 1. Monochloroacetic acid (MCAA) was purchased from Mallinckrodt (Paris, KY, USA). All other chemicals except acetonitrile (HPLC grade) were of analytical-reagent grade. Expired human plasma was purchased from North Jersey Blood Center in East Orange, NJ, USA.

Preparation of thin-layer electrode surface

A commercially available polishing kit (Model PK-3, BAS) was used to polish the Au electrode according to the manufacturer's specifications. An additional polishing step was performed using a 1-μm diamond paste. Each freshly polished Au electrode was covered by the dropwise addition of triple-distilled Hg. After amalgamation had proceeded for 10 min, excess Hg was aggressively wiped from the electrode surface with a Kimwipe. The cell was then allowed to equilibrate overnight before installation into the detector assembly.

Chromatographic conditions

HPLC was performed on either YMC Basic (YMC, Morris Plains, NJ, USA) or Zorbax R_X-C_8 (MAC-MOD Analytical, Chadds Fords, PA, USA) columns with identical dimensions (150 × 4.6 mm I.D.) and silica particle size (5 μm). Each analytical column was protected by a Resolve silica Guard-Pak (Waters) pre-column. Reversed-phase separations were accomplished at ambient temperature using a mobile phase consisting of 0.1 *M* MCAA (pH 2.8)–acetonitrile (5:3, v/v). An aqueous solution of EDTA disodium salt (0.05 *M*; 8 ml) was mixed with 18.9 g of MCAA dissolved in ≈1800 ml of deionized water before adjusting to pH 2.8 by the dropwise addition of 10 *M* NaOH. After diluting to volume with water (2000 ml), organic modifier (1200 ml) was added, the mixture filtered (Nylon-66; 0.45 μm) and then degassed under reduced pressure. A flow-rate of 1.0 ml/min generated a back-pressure approximating 100 bar.

The solvent mixture was vigorously sparged (Part No. 28915; Alltech Assoc., Deerfield, IL, USA) with helium (*ca.* 20 ml/min) during use. A Waters solvent reservoir cap (Part No. 62341) was used to restrict access from the sovent supply, sparge inlet and vent lines. To maintain a system free of dissolved oxygen, all gas-permeable PTFE solvent inlet tubing was replaced with PEEK tubing (1.59 mm I.D. × 3.18 mm O.D.). The analytical column and detector were connected using 0.254 mm I.D. × 1.59 mm O.D. PEEK tubing.

Calibration standards and quality control sample preparation

Two analysts performed separate weighings of **II** and each amount (*ca.* 10 mg) was dissolved in an appropriate volume of methanol to accurately achieve a concentration of 1.0 mg/ml. These solutions were stored at −7°C in amber-glass test-tubes (15 ml). One solution was designated for the preparation of calibration standards while the other was used for quality control (QC) samples. A single solution (1 mg/ml) of the internal standard (**III**) was similarly prepared in methanol and stored at −7°C. All subsequent dilutions were performed with drug-free human plasma fortified with EDTA (1 mg/ml) and sodium hydrogensulfite (5 mg/ml) to improve the stability of **II** and **III**. All plasma calibration standards (0.01, 0.05, 0.1, 0.2, 0.5, 0.8 and 1.0 μg/ml) were freshly prepared before each analytical run. Typically, QC samples (0.02, 0.4 and 0.9 μg/ml) were prepared coincident with the receipt of clinical plasma samples and transferred (1.5 ml) to cryogenic vials (2 ml; Corning, Corning, NY, USA) for storage at −78°C.

*Extraction procedure for **II** from plasma*

A working solution of internal standard (0.8 μg/ml) was prepared within 1 h of use by diluting 40 μl of the 1 mg/ml methanolic solution with acetone in a 50-ml low actinic glass calibrated

flask. Aliquots (200 μl) of human plasma were transferred to 1.5-ml polypropylene microcentrifuge tubes (Lux Scientific Instrument, New York, NY, USA) using a calibrated Eppendorf digital pipette (Brinkmann, Westbury, NY, USA). Plasma proteins were precipitated and drug extracted by the addition of acetone (250 μl) containing the internal standard (0.2 μg). Each sample was mixed for 10 s using a Fisher Vortex Genie-2 (Bohemia, NY, USA) and then centrifuged for 10 min at 15 800 *g*, using a refrigerated (9°C) microcentrifuge (Eppendorf Model 5402). The resulting supernatant was transferred to a clean 1.5-ml microcentrifuge tube along with an aliquot (350 μl) of 0.15 *M* MCAA (pH 2.2) containing 0.2 m*M* EDTA. Each sample was then mixed thoroughly before injection (100 μl).

Extraction efficiency

The efficiency of drug extraction from plasma was determined using the following procedure. Compound **II** was added to drug-free plasma to achieve concentrations of 0.02, 0.05, 0.5 and 1.0 μg/ml. Samples ($n = 4$) from each concentration group were processed as described earlier with added internal standard (1.0 μg/ml) and 100 μl of each extract was injected for analysis by HPLC. The recovery of **II** and **III** was calculated by comparing the peak area of both compounds from extracted samples with those obtained from the analysis of equivalent amounts of drug prepared directly in 0.15 *M* MCAA (pH 2.2) with 0.2 m*M* EDTA.

Detector response

Standard curves were generated by two analysts over a 3-day period using plasma samples freshly spiked ($n = 4$) to contain from 0.01 to 1.0 μg/ml **II** and a fixed concentration (1.0 μg/ml) of internal standard. Peak-area ratios (**II**/internal standard) *versus* concentration of **II** were evaluated by a weighted ($1/x$) linear least-squares fit analysis. Similarly prepared samples which had been spiked with **II** (0.9, 0.4 and 0.02 μg/ml) and stored frozen (−78°C) were used as quality controls over this 3-day validation period.

Selectivity

The chromatographic behaviour of the prodrug (**I**), SCH 43102 (**IV**), the deacylated free thiol derivative of **I**, SCH 42447 (**V**), the de-ethylated free acid derivative of **I**, SCH 45298 (**VI**), the symmetrical disulfide of **II** and captopril were evaluated to determine their potential for assay interference.

Stability

The stability of **II** in plasma samples which were stored frozen (−78°C) over an extended period of time was examined as follows. Drug-free plasma (25 ml) pools, with and without stabilizing agents (sodium hydrogensulfite and EDTA), were spiked with **II** to yield concentrations of 0.02, 0.1 and 1.0 μg/ml. Multiple samples (16 × 1.5 ml) from each group were transferred to 2-ml cryogenic vials and stored at −78°C. At various times (10, 23, 60 and 106 days), three samples from each concentration group were analyzed as described above.

The stability of **II** and the internal standard in processed samples stored at ambient temperature (*ca.* 20°C) were also evaluated. Extracted plasma samples containing up to 0.5 μg/ml **II** were injected, kept on the benchtop and then re-assayed 24 h later. Drug concentrations determined from initial and repeat injections were compared by correlation analysis.

The effects of freezing and thawing plasma on the stability of **II** were investigated as follows: Two separate pools of plasma (25 ml), each containing 1 mg/ml EDTA and 5 mg/ml sodium hydrogensulfite, were spiked to contain 0.1 and 1.0 μg/ml **II**. Plasma was transferred (5 × 4.5 ml for each concentration) into cryogenic tubes, quickly frozen in a dry ice–acetone bath and then stored overnight at −78°C. The following day, samples at each concentration were allowed to thaw and remain at room temperature for either 30 or 60 min before extracting. Using the same specimens, this freeze–thaw and extraction cycle was performed sequentially for a total of three times during a single day. The remaining samples, kept in frozen storage, were used to complete three freeze, thaw and analysis cycles separated by 24

h. Concentration data generated within and between freeze–thaw days and cycles were compared by ANOVA.

RESULTS AND DISCUSSION

Chromatography and detector optimization

Assay validation was performed using two different (YMC and Zorbax) reversed-phase C_8 columns marketed for the separation of basic compounds. These columns appeared to provide protection from the peak tailing of free thiols which has been extensively reported [20]. Monochloroacetic acid was selected as the conducting salt for the mobile phase as it was available in high purity (low electrochemical background) and provided better peak symmetry than several other salts which were evaluated. EDTA was determined to be an essential component of the mobile phase because it stabilized the detector background and improved peak shape at the low concentrations of drug determined by this assay. The composition of the mobile phase did not require modification to accommodate either the YMC basic or Zorbax R_x-C_8 columns. Optimum chromatographic and detector behavior was observed when the mobile phase pH ranged from 2.2 to 2.8; baseline separation was achieved between **II**

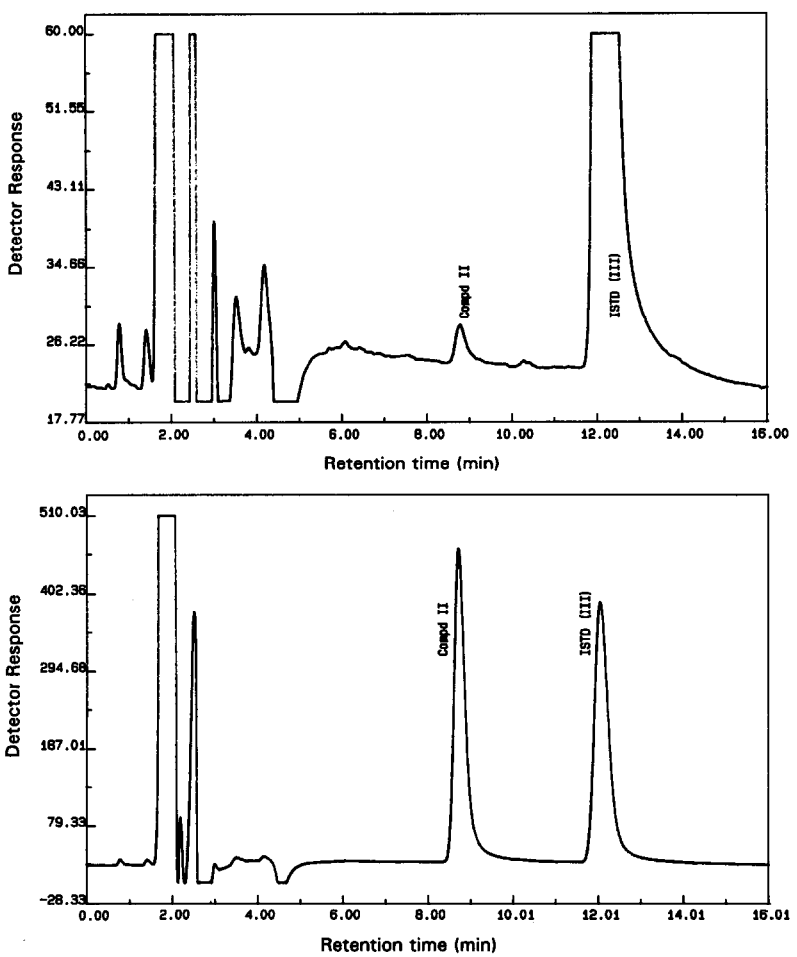

Fig. 2. Representative chromatograms of extracts from plasma spiked to contain 0.01 μg/ml (top) and 1 μg/ml (bottom) **II**. Internal standard (**III**) was added (1 μg/ml) during extraction.

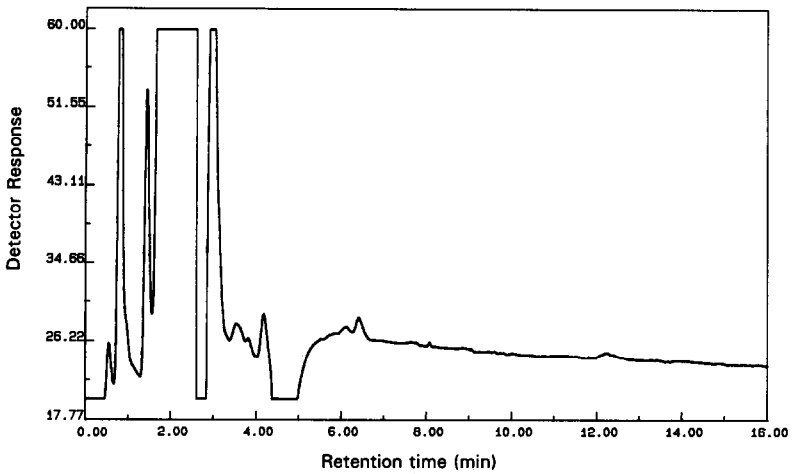

Fig. 3. Representative chromatogram of an extract from drug-free human plasma.

(≈9 min) and **III** (≈12 min). In general, there was no significant difference in the relative separation of these compounds on either column; both columns were interchangeable and have provided equivalent results. Representative chromatograms from plasma spiked to contain **II** in amounts encountered at the extreme ends of the calibration graph (0.01 and 1.0 μg/ml) are shown in Fig. 2. Extracts from drug-free plasma were devoid of endogenous interfering peaks (Fig. 3).

The source of stainless steel (*i.e.*, tubing, column, sparging element) was a major factor which affected the electrochemical background (offset current) of the LC–ED system and must be carefully evaluated. Where feasible, passivation of stainless-steel surfaces can be achieved with 6 *M* HNO_3. The final performance of the BAS thin-layer electrode is highly dependent on carefully polishing the Au surface before amalgamation; a smooth high-gloss finish produced the best result. A newly prepared electrode required at least 4–8 h of "burn-in" time before the offset current reached a normal operational range (−0.5 to +0.5 nA). We have experienced a 5–7 week lifetime for these electrodes under constant use (*ca.* 200 samples/week). The cell volume is dependent on the placement of one or more gaskets of known thickness between the steel auxiliary electrode and the working electrode block. After evaluating several combinations of gaskets ranging in thickness from 0.051 to 0.381 mm, optimum sensitivity was obtained with a single 0.051-mm gasket. A rapid loss of detector sensitivity (peak area) to **II** was observed on increasing the cell space thickness (Fig. 4).

Standard curve linearity

The integrated peak-area ratio of **II** (0.01–1.0 μg/ml) to internal standard (1 μg/ml) was chosen as the measure of detector response. These data were then subjected to weighed ($1/x$) linear regression analysis to determine the best-fit straight-line relationship between detector response and the concentration of **II**. A comparison of these mean least-squares fit parameters for five standard curves generated over a 10-week period revealed a highly linear response between 0.01 and 1.0 μg/ml ($r^2 > 0.99$). The intercepts did not significantly differ from zero at the 95% confidence interval. The sensitivity (slope) of the response curve varied by less than 13% over the same analysis period. Weighted ($1/x$) regression using either peak-height or peak-area ratios of **II** to the internal standard were found to provide equivalent results.

Standard curve accuracy and precision

Concentrations of drug in plasma were quanti-

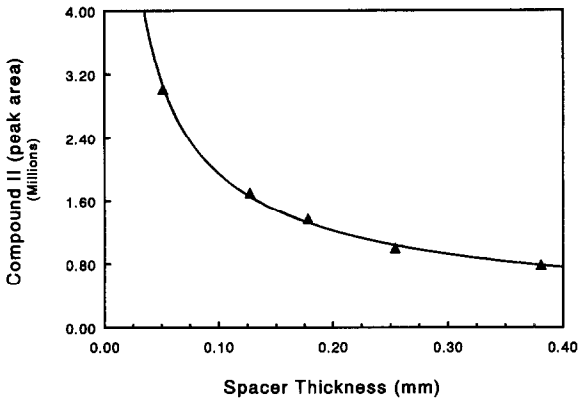

Fig. 4. Relationship between detector response (peak area) to compound **II** and Au/Hg electrode cell spacer thickness (mm).

tated by inverse-prediction following weighted $(1/x)$ least-squares fit analysis of standard curve data. Daily mean intra-assay results from calibration standards prepared by both analysts revealed that accuracy (bias) ranged from −2.8 to +3.0% whereas the precision (coefficient of variation, C.V.) varied from ±2.7 to ±14.9%. Pooled (n = 24) standard curve statistics are summarized in Table I. Inter-assay bias ranged from −2.0 to +1.0% over the entire standard curve range whereas the C.V. varied from ±3.8 to ±11.4%; excellent accuracy and precision were achieved by both analysts over this 3-day validation period. Based on these results, the lower limit of quantitation (LOQ) was established at 0.01 μg/ml.

TABLE I

INTER-ASSAY PERFORMANCE SUMMARY OF POOLED STANDARD CURVE DATA FOR COMPOUND **II** DURING THE TWO-ANALYST 3-DAY VALIDATION STUDY (n = 24)

Theoretical concentration (μg/ml)	Mean concentration found (μg/ml)	C.V. (%)	Bias (%)
1.00	0.996	4.51	−0.38
0.80	0.804	4.02	0.46
0.50	0.502	3.89	0.44
0.20	0.200	3.82	0
0.050	0.049	6.49	−2.00
0.010	0.0101	11.36	1.00

TABLE II

INTER-ASSAY PERFORMANCE SUMMARY OF POOLED (n = 36) QUALITY CONTROL DATA FOR **II** FROM THE TWO-ANALYST 3-DAY VALIDATION STUDY

Theoretical concentration (μg/ml)	Mean concentration found (μg/ml)	C.V. (%)	Bias (%)
0.90	0.94	4.90	4.49
0.40	0.41	5.75	3.50
0.020	0.022	12.18	9.50

Quality control accuracy and precision

Plasma samples which had been spiked with **II** to contain 0.9, 0.4 and 0.02 μg/ml and stored frozen (−78°C) were used for quality control over the same 3-day validation period. Between analysts, intra-assay bias ranged from −0.5 to 19.5% whereas the precision of these determinations varied from ±2 to ±8.6%. Inter-assay statistics estimated from pooled (n = 36) quality control data generated over the 3-day validation period are summarized in Table II and show that relative accuracy ranged between +3.5 and +9.5%, whereas the precision varied from ±4.9 to ±12.2%.

Extraction efficiency

The mean recovery of **II** from human plasma at concentrations ranging from 0.02 to 1.0 μg/ml was determined to be 89 ± 4.8% whereas the mean recovery of internal standard (**III**) was 81.5 ± 2.0%. A comparison of results by ANOVA detected no statistically significant ($p < 0.05$) difference in the recovery of **II** as a function of concentration.

Selectivity

A summary of retention times observed fol-

TABLE III

RETENTION TIMES FOR STANDARDS AND METABOLITES SEPARATED BY YMC OR ZORBAX C_8 HPLC COLUMNS AND DETECTED BY UV (254 nm)

Compound	Retention time (min)	
	YMC-Basic	Zorbax R_x-C_8
II	8.3	9.4
V	9.1	10.6
III	11.2	13.5
IV	22.9	24.2
I	26.0	28.6
VI	26.4	28.6
Captopril	<3	<3

lowing UV (254 nm) detection of standards and/or potential metabolites is provided in Table III. As only free thiols respond to the Au/Hg amalgam electrode, no interference was observed with disulfides or metabolites with an intact thioacetyl group. Captopril, a free thiol, is only minimally retained (retention time < 3 min); co-administration of this antihypertensive drug would not be expected to interfere in this assay. Compound **IV**, another human plasma *ex vivo* metabolite, is a deacylated co-extractant which could potentially interfere with the quantitation of either **II** or the internal standard. However, the retention time for **IV** is approximately 24 min and far removed from **II** and the internal standard peaks.

Stability on storage at low temperature

Long-term stability data for **II** added to plasma and stored frozen (−78°C) are provided in Table IV. A comparison of results from unstabilized and stabilized plasma suggest a significant improvement in the recovery of **II** at all concentrations by the addition of EDTA (1 mg/ml) and sodium hydrogensulfite (5 mg/ml). With stabilized plasma, assay values for the 1.0, 0.1 and 0.02 μg/ml concentrations of **II** on day 106 were determined to be 1.02, 0.103 and 0.022 μg/ml, respectively. Without stabilization, significantly lower concentrations of **II** were observed (0.897, 0.088 and 0.018 μg/ml), respectively, in these groups. Improved recovery is likely to be due to the mild reducing capacity of sodium hydrogensulfite and the ability of EDTA to chelate metal cations responsible for accelerating thiol oxidation. Over the 106-day study period, no pattern suggestive of drug loss was observed at any concentration.

Stability of processed samples

A statistical comparison of initial concentration data *versus* repeat results from the same extracts stored at ambient temperature and injected 24 h later demonstrated a strong degree of correlation ($r > 0.999$) and a slope (0.997) which approached theoretical unity. These findings confirmed that a stable response to the drug in processed samples was maintained at room temperature for at least 24 h.

TABLE IV

COMPARATIVE RESULTS FROM A 106-DAY STABILITY (−78°C) STUDY IN HUMAN PLASMA SPIKED TO CONTAIN 0.02, 0.1 AND 1 μg/ml COMPOUND II

n	Day No.	Concentration found (μg/ml)		
		1.00 μg/ml	0.100 μg/ml	0.020 μg/ml
Unstabilized plasma				
3	10	0.95	0.0937	0.0184
3	23	0.89	0.0860	0.0189
3	60	0.87	0.0896	0.0165
3	106	0.89	0.0829	0.0167
Mean		0.897	0.0881	0.0176
C.V. (%)		1.28	3.81	7.57
Bias (%)		−10.27	−11.90	−12.00
Plasma stabilized with EDTA and sodium hydrogensulfite				
3	10	1.11	0.114	0.0217
3	23	1.01	0.102	0.0221
3	60	1.00	0.100	0.0208
3	106	1.02	0.103	0.0216
Mean		1.036	0.105	0.0216
C.V. (%)		1.25	1.34	3.04
Bias (%)		0.98	4.80	8.00

TABLE V

RESULTS FROM A 3-DAY FREEZE–THAW STABILITY STUDY IN HUMAN PLASMA SPIKED TO CONTAIN 0.1 AND 1 μg/ml COMPOUND II

Observed	Concentration found (μg/ml)			
	30 min		60 min	
	1.00 μg/ml	0.100 μg/ml	1.00 μg/ml	0.100 μg/ml
Day 1				
Mean	1.08	0.102	1.07	0.101
C.V. (%)	5.6	9.74	8.53	4.68
Bias (%)	7.55	2.40	6.86	0.80
Day 2				
Mean	0.86	0.090	0.933	0.085
C.V. (%)	5.20	6.20	0.99	5.05
Bias (%)	−13.87	−10.30	−6.75	−14.9
Day 3				
Mean	1.01	0.098	0.991	0.101
C.V. (%)	3.53	8.67	1.61	11.97
Bias (%)	1.05	−1.80	−0.9	1.30
Pooled				
Mean	1.02	0.099	1.02	0.098
C.V. (%)	8.96	8.99	7.88	8.43
Bias (%)	1.56	−1.30	2.15	−2.3

Freeze–thaw stability

An examination of data from the freeze–thaw experiments indicated no significant decomposition of **II** in plasma followed repeated ($n = 3$) freezing and thawing (Table V). No significant ($p < 0.05$) differences were observed between samples thawed for 30 or 60 min. Even under extreme conditions, plasma samples thawed for a total of 180 min during the same day or for 60 min over each of three consecutive days exhibited excellent stability. Mean recovery of drug was within ±2.3% of each nominally prepared test concentration.

Assay feasibility

Plasma samples from hypertensive subjects treated (Q12H) with 12.5–200 mg of prodrug **I** were assayed for the active metabolite **II**. The feasibility of this HPLC assay was clearly established by the determination of significant concentrations of **II** in plasma collected from all subjects. Mean plasma concentration–time results, following the last dose of **I** administered (100 mg) to eighteen subjects, are shown in Fig. 5. A minor electrochemically active metabolite, not observed before this clinical study, appeared between **II** and the internal standard and was well resolved by the HPLC system (Fig. 6). The daily sample throughput of the assay is *ca.* 60 samples per analyst.

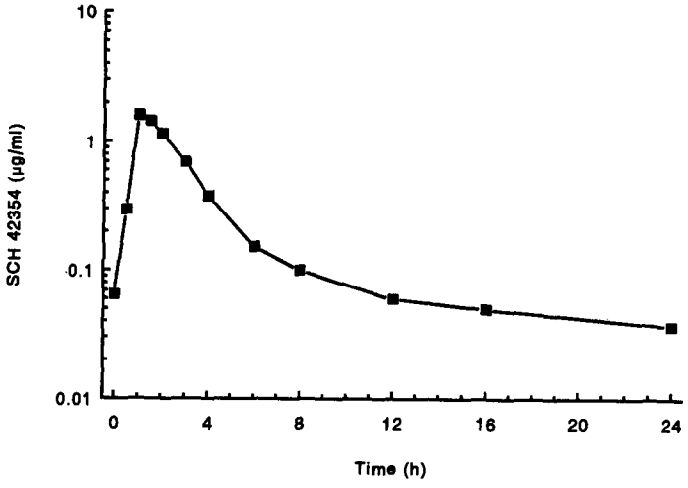

Fig. 5. Mean plasma concentrations (μg/ml) of SCH 42354 (active metabolite **II**) in mild-to-moderate hypertensive subjects orally treated with 100 mg of SCH 42495 (**I**).

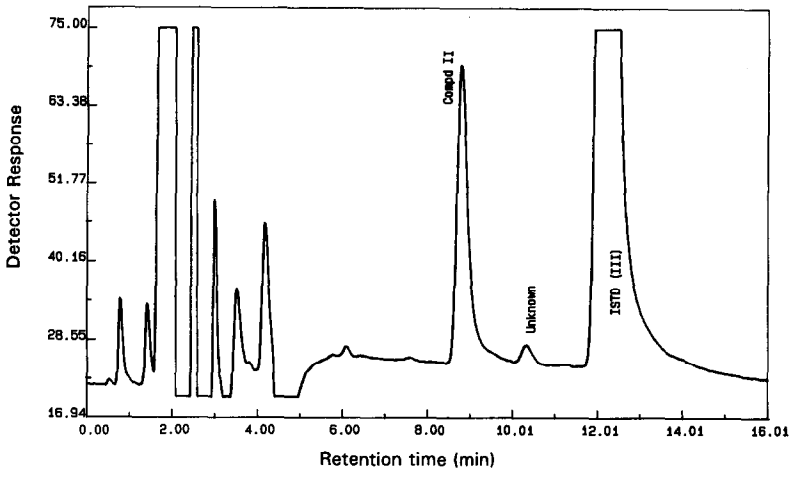

Fig. 6. HPLC chromatogram demonstrating the separation of **II** (retention time ≈ 9 min), a minor metabolite (retention time ≈ 10 min) and the internal standard (retention time ≈ 12 min) in a clinical plasma sample.

CONCLUSIONS

An HPLC assay requiring minimal sample preparation was validated in human plasma for the determination of **II**, the active metabolite of the prodrug **I**. Plasma (200 μl) was processed by protein precipitation with acetone containing the internal standard. Compound **II** was recovered (≈90%) in the supernatant after centrifugation and prepared for injection by the addition of 0.15 *M* monochloroacetic acid containing 0.2 m*M* EDTA. Separation of **II** and the internal standard (**III**) was accomplished on either of two different commercially available reversed-phase C_8 columns. Amperometric detection of **II** on a thin-layer Au/Hg amalgam electrode exhibited a linear response from 0.01 to at least 1.0 μg/ml. The lower LOQ using 200 μl of plasma was established at 0.01 μg/ml; inter-assay accuracy and precision remained within 15%. Chromatographic profiles from drug-free plasma were devoid of interfering peaks from endogenous co-extractants. The assay is specific for **II** in the presence of the parent drug and metabolites. As only thiol-containing compounds are electrochemically responsive, it is unlikely that many co-administered drugs would interfere in this assay. Compound **II** could be stabilized in human plasma for at least 106 days of storage at −78°C by the addition of EDTA and sodium hydrogensulfite. No significant decomposition of **II** in EDTA and sodium hydrogensulfite fortified plasma was observed following repetitive freeze–thaw cycles. The daily sample throughput of the assay is about 60 samples per analyst.

ACKNOWLEDGEMENTS

The authors thank Drs. J. A. F. de Silva, M. C. Scott and M. N. Cayen for their helpful suggestions during the preparation of this manuscript.

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