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Quantification of a dual angiotensin I-converting enzyme–neutral endopeptidase inhibitor and the active thiol metabolite in dog plasma by high-performance liquid chromatography with ultraviolet absorbance detection

Timothy G. Heath*, D. Darlene Massad, Janice I. Carroll, Bradley S. Mathews, Jim Chang, Dennis O. Scott, Be-Sheng Kuo[1], Paul C. Toren

Marion Merrell Dow, P.O. Box 9627, Kansas City, MO 64134-0627, USA

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Abstract

MDL 100,240 ([4*S*-[4α,7α(R^*), 12bβ]] - 7 - [[2 - (acetylthio) - 1 - oxo - 3 - phenylpropyl]amino] - 1,2,3,4,6,7,8,12*b* - octahydro - 6 - oxo - pyrido[2,1 - *a*][2]benzazepine - 4 - carboxylic acid, I) is the thioacetyl prodrug of the active thiol, MDL 100,173 (II), a dual inhibitor of angiotensin-I converting enzyme (ACE) and neutral endopeptidase (NEP). A drug which simultaneously inhibits both ACE and NEP may provide a unique therapy for hypertension and congestive heart failure. Methods based on high-performance liquid chromatography with UV absorbance detection at 200 nm were developed to support preclinical pharmacokinetic investigations. One method is used to measure unchanged I and free II, while the second method is used to quantify the total level of the thiol II after the plasma is incubated with the disulfide reducing agent, dithiothreitol. By either method, the analytes are quantified over the range of 25–1000 ng/ml with good accuracy and precision. The overall extraction efficiencies of unchanged I and free II in dog plasma were 79% and 86%, respectively, while the extraction efficiency of total II averaged 75%. Described in this report are the results obtained in validating the assay methods for measuring the compounds in plasma. Pharmacokinetic data are presented which were obtained by applying these methods to plasma collected from dogs dosed with I.

1. Introduction

Inhibition of angiotensin I-converting enzyme (ACE, EC 3.4.15.1) and neutral endopeptidase (NEP, EC 3.4.24.11) concurrently is being pursued as a novel therapeutic approach for treating hypertension and congestive heart failure [1–3]. Clinical benefits may be achieved from compounds which limit the formation of angiotensin II via ACE inhibition and prevent atrial natriuretic peptide (ANP) metabolism through NEP inhibition. The orally active thioacetyl prodrug MDL 100,240 ([4*S* - [4α,7α(R^*), 12bβ]] - 7 - [[2 - (acetylthio) - 1 - oxo - 3 - phenylpropyl]amino] - 1,2,3,4,6,7,8,12*b* - octahydro - 6 - oxo -

* Corresponding author.

[1] Present address: Parke-Davis Pharmaceutical Research Division, Warner Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, USA.

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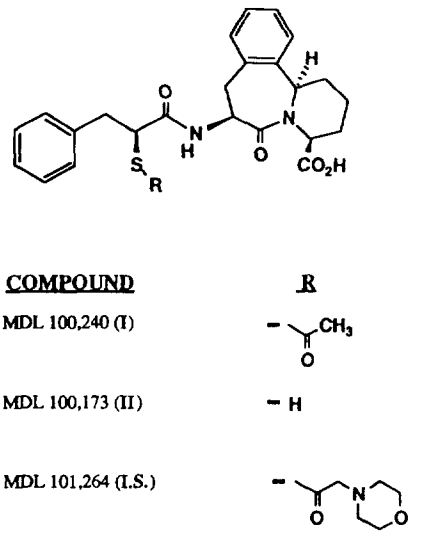

Fig. 1. Structures of MDL 100,240 (I), MDL 100,173 (II) and MDL 101,264 (I.S.).

pyrido[2,1 - *a*][2] benzazepine-4-carboxylic acid (I), Fig. 1), is being evaluated as a drug candidate in preclinical studies. The active metabolite, MDL 100,173 (II), is a competitive inhibitor of both ACE ($K_i = 0.11$ n*M*) and NEP ($K_i = 0.08$ n*M*) [1]. To support non-clinical pharmacokinetic and toxicokinetic studies, sensitive and selective methods were required for measuring levels of the active compound (II) and the parent drug (I) in plasma.

A method based on high-performance liquid chromatography (HPLC) was developed initially to quantify the levels of both unchanged I and free or unconjugated II at concentrations ranging from 25 to 1000 ng/ml in dog plasma. Following i.v. and oral dosing (12.5 and 25 mg/kg) of I in dog, however, plasma levels of both the parent drug and the active thiol, II, diminished rapidly. Plasma concentrations were measurable for <2 h. Therefore, a second method was developed to measure "total" II in dog plasma at levels of 25 to 1000 ng/ml. Determining "total" levels of thiol drugs has been reported previously, most notably with the thiol ACE inhibitor captopril [4–7]. Dithiothreitol (DTT), a reducing agent, released II covalently bound as a disulfide with endogenous thiols in plasma. For our analytical method, disulfide bound II was released by incubation of plasma with dithiothreitol. This treatment also converted unchanged I in the plasma to II. The levels of total II were measurable beyond 24 h in dogs dosed with I.

Presented in this report is a descriptive account of these two methods for measuring unchanged I, free II and total II, in dog plasma. The thioester analogue, MDL 101,264, was used in both assay methods as the internal standard (I.S.). Solid-phase extraction (SPE) was used to prepare the samples for HPLC analysis with UV absorbance detection at 200 nm. The methods were applied towards the determination of these compounds in dog plasma for defining pharmacokinetic parameters.

2. Experimental

2.1. Materials

MDL 100,240 (I), MDL 100,173 (II) and the internal standard, MDL 101,264 (I.S.) were obtained from Marion Merrell Dow (Kansas City, MO, USA). Acetonitrile, methanol, 2-propanol, and methylene chloride were all HPLC grade and purchased from the same source (Burdick and Jackson, Muskegon, MI, USA). Water was purified through a NANOpure II system (Barnstead, Dubuque, IA, USA) prior to use. The dithiothreitol (DTT), TRIZMA base and TRIZMA hydrochloride were obtained from Sigma (Sigma, St. Louis, MO, USA). The heparanized dog plasma was obtained from Pel-Freez Biologicals (Pel Freez Biologicals, Rogers, AK, USA). The C_8 Bond-Elut cartridges (Varian Analytical, Palo Alto, CA, USA) contained 100 mg of sorbent material with a 1-ml sample reservoir. The SPE vacuum manifold was a Supelco Visiprep No. 5-7030 (Supelco, Bellefonte, PA, USA) and a Turbo Vap LV Evaporator (Zymark, Hopkinton, MA, USA) was used to concentrate the extracts. Reagents used in the preparation of buffer solutions were of analytical reagent grade or better. Unless otherwise noted, solutions used in the preparation of samples were freshly prepared daily. Glassware was silanized with hexa-

methyldisilazane (HMDS) to prevent adsorption of analytes onto the surface. Glassware was placed in the vacuum oven (0.045 m^3) with HMDS (ca. 15 ml). The oven was brought under vacuum (ca. $3.4 \cdot 10^4$ Pa), isolated from the vacuum source and heated to 135°C. These conditions were maintained overnight [8].

2.2. HPLC apparatus

System A: quantification of I and free II

The HPLC system used to analyze unchanged I and unconjugated II (System A), consisted of Hitachi components, specifically L-6200A and L-6000 pumps, an AS-4000 autosampler, and a L-4250 UV detector (Hitachi Instruments, San Jose, CA, USA). The stainless steel Hypersil C_{18} HPLC column was a 250 × 4.6 mm I.D. column packed with material of 5 μm particle diameter (Phenomenex, Torrance, CA, USA).

System B: quantification of total II

System B, used in the analysis of total II, consisted of a Varian Model 2510 pump (Varian Analytical, Palo Alto, CA, USA), a Gilson Model 231 autosampler (Gilson, Middleton, WI, USA) and an LDC Analytical SpectroMonitor 4100, programmable variable wavelength detector (LDC Analytical, Riviera Beach, FL, USA). A precolumn line filter (Upchurch Scientific, Oak Harbor, WA, USA) containing a 0.5 μm frit served to filter the mobile phase prior to the HPLC column. Stainless-steel tubing with 0.178 mm I.D. was used for any precolumn interconnections, while the postcolumn tubing consisted of 0.127 mm I.D. PEEK tubing. The separation was achieved by a Hypersil C_{18} 250 × 2 mm I.D., 5 μm particle size, HPLC column (Phenomenex). The column was maintained at 30°C.

2.3. Data analysis

A VAX-based Beckman (Beckman Instruments, Fullerton, CA, USA) data acquisition system equipped with PeakPro chromatographic software was used to acquire the data. Peak heights were determined from the chromatograms for both the internal standard and the analytes. Linear regression analysis of the log-transformed data was used to fit the data where y is the peak-height ratio and x is the concentration in the equation $y = ax^b$. Unknown study sample levels were obtained by interpolation.

2.4. Chromatographic conditions

System A

The mobile phase consisted of 1 *M* phosphate buffer (pH 2.1) (KH_2PO_4)–water–acetonitrile–methanol–2-propanol (4:50:34:10:2, v/v). The mobile phase was stirred and sonicated briefly under vacuum to degas. The flow-rate was maintained at 1.0 ml/min and detection was at 200 nm. Following the separation, a step gradient introduced a second mobile phase consisting of 1 *M* phosphate buffer (pH 2.1)–water–acetonitrile–methanol–2-propanol (3:36:49:10:2, v/v). The flow was adjusted to 1.6 ml/min. After maintaining these conditions for 5 min, the system was reequilibrated with the initial mobile phase prior to the next injection. This step gradient served to elute endogenous components which were retained on the column.

System B

An isocratic mixture contained 1 *M* phosphate buffer (pH 4.1) (KH_2PO_4)–water–acetonitrile–methanol (2:53:30:15, v/v). The mobile phase was stirred and sonicated briefly under vacuum to degas. A flow-rate of 0.28 ml/min was maintained throughout the analysis. UV detection was at 200 nm.

2.5. Standard solutions

Stock solutions containing I and II (both at 1 mg/ml) and I.S. (0.1 mg/ml) were stable at 4°C for at least 2 months when stored in acetonitrile with 0.1% acetic acid. The spiking standards, prepared daily in acetonitrile (0.1% acetic acid), were at concentrations of 0.25, 1.0, and 5.0 μg/ml. The solution of I.S. used for the analyses was at a concentration of 3.2 μg/ml.

2.6. Tris–DTT solution

The 50 m*M* Tris solution pH 9, was prepared by adding 5.32 g of TRIZMA base and 0.96 g of TRIZMA hydrochloride to 1 l of water and stirring. The Tris–DTT solution was freshly prepared daily by adding DTT to 50 m*M* Tris buffer solution, pH 9, to yield a concentration of 0.7 mg/ml.

2.7. Sample preparation

Quantification of unchanged I and free II

The plasma sample volume for this method was 0.5 ml. Typically, pooled drug-free plasma was treated with additives prior to dispensing to individual silanized 12×75 mm culture tubes. Sodium fluoride (NaF) was added to plasma at a concentration of 2.5 mg/ml. For each milliliter of plasma, 100 μl of 0.1 *M* trisodium ethylenediaminetetraacetate (EDTA) and 50 μl of 1 *M* ascorbic acid were added. The sample was mixed thoroughly before each addition of stabilizer. To individual tubes, 575 μl of treated plasma was mixed with 50 μl of 6 *M* HCl. The samples were fortified with I and II at concentrations of 25, 50, 100, 200, 500 and 1000 ng/ml prepared in duplicate. A 100 μl volume of I.S. solution and 0.5 ml of water was added to each sample prior to analyte extraction.

Quantification of total II

For each milliliter of drug-free control plasma, 4 ml of freshly prepared Tris–DTT buffer solution was added and mixed thoroughly. Aliquots of 2.5 ml were added to culture tubes and each sample was treated with 0.25 ml of 6 *M* HCl and vortex-mixed. Duplicate samples were fortified with II to concentrations of 25, 50, 100, 200, 500 and 1000 ng/ml and 100 μl of I.S. solution was added.

2.8. Analyte extraction

After the samples were prepared, the solid-phase extraction (SPE) methodology for each of these analyses was identical. Samples were vortex-mixed for 1–2 min and then centrifuged (2000 *g*, 10 min) prior to SPE.

The C_8 Bond Elut SPE cartridges were placed in the vacuum manifold and preconditioned by rinsing with 1 ml of methanol and 3 ml of dilute HCl (pH 2.5). The plasma samples were transferred to the cartridges and the valves were opened half a turn with an applied vacuum of approximately $7.5 \cdot 10^4$ Pa. Cartridges were washed sequentially with 3 ml of dilute HCl (pH 2.5), and 3 ml of a blend of HCl (pH 2.5)–acetonitrile (4:1, v/v). The vacuum was increased to $5 \cdot 10^4$ Pa, and the inside of the SPE cartridge was dried with a cotton swab. Compounds I, II and I.S. were eluted under a vacuum of $8.5 \cdot 10^4$ Pa, with a 1-ml mixture of methylene chloride–methanol (95:5, v/v). The samples were dried under a stream of nitrogen in a Turbo Vap evaporator set to 40°C. The remaining residue was reconstituted in either 100 μl of acetonitrile (0.1% acetic acid) for a 20-μl injection onto the system A (unchanged I and free II), or 50 μl for a 10-μl injection onto the system B chromatograph (total II).

2.9. Assay validation

The assay methods were validated over the course of at least three days by the analysis of three concentration levels in the range of the calibration curve. Quality control (QC) samples were analyzed in triplicate; six replicates at each concentration were analyzed on one day of validation. The within-day reproducibility and accuracy were determined at three concentrations ranging from 25 to 1000 ng/ml from the measurement of six replicates. The between-day reproducibility was determined by comparing three days of analysis of samples run minimally in triplicate. The concentrations of QC samples prepared with the calibration standards and study samples were determined by the regression equation.

2.10. Extraction efficiency from dog plasma

For the analysis of unchanged I and free II, the extraction efficiency (E.E.) was determined

at concentrations of 25, 200 and 1000 ng/ml. Peak heights of replicated extracted standards were compared to the peak heights of corresponding nonextracted standards. The E.E. for the I.S. was determined at a concentration of 640 ng/ml. The E.E. of II in the assay for total II was determined at concentrations of 25, 100, 500 and 1000 ng/ml by comparing peak heights of extracted standards with peak heights of identical standards which were added to extracts of blank plasma. The E.E. for the I.S. (640 ng/ml) in the assay for total II was determined in a similar manner.

2.11. Efficiency of reducing disulfide linked II with Tris–DTT

Untreated dog plasma was fortified with II and maintained at room temperature to induce formation of disulfide linkages with endogenous plasma thiols. Aliquots of this sample were treated with Tris–DTT and incubated for 1 h at room temperature, while additional samples were treated with acidified Tris–DTT and immediately analyzed for II.

2.12. Efficiency of converting I to II with Tris–DTT

Total II includes I which has been hydrolyzed (or reduced) to II by the Tris–DTT buffer, pH 9. To determine the efficiency of this conversion, dog plasma was fortified with I to a level of 1000 ng/ml and incubated at 1 h with Tris–DTT buffer (4:1, v/v) prior to analysis for II.

3. Results and discussion

Thiols are highly reactive and can be oxidized to various products, including covalently bound disulfides [9]. Blood plasma contains endogenous thiols including peptides and proteins which may form disulfide conjugates with II. Due to the inherent instability of thiols, it was necessary to ensure that the concentration of free II at the time of sample collection was maintained until the samples were analyzed. This may be accomplished by forming a stable chemical derivative at the time of sample collection, or by adding stabilizing agents to the plasma. The method reported here is a modification of a published procedure used to stabilize the ACE inhibitor, captopril [7]; plasma was treated with an antioxidant, ascorbic acid, and a chelating agent EDTA to prevent oxidation of II. The sodium fluoride was added to inhibit esterase activity which could hydrolyze the thioester prodrug I to II. Representative chromatograms of extracts from drug-free plasma and plasma fortified with I and II are displayed in Fig. 2.

The second method was developed to measure total II in plasma. Representative separations of the components present in plasma extracts of total II are shown in Fig. 3. DTT chemically reduced disulfide bound II to the free thiol. Treatment with DTT also converted I to II. Because I was no longer measured in this assay, we sought to adjust the chromatographic conditions in an attempt to shorten the analysis time but were precluded from doing so because of matrix interferences in the plasma. Although the I.D.s of the HPLC columns were different, the C_{18} stationary phases were identical. However, as evident from Figs. 2 and 3, the elution order of II and I.S. was reversed. This reversal in elution order is attributed to pH and ionic strength differences in the mobile phases. These differences in mobile phase affected silanol interactions with the I.S. which contains a basic morpholine moiety (pK_a ca. 8.7).

The effect that ionic strength has on analyte retention at pH 2.1 is illustrated by chromatograms displayed in Fig. 4. These chromatograms were obtained when using a 250×2 mm I.D. Hypersil C_{18} column with 10, 20 and 40 m*M* phosphate buffer. Cation-exchange behavior of highly acidic residual silanols which remain unbonded on the silica surface significantly influences the retention of the basic I.S. [10–15]. The retention of the positively charged I.S. decreases with increasing ionic strength as the silanols become more effectively masked by potassium cations. This relationship of retention factor, $k_{I.S.}$, and potassium ion concentration, $[K^+]$, is illustrated graphically in Fig. 5. These data

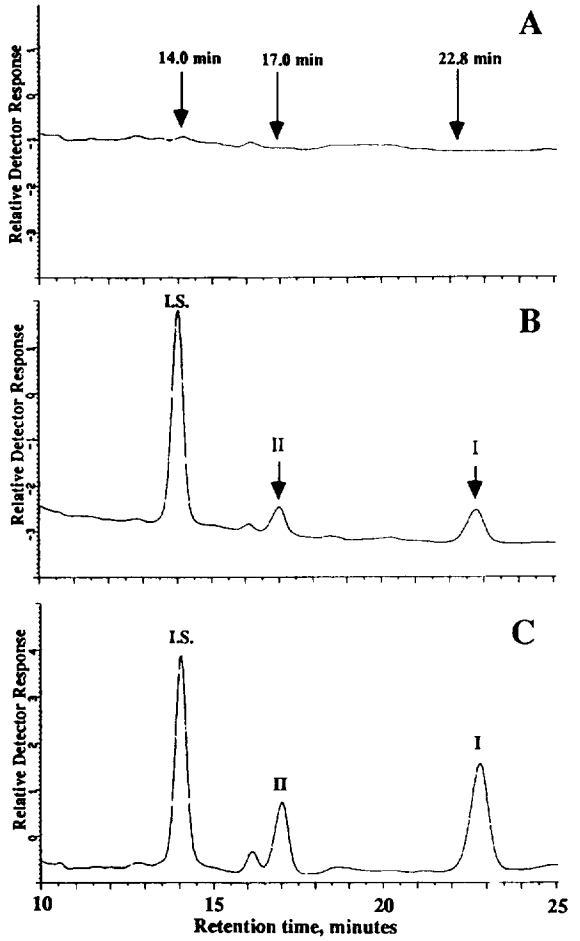

Fig. 2. Separation of components in extracts of (A) drug-free dog plasma, (B) dog plasma fortified with I and II at a concentration of 200 ng/ml and (C) dog plasma containing I and II at a concentration of 500 ng/ml, for the analysis of unchanged I and free II. The retention times of I, II and the I.S. are 22.8 min, 17.0 min and 14.0 min, respectively. These chromatograms were acquired using the system A chromatograph and conditions.

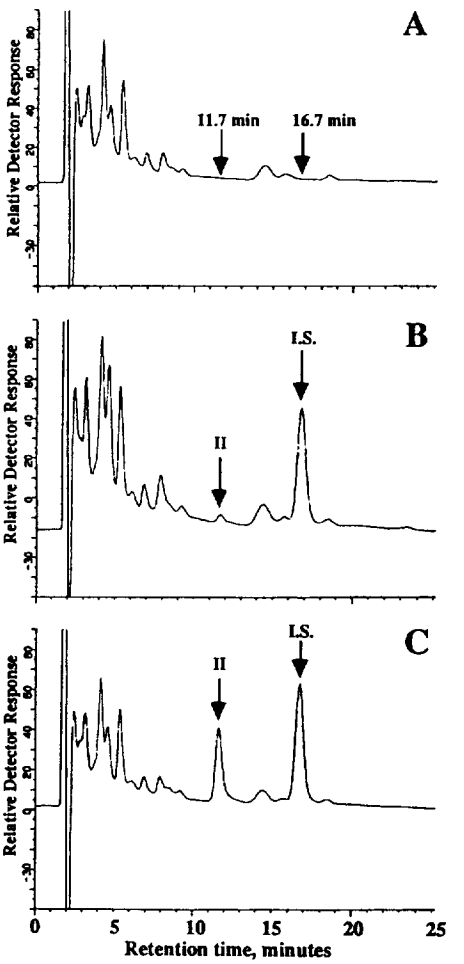

Fig. 3. Separation of components in extracts of (A) drug-free dog plasma, (B) dog plasma fortified with II at a concentration of 50 ng/ml, and (C) dog plasma containing II at a concentration of 500 ng/ml, for the analysis of total II. The retention times of II and I.S. are 11.7 and 16.7 min, respectively. These chromatograms were acquired using the system B chromatograph and conditions.

indicate that a predominant retention mechanism of the I.S. under these conditions is one of ion exchange [10,13–15]. The intercept correlates with reversed-phase retention mechanisms which should be observed at infinite potassium ion concentration [10,13–15]. Furthermore, the I.S. eluted at 41 min with poor peak symmetry when an unbuffered mobile phase (no potassium phosphate added) at pH 2.1 was used (data not shown). At pH 2.1, the retention of the I.S. could be manipulated by altering the ionic strength of the mobile phase. As seen in Fig. 4, modifications of the ionic strength while at pH 2.1 do not significantly influence the retention of II (pK_a ca. 4.3) which is not ionized at these conditions.

When the mobile phase contains 20 m*M* phosphate at pH 2.1, the I.S. elutes at 16.4 min and II elutes at 17.2 min (Fig. 4B). The I.S. elutes at 16.7 min, while II elutes at 11.7 min (Fig. 3B, C)

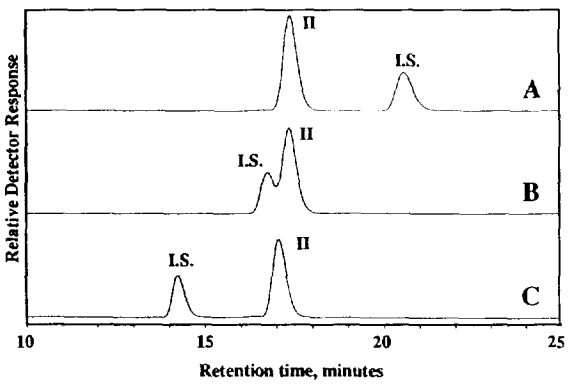

Fig. 4. Chromatograms of II and I.S. with mobile phase consisting of phosphate buffer, pH 2.1 at (A) 10 m*M* phosphate, (B) 20 m*M* phosphate, and (C) 40 m*M* phosphate. The mobile phase contained 30% acetonitrile and 15% methanol. The HPLC column was a Hypersil C_{18} 250 × 2 mm I.D., 5 μm particle size, and the flow-rate was 0.28 ml/min.

when using the same HPLC column with the mobile phase containing the identical phosphate and organic composition at pH 4.1. As the mobile phase pH is increased from 2.1 to 4.1, the retention time for II decreases due to an increase in the fraction of II which is ionized. The retention time of the I.S. does not significantly change by increasing the pH from 2.1 to 4.1, nor

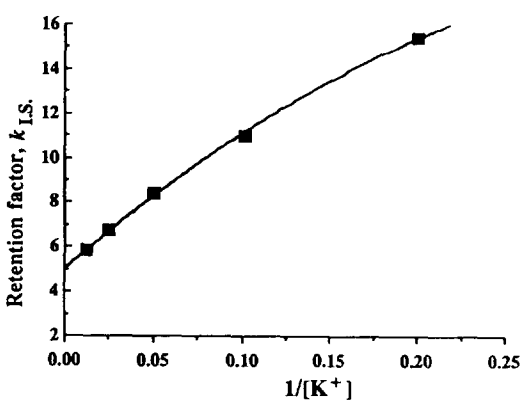

Fig. 5. Plot of retention factor, $k_{I.S.}$, of I.S. against inverse buffer concentration. The mobile phase consisted of 30% acetonitrile and 15% methanol with the aqueous phosphate buffer at pH 2.1. Determinations were made at 5 m*M*, 10 m*M*, 20 m*M*, 40 m*M* and 80 m*M* phosphate. The HPLC column was a Hypersil C_{18} 250 × 2 mm I.D., 5 μm particle size, and the flow-rate was 0.28 ml/min.

is the retention significantly influenced by varying the phosphate concentration of the mobile phase while at pH 4.1 (data not shown). However, the I.S. did not elute with an unbuffered mobile phase at pH 4.1 which clearly indicates the involvement of silanol interactions at these conditions.

3.1. Calibration curves

The calibration curves were constructed for both methods after analyzing duplicate standards and performing a logarithmic regression analysis of the peak height ratios versus the analyte concentration. The correlation coefficients of the calibration curves for the analysis of unchanged I and free II in dog plasma were typically >0.998 and >0.996, respectively. The correlation coefficient of the calibration curve generated for total II was typically >0.998.

3.2. Accuracy and precision

The accuracy and precision results determined over a three-day period for the analysis of I and free II are summarized in Table 1. The within-day relative standard deviations were less than 6% for both I and II, with relative errors ranging from −6.4% to 6.4%. For the between-day studies, the relative standard deviations were less than 16% and the relative error did not exceed 13%. The data in Table 2 present the accuracy and precision for the analysis of total II; the relative standard deviation was less than 14% and 20% for within- and between-day analyses, respectively, and the relative error was less than 11%.

3.3. Extraction efficiency

Unchanged I and free II

The extraction efficiencies for unchanged I and free II were determined by analysis of replicate samples of plasma containing both analytes at concentrations of 25, 200 and 1000 ng/ml. The overall E.E. of I determined for these concentrations was 79%, ranging from 74% at 1000 ng/ml to 91% at 25 ng/ml. The E.E. for II in

Table 1
Precision and accuracy data for determination of unchanged I and free II in dog plasma

Concentration (ng/ml)	Within-day ($n = 6$)			Between-day ($n = 12$)		
	Found	R.S.D.[a] (%)	R.E.[b] (%)	Found	R.S.D.[a] (%)	R.E.[b] (%)
Compound I						
25	23.4	5.9	−6.4	23.9	7.8	−4.4
200	196.7	3.7	−1.6	207.1	10.1	3.6
1000	1031.5	4.3	3.1	993.2	5.4	−0.7
Compound II						
25	26.6	4.0	6.4	24.1	16.2	−3.6
200	204.5	2.6	2.3	226.0	12.3	13.0
1000	998.3	3.2	−0.2	1081.8	10.4	8.2

[a] Relative standard deviation.
[b] Relative error.

plasma was 86%, with a range from 77% at 1000 ng/ml to 100% at 200 ng/ml. The E.E. for the I.S. at analyte concentrations listed above ranged from 80% at 1000 ng/ml to 87% at 25 ng/ml. The overall average for the E.E. of the I.S. which was at a concentration of 640 ng/ml, was 85%.

Total II

The mean E.E. of II in the assay method for total II at concentrations of 25, 100, 500 and 1000 ng/ml was 75% and the range was 68–79%. The E.E. of I.S. in plasma at concentrations for I indicated previously, averaged 93% with a range of 85–99%.

3.4. Freezer stability

Unchanged I and free II

Samples at concentrations of 50 ng/ml and 250 ng/ml were stored at −20°C and analyzed over the course of six weeks. The levels of unchanged I and free II measured in these samples were within ± 12% of their nominal values. These data demonstrate that the additives adequately stabilize I and II in dog plasma that is frozen at −20°C.

Total II

Early in our studies, it became apparent that storage at −20°C was not sufficient to preserve the stability of those samples which were not

Table 2
Precision and accuracy data for determination of total II in dog plasma

Concentration (ng/ml)	Within-day ($n = 6$)			Between-day ($n = 15$)		
	Found	R.S.D.[a] (%)	R.E.[b] (%)	Found	R.S.D.[a] (%)	R.E.[b] (%)
25	25.8	4.1	3.2	26.9	19.4	7.6
600	547.8	5.2	−8.7	572.1	5.2	−4.6
1000	1103.8	13.9	10.4	1066.6[c]	10.7	6.7

[a] Relative standard deviation.
[b] Relative error.
[c] $n = 12$.

treated with stabilizers. In addition, it appeared that sample stability at −20°C may be dependent on whether samples were prepared from stock plasma or collected from in vivo sources. Therefore, samples were stored at −70°C and stability was evaluated for samples fortified in vitro to 200 ng/ml and 1000 ng/ml, and for samples collected in vivo. After two months of storage at −70°C, the measured levels of total II for both in vitro and in vivo samples were within ± 10% of the day 0 concentrations.

3.5. Effect of Tris–DTT treatment

Thiol drugs in plasma readily form covalently bound mixed disulfide conjugates with low molecular mass endogenous thiols (e.g. glutathione, cysteine) or with proteins. Chemical reduction methods can be used to collectively measure all of the disulfide forms. To verify that Tris–DTT was effective in reducing disulfide conjugates of II, it was necessary to fortify dog plasma with II and induce the formation of disulfides in plasma. This was accomplished by adding II at a level of 1000 ng/ml to untreated dog plasma (no Tris–DTT or stabilizers added) and incubating at room temperature for 1 h. Analysis of these samples for free II indicated that the levels of II were <250 ng/ml. We postulated that this decrease in the concentration of II was due to formation of disulfide bonds with endogenous plasma thiols. Additional aliquots of this same fortified plasma were incubated with Tris–DTT and analyzed for II. The level of II measured was 961 ng/ml (R.S.D. 3.5%). These data suggest that incubation of dog plasma for 1 h at room temperature with Tris–DTT effectively reduces disulfide bound II.

Because we have defined that total II includes I that is hydrolyzed (or reduced) to II, we verified that this conversion goes to completion under the experimental conditions. Dog plasma was fortified with I to a level of 1000 ng/ml and treated with Tris–DTT buffer. After incubation for 1 h at room temperature, the samples were analyzed for II. If there were complete molar conversion of I to II, the concentration of II would be 913 ng/ml. Our measurement of the concentration of II was 868 ng/ml (R.S.D. 3.0%), which indicates that this Tris–DTT treatment results in complete conversion of I to II. Furthermore, there is no evidence in the chromatogram that I is present. The thioester I.S. is not converted to II because it is added after acidifying the plasma sample which precludes hydrolysis to the free thiol.

3.6. Specificity

These compounds lack strong UV chromophores and their λ_{max} are <200 nm. Nevertheless, the chromatograms of extracts of drug-free plasma (Figs. 2A and 3A) demonstrate the specificity and selectivity of the assays. No chromatographic peaks interfered with the quantification of analyte in either of the assays. The S-methyl form of II, detected as a metabolite in pharmacokinetic study samples, eluted ca. 1.5 min after II in both chromatographic methods.

3.7. Application

These methods were applied in determining drug-concentration–time curves after both i.v. and oral administration of I in male beagle dogs. The plasma concentration–time profiles after i.v. administration of 12.5 mg/kg of I in male beagle dogs are presented in Fig. 6. Levels of unchanged I and free II were below the limit of quantification (LOQ) after 0.25 h and 0.75 h, respectively. Total II averaged 83 ng/ml, 72 h after administration of the drug. The estimates of plasma half-lives derived from these data for I and free II were 0.04 h and 0.1 h, respectively. The terminal elimination half-life calculated for total II was 35.7 h. The area under the plasma concentration–time curve (AUC) determined for total II was nearly 10-fold greater than the sum of the AUCs for I and unconjugated II.

The chromatograms shown in Fig. 7 were obtained following the analysis of plasma collected 0.75 h after i.v. administration of 12.5 mg/kg of I and serve to demonstrate the significant differences in concentrations of unchanged I, free II and total II. The analysis for total II (Fig. 7B) required that the sample be diluted

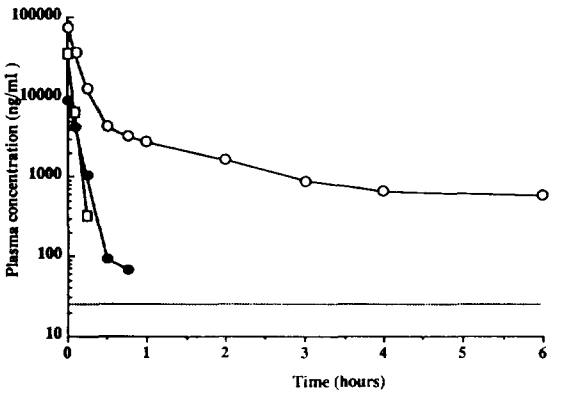

Fig. 6. Portion of the plasma concentration–time profiles of I (□), II (●), and total II (○) after i.v. adminstration of I at 12.5 mg/kg in male beagle dogs. Each point represents the mean of values measured for three dogs. The dashed line indicates the lower limit of quantification (LOQ) of 25 ng/ml. Total II (○) was detected 72 h after the dose was administered.

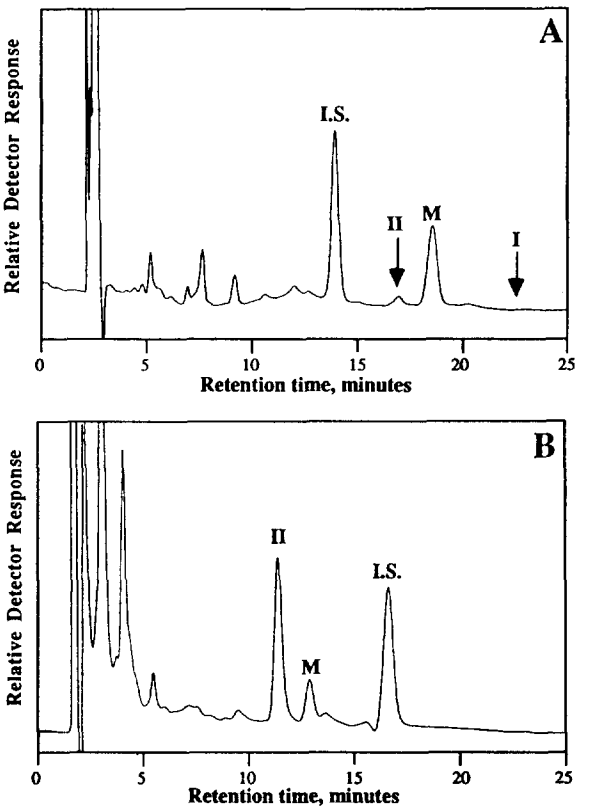

Fig. 7. Chromatograms obtained following the analysis of dog plasma collected 0.75 h after i.v. administration of 12.5 mg/kg of I: (A) unchanged I and free II, (B) total II. For the analysis of total II, the sample was diluted 5-fold with drug-free plasma so that the concentration measured was within the calibration curve. The measured concentration for I was less than LOQ, while free II was 83 ng/ml. The concentration of total II was 3690 ng/ml. The peaks labeled M represent the tentatively identified S-methyl metabolite of II. The chromatographic conditions are described in Section 2.

5-fold to ensure that the analyte concentration was within the range of the calibration curve. The concentrations of unchanged I were less than the LOQ while free II and total II concentrations were determined to be 83 ng/ml and 3690 ng/ml, respectively. A peak, M, was observed in both chromatograms and was tentatively identified as the S-methyl metabolite of II.

Previous studies with captopril, a free thiol containing ACE inhibitor, showed that captopril was eliminated rapidly from plasma due to the formation of covalently bound disulfide conjugates [16]. These data support the hypothesis that I is hydrolyzed to the active thiol in plasma and that II, like captopril, is rapidly conjugated with endogenous plasma thiols, providing a pathway for elimination. These results suggest that disulfide conjugates provide a reservoir for II, which may account for discrepancies observed in the duration of pharmacological activity with the concentration–time profile of free II in plasma.

4. Conclusion

Two similar methods are presented which rely on C_8 SPE and HPLC with UV absorbance detection at 200 nm. By appropriate adjustment of the mobile phase, silanol interactions effecting the chromatographic separation could be somewhat controlled, thereby offering a means to manipulate the retention time of the I.S., a feature which proved useful in developing the chromatographic methods. The first method provides the simultaneous measurement of the prodrug I and the free levels of active metabolite II, in dog plasma. Treatment of plasma with the reducing agent DTT permits the total level of II to be determined. These methods have been successfully applied in preclinical pharmacokinetic investigations of I in both dogs and rats.

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