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Use of dabsylation, column switching and chiral separation for the determination of a renin inhibitor in rat, marmoset and human plasma

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ABSTRACT

A high-performance liquid chromatographic method with column switching was developed for the determination of the renin inhibitor Ro 42-5892/001, (*S*)-α-[(*S*)-α-[(*tert.*-butylsulphonyl)methyl]hydrocinnamamido]-N-[(1*S*,2*R*,3*S*)-1- (cyclohexylmethyl)-3-cylcopropyl-2,3-dihydroxypropyl]imidazole-4 - propionamide methanesulphonate (1:1), in rat, marmoset and human plasma, using a Nucleosil C_8 120 (3 μm) stationary phase. Since the analyte and the internal standard are optical isomers, β-cyclodextrin was used as a mobile phase constituent for their chiral separation. The method took advantage of the characteristics of dabsyl chloride derivatives, namely sensitivity, specificity and, particularly, stability, providing a quantification limit of 5 ng/ml. The accuracy (range of inaccuracy 1–13%) and the inter-assay precision (coefficient of variation range 1.8–9.1%) were acceptable. The method was successfully applied to toxicokinetic studies in rats and marmosets.

INTRODUCTION

The renin–angiotensin system (RAS) is a multi-regulated proteolytic cascade involving renin and angiotensin-converting enzyme (ACE) [1]. It has been demonstrated that pharmacological interruption of the RAS can lower blood pressure in hypertensive patients [2]. Because of adverse effects associated with the use of ACE inhibitors, *e.g.* headache, excessive hypotension, renal impairment, hyperkalemia and angioedema [3], inhibitors of renin are under active investigation. The low systemic bioavailability and the rapid systemic clearance of renin inhibitors [4] require sensitive analytical methods for pharmacokinetic evaluation of these compounds.

The new developmental renin inhibitor Ro 42-5892/001 (I, Fig. 1) contains a secondary amino group, which is part of a histidine (His) residue. Sensitive analysis of amines is performed nowadays in many cases using derivatization. The precolumn derivatization of amino acids for high-performance liquid chromatography (HPLC) has commonly been carried out using 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride, DNS chloride) [5], 4-chloro-7-nitro-

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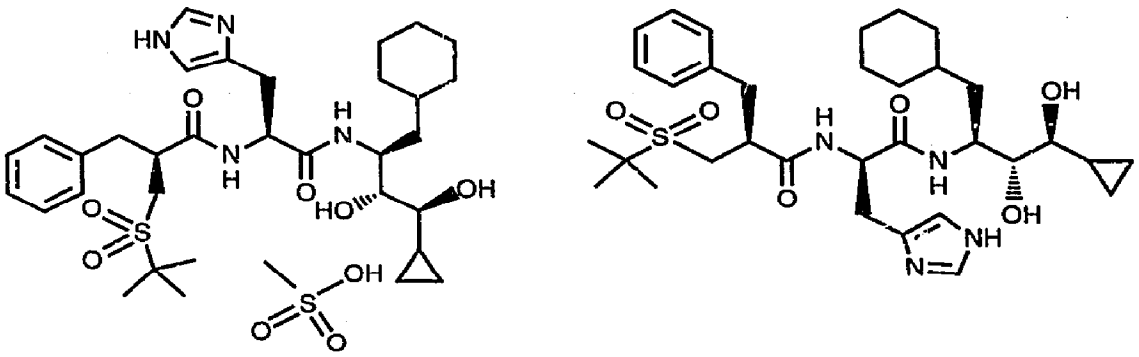

Ro 42-5892/001 (I) Ro 42-9067/000 (II)

Fig. 1. Structures of Ro 42-5892/001 (I) and Ro 42-9067/000 (II, internal standard).

benz-2,1,3-oxadiazole (NBD chloride) [6], 4-phenylspiro[furan-2-(3*H*),1′-(3′*H*)-isobenzofuran]-3,3′-diene (fluorescamine) [7], dimethylaminoazobenzene isothiocyanate (DABITC) [8], 9-fluorenylmethyl chloroformate (FMOC) [9,10] or 4-dimethylaminoazobenzene-4′-sulphonyl chloride (dabsyl chloride) [11–13].

The fluorescence intensity of dansyl chloride derivatives is markedly modified by factors such as temperature, pH, solvent polarity and exposure to light [13]. Since dansyl chloride reacts with amines and phenols, any coextracted materials or impurities containing these groups might produce interferences in chromatography [14]. NBD chloride is selective towards aliphatic amines [14] and NBD derivatives are sensitive towards light [6]. With fluorescamine, both the reaction rate and the fluorescence intensity are significantly influenced by the pH. Also, the stability of fluorescamine derivatives is not nearly as good as that of the other derivatives; thus storage of these derivatives for longer than a few hours is often impossible [14]. It appeared that even a trace amount of acetate salt could catalyse the decomposition of dimethylaminobenzene thiohydantoin (DABTH) amino acids [8]. A reduction of peak heights was noted after storage of FMOC derivatives in a refrigerator at 4°C for three days [9]. Derivatization with dabsyl chloride leads to very high sensitivity as well as selectivity. Indeed, the sensitivity of dabsyl chloride is *ca.* 60 times that of dinitrofluorobenzene (DNFB) [12].

The chromophoric properties of dabsyl derivatives allow determination in the visible region, and this avoids interferences caused by UV-absorbing substances in biological samples [13]. The stability of dabsyl derivatives is an additional advantage for the quantitative analysis of amines.

This paper describes an HPLC method comprising liquid–liquid extraction of plasma samples and chiral separation of the dabsyl derivatives of renin inhibitor I and the internal standard Ro 42-9067 (II, Fig. 1).

EXPERIMENTAL

Materials, reagents and solvents

The diastereoisomers Ro 42-5892/001 (I, renin inhibitor) and Ro 42-9067/000 (II, internal standard) were obtained from F. Hoffman-La Roche (Basel, Switzerland). Acetonitrile was purchased form Rathburn (Walkerburn, UK). Acetonitrile used for derivatization was distilled in all-glass apparatus in the laboratory. Methanol LiChrosolv, acetone, dichloromethane, potassium dihydrogenphosphate and potassium hydroxide were purchased from Merck (Darmstadt, Germany). Dabsyl chloride and glacial acetatic acid were provided by Fluka (Buchs, Switzerland). Water for the derivatization reagent (phosphate buffer) was obtained from J. J. Baker (Deventer, Netherlands). Water used for the mobile phase was obtained by distillation of purified water in all-glass apparatus. β-Cyclodextrin was supplied by Roquette (Lille, France). All reagents were of analytical grade.

All animal plasma used contained EDTA–NaF; human plasma contained sodium citrate as anticoagulant.

Preparation of standard solution of renin inhibitor I

The stock solution (M1) was prepared by dissolving 5.75 mg of renin inhibitor I (methanesulphonate salt, corresponding to 5.0 mg of free base) in 5 ml of methanol. Working solutions (M2 = 115 ng/μl; A = 11.5 ng/μl; B = 1.15 ng/μl) were obtained by successive dilution of the stock solution.

Preparation of standard solution of internal standard II

The stock solution and working solutions of the internal standard II were obtained in the same way as for renin inhibitor I. Exactly 5.00 mg were weighed, as this compound is the free base.

The stock and working solutions of both compounds were stored at 4°C. The stock solutions (M1) were used for ten weeks, M2 for one week, A for two days, and solution B for one day.

Preparation of spiked plasma samples

For analysis of unknown rat plasma samples, spiked plasma samples were prepared for calibration by using a stock solution of 1.15 mg/ml renin inhibitor I in methanol (corresponding to 1.00 mg free base per ml methanol) and a pooled rat blank plasma to obtain a plasma concentration of 10 μg/ml. Then, plasma samples containing between 3 and 100 ng free base per ml plasma were prepared by successive dilution with pooled rat blank plasma. Portions of 1 ml were transferred to glass tubes and stored at −20°C. The spiked plasma samples for the quality control (10, 20, 50 and 75 ng free base per ml plasma) and the limit of quantification samples (5 ng free base per ml plasma) were obtained by successive dilution of plasma at 10 μg/ml, prepared by using a different stock solution of

1.15 mg/ml renin inhibitor I in methanol. Portions of 1.0 ml were transferred to glass tubes and stored at −20°C.

For analysis of unknown marmoset plasma samples, calibration samples were prepared with rat plasma in the same way as for unknown rat plasma samples. However, the quality control samples used were a mixture of rat and marmoset plasma samples. First, a spiked rat plasma at 10 μg/ml was prepared with a stock solution of 1.15 mg/ml renin inhibitor I. Dilution with marmoset blank plasma gave a concentration of 1 μg/ml which, by further dilution with marmoset plasma, provided spiked plasma samples at 25 and 750 ng free base per ml plasma. Portions of 0.4 and 0.1 ml, for plasma at 25 and 750 ng/ml, respectively, were stored at −20°C. In addition, quality control samples in rat plasma were also prepared.

Sample preparation procedure

Extraction. Internal standard solution (100 μl, 1 ng II per μl methanol) was added to plasma (1.0 ml) in a conical glass tube. For marmoset samples, 0.1, 0.2 or 0.4 ml was made up to 1.0 ml with blank plasma from rats before addition of the internal standard. After thorough mixing, the pH of the mixture was raised to *ca.* 12 by addition of 85 μl of 1 *M* NaOH. Protein precipitation was then performed by addition of 800 μl of acetonitrile and 15 s mixing. After centrifugation for 10 min at 2000 *g* at room temperature, the supernatant was transferred to another tube and extracted with 2 ml of dichloromethane for 10 min on a Heidolph "head over head" agitator (Salvis, Reussbühl, Switzerland) set at speed 2. After 20 min centrifugation at 2000 *g* at room temperature, the upper layer was discarded and the organic phase was transferred to a 4-ml Chromacol 4-SV glass vial (Chromacol, London, UK) and evaporated at 70°C for 10 min under a helium stream.

Derivatization. Dabsyl chloride was not stable in solution in acetonitrile or acetone [15]. Therefore, a stock solution of dabsyl chloride in acetone (1.62 mg/ml; 5 m*M*) was prepared, portions (200 μl) of which were pipetted into 2-ml polypropylene tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany), evaporated (15 min in a vacuum centrifuge, Savant Insruments, Farmingdale, NY, USA) and stored at −20°C. Each portion of dabsyl chloride was redissolved in 200 μl of distilled acetonitrile shortly before use [15,16].

The residue of the plasma extract was dissolved in 50 μl of distilled acetonitrile and 25 μl of KH_2PO_4 buffer (0.025 *M*, pH 7.2) by mixing (15 s), and then 20 μl of dabsyl chloride solution (5 m*M* in distilled acetonitrile) were added. After mixing for 1 min, the mixture was transferred to a 0.3-ml Chromacol 03-CVG glass vial.

The vial was tightly stoppered with a crimp cap and heated for 120 min at 70°C; 70 μl of this reaction mixture were injected into the LC column.

Chromatographic system and conditions

A schematic representation of the column-switching system is given in Fig. 2. The main system, an HP 1090 liquid chromatograph (Hewlett Packard, Rock-

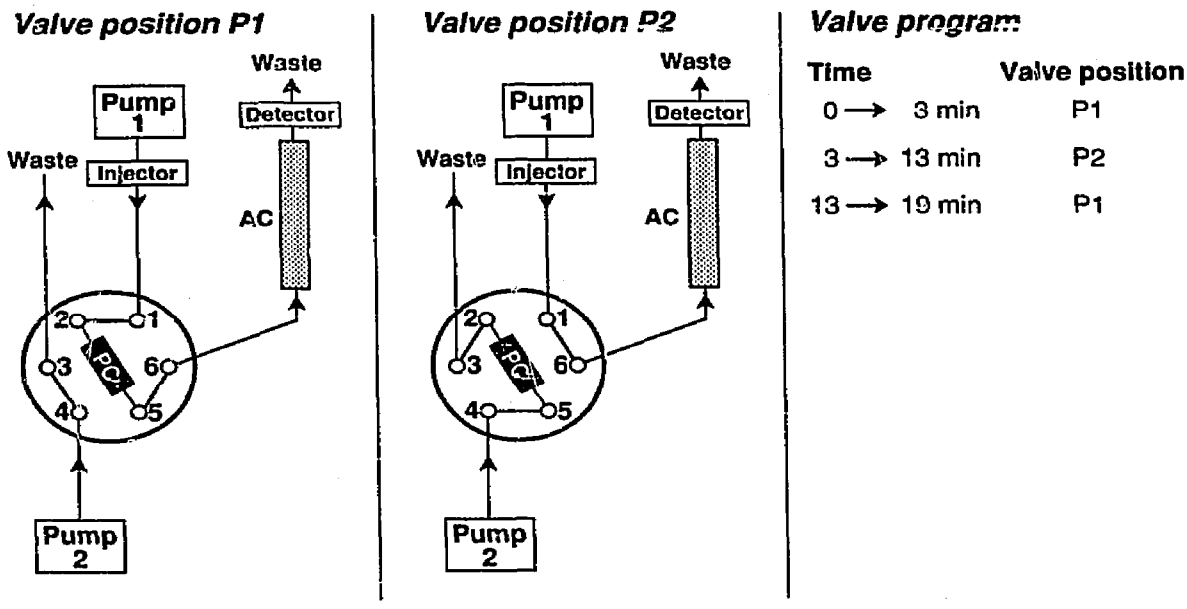

Fig. 2. Schematic representation of the column-switching system.

ville, MD, USA) delivered the mobile phase MP1 at a flow-rate of 1.5 ml/min for pre-separation and analytical separation of the compounds of interest. A Spectroflow 400 delivery system (Kratos, Karlsruhe, Germany) was used as pump P2, delivering mobile phase MP2 at a flow-rate of 1.0 ml/min for backflush elution of the retained compounds from the precolumn to waste.

An air-actuated switching valve (Model 70-00A, Rheodyne) was controlled by the external time events of a computing integrator (Model SP4200, Spectra-Physics, San José, CA, USA). A laboratory-made interface was placed between the integrator output and the valve input to achieve compatibility. The eluted compounds were detected at 478 nm with a Spectroflow 773 UV–visible detector (Kratos) equipped with a tungsten lamp, set at a rise time of 1 s and an absorption range of 0.005 a.u.f.s.

Data handling was performed by means of the computing integrator.

The guard column PC (14 mm × 4.6 mm I.D.) was packed with Nucleosil C_8 120 (3 μm) (Macherey Nagel, Düren, Germany). The analytical column AC (125 mm × 4 mm I.D.) contained the same material.

The mobile phase MP1 was acetonitrile–100 m*M* sodium acetate buffer (pH 3.5) containing 7 m*M* β-cyclodextrin (55:45, v/v). The mixture was formed in the HP 1090 liquid chromatograph. The buffer was used after filtration through a 0.45-μm filter (Millipore, Milford, MA, USA) and 10 min degassing with helium. The mobile phase MP2 had the same composition as MP1, but the acetonitrile–sodium acetate buffer mixture was prepared before use for the isocratic pump. In this case, a precipitate of β-cyclodextrin, sometimes formed during addition of acetonitrile, was redissolved by ultrasonication for 10 min.

The total sequence of automated sample analysis required 19 min and included the following three steps.

Step A (0-3 min, valve position P1): injection of the sample onto the PC. The compounds of interest were then transferred from the PC to the AC.

Step B (3–13 min, P2): MP1 directed into the AC for the analytical separation. The PC was purged with MP2 in the backflush mode.

Step C (13–19 min, P1): the PC and the AC were equilibrated with MP1.

Calibration and calculations

The concentrations of unknown samples were calculated from a linear calibration curve. This calibration curve was obtained by computing a weighted least-squares regression (weighting factor $1/y$) of the peak-height ratios y (derivatized renin inhibitor I/derivatized internal standard II) *versus* the renin inhibitor I concentrations x from six standard samples. The calculations were performed using an improved version of a special BASIC program described for the Spectra-Physics SP4100 integrator [17].

Recovery

The recovery was established for rat plasma by triplicate analyses of two renin inhibitor I concentrations, 6 and 75 ng/ml. The response for the worked-up sample (100 ng internal standard I were added before derivatization) was compared with that obtained by analysis of 1 ml of blank plasma, to which renin inhibitor I (6 or 75 ng) and internal standard II (100 ng) had been added at the end of the extraction procedure.

The extraction recovery test for marmoset plasma was performed on different volumes of plasma (0.1, 0.2 or 0.4 ml) adjusted to 1.0 ml with rat blank plasma. The results were compared with those obtained with 1.0 ml of rat plasma. Further details are given with the results.

RESULTS AND DISCUSSION

Sample preparation procedure

Extraction. Some attempts to perform rapid extraction of renin inhibitor I from plasma and to obtain cleaner extracts were made using Extrelut (Merck) and different types of solid-phase extraction cartridges with various elution conditions. The following solid-phase extraction materials were tried: C_{18}, a non-polar sorbent, the strong cation-exchange sorbent SCX, the weak cation-exchange sorbent CBA (Analytichem International, Harbor City, CA, USA), and the chiral sorbent Cyclobond I (Astec, Whippany, NJ, USA). In all cases, the result was not satisfactory owing to low recovery and/or poor selectivity.

Liquid–liquid extraction was therefore used. Because of the high protein binding of renin inhibitor I (77 ± 1% in human, 84.8 ± 0.6% in rat, 90.9 ± 0.2% in marmoset) [18], protein precipitation was performed before extraction. A 10%

(w/v) trichloroacetic acid aqueous solution (200 μl) gave good precipitation, but a poor recovery (17.6%) after extraction with dichloromethane. This could be due to the influence of an acidic pH. Different proportions of acetonitrile (10, 20 and 80% of plasma volume) were also checked for protein precipitation. For human plasma at pH 11, the highest recovery (67.7%) and the best selectivity were obtained with 800 μl of acetonitrile (corresponding to 80% of plasma sample volume), followed by extraction with dichloromethane.

The influence of the plasma pH on the recovery was investigated with human plasma. pH values form 8 to 13 were obtained by adding suitable volumes of appropriate buffer (Titrisol, Merck) to 1.0 ml of human plasma, and extraction was performed with butyl acetate or dichloromethane from the supernatant after protein precipitation with 800 μl of acetonitrile. For the two solvents, the highest recovery was obtained at pH 12–13 (Table I), and dichloromethane gave the best selectivity.

Derivatization. The following three experiments were carried out to optimize the derivatization conditions.

(1) The effect of the concentration of dabsyl chloride. It was important to use a minimum amount of dabsyl chloride (50 nmol) in the dabsylation mixture; below this concentration, the efficiency of the dabsylation was greatly reduced. A concentration of 5 m*M* was the upper limit for the dabsyl chloride solution; above this concentration, precipitation of dabsyl chloride in the reaction medium occurred.

(2) The effect of pH of aqueous buffer. The pH for dabsylation commonly lies between pH 8 and 9 [19]. The optimum pH for dabsylation of renin inhibitor I (pH 8.2) was obtained by addition of a KH_2PO_4 buffer (0.025 *M*) at pH 7.2. pH values from 8.5 to 11 were also tried, using 0.1 *M* $NaHCO_3$, but gave lower peak heights.

TABLE I

EXTRACTION RECOVERY OF RENIN INHIBITOR I FROM RAT AND HUMAN PLASMA

Plasma origin	Renin inhibitor I concentration (ng/ml)	*n*	pH	Recovery[a] (%)			
				Butyl acetate		Dichloromethane	
Rat	6	3	12			80.7 ± 9.9	
	75	3	12			75.9 ± 1.5	
Human	50	2	8	73.9	71.3	–	
	50	2	9	68.6	68.6	–	
	50	2	10	70.0	67.5	–	
	50	2	11	76.9	74.2	72.0	69.6
	50	2	12	83.9	81.2	90.2	87.9
	50	2	13	82.1	79.3	33.2	80.8

[a] For rat plasma, these values are mean ± S.D.; for human plasma, they are individual values.

(3) The effect of temperature and heating. As shown in Fig. 3, the dabsylation efficiency increased with decreasing temperature and increasing time. In contrast, the selectivity improved at higher temperatures. The best compromise was to heat the solution for 120 min at 70°C. These conditions justified the use of acetonitrile as organic solvent, instead of acetone, in spite of the better solubility of dabsyl chloride in acetone.

Analytical system and chromatography

The use of an optical isomer of renin inhibitor I as the internal standard required chiral separation. The separation of renin inhibitor I from internal standard II was performed using β-cyclodextrin in the mobile phase.

The property of cyclodextrins of cavity selectivity for many chemical species is well known. It is a function of the size of the guest molecule and the spatial disposition of its substituents [20]. The utilization of cyclodextrin inclusion processes in HPLC has been realized by two different approaches [21]. The first relies on the use of cyclodextrins chemically bonded to silica phases. One of the major limitations with this type of column is that the retention time is less reproducible than with a conventional column [22]. In the second approach, cyclodextrins are applied as mobile phase components in order to impart their selective complexation properties to reversed-phase (RP) systems. The addition of α- or β-cyclodextrin to a mobile phase in an RP system always results in a decrease in capacity factor (k') [21]. The use of β-cyclodextrin gave much better separation than α-cyclodextrin in our system.

The limit of solubility of β-cyclodextrin in water was *ca.* 18 m*M* [20]. However, because of the sparing solubility of β-cyclodextrin in a mobile phase containing more than 50% of acetonitrile as an organic modifier [23], the optimum conditions avoiding precipitation of β-cyclodextrin in the chromatographic system in

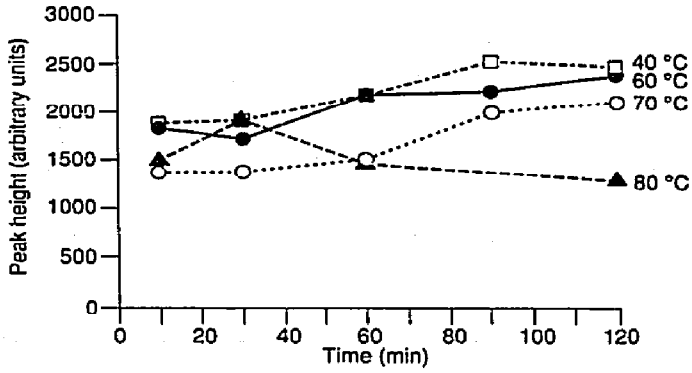

Fig. 3. Effect of temperature and time on the dabsylation efficiency. Direct dabsylation of renin inhibitor I (200 ng of free base) from a methanolic solution was performed as described in Experimental, except that four batches of ten samples were heated at 40, 60, 70 and 80°C, respectively. For each batch, two samples were heated for 10, 30, 60, 90 and 120 min. Samples of 30 μl were analysed by HPLC, and the respective peak heights were measured.

our case, was obtained with 55% acetonitrile and 45% 100 m*M* sodium acetate buffer (pH 3.5), containing 7 m*M* β-cyclodextrin. In order to reduce the time required for analysis, a column-switching technique was used for elution of late-eluting peaks (instead of gradient elution with acetonitrile).

Fig. 4 shows typical chromatograms obtained under the chromatographic conditions described for control human plasma (A), control rat plasma (B), control marmoset plasma (C), rat plasma spiked with renin inhibitor I and internal standard II (D), and rat plasma on the 48th day of a multiple oral dose of 500 mg/kg of renin inhibitor I per day (E).

Recovery

The recovery was 75–80% in the concentration range 6–75 ng per ml rat plasma (Table I).

A comparison between the recoveries from 1.0 ml of rat plasma and 0.1, 0.2 and 0.4 ml of marmoset plasma adjusted to 1.0 ml with rat blank plasma was performed. All samples contained the same amount of renin inhibitor I (50 ng). For all volumes of marmoset plasma tested, the difference in the relative recovery was less than 5% (Table II).

Selectivity

Several human control plasmas (n = 10) and marmoset (plasma pool, n = 4) and rat (plasma pool, n = 86) samples were analysed. Most of these (9 out of 10 for human plasma, 75 out of 86 for rat plasma and all of the pooled marmoset plasma) showed no interference with renin inhibitor I and the internal standard II. These results indicated that the selectivity of the chromatographic system was adequate.

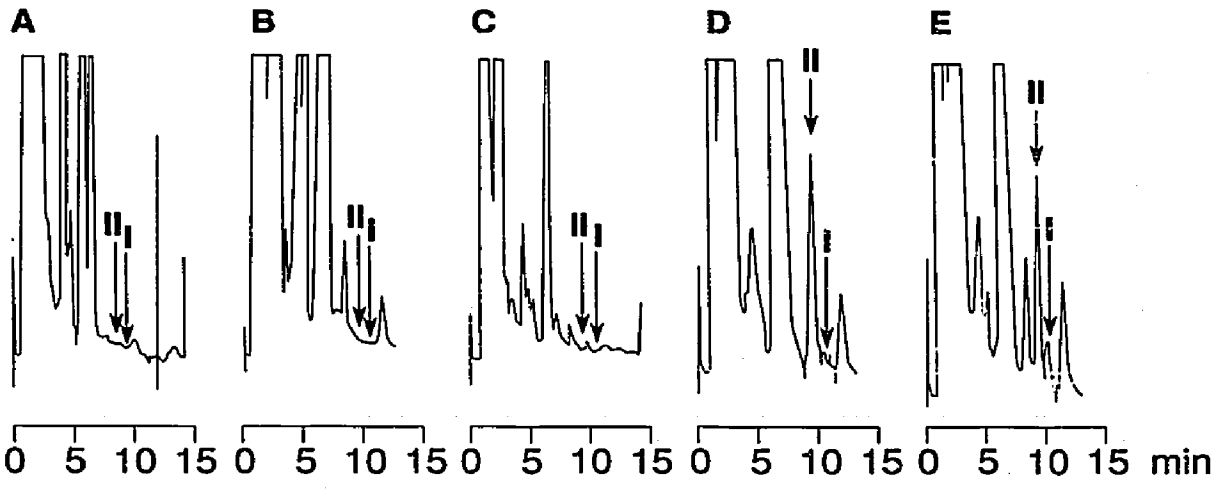

Fig. 4. Typical chromatograms from human control plasma (A), rat control plasma (B), marmoset control plasma (C), rat plasma spiked with I (5 ng/ml) and II (100 ng) (D), and rat plasma on the 48th day of multiple oral dose of 500 mg/kg per day (concentration of I: 14.3 ng/ml) (E). Peaks: I = Ro 42-5892; II = internal standard Ro 42–9067.

TABLE II

COMPARISON OF EXTRACTION RECOVERY OF RENIN INHIBITOR I FROM RAT PLASMA AND MARMOSET PLASMA

Different volumes of marmoset plasma were made up to 1.0 ml with rat plasma, and compared with 1.0 ml of rat plasma.

Volume of marmoset plasma (ml)	Difference[a] (%)	Lower limit[b] (%)	Upper limit[b] (%)
0.1	−1.1	−2.3	0.1
0.2	−4.6	−6.7	−2.5
0.4	−4.9	−7.3	−2.5

[a] Difference between mean recovered values, $n = 5$.
[b] 90% confidence interval limit.

Linearity

The linearity proved satisfactory for rat and marmoset plasma concentrations of renin inhibitor I from 5 to 100 ng/ml (Table III). Good reproducibility of the calibration curve was obtained by the use of internal standardization. During the application of the assay in toxicokinetic studies, it was also found that satisfactory results could be obtained for concentrations several times higher than 100 ng/ml, but this was not validated.

TABLE III

DATA OBTAINED FOR CALIBRATION SAMPLES OF RENIN INHIBITOR I

Species	Concentration added (ng/ml)	Mean concentration found (ng/ml)	*n*	C.V. (%)	Deviation[a] (%)
Rat	3	3.01	5	8.1	+0.4
	5	5.28	4	3.5	+5.6
	20	19.51	5	4.0	−2.4
	50	48.55	5	3.1	−2.9
	80	80.44	5	4.1	+0.5
	100	101.67	5	4.1	+1.7
Marmoset	5	5.13	12	8.4	+2.5
	10	10.07	14	7.0	+0.6
	20	19.79	14	3.9	−1.0
	50	50.09	14	2.7	+0.2
	80	79.90	14	2.2	−0.1
	100	100.26	14	2.1	+0.3

[a] Relative deviation of mean concentration found from concentration added.

TABLE IV

INTER-ASSAY PRECISION AND ACCURACY IN RAT AND MARMOSET PLASMA, DETERMINED FROM QUALITY CONTROL AND QUANTIFICATION LIMIT TEST SAMPLES

Sample type	Concentration added (ng/ml)	Mean concentration found (ng/ml)	n	C.V. (%)	Deviation[a] (%)
Rat					
QC[b]	6.0	6.06	4	9.1	+1.0
	20.0	20.88	6	6.5	+4.4
	50.0	50.9	7	6.0	+1.8
	75.0	78.35	7	4.4	+4.5
QL[c]	5.0	4.51	5	18.0	−9.8
Marmoset					
QC[b]	25	22.52	5	5.7	−9.9
	750	648.48	6	1.8	−13.0

[a] Relative deviation of mean concentration found from concentration added.
[b] QC = quality control samples.
[c] QL = quantification limit test sample.

Precision and accuracy

The inter-assay precision (given by the relative standard deviation) and the accuracy (given as inaccuracy, *i.e.* the difference between obtained and expected concentrations) were checked using quality control samples for rat plasma concentrations of renin inhibitor I ranging from 6 to 75 ng/ml and for marmoset plasma concentrations ranging from 25 to 750 ng/ml. The results (Table IV) were acceptable within these concentration ranges.

Limit of quantification

The limit of quantification was 5 ng/ml. At this concentration, the inter-assay precision and inaccuracy are less than 18 and 10%, respectively, for rat plasma (Table IV). A lower limit (*ca.* 3 ng/ml) could be achieved, depending on the quality of the plasma. A separate test was not done for marmoset plasma, since it had been observed that the extraction recovery was very similar to that from rat plasma and, also, because marmoset plasma had to be diluted (from 60 to 90%) with rat plasma before analysis.

The quantification limit of 5 ng/ml, which corresponds to 4.7 pmol injected, compares quite favourably with the detection limits reported in the literature for other dabsyl derivatives. They range from 2 pmol for polyamines in cell extracts [19] and 4 pmol for aspartylglucosamine in urine and cell homogenates [24] to 25–30 pmol for amino acids in urine [12].

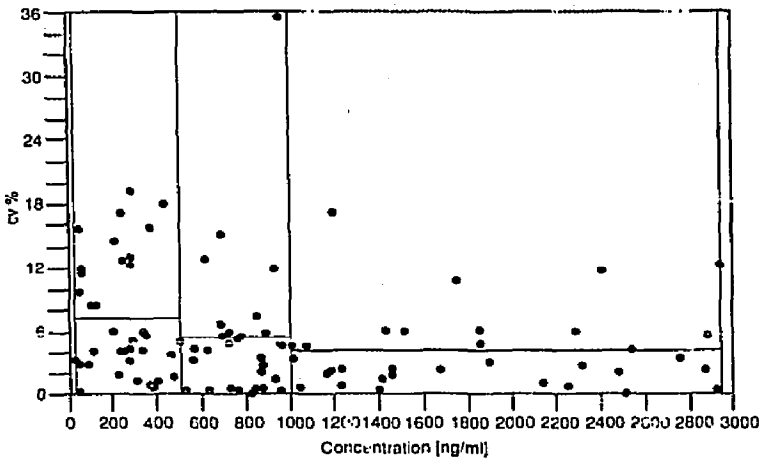

Fig. 5. Concentration dependence of the C.V. obtained from 99 duplicate determinations of renin inhibitor I in marmoset plasma samples: mean C.V. 5.58%, n = 99, between 28.9 and 2945 ng/ml (full range); mean C.V. 7.12%, n = 36, between 28.9 and 500 ng/ml; mean C.V. 5.32%, n = 30, between 500 and 1000 ng/ml; mean C.V. 4.14%, n = 33, between 1000 and 2945 ng/ml.

Application to biological samples

The method was successfully applied to toxicokinetic studies in rats and marmosets. Fig. 5 shows the concentration dependence of the coefficients of variation (C.V.) obtained from 99 duplicate determinations of renin inhibitor I in marmoset plasma samples. For a concentration range of 28.9–2945 ng/ml, the mean C.V. decreased from 7.1 to 4.1%. For the determination of amino acids in urine, C.V. values increasing from 5.2 to 7.6% were reported for concentrations decreasing from 33 to 2.75 μg/ml [12]. Aspartoylglucosamine in urine and leukocyte homogenates were measured with a C.V. of 8.3% [24].

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