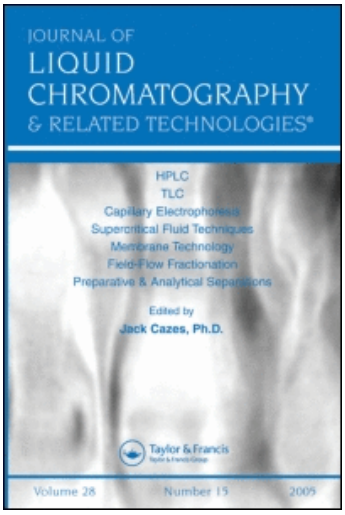

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of the New Ace-Inhibitor Quinapril and Its Active Metabolite Quinaprilate in Plasma and Urine by High-Performance Liquid Chromatography and Pre-Column Labelling for Fluorescent-Detection

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To cite this Article Hengy, Heinrich and Most, Michael(1988) 'Determination of the New Ace-Inhibitor Quinapril and Its Active Metabolite Quinaprilate in Plasma and Urine by High-Performance Liquid Chromatography and Pre-Column Labelling for Fluorescent-Detection', Journal of Liquid Chromatography & Related Technologies, 11: 2, 517 — 530

To link to this Article: DOI: 10.1080/01483918809349956

URL: http://dx.doi.org/10.1080/01483918809349956

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(2), 517-530 (1988)

DETERMINATION OF THE NEW ACE-INHIBITOR QUINAPRIL AND ITS ACTIVE METABOLITE QUINAPRILATE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRE-COLUMN LABELLING FOR FLUORESCENT-DETECTION

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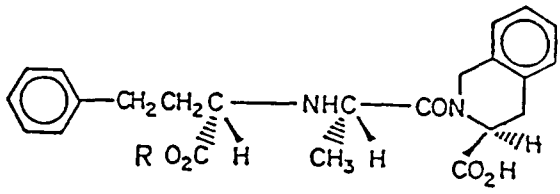
ABSTRACT

A sensitive and selective method for the determination of quinapril and its active metabolite quinaprilate in human plasma and urine is described. The method is based on isolation using C18 Bond Elut cartridges, pre-column derivatization with 9-anthryldiazomethane and purification of the reaction mixture on CBA columns followed by reversed-phase high performance liquid chromatography with fluorometric detection. Calibration curves were linear between 20 ng and 1000 ng/ml of plasma (100-2000 ng for urine) for both substances, the lower limit of detection being 5-10 ng/ml.

The present assay procedure has been applied to monotoring plasma and urine concentrations in several pharmacokinetic studies in humans.

INTRODUCTION

Quinapril, 2-[2-[[1-(ethoxycarbonyl) -3-phenylpropyl] amino] -1-oxopropyl] -1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid, monohydrochloride, [3S-[2[R*(R*)]], 3R*], CI-906, (Ia), is a new potent, orally active nonsulfhydryl, nonpeptide angiotensin-converting enzyme (ACE) inhibitor (1). It is rapidly deesterified in vivo by esterases to release its active metabolite quinaprilate, 3-isoquinolinecarboxylic acid, 2-[2- [(1-carboxy-3-phenylpropyl) amino]-1-oxopropyl] -1,2,3,4-tetrahydro-, [3S-[2[R*(R*)]],3R*]-, CI-928 (Ib)



Ia R = CH_3CH_2 Quinapril
Ib R = H Quinaprilate

Most ACE-inhibitors possessing a similar structure to quinapril or quinaprilate are currently determined by means of GLC or GLC-MS after appropriate derivatization (2,3) or by radioimmuno - and radioenzymatic assay methods (4-7). A capillary-GLC-ECD-method exists for quinapril and quinaprilate also comprising a 2 fold derivatization step prior to gaschromatographic analysis (8).

This report describes a novel high-performance liquid chromatographic method based on pre-column derivatization of the carboxylic acid group(s) with 9-anthryldiazomethane utilising spectrofluorometric detection. It proved to be well-suited for pharmacokinetic studies of quinapril in man.

The method should also be useful for the determination of other ACE-inhibitors with similar structures.

MATERIALS AND METHODS

Reagents and Standards

Quinapril·HCl (CI-906) and quinaprilate (CI-928), as well as the internal standard, 1H- indole-2-carboxylic acid, 1- [2- [[(1-carboxy-3-phenyl) propyl] amino] -1- oxopropyl]-octahydro-, [2S- [1 [R*(R*)]] , 2R*], PD 110021, a diacid structurally related to quinaprilate, were obtained from Warner-Lambert, (Ann Arbor, Mich. U.S.A.). All chemicals were of the highest grade commercially available. They were purchased from E. Merck (Darmstadt, F.R.G.), except for 9-anthraldehyde, hydrazine hydrate and manganese dioxide which were obtained from Aldrich Chemicals (Steinheim, F.R.G.). For aqueous solutions, water was purified by reverse osmosis and additionally passed through a water purification system for adsorption of organic substances, (Millipore, Neu Isenburg, F.R.G.). C18 and CBA-Bond Elut disposable solid phase columns (100 mg/1.0 ml) were obtained from ICT (Frankfurt, F.R.G.).

Preparation of 9-Anthryldiazomethane (Derivatization Reagent)

9-Anthraldehyde hydrazone was synthesized by the reaction of 9-anthraldehyde with hydrazine hydrate according to a method of Nakaya et.al. (9).

9-Anthraldehyde hydrazone (0.44 g, 0.002 mol) was dissolved in 200 ml of anhydrous (distilled) diethylether and 1.6 g of activated manganese dioxide as well as 1.2 ml of a saturated ethanol solution of potassium hydroxide were then added. The solution was stirred vigorously and the reaction stopped by filtering off

the manganese dioxide after 15 min. The solution was evaporated and the solid crystals kept at -20°C.

The derivatization reagent was dissolved in methyl-t-butylether to obtain a concentration of 2 mg/ml and was found to be stable for at least a week at -20°C.

Stock Solutions

Stock solutions of quinapril and quinaprilate (50 ng and 500 ng/10 µl) and the internal standard PD 110021 (250 ng/10 µl) were prepared weekly in 50% acetonitrile and stored at 5°C.

For calibration analysis, 1 ml drug-free plasma samples were spiked with 20, 50, 100, 250, 500 and 1000 ng of quinapril and quinaprilate, in the case of urine 0.5 ml were spiked with 50, 100, 250, 500 and 1000 ng.

Quality Control Samples

Drug free plasma or urine were spiked with known concentrations of quinapril and quinaprilate. Three quality control levels (50, 250 and 1000 ng/ml plasma and 0.5, 2 and 5 µg/ml urine) were prepared, aliquoted, and stored at -20°C until needed for use. After the samples were brought to room temperature, the samples were carried through the plasma or urine assay. The amount of quinapril and quinaprilate found in the quality control samples was calculated by comparison to a standard curve prepared daily.

Assay Procedure

Plasma (1 ml, in some cases, where not sufficient plasma was available, 0.5 ml was used) diluted 1:1 with KCl/HCl buffer pH 2 (3.73 g KCl and 134 ml 0.1 N HCl per 1000 ml H_2O) and 250 ng internal standard (PD 110021) is applied to C18 Bond-Elut columns (0.1 mg/1 ml) preconditioned with 2 x 1 ml methanol, 2 x 1 ml "Millipore" water and 2 x 1 ml 0.1 N HCl at a pressure of 20 kPA. Stopcocks are used to avoid unequal flow in the C18 columns. For urine analysis a volume of 0.05-0.5 ml and 500 ng int. st. was used.

Wash columns with 2 x 1 ml water pH 3.4 and 2 x 1 ml dist. n-hexane at the same reduced pressure. Dry columns for 20-30 minutes at a pressure of 60 kPA.

Elute compounds into a 5 ml tapered flask with 3 x 1 ml $CHCl_3$/MeOH 2:1 by applying slight pressure to the Bond-Elut columns with a rubberball.

Evaporate the solvent with the aid of a rotary evaporator or a stream of nitrogen.

50 µl of a mixture of $CHCl_3$/MeOH 1:1 and 50 µl derivatizing agent 9-anthryldiazomethane in methyl-t. butylether is added, vortexed for a few seconds, tightly stoppered and heated for 90 minutes at 40°C in an incubation oven. Afterwards the solvent was evaporated on a micro-evaporator.

To remove excess reagent and other interfering compounds, the residue was applied to CBA-Bond-Elut columns preconditioned with methanol and water, using 2 x 100 µl acetonitrile. The CBA-columns were washed twice with 1 ml acetonitrile and eluted 3 x with 1 ml acetonitrile containing 0.2% triethylamine. After evaporation to dryness using a micro rotary evaporator, the sample was redissolved in 200 µl acetonitrile prior to injection of 20-50 µl onto the HPLC-column.

Chromatographic System

A series 10 liquid chromatograph (Perkin-Elmer, Überlingen, F.R.G) fitted with a model 231 diluter-autosampler (Gilson/Abimed, Langenfeld, F.R.G.) was used with a Hyperchrome column packed with 5 µm Spherisorb ODS II, 12.5 cm x 4.6 mm (Bischoff, Leonberg, F.R.G.).

The mobile phase consisted of acetonitrile-methanol-water (45:40:15) containing 0.24% NH_4ClO_4 and 0.02% triethylamine, at a flow rate of 1.6 ml/min.

Elution peaks were detected by means of a 650-10 LC fluorimeter (Perkin-Elmer, Überlingen, F.R.G.) set at an excitation wavelength of 360 nm and emission at 440 nm. The slit-width in both cases was 10 nm.

Retention times and peak heights were determined using either a D-2000 Chromato Integrator (Merck-Hitachi, Darmstadt, F.R.G.) or a BD 41 Kipp a. Zonen recorder (Bischoff, Leonberg, F.R.G.).

Validation

Triplicate samples of 6 plasma standards ranging from 20 ng - 1000 ng/ml as well as 5 urine standards of 50, 100, 250, 500 and 1000 ng/0.5 ml were analyzed on 3 days. Peak height ratio vs. concentration plots of the standards were constructed. The best fit straight line was determined using the method of least squares and weighting factors of 1/concentrations squared. Using the results of the regression analysis, the concentrations of the standards were back-calculated.

The intraday and interday relative standard deviations were determined as well as the relative error of the back-calculated standards.

RESULTS

Specificity

Typical chromatograms of human plasma samples obtained from blank plasma, spiked plasma (250 ng) and a real plasma sample obtained 0.5 h after oral administration of 40 mg quinapril to a young patient are depicted in Figure 1. Chromatograms of blank urine, a spiked urine sample and a sample from a 0-24 h fraction of a subject having received 40 mg quinapril are shown in Figure 2.

The anthryl derivatives of quinapril and quinaprilate and of the internal standard PD 110021 are eluted after approximately 3.8, 12 and 16 minutes, respectively, in an interference-free part of the chromatogram.

Recovery

It was difficult to determine the direct recovery of quinapril and quinaprilate and the internal standard

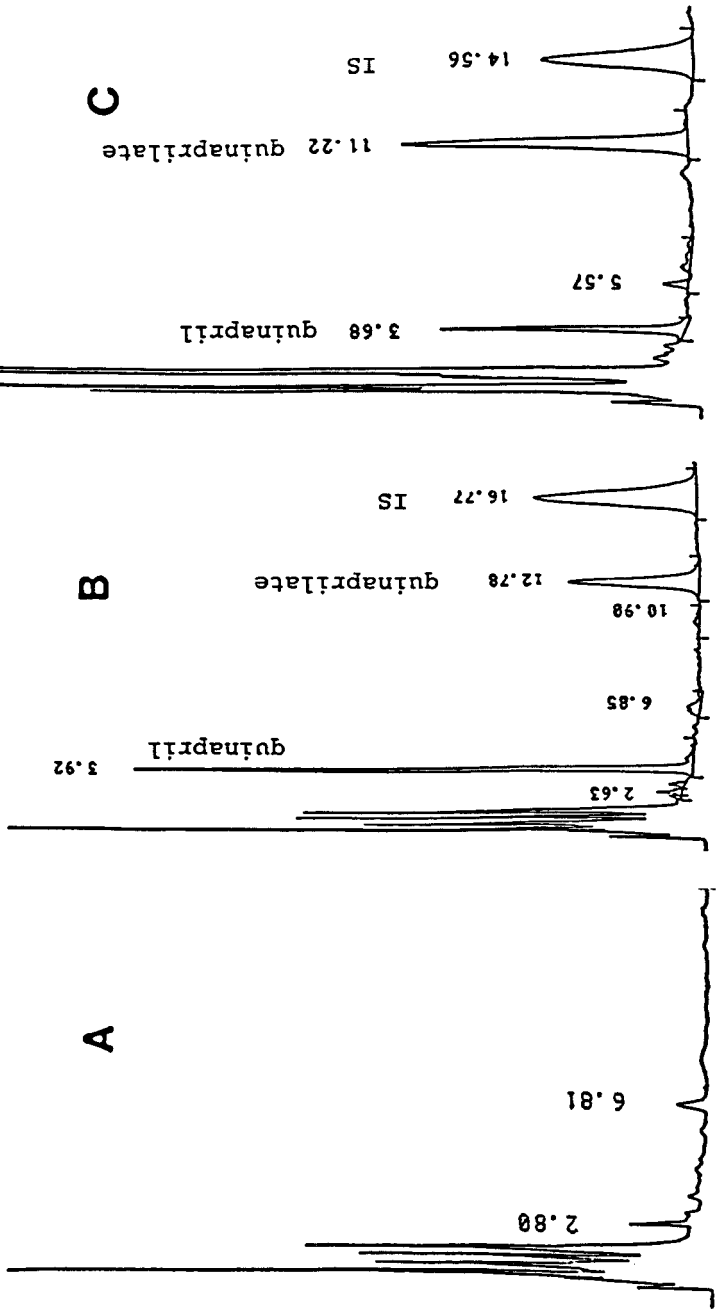


FIGURE 1

Representative chromatograms of blank human plasma (A), 250 ng/ml calibration standard (B) and a human plasma sample derived 0,5 h after oral administration of 40 mg quinapril to a young human volunteer/amount equal to 256 ng CI-906 and 1057 ng/ml CI-928 (0,5 ml plasma analyzed)(C)

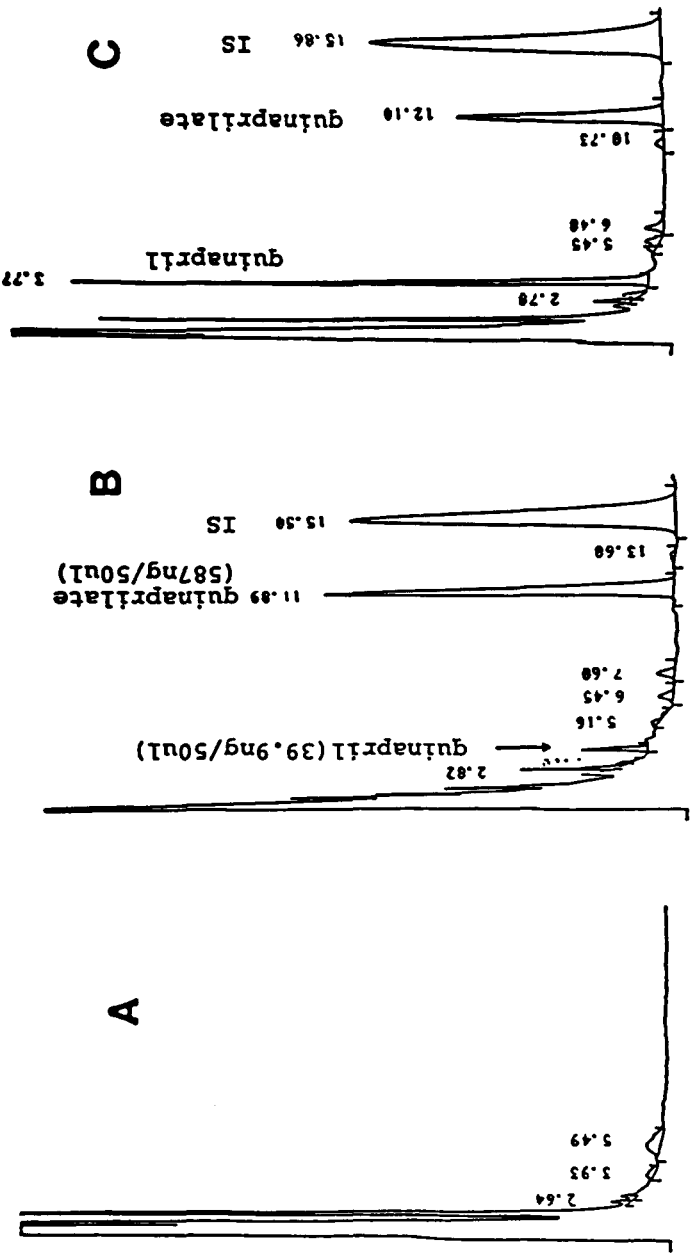


FIGURE 2

Chromatograms of blank urine (200 µl)(A), a sample (50 µl) from a 0-24 h fraction of a subject having received 40 mg quinapril (B) and a urine sample spiked with 2 µg/ml (200 µl analyzed)(C)

because the anthryl derivatives of the above substances were not available in their pure form. Only the approximate recovery from plasma could be determined when analysing the above pure substances directly and after subsequent clean up on C18 Bond Elut columns.

The recovery of quinapril, quinaprilate and the internal standard from plasma was approximately 80-90% over the range of concentrations studied, assuming a 100% yield during derivatization.

Linearity

The peak height ratios of quinapril and quinaprilate were linearly related (r=0,999) to the amount of quinapril and quinaprilate added to blank plasma over a range from 20 ng/ml to 1000 ng/ml and in the case of urine from 100 ng -2000 ng/ml respectively.

Precision and Accuracy

The between day precision in plasma over a three day period of the back-calculated data of the quinapril and quinaprilate calibration standards is given in Table 1.

The relative standard deviations for the back-calculated values varied for quinapril from 1.0 to 12.9% and for quinaprilate from 1.1 to 7.0%.

The intraday precision of the back-calculated values had a R.S.D. varying for quinapril and quinaprilate from 2.2 to 15.5% and 1.1 to 11.7%, respectively.

Quality control samples were analyzed each day with the patient or human volunteer samples. The quality control samples results demonstrate good stability of quinapril and quinaprilate in frozen samples as well as the accuracy of the method. Plasma data are summarized in Table 2.

Results of the precision study with urine, were similar.

TABLE 1

PRECISION STUDIES

Values represent back-calculated concentrations of quinapril and quinaprilate; mean values over 3 days (plasma). Values in parentheses are relative standard deviations (%)

Added	Found (ng/ml)			
(ng/ml)	Day 1(n=3)	Day 2(n=3)	Day 3(n=3)	Mean
Quinapril				
20	20 (3.9)	21 (15.5)	21 (9.2)	20.5 (3.6)
50	57 (2.2)	46 (8.5)	46 (8.5)	49.8 (12.9)
100	92 (12.9)	96 (9.6)	109 (6.8)	99.0 (9.1)
250	251 (10.3)	252 (4.2)	236 (7.4)	246.5 (3.6)
500	500 (9.3)	532 (10.3)	514 (5.0)	515.4 (3.1)
1000	999 (7.3	1016 (6.1)	1018 (2.5)	1011.1 (1.0)
Quinaprilate				
20	21 (7.2)	20 (11.7)	20 (10.5)	20.5 (2.2)
50	46 (3.3)	48 (2.2)	47 (1.1)	47.0 (2.0)
100	95 (9.7)	107 (3.2)	107 (5.3)	103.2 (7.0)
250	263 (2.9)	249 (2.3)	262 (7.4)	258.0 (3.0)
500	525 (3.1)	486 (3.4)	479 (2.9)	496.8 (5.0)
1000	1012 (1.5)	1008 (3.0)	991 (1.4)	1003.7 (1.1)

TABLE 2

Accuracy of quality control samples of quinapril and quinaprilate added to human plasma

Compound	Concentration added (ng/ml)	Mean concentration over 3 days (n=9) found (ng/ml)	Accuracy (%)	Coefficient of variation (%)
quinapril	50	52.9	106	13.5
	250	246.3	99	10.6
	1000	896.0	90	12.8
quinaprilate	50	50.6	101	4.1
	250	263.4	105	12.7
	1000	992.5	99	9.2

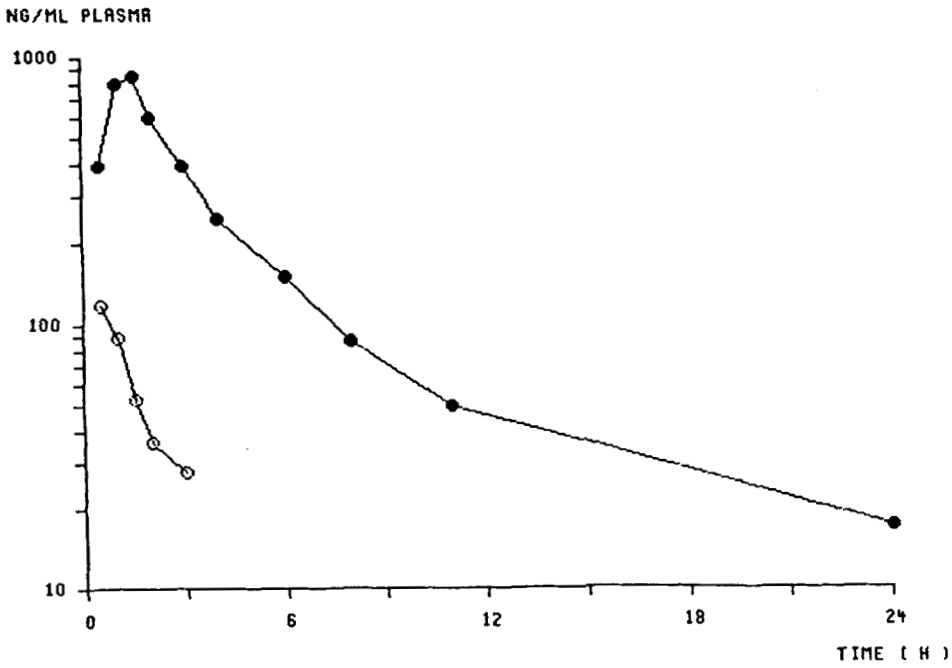


FIGURE 3

Plasma concentration versus time profile for quinapril (o) and quinaprilate (●) after administration of a single 20 mg oral dose of quinapril to a human subject.

Limits of Detection and Quantitation

The limit of detection for quinapril and quinaprilate in plasma was in both cases approximately 5 ng/ml. The limit of quantitation was 20 ng/ml for both compounds. In urine, the minimum quantifiable concentration was approximately 100 ng/ml, depending somewhat on the purity of the urine.

Applicability of the method

The new HPLC-assay has been applied to numerous pharmacokinetic studies of quinapril in human volun-

teers and patients. A characteristic plasma concentration versus time profile of both quinapril and quinaprilate following a single oral administration of 20 mg quinapril to a human volunteer is shown in Fig. 3.

DISCUSSION

Due to the amphoteric properties of quinapril and quinaprilate it was not possible to isolate these compounds from biological matrices by means of a conventional extraction technic. A solid-phase extraction procedure similar to that of Hajdú et.al.(2) was the method of choice, using Bond-Elut C18 cartridges. However, in a few cases it was noted that the recoveries of the substances from the C18 material varied from cartridge to cartridge, especially when using different batches. This effect could somewhat be minimized when ensuring an equal flow through the columns by using stop-cocks. The lack of constant quality of commercially available solid-phase extraction columns seems occasionally to be a problem, also pointed out by other authors (10).

The direct determination of quinapril and quinaprilate as well as other ACE-inhibitors with similar structures, by means of HPLC using UV-detection, was hampered because of the lack of a chromophoric group in the molecule. To facilitate its sensitive detection, we attempted to form derivatives containing a chromophoric or fluorescent group. A number of UV-visualizing reagents for the derivatisation of carboxylic acid groups e.g. phenacylbromide, p-bromophenacylbromide and -bromo-2-acetonapthone or fluorescent labelling with e.g. 4-methyl-7-methoxycoumarin or 4-bromoethyl-7-methoxycoumarin were investigated without success. This was mainly due to decomposition of the compounds of inter-

est during the reaction procedure or the vast amount of interfering reaction products resulting from the plasma matrix, or excess reagent.

9-Anthryldiazomethane proved to be a suitable fluorescent derivatizing reagent being highly reactive with carboxylic acid groups whereby esterification proceeds at mild temperature without catalyst, the reaction being complete after a period of approximately 1 hour. To remove unreacted reagent as well as other byproducts contained in the reaction mixture, an additional purification step was needed. This was achieved by applying the reacted sample mixture to a CBA-Bond Elut column and washing off the excess reagent and anthracyl derivatives of other carbonic acids with acetonitrile. Because of the free amino group present in the anthryl-derivative of quinapril or quinaprilate, these compounds are retained on the CBA-column and can subsequently be eluted with acetonitrile containing triethylamine.

The intense fluorescent derivatives could easily be separated by means of HPLC using a reversed phase column (Spherisorb-ODS) and a basic eluent comprising a counter ion.

It should be possible to use the described method to determine other ACE-inhibitors having a similar structure as quinapril or quinaprilate. Initial experiments with enalapril and enalaprilate showed the possibility of determining these compounds in biological fluids. They also form the corresponding 9-anthrylderivatives with a retention time of 2.90 min. for enalapril and 8.7 min. for enalaprilate, using the described procedure.

In conclusion the method described proved to be well suited for pharmacokinetic studies to determine quinapril and its active metabolite quinaprilate in

plasma and urine. Approximately 30-40 samples can be analyzed by one person in the course of a working day. It could also be useful for the quantitative determination of other ACE-inhibitors with similar structures.

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