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Interference from a glucuronide metabolite in the determination of ramipril and ramiprilat in human plasma and urine by gas chromatography–mass spectrometry

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Abstract

In the course of development and validation of a gas chromatography–mass spectrometry (GC–MS) method for ramipril and its biologically active metabolite ramiprilat, evidence was found for an unknown interfering metabolite. Sample treatment included isolation from plasma or urine by solid-phase extraction, methylation with trimethylsilyldiazomethane and acylation with trifluoroacetic anhydride (TFAA). When liquid chromatography was used to fractionate plasma extracts prior to derivatization, the alkyl, acyl-derivative of ramipril was obtained from two separate LC fractions. Electrospray ionization mass spectral data, together with circumstances for the derivatization, were consistent with the presence of an *N*-glucuronide of ramipril. Interference from the metabolite was eliminated by including a wash step after extraction/alkylation, prior to acylation. The final assay had a lower limit of quantification at 1.0 nmol/L and a linear range of 1–300 nmol/L. Intra- and inter-batch precision for ramipril and ramiprilat in plasma or urine were better than 10 and 5% at 2 and 80 nmol/L, respectively.
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Keywords: Ramipril; Ramiprilat; Metabolite; Glucuronide; Interference; Gas chromatography; Liquid chromatography; Mass spectrometry

1. Introduction

Ramipril (Fig. 1), 2-[*N*-[(*S*)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(l*S*, 3*S*, 5*S*)-2-azabicyclo[3,3,0]octane-3-carboxylic acid, is since a number of years used as a drug for treatment of hypertension and related cardiovascular diseases [1]. Ramipril is the prodrug for the major metabolite formed by ester hydrolysis, ramiprilat (Fig. 1), which is a highly active inhibitor of the angiotensin-converting enzyme (ACE). Analytical methods using enzymatic assay [2] and radio immunoassay [3] were early on described, where the concentration of ramipril was estimated as the difference between two assays of ramiprilat, before and after hydrolysis. Gas chromatography (GC) after derivatization reactions using a nitrogen selective detector has been reported for ramipril and ramiprilat in urine [2] but the method lacked sensitivity for low concentration plasma samples. Higher selectivity and sensitivity was obtained by using GC with mass spectrometry (MS) detection for the determination of related ACE-inhibitors in human plasma and urine [4–10]. Recently liquid chromatography–mass spectrometry (GC–MS) has been employed for the determination of ramipril and ramiprilat without derivatization [11].

In this paper, we describe a method based on GC with selected ion monitoring for determination of ramipril and ramiprilat in plasma and in urine after solid-phase extraction followed by methylation of the carboxylic acid functions and trifluoroacetylation of the amino group. Validation of the method revealed that in certain plasma samples an unknown metabolite was co-determined and contributed to the measured amount of ramipril. These findings demonstrated, as emphasized by others before [12–14], that validation of bioanalytical methods should include tests on authentic samples in order to detect potential interferences from major metabolites. A study was undertaken to obtain more information on the metabolite and to eliminate the interference in the assay. The final analytical method was used for numerous plasma samples and also for urine samples in clinical studies.

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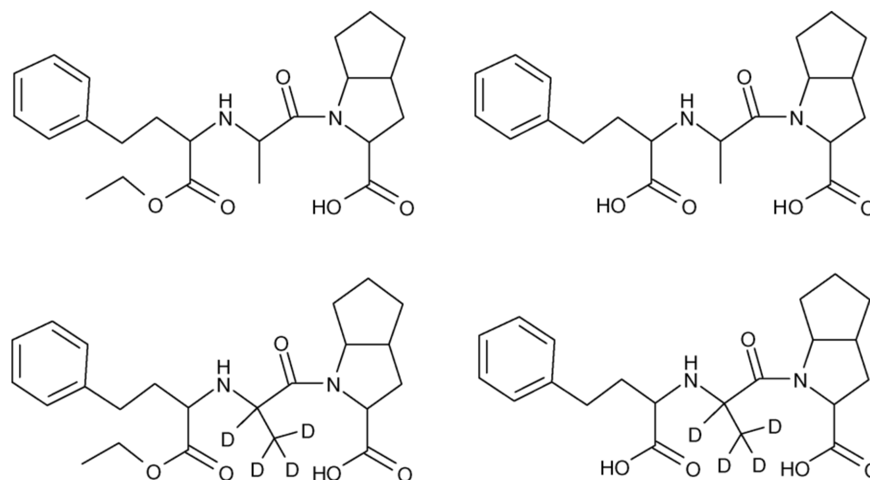

Fig. 1. Structural formula for ramipril (upper left) and ramiprilat (upper right) and their deuterated internal standards (lower left and right).

2. Experimental

2.1. Chemicals and materials

Ramipril and ramiprilat were obtained from Hoechst AG (Frankfurt, Germany). Ramipril-D_4 and ramiprilat-D_4 (Fig. 1) were used as internal standards and were supplied by AstraZeneca R&D, Mölndal, Sweden. Standard solutions were prepared in 0.1 mol/L sodium dihydrogenphosphate solution adjusted to pH 4.0. Methanol, 2-propanol, hexane and dichloromethane from Rathburn Chemical Ltd. (Walkerburn, Scotland), all glass-distilled grade were used without further purification. Ethyl acetate, distilled grade from Rathburn Chemical Ltd., was purified by distillation and stored in a refrigerator. 1-Butanol p.a. from E. Merck (Darmstadt, Germany) was purified by distillation. Trifluoracetic anhydride (TFAA) purum from Fluka AG (Buchs, Switzerland) was stored in a refrigerator. Trimethylsilyldiazomethane was obtained as a solution in hexanes (2 mol/L) from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). C_{18} Bond Elut® SPE columns (500 mg, 6 mL) were obtained from Varian (Harbor City, CA, USA).

2.2. Gas chromatography–mass spectrometry

Chromatographic experiments were performed using a Hewlett-Packard 5890 gas chromatograph equipped with a split/splitless capillary inlet system and a Hewlett-Packard 7673 autosampler. Separation was made in a 10 m long fused silica capillary column 0.25 mm i.d. with methyl phenyl silicone (SE-54) stationary phase (0.15 μm film thickness) with helium as carrier gas at an inlet pressure of 0.5 bar. The injector was operated in the splitless mode at 260 °C. The column temperature was held at 120 °C for 1 min, rose at 20 °C/min to 260 °C where it was held for 4 min and further raised at 30 °C/min to 300 °C where it was held for 4 min. The retention times for ramiprilat and ramipril were about 8.8 and 8.9 min, respectively. The Hewlett-Packard 5970B mass-selective detector was operated in the selective-ion monitoring (SIM) mode at *m/z* 316, 320, 330 and 334 for ramiprilat, ramprilat-D_4, ramipril and ramipril-D_4, respectively. The mass numbers *m/z* 316 and 330 gave the most intense ions for ramiprilat and ramipril, respectively (Fig. 2). The open-split and connection-line temperature was 300 °C.

2.3. Analytical procedure

Thawed plasma samples were mixed and centrifuged for 5 min before extraction. The SPE tubes (500 mg, 6 mL) were activated by addition of 5 mL methanol followed by 5 mL, 0.01 mol/L HCl containing 1% methanol. One milliliter plasma was transferred to centrifuge tubes, volume adjusted to 1.0 mL with blank plasma if needed. One hundred microliters internal standard solution (600 nmol/L) was added and 250 μL phosphoric acid solution 1 mol/L. After mixing, the mixture was transferred to the top of the SPE column and was allowed to elute by gravitational flow. After washing with 5 mL HCl 0.01 mol/L containing 15% methanol, followed by 4 mL hexane containing 5% 2-propanol the analytes were eluted with 15 mL dichloromethane containing 5% methanol. The resulting eluate was evaporated to dryness at room temperature under a gentle stream of dry nitrogen and the residue was redissolved in 100 μL of methanol. Alkylation was performed by adding 25 μL or more of the trimethylsilyldiazomethane solution until the solution remained yellow. After 20 min at room temperature the reaction mixture was evaporated to dryness at room temperature under a gentle stream of dry nitrogen as above. The residue was dissolved in 6.0 mL hexane and 2 mL 5% sodium hydrogencarbonate solution was added. After shaking for 10 min and centrifugation (5 min at 2500 rpm), the hexane phase was transferred to another tube and evaporated to dryness as above. The residue was dissolved in 200 μL ethyl acetate, 100 μL trifluoroacetic anhydride was added and the mixture was held at 60 °C for 20 min. This reaction mixture was then evaporated to dryness and the residue dissolved in 50 μL 1-butanol and 3 μL of this solution was injected into the gas chromatograph. Urine samples were treated exactly as described for plasma above except for 0.5 mL sample volume, blank urine added up to 0.5 mL if needed, was mixed with 1 mL 0.25 mol/L citric acid solution. Plasma standards and urine standards for daily calibration were

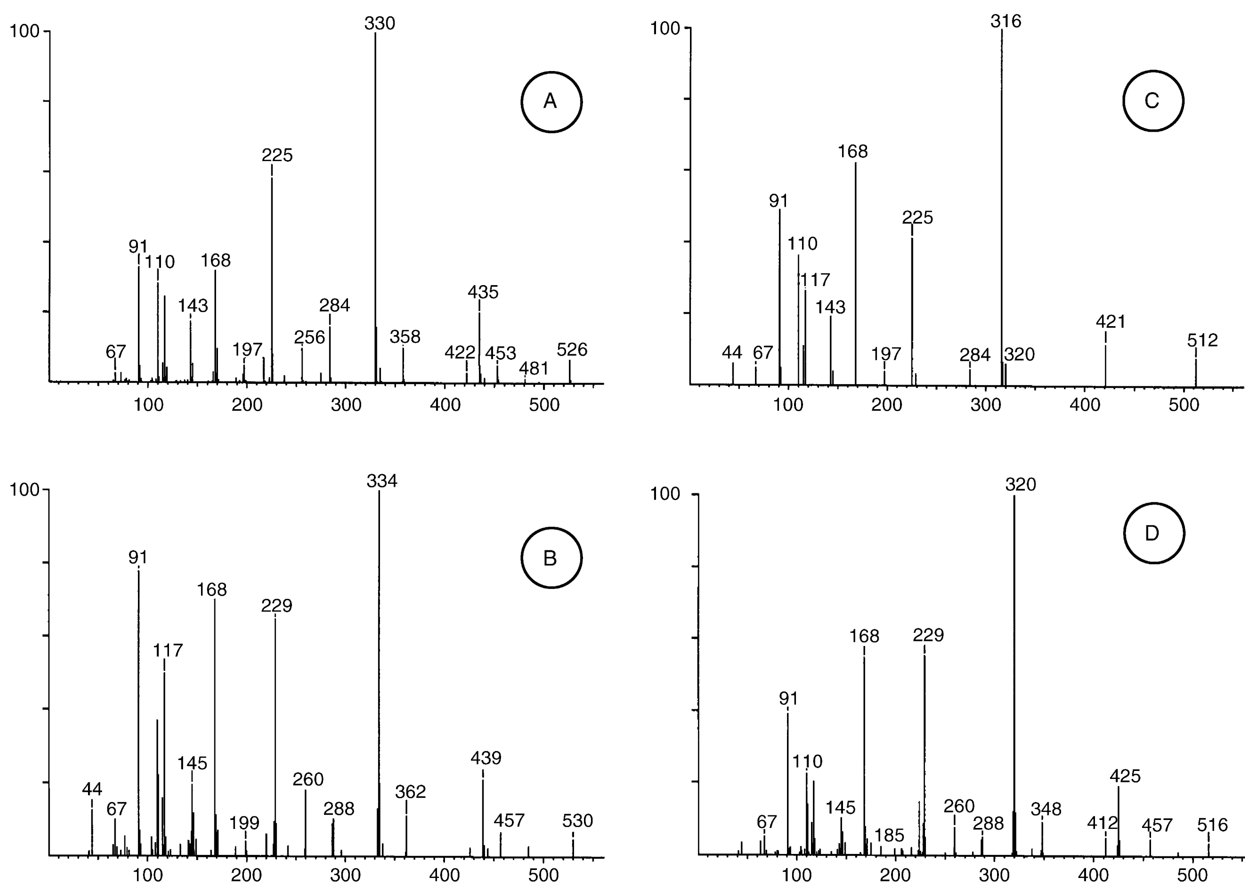

Fig. 2. Electron impact mass spectra for the derivatization products of ramipril (A), ramipril-D_4 (B), ramiprilat (C) and ramiprilat-D_4 (D).

prepared from 100 μL working standard solution and 900 μL blank plasma or 400 μL blank urine, respectively, and were run in parallel to the authentic samples.

3. Results and discussion

3.1. Extraction of ramipril and ramiprilat

Ramipril is a peptidomimetic compound with one carboxylic group and one secondary amino group, with pK_a values of 3.1 and 5.5, while ramiprilat has an additional carboxylic group and pK_a values of 2.2, 3.4 and 8.0 [15]. Both ramipril and ramiprilat are zwitterionic compounds not easily isolated from aqueous media by liquid–liquid extraction. SPE was therefore a natural choice and one parameter to consider was pH of the applied sample. According to the pK_a values given above ramipril should have a net charge of zero at around pH 4.0 while for ramiprilat this should occur at around pH 2.5. Since ramiprilat is more hydrophilic and is expected to be less easily retained than ramipril during solid-phase extraction, we chose to adjust the sample to pH 2.5–3.0 with a phosphate buffer before application to the SPE cartridge. The extraction recoveries for ramipril and ramiprilat were determined at concentrations above 3 μmol/L using liquid chromatography and UV-detection. They were found to be >90% for ramipril and >80% for ramiprilat, when compared with direct injection of reference solutions.

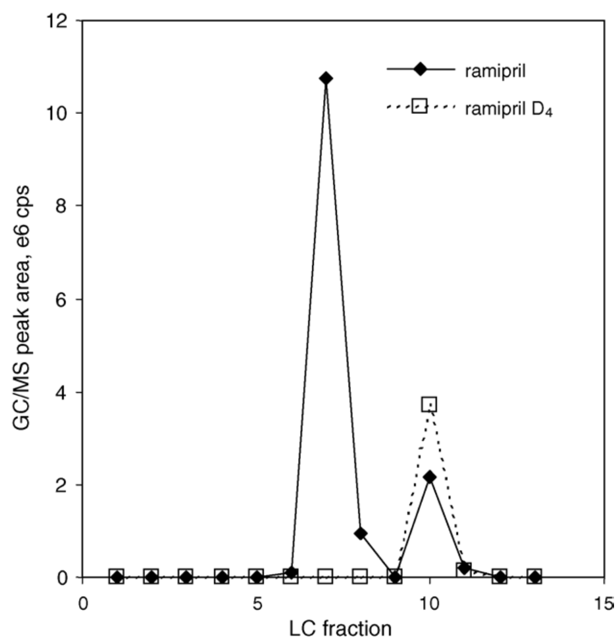

Fig. 3. Amounts of *O*-methyl, *N*-trifluoroacetyl derivatives of ramipril (solid line) and added internal standard (dashed line) measured by GC–MS after solid-phase extraction, LC fractionation and two-step derivatization of an authentic human plasma sample.

3.2. Derivatization

Initially, methylation of the free carboxylic groups of ramipril and ramiprilat was carried out using diazomethane as alkylating agent. In order to avoid the hazards of diazomethane, which is both highly toxic and unstable, we employed trimethylsilyldiazomethane, a thermally stable reagent [16,17], which is commercially available as a hexane solution. The 25-μL volume of reagent solution used was in general enough for complete reaction and we checked that the reaction mixture stayed yellow and no more reagents was needed. The final acylation of the amino function was readily accomplished with TFAA. The ruggedness of the acylation procedure was optimized by varying the reaction time and using different mixtures of ethylacetate and hexane for the reaction at a temperature of 60 °C. We chose 1-butanol as reconstituting solvent for the final extract since this was found to dissolve the evaporation residue easily.

3.3. Characterization of interfering metabolite

An early version of the method was intercalibrated with a GC–MS method in another laboratory. While fortified blank plasma samples showed good agreement between methods, authentic plasma samples taken after administration of ramipril to healthy volunteers revealed a discrepancy between the methods, our method yielding significantly higher concentrations of ramipril and a tendency to higher concentrations of ramiprilat, in samples taken shortly after administration. This indicated the presence of conjugates that were at least partially extracted and thereafter transalkylated or transacylated to yield the same final derivatives as the analytes. Such conjugates had not been found in animal plasma or urine [18] and were not anticipated in human plasma at this stage. Glucuronide conjugates were later identified in human urine [19,20]. One difference between the two methods was that the method giving lower results for ramipril included a washing step after alkylation prior to acylation, the

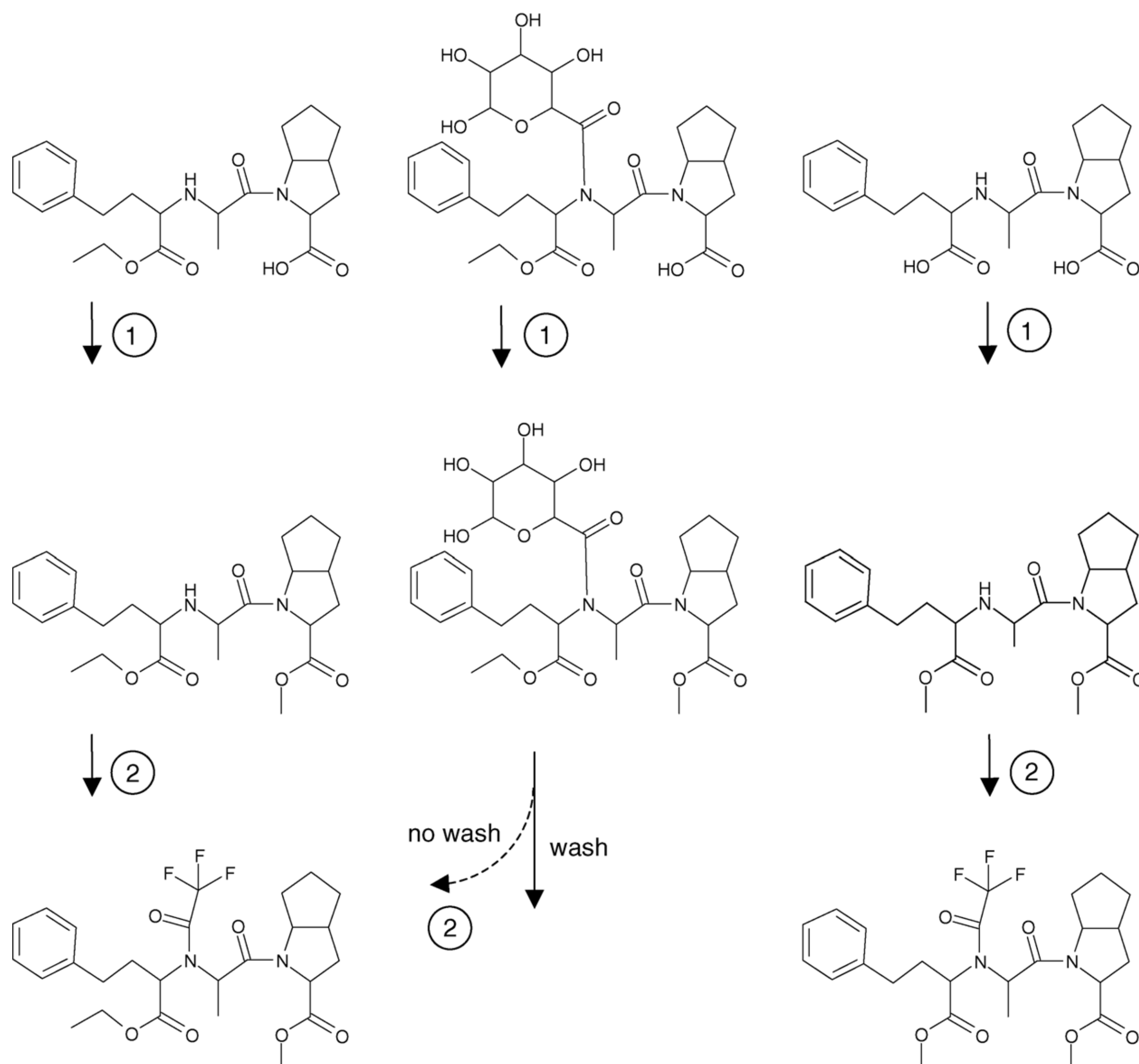

Fig. 4. Alkylation (1) and acylation (2) of ramipril (left), ramipril *N*-glucuronide (centre) and ramiprilat (right).

other one did not. This washing step seemed to help removing the interfering metabolite.

An attempt was made to gain proof of the existence and identity of an interfering ramipril metabolite. A single therapeutic dose of 15 mg ramipril in tablets was given to a healthy male volunteer and two 3 mL blood samples were taken (30/60 min after dose). The samples were carried through the initial solid-phase extraction step and then subjected to LC separation on an octadecyl silica column (Supelcosil C18 DB 4.6 mm × 100 mm) using a formic acid/ammonium acetate buffer/acetonitrile gradient. Flow rate was 1.00 mL/min. Three percent of the flow was used for mass spectrometric monitoring using a Sciex API-3 triple quadrupole mass spectrometer with electrospray ion source (ESI–MS) (Fig. 3). The remainder of the LC effluent was collected in 1-min fractions. One set of fractions was carried through the remaining steps of the sample treatment procedure and each fraction was analyzed by GC–MS. A duplicate set of fractions was collected for further ESI–MS measurements. The derivatives of ramipril and the internal standard ramipril-D4 were found in fractions 10 and 11 by GC–MS. Furthermore, derivatized ramipril was found in fractions 7 and 8, where no internal standard appeared (Fig. 3). Ramipril eluting together with the deuterated internal standard amounted to only about 20% of the total content of ramipril derivative found. ESI–MS spectra recorded during LC elution of fractions 10–11 showed peaks at *m/z* 417 and 421, corresponding to the protonated molecules of ramipril and ramipril-D4, respectively. Spectra from fractions 7 to 8 showed an intense ion at *m/z* 593. Fraction 7 was infused at 30 μL/min and product ions of the precursor ion at *m/z* 593 were recorded, using collision energy of 40 V.

Major fragments were found at *m/z* 417, corresponding to cleavage of the glucuronide amide bond yielding ramipril, and at *m/z* 234, corresponding to further elimination of 1-formyloctahydrocyclopenta[*b*]pyrrole-2-carboxylic acid. Since the derivatization route started with methylation, the glucuronide should also be O-methylated, leaving glucuronation at the N-position as the only alternative. Partitioning between hexane and bicarbonate buffer would keep methylated ramipril in the organic phase while removing the more polar methylated glucuronide together with the discarded aqueous phase. At the same time, any glucuronide conjugate of ramiprilat would be removed. The fact that only slightly higher concentrations of ramiprilat were observed in the GC–MS assay, when the wash step was omitted, could be explained by a low recovery of ramiprilat glucuronide in the solid-phase extraction step, and/or by low levels of this metabolite in the plasma samples (Fig. 4).

3.4. Quantification

Using peak area ratios between analytes and their isotope labeled internal standards, linear response was obtained for ramipril and ramiprilat from plasma samples ($n = 3$) at nine concentrations over the range 1–300 nmol/L, with accuracy at each level within 85–115%. For plasma samples, intra-batch variation was 8.8 and 1.2% for ramipril and 3.5 and 2.1% for ramiprilat at 2 and 80 nmol/L, respectively. For urine samples, intra-batch variation was 5.6 and 3.4% for ramipril and 3.6 and 2.9% for ramiprilat at 4 and 160 nmol/L. Inter-batch variation, obtained for quality control plasma samples run at 37 occasions, was 7.0 and 2.2% for ramipril and 5.9 and 2.5% for ramiprilat at 2 and 85 nmol/L.

4. Conclusions

A ramipril metabolite was found in human plasma giving the same final reaction product as ramipril after alkylation and acylation. Electrospray mass spectra indicated a glucuronic acid conjugate. The fact that the metabolite could be removed by liquid–liquid extraction after alkylation, prior to acylation, indicated conjugation at the amino group and not at the carboxylic group. By introducing a wash step in the sample treatment procedure, the GC–MS assay was made selective against glucuronide metabolites, and adequate accuracy and precision was obtained over the analytical range, 1–300 nmol/L.

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