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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.

Keywords: Ramipril; Ramiprilat; Metabolite; Glucuronide; Interference; Gas chromatography; Liquid chromatography; Mass spectrometry

1. Introduction

Ramipril (Fig. 1), 2-[*N*-[(*S*)-1-ethoxycarbonyl-3-phenyl-propyl]-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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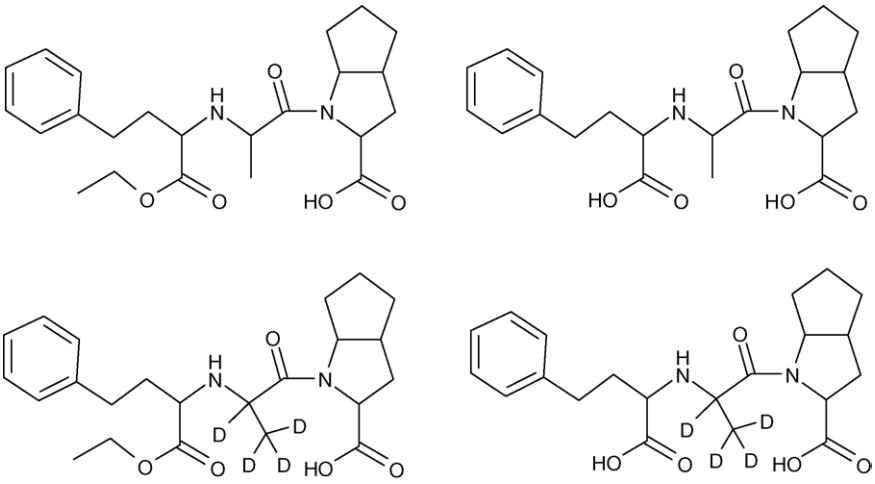

Fig. 1. Structural formula for ramipril (upper left) and ramiprilat (upper right) and their deuterated internal standards (lower left and right).

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