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Use of high-performance liquid chromatography–thermospray mass spectrometry and gas chromatography–electron-impact mass spectrometry in the identification of the metabolites of α-methylacetohydroxamic acids, potential anti-asthmatic agents

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ABSTRACT

Two potential anti-asthmatic α-methylacetohydroxamic acids, compound I and compound II were metabolised to two major products (metabolite 1 and metabolite 2) after oral dosing to rabbits. Metabolite 1, extracted under acid conditions from the plasma and urine of dosed animals, was identified as a glucuronide by incubation with β-glucuronidase and subsequent high-performance liquid chromatographic–mass spectrometric (HPLC–MS) analysis of the aglycone. HPLC–MS analysis of metabolite 2 suggested that it was the acetamide, however, unequivocal identification was obtained by further analysis using gas chromatography–mass spectrometry (GC–MS) of its trimethylsilyl derivative and by comparison with the mass spectra of the authentic acetamides. This study shows the advantages of combining HPLC–MS with other techniques such as GC–MS for the identification of metabolites.

INTRODUCTION

Leukotrienes are a class of biologically active compounds which have been implicated as mediators in various inflammatory diseases such as rheumatoid arthritis [1] and in asthma [2]. Leukotrienes are synthesised biologically from precursor fatty acids, in particular arachidonic acid, after its release from membrane phospholipids. Arachidonic acid is first converted to a 5-hydroperoxy metabolite by the 5-lipoxygenase enzyme and then to an unstable intermediate from which a number of leukotrienes can be formed [3]. Compounds inhibiting the 5-lipoxygenase enzyme therefore may have therapeutic potential as anti-asthmatic drugs.

Earlier investigations showed that several novel hydroxamic acid compounds, for example the N-(3-phenoxycinnamyl)acetohydroxamic acid (compound III[a]),

[a] Wellcome compounds: compound I, BW B218C, ±(*E*)-N-[1-methyl-3-(3-phenoxyphenyl)prop-2-enyl]acetohydroxamic acid; compound II, BW A879C, ±(*E*)-N-{3-[3-(4-chlorophenoxy)phenyl]-1-methylprop-2-enyl}-acetohydroxamic acid; compound III, BW A4C, N-(3-phenoxycinnamyl)acetohydroxamic acid.

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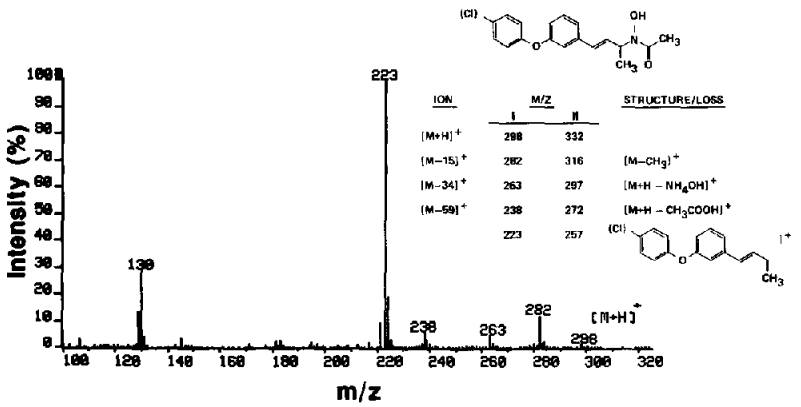

Fig. 1. Thermospray mass spectrum of compound I. The structure of I and its 4-chloro analogue, II (brackets), are shown. The ions observed for both compounds are indicated.

were effective inhibitors of leuktrotriene B_4 production *in vitro* [4]. However, the inhibitory activity of these compounds *in vivo* was short lived due to the formation of several inactive metabolites [5]. In particular compound III, which lacks the α-methyl group to the hydroxamate of I (see Fig. 1), was oxidised at this point to the corresponding carboxylic acid. Indeed no carboxylic acid metabolites were detected in the plasma of animals dosed with analogues containing this α-methyl group. As a consequence the parent compounds reached higher plasma concentrations which were maintained for longer [5]. In addition a glucuronide conjugate of III has been detected. With the substitution of the α-methyl group it was proposed that inhibition of glucuronidation or stereoselective metabolism may occur. High-performance liquid chromatographic (HPLC) analysis of plasma extracts, however, still indicated the formation of two major metabolites, one of which was extracted from urine more efficiently under acid conditions, and was predicted to be a conjugate.

This paper describes the identification of these metabolites extracted from the urine and plasma of rabbits dosed with the α-methylacetohydroxamic acids I and II (4-chloro analogue). It was anticipated that the latter compound would aid in identifying the metabolites due to the recognisable chlorine isotope pattern in its mass spectra. Thermospray mass spectrometry (MS) was selected as the initial analytical detection system as this enabled the HPLC systems already developed to be used directly. Thus the chromatographic characteristics and metabolite retention times were comparable with previous experiments.

EXPERIMENTAL

Administration of compounds

New Zealand white rabbits (2.0–3.5 kg) were deprived of food, but not water, for 12 h prior to administration of compounds. The acetohydroxamic acids I and

II were administered orally (50 mg/kg) as a suspension in 0.25% methyl cellulose (2.0 ml/kg Celacol gum, British Celanese, Spondon, U.K.). Blood was collected from the marginal vein of the ear at 1 and 8 h after administration and plasma was obtained by centrifugation. Urine was collected from rabbits confined in metabolism cages between 4 and 8 h after administration of compounds. All biological samples were stored at −20°C until extraction.

Extraction of metabolites from plasma and urine

An aliquot (0.5 ml) of plasma or urine was vortexed with ice-cold acetone (1.2 ml) to precipitate proteins. After centrifugation (1500 *g*, 5 min) the supernatant was decanted and saturated sodium chloride solution (0.2 ml) was added. The aqueous acetone was then extracted with chloroform (2 × 1.5 ml) and the organic phases were combined. The aqueous phase was then adjusted to pH 3 with hydrochloric acid (0.5 *M*) and re-extracted with chloroform (2 × 1.5 ml). These organic phases were pooled. Both neutral and acid extracts were evaporated to dryness under a stream of nitrogen at 40°C prior to dissolution in tetrahydrofuran (THF)–water for HPLC analysis. For the identification of metabolite 2 a larger volume of plasma (10 ml) was extracted in 1-ml aliquots with appropriate doubling of the reagents, and the neutral organic extracts were subsequently pooled and evaporated to dryness.

High-performance liquid chromatography

Metabolite 1 was purified by HPLC on a Spherisorb C_{18} (ODS) column (250 mm × 4.6 mm I.D.; LDC/Milton Roy, Stone, U.K.) at 40°C eluted with a mobile phase consisting of THF–water–trifluoroacetic acid (TFA) (47.5:52.5:0.1, v/v) containing 0.5 m*M* oxalic acid (BDH, Poole, U.K.) at a flow-rate of 1 ml min^{-1}. Metabolite 2 was purified using a mobile phase of THF–water–TFA (40:60:0.1, v/v) containing 0.5 m*M* oxalic acid. The HPLC eluent containing the metabolites was collected manually and was re-extracted using chloroform (2 × 2 volumes). After evaporation of the chloroform the residues were reconstituted in methanol (300 μl) prior to HPLC–MS.

Treatment of extracts

The extract of metabolite 2 obtained from 10 ml of plasma was divided into two equal volumes and one portion was reserved for HPLC–MS analysis. The other portion was evaporated to dryness under nitrogen, the residue reconstituted in 12 μl of methanol and an aliquot (2 μl) analysed by gas chromatography–mass spectrometry (GC–MS). The remaining solution was taken to dryness under nitrogen; the residue was resuspended in N,O-bis(trimethylsilyl)trifluoracetamide + 1% trimethylsilyl chloride (BSTFA; Pierce and Warriner, Chester, U.K.) (10 μl) and heated at 60°C for 1 h. An aliquot (2 μl) of the derivative in silylating reagent was re-analysed by GC–MS.

Enzymic hydrolysis

An aliquot of the methanolic solution of purified metabolite 1 (approximately 1 μg of the presumed glucuronide of the α-methylacetohydroxamic acid) was dried under nitrogen. To the residue a solution (1 ml) containing β-glucuronidase (1000 Fishman units; EC 3.2.1.31 from bovine liver; Sigma, Poole, U.K.) in 0.2 *M* sodium acetate buffer (pH 5.4) was added and mixed. This was incubated overnight (16–18 h) at 37°C. As a control compound I (1 μg) was incubated in buffer alone and extracted in the same manner.

The reaction mixture was extracted without adjusting the pH with 2 volumes of chloroform. The chloroform was evaporated and the residue reconstituted in THF–water prior to HPLC–MS.

Mass spectrometry

MS was carried out using a quadrupole VG Trio 2 mass spectrometer (VG Biotech, Altrincham, U.K.) interfaced with either a thermospray probe for HPLC–MS or to a Hewlett-Packard Model 5890 gas chromatograph (Hewlett-Packard, Wokingham, U.K.).

HPLC–thermospray MS

Reversed-phase HPLC was performed using a Spherisorb C_{18} column (250 mm × 4.6 mm I.D.) and a mobile phase consisting of THF–water TFA (45:55:0.1, v/v) containing 0.1 *M* ammonium acetate at a flow-rate of 1 ml min^{-1}. Previous investigations have shown that the inclusion of oxalic acid is necessary for satisfactory chromatography [4] and 0.5 m*M* oxalic acid was included in the mobile phase without deleterious effect to the MS detection. Samples in 50 μl of THF–water (40:60, v/v) were injected manually. The UV absorbance (260 nm) of the eluent was monitored (Waters Model 490 UV detector, Millipore, Watford, U.K.) before being introduced into the mass spectrometer. A thermospray probe temperature of 275°C and a repeller voltage of 300 V were employed. Satisfactory positive-ion mass spectra could be obtained from injection of approximately 200 ng of the authentic parent acetohydroxamic acids which exhibited an intense $[M-CH_3(CO)NOH]^+$ ion at *m/z* 223 (I) (Fig. 1) and *m/z* 257 (II). $[M+H]^+$ ions (*m/z* 298 and 332) were of low intensity.

Gas chromatography–mass spectrometry

Gas chromatography was performed using a cross-linked methylsilicone fused-silica capillary column (25 m × 0.31 mm I.D., 0.17 μm film thickness, HP1, Hewlett-Packard). Splitless injections were performed at an oven temperature of 120°C and after 2 min the temperature was ramped at 15°C min^{-1} to 240°C, held for a further 2 min and then ramped at 5°C min^{-1} to 270°C.

The GC column was interfaced by direct insertion into the mass spectrometer source. Positive-ion electron-impact mass spectra were obtained at an electron energy of 25 eV. Samples were injected in methanol or, after derivatisation, in

BSTFA. However, GC of the underivatised hydroxamic acids led to their thermal decomposition on the column: the mass spectra obtained were consistent with formation of the acetamide derivatives.

RESULTS AND DISCUSSION

Reversed-phase analysis of plasma extracts from rabbits dosed orally with compound I or II (50 mg/kg) revealed the presence of two significant fluorescent and UV-absorbing drug-related peaks. While metabolite 2 (relative retention time 0.9 *cf.* parent compound) was extracted efficiently under the same conditions as the parent compound, metabolite 1 (relative retention time 0.6 *cf.* parent compound) was extracted more efficiently after acidification of the plasma to pH 3. Extraction of rabbit urine collected at 8 h after dosing also revealed a compound with the same characteristics as metabolite 1. A similar metabolite had been identified earlier as the glucuronide conjugate of the N-hydroxy group after oral dosing of compound III (results not shown) and therefore metabolite 1 was proposed to be the glucuronide of the α-methylacetohydroxamic acids. The thermospray mass spectrum of metabolite 1 did not exhibit an $[M+H]^+$ ion (Fig. 2), however, $[M+H]^+$ ions were weak or absent on analysis of a number of commercially available glucuronide conjugates (*e.g.* phenolphthalein mono-, 8-hydroxyquinoline and 4-methylumbelliferyl glucuronides; results not shown). HPLC–MS of metabolite 1 after treatment with β-glucuronidase showed that it was converted to the parent compound confirming its identity as the N,O-glucuronide.

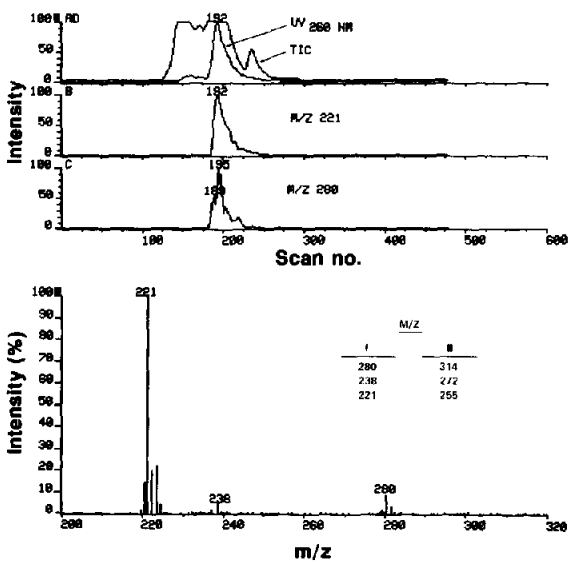

Fig. 2. HPLC–MS of metabolite 1 obtained from compound I. $UV_{260\ nm}$ and ion chromatograms (upper) and thermospray mass spectrum (lower) are shown. No $[M+H]^+$ ion was observed.

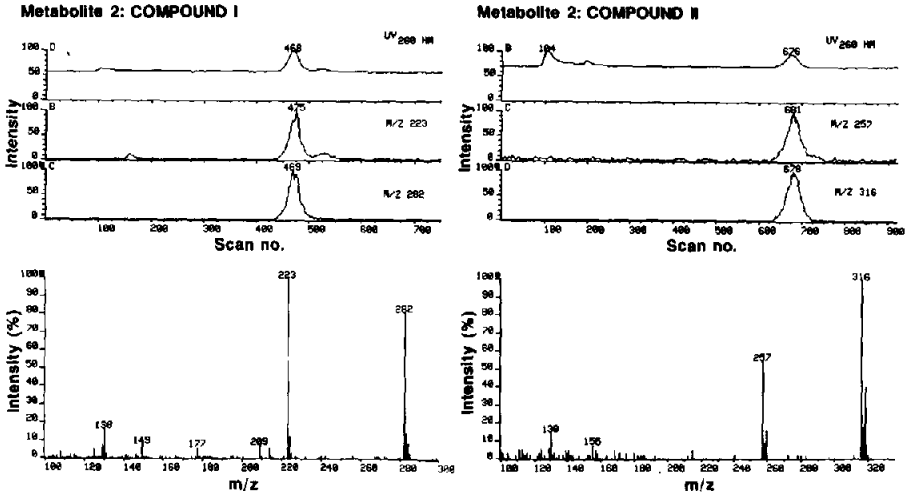

Fig. 3. HPLC MS of metabolite 2 obtained from compounds I and II. $UV_{260\ nm}$ and ion chromatograms (upper) and thermospray mass spectra (lower), are shown.

This demonstrates that the substitution of the α-methyl group does not abolish glucuronide formation, however, further experiments have shown that stereoselective metabolism occurs and that the preferred substrate is the (+)-α-methylacetohydroxamic acid [5].

The thermospray mass spectrum of metabolite 2 exhibited an intense ion at 16 a.m.u. less than the $[M+H]^+$ ion of the parent compound. In addition a fragment ion derived by cleavage α to the hydroxamate group was observed as in the unchanged compound (Fig. 3). This relative loss of 16 a.m.u. suggested that the ion was either formed by fragmentation of the terminal methyl group, possibly metabolised to the alcohol (*i.e.* $[M-CH_2OH]^+$) or, more likely, by conversion of the hydroxamate (R_2NOH) to the acetamide (R_2NH) and the formation of an intense $[M+H]^+$ ion. The formation of a molecular ion could not be assumed, however, due to the ease of fragmentation of these compounds. GC–MS of metabolite 2 suggested that it was the acetamide, exhibiting molecular ions at *m/z* 281 (I) and *m/z* 315 (II) (Fig. 4). Fragmentation to form the ion $[M-CH_3CO]^+$ (*m/z* 238 and 272; I and II, respectively) indicates the integrity of the terminal methyl group and therefore that metabolism has occurred at another site. However, the retention times and mass spectra were identical to those obtained after decomposition 'on-column' of the parent compounds to the presumed acetamides with concomitant chromatographic tailing. Little decomposition though was observed during GC of metabolite 2.

GC–MS after reaction with BSTFA was consistent with the formation of the N-trimethylsilyl derivative of the acetamide, having molecular ions at *m/z* 353 and *m/z* 387 (I and II respectively) (Fig. 5). The GC profiles clearly show that

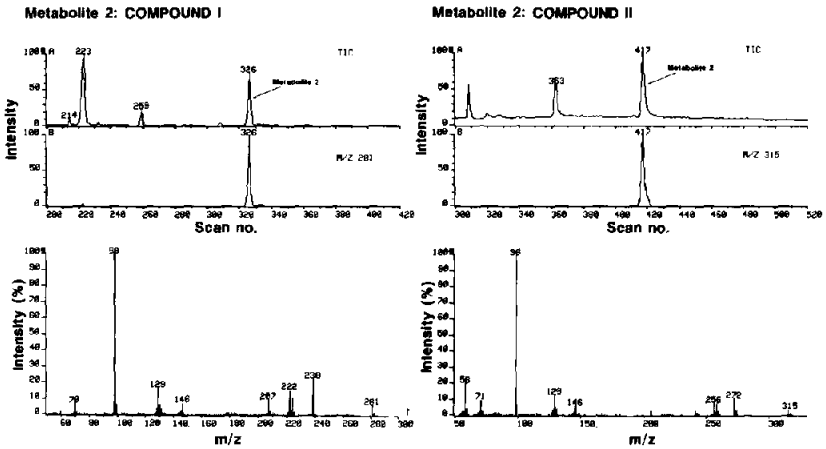

Fig. 4. Gas chromatograms (upper) and electron-impact mass spectra (lower) of metabolite 2 obtained from compounds I and II. The parent compounds decompose when subjected to GC but yield peaks with the same retention time and mass spectra as shown, though pronounced peak tailing occurs.

residual amounts of parent compound formed O-trimethylsilyl derivatives which were stable and well resolved on the chromatogram. A small amount of underivatised acetamide was also present.

The identity of metabolite 2 was confirmed by comparison with the mass spectra obtained by HPLC–MS and GC–MS analysis of the chemically synthesised acetamides.

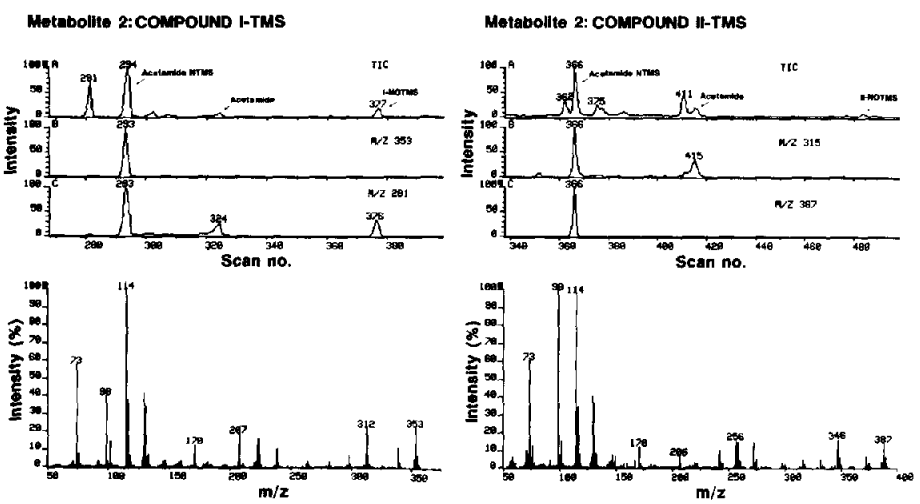

Fig. 5. Gas chromatograms (upper) and electron-impact mass spectra (lower) of the trimethylsilyl (TMS) derivatives of metabolite 2 obtained from compounds I and II. The chromatograms show that residual parent compound is well resolved as the TMS derivative from that of the metabolite.

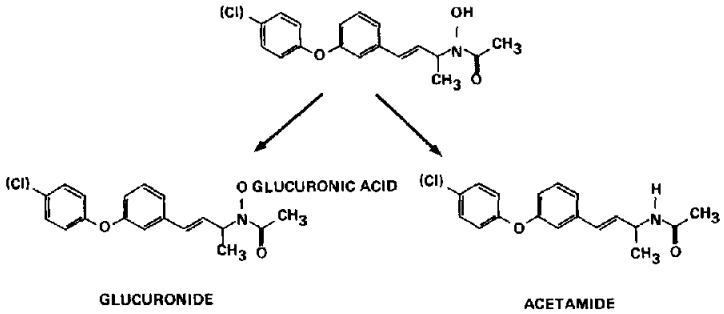

Fig. 6. Metabolism of the α-methylacetohydroxamic acids to the glucuronide conjugate and the acetamide.

CONCLUSIONS

Due to the facile fragmentation of the α-methylacetohydroxamic acids during thermospray MS, identification of their metabolites using HPLC–MS alone was equivocal. In addition an earlier observation (unpublished) that the $[M+H]^+$ ions for a number of glucuronide conjugates were weak or absent (see Results and discussion) necessitated the use of HPLC–MS with other techniques. Thus, the combination of MS interfaced to HPLC and GC has enabled identification of two metabolites of the putative anti-asthmatic α-methylacetohydroxamic acids as the corresponding glucuronide and acetamide (Fig. 6).

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