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Analysis of α-Hydroxy Metabolites of Metoprolol in Human Urine after Phosgene/Trimethylsilyl Derivatization†

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Three metoprolol metabolites containing an α-hydroxy group were identified in human urine by capillary column gas chromatography/mass spectrometry. After aqueous phase cyclization with phosgene the neutral or acidic derivatives formed were isolated by solvent extraction at pH 10 or 3, respectively. Following silylation the electron impact mass spectra of the metabolites exhibited a characteristic ion at *m/z* 336 of high abundance which originated from cleavage of the bond adjacent to the α-OTMS group. Most probably the identified compounds were formed by further biotransformations of α-hydroxy metoprolol, which is a primary metabolite. The analytical method is applicable to detect the metoprolol metabolites reported so far. A quantitative assay for one of the metabolites (H 119/72) with nitrogen selective detection is described. The total amount of this metabolite excreted by one subject within 24 h after dosing was about 0.25% of the given dose.

INTRODUCTION

Metoprolol is mainly metabolized by *O*-dealkylation of the methoxyethyl *para* substituent with subsequent oxidation of the alcohol to the corresponding aliphatic acid, which is the major metabolite in man. Furthermore, benzylic hydroxylation and oxidative deamination of the long side chain are important metabolic pathways in all species studied.[1] Additionally, three minor metabolites of metoprolol have been identified in rat urine and all of them have the hydroxylated benzylic carbon in common.[2]

Methods for the determination of the metabolites were based on work with radioactively labelled metoprolol and isolation on XAD-2 resin followed by separation and detection by radio gas chromatography.[1] Gas chromatography/mass spectrometry (GC/MS) has been used to measure metoprolol and two of the basic metabolites simultaneously in plasma after trifluoroacetylation.[3] The major urinary metabolite, the amino acid H 117/04, can be measured by gas chromatography (GC) after derivatization with mixed anhydrides.[4] Recently liquid chromatographic systems have been described to quantify the main metabolites simultaneously[5,6] with fluorescence detection[6] or separately on different columns.[7] The wide difference in the lipophilic character of metoprolol and its metabolites required gradient elution systems to determine all compounds in a single analysis.[6]

Recently a method was reported for the simultaneous determination of metoprolol and four of its metabolites in urine.[8] After cyclization of the compounds with phosgene at pH 12 and trimethylsilylation of pH 3 extractable derivatives, capillary column GC with flame ionization detection was used. The limit of determination was in the range 5–20 μmol l^{-1} of urine. In this report the further development of this method is presented, aimed at increased selectivity in the isolation and the detection of mainly α-hydroxylated metoprolol metabolites in human urine.

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EXPERIMENTAL

Apparatus

Gas chromatography. A Varian Model 3700 gas chromatograph was used, equipped with a nitrogen-selective detector. The capillary column (22 m × 0.32 mm i.d.) was coated with CP-Sil 8 from Chrompack (Middleburg, The Netherlands) after deactivation with diphenyltetramethylsilazane. Nitrogen was used as carrier gas with an inlet pressure of 100 kPa. The split and the make-up flow rate were both 20 ml min^{-1}. Samples were introduced in the splitless mode, the split vent being opened after 90 s. The injector and the detector were maintained at 300 °C. The oven temperature was kept at 150 °C for 1 min after the injection and was then increased by 10 °C min^{-1} to 280 °C. Samples were introduced by a Varian Model 8000 Autosampler, or manually for the concentrations below 5 μmol l^{-1}.

Gas chromatography/mass spectrometry. Mass spectra were recorded in a Finnigan MAT 44S instrument equipped with a Varian 3700 gas chromatograph. The fused silica capillary column as described above was connected to the mass spectrometer by an open split interface. Mass spectra were recorded and selected ion monitoring was performed under electron impact (70 eV) conditions. Data were aquired by a Finnigan MAT SS 200 data system. The same injection technique and temperature programme was used as given above.

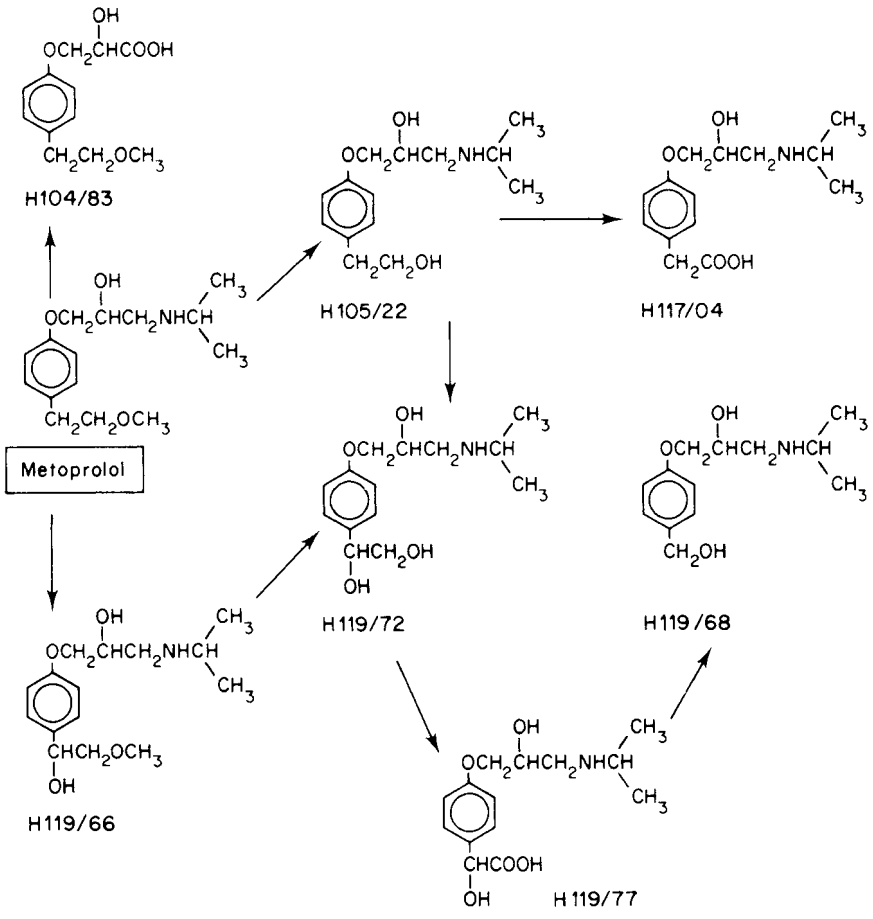

Figure 1. The metabolic pathway of metoprolol. All compounds depicted can be isolated and detected by the present methods.

Reagents and chemicals

Metoprolol and the metabolites were synthesized by the Department of Organic Chemistry, AB Hässle.[2] The structures are shown in Fig. 1 and their nomenclature is adapted from Ref. 2, which describes the synthetic procedures.

Phosgene, 2 M in toluene (purum), was purchased from Fluka (Buchs, Switzerland) and bis(trimethylsilyl)acetamide (BSA) from Macherey and Nagel (Düren, FRG).

Methods

Isolation of metabolites from urine. The urine sample (1 ml) was mixed with buffer (1 ml, ionic strength 1, final pH 12) and the sample was reacted with 2 M phosgene in toluene (3 × 20 μl) by vibrating the tubes mechanically in a VX 2 apparatus (Janke & Kunkel, Staufen, FRG) for 6 min. The organic phase consisted of 3 ml of diethyl ether and 2 ml of dichloromethane. The solution was added and the mixture was stirred for 2 min. After centrifugation the organic phase was collected and the aqueous phase was extracted for a second time. The combined organic extracts were evaporated to dryness under a stream of nitrogen. The aqueous phase was made acidic with 0.5 ml of 1 M sulphuric acid and was extracted as described above, and the separated organic phase was removed under nitrogen. Both residues were dissolved in 30 μl of BSA and analysed without further purification by GC/MS for the qualitative determination of derivatized metoprolol metabolites. Identification was based on identical GC retention times and mass spectrometric data of both metabolite and a synthetic reference.

Quantitative determination of H 119/72 in urine. Metabolite H 119/72 in urine was reacted with phosgene and the derivatized metabolite was extracted twice under alkaline conditions as given above. During the first extraction the organic phase contained 1 μg of propranolol oxazolidone as a marker. After separation and evaporation of the organic phase the residue was dissolved in 30 μl of BSA and 120 μl of ethyl acetate before gas chromatographic analysis using automatic injection and nitrogen-selective detection. Blank urine with 25–200 μl of a 37.4 μmol l^{-1} solution of H 119/72 in 0.01 M HCl added was analysed. The standard curve was constructed by plotting the area ratio of H 119/72 to the marker versus the known concentrations of the metabolite.

RESULTS

Figure 1 summarizes the metoprolol metabolites identified in human urine. All of them, including the parent compound, can be analysed by the method presented here. In addition the structural elucidation of H 119/72,

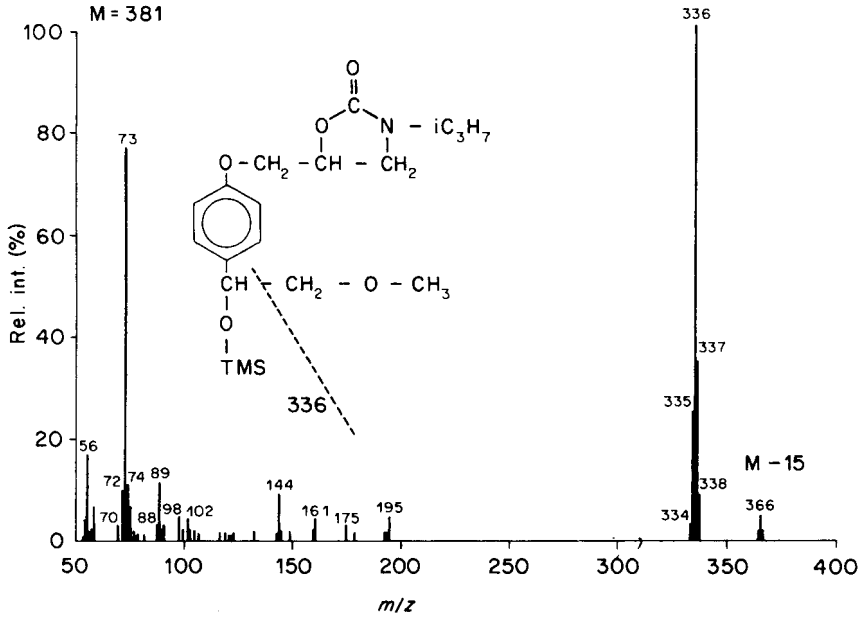

Figure 2. The mass spectrum of α-hydroxymetoprolol (H 119/66) after phosgene derivatization followed by trimethylsilylation.

H 119/77 and H 119/68 is reported in this study. Most probably, the new metabolites were formed by consecutive transformations of α-hydroxymetoprolol, H 119/66, which is an important primary metabolite in healthy subjects, accounting for about 10% of the given dose.[9] Alternative routes for their formation are indicated by arrows.

In Figs 2–5 the electron impact (EI) mass spectra of four α-hydroxylated metoprolol metabolites as oxazolidone/trimethylsilyl derivatives are given. The molecular ion was detected only in the spectrum of H 119/68 (Fig. 5) but $[M-15]^+$ ions were measurable for all compounds. The key ion of interest, m/z 336, was present in all spectra. It originated from cleavage of the carbon–carbon bond in the *para* substituent, being α to the aromatic ring with charge retention on the *O*-TMS-containing moiety. Other diagnostic fragments were observed only in the mass spectrum of H 119/68 (Fig. 5). The base peak at m/z 194 was probably due to loss of the oxazolidone ring and charge retention on the aromatic ring. The fragmentation pathway of the oxazolidone ring (m/z 143) gave rise to m/z 100 by elimination of the isopropyl group and m/z 56 after carbon dioxide expulsion.[11]

Both the basic and the acidic fractions were analysed by selected ion monitoring (SIM) based on specific fragments of these derivatives. The selected ion chromatogram of the basic fraction is given in Fig. 6, showing the retention time of H 119/68 (9.05 min), α-hydroxymetoprolol (H 119/66) (9.53 min) and H 119/72

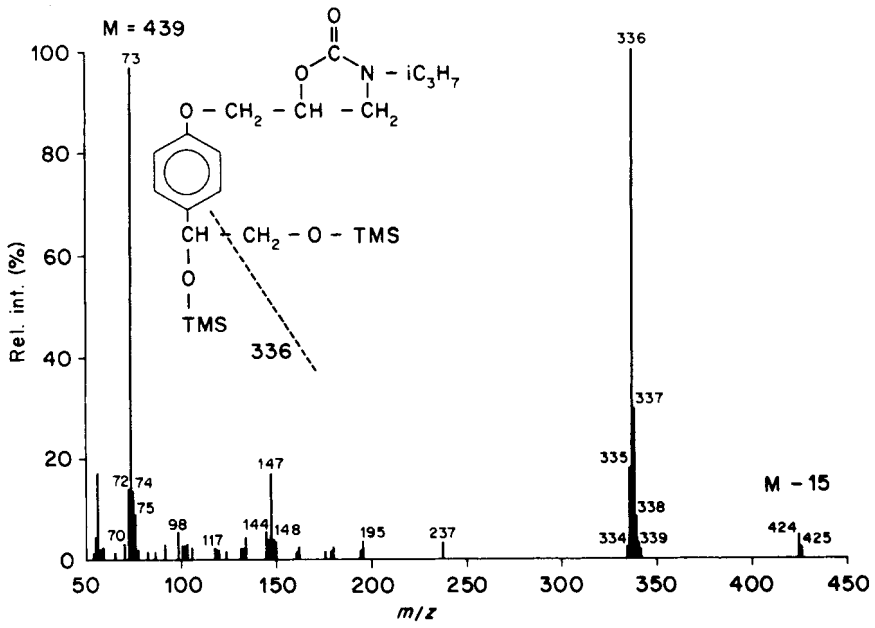

Figure 3. The mass spectrum of H 119/72 after phosgene derivatization followed by trimethylsilylation.

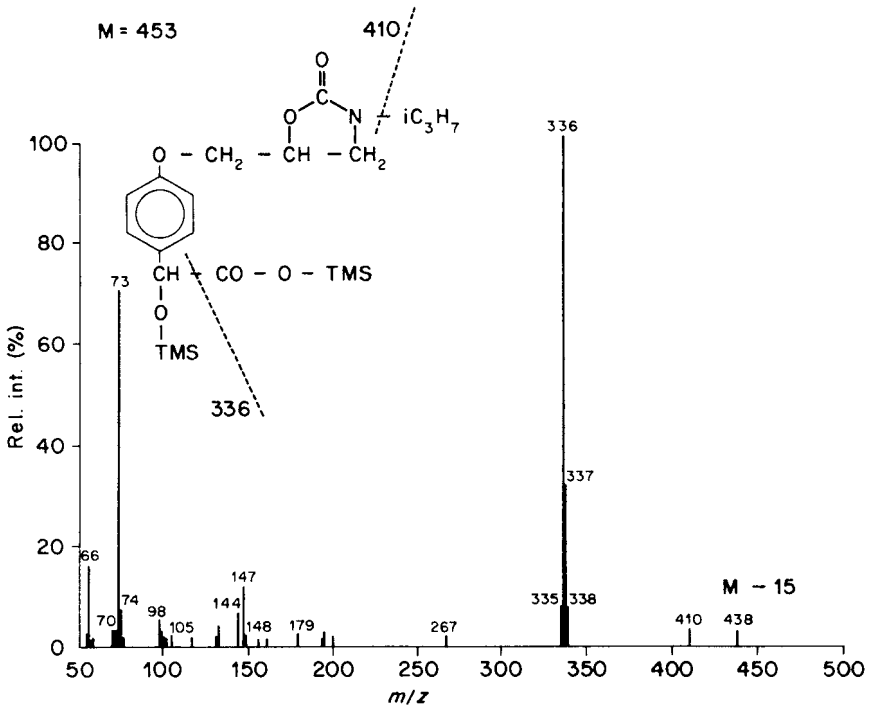

Figure 4. The mass spectrum of H 119/77 after phosgene derivatization followed by trimethylsilylation.

(10.15 min). Metabolite H 119/77 was detected in the acidic fraction (Fig. 7, t_R 8.00 min). The major metabolite H 117/04 eluted from the column after 7.33 min (m/z 365/350) and the third acidic metabolite H 104/83 was detected 4.52 min after injection using the $M^{+\cdot}$ and $[M-15]^+$ ions (m/z 384/369).

Nitrogen-selective detection was used in the quantitative assay for H 119/72, and the chromatograms are given in Fig. 8, showing blank urine (b), H 119/72 added (c) and an authentic sample (a). Interestingly, both metoprolol and α-hydroxymetoprolol were detectable in the sample, 22 and 50 μmol l^{-1}, respectively. Calibration graphs were in the range 7.5–0.94 μmol l^{-1} and the linear regression was typically about 0.99. Upon repeated analysis of a standard sample the precision was 3.6% ($n = 8$) at the 0.94 μmol l^{-1} level. The method was used to determine H 119/72 in 0–24 h urine from a volunteer who had taken a standard tablet of metoprolol tartrate (292 μmol, 100 mg). The concentration varied between 0.47 and 1.43 μmol l^{-1} at different collection

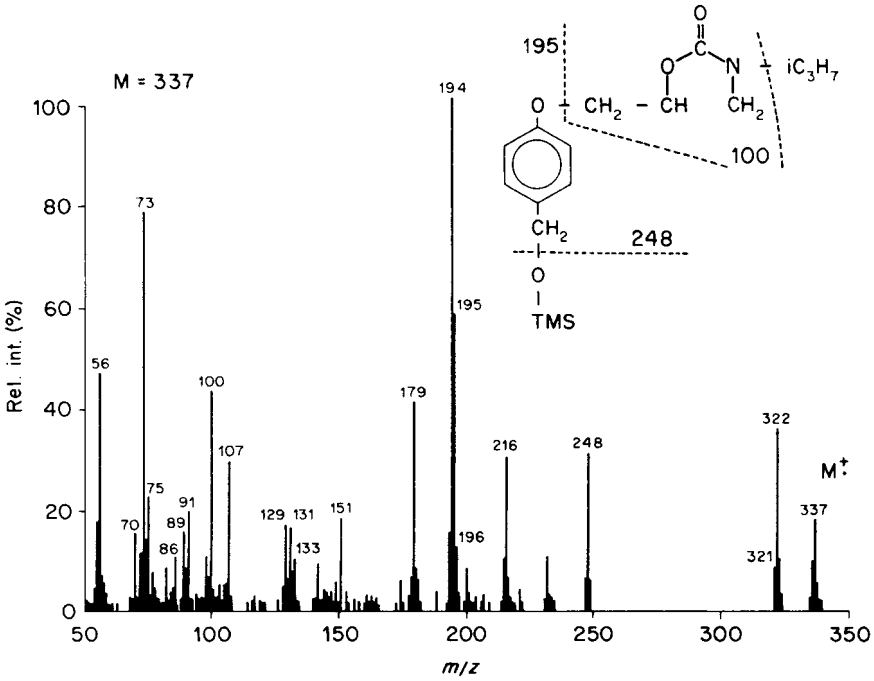

Figure 5. The mass spectrum of H 119/68 after phosgene derivatization followed by trimethylsilylation.

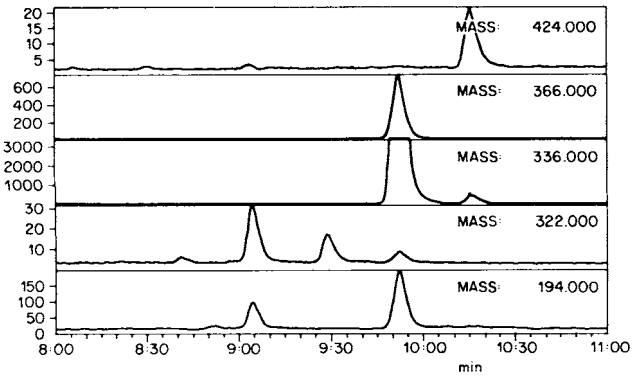

Figure 6. Selected ion monitoring of metoprolol metabolites after phosgene derivatization, basic extraction and trimethylsilylation. Peak identification: 9.05 min = H 119/68, 9.53 = α-hydroxymetoprolol (H 119/66) and 10.15 = H 119/72.

intervals. The recovery of excreted H 119/72 was 0.25% of the administered dose, indicating that this metabolic pathway is probably a minor one in man.

DISCUSSION

Derivatization with phosgene of the 2-aminopropanol side-chain in β-adrenoceptor antagonists produces oxazolidineones and the reaction offers several advantages. The formation of a cyclic derivative gives some degree of selectivity as monofunctional substances will react more slowly or form derivatives with low stability or poor chromatographic properties.[10] The applicability of gas chromatographic analysis with phosgene as reagent has been shown for the determination of metoprolol in plasma,[11] metoprolol and some of its metabolites in urine[8] and alprenolol and its phenol metabolite in urine.[12] A number of enantiomers have been separated on a modified chiral XE-60 gas chromatographic column following reaction with phosgene.[13-16] The oxazolidones of optically active compounds have also been shown to be suitable for enantiomer separation by liquid chromatography with Pirkle columns,[17] α_1-acido glycoprotein-coated silica columns[18] or on triacetyl cellulose.[19]

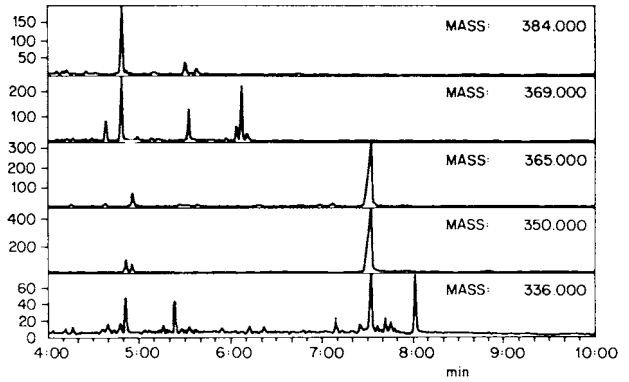

Figure 7. Selected ion monitoring of metoprolol metabolites after phosgene derivatization, acid extraction and trimethylsilylation. Peak identification: 4.52 min = H 104/83, 7.33 = H 117/04 and 8.00 = H 119/77.

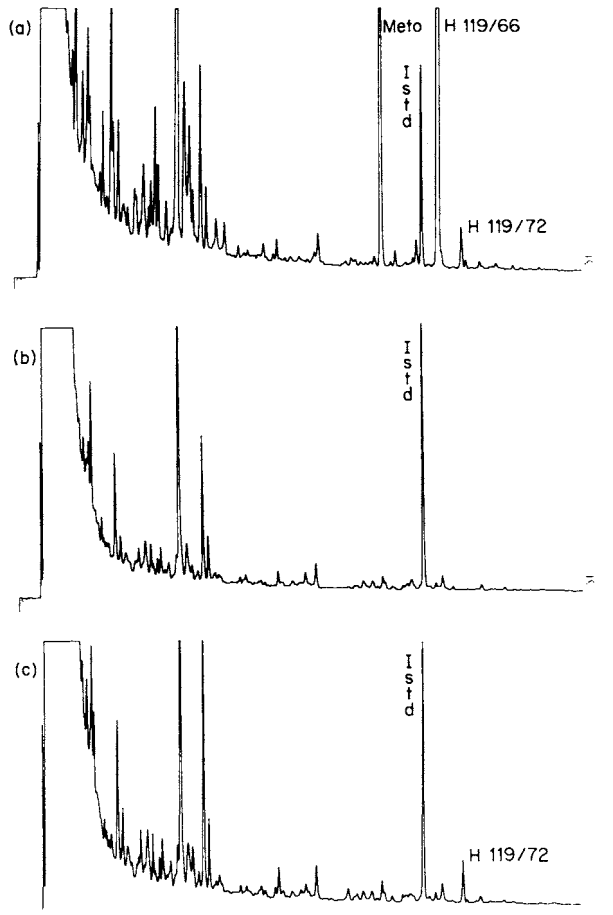

Figure 8. Nitrogen-selective detection gas chromatogram of the basic fraction after phosgene/trimethylsilylation: (a) real sample, (b) blank urine with internal standard added and (c) urine with H 119/72 added, 0.94 μmol/l. Peak identification: Meto = metoprolol, Istd = internal standard. Retention time of H 119/72 = 11.7 min.

The present study represents an extension of the analytical protocol for analysis of urinary metoprolol metabolites reported earlier.[8] The first extraction step after reaction with phosgene under alkaline conditions recovered the neutral derivatives of interest. The selectivity of both the chemical reaction and the nitrogen-selective detector was sufficient to determine H 119/72 in authentic samples. Compared to the previous method there were fewer peaks eluting early from the gas chromatographic column, which was mainly due to the absence of extracted and silylated endogenous acids in the urine. Owing to the more polar nature of some of these metabolites the extraction was repeated once. Using an organic phase ratio of 5:3, more than 95% of H 119/72 was recovered in the collected organic extracts as the oxazolidone derivative.

Metabolite H 119/77 was found in the acidic extract with an extraction recovery of about 90%. It was preferable to analyse this fraction by SIM of ions specific for these acidic metabolites: H 117/04, H 104/83 and H 119/77. As demonstrated in Fig. 7, they are easily measured in a single run without significant interference from the endogenous acids. This disturbance was a major problem in the previous method[8] with flame ionisation detection (FID), where numerous peaks eluted early from the column at retention times close to H 104/83.

In conclusion, the method presented here is capable of isolation and derivatization of metoprolol and seven urinary metabolites. The characteristic fragment at m/z 336 for α-hydroxylated metabolites enables a specific profiling of this class of compounds. This metabolic route might be of interest from the genetic point of view[20] in studies on the polymorphic metabolism of β-adrenoreceptor blocking drugs.

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