Selected Ion Monitoring of Metoprolol and Two Metabolites in Plasma and Urine Using Deuterated Internal Standards

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A highly sensitive and specific quantitative assay for metoprolol and two of its metabolites, containing an unchanged 2-hydroxy-4-isopropylaminopropoxy sidechain, has been developed. The compounds are isolated from the alkalized sample (plasma or urine) by extraction with dichloromethane, and converted to trifluoroacetyl derivatives by reaction with methyl-bis-(trifluoroacetamide). The reaction mixture is gas chromatographed on an OV-17 column and each substance is assayed by electron impact mass spectrometry using selected ion monitoring, and quantified by comparing the intensity of fragment ion *m/z* 266 with the intensities of corresponding fragment ions from the deuterated internal standards (*m/z* 270 and 271). It is possible to determine concentrations as low as 1 nmol l^{-1} (0.3 ng ml^{-1}) in 1 ml of sample with a relative standard deviation of less than 10%.

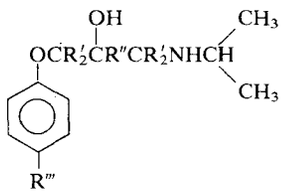
INTRODUCTION

Metoprolol is a selective β_1 -receptor antagonist which is extensively metabolized in man, dog and the rat.[1–3] Two of the metabolites, *O*-demethylmetoprolol (H 105/22) and α-hydroxymetoprolol, (H 119/66), have selective β_1-receptor blocking activity, although these substances have to be given in 5–10 times higher doses intravenously than metoprolol.[2–4] These metabolites cannot be determined in plasma and urine by the same method as metoprolol using electron capture gas chromatographic detection[5] because the plasma level of *O*-demethylmetoprolol is in most cases too low, and the derivative of α-hydroxymetoprolol formed with trifluoroacetic anhydride is unstable. In order to monitor low plasma and urine concentrations of both unchanged metoprolol and the two metabolites, an assay method with increased sensitivity is needed, which avoids the degradation of the products formed in the derivatization procedure.

EXPERIMENTAL

Chemicals

Metoprolol, the two metabolites *O*-demethylmetoprolol and α-hydroxymetoprolol, and the internal standards deuterated metoprolol and deuterated α-hydroxymetoprolol, were all supplied by the Department of Organic Chemistry, AB Haessle. Structural formulae are given below. The isotopic purity of fragment ion *m/z* 266 in the trifluoroacyl derivatives of the internal standards was found to be better than 99.9%, calculated from the peak height measurements of *m/z* 266, *m/z* 270 (deuterated metoprolol) and *m/z* 271 (deuterated α-hydroxymetoprolol). Dichloromethane, obtained from Fisher Scientific Co., was purified by distillation. Methyl-bis-(trifluoroacetamide) (MBTFA) was purchased from Macherey-Nagel+Co. GmbH (Germany). *N*-Methylimidazole (NMIM) (purum) was purchased from Fluka AG.



	R′	R″	R‴
Metoprolol	H	H	$-CH_2CH_2OCH_3$
O-demethylmetoprolol	H	H	$-CH_2CH_2OH$
α-hydroxymetoprolol	H	H	$-CHCH_2OCH_3$ (OH)
[$^2H_4$]Metoprolol	D	H	$-CH_2CH_2OCH_3$
α-[$^2H_5$]Hydroxymetoprolol	D	D	$-CHCH_2OCH_3$ (OH)

Abbreviations: MBTFA = methyl-bis-(trifluoroacetamide); NMIM = N-methyl imidazole.

Instrumentation

Mass spectra were recorded on a Varian MAT gas chromatograph mass spectrometer system 44S equipped with a 2 m glass column (2 mm i.d.) packed with 3% OV-101 on Gas Chrom Q, 120–140 mesh (Applied Science Lab.). Helium was used as carrier gas at a flow rate of 15 ml min^{-1}. Column oven temperature was 180 °C. Injector block, separator and transfer line temperatures were all 200 °C, while the ion source temperature was 220 °C. Mass spectra were recorded at an electron energy of 70 eV in the EI mode and 170 eV in the CI mode. Data acquisition and reduction were performed by Varian MAT Spectro System 200.

Quantitative analyses were performed on a Varian MAT gas chromatograph mass spectrometer system

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112. Chromatography was performed with a 1 m glass column (2 mm i.d.) packed with 3% OV-17 on Gas Chrom Q, 100-120 mesh (Applied Science Lab.), operated at 200 °C. Injection block, separator, transfer line and ion source temperatures were all 250 °C. The mass spectrometer was operated in the electron impact (EI) mode with an electron energy of 60 eV and an emission current of 1.5 mA. Selected ion monitoring at m/z 266, 270 and 271 was performed under software control by Varian MAT Spectro System 100, including peak area or peak height calculations.

Glassware

All tubes, pipettes and other glassware were washed in a laboratory dishwasher with detergent at pH 12, rinsed with phosphoric acid solution (pH 2) and with deionized water and finally dried at 60 °C.

Standard and internal standard solutions

Stock solutions of metoprolol, *O*-demethylmetoprolol and α-hydroxymetoprolol in hydrochloric acid (0.01 mol l^{-1}) were prepared in concentrations of 1×10^{-3} mol l^{-1}. Appropriate volumes of these solutions were brought together in one volumetric flask and diluted with hydrochloric acid (0.01 mol l^{-1}) to give a working standard solution with a concentration of 1×10^{-6} mol l^{-1} of each substance. The same procedure was used to produce a working internal standard solution with a concentration of both deuterated metoprolol and deuterated α-hydroxymetoprolol of 5×10^{-6} mol l^{-1}.

Analytical procedure

Urine or plasma, 0.1–1.0 ml, was transferred to a 15 ml tube (fitted with a Teflon-lined screw cap) containing 100 μl of the working internal standard solution. A sample volume of less than 1.0 ml was corrected by adding water. The aqueous phase was made alkaline with 0.25 ml of sodium carbonate solution (0.5 mol l^{-1}) and extracted with 8 ml of dichloromethane. After shaking for 10 min and centrifugation, the organic layer was transferred to a second screw-capped tube and evaporated to dryness under a gentle flow of dry nitrogen. After the addition of 95 μl of methyl-bis-(trifluoroacetamide) (MBTFA) and 5 μl of *N*-methylimidazole (NMIM) the reaction mixture was allowed to stand for at least 10 min at room temperature and then 2–5 μl of this mixture was injected into the gas chromatograph.

Quantitation

Three reference samples were prepared by adding 150 μl of the working standard solution to 1 ml of blank plasma or urine. These samples were analysed according to the analytical procedure. The peak height ratio for each substance (derivatives of *O*-demethylmetoprolol, metoprolol and α-hydroxymetoprolol) over the respective internal standard derivative was calculated for each chromatogram. Deuterated metoprolol was used as internal standard for both metoprolol and the metabolite *O*-demethylmetoprolol. The average of the peak height ratios for the three reference samples was used for the quantitative evaluation of the authentic samples.

Formation of α-hydroxymetoprolol-TFA

The formation of the α-hydroxymetoprolol-TFA derivative was studied starting with 1.0 ml of a water solution of α-hydroxymetoprolol (1.5×10^{-7} mol l^{-1}). Extraction and solvent evaporation were performed according to the analytical procedure described above. The residue was dissolved in MBTFA and the reaction was performed in this solution, or after dilution with toluene, ethyl acetate or pyridine (total volume 100 μl). The reaction was performed at 65 °C. At different time intervals 0.5 nmol of deuterated α-hydroxymetoprolol-TFA dissolved in 50 μl of toluene was added as internal marker before the selected ion monitoring assay. This method was also used to study the catalytic activity of *N*-methylimidazole at different concentrations in MBTFA. The reaction was run at room temperature for 5 min.

RESULTS AND DISCUSSION

Extraction

Benzene has been used previously as an extraction solvent for metoprolol.[5] Due to the more hydrophilic character of the metabolites of metoprolol this solvent will not in this instance give sufficient extraction. A more efficient solvent was dichloromethane for which distribution ratios and calculated extraction recoveries are presented in Table 1.

Derivatization

The acylated derivative of *O*-demethylmetoprolol is easily produced by standard methods, e.g. that used for metoprolol.[5] The trifluoroacetyl derivative of α-hydroxymetoprolol is also formed on treatment with trifluoroacetic anhydride, although the reaction is so slow that it has to be catalysed by for example triethylamine. A reaction mixture containing a tertiary amine as catalytic agent has to be washed with a buffer solution before injection, as described by Walle and Ehrsson.[6] However, the trifluoroacyl derivative of α-hydroxymetoprolol is unstable if treated in this way. On the other hand no washing will rapidly ruin the column, probably because the reaction mixture contains a salt

Table 1. Distribution ratios and extraction recoveries with organic phase = dichloromethane of pH = 13

Substance	D	Calculated extraction recovery ($V_{org}/V_{aq} = 6$)
Metoprolol	270	100
O-Demethylmetoprolol	3.5	95.4
α-Hydroxymetoprolol	3.9	95.9

between the tertiary amine and the fluorinated acid formed. In our experience the column lasted only one day and was destroyed by standing at an elevated temperature overnight. Similar results have been observed by injecting solutions containing ammonium chloride onto capillary columns.[7] Quarterman *et al.*[8] avoided these problems by using the more reactive heptafluorobutyrylimidazole without a catalyst to derivatize α-hydroxymetoprolol, and trifluoroacetic anhydride to derivatize metoprolol and *O*-demethylmetoprolol. Their method made use of electron capture detection. In order to perform the reaction in a non-acidic environment we have used another approach introduced by Donike,[9] utilizing *N*-methyl-bis-(trifluoroacetamide) (MBTFA) as trifluoroacylation reagent for amine and hydroxyl groups. This reagent gives neutral trifluoroacetamides as by-products. Therefore, in a gas chromatographic mass spectrometric (GCMS) procedure the reaction mixture can be injected directly onto the column without any clean-up.

The use of MBTFA as acylating reagent for α-hydroxymetoprolol was studied separately in more detail as this substance was the one that caused difficulties. The reaction profiles of α-hydroxymetoprolol in different mixtures are shown in Fig. 1. The reaction takes place at 65 °C in both pure reagent and in a mixture with other solvents. The fastest reaction is achieved in mixtures with pyridine which suggests that the reaction is base-catalyzed.[10,11] An even more effective catalyst, *N*-methylimidazole, introduced by Connors *et al.*[12,13] was also studied and was found to give a still faster reaction. Concentrations of *N*-methylimidazole (NMIM) from 5–20% (v/v) in MBTFA will give complete acylation of α-hydroxymetoprolol even at room temperature (Fig. 2). The reaction seems to be complete at the same rate as the substance dissolves.

There were no difficulties in applying this derivatization technique to the mixture of *O*-demethylmetoprolol, α-hydroxymetoprolol and metoprolol. The TFA-derivatives were stable in the reagent mixture for several days at room temperature, although the solution became slightly brownish upon storage. Structures of the derivatives were confirmed by mass spectrometry in both EI (Fig. 3) and chemical ionization (CI) mode with ammonia as ionization gas (Fig. 4).

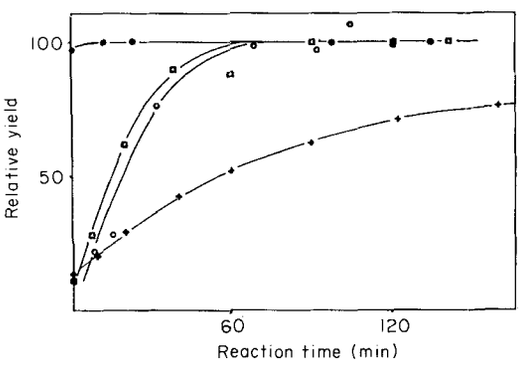

Figure 1. Formation rate of α-hydroxymetoprolol-TFA derivative at 65 °C with MBTFA in different solvents. □ MBTFA; ○ MBTFA + toluene (1+1); + MBTFA + ethyl acetate (1+1); ● MBTFA + pyridine (4+1).

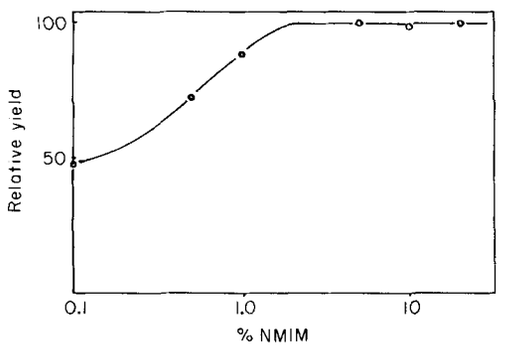

Figure 2. The influence of different concentrations of *N*-methylimidazole (NMIM) in MBTFA on the formation rate of α-hydroxymetoprolol-TFA (at room temperature for 5 min).

Quantitative evaluation

Trifluoroacetyl derivatives of substances with a 2-hydroxy-4-isopropylaminopropoxy sidechain will always give intense fragment ions at *m/z* 308 and *m/z* 266 in an EI mass spectrum, as described by Garteiz and Walle,[14] and these fragments have been used in determination methods for propranolol.[15,16] As the 2-hydroxy-4-isopropylaminopropoxy sidechain of metoprolol is intact in the two metabolites, α-hydroxymetoprolol and *O*-demethylmetoprolol, their mass spectra have these fragment ions in common (Fig. 3) and the ion *m/z* 266 has been selected for the quantitative work. The deuterium labelling of the internal standards in the propanol moiety in the sidechain is the most favourable and a shift of four mass units for [$^2H_4$]metoprolol-TFA (*m/z* 270 and 312) and five mass units for α-[$^2H_4$]hydroxymetoprolol-TFA (*m/z* 271 and 313) is obtained. The quantitative evaluation of *O*-demethylmetoprolol and metoprolol was made by comparing the intensities of the ions *m/z* 266 and *m/z* 270 and of α-hydroxymetoprolol by using the intensities of ions

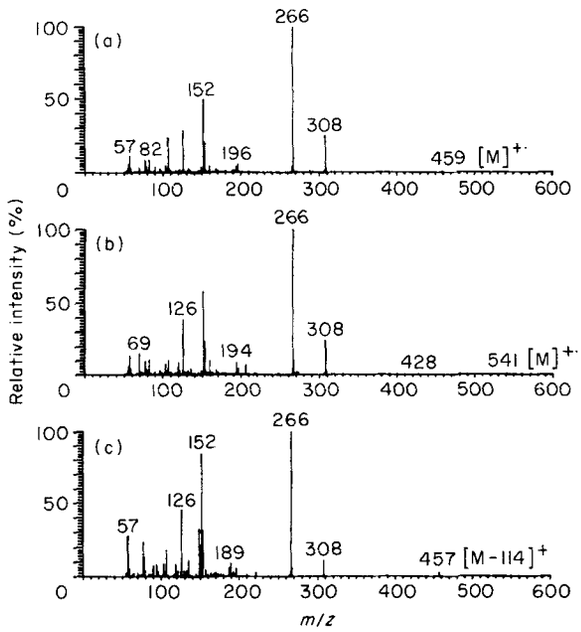

Figure 3. Electron impact mass spectra of the TFA-derivatives of metoprolol (a), *O*-methylmetoprolol (b) and α-hydroxymetoprolol (c).

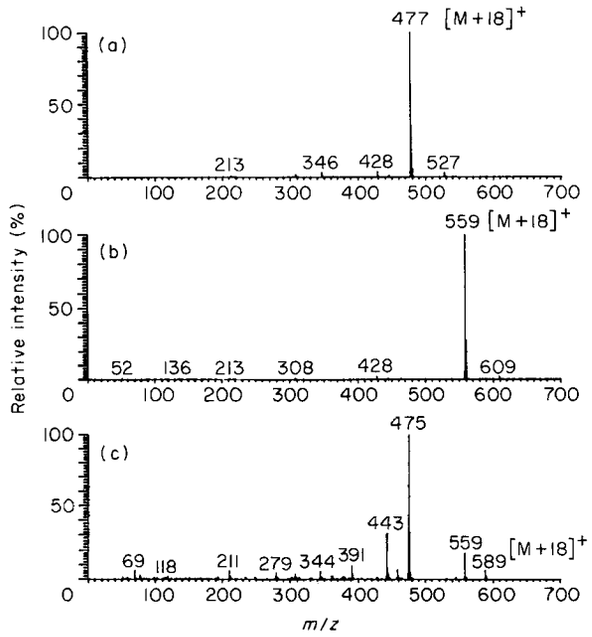

Figure 4. Chemical ionization (NH_3) mass spectra of the TFA-derivatives of metoprolol (a), *O*-demethylmetoprolol (b) and α-hydroxymetoprolol (c).

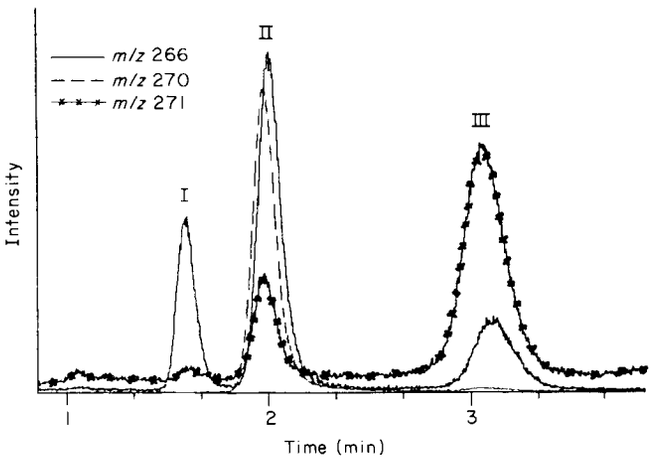

Figure 5. Selected ion recording of the TFA-derivatives of *O*-demethylmetoprolol (**1**), metoprolol (**2**) and α-hydroxymetoprolol (**3**) (stationary phase: OV-17). The amount injected on the column corresponds to 2.5 pmol each of *O*-demethylmetoprolol and α-hydroxymetoprolol, 5 pmol of metoprolol, 10 pmol of α-[$^2H_5$]hydroxymetoprolol and 20 pmol of [$^2H_4$]metoprolol.

m/z 266 and m/z 271. A typical chromatogram obtained from analysing a plasma sample is given in Fig. 5. By using a separate internal standard for α-hydroxymetoprolol the repeatability for the determination of this substance was improved significantly. At a concentration level of 100 nmol of α-hydroxymetoprolol l^{-1} the relative standard deviation was determined to 3%. The corresponding figure with deuterated metoprolol alone as internal standard for all three compounds was 9%. A reason for this improvement may be that the trifluoroacetyl derivative of α-hydroxymetoprolol is not thermally stable, and will to some extent decompose in the injection block and the column. This is not easily seen in the chromatogram since the decomposed substance does not separate from the intact derivative. However, by using a less polar stationary phase, OV-101, separation takes place as can be seen in the reconstructed mass chromatogram in Fig. 6. The relationship between these two peaks can be altered by varying the temperature of the injection block. Thus, an increase in temperature of the injection block will lead to decreased intensity of peak 2, which corresponds to the tri-trifluoroacyl derivative of α-hydroxymetoprolol, and an increase in the intensity of peak 1 (degradation product). The two substances have almost identical mass spectra in the El mode (Fig. 7), especially in respect to the relative intensity to m/z 266 (10.1%, $\delta_{rel} = 5\%$ for peak 1 and 9.9%, $\delta_{rel} = 4\%$ for peak 2, $n = 10$) (Fig. 6). Consequently, the quantitative evaluation of α-hydroxymetoprolol can be made using OV-17 as stationary phase and adding the intensities of the two unresolved peaks in the detector. By using chemical ionization (NH_3) the highest mass ion in the mass spectrum of the decomposed substance (peak 1 in Fig. 6) is determined to m/z 475 $[457 + NH_4]^+$ corresponding to the $[M-114]^+$ ion in the mass spectrum of the tri(trifluoro)acyl derivative of α-hydroxymetoprolol. It should be emphasized that the ion m/z 475 is the base peak even in the Cl mass spectrum of the intact TFA-derivative of α-hydroxymetoprolol (Fig. 4(c)) indicating an extremely labile functional group in the molecule. The derivatized aminohydroxy chain must be intact in the decomposed substance as rationalized by the abundant ions m/z 308 and 266 in the mass spectrum (Fig. 7). The degradation must then take place in the derivatized *para* substituent of α-hydroxymetoprolol and is most likely to involve a loss of trifluoroacetic acid (m/z 114) to form the side chain $-CH{=}CHOCH_3$. However, this compound is not available as a reference substance for confirmation of the structure.

The standard curve for all three substances, shown in Fig. 8, is straight and goes through the origin. No significant difference was observed between standard curves from plasma and urine samples within the concentration range studied. Repeatability measurements were performed at two concentration levels, at 200 nmol l^{-1} and at 10 nmol l^{-1}, and the results are

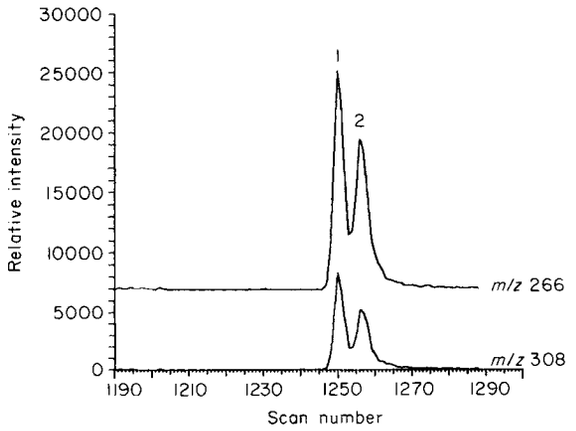

Figure 6. Reconstructed mass chromatogram of fragment ions m/z 266 and 308 from the gas chromatographic analysis of α-hydroxymetoprolol-TFA (reference substance) on an OV-101 column. Peak 2 corresponds to the intact tri-TFA derivative of α-hydroxymetoprolol (mass spectrum in Fig. 3 (c)), and peak 1 is the decomposition product formed during analysis (mass spectrum in Fig. 7).

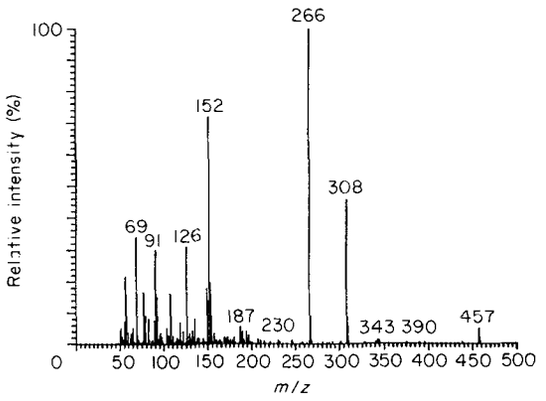

Figure 7. Electron impact mass spectrum of decomposition product formed during gas chromatographic analysis of α-hydroxymetoprolol-TFA (peak 1 in Fig. 6).

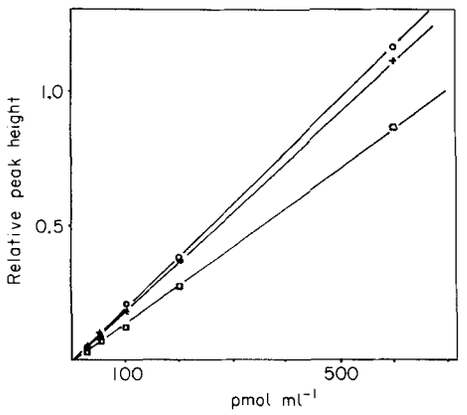

Figure 8. Standard curves for metoprolol (+), *O*-demethyl-metoprolol (□) and α-hydroxymetoprolol (o). These curves were obtained by analysing plasma samples with known concentrations of the substances.

Table 2. Repeatability of the method at different concentration levels ($n = 8$)

Concentration nmol l^{-1} \ Substance	Relative standard deviation (%) Metoprolol	*O*-demethyl-metoprolol	α-Hydroxy-metoprolol
200	2.5	4.0	2.0
10	5.0	5.0	7.0

summarized in Table 2. Using a maximum relative standard deviation of 10% as the definition of minimum determinable concentration, this could be set to 1 nmol l^{-1} for all three substances. This method has been used successfully for the analyses of plasma and urine samples from patients with impaired renal function treated with metoprolol[17–19] and from dogs[4] to evaluate the pharmacological activity of α-OH-metoprolol in this animal species.

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