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## Note

# Determination of metoprolol and its α-hydroxy metabolite in urine by direct-injection reversed-phase high-performance liquid chromatography with fluorimetric detection

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Oxidative drug metabolism is a major means of drug deactivation and clearance from the body. This enzymatic metabolism is often controlled by a single gene product which may exhibit polymorphism and so result in individuals with either slow or fast metabolism of the drug.

Mahgoub et al. [1] and Tucker et al. [2] discovered polymorphism of the 4-hydroxylation of debrisoquine, an anti-hypertensive drug, resulting in about 9% of Caucasians being poor metabolizers [3] whilst no poor metabolizers were found in the Japanese [4]. Lennard et al. [5] claimed that metabolism of the β-blocker, metoprolol, to α-hydroxymetoprolol also exhibits debrisoquine-type polymorphism, which McGourty et al. [6] later showed to have a 9% incidence in the British population.

To investigate this polymorphism in various population groups, a sensitive and selective assay method for metoprolol and its α-hydroxy metabolite is necessary.

Derivatized metoprolol in plasma has been assayed using gas chromatography (GC) with electron-capture detection (ECD) [7] and in conjunction with mass spectrometry [8] and Kinney [9] used GC–ECD to determine metoprolol in both plasma and urine. Reversed-phase (RP) high-performance liquid chromatography (HPLC) with fluorimetric detection was used by Harrison et al. [10] and Rosseel et al. [11] to assay metoprolol in plasma and for counter-ion separation of propranolol, metoprolol and atenolol after extraction from plasma and tissue [12]. None of these methods are applicable to the separation of α-hydroxymetoprolol from all the other metoprolol metabolites and matrix components in urine. Balmér et al. [13] used a 3-μm ODS column and fluorimetric detection for

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the counter-ion separation of metoprolol, α-hydroxymetoprolol and metoprolol acid in urine, with a determination limit of 0.5 μmol/l (ca. 100 ng/ml).

Gyllenhaal and Hoffmann [14] used capillary GC with flame ionization detection (FID) to separate and quantify metoprolol and its four polar metabolites in urine, but only after extensive sample extraction and derivatization.

More direct methods have utilized HPLC [15–18]. Godbillon and Duval [15] used RP-HPLC with UV detection to assay the required analytes, but the method proved too insensitive for our purposes of measuring the α-hydroxy metabolite in poor metabolizers. Pautler and Jusko [16] employed normal-phase HPLC on a 5-μm silica column and fluorimetric detection to assay metoprolol and α-hydroxymetoprolol in urine, whilst Lennard and Silas [17] used RP-HPLC with fluorimetric detection for the same separation in both plasma and urine. Lennard later [18] improved the assay method and applied it to the separation of metoprolol and its three major metabolites in urine and liver microsomes. We found that, as well as requiring tedious sample preparation, none of the above HPLC methods gave satisfactory resolution of metoprolol and its major metabolites from each other and interfering matrix components.

This paper describes a method for the assay of metoprolol and α-hydroxymetoprolol in urine using isocratic RP-HPLC separation on a phenyl column with fluorimetric detection. Sample preparation is minimal, being limited to the addition of internal standard and buffer to the sample before direct sample injection.

## EXPERIMENTAL

### *Reagents*

All solvents used were spectrographic grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and all water was purified by the Milli-Q system (Millipore, Bedford, MA, U.S.A.). All other reagents were analytical grade. Ammonium dihydrogenphosphate (0.05 *M*, pH 4.6) was used as buffer. The pure standards were donated by Ciba-Geigy (Basle, Switzerland) and atenolol was a gift from ICI (Pharmaceuticals ) S.A.

### *Internal standards*

Atenolol was found to be satisfactory as an internal standard in the elution and detection conditions used. Atenolol (5 μg/ml) in buffer was used as the internal standard solution.

### *Sample collection*

Prior to dosing, blank urine was collected from each subject and a 10–20 ml sample frozen. Metoprolol (100 mg metoprolol tartrate; Lopressor) was then administered orally to each fasting subject and the total urine collected for the 0–8 h period. Samples (10–20 ml) of the measured 8-h collection were rapidly frozen in liquid nitrogen and stored at −18 °C until assay.

*Sample preparation*

A 200-$\mu$l volume of urine was added to 100 $\mu$l of buffer plus 100 $\mu$l of internal standard solution (5 $\mu$g/ml atenolol in buffer), vortex-mixed for 5 s, then injected on-column through a 100-$\mu$l loop.

*Standards preparation*

Standards (100 $\mu$l) in buffer (range 0.2–20 $\mu$g/ml) were added to 200 $\mu$l of blank urine with 100 $\mu$l of internal standard solution, vortex-mixed and injected as for the sample urines.

*Chromatography*

The HPLC was performed on a Spectra Physics 8100 liquid chromatograph with a Valco auto-injector loop valve (100-$\mu$l loop). Separation was achieved with a 250 mm $\times$ 4.6 mm I.D. Spherisorb 5-$\mu$m phenyl column. Isocratic binary elution was performed with a mobile phase of acetonitrile–0.05 *M* ammonium dihydrogenphosphate (pH 4.6) buffer (8:92). The flow-rate was 2-ml/min and the column temperature was 34°C.

*Detection*

A Perkin-Elmer 650-10 dual-monochromator fluorescence detector was used with an excitation wavelength of 220 nm and an emission wavelength of 318 nm. The detector output was monitored simultaneously on a Perkin-Elmer 56 strip-chart recorder and a Spectra Physics SP 4200 integrator. The concentrations of metoprolol and $\alpha$-hydroxymetoprolol in urine were estimated on the basis of peak-height ratio from the standard calibration curves.

## RESULTS

Metoprolol is oxidatively metabolized by three routes in humans yielding two active metabolites, $\alpha$-hydroxymetoprolol ($M_1$) and O-desmethylmetoprolol ($M_4$), and two very polar inactive acid metabolites ($M_2$ and $M_3$) [19]. Using the above technique good separation and detectability of the active analytes in urine could be achieved, free of interference from the other metabolites or matrix components, as can be seen in the chromatograms (Fig. 1). No interfering components were found in the pre-dosing urine of 200 volunteers.

The standard response curves were linear over the ranges tested, 20–400 ng/ml (1 $\mu$g/ml atenolol as internal standard) and 0.2–20 $\mu$g/ml (5 $\mu$g/ml atenolol as internal standard), although only the upper range was used for the assays. The inter-sample standard deviations were 1.15 and 1.85% for metoprolol and $\alpha$-hydroxymetoprolol, respectively, over five samples at 1 $\mu$g/ml. The limit of determination was ca. 20 ng/ml for metoprolol and 10 ng/ml for $\alpha$-hydroxymetoprolol, ca. 200 times lower than the concentrations found in normal metabolizers.

Although no guard column was used, the resolution of the column was maintained by exchanging the first 5 mm of the column packing with fresh packing after every 100 assays. Using this technique, the resolution was still satisfactory after more than 450 assays.

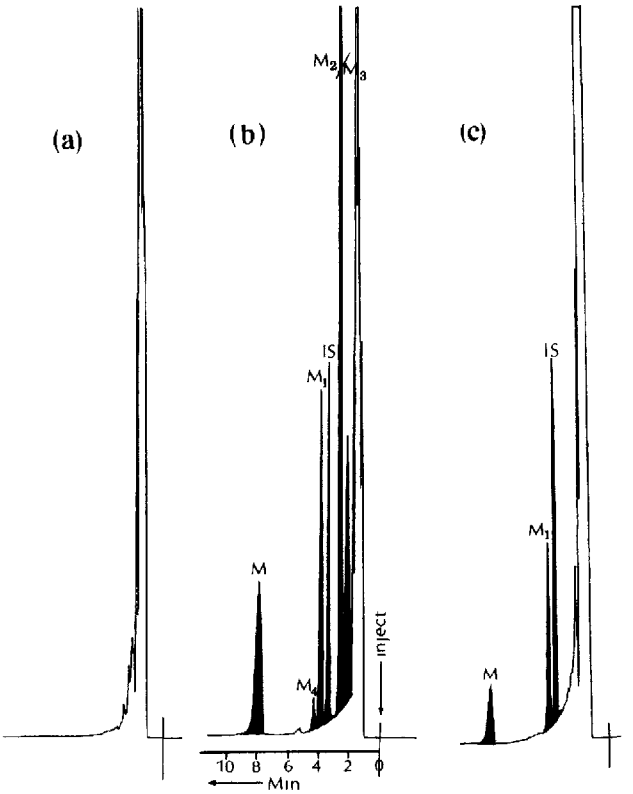


Fig. 1. Chromatograms of (a) blank urine, (b) 0–8 h urine sample after oral administration of 100 mg metoprolol tartrate and (c) 1 $\mu$g/ml spiked urine standard. The chromatograms were obtained with the ODS guard and phenyl analytical columns. Peaks: IS = atenolol (internal standard); M = metoprolol; $M_1$ = $\alpha$-hydroxymetoprolol; $M_2$ and $M_3$ = acid inactive metabolites; $M_4$ = O-desmethylmetoprolol.

## DISCUSSION

Modern techniques of double end-capping reversed-phase columns result in ODS columns with a very lipophilic nature. Whilst this yields much greater reproducibility between columns, it also means the columns no longer have the mildly polar–lipophilic mixed-phase effect which resulted from the residual silanol groups which the older-type ODS columns variably contained. Bonded phenyl, nitrile and amine columns offer a more reproducible replacement of this mixed-phase effect and are being increasingly utilized when a mildly polar separation is required.

Because of the difference in polarity of moderately lipophilic metoprolol and its hydrophilic metabolites, a phenyl column was chosen. It was hoped that the mild cyclic $C_6$ lipophilic interaction would give a sufficiently short retention time for metoprolol, whilst the polar aromaticity would increase the retention of the polar metabolites and thus permit elution and separation of the analytes in a

suitable time span. Applying the above method, the elution time for the analytes was less than 10 min.

Whilst fluorescence detection was not necessary for detection sensitivity purposes, it was superior to UV–VIS detection because of its better selectivity and resultant decrease of interfering matrix components.

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