Journal of Chromatography, 342 (1985) 199—205
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2616

Note

Quantitative analysis of metoprolol and three of its metabolites in urine and liver microsomes by high-performance liquid chromatography

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(First received December 18th, 1984; revised manuscript received March 6th, 1985)

Metoprolol is a lipophilic β-adrenoceptor antagonist which undergoes extensive first-pass metabolism in man [1]. Only about 3% of an oral dose is excreted unchanged in the urine, the rest being oxidatively metabolised by one of three major routes (Fig. 1) [2]. Studies using an assay developed in this

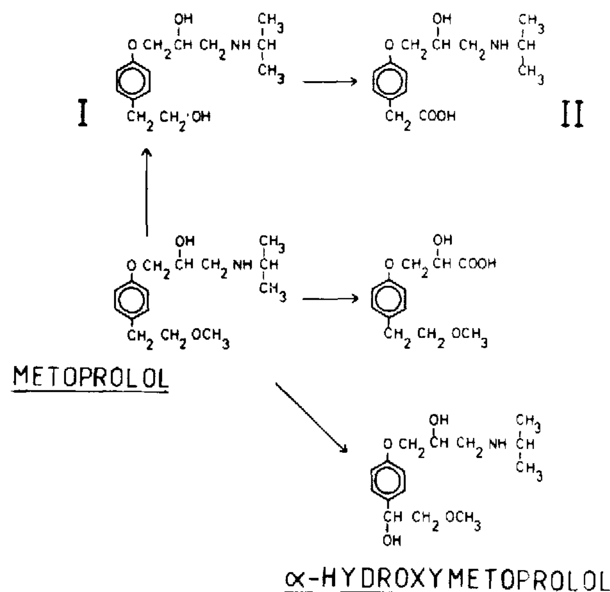

Fig. 1. Major pathways of metoprolol metabolism in man.

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department [3] have demonstrated that plasma concentrations of metoprolol are elevated and formation of α-hydroxymetoprolol (HM) is impaired in poor metabolisers of the polymorphically oxidised drug, debrisoquine [4, 5]. In extending this work to the study of hypertensive patients taking other drugs, the method was found to be lacking in selectivity and further refinement was necessary.

HM is a relatively minor metabolite of metoprolol (10% of dose) and it was decided to investigate the major route, O-dealkylation, in relation to polymorphic oxidation. The O-dealkylated product (I, Fig. 1) is almost completely oxidised to the carboxylic acid (II, Fig. 1), which is reported to account for 65% of the dose [2]. The zwitterionic nature of II probably accounts for the paucity of methods available for its analysis in urine. Attempts at extraction of II into organic solvents have been unsuccessful. The original metabolic data [2] were obtained by separation of tritiated material on a XAD-2 column followed by silylation and radio gas chromatography. Two further gas—liquid chromatographic (GLC) methods have been described [6, 7]. Both, however, incorporate complex, time-consuming sample work-up procedures involving sequential derivatisation of the secondary amino (performed in situ) and carboxylic acid moieties of II. The high-performance liquid chromatographic (HPLC) method of Godbillon and Duval [8] represents a considerable advance on the above GLC assays. The conditions allow direct injection of urine on to the chromatograph, with simultaneous determination of II, HM and metoprolol. The lower limit of sensitivity (5 μg/ml), however, would be insufficient for our studies.

In this paper, rapid and sensitive HPLC methods possessing good selectivity are described for the analysis of metoprolol, HM, I and II in urine (the latter by direct injection) down to concentrations of 0.1, 0.01, 0.02 and 0.5—1.0 μg/ml, respectively.

A further aim of our work is to explore the enzymatic basis of the defective oxidation of metoprolol and, to this end, the assays for urine have been modified to allow the estimation of metoprolol and metabolites in rat and human liver microsomes.

Data are presented which show the applicability of these methods to in vivo and in vitro studies of metoprolol metabolism.

EXPERIMENTAL

Drugs and chemicals

Metoprolol tartrate, α-hydroxymetoprolol *p*-hydroxybenzoate, II · HCl and I base were generous gifts from Hässle (Mölndal, Sweden). Nadolol base was donated by E.R. Squibb (London, U.K.). Dichloromethane (glass-distilled) and acetonitrile (HPLC S grade) were purchased from Rathburn Chemicals (Walkerburn, U.K.). All other reagents were of analytical-grade purity.

Standard solutions

Stock solutions (100 μg/ml, metoprolol base equivalent) of drugs and metabolites were prepared in distilled water and were found to be stable for at least one year at 4°C.

Source of human liver

Tissue was obtained from renal transplant donors being maintained on life support systems until the kidneys were required. The local Coroner and the Ethics Committee of the Royal Hallamshire Hospital gave approval for this tissue to be used in studies on the metabolism of metoprolol and other drugs.

Preparation of microsomes

Microsomes were prepared from rat or human liver and incubated with drug as described by Boobis et al. [9]. The reaction was terminated by the addition of 6% (w/v) perchloric acid (0.2 vol. to 1 vol. incubation mixture).

Sample preparation: urine (method I)

Metoprolol, HM and I Sample (0.5–1.0 ml), internal standard (nadolol; for amount see Table I) and sodium carbonate (0.5 ml, 0.5 *M*) were gently mixed with dichloromethane (5 ml) for 10 min. After centrifugation (900 *g*, 2 min) the upper aqueous layer was discarded and the organic extract was evaporated to dryness at 40°C on a Buchler vortex evaporator (Baird and Tatlock, Romford, U.K.). The residue was reconstituted in mobile phase (0.5—2 ml) and an aliquot (20—100 μl) was injected into the chromatograph.

Compound II. The sample was diluted with an equal volume of mobile phase and an aliquot (50 μl) was injected into the chromatograph.

Sample preparation: microsomes (method II)

HM and I. The incubation mixture (100 μl) was mixed with distilled water (40 μl) and internal standard (200 ng nadolol) to give a total volume of 160 μl. After centrifugation (900 *g*, 5 min) an aliquot (10—30 μl) of the resulting supernatant was injected into the chromatograph.

HPLC instrumentation

The chromatograph comprised a Model 6000A pump (Waters Assoc., Northwich, U.K.), a WISP Model 710B automatic sample injector (Waters), a Guard-Pak pre-column module (Waters), a Z-Module column system (Waters) and a Model 970 FS Schoeffel fluorimeter detector (HPLC Technology, Macclesfield, U.K.). Peaks were monitored at an excitation wavelength of 193 nm with no emission filter.

Column packings and mobile phases

Metoprolol, HM and I. A Radial-Pak cartridge (10 cm × 8 mm I.D.) containing Nova-Pak C_{18} reversed-phase material (5 μm particle size) (Waters) and a plastic insert pre-packed with C_{18} (40 μm particle size) were used for the analytical column and pre-column, respectively. The mobile phase was water—acetonitrile (88:12) containing 1% (w/v) triethylamine adjusted to pH 3 with orthophosphoric acid and was pumped through the column at a flow-rate of 3 ml/min.

Compound II. A Radial-Pak cartridge containing Partisil SCX (Merck) strong cation-exchange material (10 μm particle size) and a plastic insert pre-packed with CN material (40 μm particle size) were used for the analytical column and pre-column, respectively. The mobile phase comprised of 0.05 *M* sodium di-

hydrogen phosphate—methanol (98:2) adjusted to pH 5.5 with 10 *M* sodium hydroxide. The flow-rate was 3 ml/min.

All chromatography was performed isocratically and at ambient temperature.

Calibration

Calibration graphs were constructed by spiking urine or microsome samples with known amounts of drug and metabolites. A range of standards was included with each batch of samples.

RESULTS

Sharp symmetrical peaks were observed for HM, I, internal standard (I.S.), metoprolol and II (retention times 105, 120, 170, 504 and 270 sec, respectively) using either reversed-phase or ion-exchange chromatography (Fig. 2). HM, I and metoprolol did not elute from the cation-exchange column. Compound II had a retention time of 150 sec under the reversed-phase conditions. Many assays have been performed on the urine of patients taking a variety of cardiovascular and other drugs which included atenolol, propranolol, hydrochlorthiazide, triamterene, methyldopa, spironolactone, hydralazine, bendrofluazide, carbidopa, levodopa and flurbiprofen. None of these drugs caused interference. When present, spurious peaks were fully resolved from metoprolol and metabolites.

Calibration curves for metoprolol and each metabolite were linear and passed through the origin (Table I). Standards prepared from water instead of urine or microsomes gave identical absolute peak heights and peak height ratios

TABLE I

CALIBRATION DATA FOR THE MEASUREMENT OF METOPROLOL, HM, I AND II IN URINE AND LIVER MICROSOMES

Drug/ metabolite	Amount of internal standard (μg)	Calibration range (μg/ml)	Linearity (r^2)	Coefficient of variation (n = 6)	Minimum detectable concentration (μg/ml)
Urine					
Metoprolol	5	0.5 –5 0	>0.99	1.7 (2.5)*	0.05
HM	5	0.5 –5.0	>0.99	1.5 (2.5)	0.01
	1	0.05–1.0	>0.99	3.0 (0.2)	
I	1	0.05–1.0	>0 99	—	0 02
II	—	5 –100	>0.99	2.0 (10) 1.2 (60)	0 5–1.0
Microsomes					
HM	0.2	0.05–4.0	>0.99	5.4 (0 1) 1.5 (2.0)	0.02
I	0.2	0.05–4.0	>0.99	5.1 (0.1) 1.1 (2.0)	0 04

*Values in parentheses represent concentrations at which coefficients of variation were estimated.

indicating that the presence of a biological matrix did not affect drug or metabolite recoveries. Values for intra-assay coefficients of variation and estimates of lowest measurable concentrations are also shown in Table I.

DISCUSSION

Using a previous method [3] to measure metoprolol and HM in urine, most pre-dose samples from hypertensive patients gave a peak with an identical retention time to that of HM. The hydrophilic β-blocker, atenolol, was suspected of causing this interference because the majority of these patients were taking atenolol, and atenolol co-eluted with HM. By substitution of a C_{18} for the phenyl stationary phase, acetonitrile—triethylamine for methanol—heptanesulphonic acid as eluent and an excitation wavelength of 193 nm for one of 222 nm, a baseline separation of both atenolol and HM and the interfering peak and HM was achieved. Atenolol and the interfering peak had identical retention times under the new conditions (78 sec). These modifications also led to a considerable enhancement in the assay sensitivity of about twenty-fold for HM and five-fold for metoprolol.

Of the other HPLC methods available for metoprolol and HM in urine Gengo et al. [10] quoted lower limits of detection than in the present assay but do not indicate whether their chromatography is likely to be affected by concomitant drug therapy. Godbillon and Duval [8] described a procedure which does not require a solvent extraction step. However, the analysis time is

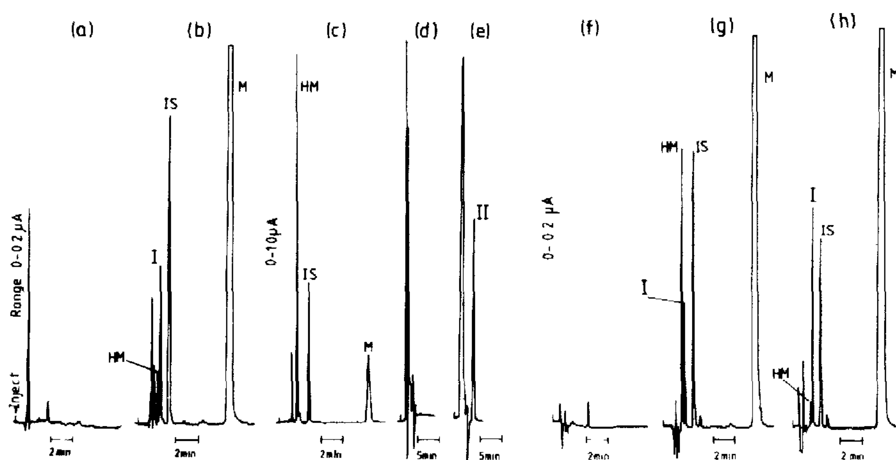

Fig. 2. Chromatograms of metoprolol and metabolites assayed in urine and liver microsomes: (a) pre-dose urine (reversed-phase conditions); (b and c) 0—8 h urine from subjects who had taken 100 mg metoprolol containing (b) 0 058 µg/ml HM, 0.17 µg/ml I, 7.6 µg/ml metoprolol and 1 µg internal standard, (c) 13.8 µg/ml HM, 7.3 µg/ml metoprolol and 5 µg/ml nadolol, internal standard; (d) pre-dose urine (ion-exchange conditions); (e) 0—8 h urine containing 24.6 µg/ml II; (f) liver microsomes before incubation, (g) rat liver microsomes incubated for 5 min with 40 µM metoprolol at 37° C and pH 7.25 containing 0.25 µg/ml HM, 0.16 µg/ml I, and 1 µg internal standard; (h) human liver microsomes incubated for 20 min with 400 µM metoprolol containing 0.11 µg/ml HM, 1.28 µg/ml I, and 1 µg internal standard. Peaks: M = metoprolol; HM = α-hydroxymetoprolol; I = O-dealkylated metoprolol; II = carboxylic acid of I; IS = internal standard.

about twice as long and the sensitivity at least two orders of magnitude lower than the present method reported here and would not be adequate for HM estimation in samples from defective metabolisers of metoprolol.

Because almost all of compound I formed by O-demethylation of metoprolol is further oxidised, this metabolite represents only a minor fraction of the dose (less than 0.4%) [6, 11]. The present assay was sufficiently sensitive to detect I in the urine of subjects taking metoprolol (Fig. 2). However, in those individuals who had relatively high concentrations of HM in their urine estimation of I was less than ideal under the HPLC conditions used, owing to the similarity of retention times of the two metabolites. It was found that the determination of I could be improved by increasing the water content of the mobile phase, thereby increasing resolution but this was at the expense of a longer analysis time.

Gyllenhaal and Hoffman [7] have questioned the suitability of HPLC for the analysis of II in urine. Since II cannot be extracted from aqueous media, the sample would have to be injected directly on to the column unless a derivatisation step is included. Following this approach and using a reversed-phase column and UV detection these workers were not able to obtain sufficient selectivity even though urinary concentrations of II are known to be high [6]. Using a C_8 column and an acetonitrile—buffer mobile phase Godbillon and Duval [8] have essentially overcome this problem enabling II to be analysed by direct injection of diluted urine. In the present work selectivity was improved by using a strong cation-exchange column with fluorescence detection. The resulting ten-fold increase in sensitivity was found to be necessary for monitoring II over a post-dose period of 48 h in some subjects. Fig. 3 shows the urinary excretion of metoprolol, HM and II in a healthy volunteer.

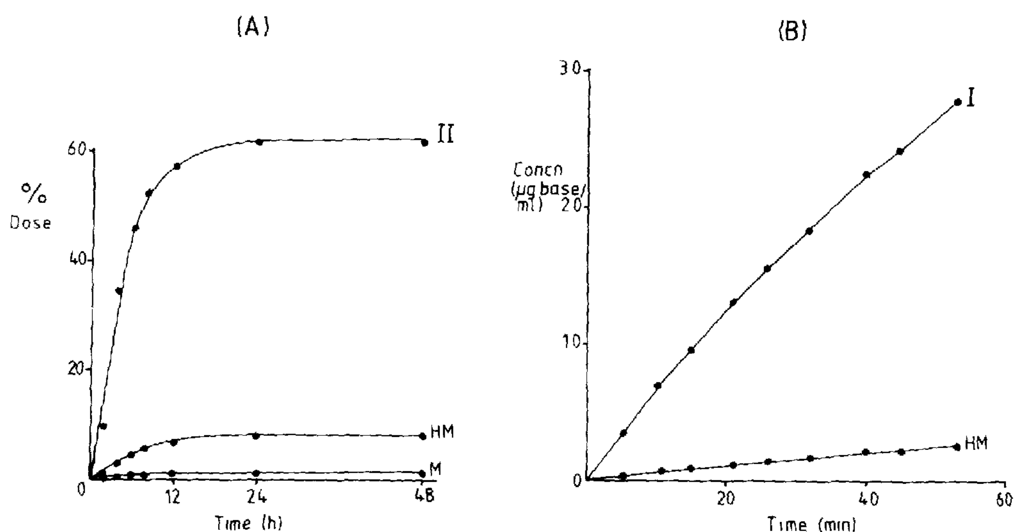

Fig. 3. (A) Cumulative urinary excretion over 48 h of metoprolol (M), HM and II in a subject after a single oral dose of 100 mg metoprolol. (B) Appearance of HM and I with time in human liver microsomes incubated with 400 μM metoprolol at 37°C, pH 7.25, protein concentration = 2.5 mg/ml.

Initially the above urine methods were applied without significant modification to the analysis of metoprolol, HM and I in incubations of liver microsomes. Since the latter contains far fewer endogenous materials than urine the use of a simplified sample clean-up procedure was investigated. Protein precipitation with perchloric acid and injection of the resulting supernatant gave clean chromatograms and did not affect the peak shape of the metabolites adversely. Detector sensitivity was sufficient to allow the volume of sample to be reduced from 1.0 to 0.1 ml.

When incubated with metoprolol, rat and human liver microsomes generated differing metabolic profiles (Fig. 3). In the rat α-hydroxylation was predominant over O-demethylation at the substrate concentration used. In contrast metabolism by human liver microsomes was esssentially restricted to O-demethylation and only small amounts of HM were formed. The appearance of II, which eluted between I and the internal standard, was negligible using microsomes from both species.

ACKNOWLEDGEMENTS

The author is grateful to Mrs. H.K. Crewe, Mrs. J. Harlow and Mr. L. Shaw for laboratory assistance, to Dr. G.T. Tucker for reviewing the manuscript, and to Professor H.F. Woods for provision of laboratory facilities. M.S.L. is a Wellcome Trust Lecturer.

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