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## Note

# Timolol determination in plasma and urine by high-performance liquid chromatography with ultraviolet detection

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The β-adrenoceptor antagonist, timolol, is a potent antihypertensive and antianginal drug which has also proved to be effective for the secondary prevention of myocardial infarction [1].

The analysis of timolol in human plasma following therapeutic doses (10—20 mg) requires methods capable of measuring the drug in the lower ng/ml range. Gas—liquid chromatography (GLC) with electron-capture or alkali-flame-ionization detection have been used most commonly for this purpose and the methods have lower limits of assay of 1—5 ng/ml [2—4]. Greater sensitivity (0.5 ng/ml) has been obtained using GLC—mass spectrometry [5, 6]. However, this technique is not available in many laboratories. Furthermore, all the above GLC procedures involve derivatisation of the drug and in some cases they incorporate tedious sample and reagent clean-up steps.

High-performance liquid chromatography (HPLC) allows analysis of underivatised β-adrenoceptor antagonists. However, a lack of suitably sensitive detectors has been a limiting factor in the use of this technique to analyse timolol concentrations in plasma after therapeutic doses. Thus, the method of Lefebvre et al. [7] can only detect peak plasma concentrations of the drug. Recently, however, Gregg and Jack [8] have reported a limit of sensitivity of 2 ng/ml in plasma and breast milk, using an electrochemical detector.

We now describe an alternative HPLC method for the determination of timolol in plasma and urine which is comparable in sensitivity to that of Gregg and Jack [8] but which uses a very stable UV detector. Although the capital cost of this UV detector is about twice that of most electrochemical detectors, the latter tend to require more attention during routine use.

This method was developed to explore the relationship between oxidation phenotype and the metabolism of timolol in man [9]. Plasma concentration

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and urine excretion data from one extensive and one poor metaboliser of debrisoquine are presented.

## MATERIALS AND METHOD

### *Chemicals and drugs*

Timolol maleate was a gift from Merck Sharpe & Dohme (Hoddesden, U.K.). The internal standard, phenacetin, was obtained from BDH (Poole, U.K.). HPLC-grade acetonitrile and glass-distilled methyl *tert.*-butyl ether were purchased from Rathburn Chemicals (Walkerburn, U.K.). All other chemicals were of analytical grade.

Stock solutions of timolol (1 μg base per ml) prepared in distilled water were found to be stable at 4°C for at least three months.

### *HPLC instrumentation and conditions*

The chromatograph consisted of a Model 3000 Applied Chromatography Systems pump, a Model 773 Kratos Spectroflow UV—VIS absorbance detector and a Model 7125 Rheodyne injector (0.5-ml loop), all purchased from HPLC Technology (Macclesfield, U.K.). The detector wavelength was set at 295 nm. The stainless-steel column (10 cm × 5 mm I.D.) used was packed with Hypersil 5-ODS reversed-phase material (5 μm particle size) (HPLC Technology). A standard pre-column (5 cm × 5 mm I.D.) containing reversed-phase guard column material (40 μm; Waters Assoc., Northwich, U.K.) was fitted between the injector and the analytical column.

Water—acetonitrile (87:13) containing 1% triethylamine and adjusted to pH 3 with orthophosphoric acid was used as the mobile phase. Chromatography was performed isocratically at a flow-rate of 2 ml/min and at ambient temperature.

### *Extraction procedure*

Heparinised plasma or diluted urine (1.0 ml of a ten-fold dilution in water) was shaken gently with sodium hydroxide (4 *M*, 0.1 ml), internal standard (0.25 and 1.0 μg for plasma and urine, respectively) and methyl *tert.*-butyl ether (5 ml) for 10 min. After centrifugation (900 *g*, 5 min), as much of the upper layer as possible was transferred to a 10-ml conical centrifuge tube and evaporated to dryness at 40°C on a Buchler Vortex Evaporator (Baird and Tatlock, Romford, U.K.). The residue was reconstituted in mobile phase (100 μl) and an aliquot (50—100 μl) was injected into the chromatograph.

## RESULTS AND DISCUSSION

Owing to limitations in the sensitivity of their method, Lefebvre et al. [7] concluded that timolol could not be monitored effectively in plasma by HPLC with UV detection. The UV monitor used in the present work, however, was operated routinely at very high sensitivity settings allowing detection of as little as 0.5 ng of the drug on column.

As in previous work with metoprolol [10], addition of a triethylamine—phosphoric acid modifier to the HPLC mobile phase resulted in sharp, sym-

metrical peaks for timolol (Fig. 1). Under the chromatographic conditions used retention times for timolol and the internal standard were 3.7 and 8.6 min, respectively.

Dichloromethane, the solvent most frequently employed in this laboratory for the extraction of β-blocking drugs from biological fluids, gave unexpectedly poor recoveries for timolol. Replacement of dichloromethane with methyl *tert.*-butyl ether [11] overcame this problem giving yields of > 90%.

Neglible interference was observed in extracts of samples which did not contain timolol (Fig. 1). In addition the following drugs and metabolites when injected directly were either undetectable or had substantially different retention times from those of timolol and phenacetin: atenolol, acebutolol, sotalol,

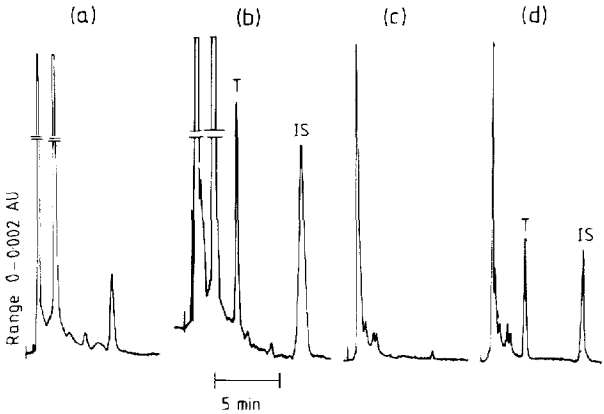


Fig. 1. Chromatograms of extracted plasma and urine. Plasma containing no drug (a); 20 ng/ml timolol base (b). Urine containing no drug (c); 700 ng/ml timolol base (d). Peaks: T = timolol; IS = internal standard.

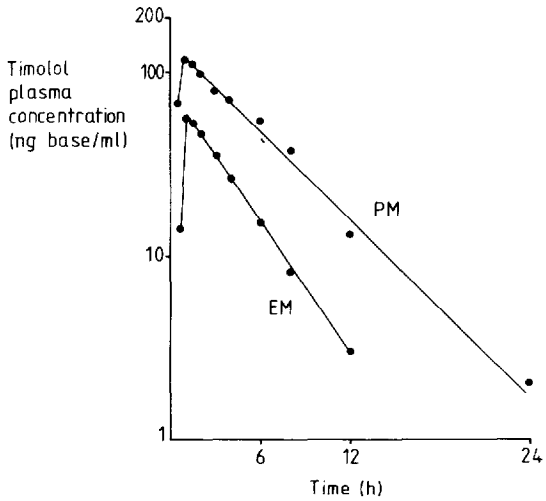


Fig. 2. Plasma concentrations of timolol in a poor (PM) and an extensive metaboliser (EM) of debrisoquine following a single oral dose of 20 mg timolol maleate. The PM and EM subjects excreted 20.6% and 6.6%, respectively, of the dose unchanged in the urine after 24 h.

nadolol, propranolol, labetalol, verapamil, norverapamil, paracetamol, warfarin, disopyramide, canrenone, nifedipine, lignocaine, hydralazine and three of its metabolites (methyltriazolophthalazine, 3-hydroxymethyltriazolophthalazine and phthalazine). A fourth metabolite of hydralazine, triazolophthalazine, had a retention time sufficiently close to that of timolol to be a possible source of interference.

Calibration plots of the peak height of timolol to that of the internal standard versus timolol concentration were linear over the range 5—200 ng/ml for plasma ($r^2 > 0.99$) and 50—2000 ng/ml for urine ($r^2 > 0.99$) and passed through the origin. Calibration standards were included in each batch of analyses. The intra-assay coefficients of variation for plasma were 5.9% and 5.7% at 5 and 50 ng/ml, respectively, and for urine were 5.0% and 9.3% at 500 and 1000 ng/ml, respectively. The lowest assayable concentration of timolol in plasma was about 2 ng/ml.

Plasma concentrations of timolol were found to be significantly higher in poor metabolisers than in extensive metabolisers of debrisoquine, indicating a close link between the metabolism of the two drugs [9]. The plasma drug concentration versus time profiles of one subject from each phenotype group are shown in Fig. 2. Timolol was detectable up to 24 h in the plasma of most poor metabolisers but could not be followed beyond 12 h in the extensive metabolisers. The 24-h urine concentrations of the drug were approximately ten times higher than peak plasma concentrations.

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