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Short Communication

Assay of timolol in human plasma using gas chromatography with electron-capture detection

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ABSTRACT

A simple and sensitive gas chromatographic method has been developed for the determination of timolol in plasma using electron-capture detection and propranolol as internal standard. Timolol was extracted using butyl chloride and derivatized using trifluoroacetic anhydride in butyl acetate. The lower detection limit for the assay was found to be 1 ng/ml from 1 ml of plasma. Extracted standards gave within-day precision of 12.55, 9.68 and 3.78% for 1, 20 and 100 ng/ml plasma samples, respectively. A recovery of at least 80% of timolol was found using the extraction method described. The assay was used in a randomized cross-over bioequivalence trial using an oral administration of 20 mg of timolol. Pharmacokinetic parameters compare favourably with other literature values.

INTRODUCTION

Timolol maleate is a β_1 and β_2 non-selective adrenergic blocking agent. It is non-cardioselective and used in the treatment of glaucoma, hypertension and angina. Chromatographic procedures previously reported for the analysis of timolol in plasma are insensitive [1,2] or have involved the use of uncommon reagents [3,4].

The high-performance liquid chromatographic (HPLC) procedure described by Kubota [5] requires 2 ml of plasma and maximum detector sensitivity to achieve the required sensitivity. The maintenance of the necessary precision required at low concentrations for bioequivalence studies is difficult with such procedures when large numbers of samples are analysed.

Mass-selective detection gas chromatography (GC) procedures also have the required sensitivity [6] but are relatively expensive and derivatization of the aminoalcohol side-chain is still required for good chromatography. We report here a simple inexpensive gas chromatographic procedure that uses a common derivatising reagent, trifluoroacetic anhydride, for the analysis of timolol in plasma. The method has been used in a bioequivalence study to estimate plasma levels as low as 1 ng/ml in plasma.

EXPERIMENTAL

Chemicals

Redistilled analytical-reagent (AR)-grade bu-

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tyl chloride was obtained from Merck (Darmstadt, Germany). Propranolol hydrochloride (internal standard) was obtained from Pacific Pharmaceuticals (Ellerslie, New Zealand). Timolol maleate was obtained from Merck, Sharpe and Dohme (Papatoetoe, New Zealand). Trifluoroacetic anhydride was obtained from Sigma (St. Louis, MO, USA). All other chemicals were AR grade. Glass extraction tubes were silanized prior to use using dimethylmonochlorosilane vapour.

Standard solutions

Timolol and propranolol standards were made up as aqueous stock solutions and diluted daily. The stock solutions were stable for at least eight weeks when stored at 4°C. Plasma standards were prepared by adding aqueous standards to 1 ml of plasma, resulting in concentrations of 1, 2, 5, 7.5, 10, 20, 42, 50, 84, 100 and 151 ng/ml free base in plasma. The working internal standard (propranolol) was used at final concentrations of 10 ng/ml for analysis of timolol concentrations below 20 ng/ml, and at 100 ng/ml for analysis of higher concentrations.

Chromatography

An HP 5890A gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with an HP 7673A autoinjector and an electron-capture detector (maintained at 300°C) was set on range 3 for analysis of samples between 10 and 150 ng/ml and range 1 for samples between 1 and 20 ng/ml. The necessary separation was achieved using splitless injection at 280°C (split vent time 30 s) onto a DB1 polymethyl siloxane, 30 m × 0.32 mm I.D. capillary column, 0.25 μm film thickness with an injection volume of 2 μl. A temperature programme from 160 to 290°C over 12 min was used to accomplish separation from co-extracted compounds.

Assay

Plasma samples (1 ml) were placed in silanized glass extraction tubes, and then internal standard (100 μl), saturated sodium borate (400 μl) and butyl chloride (3 ml) were added and the tubes vortex-mixed for 15 min. After centrifugation at 1600 *g* for 5 min, the organic layer was transferred to a clean silanized tube, the butyl chloride evaporated and the residue redissolved in 0.05 *M* sulphuric acid (400 μl). The aqueous layer was washed with 1 ml of heptane which was then discarded. Concentrated ammonia (400 μl) and butyl chloride (3 ml) were added and the sample was again vortex-mixed. The organic layer was transferred to a clean silanized tube, evaporated and the residue redissolved in dry butyl acetate (100 μl). Trifluoroacetic anhydride (100 μl) was added, the tubes were tightly capped and the reaction took place at 60°C for 20 min. The derivatised extracts were dried under a stream of nitrogen and redissolved in 25 μl of butyl acetate for injection into the gas chromatograph.

RESULTS AND DISCUSSION

Timolol could not be derivatized with pentafluoropropionic anhydride, the commonly used agent for other β-blockers, presumably because of steric hindrance from the *tert.*-butyl group in the side-chain. Trifluoroacetic anhydride was found to be a suitable derivatizing agent for timolol and other β-blockers. Metoprolol, propranolol and pindolol were assessed as potential internal standards and the first two were found to react reproducibly under mild conditions. Pindolol did not derivatise to a single compound and metoprolol was found to be unsuitable because of its poor stability in solution. Although these compounds were derivatized in the presence of more non-polar solvents, or in the case of metoprolol in the absence of solvent, the more polar nature of timolol required the use of the butyl or ethyl acetate for effective derivatization. It was found that a temperature of 60°C for 20 min was sufficient to complete the reaction without the side-reactions observed at higher temperatures. The derivative was found to be stable for at least 24 h in butyl acetate.

The retention time for timolol using the chromatographic system described is 7.9 min. The other β-blockers have retention times of 5.9 min (metoprolol) and 6.7 min (propranolol). No interference with timolol or propranolol was ob-

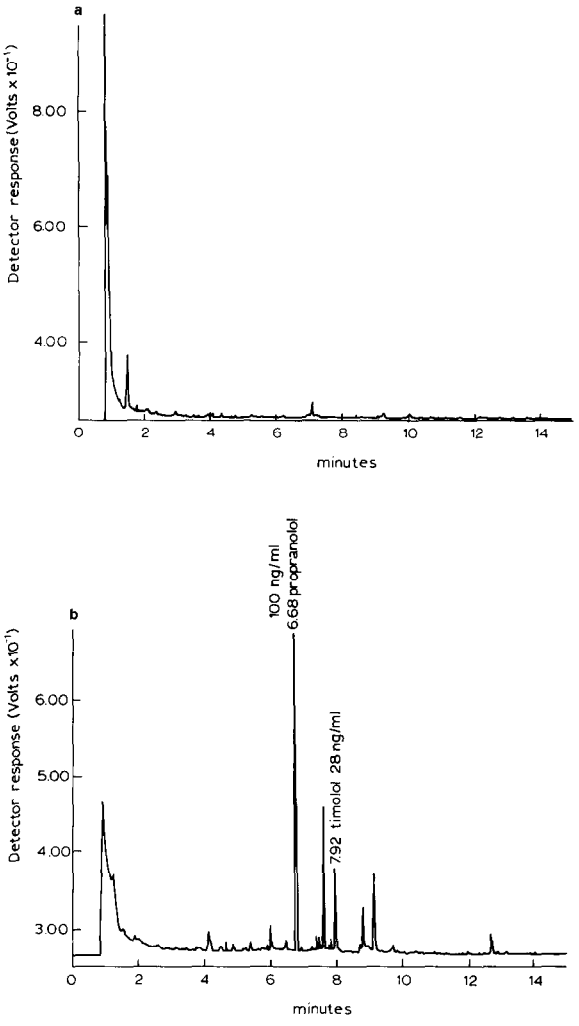

Fig. 1. (a) Chromatogram of extract of drug-free plasma; (b) example chromatogram from subject J.S. (timolol concentration 28 ng/ml).

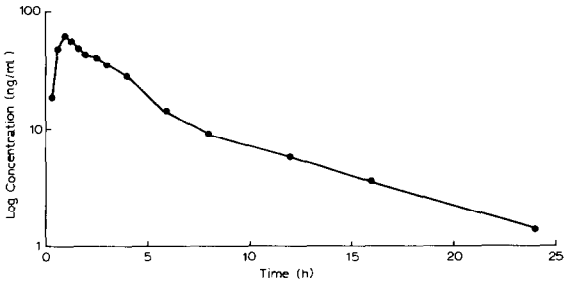

Fig. 2. Mean concentration–time profile obtained for the reference formulation.

served from metabolites or coextracted material including plasticisers and other extractants from the tubes in which the samples were stored (Fig. 1). A back-extraction step was found necessary to ensure adequate clean-up of the sample.

A recovery of timolol at 5, 42 and 100 ng/ml of at least 80% was found by comparing peak heights (correcting for solvent volumes by the use of ratios with the internal standard) of standards extracted from plasma to peak heights of unextracted standards. Two standard curves, 1–20 and 10–150 ng/ml, were required at different range settings to ensure sufficient sensitivity without quenching the detector response at each range. High-concentration standard curves were linear with coefficients of determination (r^2) generally greater than 0.995. Although more scatter was observed in the low-concentration curves, the best fit was linear and coefficients of determination of 0.99 or greater were obtained.

The minimum quantifiable level, for the purpose of this study, was defined as the quantity of timolol that gave a maximum relative standard deviation of 20%. Extracted standards gave within-day precision (n = 5) of 13% (1 ng/ml), 10% (20 ng/ml) and 3.8% (100 ng/ml). Between-day precision over four months gave relative standard deviations of 9.0% (42 ng/ml, n = 70), 14% (10 ng/ml, n = 27) and 11% (5 ng/ml, n = 35).

The assay was used for a randomised cross-over bioequivalence trial using eighteen subjects with eighteen samples collected over a 72-h period. The mean results from one arm of the study are illustrated in Fig. 2. Plasma levels of samples collected beyond 24 h were below the minimum quantifiable level.

The pharmacokinetic parameters calculated in this study, elimination half-life ($t_{1/2}$), area under the curve (AUC_{0-inf}) and maximum observed concentration (C_{max}), are given in Table I. Results from literature studies using similar doses of timolol undertaken by Bobik *et al.* [7] and Ishizaki *et al.* [8] are also given in Table I. One subject in the present clinical trial is probably a slow me-

TABLE I

TIMOLOL PHARMACOKINETIC PARAMETERS

Study	n	$t_{1/2}$ (h)	$AUC_{0\ inf}$ (ng/ml h)	C_{max} (ng/ml)
Bobik *et al.* [7]	7	2.5 ± 0.05	394 ± 119	93 ± 18
Ishizaki *et al* [8][a]	4	3.7	304 ± 71	54 ± 73
Present study[b]				
Reference formulation	18	4.1 ± 2.3	292 ± 105	77 ± 31
Test formulation	18	4.4 ± 3.3	289 ± 103	73 ± 28

[a] Dose of 20 mg timolol maleate.
[b] Dose of 20 mg timolol.

TABLE II

PHARMACOKINETIC PARAMETERS FROM PRESENT STUDY: SLOW METABOLISER DATA REMOVED

Study	n	$t_{1/2}$	AUC_{0-inf}
Reference	17	3.7 ± 1.7	285 ± 103
Test	17	3.7 ± 1.9	281 ± 101

taboliser ($t_{1/2} > 10$ h). Elimination of this patient from the data set results in the pharmacokinetic parameters in Table II. It is also worth noting that enterohepatic recycling makes accurate estimation of $t_{1/2}$ difficult for β-blockers.

Although timolol can be detected at 10 ng/ml using a nitrogen–phosphorus detector in the underivatized state, the aminoalcohol side-chain results in adsorption, peak tailing and poor quantitation under the conditions described. In addition, split peaks are observed under some injection conditions, probably due to loss of water from the side-chain. For these reasons derivatization is the method of choice in analysis of timolol by GC. The heptafluorobutyrylimidazole derivatization previously reported for electron-capture detection [3,4] was found difficult to achieve in our hands. In addition, heptafluorobutyrylimidazole is expensive and transportation requirements for the reagents are stringent. Trifluoroacetic anhydride is a common reagent, found in most GC laboratories which, under mild conditions, effectively derivatises β-blockers.

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