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Note

Determination of timolol in plasma and breast milk using high-performance liquid chromatography with electrochemical detection

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Timolol maleate, (—)-1-(*tert.*-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol maleate, is a potent non-selective beta-adrenoceptor antagonist [1] effective in the management of systemic hypertension [2], angina pectoris [3] and glaucoma [4]. Because of its high potency timolol is administered in small oral doses, usually 10—45 mg per day [5] and this, in addition to its large apparent volume of distribution, results in the low plasma concentrations generally found in man.

Several methods are available for its measurement in biological fluids, the most widely used being that of Tocco et al. [6] which involves gas chromatography (GC) with electron-capture detection (ECD) and has a limit of sensitivity of 2 ng/ml. Else et al. [7] have used GC with nitrogen-selective flame ionization detection (NFID) and have achieved a similar sensitivity, while Fourtillan et al. [8] have been able to measure plasma concentrations of 0.5 ng/ml using GC–mass spectrometry (MS). Lefebvre et al. [9] have developed a high-performance liquid chromatographic (HPLC) method with UV detection but this displayed a rather poor sensitivity of 40 ng/ml which limited its usefulness in pharmacokinetic studies using normal dosing regimens.

In this paper we describe an accurate and selective HPLC method for the measurement of timolol in plasma and breast milk. It is important to be able to measure timolol in the latter fluid since a number of beta-adrenoceptor antagonists are now used to control high blood pressure in the later stages of pregnancy and it is vital to know the concentration of these drugs in milk since they may be transferred to the new-born during breast feeding. The method uses electrochemical detection and is as sensitive as the GC methods above, with the exception of GC—MS.

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MATERIALS AND METHODS

High-performance liquid chromatography

A Waters Model 6000A solvent delivery system was attached to a reversed-phase column 25 cm × 4.6 mm I.D. The column was packed with Whatman PXS 5/25 Partisil ODS 3, particle size 5 μm (Whatman Chemical Separations, Maidstone, U.K.). Samples were introduced onto the column by means of a Rheodyne loop injector, the volume of the loop being 100 μl. Sample detection was carried out electrochemically using a TL5A thin-layer cell assembly with an LC2A controller (Bioanalytical Systems).

Voltammetry

It was necessary to establish the optimum applied potential to give maximum sensitivity, low signal-to-noise ratio and rapid equilibration. Repeated injections of 50 ng of timolol were made under the conditions described below and the amplitude of the response compared with the applied potential (Fig. 1). The optimum applied potential was found to be +1.2 V and this was maintained throughout the study.

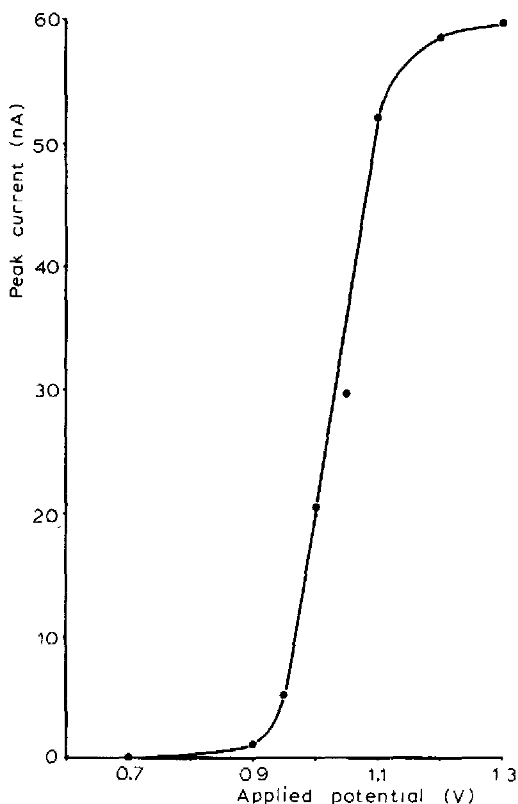

Fig. 1. Voltammogram of repeated injections of 50 ng of timolol.

Reagents and materials

Timolol maleate was supplied by Merck, Sharpe and Dohme, U.K. and

propranolol hydrochloride by ICI (Macclesfield, U.K.). Both drugs were dissolved in glass-distilled water to give stock solutions of 100 μg/ml; from these timolol dilutions of 0.1 and 1.0 μg/ml were made and a propranolol dilution of 1.0 μg/ml. Pindolol was supplied by Sandoz Products (Leeds, U.K.) and was dissolved in methanol to give a stock solution of 100 μg/ml from which a dilution of 1.0 μg/ml in water was made. The stock solutions were replaced after four weeks, 1.0 μg/ml solutions after one week and the 0.1 μg/ml solutions daily. All standards were kept at 4°C when not in use.

Methanol (HPLC grade) was supplied by Fisons Scientific Apparatus (Loughborough, U.K.). All other chemicals were of analytical grade. Glassware was silanized before use.

Extraction of timolol from plasma or serum

To 1.0 ml of plasma or serum was added 0.1 ml of propranolol, internal standard (1.0 μg/ml), followed by 1.0 ml of saturated sodium chloride. The pH was adjusted by the addition of 0.2 ml buffer (1 *M* sodium hydroxide—1 *M* sodium carbonate, 1:2), 3 ml of water-satured diethyl ether were added and the mixture was shaken gently for 15 min. After centrifugation at 300 *g* for 10 min, the organic phase was transferred to a test-tube containing 0.15 ml of 0.1% (v/v) orthophosphoric acid. The tube was vortexed for a few seconds, centrifuged at 300 *g* for 10 min and the organic phase discarded; 1.5 ml of water-saturated hexane were then added to the aqueous phase and the tube was vortexed for 2 min, centrifuged at 300 *g* for 10 min and the organic phase again discarded. Aliquots, usually 0.1 ml, of the orthophosphoric acid phase were then injected onto the chromatographic column. The mobile phase consisted of a mixture of methanol—0.2 *M* sodium dihydrogen phosphate—88% orthophosphoric acid (sp. gr. 1.75)—water (500:200:3:297) pumped at a flow-rate of 1.0 ml/min.

Extraction of timolol from breast milk

To 1.0 ml of breast milk was added 0.05 ml of pindolol, internal standard (1.0 μg/ml), followed by 1.0 ml of saturated sodium chloride and 3.0 ml of water-saturated diethyl ether. The mixture was shaken for 15 min, then centrifuged at 300 *g* for 10 min. The organic phase was transferred to a tube containing 2.0 ml of 0.1% (v/v) orthophosphoric acid, shaken for a further 15 min and centrifuged at 300 *g* for 10 min. The organic phase and the cloudy interface were carefully removed and discarded. To the aqueous phase were added 0.2 ml of the sodium hydroxide—carbonate buffer and 3.0 ml water-saturated diethyl ether. After shaking for 15 min and centrifuging for 10 min as described above, the organic phase was transferred to a test-tube containing 0.15 ml of 0.1% orthophosphoric acid. The tube was vortexed for a few seconds, centrifuged for 10 min as above and the organic phase discarded; 1.5 ml of water-saturated hexane were then added to the acid phase and the mixture vortexed briefly and centrifuged as described. The organic phase was again discarded and an aliquot of the acidic aqueous phase injected onto the column. In this case the mobile phase was composed of the same ingredients in the proportions 300:200:3:497 at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Typical chromatograms of blank and spiked plasma are shown in Fig. 2. Under the conditions described in the text the retention times for timolol and propranolol were 4.8 and 8.2 min, respectively. The chromatogram of patient plasma was similar to that of spiked plasma in all respects. The calibration curve was linear from the limit of detection, 2 ng/ml, up to 100 ng/ml and could be described by the equation: $y = 0.02356x + 0.0075$. Typical chromatograms from breast milk are shown in Fig. 3. Under the conditions described in the text the retention times of pindolol and timolol were 7.0 and 13.5 min respectively. Again the chromatogram of patient breast milk was similar to that of spiked breast milk in all respects. The calibration curve for breast milk could be described by the equation: $y = 0.01125x - 0.00563$. The limits of detection and linearity were as for plasma.

The precision and accuracy of the method applied to plasma and breast milk are shown in Table I and the coefficient of variation (C.V.) is less than 10% at the 10 and the 50 ng/ml level. Recoveries of timolol were approximately 80% from plasma and 60% from breast milk.

Plasma concentrations of timolol following a single oral dose of 20 mg were monitored in a young healthy volunteer over a 7-h period (Fig. 4) and the results were consistent with those reported in earlier studies [5, 8]. A peak plasma concentration of 58 ng/ml was achieved after 2 h.

Most pharmacokinetic studies with timolol have used GC as the analytical tool, the method of Tocco et al. being most widely used [6]. That method involves a derivatization step with heptafluorobutyrylimidazole and we feel

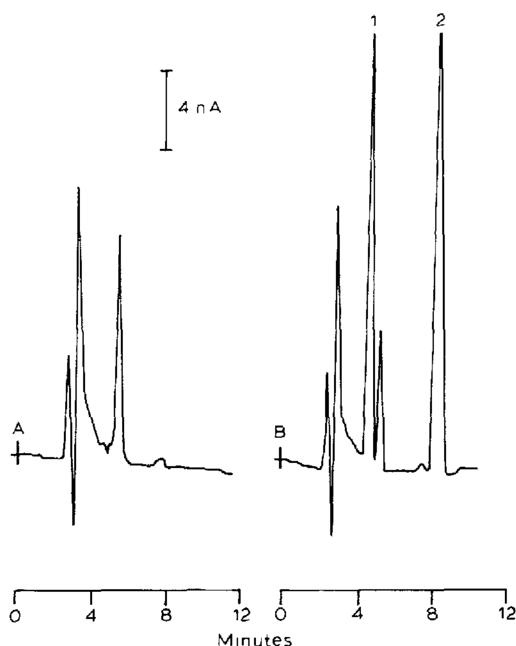

Fig. 2. Timolol in plasma. A, blank plasma; B, plasma containing 50 ng of timolol (1) and 100 ng of propranolol, internal standard (2).

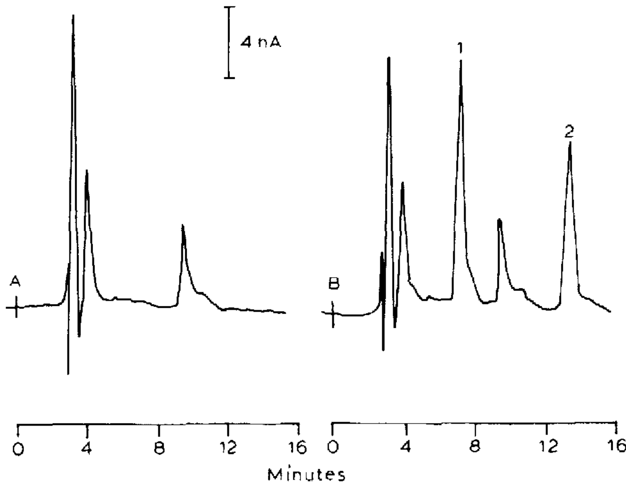

Fig. 3. Timolol in breast milk. A, blank milk; B, milk containing 50 ng pindolol, internal standard (1) and 50 ng timolol (2).

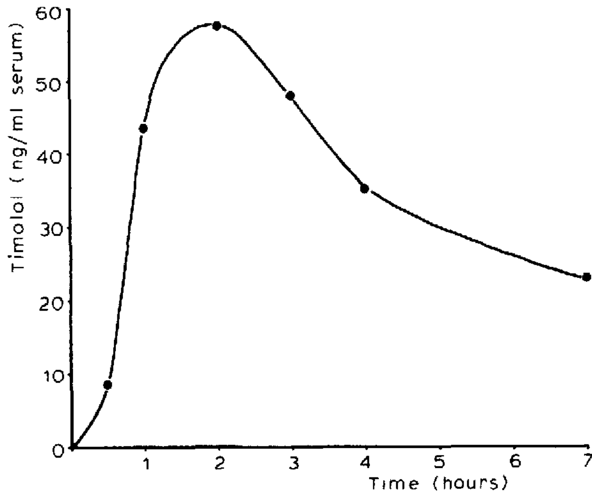

Fig. 4. Plasma concentrations of timolol in a volunteer following a single oral dose of 20 mg timolol maleate.

TABLE I

PRECISION AND ACCURACY OF THE METHOD APPLIED TO PLASMA AND BREAST MILK

	Spiked concentration (ng/ml)	Detected concentration ± S.D. (ng/ml)	C.V. (%)	No. of assays (*n*)
Plasma	10.0	9.8 ± 0.8	8.2	10
	50.0	50.1 ± 3.7	7.4	9
Breast milk	10.0	9.8 ± 0.5	5.1	10
	50.0	50.3 ± 2.3	4.6	9

that the present method is simpler and offers similar sensitivity. The only published method of greater sensitivity is by mass fragmentography and is beyond the reach of most laboratories.

The method described here is both sensitive and accurate and provides a useful alternative to the established GC method for timolol in plasma and breast milk.

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