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Note

Simple and sensitive determination of timolol in human plasma and urine by high-performance liquid chromatography with ultraviolet detection

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Timolol, a β-adrenoceptor antagonist, is effective against hypertension, arrhythmias and angina pectoris [1,2] as well as for the secondary prevention of myocardial infarction [3]. Recently, transdermal application of timolol has been reported to produce an appropriate β-blocking effect [4]. However, if the detailed examination of the post-application plasma concentration–time data resulting from this new route of absorption is desired, an assay method capable of measuring plasma timolol concentrations as low as 1–2 ng/ml appears to be required [4].

Gas chromatography (GC) with electron-capture or alkali-flame ionization detection with the lower limits of assay of 1–5 ng/ml [5–7] and GC–mass spectrometry with the better detection limit of 0.5 ng/ml [8,9] have been used to measure timolol in human plasma. However, these assays require derivatization of the drug, and, in some cases, tedious sample and reagent clean-up steps. Fur-

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thermore, all the reported GC procedures [5–9] employ equipment that is not readily available to many laboratories.

High-performance liquid chromatography (HPLC) permits analysis of underivatized β-adrenoceptor anatagonists [10]. Indeed, an HPLC method with electrochemical detection has been reported to provide the lowest detection limit of 2 ng/ml of timolol in plasma [11]. Recently, however, HPLC assays with ultraviolet (UV) detection have been reported to provide a simple, rapid, sensitive and inexpensive method for measuring timolol concentrations in plasma and urine [12] or in aqueous humor [13,14].

We therefore intended to develop a simple, rapid and more sensitive HPLC–UV assay method for determining timolol in human biological fluids, which can be used for a study of the kinetic profile of this β-antagonist after the topical or transdermal application to humans.

EXPERIMENTAL

Chemicals and reagents

Timolol maleate and metoprolol tartrate as an internal standard were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile was of HPLC grade, and dichloromethane, phosphoric acid, sodium hydroxide and triethylamine were of reagent grade. All these chemicals were purchased from Wako (Osaka, Japan). Amounts and concentrations of timolol and metoprolol in stock solution, and in plasma and urine, are expressed as those of their respective free bases throughout the text. Standard stock solutions containing timolol (10 μg/ml) and metoprolol (1 mg/ml) were prepared in distilled water and stored at 4°C.

Instrumentation and chromatographic conditions

The HPLC analysis was performed by a Hitachi L-6000 pump (Tokyo, Japan) equipped with a Hitachi 655A-40 autosampler system, a Hitachi L-4000 UV detector and a Hitachi D-2500 chromato-integrator. The reversed-phase HPLC separation was carried out with a Wakosil $5C_{18}$ column (250 mm × 4.0 mm I.D., 5 μm particle size) (Wako, Osaka, Japan), and the UV wavelength was set at 295 nm with a sensitivity of 0.001–0.005 a u.f.s. The mobile phase was acetonitrile–water–triethylamine (18:81:1, v/v/v) and the pH was adjusted to 3.0 with phosphoric acid; it was supplied at a flow-rate of 1 ml/min at room temperature.

Sample preparation

A sample of 1–2 ml of human plasma was mixed with 100 μl of 4 *M* sodium hydroxide, 100 μl of internal standard solution (100 μg/ml) and 5 ml of dichloromethane in a 10-ml conical glass centrifuge tube. The tube was capped and shaken by hand for 10 s, followed by vortex-mixing for 180 s. After centrifugation for 5 min at 1500 *g*, the upper aqueous layer was discarded, and the mixture was transferred to a small glass tube and evaporated to dryness with a gentle air

stream at 37°C. The residue was reconstituted with 100 μl of mobile phase and filtered with an ACRO LC3S disposable filter assembly (0.45 μm, Gelman Sciences, Ann Arbor, MI, U.S.A.), and 40–60 μl of the filtered solution were injected into the chromatograph through the autosampler.

In the same manner as the plasma sample, 0.1–1 ml of urine was mixed with 100 μl of 4 *M* sodium hydroxide, 100 μl of internal standard solution (1 mg/ml) and 5 ml of dichloromethane in a 10-ml conical glass centrifuge tube. The tube was capped and shaken by vortex-mixing for 60 s. After centrifugation for 5 min at 1500 *g*, the aqueous layer was discarded, and the organic layer was transferred to another 10-ml conical glass centrifuge tube. A 1-ml volume of 0.1 *M* phosphoric acid was added and shaken by vortex-mixing for 60 s. After centrifugation for 5 min at 1500 *g*, 40 μl of the upper aqueous phase were injected into the chromatograph through the autosampler.

Results were calculated from the peak-height ratios. The calibration curves were obtained daily from spiked plasma and urine samples.

Clinical application

A healthy 25-year-old male volunteer participated in the preliminary pharmacokinetic study of timolol administered transdermally after providing an informed, written consent. A 10 × 6 cm patch, 80 μm in thickness, containing 10% (w/v) timolol (total dose as the free base = 48 mg) in an acrylic copolymer adhesive [15], was applied to the inner surface of his left upper arm and worn continuously for 48 h. At the end of this time, the adhesive was removed from the skin. Multiple venous blood and urine samples were collected up to 76 and 84 h, respectively, after the patch application. Blood samples were immediately centrifuged and the plasma samples were separated. Plasma and urine samples were stored at −20°C until the time of analysis.

RESULTS

Representative chromatograms obtained from the drug-free plasma and urine and their standards, together with those samples from a male volunteer who received a transdermal patch containing the free base of 48 mg of timolol over 48 h, are shown in Fig. 1. Retention times for timolol and the internal standard, metoprolol, were 7.36 and 8.69 min, respectively. The chromatograms from plasma extracts contained no unknown peaks that would interfere with the analysis (Fig. 1E–G). Although a few unknown peaks appeared in the chromatograms from urine extracts (Fig. 1C and D), the compounds were well separated and were not affected by these unknown peaks. The unknown peaks close to the anticipated retention times of the analytes derived from urine samples, which had appeared before the back-extraction (Fig. 1A), disappeared when the extraction into dichloromethane was followed by back-extraction into phosphoric acid (Fig. 1B).

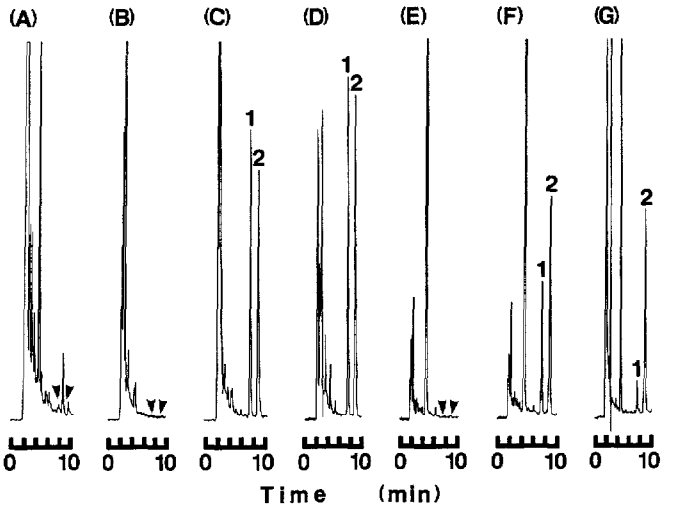

Fig. 1. Chromatograms of extracts from drug-free urine (A) before and (B) after back-extraction into phosphoric acid, (C) urine standard containing 400 ng/ml timolol and 100 μg/ml metoprolol (internal standard), (D) urine of a healthy volunteer who received a transdermal patch containing 48 mg of timolol as the free base, (E) drug-free plasma, (F) plasma standard containing 20 ng/ml timolol and 10 μg/ml metoprolol, (G) plasma sample of the same healthy subject as noted in (D) Peaks 1 = timolol, 2 = metoprolol Arrows indicate the anticipated peak appearance times of analytes

The absolute extraction recoveries of timolol and metoprolol from plasma and urine were assessed by comparing the peak heights obtained from the compounds' standard stock solutions with those of drug-free plasma or urine spiked with the respective drugs. The extraction recoveries from 1 ml of plasma averaged 104 1 and 104.2% for timolol concentrations of 10 and 40 ng/ml, respectively, and 100.1% for metoprolol at 10 μg/ml. The recoveries from 1 ml of urine averaged 103.1 and 99.6% for timolol at concentrations of 100 and 400 ng/ml, respectively. For the internal standard, 100.6% was recovered at a concentration of 100 μg/ml.

Calibration curves were obtained by plotting the peak-height ratios of timolol to the internal standard with different concentrations for plasma and urine samples expected from dose sizes of timolol clinically used (*e g.* 5–30 mg as timolol maleate orally [16,17] and 30–60 mg as timolol base transdermally [4]). The regression lines were linear over the concentration ranges examined (5–80 ng/ml for plasma and 50–800 ng/ml for urine), and their intercepts passed through the origin. The correlation coefficients of the calibration curves for both plasma and urine analyses ranged between 0.9991 and 0.9999.

To assess the precision of this analytical procedure, reproducibilities for both within-day and day-to-day variations were determined (Table I). The coefficients of variation (C.V.) for five different concentrations in the within-day study varied between 1.0 and 2.0% for plasma (1 ml) and between 0.9 and 2.4% for urine (1

TABLE I

PRECISION AND ACCURACY IN THE DETERMINATION OF TIMOLOL IN PLASMA AND URINE

Plasma				Urine			
Concentration given (ng/ml)	Concentration determined (mean ± S.D.) (ng/ml)	C.V. (%)	Relative error (%)	Concentration given (ng/ml)	Concentration determined (mean ± S.D.) (ng/ml)	C.V. (%)	Relative error (%)
Within-day variation (n = 9)							
5	5.08 ± 0.08	1.6	1.7	50	49.06 ± 1.17	2.4	−1.9
10	9.92 ± 0.20	2.0	−0.8	100	97.88 ± 0.83	0.9	−2.1
20	19.86 ± 0.18	1.0	−0.7	200	201.59 ± 1.75	0.9	0.8
40	39.92 ± 0.49	1.2	−0.2	400	403.23 ± 3.56	0.9	0.8
80	80.05 ± 0.94	1.2	0.1	800	798.04 ± 13.41	1.7	−2.5
Day-to-Day variation (n = 7)							
5	5.09 ± 0.38	7.4	1.8	50	53.51 ± 3.73	7.0	7.0
10	9.94 ± 0.39	4.0	−0.6	100	103.91 ± 4.81	4.6	3.9
20	20.24 ± 0.54	2.7	1.2	200	197.23 ± 4.74	2.4	−1.4
40	39.82 ± 2.05	2.1	−0.4	400	390.85 ± 13.13	3.4	−2.3
80	79.84 ± 4.72	4.5	0.2	800	802.33 ± 41.23	5.1	0.3

ml) samples; those in the day-to-day study ranged from 2.1 to 7.4% for plasma (1 ml) and from 2.4 to 7.0% for urine (1 ml) samples. The accuracy of our measurements was concurrently evaluated by comparing the given amounts of drug with their estimated amounts. The relative errors ranged from −0.8 to 1.8% for plasma and from −2.5 to 7.0% for urine samples (Table I).

The detection limit of timolol in plasma was 0.5 ng/ml at a detector attenuation of 0.001 a.u.f.s. (signal-to-noise ratio of 5·1) when a 2-ml plasma sample was used and a 50-μl aliquot was injected onto the chromatograph.

The possible interference(s) from several other β-antagonists, calcium channel blocking agents and antiarrhythmic drugs (which might be concurrently administered with timolol or metoprolol), including some of their active metabolites, was tested, and the data are listed in Table II. All the drugs, except for acebutolol and nicainoprol, which could be extracted and eluted within 60 min on the chromatogram under the conditions described, had substantially different retention times from those of timolol and metoprolol. Acebutolol had a retention time almost identical with that of timolol, whereas nicainoprol had a retention time rather close to that of metoprolol (Table II). Thus, these two drugs would be a possible source of interference in the assay.

The preliminary data on the clinical applicability of the proposed HPLC–UV

TABLE II

RETENTION TIMES OF OTHER β-ADRENOCEPTOR ANTAGONISTS, CALCIUM-CHANNEL BLOCKING AGENTS, ANTIARRHITHMIC DRUGS AND THEIR ACTIVE METABOLITES

Drug	Retention time (min)	Drug	Retention time (min)
Procainamide	2 34	Indenolol	21 26
Carteolol	3.47	Bufetolol	22 78
Quinidine	4.30	Atenolol	27.04
N-Acetylprocanamide (NAPA)[a]	4 40	Nifedipine	37.56
Pindolol	4 84	Propranolol	42.14
Mono-N-dealkyldisopyramide (MND)[a]	5 89	Bupranolol	49 66
Acebutolol	7 34	Diltiazem	–[b]
Timolol	7 36	Glycinexylidide (GX)[a]	–[b]
Metoprolol	8 69	Mexiletine	–[b]
Nicainoprol	8 72	Monoethylglycinexylidide (MEGX)[a]	–[b]
Disopyramide	11 76	Nicardipine	–[b]
Lidocaine	17 08	Tocainide	–[b]
Alprenolol	21 07	Verapamil	–[b]

[a] NAPA, MND, and GX and MEGX are pharmacologically active metabolites of procainamide, disopyramide, and lidocaine, respectively

[b] Not eluted on the chromatogram within 60 min under the conditions described

method for studying the pharmacokinetic behaviour of timolol administered transdermally are shown in Fig. 2, which provides the plasma concentration–time and urinary excretion–time profiles (Fig. 2A and B, respectively). The derived pharmacokinetic data were: maximum plasma concentration (C_{max} = 6.8 ng/ml; time to C_{max} (t_{max}) = 24 h; the area under the plasma concentration–time curve from 0 to 76 h (AUC_0^{76}) = 369 ng/ml h; mean residence time (MRT) = 47 2 h;

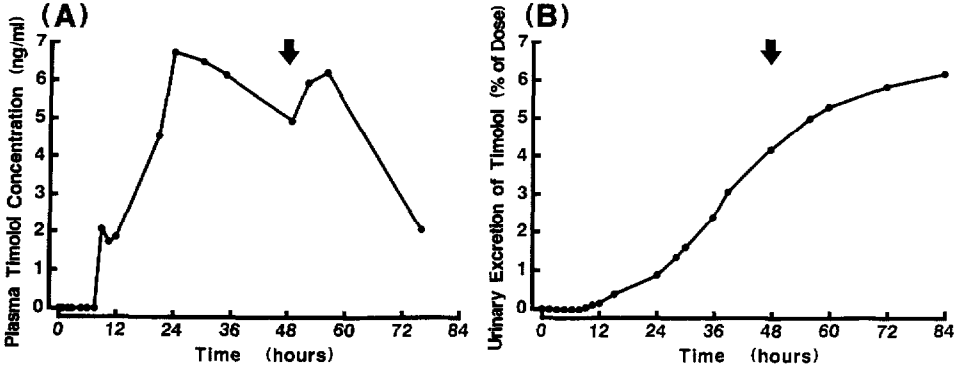

Fig 2 Plasma concentration–time (A) and cumulative urinary excretion–time (B) profiles of timolol following a topical patch application of 48 mg of timolol as the free base to the inner surface of left upper arm of a healthy volunteer Arrows indicate the time (48 h) when the patch was removed from the skin

and cumulative urinary excretion from 0 to 84 h (Xu_0^{84}) = 2.97 mg, which corresponded to *ca.* 6.2% of the free-base dose (48 mg) topically applied to the skin (Fig. 2B)

DISCUSSION

Lefebvre *et al.* [10] originally reported the HPLC analysis of underivatized β-antagonists. However, owing to the detection limit for timolol in plasma (40 ng/ml), they concluded that timolol could not be monitored in plasma after therapeutic doses [10]. Thereafter, Lennard and Parkin [12] reported a sensitive HPLC–UV method, capable of detecting a much lower concentration in plasma (2 ng/ml), in which timolol in plasma or in ten-fold diluted urine was extracted into methyl *tert.*-butyl ether. The present HPLC–UV assay gave a lower detection limit of 0.5 ng/ml timolol in plasma. The UV detector in the present assay was operated at very high sensitivity settings (0.001–0.005 a.u.f.s.), as used by Lennard and Parkin [12], allowing the detection of 0.5 ng/ml of the drug.

Lennard and Parkin [12] reported that extraction into dichloromethane gave unexpectedly poor recoveries for timolol and that replacement of dichloromethane with methyl *tert.*-butyl ether overcame such problems and gave recoveries of more than 90%. In contrast, we found that use of dichloromethane provided good recoveries for timolol in plasma (*ca.* 104%). Although unknown peaks close to those of the analytes existed in the chromatogram from urine samples when extracted into dichloromethane (Fig 1A), back-extraction into phosphoric acid overcame this problem (Fig. 1B), giving excellent recoveries for timolol in urine samples (99.6–103.1%). For the internal standard, metoprolol, 100.1 and 100.6% were recovered from 1 ml of plasma and urine, respectively. These data on recoveries for timolol and metorpolol using dichloromethane are fairly consistent with those reported by Lefebvre *et al.* [10]: they reported 99.9% recoveries of both timolol and metoprolol using dichloromethane, as in the present assay.

There appear to be some possible explanations for good recoveries in the present assay, even though timolol was extracted into dichloromethane. In the previous report using extraction into methyl *tert.*-butyl ether [12], the plasma and urine samples were gently shaken for 10 min. In contrast, after a 10-s manual shaking to avoid emulsification, we used vigorous vortex-mixing for 180 s for the plasma samples. A short (60 s) vigorous vortex-mixing was also used for the urine samples before and after the back-extraction into phosphoric acid Good recoveries for timolol in both the samples appear to be related to this analytical manipulation. Indeed, Lefebvre *et al.* [10] reported that a short (2 min) manual agitation was better than an automatic gentle shaking for the extraction.

During the development of the present assay, we looked for the appropriate time for vortex-mixing. We noticed that the magnitudes of the C.V. for timolol and metoprolol recoveries by vortex-mixing for 180 s (2.3 and 2.1%, $n = 7$) were significantly ($p < 0.01$) smaller than those for 60-s mixing (13.2 and 5 2%, $n = 7$),

when 2-ml plasma samples at concentrations of 20 ng/ml timolol and 5 μg/ml metoprolol were used, respectively. Based on this finding, we selected the 180-s vortex-mixing. However, in the case of urine samples, vortex-mixing times of 60 to 180 s did not result in any difference in the recoveries for timolol and metoprolol, and therefore the 60-s vortex-mixing was chosen.

Our recoveries of timolol and metoprolol from urine samples were much better than those described in the previous paper [10] (*i.e.* 53% for timolol and 41% for metoprolol) using extraction into chloroform–*n*-pentanol followed by back-extraction into 0.01 to 1 *M* hydrochloric, sulphuric and acetic acids. This indicates that extraction into dichloromethane followed by back-extraction into phosphoric acid resulted in the much better recoveries of both β-antagonists from urine samples.

We observed that the plasma concentrations of timolol attained after transdermal application were comparable with those not only after use of a different transdermal patch [4] but also after the usual oral doses [16,17]. Our transdermal patch also caused a β-blocking effect, as assessed by an exercise test (data are not shown). It is of interest to see that the plasma concentration of timolol increased transiently following the removal of the patch (Fig. 2A). The exact reason for this observation is unclear. However, such a transient increase in plasma drug concentration after patch removal appears to resemble a phenomenon known as "washing-in effect", an increase in the urinary excretion rate of a permeant which occurs several hours after the removal of an ointment using soap and water [18] or water and acetone [19], and after the removal of a cream preparation of topical prednisolone 17-valerate 21-acetate using a warm wet towel [20]. Thus, physical stimuli induced by the removal of a vehicle may sometimes alter the percutaneous drug absorption profile. Further study is required to clarify such an assumptive explanation.

In conclusion, the proposed HPLC–UV method is of sufficient sensitivity, precision and accuracy, and of clinical applicability to the study of the kinetic profile of timolol administered transdermally in humans. By using this assay, a study of the pharmacokinetics and pharmacodynamics of timolol from the transdermal patch [15] is underway in our laboratory, and the results will be reported elsewhere.

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