diphenoxylate hydrochloride has been suggested previously, the present studies provide *in vitro* as well as *in vivo* evidence to support the possibility that activated charcoal may significantly modify the bioavailability of this drug.

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Electron-Capture GLC Determination of Timolol in Human Plasma and Urine

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Abstract □ A GLC procedure was developed for measuring nanogram quantities of timolol in plasma and urine. The unchanged drug was extracted into heptane–4% isoamyl alcohol from alkalinized plasma or urine, together with a homolog of timolol which served as the internal standard. The compounds were subsequently back-extracted into 0.1 *N* HCl and then into chloroform following adjustment of the acid phase to an alkaline pH. The compounds in the chloroform extract were derivatized with heptafluorobutyrylimidazole to form the diheptafluorobutyryl derivatives; these were quantitated by electron-capture GLC. Recovery of timolol added to normal plasma and urine was quantitative and reproducible, and no interfering substances were observed in normal biological samples. The method is capable of measuring concentrations as low as 2 ng/ml in plasma or 20 ng/ml in urine. After a 10-mg oral dose of ^{14}C-timolol, peak plasma levels of approximately 30 ng/ml were observed in 1–2 hr.

Keyphrases □ Timolol—extraction, derivatization, GLC analysis, biological fluids □ Adrenergics—timolol, extraction, derivatization, GLC analysis, biological fluids □ GLC—analysis, timolol, biological fluids

Timolol maleate, (−)-1-(*tert*-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol maleate, is a new β-adrenergic receptor blocking agent (1). More recently, it was shown to reduce the frequency of anginal episodes and thus have a place in the treatment of angina pectoris (2). When administered orally to rats and dogs, the compound is several times more potent than propranolol in blocking isoproterenol-induced cardiac acceleration (3). Its activity in humans also has been demonstrated (4, 5).

This report is concerned with the determination of timolol in biological fluids. In view of the small therapeutic doses used (generally 5–15 mg po), a sensitive as well as a specific analytical method is required. The procedure involves extraction of the compound from biological fluids followed by derivatization to form the diheptafluorobutyryl analog and determination by electron-capture GLC. The procedure is capable of quantitating timolol in concentrations as little as 2 ng/ml in plasma and 20 ng/ml in urine. Accordingly, the method should prove useful in clinical applications where quantitation and diagnostic confirmation are necessary.

EXPERIMENTAL

Reagents—Pesticide quality *n*-heptane[1] was used without further purification. Reagent grade isoamyl alcohol[2] was glass distilled prior to use. Methylene chloride[3], reagent grade, was washed successively with 1 *N* HCl, 1 *N* NaOH, and three times with water and then glass distilled. Distilled, deionized water was used in the preparation of 0.1 *N* HCl and 2.0 *N* NaOH. Heptafluorobutyrylimidazole[4], in 1-ml ampuls stored under nitrogen, was diluted 1:10 with pesticide quality ethyl acetate[5] immediately before use. Timolol (I) was used as the maleate salt, and desmethyltimolol, 1-isopropylamino-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol hydrochloride (II) served as the internal standard. All concentrations were expressed in terms of the free base.

Apparatus—Samples were analyzed on a gas chromatograph[6]

[1] Matheson, Coleman and Bell.
[2] J. T. Baker.
[3] Fisher Certified, ACS Spectranalyzed.
[4] Pierce Chemical Co.
[5] Fisher Certified, ACS.
[6] Hewlett-Packard model 5750.

Table I—Recovery of Timolol from Human Plasma

Timolol Added, ng/ml	Found, ng/ml	Percent Recovery, Mean ± *SD*
50	49.0 ± 4.0	98 ± 7
40	38.0 ± 5.0	95 ± 12
20	18.0 ± 2.0	90 ± 9
10	8.5 ± 2.0	85 ± 18
5	5.7 ± 0.9	120 ± 18
2	1.9 ± 0.8	95 ± 40

equipped with a ^{63}Ni (2 mCi) electron-capture detector and a 1.83-m × 0.64-cm (6-ft × 0.25-in.) column packed with 1% OV-17 on Gas Chrom Q[7] (80–100 mesh). The instrument was operated isothermally with oven, detector, and injection port temperatures maintained at 185, 300, and 250°, respectively; the carrier gas (helium) and the purge gas (10% methane in argon) were each maintained at 75 ml/min. The pulse mode of voltage was used with a pulse interval of 50 μsec. Gas lines were fitted with filters containing molecular sieve 4A.

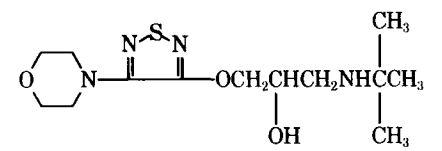
Procedure—In a 30-ml glass-stoppered centrifuge tube were placed 1.0 ml of plasma or 0.1 ml of urine, 0.1 ml of desmethyltimolol internal standard (stock solution, 500 ng/ml of water), 0.1 ml of 1.0 *N* NaOH, and 10 ml of heptane–4% isoamyl alcohol. The mixture was shaken mechanically for 10 min and centrifuged. In a clean 13-ml glass-stoppered centrifuge tube were placed 1.0 ml of 0.1 *N* HCl and 8.0 ml of the organic phase.

After the tube was shaken for 10 min and centrifuged, the organic phase was discarded by aspiration and the aqueous phase was washed three times with 10 ml of *n*-heptane to remove traces of the isoamyl alcohol. To the aqueous phase were added 0.2 ml of 2 *N* NaOH and 5.0 ml of methylene chloride. The samples were shaken for 10 min and centrifuged. The aqueous phase was carefully aspirated, and 4.0 ml of the organic phase was added to a clean 13-ml glass-stoppered tube. The contents were evaporated to dryness in a 60° water bath under a stream of nitrogen.

The residue was dissolved in 0.1 ml of the heptafluorobutyrylimidazole–ethyl acetate reagent, and the stoppered tube was immersed in a boiling water bath for 1 hr. The tubes were cooled to room temperature, 3.0 ml of *n*-heptane plus 1.0 ml of 0.1 *N* HCl was added, and the tubes were then shaken for 10 min. After centrifugation, 2.0 ml of the *n*-heptane phase was transferred to a clean 13-ml conical centrifuge tube and the contents were evaporated to dryness at 60° using a stream of nitrogen.

The residue was dissolved in 0.1 ml of *n*-heptane, and 1–5 μl was chromatographed. The retention times of derivatized timolol and derivatized internal standard were 7.6 and 6.0 min, respectively. GC–mass spectrometric methods showed that the products of acylation of these compounds are the diheptafluorobutyryl derivatives[8].

Peak heights for derivatized timolol and internal standard were measured to the nearest millimeter. A standard curve was constructed by analysis of samples of control plasma containing known quantities of timolol and internal standard. The ratio obtained by dividing the timolol peak height by the internal standard peak height was plotted against the concentration of timolol in



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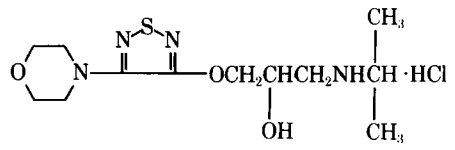

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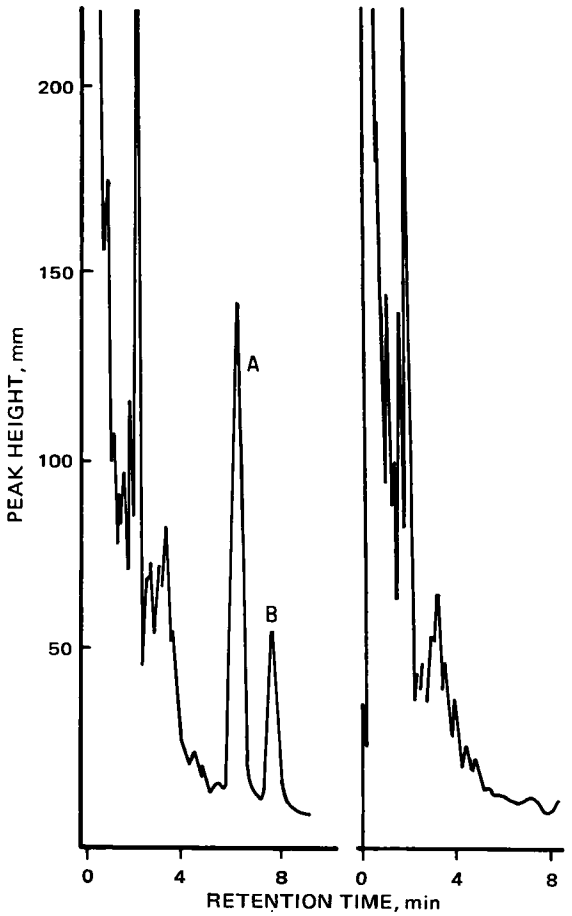

Figure 1—*Gas–liquid chromatograms (electron-capture detector) of human plasma extracts. Left: 50 ng of desmethyltimolol (A) and 20 ng of timolol (B) added to 1.0 ml of plasma. Right: control human plasma.*

nanograms per milliliter of plasma or urine. The line that best fit the data was drawn through the experimental points and the origin. Concentrations of timolol in biological samples were determined by interpolation from the standard curve.

Timolol in Human Plasma—Three healthy human subjects were given a single oral dose (either a tablet or solution) containing 10 mg of ^{14}C-timolol in which both carbons of the thiadiazole ring were labeled. Fourteen days later, the same subjects were given a second 10-mg dose in a simple crossover design. Blood was collected in heparinized containers at intervals during 24 hr following each dose, and plasma was separated by centrifugation and frozen until assayed.

"Apparent timolol" in plasma was determined by measuring the radioactivity in the heptane–4% isoamyl alcohol extract of plasma. Aliquots (0.5 ml) were evaporated to dryness in counting vials. The samples were counted in 16 ml of a toluene medium containing 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (8 g/liter), 2-(4-biphenylyl)-6-phenylbenzoxazole (0.5 g/liter), and solubilizer[9] (125 ml/liter).

At least 5000 total counts were made on all samples[10]. The counting efficiency was determined by external standardization and corrected for a background counting rate of approximately 40 cpm.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram obtained when plasma was assayed by the electron-capture procedure. In this sample, 50 ng of internal standard (peak A) and 20 ng of timolol (peak B)

[7] Applied Science Laboratories.
[8] W. J. A. VandenHeuvel, unpublished data.
[9] Beckman Biosolve, Beckman Instruments.
[10] Beckman LS-100 liquid scintillation spectrometer.

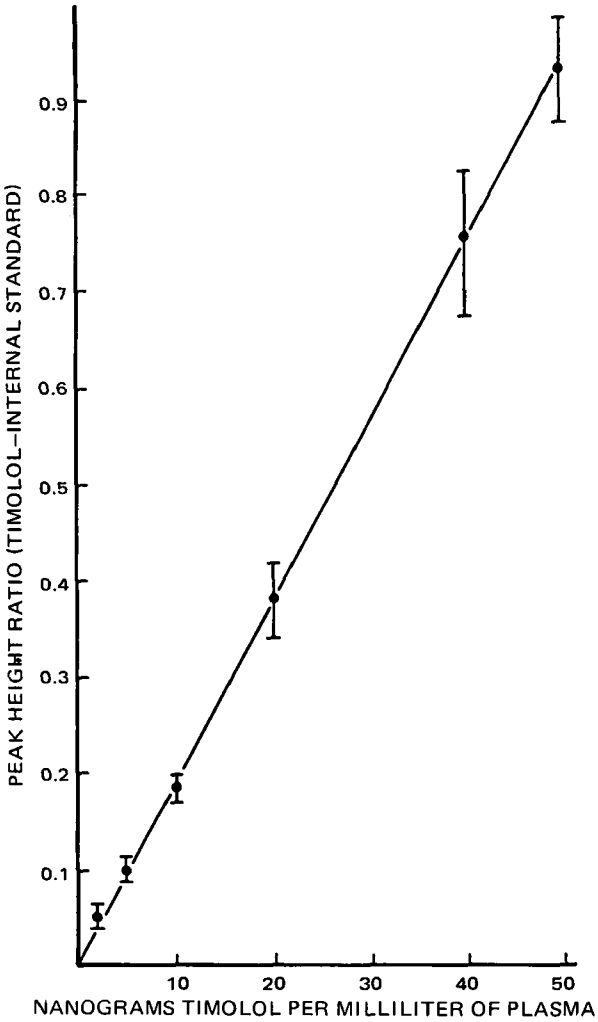

Figure 2—*Calibration curve showing the relationship between the peak height ratio of the diheptafluorobutyryl derivatives of timolol and desmethyltimolol and the amount of timolol in plasma. Each point represents the mean ± SD for four determinations.*

were added to 1.0 ml of human plasma and carried through the procedure. This concentration of timolol gave a peak height of approximately 50 mm (32 pamp) at a range setting of 10 and an attenuation of 16. Blank plasma samples assayed in the same manner gave no interfering peaks on the chromatogram.

Quadruplicate samples, containing 2.0–50 ng of timolol/ml of plasma, were adequately recovered (Table I). The concentrations found were equal to 98 ± 22% (*SD*) of the actual values. A calibration curve prepared from these data indicates that the peak height ratios of timolol–internal standard were linear when plotted against the concentration of timolol, and the curve passed through the origin (Fig. 2). The lower limit of sensitivity was 2.0 ng/ml, and the upper limit of linearity was at least 50 ng/ml.

Plasma levels of timolol were determined in six human subjects over 24 hr following a single oral 10-mg dose of ^{14}C-timolol (Table II). Apparent timolol, *i.e.*, radioactive equivalents of timolol that were extracted by heptane–4% isoamyl alcohol, showed reasonably

Table II—Mean Plasma Levels of Timolol in Six Human Subjects after a Single Oral 10-mg Dose of ^{14}C-Timolol[a]

Time after Dose, hr	Timolol in Plasma, ng/ml ± *SD*		Ratio of Apparent Timolol–Timolol
	"Apparent Timolol"[b]	Timolol	
0.5	15.0 ± 9.6	16.2 ± 10.8	0.93 ± 0.15
1.0	30.0 ± 11.7	30.3 ± 11.2	1.00 ± 0.16
2.0	34.3 ± 12.6	31.2 ± 12.5	1.09 ± 0.08
3.0	34.0 ± 17.2	28.4 ± 11.2	1.20 ± 0.20
4.0	27.3 ± 16.4	25.0 ± 13.6	1.09 ± 0.14
5.0	22.0 ± 14.2	21.2 ± 14.7	1.04 ± 0.14
6.0	16.2 ± 11.4	13.5 ± 10.6	1.20 ± 0.27
8.0	11.3 ± 9.0	10.3 ± 9.2	1.10 ± 0.37
24.0	0	0	0.98 ± 0.05

[a] Timolol labeled with ^{14}C in both carbons of the thiadiazole ring had a specific activity of 3996 dpm/μg. [b] The radioactive equivalents of timolol, expressed as nanograms per milliliter, that were extracted from plasma during the analytical procedure for timolol.

good agreement with the timolol concentration as measured by the electron-capture procedure. The ratio of apparent timolol–timolol was 1.11 ± 0.24 (mean ± *SD*). Peak plasma levels of 31 ng/ml occurred at 2 hr after drug administration. The half-life of the drug in plasma was approximately 3 hr.

The electron-capture method was applied to numerous samples of urine as well as plasma. Electrophoretograms of human urine have shown that timolol is the only drug-related base in the urine[11]. Hence, the method is presumed to be specific for determining the intact compound in plasma and urine. In humans, 10–20% of an oral 10-mg dose of timolol was excreted unchanged. Frequently the concentration of drug in the urine was between 500 and 2000 ng/ml. Therefore, to avoid unnecessary dilution at the end of the assay, a smaller aliquot (0.1 ml of urine) should be processed at the beginning. As with plasma, blank urine samples gave no interfering peaks on the chromatogram.

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