New and Expedient Determination of Atenolol in Biological Samples

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Abstract □ A method is described for the GLC determination of atenolol BP in plasma and urine. Extraction is accomplished under dehydrating conditions, and interfering impurities are removed by using an acidified cyclohexane–isopropanol mixture (2:1) and charcoal-treated paper disks. The drug thus isolated appears to react more efficiently with heptafluorobutyric anhydride, increasing the sensitivity of GLC electron-capture analysis. Concentrations as low as 0.02 μg/ml were measured using 0.5-ml aliquots of plasma or 0.1 ml of urine. Amino alcohols such as atenolol may form hydrates or alcoholates, precluding complete derivatization with heptafluorobutyric anhydride.

Keyphrases □ Atenolol—GLC analysis, biological samples □ GLC—analysis, atenolol, biological samples □ Adrenergic agents—atenolol, GLC analysis, biological samples

Atenolol[1] [4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide] (I) is a new β-adrenergic blocking agent. The results of animal studies indicate that atenolol differs from other β-blocking drugs currently available for study in humans because of its cardioselectivity, absence of intrinsic sympathomimetic activity, and membrane-stabilizing activity (1–4). The compound may be valuable as an antihypertensive agent in humans (5, 6).

A GC method for the determination of atenolol in whole blood, serum, tissue, and urine was recently reported (7). The method required an initial aqueous–solvent extraction followed by purification steps involving back-extraction techniques. This extraction procedure was tedious, and a great chance for error resulted from the number of transfer steps involved. As a result, a simplified extraction procedure was developed.

The extraction procedure for both plasma and urine, as developed in this laboratory, is performed under dehydrating conditions. It is both expedient and efficient, requiring only 0.5 ml of plasma or blood and 0.1 ml or less of urine. The method is of sufficient sensitivity (0.02 μg/ml) to determine plasma levels of atenolol in humans after a single 25-mg oral dose.

EXPERIMENTAL

Reagents—Ethyl acetate[2], cyclohexane[2], isopropyl alcohol[2], and acetone[2] were glass-distilled, reagent grade solvents. Acetic acid[3], ammonium hydroxide[3] (14.8 *N*), anhydrous sodium sulfate[3], and anhydrous ether[3] were all reagent grade, ACS certified.

Heptafluorobutyric anhydride was freshly prepared from heptafluorobutyric acid[4] (sequenation grade) by refluxing over excess phosphorus pentoxide[5] for 3 hr. The mixture was then fractionally distilled through a 25.4-cm (10-in.) Vigreux column, and the anhydride was collected at 107–108°. The acidic wash solvent was prepared by adding 128 ml of isopropyl alcohol to a mixture of 250 ml of cyclohexane and 5 ml of acetic acid.

Table I—Effect of Acetone in Removing Solvate from Atenolol Residue prior to Reaction with Heptafluorobutyric Anhydride[a]

Original Solution	Relative GLC Peak Area	
	Control	Acetone
Water	0.589	0.955
Absolute ethanol	0.603	0.955

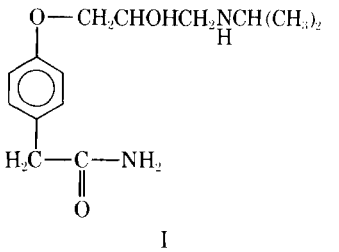
[a] Concentration of atenolol was 0.4 μg/ml. Conditions were as described in the text.

Plasma Level Study—Atenolol was administered to two healthy male volunteers[6]. Subject 1 (24 years, 75.5 kg) received a 25-mg tablet[7], and Subject 2 (31 years, 71.9 kg) received a 100-mg tablet[7]. Blood was withdrawn at various time intervals from an indwelling needle attached to a plastic catheter[8] in a superficial vein of the forearm. The blood was collected in oxalated containers and centrifuged, and the plasma was transferred and stored in plastic vials at −10°. Urine samples were also collected during 24-hr intervals within a 72-hr period and pooled.

Extraction of Atenolol—A premeasured volume of anhydrous sodium sulfate (1.0 ml) was added to a 15.0-ml graduated conical centrifuge tube containing 0.5 ml of plasma. The tube was mixed[9], and 2.5 ml of the acidic wash solvent was added to the resulting paste. The tube was again mixed until the solid sodium sulfate was dispersed and then was centrifuged at 2000 rpm in an angular head, clinical centrifuge for 3 min.

The organic phase was removed and discarded using a disposable Pasteur pipet. One drop (0.05 ml) of 14.8 *N* NH_4OH was added to the residue, followed by 4.0 ml of freshly distilled ethyl acetate. The tube was mixed, and two 6.5-mm diameter charcoal-treated paper disks[10] were added. After mixing for 30 sec, the tube was centrifuged as before.

The ethyl acetate layer was carefully transferred, using a Pasteur pipet, into a tube and flash evaporated *in vacuo*[11] to dryness. To the residue was added 0.4 ml of heptafluorobutyric anhydride–ether (1:4). After reacting at ambient temperature for 5 min, the contents of the tube were dried under a nitrogen stream (using an in-line activated silica gel desiccant filter) for 15 min. The residue was dissolved in 1.0 ml, or some other suitable volume, of freshly distilled ethyl acetate. Atenolol extracts from plasma or urine that



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[1] British Pharmacopoeia approved name.
[2] Burdick-Jackson Labs., Inc., Bodman Chemicals, Narberth, Pa.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Eastman Kodak, Rochester, N.Y.
[5] Fisher Scientific Co., Fairlawn, N.J.
[6] Plasma and urine samples were obtained from Dr. R. F. Maronde, University of Southern California School of Medicine.
[7] Preliminary experimental formulations.
[8] Butterfly-19, Abbott Laboratories.
[9] Vortex Genie, Fisher Scientific Co.
[10] Made with a paper punch-out from S & S charcoal-treated filter paper No. 505.
[11] Evapo-Mix, Buchler Instruments, Division of Searle Analytic Inc., Fort Lee, N.J.

Table II—Peak Area (GLC) of Atenolol Extracted from Plasma at Different Concentrations and Times of Day over a 3-Day Period[a]

Atenolol, μg/ml	Day 1		Day 2		Day 3	
	am	pm	am	pm	am	pm
0.2	0.338	0.295	0.283	0.233	0.368	0.341
0.3	0.383	0.435	0.379	0.421	0.391	0.398
0.4	0.559	0.477	0.447	0.453	0.435	0.455
0.6	0.724	0.702	0.628	0.571	0.622	0.556

[a] Each value represents a separate plasma extraction and is the average of a duplicate run on GLC. The maximum volume of atenolol used to spike plasma was 25 μl.

were not reacted immediately after extraction were stored in a desiccator over phosphorus pentoxide.

Urine—A fresh solution of urease[12] was prepared just before use (25 mg/ml, approximately 3500 units/g in 1 *M* KH_2PO_4 buffer, pH 7.4). To 0.5 ml of diluted urine (1:5 dilution with distilled water) in a 15-ml conical centrifuge tube was added 0.2 ml of the urease solution. The mixture was then incubated at 30° for 10 min. After incubation, the mixture was analyzed as previously described for plasma except that 1.2 ml of premeasured anhydrous sodium sulfate was used instead of 1.0 ml.

Electron-Capture GLC—The derivatized samples were analyzed using a gas chromatograph[13] equipped with a ^{63}Ni-electron-capture detector. A glass column (1.2 m × 6 mm o.d.) was packed with a 3.8% UCW 98 on Gas Chrom Q, 80–100 mesh. The injection port and oven temperatures were maintained at 225 and 208°, respectively. The detector temperature was set at 320° and operated with a pulse interval of 150 μsec. The carrier gas was argon with 5% methane at a flow rate of 60 ml/min. Under these conditions, the retention time of the atenolol derivative was approximately 3 min. The injection volume was 2 μl.

Calculations—A stock solution of atenolol in absolute ethanol (1.0 mg/ml) was used to prepare daily working standards (0.02–1.0 μg/ml) by spiking plasma or urine obtained from undosed controls. From these standards, a calibration curve of concentration *versus* peak area was determined. The overall recovery of atenolol from plasma or urine was determined by comparing standards extracted from plasma or urine by the described procedure with nonextracted acetone-stripped standards. The latter procedure involved flash evaporating a standard solution *in vacuo*[11], then adding 5.0 ml of acetone, and flash evaporating once again. This procedure with acetone was repeated one more time. The stock solution of atenolol was stored at 5°, protected from light, and was stable for several months.

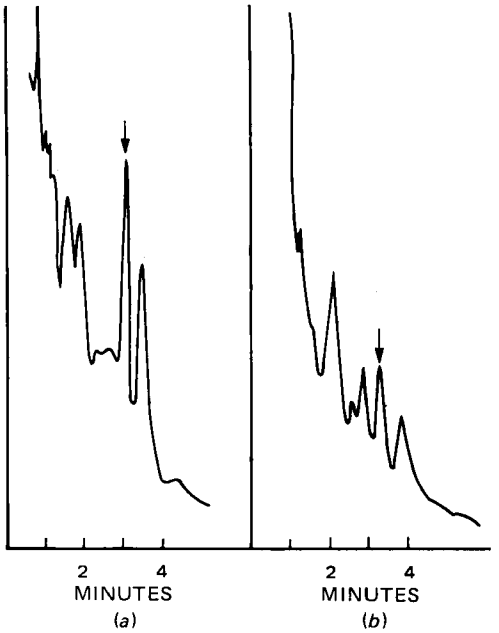

Figure 1—*(a) GLC trace of extract from plasma containing 0.10 ng/μl of atenolol. (b) GLC trace of extract from urease-treated urine containing 0.05 ng/μl of atenolol. (Position of atenolol is designated by the arrow.)*

[12] Sigma, Type III.
[13] Hewlett-Packard HP7620A.

Table III—Slope and Intercept Variations within the Day and from Day to Day

Time	Day 1	Day 2	Day 3
am:			
Slope	1.02	0.85	0.65
Intercept	0.12	0.11	0.21
pm:			
Slope	0.98	0.79	0.54
Intercept	0.11	0.12	0.24
Average:			
Slope	1.00	0.82	0.59
Intercept	0.11	0.12	0.22

RESULTS AND DISCUSSION

The GLC peak of the heptafluorobutyrate derivative of atenolol isolated from human plasma and urine is shown in Fig. 1. The GLC recorder trace for the blank plasma and urine is shown in Fig. 2. The electron-capture detector response was very high for this derivatized amino alcohol. The structure of the atenolol heptafluorobutyrate derivative was confirmed by mass spectrometry (7), which showed that the secondary alcohol was esterified and the amino group was acylated. The amide group on the phenyl ring was dehydrated on reaction with heptafluorobutyric anhydride to form the nitrile.

The relative GLC peak area was diminished significantly if an aqueous or ethanol solution of atenolol was reacted with heptafluorobutyric anhydride without prior removal of solvate from residue with acetone, as described under *Experimental*. Typical data are shown in Table I; the GLC peak area was enhanced as much as

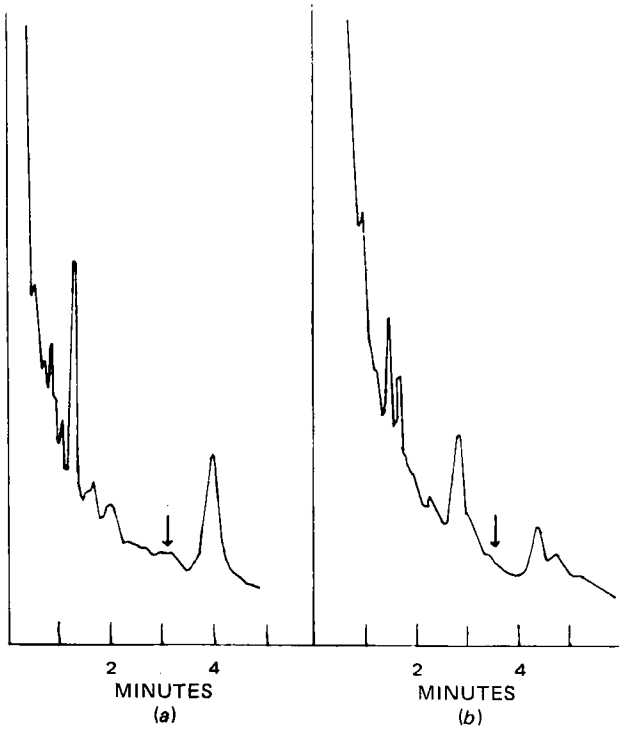

Figure 2—*(a) GLC trace of extract from plasma blank. (b) GLC trace of extract from urine blank. (Position of atenolol is designated by the arrow.)*

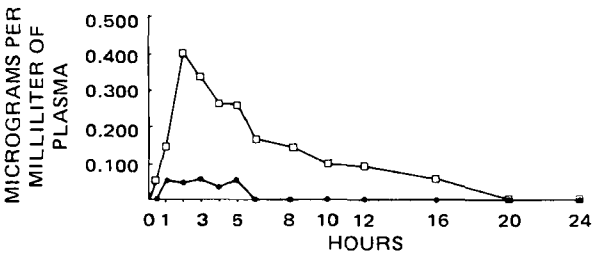

Figure 3—*Plasma concentrations of atenolol in two subjects administered a single oral dose. Key:* ●, *Subject 1, 25 mg; and* □, *Subject 2, 100 mg.*

38% by using acetone to remove solvate.

The overall recovery of atenolol extracted from plasma or urine was 60 ± 4% over the concentration range of 0.05–1.0 μg/ml[14]. This range is comparable to what was previously reported (7). A consistent 27–30% of the drug, independent of the concentration, was lost in the initial wash step. Attempts to minimize this loss by using solvents of different polarities resulted in an increase in interfering impurities on GLC. Preextraction with the acidic wash solvent resulted in the removal of some plasma components which, if carried to the derivatization step, would elute from the GLC column approximately 50 min after an initial sample injection. Other interfering plasma impurities were found to absorb preferentially onto charcoal-coated paper disks.

The somewhat lower recoveries (approximately 40–50%) obtained using the aqueous–solvent extraction procedure previously reported (7) may be attributed to an apparent hydrated form of the amino alcohol and also possibly to a less efficient extraction procedure.

Plasma and urine samples containing atenolol (0.05 and 0.2 μg/ml) and stored at −10° were stable for at least 1 month, with no significant change in concentration noted.

The high and variable concentration of urea in urine interfered with the heptafluorobutyric anhydride derivatization step. Pretreating a urine aliquot with urease and incubating for the prescribed period eliminated this interference.

Within-day and day-to-day variation and reproducibility of the extraction procedure were evaluated at four different concentrations of atenolol in plasma (Table II). From these data, the slope and intercept variations within the day and from day to day were obtained (Table III). As described, the am and pm slopes within the day were not significantly different. However, the day-to-day slope and intercept variation were significantly different ($p < 0.01$). For this reason, a fresh control plasma standard calibration curve was prepared daily. Calibration plots were strictly linear over the range of 0.0–1.0 ng injected onto the column.

[14] Based on aqueous standard, dried *in vacuo*, and stripped with acetone as described in the text prior to reacting with heptafluorobutyric anhydride.

Table IV—Atenolol Excreted in Urine after a Single Oral Dose

Subject	Dose, mg	Atenolol Excreted, mg			Percent of Dose
		0–24 hr	24–48 hr	48–72 hr	
1	25	7.15	0.39	0.06	30.4
2	100	25.15	0.92	0.13	26.2

Plasma levels of atenolol (preliminary experimental formulation) administered to two human volunteers following a single oral dose are shown in Fig. 3. The amount of atenolol excreted in urine over 72 hr is shown in Table IV. A 25-mg oral dose resulted in plasma levels close to the limit of detection (0.02 μg/ml).

The method described is sensitive for the determination of atenolol concentrations as low as 0.02 μg/ml in plasma or urine. It offers several advantages over aqueous–solvent extractions, employing back-extraction techniques. First, the number of transfer steps is kept to a minimum by extracting under dehydrating conditions. Second, under these same conditions, an apparent hydrate of atenolol is prevented from forming, thus favoring a more complete reaction with heptafluorobutyric anhydride.

Acetone was effective in removing apparent bound water or alcohol to drug molecules when prepared for standard use. Preliminary data indicate that this phenomenon may be peculiar only to amino alcohols when used for derivatization by heptafluorobutyric anhydride.

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