GLC Determination of Atenolol and β-Blocking Agents in Biological Fluids

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Abstract □ A rapid, sensitive, and specific method of analysis for atenolol is described. Metoprolol is used as the internal standard. Atenolol and metoprolol are extracted into 1-butanol–benzene. Interfering components present in plasma and urine, but not discolored saliva, are removed during an acid wash and reextraction into ether. Drug and internal standard are converted to the pentafluoropropionate derivatives, which are quantitated by GLC with electron-capture detection and characterized by chemical-ionization mass spectrometry. The method should be applicable to measurement of other β-adrenergic blocking agents with similar structures.

Keyphrases □ Atenolol—GLC analysis in biological fluids □ GLC—analysis, atenolol in biological fluids □ Antiadrenergic agents—atenolol, GLC analysis in biological fluids

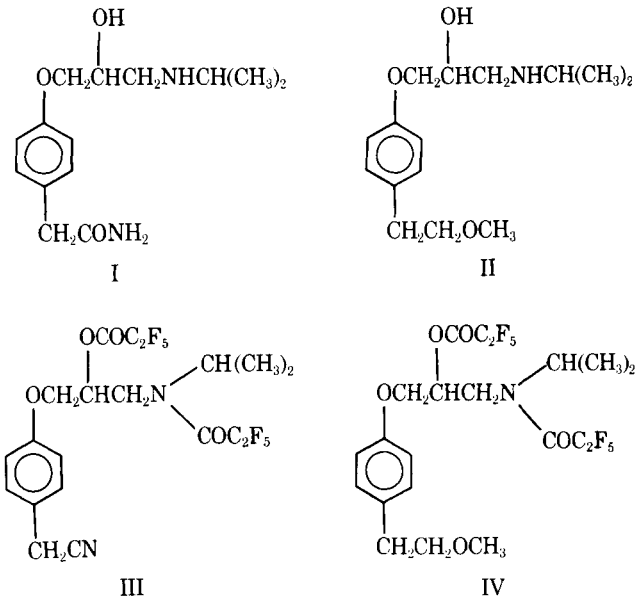
Recently, some β-adrenergic receptor blocking agents having similar structures and physicochemical properties were synthesized. Atenolol[1], 4-[2-hydroxy-3-(1-methylethyl)aminopropoxy]benzeneacetamide (I), differs from most compounds in this group by having a polar acetamide substituent that confers low partitioning into organic solvents. Thus, extraction procedures used in the analysis of β-blockers such as propranolol, alprenolol, and pronethanol must be modified considerably for application to polar compounds like atenolol.

A fluorescence assay for atenolol is rapid but lacks specificity and sensitivity (1). GLC methods (2, 3) have improved both specificity and sensitivity. A disadvantage in these methods is that the derivatizing agent used, heptafluorobutyric anhydride, must be prepared by a lengthy refluxing process because the commercially available compound is unsuitable. Moreover, the background becomes excessive at low tissue levels, resulting in high variability and low accuracy and reproducibility.

The described GLC method is rapid, specific, and sensitive to 10 ng of atenolol/ml of biological fluid. It can also be used for the analysis of other β-blocking agents such as propranolol and metoprolol. GLC coupled with a chemical-ionization mass spectrometer was used to characterize the derivatives formed.

EXPERIMENTAL

Reagents—Commercially available pentafluoropropionic anhydride[2] was used as the derivatization reagent. Sodium hydroxide (5 *N*), sulfuric acid (0.2 *N*), and pH 8 phosphate buffer (0.067 *M*) were prepared with deionized water[3]. Glass-distilled[4] 1-butanol, benzene, hexane, and ethyl acetate were used. Spectrophotometric grade pyridine[5] and nanograde ether[6] were used without prior distillation. Metoprolol[7], 1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]-2-propanol (II), 50 ng in 0.1 ml of water, was used as the internal standard. Standard aqueous solutions were prepared every 2 weeks and were kept at 4°.



Glassware—The glassware was cleaned by soaking in 10% nitric acid overnight. It was then washed and dried by heating at approximately 400° for 3 hr.

Extraction and Derivatization—To 1 ml of standard solution, plasma, saliva, or appropriately diluted urine in a 7-ml, polytef-faced, screw-capped test tube were added 50 ng of the internal standard and 0.1 ml of 5 *N* NaOH. Atenolol and metoprolol were extracted into 5 ml of a solution of 30% benzene in 1-butanol by shaking for 15 min. The two liquid phases were separated by centrifugation, and the organic layer was transferred to another tube containing 1 ml of 0.2 *N* H_2SO_4. After shaking and centrifugation, the aqueous layer was transferred to a third tube. The solution was made basic by addition of 0.2 ml of 5 *N* NaOH, and the compounds of interest were extracted into 5 ml of ether. The ether layer was transferred to a clean tube and evaporated to dryness at 70° under a nitrogen stream.

To the residue were added 0.5 ml of hexane, 0.025 ml of ethyl acetate containing 1.5% pyridine, and 0.025 ml of pentafluoropropionic anhydride. The tube was capped and heated at 70° for 15 min. At the end of this reaction time, solvents and excess pentafluoropropionic anhydride were removed by flushing with nitrogen. To the residue were added 1 ml of phosphate buffer and 0.05 ml of hexane, and the contents of the capped tube were mixed on a vortex mixer for 15 sec. The phases were then separated by centrifugation, and 1–3 μl of the hexane layer was injected into the gas chromatograph.

GLC Conditions—A gas chromatograph[8] equipped with a ^{63}Ni-detector was used. The glass column, 180 cm × 2 mm i.d., was packed with 100–120-mesh Gas Chrom Q coated with 3% OV-1[9]. The carrier gas was 5% methane in argon, and the flow rate was maintained at 30 ml/min. Injector port, column, and detector temperatures were maintained at 190, 190, and 300°, respectively.

Mass Spectrometric Conditions—A gas chromatograph interfaced with a chemical-ionization mass spectrometer[10] was used in the characterization of atenolol and metoprolol derivatives. The GLC column was

[1] Imperial Chemical Industries Ltd., Macclesfield, England.
[2] PCR Inc., Gainesville, Fla.
[3] Millipore, Bedford, Mass.
[4] Burdick & Jackson, Muskegon, Mich.
[5] Aldrich Chemical Co., Milwaukee, Wis.
[6] Mallinckrodt Chemical Works, St. Louis, Mo.
[7] A. B. Hassle, AB Astra, Sweden.
[8] Hewlett-Packard model 5710A.
[9] Applied Science Laboratories, State College, Pa.
[10] Finnigan model 3200.

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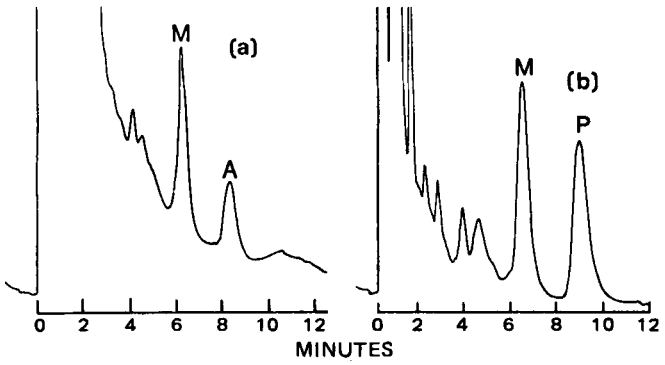

Figure 1—(a) *Spiked plasma sample containing 40 ng of atenolol and 50 ng of metoprolol/ml of sample. Injection volume was 2 μl; attenuation was 32. Key: peak M, metoprolol; and peak A, atenolol.* (b) *Spiked plasma sample containing 60 ng of metoprolol and 50 ng of propranolol/ml of sample. Injection volume was 2 μl; attenuation was 64. Key: peak M, metoprolol; and peak P, propranolol.*

maintained at 160°. Methane was both the carrier and reagent gas. The chemical-ionization source was operated at 250°, and pressure was maintained at 1 torr.

RESULTS AND DISCUSSION

Under the described GLC conditions, metoprolol and atenolol eluted with retention times of 6.5 and 8.5 min, respectively (Fig. 1*a*). Blank plasma and urine samples from healthy volunteers did not contain any interfering peaks with retention times similar to atenolol or metoprolol (Fig. 2). However, discolored saliva samples contained multiple interfering peaks, and such samples could not be analyzed by this technique. Standard curves were obtained by spiking plasma, saliva, or urine with known amounts of atenolol and measuring the peak height ratios of atenolol to metoprolol. The standard curves were linear in the range of 10–500 ng of atenolol/ml of sample.

The coefficients of variation at 10 and 100 ng/ml were 8.50 and 5.43%, respectively (n = 5). The daily variability was determined from the slopes and intercepts of five standard curves and indicated only minor fluctuations (Table I). The slopes of standard curves from water or biological fluids were similar, indicating little difference in extractability of drug from aqueous solutions of different composition. No interference was observed for plasma and urine samples from patients with renal failure who were receiving other medication including guanethidine, furosemide, clonidine, hydrochlorothiazide, digoxin, allopurinol, methyldopa, and hydralazine (Fig. 3).

The pentafluoropropyl derivative of atenolol, when subjected to chemical-ionization mass spectrometry, provided an M + H ion at mass 541 (Fig. 4*a*). The mass of this ion is consistent with Structure III and is confirmed to be the molecular ion by the presence of M + 29 = 569 and M + 41 = 581 ions, the characteristic ions formed with methane under chemical-ionization conditions. The structure of III is further supported by the formation of the doubly derivatized side chain with mass 408. The thermal conversion of the amide moiety of atenolol to cyanide under GLC conditions was reported previously (2).

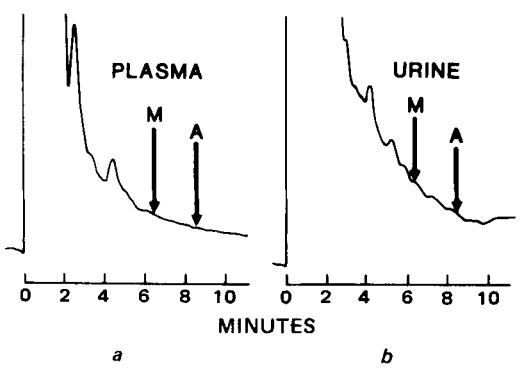

Figure 2—*Blank plasma and urine samples from healthy volunteers. Injection volume was 2 μl; attenuation was 32. Arrows M and A indicate retention times of metoprolol and atenolol, respectively.*

Table I—Daily Variability in Slopes and Intercepts of Standard Curves in Water (Atenolol Concentration of 10–100 ng/ml)

Day	Slope	Intercept
1	0.00635	−0.007
2	0.00628	0.000
3	0.00695	−0.010
4	0.00705	0.008
5	0.00634	0.008
Mean (*SE*)	0.00659 (0.00017)	0.000 (0.0037)

Figure 4*b* shows the chemical-ionization mass spectrum of derivatized metopropolol (IV). The compound formed an abundant ion at mass 528, which was equivalent to the loss of the CH_3OH molecule from the protonated compound (M + H = 560). The presence of a small peak at 560 supports this observation. The formation of doubly derivatized metoprolol is established by the formation of an ion at mass 408 and an ion at mass 396.

The solvent mixture used in the first extraction step was similar to that described by Scales and Copsey (2) who used 1-butanol containing 30% cyclohexane and obtained an overall extraction efficiency of 55% for atenolol (2). The acid wash reduced background detector response and variability of measurement at low drug levels. Ether was used in the final extraction step because it was readily evaporated and expedited sample

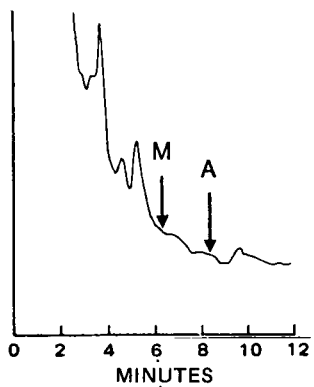

Figure 3—*Blank plasma from a subject with renal failure and receiving clonidine, hydrochlorothiazide, hydralazine, and insulin. Injection volume was 1.8 μl; attenuation was 16. Arrows M and A indicate retention times of metoprolol and atenolol, respectively.*

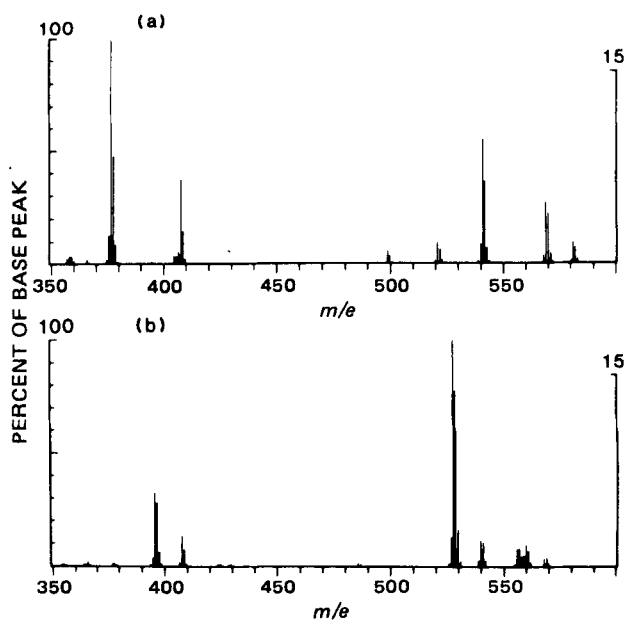

Figure 4—*Chemical-ionization mass spectrum of pentafluoropropionate derivatives of atenolol* (a) *and metoprolol* (b).

preparation. In this method, urea in urine did not interfere with derivatization by pentafluoropropionic anhydride, and it was unnecessary to destroy urea by incubation with urease (3). Derivatization of atenolol and metoprolol with pentafluoropropionic anhydride yielded products with good GLC properties, low retention times, and high electron-capture response. Back-extraction procedures ensured removal of extraneous components which normally take a long time to elute, reducing the time between injections to 15 min.

The method was applicable to the measurement of propranolol, which had a retention time of 9 min (Fig. 1*b*).

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New Compounds: Antitumor Activity of 3β-Hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam 4-[*p*-[Bis(2-chloroethyl)amino]phenyl]butyrate

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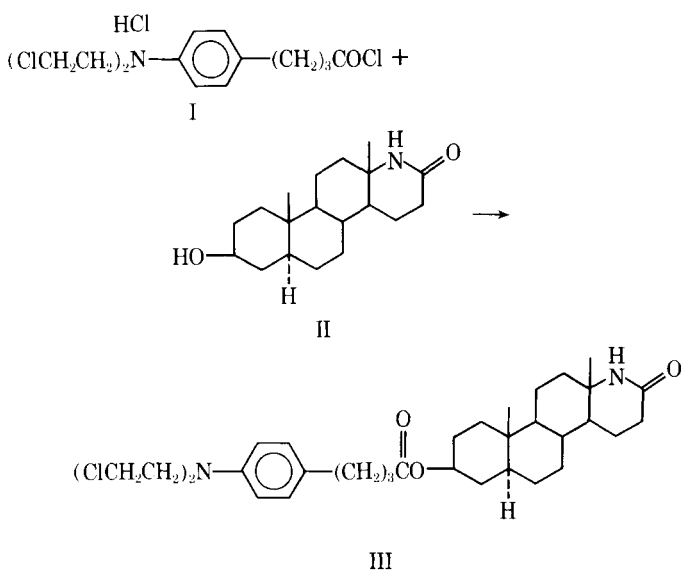
Received October 21, 1977, from the **Department of Chemistry, Nuclear Research Center "Demokritos," Aghia Paraskevi Attikis, Greece,* and the ‡*Theagenion Cancer Institute, Thessaloniki, Greece.* Accepted for publication January 6, 1978.

Abstract □ 3β-Hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyrate was prepared by reacting 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyryl chloride hydrochloride with 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam. The cytostatic action of the ester was investigated on two tumor systems (B16 melanoma on C57 bl mice and T8-Guerin on rats).

Keyphrases □ 3β-Hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam ester of chlorambucil—synthesized, antineoplastic activity evaluated, mice and rats □ Chlorambucil, 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam ester—synthesized, antineoplastic activity evaluated, mice and rats □ Antineoplastic activity—3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam ester of chlorambucil evaluated in mice and rats

3β - Hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam *p*-[bis(2-chloroethyl)amino]phenylacetate exhibited significant antitumor activity against P-388 and L-1210 leukemia in mice (1) and T8-Guerin, B16 melanoma, and Theagenion-Bahner angiosarcoma (2).



Scheme I

Table I—Effect of III on T8-Guerin Tumor and B16 Melanoma

Treatment	Number of Animals	Mean Survival of Noncured Animals, Days	Survivors
T8-Guerin Tumor (on Wistar Rats)			
Controls	10	55	0/10
Chlorambucil, 12.5 mg/kg ip			
Day 2	5	—	5/5
Days 2 and 10	4	65	3/4
Day 10	5	66	1/5
Days 10 and 20	5	60	1/5
III, 70 mg/kg ip			
Day 2	4	54	1/4
Days 1 and 10	5	60	3/5
Day 10	7	58	2/7
Days 10 and 20	4	76	3/4
B16 Melanoma (on C57 Black Mice)			
Controls	10	30	0/10
Chlorambucil, 12.5 mg/kg ip			
Day 2	5	52	0/5
Days 2 and 10	5	49	0/5
Day 10	5	43	0/5
Days 10 and 20	5	56	0/7
III, 70 mg/kg ip			
Day 2	7	48	0/7
Days 2 and 10	9	44	0/9
Day 10	6	47	0/6
Days 10 and 20	5	57	0/5

This fact created an interest in this compound that led to the synthesis of a number of derivatives (3–5).

DISCUSSION

These findings prompted the study of compounds that might have a favorable ratio between lethal and minimum effective doses and at the same time a lower toxicity than the nitrogen mustards. Therefore, 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyrate (III, Scheme I) was synthesized by the action of 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyryl chloride hydrochloride (I) on 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam (II) (6).

Compound III was isolated in pure form after silica gel column chromatography. It gave a 50% increased lifespan over controls in the treatment of L-1210 leukemia in mice by the intraperitoneal, subcutaneous, and oral routes. In contrast, unmodified steroidal esters were inactive

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