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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS IN BIOLOGICAL FLUIDS

V. ANALYSIS OF ACEBUTOLOL AND ITS MAJOR METABOLITE

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SUMMARY

A high-pressure liquid chromatographic analysis for acebutolol and its major metabolite in blood, plasma and urine is reported. The analysis, in which the above mentioned compounds are chromatographed as ion pairs with dodecyl sulfonic acid, uses a simple and rapid method of sample preparation. The technique is more sensitive and rapid than those previously reported and it has equivalent or better reproducibility. The method is applied to the measurement in blood of acebutolol and its acetyl metabolite after a single oral dose.

INTRODUCTION

Acebutolol (Fig. 1) is a competitive β-adrenergic receptor antagonist which has been shown to be clinically effective in the treatment of cardiac arrhythmias[1–3]. Previous reports from our laboratory have described an acetyl homologue metabolite (see Fig. 1) in the plasma of patients receiving oral dosage regimes of acebutolol for the treatment of cardiac arrhythmias, in concentrations of up to ten times those of the unchanged drug[3,4]. Studies in animal models indicate that the acetyl metabolite is approximately as pharmacologically potent as acebutolol[5]. If the metabolite is also found to have pharmacologic activity in man, it may become clinically important to be able to quantitate separately both acebutolol and its metabolite in patients receiving the drug.

A number of methods have been described for the analysis of acebutolol which involve its acid hydrolysis to the primary arylamine (see Fig. 1) and subsequent measurement of visible absorbance[6,7] or fluorescence[8] of a derivative. Such methods are non-specific and detect not only the unchanged drug, but also metabolites containing the amide or primary arylamine function, including the major metabolite. A specific thin-layer chromatographic (TLC) method which uses the fluorescence of acebutolol and its acetyl metabolite has been described[9]. However, this method has a disadvantage in that it utilizes a commonly used and anti-arrhythmic drug, quinidine,

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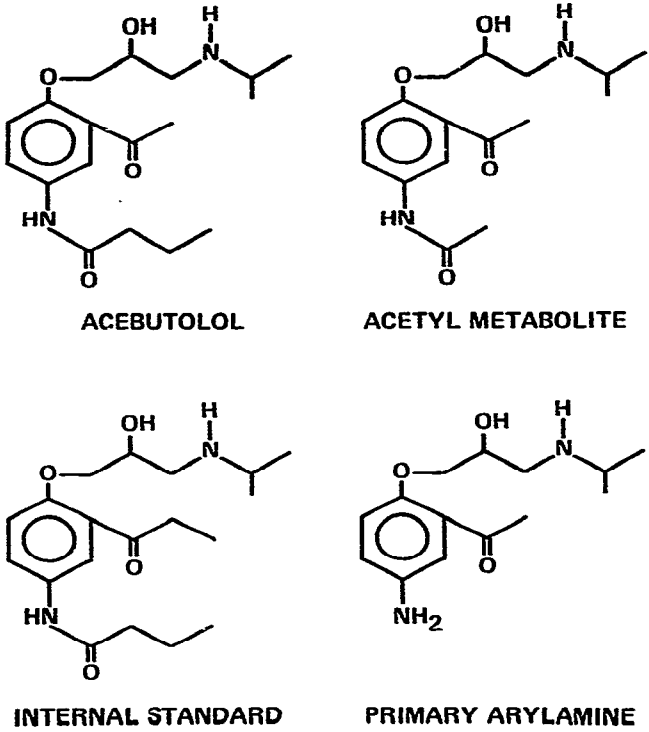

Fig. 1. The chemical structures of acebutolol, its acetyl metabolite, the internal standard used in the analysis and the primary arylamine hydrolysis product of acebutolol.

as an internal standard. In clinical practice, patients are often treated with more than one anti-arrhythmic drug[10] which could result in serious errors in acebutolol determination if quinidine were also being administered. A gas chromatographic (GC) method for acebutolol and its acetyl metabolite has previously been reported from this laboratory[4]. Although this technique was useful in allowing the unequivocal identification of the acetyl metabolite in the plasma of patients receiving acebutolol using combined GC and mass spectrometry, it is complicated and time consuming and has poor reproducibility for the acetyl metabolite. The analytical method described in this paper uses ion-paired reversed-phase high-pressure liquid chromatography (HPLC) for the analysis of acebutolol and its acetyl metabolite. This technique is simple and more rapid than the GC method previously reported by us and has good reproducibility and sensitivity.

EXPERIMENTAL

Reagents and materials

Acebutolol (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane hydrochloride; the acetyl metabolite, (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane; and the internal standard, (±)-1-(2-propyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (see Fig. 1)

were kindly supplied by May & Baker (Dagenham, Great Britain). Dodecyl sodium sulfate was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.). The methanol and ethyl acetate were of "distilled-in-glass" quality and were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All other solvents and reagents were reagent grade.

Sample preparation

A schematic representation of the procedure is shown in Fig. 2. Whole blood, plasma or urine, 0.1–2.0 ml, is placed in a 15-ml capacity PTFE-lined screw-cap culture tube which contains 100 μl of internal standard solution (containing 500 ng of internal standard), 200 μl of 2 *N* NaOH and sufficient water to produce a total volume of 2.3 ml. Ethyl acetate (10 ml) is added and, when the biological fluid is whole blood, the tube is immediately mixed to prevent the formation of solid aggregates. Samples are mixed using a gentle tilting action for 10 min and the organic and aqueous phases are separated by centrifugation. The aqueous phase is frozen by immersion in a dry ice–acetone bath and the organic layer decanted into a second tube which has an elongated cone at its base of approximately 50-μl capacity. A 10-μl volume of 0.1 *N* H_2SO_4 is added to the tube and the mixture is extracted on a vortex mixer for 2 min. The tube is then chilled at 4° (refrigerator) for 10 min, and the aqueous and organic phases separated by brief centrifugation. Depending on the ambient temperature, a shorter or longer period of chilling may be needed to produce

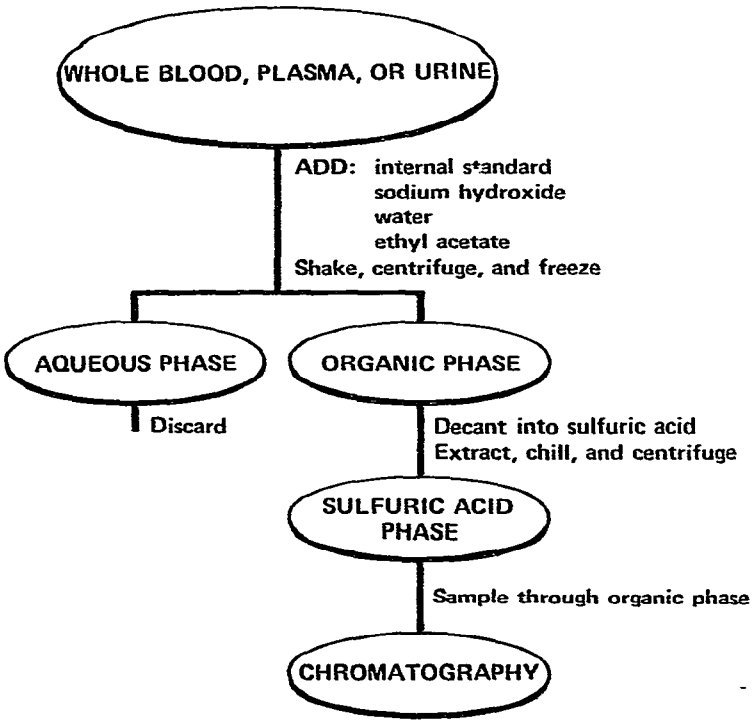

Fig. 2. Schematic outline of the sample preparation used in the analysis of acebutolol and its acetyl metabolite in blood, plasma and urine.

the required 30–40 μl of aqueous phase in the conical section of the tube. The aqueous phase is sampled through the ethyl acetate with a 50-μl syringe and injected into the chromatograph.

Chromatography

A Varian Model 85000 high-pressure liquid chromatograph fitted with a Varian Micro-Pack MCH-10 reversed-phase column (25 cm × 2.0 mm I.D.) was used for the analysis. Absorbance was measured at 240 nm, with a 16-nm band pass, using a Varian Vari-Chrome detector. One pump of the dual-pump gradient-elution chromatograph contained a 0.01 *M* solution of dodecyl sodium sulfate in water adjusted to pH 3.5 with glacial acetic acid. The other pump contained the same concentrations of dodecyl sodium sulfate and acetic acid in methanol. An isocratic mixture containing 50% methanol was used for the analysis. Minor adjustments (1–5%) of the solvent composition were required from time to time to maintain constant retention times. With a dual-pump chromatograph, it was convenient to use two pumps operating under isocratic conditions. However, an acceptable alternative is to use the desired mixture of solvents in a single pump. The flow-rate of the solvent mixture was 40 ml/h with a column input pressure of 135 atm (2000 p.s.i.). Column temperature was maintained at 30° with the aid of a column jacket through which water was circulated. Previously described techniques to suppress detector noise were used[11]. Chromatograms were recorded on Varian A-25 dual-pen recorder with 0–100- and 0–200-mV spans.

Calibration and accuracy

Calibration curves were constructed by adding known amounts of acebutolol, the acetyl metabolite and internal standard to control blood, plasma, and urine which were then analyzed. The peak height ratio of acebutolol or the acetyl metabolite to the internal standard was plotted against the amount of acebutolol or the acetyl metabolite added. In order to calibrate the method and determine accuracy for each batch of unknown samples, standards containing 50, 100, 500, 1000 and 2000 ng of acebutolol and the acetyl metabolite were added to control samples, which were then assayed concurrently with unknown samples. The peak height ratio of each standard was divided by the amount of acebutolol or the acetyl metabolite added to give normalized peak height ratios. The mean normalized peak height ratio was used to calculate the amount of acebutolol or its acetyl metabolite in unknown samples, and the standard deviation of normalized height ratio was used to determine the accuracy of the method over the range of standard concentrations used. The reproducibility of the method was also studied by carrying out replicate analyses of whole blood samples containing 50 and 500 ng of acebutolol and its acetyl metabolite. The effect of sample size on the method was investigated by adding 500 ng of acebutolol and its acetyl metabolite to tubes containing different volumes of biological fluid (0.1–2.0 ml) which were then assayed for acebutolol and its acetyl metabolite. The volumes of internal standard solution, sodium hydroxide and ethyl acetate were kept constant.

To obtain an estimate of the efficiency of the analytical procedure, three control samples, each containing 500 ng of acebutolol and its acetyl metabolite were taken through the analysis, and the mean peak height of the acebutolol and the acetyl metabolite peak was compared to the mean peak height of three peaks obtained by

injecting the same amount of acebutolol and its acetyl metabolite directly into the chromatograph.

Application of the method

A healthy male volunteer received an oral dose of acebutolol of 350 mg, and samples of venous blood were collected at frequent intervals during the next 24 h. The blood was collected into heparinized Venoject tubes (Scientific Products), and was stored at −15° until analyzed.

RESULTS AND DISCUSSION

Under the chromatographic conditions described in Experimental, the retention times of acebutolol, its acetyl metabolite and the internal standard were 5.9, 4.1 and 7.4 min, respectively. Fig. 3A shows the chromatogram of an extract of 1 ml of whole blood containing 500 ng each of acebutolol, its acetyl metabolite and internal standard. Figs. 3B and 3C show chromatograms resulting from the analysis of 1 ml of control blood and urine, respectively. No peaks corresponding to the peaks shown

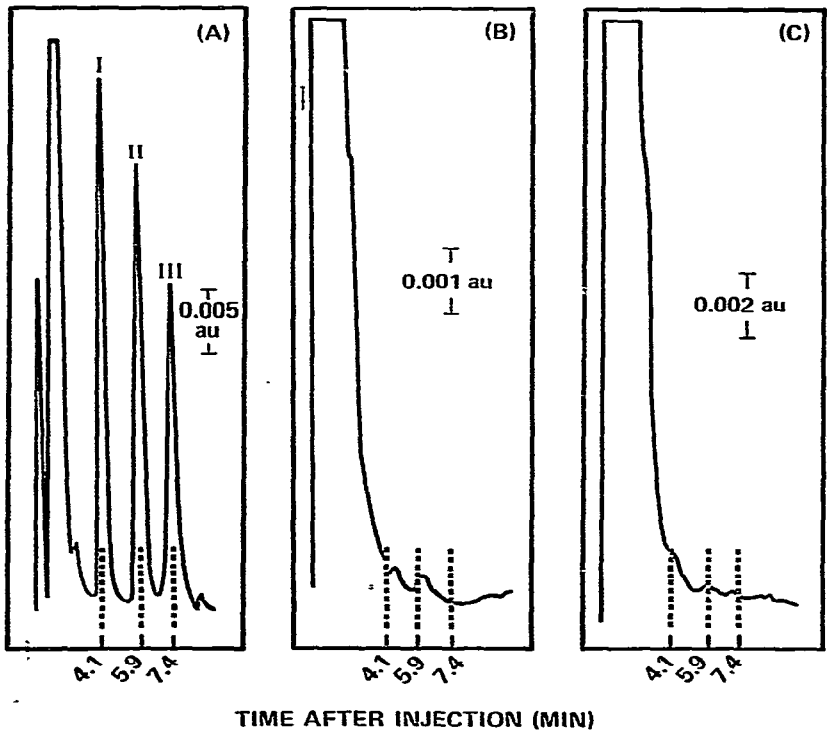

Fig. 3. A: chromatogram resulting from the analysis of 1 ml of blood containing 500 ng each of the acetyl metabolite (peak I), acebutolol (II) and internal standard (III). B: chromatogram resulting from the analysis of the same volume of control blood at five times the detector sensitivity. C: chromatogram resulting from the analysis of 1 ml of control urine. The marks in B and C correspond to the retention times of peaks I, II and III in A. For visual clarity only one tracing of the dual-pen recorder is shown.

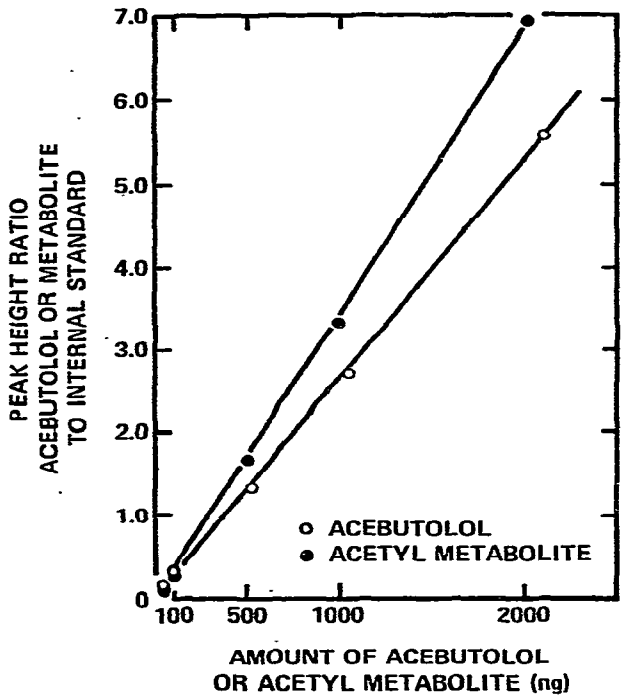

Fig. 4. A typical calibration curve from whole blood for acebutolol and its acetyl metabolite. The coefficient of variation for the normalized peak height ratio is 3.6% for acebutolol and 5.4% for the acetyl metabolite.

in Fig. 3A have been observed in the analysis of control samples of whole blood, plasma and urine from approximately 20 subjects.

A typical calibration curve from whole blood is shown in Fig. 4. The curves for both acebutolol and its acetyl metabolite are linear in the range of 50–2000 ng and pass through the origin. Tables I and II list the estimates of accuracy for the method for blood, plasma and urine. Calibration curves in the range of 50–2000 ng per sample had mean coefficients of variation for the normalized peak height ratio of 3.4% for acebutolol and 5.4% for the acetyl metabolite. Reproducibility studies

TABLE I

CALIBRATION CURVE DATA

C.V. = coefficient of variation.

Biological fluid	*Range (ng)*	*No. of samples*	*% C.V. in normalized peak height ratio*	
			Acebutolol	*Metabolite*
Blood	50–2000	4	3.6	5.4
Blood	50–2000	6	3.0	3.9
Plasma	50–2000	4	5.4	6.8
Urine	50–2000	4	1.5	5.4
		Average	3.4	5.4

TABLE II

REPRODUCIBILITY AT A GIVEN CONCENTRATION (BLOOD)

C.V. = coefficient of variation.

Concentration (ng per sample)	*No. of samples*	*% C.V. in normalized peak height ratio*	
		Acebutolol	*Metabolite*
50	7	2.8	4.0
50	10	2.6	3.5
500	7	2.7	4.5
500	10	2.5	3.8
500	10	2.0	4.4
	Average	2.5	4.0

from whole blood at a given concentration (50 and 500 ng per sample) gave slightly better reproducibility, the mean coefficient of variation in the peak height ratio determined on five separate occasions being 2.5% for acebutolol and 4% for the acetyl metabolite. Although reproducibility studies were carried out down to 50 ng per sample only, concentrations in the order of 15–20 ng/ml (2 ml sample) could be measured in whole blood with fair accuracy.

Because it is often necessary to take variable volumes of biological fluid for analysis in order to optimize the application of the method, the effect of the sample volume on the peak height ratio of acebutolol and its acetyl metabolite was examined. In order to obtain reproducible peak height ratios, it was found necessary to have a constant ratio of aqueous to organic phase present during the initial extraction. The efficiency of the extraction procedure was 85% for acebutolol and 60% for the acetyl metabolite.

Fig. 5 shows the blood concentrations of acebutolol and its acetyl metabolite in a healthy volunteer at various times following a single oral dose of 350 mg of acebutolol. The method enabled the blood concentrations of acebutolol to be measured for 20 h following the dose, or approximately 6 half-lives, at which time virtually all drug had been eliminated from the body. The blood concentrations of the acetyl metabolite could have been measured for a considerably longer period. The sensitivity of the method is therefore sufficient for most pharmacokinetic studies. Typical therapeutic regimes with acebutolol are 200–400 mg, three times a day, and so the method has a large excess of analytical sensitivity for the monitoring of patient samples.

Acebutolol and its acetyl metabolite have poor GC characteristics, presumably due to their poor thermal stability. In order to analyze these compounds by GC a cor- derivatization procedure involving both trimethylsililation and trifluoroace- ion is required[4]. In contrast, ion-paired HPLC allows the analysis of acebutolol and its acetyl metabolite underivatized at near ambient temperature. The advantages of ion-paired chromatography for the analysis of basic drugs in biological fluids has previously been discussed[11]. Briefly, ion-paired chromatography allows the use of rapid and simple sample preparations such as that outlined in Fig. 2, and enables the separation of basic compounds on reversed-phase columns under conditions consistent with column stability. The choice of dodecyl sodium sulfate as the counter-

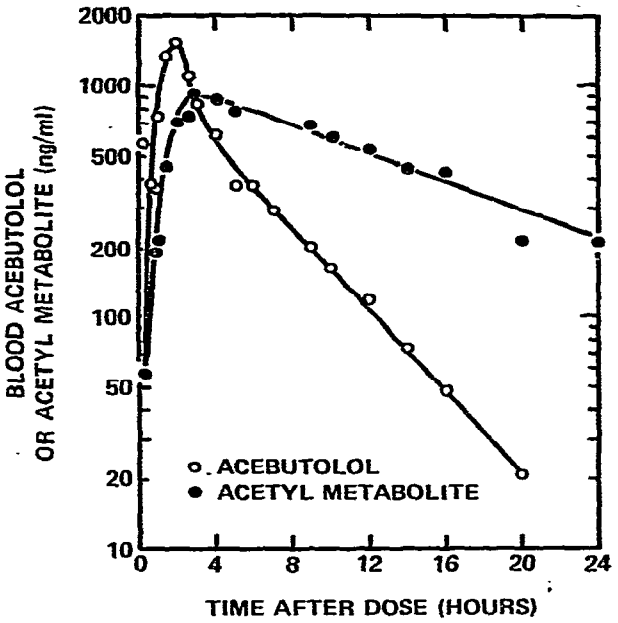

Fig. 5. Blood concentrations of acebutolol and its acetyl metabolite at various times following a single 350-mg oral dose of acebutolol.

ion in this analysis was made on the basis of the convenient retention times with approximately equal volumes of water and methanol. Such a solvent mixture gives the greatest degree of flexibility and requires the least frequent filling of the chromatograph pumps. The peaks were detected at 240 nm, the absorbance maximum for acebutolol in the solvent mixture used. Similar sensitivity and reproducibility were obtained at 254 nm with a fixed-wavelength detector (Varian).

Table III compares the characteristics of the specific chromatographic methods for the analysis of acebutolol and its acetyl metabolite. The use of ion-paired reversed-

TABLE III

COMPARISON OF ANALYTICAL CHARACTERISTICS OF CHROMATOGRAPHIC METHODS FOR ACEBUTOLOL AND ITS ACETYL METABOLITE IN BIOLOGICAL FLUIDS

C.V. = coefficient of variation.

	Ref. 4	*Ref. 9*	*This paper*
Technique	GC	TLC	Ion-paired HPLC
Biological fluid	Plasma, urine	Serum	Blood, plasma, urine
Sample size (ml)	0.05–2	2	0.1–2
Sample preparation	Complex extraction and derivatization	Extraction, evaporation and fluorescence development	Simple two-stage extraction
Range of analysis (ng/ml)	50–1000	100–1000	50–2000 (25–2000 with 2-ml samples)
C.V. (%)			
Acebutolol	2.7	2.3	2.5
Acetyl metabolite	6.2–20	—	4.0

phase HPLC and ultra-violet absorbance detection results in a method that is more rapid, simple and sensitive than previously published methods. Furthermore, the analysis can be applied to a greater range of biological samples, and for clinical applications, would require smaller sample volumes than previous methods. During a period of several months this method has been applied by us to the analysis of approximately 2000 biological samples. Using this technique an analyst can routinely extract and chromatograph 25 samples in a day.

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REFERENCES

1 B. S. Lewis, A. S. Mitka and M. S. Gotsman, *S. Afr. Med. J.*, 48 (1974) 821.
2 F. Biron, A. Proulx, L. Lapointe, R. Nadeau and S. Tremblay, *Eur. J. Clin. Pharmacol.*, 8 (1975) 11.
3 A. H. Gradman, R. A. Winkle, J. W. Fitzgerald, P. J. Meffin, J. Stoner, P. A. Bell and D. C. Harrison, *Circulation*, 55 (1977) 785.
4 P. J. Meffin, S. A. Harapat and D. C. Harrison, *Res. Comm. Chem. Path. Pharmacol.*, 15 (1976) 31.
5 R. F. Collins, *Nouv. Presse Med.*, 4 (1975) 3223.
6 M. F. Cuthbert and R. F. Collins, *Brit. J. Pharmacol.*, 2 (1975) 49.
7 C. M. Kaye, C. R. Kumana, M. Leighton and P. Turner, *Clin. Pharmacol. Ther.*, 19 (1976) 416.
8 A. Roux, B. Flouvat and P. Delaveau, *Ann. Biol. Clin.*, 33 (1975) 281.
9 J. M. Steyn, *J. Chromatogr.*, 120 (1976) 465.
10 J. T. Bigger and E. S. V. Giardina, in K. L. Mehnon (Guest Editor), *Cardiovascular Drug Therapy*, in A. N. Brest (Editor), *Cardiovascular Clinics*, F. A. Davis Co., Philadelphia, Pa., 1974, pp. 103–117.
11 P. J. Meffin, S. A. Harapat and D. C. Harrison, *J. Chromatogr.*, 132 (1977) 503.