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Note

High-performance liquid chromatography assay of acebutolol and two of its metabolites in plasma and urine

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Acebutolol, a β-adrenergic receptor antagonist, is used in Europe and is undergoing clinical testing in the U.S.A. We report a high-performance liquid chromatography (HPLC) assay which has advantages over previously published assays [1,2] in increased sensitivity, simultaneous separate quantitation of acebutolol and two major metabolites, and applicability to both plasma and urine samples with minor variations in the sample processing.

EXPERIMENTAL

Materials

Acetonitrile and ethyl acetate were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled using an all-glass still. Acebutolol, (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane); metabolite I, (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane; metabolite II, (±)-1-(2-acetyl-4-aminophenoxy)-2-hydroxy-3-isopropylaminopropane; and internal standard, (±)-1-(2-propyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane were kindly supplied by Ives Labs. (New York, NY, U.S.A.) and May & Baker (Dagenham, Great Britain). All other chemicals were analytical reagent grade.

Sample preparation

For plasma samples, 1 ml of plasma is added to a test tube containing 1 μg of internal standard in 1 ml of 0.01 *M* phosphate buffer at pH 6.0 (KH_2PO_4—Na_2HPO_4). The phosphate buffer also contains appropriate amounts of acebutolol and metabolites where required for standard curve calibrators. After addition of 200 μl of 2 *M* sodium hydroxide and 10 ml of ethyl acetate,

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tubes are vortexed for 90 sec and centrifuged for 5 min at 500 *g*. The organic phase is transferred to a tapered test tube (a test tube with a capillary of 300 μl capacity fused to the bottom). A back-extraction is accomplished by addition of 150 μl of 0.005 *M* sulfuric acid, vortexing for 90 sec, immersing the tubes in a dry ice—acetone bath until the aqueous phase solidifies and then centrifuging for 5 min. The aqueous phase, by now thawed and approximately 250 μl, is transferred to disposable polyethylene limited-volume inserts (Brinkman, Westbury, NY, U.S.A.) for the injection vials of the HPLC automatic sampler.

Urine samples are processed similarly, with the following exceptions: 0.1 ml of urine and 4 μg of internal standard are used, extraction of urine is into 10 ml of diethyl ether instead of ethyl acetate, back-extraction uses 200 μl of sulfuric acid and the freezing step is omitted.

Chromatography

The apparatus used consists of a 6000A pump, with eluent flow-rate at 1.0 ml/min, a 710B automatic sample injector (both Waters Assoc., Milford, MA, U.S.A.), a VUV-10 ultraviolet detector set at 243 nm (Varian, Palo Alto, CA, U.S.A.) and an SP 4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). A 2-μm inline filter (Alltech, Deerfield, IL, U.S.A.) is used before the Spherisorb ODS 5-μm, 250 × 4.6 mm, column (Altex, Berkeley, CA, U.S.A.). The mobile phase consists of an aqueous solution containing 6% of a 0.1 *M* phosphate buffer at pH 4.0 (H_3PO_4—KH_2PO_4) filtered through a 0.45-μm filter (type HA, Millipore, Bedford, MA, U.S.A.) and 55% of acetonitrile. The operating pressure is approximately 90 bar. Injection volume varies from 10 to 150 μl.

Quantitation

Standard curves are run daily. For plasma samples, they include calibrators of acebutolol and both metabolites at 0, 20, 40, 60, 80, 100, 200, 500, 1000, 2000 and 3000 ng/ml. A 5000 ng/ml calibrator is also used when samples higher than 3000 ng/ml are encountered. The standard curve is divided into three ranges to avoid undue weighting by high standards. Within each range a least-squares regression fit of peak height ratio versus concentration is used to compute concentrations of samples within that range.

For plasma, the low range contains the calibrators up to 100 ng/ml and is used to quantitate samples containing 20—100 ng/ml; the interim range includes the calibrators from 80—1000 ng/ml and is used for samples from 100—1000 ng/ml; the high range has the calibrators over 500 ng/ml and is used for samples of 1000—5000 ng/ml. The urine standard curve consists of calibrators at 0, 1, 2, 4, 10, 20, 40, 100, 150, 200 and 250 μg/ml. It also is divided into three ranges; calibrators from 0—10 μg/ml are used for quantitating urine samples from 1—10 μg/ml, calibrators from 4—100 μg/ml for quantitating samples from 10—100 μg/ml, and calibrators from 40—250 μg/ml for quantitating samples from 100—250 μg/ml. (All ranges described are inclusive.)

RESULTS AND DISCUSSION

Linearity, precision and accuracy specifications for the assay of acebutolol

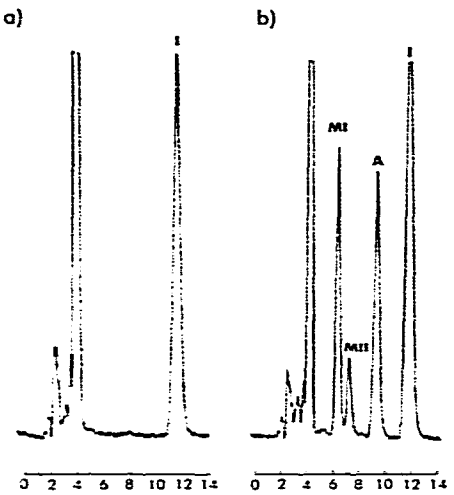

Fig. 1. Chromatograms from a subject (a) before and (b) 8 h after taking a 400-mg oral dose of acebutolol. Peaks: MI = metabolite I (6.6 min); MII = metabolite II (7.5 min); A = acebutolol (9.6 min); I = internal standard (12.0 min).

and both metabolites in plasma and in urine, are shown in Table I. Fig. 1 shows chromatograms resulting from plasma samples collected (a) before and (b) 8 h after a subject took a single oral dose of 400 mg of acebutolol. Fig. 2 shows (a) plasma concentrations and (b) urinary excretion rates in an individual following a single 400-mg oral acebutolol dose.

The modificiations in sample prepartion described for urine were found to be necessary for consistent assay, particularly amongst urines from different subjects. For reproducible urine assay also, we recommend use of one injection volume only, for all standards and samples within each concentration range. In practice, this may mean that scaling considerations necessitate some repeat injections when assaying samples of widely differing concentrations.

Meffin et al. [1] reported an HPLC assay for acebutolol and metabolite I

TABLE I

ASSAY PERFORMANCE SPECIFICATIONS

Type of sample	Assay range	Range linearity*			Concentration of samples spiked for precision and accuracy tests (μg/ml)	Precision**
		Acebutolol	Metabolite I	Metabolite II		Acebutolol
Plasma	Low	8.0±3.2 (27)	7.0±4.4 (27)	5.3±2.4 (7)	0.020	17 (16)
	Interim	3.7±2.2 (27)	4.2±2.5 (27)	3.6±1.3 (7)	0.120	5.1 (20)
	High	2.4±2.0 (23)	3.2±1.7 (23)	4.5±3.5 (3)	1.200	5.4 (19)
Urine	Low	6.0±2.2 (6)	5.2±3.6 (6)	6.0±1.4 (3)	5	3.0 (9)
	Interim	5.2±2.6 (6)	3.3±1.6 (6)	4.6±1.1 (3)	50	7.8 (9)
	High	3.5±2.5 (6)	2.4±1.1 (6)	1.4±0.5 (3)		

*Coefficient of variation of concentration—normalized peak height ratios (%), mean ± S.D. of n (in parentheses) standard curves.

**Coefficient of variation of n (in parentheses) determinations (%).

***Deviation of mean determination from concentration spiked (%); n as for precision.

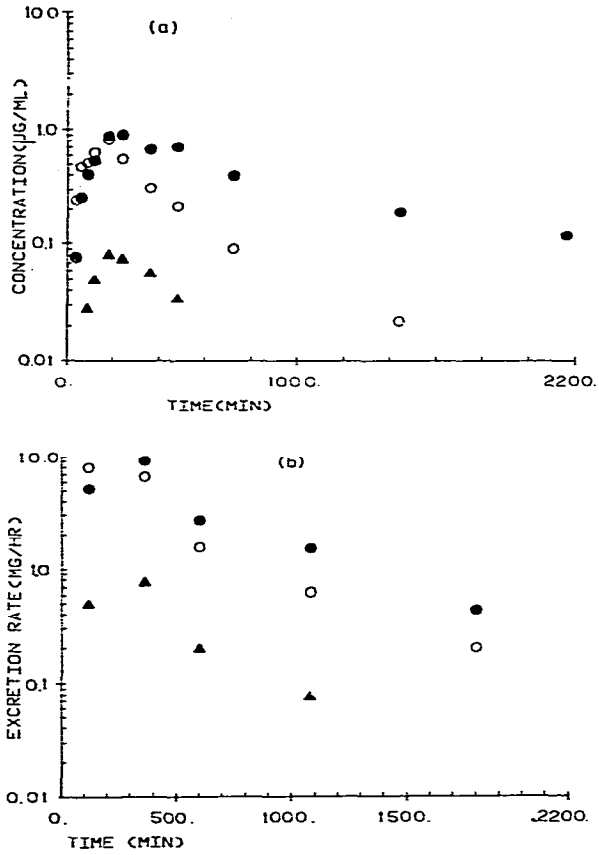

Fig. 2. (a) Plasma concentration of acebutolol and two major metabolites after a single oral dose of 400 mg and (b) urinary excretion rates for the same subject. (○) Acebutolol; (●) metabolite I; (▲) metabolite II.

		Accuracy***		
Metabolite I	Metabolite II	Acebutolol	Metabolite I	Metabolite II
15 (16)	11 (3)	−0.4	−6.5	−0.3
6.4 (20)	8.0 (7)	−1.5	−3.5	−1.0
9.6 (19)	12 (3)	+1.5	−6.5	−11
3.3 (9)	1.7 (3)	−0.6	−1.8	+2.2
9.2 (9)	6.9 (3)	−3.7	−5.1	−0.3

in blood, plasma and urine. They reported a sensitivity of 50 ng per sample but they did not give any accuracy specifications and gave precision specifications for blood only. Guentert et al. [2] reported a modified assay for these compounds in blood and plasma with a sensitivity of 50 ng/ml. With further modifications in chromatography and in sample processing we have extended the sensitivity of this assay to 20 ng/ml in plasma and have compiled specifications for assay of urine. Furthermore, we can simultaneously but separately measure a second major metabolite of acebutolol [3] in both plasma and urine. While sensitivity down to 5 ng/ml of acebutolol and metabolite I can be obtained with fluorescence rather than UV detection, as reported by Lefebvre et al. [4], simultaneous quantitation of the second metabolite, with very different fluorescence properties, is then impaired.

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