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Note

Specific and sensitive assay of celiprolol in blood, plasma and urine using high-performance liquid chromatography

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Celiprolol (Fig. 1) is a new β-adrenergic antagonist undergoing clinical trials in Europe, for use in hypertension and in angina pectoris. Little is known about its metabolism and assays to-date have been based upon radiotracer or other potentially non-specific or insensitive methods. A modification of previous high-performance liquid chromatographic (HPLC) assays for acebutolol [1—3] provides a convenient and specific assay for celiprolol in whole bood, plasma or urine, with excellent precision and accuracy specifications and with sufficient sensitivity (10 ng/ml in plasma) for pharmacokinetic studies. Quantitation of one and possibly even a second metabolite, appears possible with this method.

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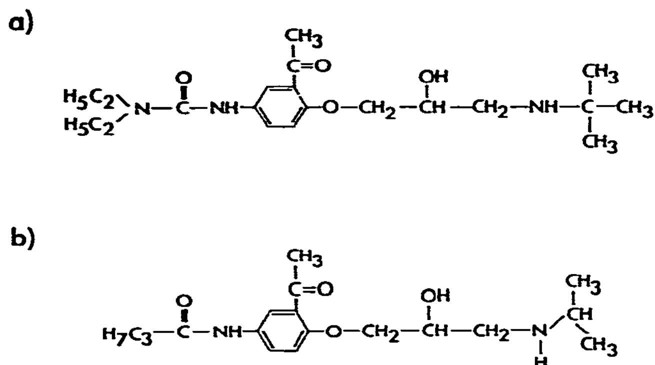

Fig. 1. Molecular structures of (a) celiprolol and (b) acebutolol, the internal standard used.

EXPERIMENTAL

Materials

Acetonitrile and ethyl acetate were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled using an all-glass still. Fig. 1 shows the internal standard, acebutolol, (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (Ives Laboratories, New York, NY, U.S.A.) and celiprolol, N-4-(3-*tert.*-butylamino-2-hydroxypropoxy)-3-acetylphenyl-N',N'-diethylurea (Chemie Linz, Linz, Austria). All other chemicals used were analytical reagent grade. Acebutolol and celiprolol solutions were made in 0.01 *M* phosphate buffer (KH_2PO_4/Na_2HPO_4) at pH 6.0. All concentrations and amounts of celiprolol in this paper refer to celiprolol · HCl equivalents.

Sample preparation

Blood samples were prepared by adding 1 ml of blood to glass test tubes in which had been placed 1 ml of 0.01 *M* phosphate buffer at pH 6.0 containing 1 μg of acebutolol and for standard curve calibrators, appropriate amounts of celiprolol. To this were added 2 ml of acetonitrile. After vortex-mixing the contents for 1 min, the tubes were centrifuged for 5 min at 500 *g*. The supernatants were decanted into test tubes and the volume reduced to approximately 1.5 ml at 40°C under a nitrogen stream. To these tubes, 200 μl of 2 *M* sodium hydroxide and 10 ml of ethyl acetate were added. The tubes were vortexed for 90 sec and centrifuged for 5 min, after which the organic phases were transferred to tapered extraction tubes (test tubes with capillaries of 300-μl capacity, fused to the bottom) and 150 μl of 0.01 *M* sulfuric acid were added. After capping and vortexing for 90 sec, the tubes were placed in a dry ice—acetone bath for 1 min to solidify the contents. They were then centrifuged for 5 min. The aqueous phases (approximately 250 μl), now thawed, were transferred to disposable polyethylene limited-volume inserts (Brinkmann, Westbury, NY, U.S.A.) for the injection vials of the HPLC automatic sampler.

Plasma samples were prepared by the same method, except that the aceto-

TABLE I

ASSAY PERFORMANCE SPECIFICATIONS

Type of sample	Assay range*	Range linearity: coefficient of variation of concentration-normalized peak height ratios (%)	Concentration of samples spiked** for precision and accuracy tests (ng/ml)	Number of samples	Precision: coefficient of variation of determinations (%)	Bias: deviation of mean from concentration spiked (%)
Blood	Low	4.8	20	5	9.1	−18
	Interim	4.5	100	5	2.1	−7.2
	High	3.1	1000	5	0.5	−1.7
Plasma	Low	13.9	10	6	28	+6.3
			20	6	8.9	−4.3
	Interim	2.1	100	6	1.5	−3.8
	High	6.6	1000	6	1.2	−2.7
Urine	Low	6.8	200	5	5.0	+23
	Interim	10.9	2000	5	3.7	−2.4
	High	11.4	20,000	5	5.4	+8.0

*Range defined in text under Quantitation.
**Not standard curve calibrators but samples run in addition.

nitrile protein-precipitation step and subsequent evaporation were omitted.

For urine samples, the plasma processing method was employed using only 200 μl of sample and 800 μl of phosphate buffer containing the appropriate standards (2 μg of acebutolol). Diethyl ether (10 ml) rather than ethyl acetate was the extracting solvent and the back-extraction was accomplished by adding 200 μl of 0.01 *M* sulfuric acid and omitting the freezing step.

Chromatography

The mobile phase was an aqueous solution containing 55% of acetonitrile and 6% of a 0.1 *M* phosphate buffer (H_3PO_4/KH_2PO_4) at pH 4.0, filtered through a 0.45-μm filter (type HA, Millipore, Bedford, MA, U.S.A.). The eluent was pumped at 1.0 ml/min at about 86 bar (Model 6000A pump, Waters Assoc., Milford, MA, U.S.A.), through a 2-μm inline filter (Alltech, Deerfield, IL, U.S.A.) before reaching a Spherisorb ODS 5 μm, 250 mm × 4.6 mm column (Altex, Berkeley, CA, U.S.A.). Samples were injected by automatic sample injector (Waters 710B). Injection volume was 150 μl for low- and intermediate-range samples and 40 μl for high-range samples. These ranges are defined below. Absorbance of the effluent was monitored at 237 nm (by either a VUV-10 or a UV-50 detector, Varian, Palo Alto, CA, U.S.A.). Peak heights were measured by a computing integrator (Spectra-Physics 4100, Santa Clara, CA, U.S.A.).

Quantitation

Standard curves for blood and plasma samples used calibrators with concentrations of 0, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml. To avoid undue weight being assigned to high-concentration points, the standard curves were divided into three ranges. For each range a least-squares linear regression was fitted to peak height ratio versus concentration. The low range contained the calibrators up to 100 ng/ml, the interim range calibrators between 100 and 1000 ng/ml and the high range calibrators between 500 and 5000 ng/ml. These three ranges were used to quantitate samples with 10—100 ng/ml, 100—1000 ng/ml and 1000—5000 ng/ml of celiprolol, respectively. The urine standard curve included calibrators at 0, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 μg/ml and was also divided into three ranges. Calibrators from 0—2 μg/ml were used for quantitating urine samples from 0.2—2 μg/ml, calibrators from 1—20 μg/ml for quantitating samples from 2—20 μg/ml and calibrators from 10—200 μg/ml for quantitating samples from 20—200 μg/ml.

RESULTS AND DISCUSSION

Linearity, precision and accuracy specifications for the assay of celiprolol in blood, plasma or urine are shown in Table I. For the urine data, five different blank urines were used in order to assess any variability due to differences in urine composition.

Chromatograms arising from plasma and blood samples are almost identical in appearance. Fig. 2 shows chromatograms from a plasma sample and a blood sample taken before a celiprolol dose and from a blood sample taken after administration of celiprolol. A chromatogram from a pre-dose blood sample

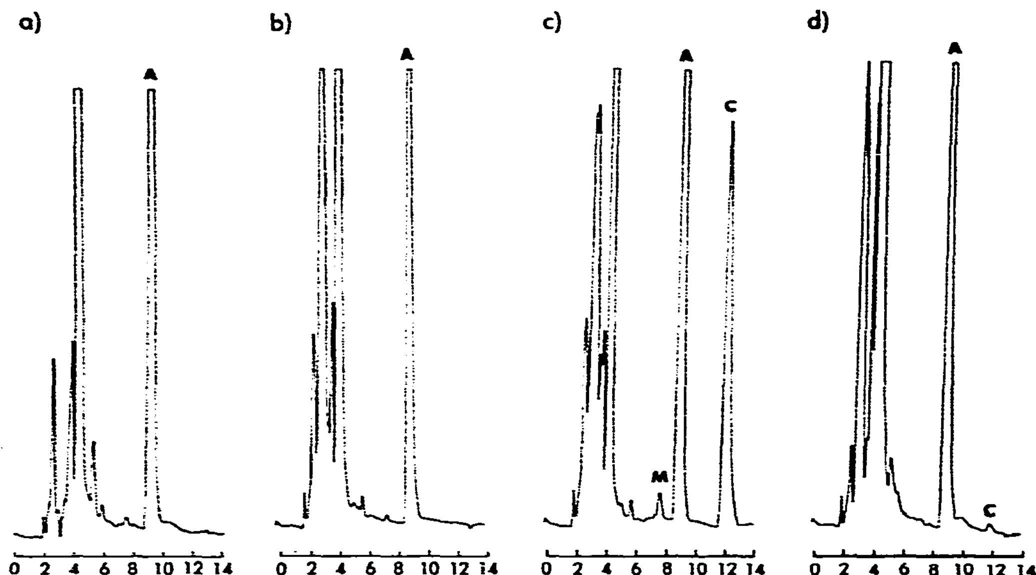

Fig. 2. Chromatograms from (a) a plasma sample and (b) a blood sample both taken before dosing, (c) a blood sample taken after administration of a 300-mg oral dose of celiprolol, (d) a pre-dose blood sample spiked with 10 ng/ml of celiprolol. Peaks: A, acebutolol, the internal standard; C, celiprolol; M, a possible metabolite of celiprolol.

spiked with 10 ng/ml of celiprolol (the lowest calibrator) is also shown. Retention times are 9.0 and 12.1 min for acebutolol and celiprolol, respectively. The peak at 7.8 min is designated M on the chromatogram from the post-dose blood sample. Because this peak is not noticeable in blank or spiked samples and because its amplitude from both plasma and blood rises and falls with celiprolol levels, this peak may represent a metabolite of celiprolol.

Fig. 3 shows chromatograms from urine samples collected before and after a dose of celiprolol and from a spiked pre-dose sample at 0.2 μg/ml which is the lowest calibrator. Peaks marked in the post-dose sample include the possible metabolite at 7.5 min (designated M), the internal standard at 8.7 min, celiprolol at 11.5 min, and a further peak at 8.1 min (designated N), not well resolved from the acebutolol peak, which may represent another metabolite of celiprolol.

Fig. 4 shows celiprolol concentrations in plasma and in blood of a healthy adult subject at various times after a single oral dose of 300 mg. Also shown are "relative" concentrations of the compound with a 7.8-min chromatographic retention time.

These "relative" concentrations were estimated using the peak height ratio for this compound and the relationship of concentration to peak height ratio for celiprolol. If the molecular weights and absorbance coefficients are indeed identical, the calculated concentrations of the potential metabolite would be an overestimate since there appears to be less peak spread associated with the earlier peak. Fig. 5 shows urinary excretion rate plots for celiprolol and the two potential metabolites, estimated similarly.

Previous studies with celiprolol have been based on radiolabelled drug or

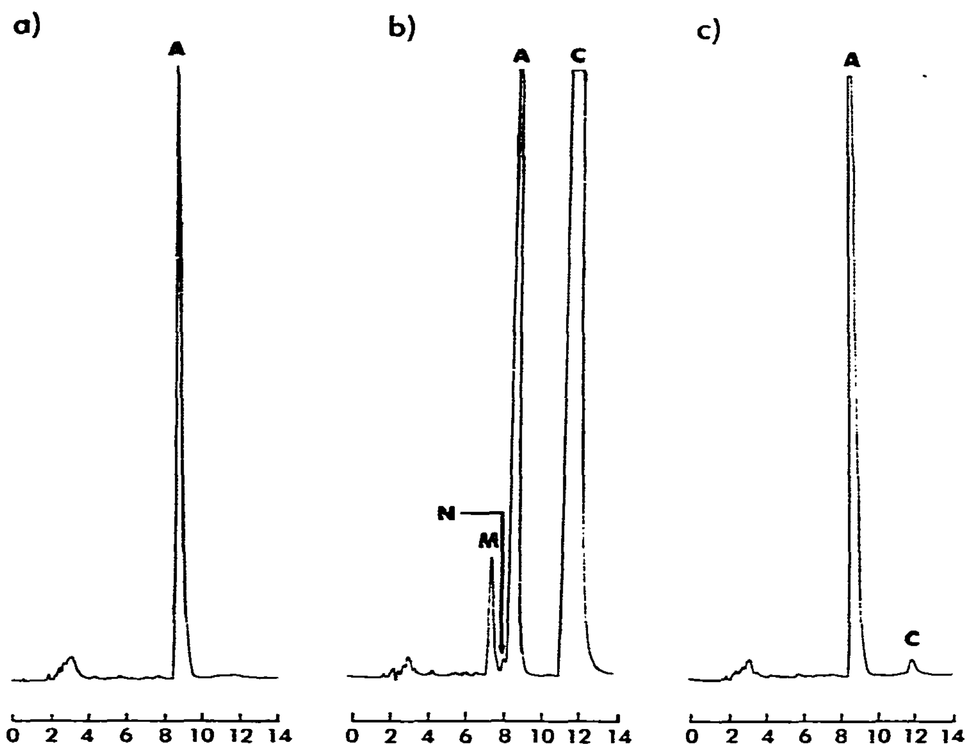

Fig. 3. Chromatograms from urine samples collected (a) prior to and (b) after administration of a 300-mg oral dose of celiprolol and (c) from a urine sample spiked with 0.2 μg/ml of celiprolol. Peaks: A, acebutolol, the internal standard; C, celiprolol; M, N, possible metabolites of celiprolol.

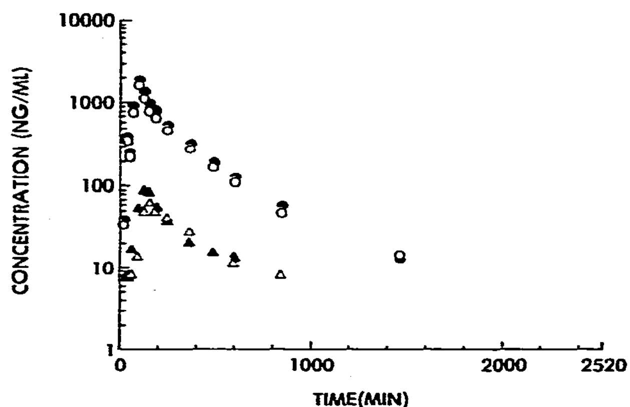

Fig. 4. Blood (●) and plasma (○) concentrations of celiprolol in a subject following an oral dose of 300 mg of celiprolol. Also shown are "concentrations" of a possible metabolite of celiprolol in blood (▲) and in plasma (△).

on nonspecific or insensitive assay procedures. Detailed specifications are given here of a specific and convenient HPLC assay for celiprolol in blood, plasma and urine. This assay has sufficient sensitivity, accuracy and precision to be of use in pharmacokinetic studies. In addition, it appears to quantitate a potential

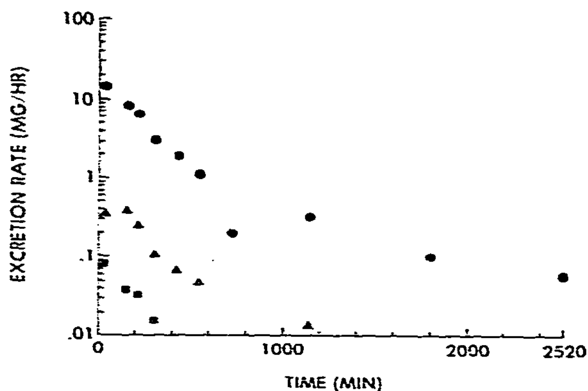

Fig. 5. Urinary excretion rate of a subject following an oral dose of 300 mg celiprolol. Shown are rates of celiprolol elimination (●) as well as "elimination" of two possible metabolites of celiprolol. These compounds correspond to the peaks designated M (▲) and N (■) in Fig. 3.

metabolite apparent in all three biological fluids and a second potential metabolite which was observed only in urine.

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NOTE ADDED IN PROOF

Since submission of this paper, we have investigated the use of fluorescence detection with this assay. Using a fluorescence detector (Model 650-10LC; Perkin Elmer, Norwalk, CT, U.S.A.) set at 335 nm excitation wavelength and 472 nm emission wavelength (20-nm slit widths) and quinidine as an internal standard, the sensitivity of the technique described above can be extended down to 5 ng/ml allowing celiprolol plasma concentrations to be traced for an extra half-life. The following linearity specifications were obtained: coefficient of variation of concentration—normalized peak height ratios in the low range (calibrators at 0, 5, 8, 10, 20, 50 ng/ml) = 5.4 ± 1.5% (n = 3 standard curves on 3 days); in the intermediate range (calibrators as before) = 4.0 ± 0.6%; in the high range (calibrators as before) = 6.2 ± 1.3%. Interday bias/precision specifications (defined in Table I) for controls spiked at 5, 10, 100 and 1000 ng/ml were −2.8/4.1% (n = 4), +9.3/7.4% (n = 3), −2.0/5.2% (n = 3) and +1.9/5.2% (n = 3), respectively. The metabolite detected in plasma by ultraviolet monitoring can be similarly detected by fluorescence monitoring.

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