High-Pressure Liquid Chromatographic Determination of 8-Hydroxycarteolol in Plasma and Urine Using Electrochemical Detection

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Abstract □ Assay of 8-hydroxycarteolol (a metabolite of carteolol) was achieved using high-pressure liquid chromatography with electrochemical detection. Plasma or urine samples alkalinized by addition of a sodium carbonate solution were extracted with ethyl acetate or chloroform. The residues from evaporation of the organic extracts were redissolved in pH 2.1 phosphate buffer, and the solutions were chromatographed on a Partisil 10 SCX chromatographic column. The detection of 8-hydroxycarteolol was accomplished using an electrochemical detector. The procedure is rapid, specific, and highly sensitive. Reproducible results can be obtained, with relative standard deviations from analysis of replicate samples within ±8%. With 1-ml samples, the lower quantifiable concentrations of 8-hydroxycarteolol in plasma and urine are approximately 5 and 25 ng/ml, respectively.

Keyphrases □ 8-Hydroxycarteolol—high-pressure liquid chromatographic analysis in plasma and urine □ High-pressure liquid chromatography—analysis, 8-hydroxycarteolol in plasma and urine □ Antiadrenergics—8-hydroxycarteolol, high-pressure liquid chromatographic analysis in plasma and urine □ Metabolites—8-hydroxycarteolol, high-pressure liquid chromatographic analysis in plasma and urine

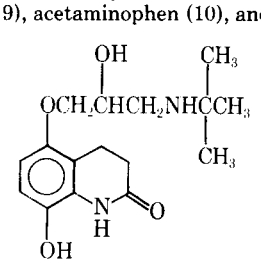
Carteolol hydrochloride [5-(3-*tert*-butylamino-2-hydroxypropoxy)-3,4-dihydrocarbostyril hydrochloride] is a potent β-adrenergic blocking agent (1). One of its major metabolites in animals and humans was identified as 8-hydroxy-5-(3-*tert*-butylamino-2-hydroxypropoxy)-3,4-dihydrocarbostyril, 8-hydroxycarteolol (I) (2). This metabolite also possesses β-adrenergic blocking activity (3).

BACKGROUND

To assess the correlation between plasma drug levels and responses of β-adrenergic blockade following administration of carteolol, the plasma concentrations of the active metabolite, I, must be considered together with the levels of the parent compound. Furthermore, in conjunction with pharmacokinetic and bioavailability studies, information on the time course and extent of formation of such an active metabolite should be highly pertinent. Therefore, the development of a sensitive method for the determination of I in biological fluids was undertaken.

Data from high-pressure liquid chromatographic (HPLC) analysis of I solutions showed that the compound could be readily eluted from both reversed-phase and strong cation-exchange columns. Detection using a UV (254 nm) photometer had a lower sensitivity limit of about 5 ng of I injected on-column. This method was inadequate for the measurement of clinically achievable plasma levels of I.

Recently, a simple design for an electrochemical detector for liquid chromatography was described (4, 5). The usefulness of this detector was demonstrated in the HPLC analysis of catecholamines (6–8), uric acid (5), ascorbic acid (5, 9), acetaminophen (10), and β-cetotetrine (11) in tissues, body fluids, and pharmaceutical preparations. In all reported cases, the electrochemical detections were characteristically highly sensitive, with lower detection limits at picomole quantities. Additionally, because only electrochemically active compounds can evoke responses from the detector, the electrochemical HPLC technique frequently offers an opportunity to mask interfering peaks appearing in HPLC–UV chromatograms.



I

Based on these published observations, it was conceived that the electrochemical HPLC technique should be of great potential in the analysis of phenolic drug metabolites in biological fluids, especially when highly efficient microparticulate columns were utilized in combination with this sensitive detector. In the present study, this approach was examined for the analysis of I. The detection of I in HPLC effluent by the electrochemical technique was considerably more sensitive than that by a UV photometer, with a minimum detectable quantity of about 0.22 ng. A simple and specific method was developed for the determination of I in plasma and urine using the electrochemical HPLC technique.

EXPERIMENTAL

Reagents—8-Hydroxycarteolol maleate[1] was at least 98% pure, based on HPLC–UV analysis on μBondapak-C_{18} and Partisil 10 SCX columns. Its electron-impact and chemical-ionization mass spectral data were consistent with the proposed 8-hydroxycarteolol structure. A stock solution of I maleate was prepared at 1 mg/ml in freshly prepared 0.01 *M* sodium bisulfite. Standard solutions of I maleate at 10 and 1 μg/ml were prepared by serial dilutions of the stock solution with the sodium bisulfite solution. All standard solutions were refrigerated until used.

All chemicals were analytical reagent grade[2]. A 1 *M* sodium bisulfite solution in distilled water was prepared fresh weekly and stored under refrigeration. This solution was used in the preparation of the eluent and the 0.01 *M* sodium bisulfite. A 3 *M* sodium carbonate solution was prepared in distilled water to which was added sodium metabisulfite to a concentration of 2% (w/v). The reconstituting buffer was prepared fresh by adding 100 μl of 1 *M* sodium bisulfite to 10 ml of an acidic phosphate buffer (0.1 *M* monobasic ammonium phosphate–0.1 *M* phosphoric acid, pH ~2.1).

Chromatographic System—The HPLC system consisted of a solvent delivery pump[3], a Partisil 10 SCX chromatographic column[4] (4.6 mm i.d. × 25 cm), and an electrochemical detector[5]. Injection was accomplished with a sample injection valve[6] modified with a homemade sample loading port to allow syringe loading of variable sample sizes at atmospheric pressure. The electrochemical potential of the working electrode was set at +0.65 ± 0.02 v *versus* a silver–silver chloride reference electrode.

The aqueous mobile phase was composed of sodium sulfate (0.2 *M*), 2-propanol (5% v/v), sodium dihydrogen phosphate (0.02 *M*), phosphoric acid (0.01 *M*), and sodium bisulfite (1 m*M*). The mobile phase was degassed under reduced pressure before use. The chromatographic system was operated at ambient temperature, with an eluent flow rate of 1.0 ml/min.

Procedures—To minimize the loss of 8-hydroxycarteolol because of oxidation, the centrifuge tubes (which had been washed with chromic acid) were rinsed with 5% (w/v) sodium bisulfite and dried before use.

Assay of Plasma Samples—A 1.0-ml aliquot of plasma, with sodium bisulfite preservative added to a concentration of about 100 mg/ml, was transferred to a screw-capped centrifuge tube. To the sample were added

[1] Otsuka Pharmaceutical Co., Osaka, Japan.
[2] Mallinckrodt, St. Louis, Mo.
[3] Model M-6000, Waters Associates, Milford, Mass.
[4] Whatman Co., Clifton, N.J.
[5] Model LC-2, Bioanalytical Systems, West Lafayette, Ind.
[6] Model 70-10, Rheodyne, Berkeley, Calif.

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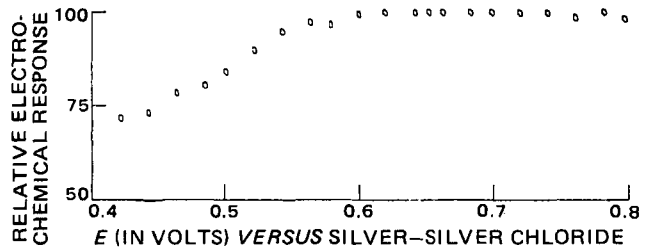

Figure 1—*Relative electrochemical response of I at various potentials.*

1 ml of 3 *M* sodium carbonate and 4.0 ml of ethyl acetate. The mixture was shaken for 5 min at low speed on a reciprocating shaker and centrifuged for 10 min at ~1000×*g*. A 3.0-ml aliquot of the organic layer was transferred to a clean conical centrifuge tube, and the solution was evaporated to dryness at 45° under a nitrogen stream. The residue was mixed with 100 μl of the reconstituting buffer; then a 20-μl aliquot of this mixture was injected into the HPLC system and analyzed.

A set of standard samples prepared from blank plasma spiked with known amounts of I was analyzed along with the unknown samples. Typically, 20-, 40-, 60-, 100-, and 200-μl aliquots of a standard solution of I maleate at 1 μg/ml were transferred to centrifuge tubes. To each tube plus a blank were added 1.0 ml of blank control plasma and 100 mg of sodium bisulfite. After the volumes were equalized by addition of distilled water, these standard samples were carried through the analytical procedure. A calibration curve was constructed from resulting data, and the concentrations of the unknown samples were derived.

Assay of Urine Samples—A procedure identical to the plasma assay was followed, except that chloroform (4 ml) was used instead of ethyl acetate. A set of standard urine samples prepared from blank urine spiked with known quantities of I was analyzed along with the unknown samples. The levels of I added to the blank urine may be varied to suit the concentration ranges of the anticipated analyses. Typically, 5-, 10-, 50-, 100-, and 200-μl aliquots of the standard I maleate solution at 10 μg/ml were transferred to centrifuge tubes. To each of the tubes plus a blank were added 1.0 ml of blank control urine and 100 mg of sodium bisulfite powder. These samples were carried through the analytical procedure, and their results were used to construct a calibration curve of peak height *versus* the corresponding urinary concentration of I.

RESULTS AND DISCUSSION

When I was subjected to electrochemical HPLC analysis, the compound evoked a large electrochemical response. To study the effect that varying the potential had on the response, an aqueous solution of I maleate was analyzed at various working electrode potentials. The peak heights observed were compared to those obtained at 0.66 v, which was arbitrarily set at 100%. The results (Fig. 1) indicated that electrochemical responses of I increased as the potential on the carbon paste electrode was raised from +0.4 to +0.6 v, with no significant changes at potentials between +0.6 and +0.8 v. Based on these observations and on the rationale that the potential should be at the lower end of the optimum potential ranges to increase the electrochemical specificity, a potential of +0.65 ± 0.02 v was chosen.

The relative sensitivities of the two modes of detection were examined by subjecting the column effluent to both UV and electrochemical de-

Table I—Data for the Determination of Linearity of the Electrochemical HPLC Assay of I in Plasma

Theoretical Plasma I Concentration, ng/ml	I Peak Height, Arbitrary Units	Recalculated Plasma I Concentration, ng/ml	Percent of Theory
15.5	24.4[a]	14.0	90.2
31.1	55.0[a]	30.9	99.5
93.3	155	86.3	92.5
155	316	176	113.3
311	585	325	104.4
933	1820	1009	108.1
1860	3330	1846	99.2
3110	5450	3020	97.1
6219	11250	6234	100.2

[a] These are mean values obtained from replicate plasma samples, five each spiked with I at 15.5 and 31.1 ng/ml.

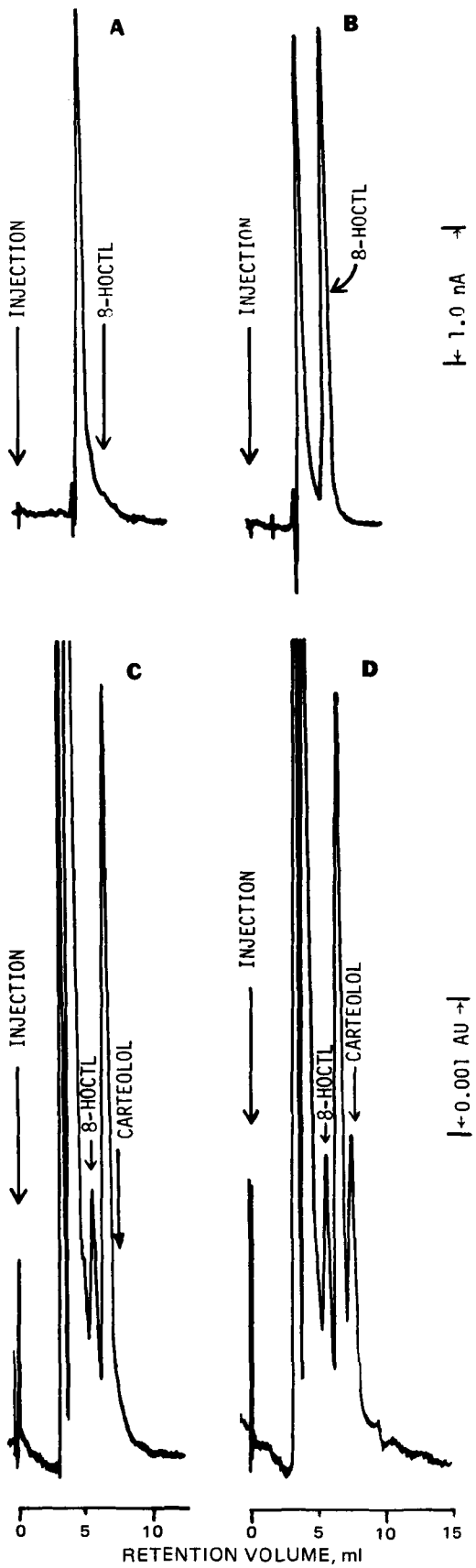

Figure 2—*HPLC chromatograms of samples prepared from blank plasma (A and C) and blank plasma spiked with I (8-HOCTL) at 155 ng/ml and carteolol hydrochloride at 492 ng/ml (B and D), analyzed according to the HPLC conditions specified under* Experimental. *Chromatograms A and B are tracings of the electrochemical detection, and chromatograms C and D are recordings of UV absorption.*

Table II—Data for the Determination of Linearity of the Electrochemical HPLC Assay of I in Urine

Spiked Urine I Concentration, ng/ml	I Peak Height, Arbitrary Units	Recalculated I Concentration in Urine, ng/ml	Percent of Theory
36.3	15	33.0	90.9
72.7	40	73.4	100.4
109	64[a]	113	103.8
182	100	170	93.4
363	211[a]	350	96.4
727	450	736	101.2

[a] These are mean values from analysis of replicate urine samples, five each spiked with I at 109 and 363 ng/ml.

tections. The column outlet was connected to the UV detector, from which the effluent was led to the inlet of the electrochemical detector, using a zero dead volume union and a short piece of 0.23-mm i.d. stainless steel tubing. HPLC analyses of both aqueous solutions of I and samples from plasma spiked with I were performed using the dual-detection system. The results indicated that the electrochemical detection was about 15-fold more sensitive than UV detection and that approximately 0.22 ng (0.7 picomole) of I on-column would be detected.

Figure 2 shows typical chromatograms of samples prepared from plasma using this dual-detection system. When UV detection was used, the I peak was lost in the background peaks as shown in chromatograms C and D. In contrast, the electrochemical detector provided more selectivity, yielding a well-resolved I peak, with cleaner backgrounds (chromatograms A and B). Thus, the use of the electrochemical HPLC technique eliminates the need for extensive sample cleanup procedures which would be required for UV detection.

As mentioned previously, I could be eluted readily under reversed-phase conditions; however, the increased concentrations of organic solvents required for the elution caused a considerable reduction in the electrochemical detector sensitivity. Furthermore, the resolution of the desired peak of I from the background peaks was inferior to that attainable using the proposed procedure with a strong cation-exchange column.

Because of the susceptibility of I to oxidative degradation, addition of preservative to plasma and urine samples was required to obtain reproducible results and good linear standard curves. Among the antioxidants tested, sodium bisulfite at 100 mg/ml of plasma or urine was most satisfactory. Based on these observations, some precautions are recommended in handling plasma or urine specimens when assaying for their I contents. Sodium bisulfite should be added at 100 mg/ml, and the samples should be stored immediately at about −10°.

To assess the precision and range of linearity of the proposed assay, 17 plasma samples spiked with known amounts of I were prepared from blank plasma and carried through the proposed analytical procedure (Table I). The method was reproducible with relative standard deviations of ±7.8 and ±3.6% from analysis of replicate plasma samples at levels of 15.5 and 31.1 ng/ml, respectively. Linear regression was performed on the data of Table I. The equation y (peak height) = $1.805x$ (concentration) − 0.821 was obtained, with the y-intercept of −0.821 being not significantly different from zero. The correlation coefficient was 0.9995, clearly demonstrating the linearity of the data. The observed peak heights were then inserted back into the equation to calculate the determined concentrations. The recalculated concentrations were all within 90–113% of theoretical values.

For the assay of I in urine, chloroform was used for the extraction of I instead of ethyl acetate. This modification was necessary since ethyl acetate extracts of urine samples gave broad background peaks that interfered with the analyses. After several unsuccessful attempts to clean up the sample by back-extraction steps, chloroform was chosen as the extraction solvent. Typical chromatograms are shown in Fig. 3. Assay of replicate urine samples to which I had been added to levels of 109 and 363 ng/ml were performed using the proposed procedure. The results showed relative standard deviations of ±2.7 and ±3.2%, respectively. Data for the determination of the linearity of the assay are listed in Table II. From these results, a linear equation was derived by least-squares analysis: y (peak height) = $0.619x$ (concentration) − 5.432, with the y-intercept of −5.432 being not significantly different from zero. The correlation coefficient was 0.9992, indicating linearity of the data.

The method was used for the analysis of plasma and urine samples collected from dogs or humans that had been orally dosed with carteolol.

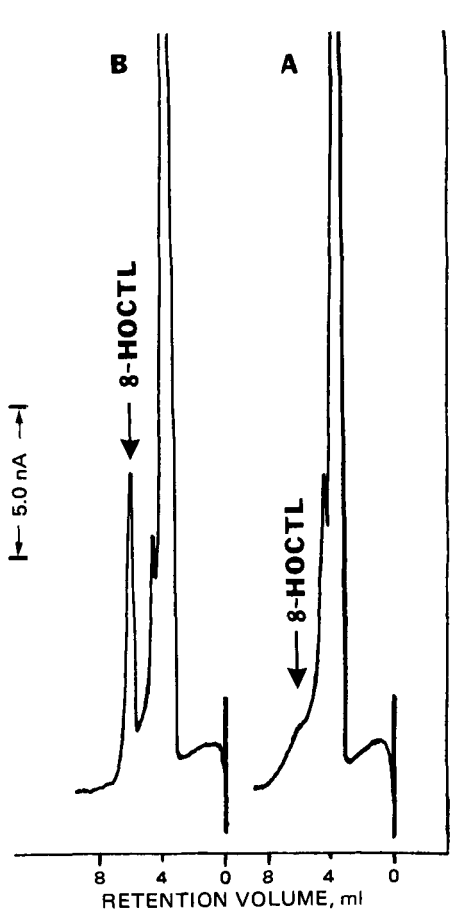

Figure 3—*Electrochemical HPLC chromatograms of samples prepared from blank urine (A) and blank urine spiked with I (8-HOCTL) at 182 ng/ml (B).*

Chromatograms were identical to those shown in this report, and no interfering peaks were observed. Minor metabolites such as 5-hydroxy-3,4-dihydrocarbostyril and 5,8-dihydroxy-3,4-dihydrocarbostyril have been found in dog urine (2); however, these compounds do not interfere with the assay of I because they are well resolved from I.

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