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DETERMINATION OF β-CETOTETRINE IN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A sensitive and specific method for the determination of β-cetotetrine in biological fluids has been developed using reversed-phase high-performance liquid chromatography and thin-layer electrochemical detection. The limit of detection for β-cetotetrine was less than 0.25 pmoles, or approx. 100 pg injected on-column. β-Cetotetrine could be analyzed in plasma at concentrations of 25 ng/ml. Linear responses were observed up to 2.5 μg/ml, and the relative standard deviation of the procedure was ±5%. The method was also employed for the determination of similar levels of β-cetotetrine in urine.

INTRODUCTION

β-Cetotetrine hydrochloride* (Fig. 1) is a broad-spectrum antibiotic with a novel tetracycline-like structure. It was first introduced in 1962[1,2] and later characterized as 2-acetyl-4a-amino-4ab,12a-dihydro-3,10,11,12ab-tetrahydroxy-6,9-dimethyl-1,12 (4H,5H)-naphthacenedione hydrochloride[3,4]. Plasma levels of β-cetotetrine can be measured by microbiological or fluorometric[5] procedures, but neither method provides the sensitivity and specificity required for pharmacokinetic evaluation of the drug.

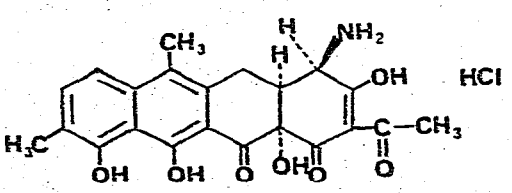

Fig. 1. Structure of β-cetotetrine hydrochloride.

To obtain specificity, a chromatographic separation of β-cetotetrine from

* β-Cetotetrine hydrochloride (Abbott-40728) was formerly called β-chelocardin hydrochloride

plasma and urine constituents was undertaken. Although a number of reversed-phase high-pressure liquid chromatographic (HPLC) separations have been reported for tetracyclines[6–8], application of these to the analysis of such drugs in biological fluids appears to have received little attention. Initial experiments with HPLC using UV (254 nm) detection were encouraging; however, when plasma and urine extracts were applied to the HPLC column, high backgrounds limited the sensitivity of the procedure. Additional clean-up steps further reduced recovery, sensitivity, and reproducibility.

To overcome these problems, a new electrochemical detector was tested. The combination of HPLC and thin-layer electrochemistry (LCEC) has recently been introduced as a highly sensitive and selective technique for the analysis of electroactive compounds[9,10]. This approach has been applied to the analysis of several compounds of clinical interest, including catecholamines, acetaminophen, uric acid, and ascorbic acid, in amounts as small as 50 pg[11–13]. The tetracyclines have several functional groups capable of being oxidized or reduced, making them excellent candidates for electrochemical detection.

In the present study, the oxidation of β-cetotetrine at the carbon paste electrode was found to permit highly sensitive detection and to be compatible with the chromatographic conditions previously established for β-cetotetrine. A simple method for the analysis of nanogram amounts of β-cetotetrine in plasma and urine using the technique of LCEC is described in this report.

EXPERIMENTAL

Reagents and materials

Unless otherwise stated, all chemicals were analytical reagent grade and were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Absolute ethanol was obtained from U.S. Ind. Chem. (New York, N.Y., U.S.A.). Benzene was distilled over NaOH or KOH and used within 3 days. Nanograde® benzene could also be used. Distillation of benzene was needed to eliminate an interfering peak which appeared when benzene and trichloroacetic acid (TCA) were mixed and evaporated to dryness.

Eluent. A buffer of dipotassium hydrogen phosphate (0.025 *M*) and disodium EDTA (0.1%) was prepared and adjusted to pH 7.8. Ethanol was added to the buffer to give a 7% (v/v) solution. The solution was degassed under vacuum for 10 min.

Alcoholic TCA. Trichloroacetic acid (1.75 g) was dissolved in 100 ml of isopropanol.

Citric acid–EDTA. A buffer of citric acid (0.025 *M*) and disodium EDTA (0.02 *M*) was prepared and adjusted to pH 4.0 with 10 *N* NaOH.

Chromatographic system

A Waters Assoc. (Milford, Mass., U.S.A.) Model M6000 pump or a Milton Roy Constametric® pump (Laboratory Data Control) was used in these experiments. The mobile phase was pumped through a septum injector fitted with silicone septa.

An empty stainless-steel column (2 ft. × 2 mm I.D. × 1/8 in. O.D.; Waters Assoc.) was rinsed with chloroform, methanol, 0.05 *M* disodium EDTA, water, and methanol before use. The column was dry packed with Bondapak Phenyl Corasil

reversed-phase packing, 37–50 μm (Waters Assoc.), and pretreated with successive rinses of 0.05 *M* disodium EDTA (about 50 ml) and water prior to mobile phase. EDTA treatments reduced tailing of the cetotetrine peak. The column was regenerated by eluting with methanol (about 15 ml) as needed to maintain resolution; when plasma assays were run, the column was cleaned daily.

The column was operated at ambient temperature at a constant flow-rate of 0.5–0.6 ml/min (*ca.* 600 p.s.i.). The recorder (Varian, Model A-25) was set at a span of 1 V and a chart speed of 12 min/in.

Connections between the metal column and the Lucite carbon paste cell consisted of a short length of 0.009 in. I.D. × 1/16 in. O.D. stainless-steel tubing coupled to a short length of 0.3 mm I.D. × 1/16 in. O.D. PTFE tubing via an Altex adapter (Cat. No. 200-41). No other precautions were needed to insulate the metal column from the electrochemical detector, although the pump and the controller box were grounded to different electrical circuits.

Electrochemical detector

The detector electronics and the LCEC cell were constructed as described in the literature[9,10]. The electrochemical controller (Model LC 2) and thin-layer carbon paste cell are now available from Bioanalytical Systems (West Lafayette, Ind., U.S.A.). The reference electrode and auxiliary platinum electrode were housed in a separate cylindrical Lucite flow vessel, downstream from the thin-layer cell. The inlet of the reference section was connected to the thin-layer cell outlet via a 2-in. length of 0.8 mm I.D. × 1/16 in. O.D. PTFE tubing and appropriate end fittings. The reference electrode (miniature S.C.E., fibre-type; Corning, Corning, N.Y., U.S.A.) was positioned in the top half of the flow cell via an O-ring seal, so that the tip of the electrode extended into the effluent about 1/2 in. above the auxiliary electrode and 1 in. below the outlet port. Carbon paste for the working electrode was obtained from Princeton Applied Research (Princeton, N.J., U.S.A.).

The sensitivity of the detector decreased after 100 or more plasma assays were run and returned to normal when the carbon paste was replaced. Analysis of urine extracts did not affect the carbon paste and the sensitivity of the detector remained stable for several weeks.

Procedures

Preparation of β-cetotetrine standard solutions. Stock solutions of β-cetotetrine hydrochloride (Abbott-40728; Lot No. 2022-270-1-1A) were prepared in methanol and used within 24 h. Standards for spiking plasma or urine were also made up in methanol and diluted appropriately.

Assay of urine samples. An aliquot of urine (2.0 ml) was buffered by the addition of 1.0 ml of citric acid–EDTA solution (pH 4.0) and extracted with 7.0 ml of chloroform. The mixture was centrifuged briefly at 1000 *g* and 5.0 ml of the chloroform layer were transferred to a conical centrifuge tube. The sample was placed in a 40° water bath and the solvent evaporated to dryness with a stream of nitrogen. The residue was reconstituted in 100 μl of methanol and 5 μl injected on the HPLC column for analysis. A set of standards (0.5–7.5 μg of β-cetotetrine added per ml of urine) was prepared and analyzed with the unknowns.

Assay of plasma samples. To a 1.0-ml portion of plasma or serum was added

0.1 ml disodium EDTA (0.1 *M*), 0.1 ml methanol or methanol standard of β-cetotetrine, and 2.0 ml of alcoholic TCA. The sample was stirred briefly on a vortex mixer and then 4.0 ml of distilled benzene was added. The sample was extracted 5 min on a shaker and then centrifuged at 1000 *g* for 2 min. A 5.0-ml portion of the upper layer was transferred to a conical centrifuge tube, placed in a 45° water bath, and evaporated to dryness with nitrogen. The sample residue was reconstituted in 100 μl of methanol and a 10-μl portion was introduced onto the HPLC column for analysis.

A set of samples prepared from control plasma spiked with β-cetotetrine hydrochloride at levels of 0.1–2.0 μg/ml was prepared and analyzed along with the unknown samples. The concentrations of the unknowns were calculated from a calibration curve constructed from peak heights of the plasma standards and their concentrations.

To extend the sensitivity of the plasma assay to *ca.* 0.025 μg/ml, sample volumes of 1.5 ml were analyzed. Corresponding changes were made in the reagent volumes as follows: EDTA, 0.2 ml; alcoholic TCA, 3.0 ml; benzene, 5.0 ml. A 7.0-ml portion of the top layer was evaporated to dryness and reconstituted in 100 μl of methanol for analysis.

RESULTS AND DISCUSSION

Detector sensitivity and performance

To determine the oxidation potential for β-cetotetrine a current–voltage curve was generated in a stepwise fashion by repeatedly injecting the compound onto the chromatographic column as the applied potential of the LCEC cell was varied. The electrochemical detector is generally operated at an applied potential within 100 mV of the lowest potential at which a limiting current is obtained. In these experiments, maximum sensitivity was achieved by operating the detector at an applied potential of +0.58 V *vs.* S.C.E.

To test the sensitivity and linearity of the LCEC detection system, a series of methanolic β-cetotetrine solutions were analyzed. The lower detection limit was estimated to be 0.05–0.10 ng of injected β-cetotetrine or less than 0.25 pmoles. Linear responses were obtained from 0.5–500 ng of β-cetotetrine on-column.

Chromatographic behavior of β-cetotetrine

Several reversed-phase chromatographic columns were studied for optimum resolution of β-cetotetrine from backgrounds. A μBondapak C_{18} column (Waters Assoc.) gave a highly efficient separation of the compound, but the level of acetonitrile in the mobile phase precluded use of the LCEC detector. Significant decreases in detector response were noted when mobile phases containing greater than 20% methanol or acetonitrile were used. Vydac reversed-phase packing (Applied Science Labs.) was also tested, but gave broader cetotetrine peaks and more tailing than Phenyl Corasil for the same solvent strength. The Phenyl Corasil column resolved β-cetotetrine from all components in both plasma and urine which eluted before β-cetotetrine. Typical elution patterns for β-cetotetrine in plasma and urine are presented in Figs. 2 and 3, respectively. The retention volume calculated for β-cetotetrine on Phenyl Corasil was about 3.6 ml. The chromatograms obtained with electrochemical detection were far cleaner than those obtained with UV (254 nm) detection.

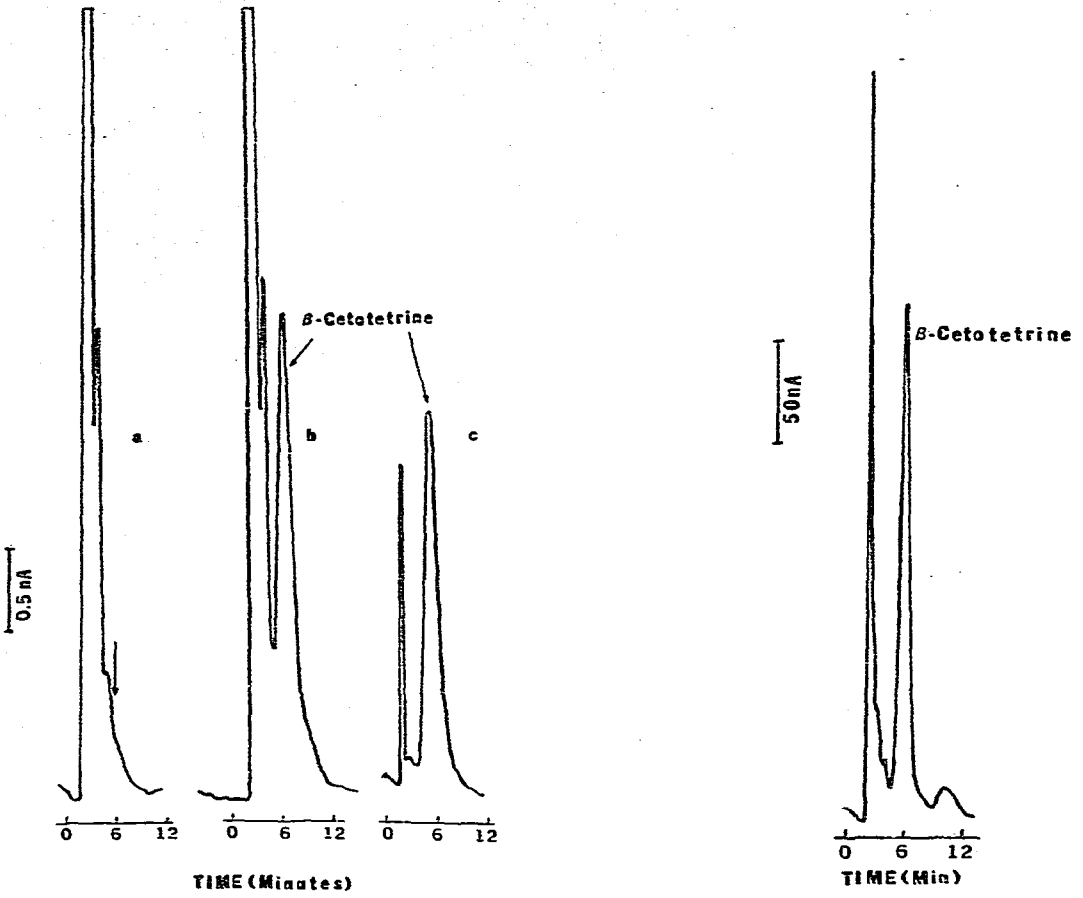

Fig. 2. High pressure liquid chromatograms of samples prepared from (a) plasma blank (b) plasma spiked with 200 ng of β-cetotetrine per ml and (c) 20 ng β-cetotetrine standard, equivalent to 100% recovery for (b).

Fig. 3. Elution pattern of sample prepared from urine spiked with 10.0 μg of β-cetotetrine per ml. The amount injected on-column is about 0.5 μg.

The separation of β-cetotetrine froms its α-isomer was also of concern, since the isomer is a degradation product and possible metabolite. The separation of these isomers on Phenyl Corasil (Fig. 4) was nearly complete. The separation factor, α, was calculated as 1.92 and the resolution, R, was 1.0. The separation factor is defined as $(V_\alpha - V_0)/(V_\beta - V_0)$, where V_0 is the void volume and V_α and V_β are the retention volumes of α- and β-cetotetrine, respectively. Resolution is defined as $2(V_\alpha - V_\beta)/(W_\alpha + W_\beta)$, where W_α and W_β are baseline peak widths.

Assay of β-cetotetrine in urine and plasma

Urine levels of 0.5 μg/ml were routinely detected using 2.0-ml samples. More sensitivity can be achieved by adjusting the size of injection, sample volume, or reconstitution volume. A typical standard curve was linear from 0.5–10.0 μg/ml β-

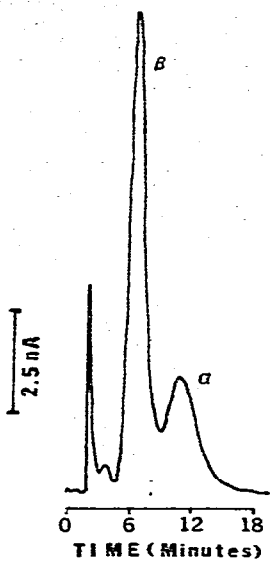

Fig. 4. Resolution of α and β isomers of cetotetrine on Phenyl Corasil reverse-phase column. Mobile phase, 7% ethanol in phosphate–EDTA buffer (pH 7.8); flow-rate, 0.6 ml/min.

cetotetrine, and the line had a correlation coefficient of 0.998. Analysis of replicate samples to which β-cetotetrine had been added was conducted to evaluate the reproducibility of the assay. The results of the analysis are presented in Table I. The relative standard deviations were less than $\pm 6\%$ at both 2.5- and 14.8-μg/ml levels of added drug.

The selectivity of both the HPLC column and the electrochemical detector and the superior sensitivity of this detector contributed greatly to the development of a suitable plasma assay for β-cetotetrine. One problem which had to be overcome was the difficulty of extracting this compound from plasma while minimizing plasma blanks and sample handling. The combination of TCA and alcohol appeared to solubilize the drug while destroying protein binding. Although β-cetotetrine could not be extracted from plasma into benzene, it was extracted with an efficiency greater than 80% into a mixed organic phase of benzene, alcohol, and TCA (see Table I). There is some evidence that tetracyclines form ion pairs with TCA which are readily extracted into alcohol-containing organic phases via a solvation effect[14,15]. Experiments conducted in this study suggest that this effect may also apply to β-cetotetrine.

Typical chromatograms of the plasma blank and plasma spiked with β-cetotetrine at 200 ng/ml are shown in Fig. 2. Although unretained components of plasma do tail into the cetotetrine peak, levels of 25 ng/ml of β-cetotetrine can be detected. The β-cetotetrine peak in Fig. 2 represents about 16 ng of cetotetrine on-column at a detector setting of 10 nA/V. More detector sensitivity is available, with a setting of 5 nA/V (full scale) being the lowest practical setting applicable to plasma extracts.

Linear standard curves with correlation coefficients of 0.999 were obtained for 0.075–2.5 μg of β-cetotetrine per ml of plasma. Plasma standards were injected after six samples to adjust for any changes in detector response. Analysis of replicate plasma samples spiked with β-cetotetrine at 0.20 and 2.00 μg/ml indicated that the

TABLE I

PRECISION AND RECOVERY DATA FOR β-CETOTETRINE ASSAY
S.D. = standard deviation; R.S.D. = relative standard deviation.

Sample	*β-Cetotetrine added (μg/ml)*		*Found (μg/ml)*	*Recovery (%)*
Urine	2.50		2.41	96.0
			2.78	111.0
			2.41	96.0
			2.55	101.7
			2.35	93.6
			2.52	100.6
			2.41	96.0
			2.37	93.6
		Mean	2.47	98.6
		S.D.	±0.14	±5.8
		R.S.D.	±5.7%	
	14.80		12.43	84.0
			12.36	83.5
			13.10	88.5
			13.54	91.5
			12.14	82.0
			11.77	79.5
			12.43	84.0
			11.91	80.5
		Mean	12.46	84.2
		S.D.	±0.59	±4.0
		R.S.D.	±4.7%	
Plasma	2.00		1.64	82.0
			1.73	86.5
			1.85	92.5
			1.67	83.5
			1.64	82.0
			1.67	83.5
		Mean	1.70	85.0
		S.D.	±0.08	±4.0
		R.S.D.	±4.7%	
	0.20		0.176	88.0
			0.166	83.0
			0.169	84.5
			0.162	81.0
			0.152	76.0
			0.172	86.0
		Mean	0.166	83.0
		S.D.	0.008	±4.2
		R.S.D.	±4.8%	

relative standard deviation of the procedure was less than ±5%. These data are presented in Table I. Precision at levels near the limit of detection was estimated to be less than ±10%.

The LCEC plasma assay for β-cetotetrine offers more sensitivity than the

microbiological serum disc assay which has a limit of detection of 0.15 μg/ml. The urine assays by both techniques are comparable with sensitivities of 0.5 μg/ml. The anhydrotetracycline fluorescence procedure[5] adapted for β-cetotetrine was less sensitive than either the microbiological or the LCEC plasma assays and could not be used for the analysis of drug in urine.

CONCLUSION

The electrochemical detector has operated reliably over a period of 12 months and performed the analysis of several thousands of clinical samples. The detector offers excellent sensitivity and selectivity, is compatible with commercially available HPLC instrumentation, and is relatively simple to operate. Both the performance and potential applications of this electrochemical detector make it an attractive alternative to the HPLC detectors currently available.

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