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APPLICATION OF AN ELECTROCHEMICAL DETECTOR TO THE DETERMINATION OF PROCARBAZINE HYDROCHLORIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An amperometric flow-through detector with a carbon paste working electrode was utilized as a high-performance liquid chromatographic (HPLC) detector to determine procarbazine hydrochloride, an antineoplastic agent, in both buffer solution and biological fluids. The HPLC system included an amino-cyano stationary phase and an aqueous (pH 7)–methanolic mobile phase which enabled the separation of procarbazine from its only electroactive degradation product, N-isopropyl-α-(2-methylhydrazono)-*p*-toluamide. The electrochemical detector, with an approximate limit of detection of 2 ng procarbazine injected, was 20 times more sensitive to procarbazine than a typical UV detector. The low dead volume (1 μl) and superior selectivity of the electrochemical detector enabled the HPLC determination of procarbazine in untreated human urine and plasma.

INTRODUCTION

Procarbazine hydrochloride [N-isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride; I] is a methylhydrazine derivative which has demonstrated an antineoplastic effect against Hodgkin's disease[1]. In the presence of moisture or in aqueous solution, procarbazine hydrochloride undergoes oxidative degradation to yield N-isopropyl-α-(2-methylazo)-*p*-toluamide (II), N-isopropyl-α-(2-methylhydrazono)-*p*-toluamide (III), and, to a lesser extent, 4-formylbenzoic acid isopropylamide (IV)[2,3].

The major metabolites of procarbazine hydrochloride are II and N-isopropyl-terephthalamic acid {V; produced by the oxidation of IV by NAD-linked dehydrogenase}[4–6] (Fig. 1). While a large portion of procarbazine is excreted in urine as V and in blood as II and V[4,7,8], a small amount (less than 5% of dose) is excreted as unchanged compound[7]. Reported methods for the quantitative determination of procarbazine hydrochloride in biological fluids have generally utilized multiple-extraction steps followed by ultraviolet spectroscopy[7], thin-layer chromatography[9], oxidation of the hydrazine group with ferricyanide[4] and thin-layer radiochromatography[6]. More recently, a rapid and accurate method for determining procarbazine
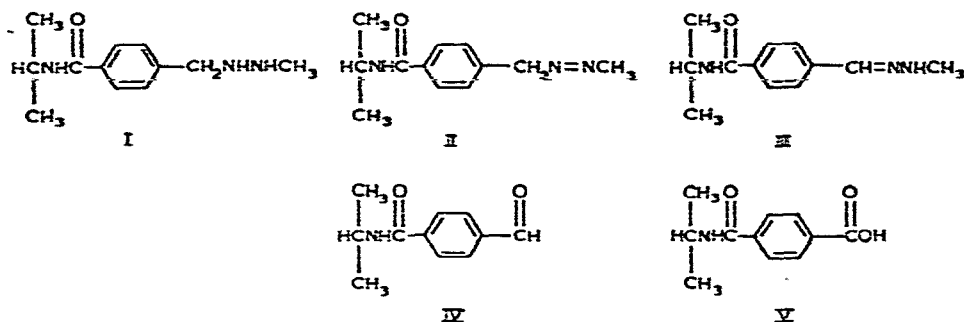
Fig. 1. Structural formulae for procarbazine hydrochloride and its degradation products.

and its degradation products in procarbazine hydrochloride capsules using high-performance liquid chromatography (HPLC) with UV detection has been reported[10].

In the present study, a commercially available electrochemical detector for HPLC was utilized as a selective as well as sensitive detector for procarbazine in both buffer solution and biological fluids. The use of this detector design has been reported for the determination of both natural and synthetic materials in biological fluids, tissues, plant matter, pharmaceutical preparations, pesticides and food-stuffs[11-17]. The method presented in this report enables the rapid and accurate determination of procarbazine at the nanogram level after HPLC separation from its only electro-oxidizable degradation product, III.

EXPERIMENTAL

Reagents

Procarbazine hydrochloride and compounds II–IV were reference standard quality (Hoffmann-La Roche, Nutley, N.J., U.S.A.). Ammonium phosphate mono-basic, used as the electrolyte in the mobile phase, was analytical reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.). An aqueous solution of 0.1 M ammonium phosphate monobasic was prepared by dissolving the appropriate amount of salt in distilled, deionized water. The pH of this solution was adjusted to pH 7.0 ± 0.1 with small amounts of reagent grade (29%) ammonium hydroxide (J. T. Baker, Phillips-burg, N.J., U.S.A.). The methanol used in the mobile phase was certified ACS grade (Fisher Scientific, Fair Lawn, N.J., U.S.A.).

Chromatographic system

The HPLC system consisted of a Milton Roy MiniPump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.), a Model 7120 sample injector valve with 10-μl loop (Rheodyne, Berkeley, Calif., U.S.A.), a Partisil PXS 10/25 PAC (250 × 4.6 mm I.D.) column (Whatman, Clifton, N.J., U.S.A.) and a Model TL-3 electrochemical detector with carbon paste working electrode (Bioanalytical Systems, West Lafayette, Ind., U.S.A.). The applied potential of +0.75 V *versus* silver/silver chloride reference electrode was controlled with a Model 174 polarographic analyzer (EG & G Princeton Applied Research, Princeton, N.J., U.S.A.).

The sensitivity and selectivity of the electrochemical detector was compared to

that of a Model SF770 Spectroflow Monitor 254-nm ultraviolet detector (Schoeffel, Westwood, N.J., U.S.A.).

The mobile phase consisted of 0.1 M ammonium phosphate monobasic, pH 7–methanol (9:1) and was deaerated with nitrogen before use. Ambient temperature and an eluent flow-rate of 0.4–0.5 ml/min were employed.

Procedures

Amber glassware was used for all procarbazine hydrochloride solutions because of the compound's light sensitivity[2].

Standard solutions of procarbazine hydrochloride were prepared by accurately weighing the bulk compound, dissolving it in deaerated mobile phase and making appropriate subdilutions with deaerated mobile phase for the desired concentration. For an injection of 100 ng of procarbazine hydrochloride (equivalent to about 86 ng of procarbazine, the species actually detected), 5 mg of the bulk compound was dissolved in 25 ml of mobile phase and 5 ml of the resulting solution was subdiluted to 100 ml with mobile phase. A 10-μl volume of this final solution was injected onto the column.

Mixtures of procarbazine hydrochloride and its electroactive degradation product, III, were prepared by weighing an appropriate amount of each compound into the same vessel and dissolving the solid mixture in deaerated mobile phase.

For the detection of procarbazine in biological fluids, pools of normal human urine and normal human plasma (Hoffmann-La Roche) were filtered through a 0.45-μm filter (Millipore, Bedford, Mass., U.S.A.). Known amounts of procarbazine hydrochloride were then dissolved in the filtered fluid and an injection into the HPLC system was made. The urine and plasma were refrigerated between uses.

RESULTS AND DISCUSSION

Fig. 2 depicts a typical chromatogram of procarbazine utilizing the described HPLC system with electrochemical detection. Baseline separation was achieved between the parent compound and its only electro-oxidizable degradation product, III. By spiking samples of procarbazine hydrochloride with various amounts of III, it was shown that good resolution was retained even with very large amounts of III present (Fig. 3).

Since a large portion of the procarbazine hydrochloride dosage is rapidly converted *in vivo* to several metabolic products, detector sensitivity is critical for the determination of parent compound. Clinical reports[4,7] have indicated that typical amounts of unchanged procarbazine found in human blood and urine are in the region of a few to several hundred ng per 10 μl. Oliverio *et al.*[7] reported that the method using multiple chloroform and aqueous extractions followed by UV spectroscopy yielded accurate results for procarbazine concentrations greater than 100 ng/10 μl. Utilizing HPLC with electrochemical detection, amounts below 100 ng/10 μl can be precisely and routinely determined.

The precision of the electrochemical detector response to procarbazine was determined by making a number of sample injections at the 70–80 ng procarbazine level and measuring the resulting peak heights. A relative average deviation of $\pm1.8\%$ and a relative standard deviation of $\pm2.3\%$ were obtained (Table I). Replicate
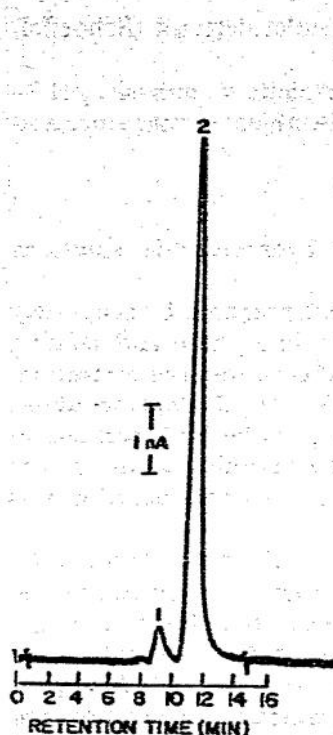
Fig. 2. Typical electrochemical HPLC chromatogram of procarbazine hydrochloride in deaerated mobile phase. Peaks: 1 = III; 2 = procarbazine. Amount injected, 80 ng procarbazine. Flow-rate, 0.5 ml/min.
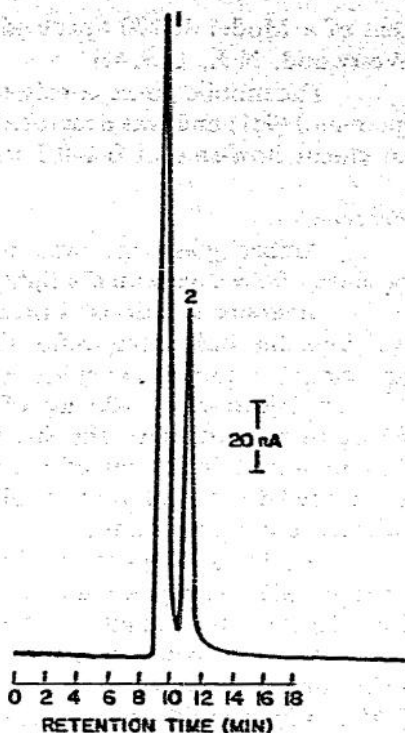
Fig. 3. Electrochemical HPLC chromatogram of 1:1 (w/w) mixture of procarbazine and III in deaerated mobile phase. Amount injected = 1 μg of each compound. Flow-rate, 0.5 ml/min.

TABLE I

PRECISION OF ELECTROCHEMICAL DETECTOR RESPONSE TO PROCARBAZINE

Current range of polarograph = 1 nA per 2.5 cm; flow-rate = 0.5 ml/min.

Amount injected (ng)	Peak height (nA)	Response (nA/ng)
76.91	7.66	0.0996
75.85	7.28	0.0960
73.12	7.44	0.1018
77.47	7.44	0.0960
78.39	7.58	0.0967
78.39	7.52	0.0959
78.75	7.64	0.0970
Average response	0.0976	
Relative average deviation	±1.8%	
Relative standard deviation	±2.3%	

injections of 10 ng procarbazine yielded results almost as precise (relative average deviation of ±2.2%). Detector response was determined to be linear with procarbazine concentration between 10 and 1000 ng injected (Fig. 4).
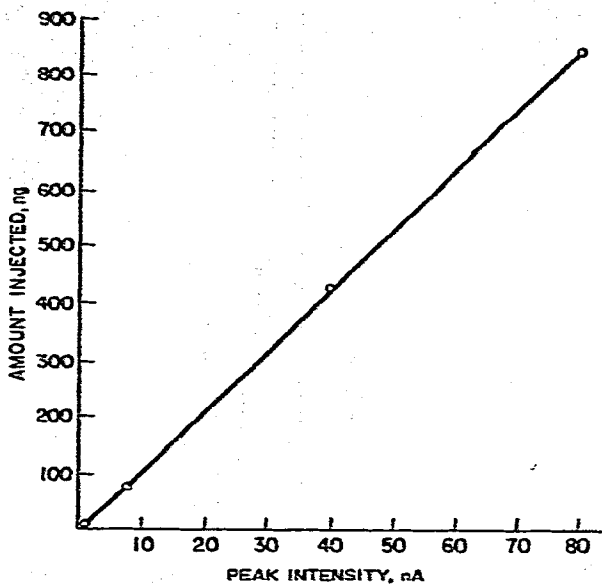

Fig. 4. Calibration curve indicating linearity of electrochemical detector response with procarbazine concentration between 10 and 1000 ng injected. Flow-rate, 0.5 ml/min.

By passing the HPLC eluents through both the UV detector set at 254 nm and the electrochemical detector, it was determined that electrochemical detection was about 20 times more sensitive to procarbazine than UV detection. Using a signal-to-noise ratio of 2 as the minimum criterion for a measurable response, the limit of electrochemical detection of procarbazine was calculated to be approximately 2 ng injected.

In addition to sensitivity, the electrochemical detector exhibited superior selectivity to that of the UV detector. While a vast number of compounds yield UV spectra, far fewer compounds are electroactive in an easily accessible potential range. The potential applied to the electrochemical detector can be adjusted to discriminate further between two or more compounds with different oxidation or reduction potentials. The low dead volume of the electrochemical detector (1 µl as opposed to approximately 10 µl for the UV detector) resulted in sharper HPLC peaks and thus better peak resolution. The combination of superior selectivity and resolution enabled the detection of procarbazine in spiked human plasma and urine with no pretreatment of these fluids other than filtering (Figs. 5 and 6). With the UV detector, the procarbazine peak was masked by components in the biological fluids. Since the composition of biological fluids varies from batch to batch, some pretreatment of the samples may be required in certain cases; however, the selectivity and resolution benefits of the electrochemical detector will remain and should minimize or simplify required pretreatment steps.
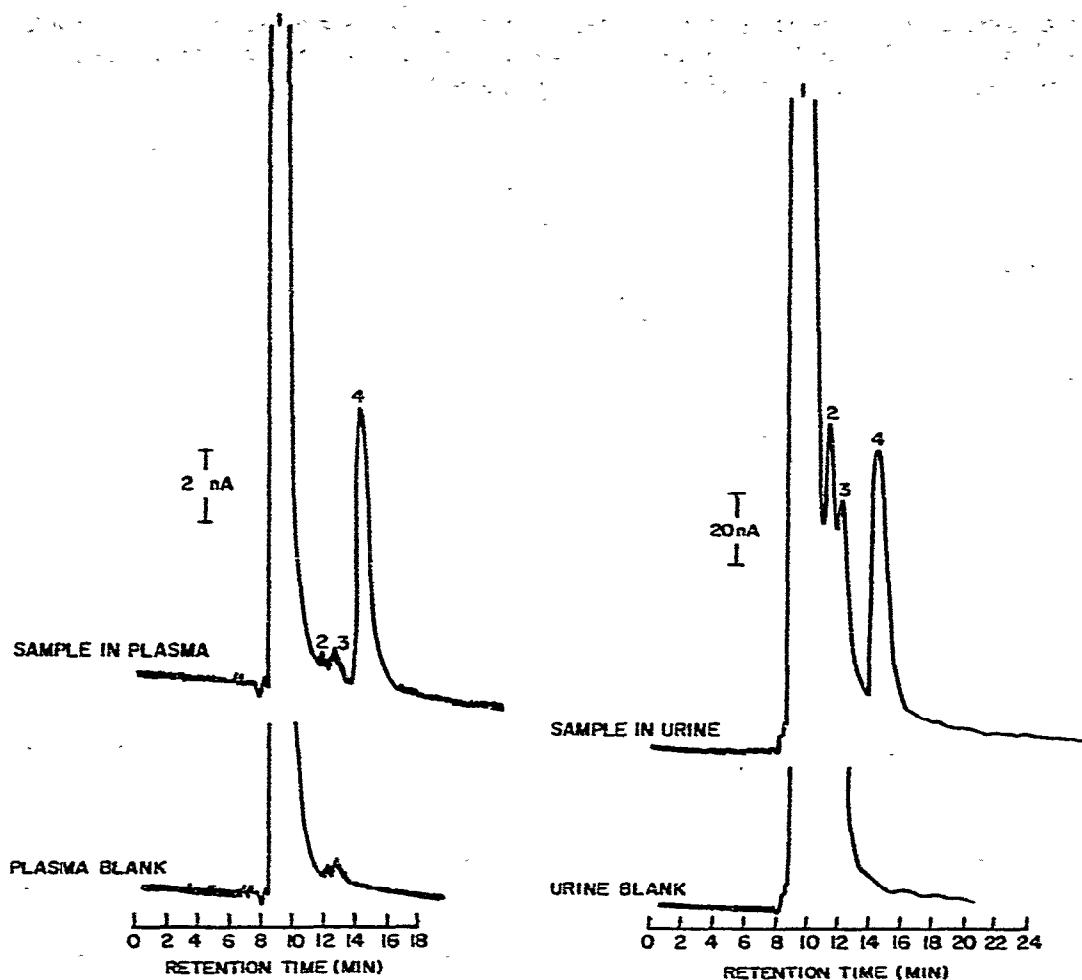
Fig. 5. Electrochemical HPLC chromatogram of procarbazine in filtered human plasma. Peaks: 1, 2, 3 = plasma components; 4 = procarbazine (100 ng injected). Flow-rate, 0.4 ml/min.

Fig. 6. Electrochemical HPLC chromatogram of procarbazine in filtered human urine. Peaks: 1, 2, 3 = urine components; 4 = procarbazine (930 ng injected). Flow-rate, 0.4 ml/min.

Two drawbacks of the carbon paste electrochemical detector should be noted. First, organic solvents, especially if greater than 20% of the mobile phase, tend to degrade the carbon paste rapidly. Even with the system in this report (10% methanol), changes in the electrode response were noted after a few days of use. Daily resurfacing of the electrode (a quite simple task requiring about 2 min) eliminated this problem. Second, the carbon paste electrode was rather slow to attain a constant background current; equilibration of this current required from several minutes for changes in flow-rate to several hours for daily start-up at high sensitivity settings.

Since the completion of this work, an electrochemical detector with a glassy carbon electrode, similar in design and electrochemical response as the carbon paste detector but relatively free of the above-mentioned problems, has been commercially

introduced (Bioanalytical Systems). Glassy carbon electrodes are more stable to non-aqueous solvents and equilibrate considerably more rapidly with solvent changes, flow-rate changes and daily start-up.

CONCLUSION

The method described permits the rapid determination of procarbazine hydro-chloride in buffer solution, plasma or urine down to 2 ng on-column. The electro-chemical detector exhibits linear response to concentration and good precision at the nanogram level and demonstrates superior sensitivity (factor of 20) to a 254-nm UV detector. The excellent resolution and selectivity features of the electrochemical detector minimize or, in some cases, eliminate the pretreatment of biological samples prior to chromatography.

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