Journal of Chromatography, 343 (1985) 99—108
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2653

DETERMINATION OF HYDRALAZINE AND METABOLITES IN URINE BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

K. RAVICHANDRAN and RICHARD P. BALDWIN*

Department of Chemistry, University of Louisville, Louisville, KY 40292 (U.S.A.)

(First received October 31st, 1984; revised manuscript received March 27th, 1985)

SUMMARY

The cyclic voltammetric behavior of hydralazine and its primary metabolites, the pyruvate and acetone hydrazones, was examined in the positive potential range at both conventional and electrochemically pretreated glassy carbon electrodes. The enhanced oxidations observed at the treated surface were used as the basis of amperometric electrochemical detection of the compounds following reversed-phase liquid chromatography. The detection limits so obtained at +0.75 V vs. Ag/AgCl (1, 3, and 5 ng injected, respectively) were comparable to those previously reported for absorption and fluorescence detection approaches employing derivatization/preconcentration procedures. For liquid chromatography with electrochemical detection, however, direct quantitation of all three species in urine samples was readily accomplished without any chemical derivatization or sample treatment operations other than particulate filtration.

INTRODUCTION

Hydralazine (1-hydrazinophthalazine, HDZ) is the active ingredient of the direct-acting vasodilator Apresoline® (Ciba-Geigy, Basle, Switzerland) and, as such, has experienced extensive clinical application in the treatment of both hypertension and chronic, resistant heart failure. Although HDZ has been in clinical use for over twenty years, the unavailability of assay procedures possessing adequate selectivity and sensitivity for determination of the drug in biological fluids has hampered investigations of its physiological disposition and pharmacokinetic behavior. Because of the potential for conversion of its principal metabolites the acetonehydrazone (HAH) and the pyruvatehydrazone (HPH) back to HDZ during the assay procedure, many earlier quantitation methods have been shown to provide only an apparent hydralazine level [1].

0378-4347/85/$03.30

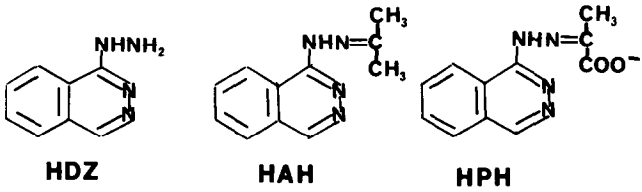

Fig. 1. Structural formulae of 1-hydrazinophthalazine (HDZ), acetonehydrazone (HAH), and pyruvatehydrazone (HPH).

(See Fig. 1 for the structures of all three compounds.) Recently, Jack et al. [2] reported a gas chromatographic–mass spectrometric procedure for detecting the parent drug at the 10 ng/ml level after initial conversion to tetraazolo(1,5-*a*)phthalazine. Competing high-performance liquid chromatographic (HPLC) assays developed for both HDZ and the metabolites have employed direct UV absorption and fluorescence detection [3], formation of fluorescent derivatives by treatment with HNO_2 [4], and UV detection at 365 nm following conversion to the *p*-anisaldehyde derivatives [5, 6].

Unfortunately, all these assay procedures require elaborate sample preparation, usually including both extractive preconcentration and derivatization prior to the quantitation step. As a consequence, the development of an analytical method that does not require extensive sample manipulation prior to detection and yet retains adequate sensitivity and selectivity to be of use in physiological sample matrices would constitute an important advance. Previous work in our laboratory has shown that oxidation of simple hydrazines at carbon electrodes can provide a useful detection method for these compounds in HPLC [7]. In particular, the use of electrochemically pretreated glassy carbon electrodes enabled direct detection of underivatized hydrazine and methyl hydrazines in physiological fluids with sensitivities two to three orders of magnitude better than that obtained via the conventional derivatization/UV absorption approaches. Recently, Shah and Stewart [8] showed that the HDZ level in pharmaceutical preparations can be detected amperometrically. However, to this point in time, the actual use of liquid chromatography (LC) with electrochemical detection (ED) for detecting HDZ in physiological matrices or for detecting the metabolites has not been reported in the literature. In the work described here, we have characterized the electrochemical activity of HDZ, HAH, and HPH, and subsequently devised and evaluated an LC–ED method for the direct detection of these species in physiological samples. The suggested assay procedure, which utilizes electrochemically pretreated glassy carbon as the sensing electrode, possesses sensitivity and selectivity for HDZ and its ketone metabolites at least comparable to the previously reported methods and yet requires no sample treatment or derivatization operations of any kind.

EXPERIMENTAL

Reagents

Hydralazine hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.) and was used as received without further purification. The acetone and

pyruvate derivatives were synthesized from the parent compound according to previously described procedures [9, 10]; their structures were confirmed by NMR, IR, and mass spectrometric measurements. Cyclic voltammetry (CV) experiments were carried out in a 0.50 *M* ammonium phosphate buffer system of either pH 7 or 4. All solutions were prepared with deionized water.

Instrumentation

CV experiments and electrode pretreatment were performed with a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model CV-1B potentiostat and employed a Model MF-2012 glassy carbon working electrode. A three-electrode cell was used with Ag/AgCl reference and platinum wire counter electrodes. The scan rate for all CV experiments was 10 mV/sec. The pretreatment procedure used for the glassy carbon electrode was similar to that reported by Engstrom [11]. First, the electrode was polished three times with a 5-μm alumina slurry for 1 min and rinsed thoroughly each time with deionized water. To eliminate the possibility of aluminium oxide catalysis as reported by Zak and Kuwana [12], the electrode was then thoroughly sonicated in deionized water. The electrochemical pretreatment itself consisted of holding the electrode first at +1.75 V vs. Ag/AgCl for 5 min and then at −1.2 V for 10 sec. Unless otherwise indicated, the electrode was immersed in pH 7 phosphate buffer during the electrochemical conditioning sequence.

The liquid chromatograph employed consisted of a Waters Assoc. Model M-45 pump, a Rheodyne Model 7125 injector equipped with a 20-μl sample loop, a Waters Model 440 UV detector (254 nm), and an IBM Model EC-230 or a Bioanalytical Systems Model LC-3 electrochemical detector with a Bioanalytical TL-5 thin-layer glassy carbon electrode assembly. All chromatography was performed on a 25 cm × 4.6 mm I.D. octadecylsilane column of 5-μm spherical particles (Alltech Assoc., Deerfield, IL, U.S.A.). A 50 mm × 4.6 mm guard column, packed with 40-μm pellicular octadecylsilane packing material (C_{18} guard column kit, Alltech Assoc.) was inserted before the analytical column when urine samples were injected. The only treatment applied to urine samples prior to injection consisted of filtration through a 3-μm glass filter in order to remove particulate matter.

RESULTS AND DISCUSSION

Voltammetry of hydralazine and metabolites

The current—potential curves obtained for HDZ upon cyclic voltammetry at positive potentials are shown in Fig. 2. On the first CV scan at pH 7 at a freshly polished glassy carbon electrode, the drug exhibited a broad irreversible oxidation wave at +0.25 V vs. Ag/AgCl (Fig. 2A, dashed line). A shift of the redox process to more positive potentials occurred as the pH of the electrolysis medium was decreased (Fig. 2B, dashed line). Upon continued scanning, subsequent voltammograms yielded currents much reduced in magnitude and shifted to higher potentials. Apparently, fouling of the electrode surface occurred relatively rapidly upon HDZ oxidation, and re-polishing of the glassy carbon was required to restore the initial electrode activity. In the case of HAH, two irreversible oxidations were seen, the first at +0.05 V vs. Ag/AgCl

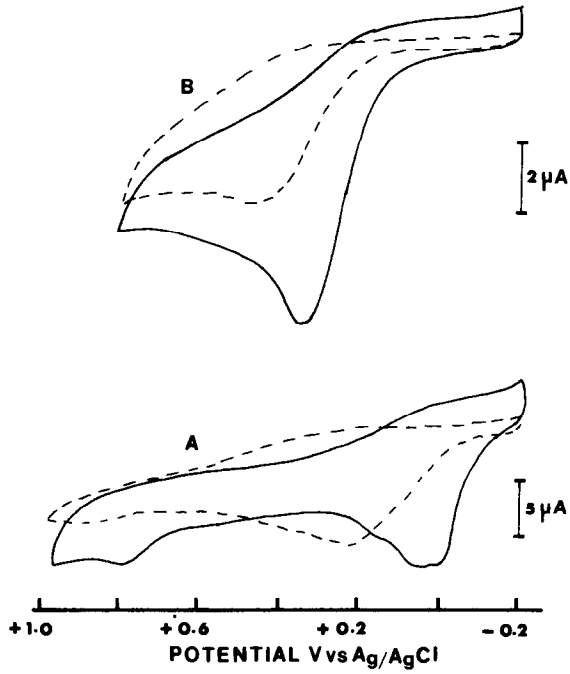

Fig. 2. Cyclic voltammograms of $1.1 \cdot 10^{-3}$ *M* hydralazine hydrochloride at untreated (- - -) and electrochemically pretreated (——) glassy carbon electrodes. (A) In pH 7 buffer; (B) in pH 4 buffer.

and the second at +0.63 V. For the pyruvate compound, a single broad wave occurred at +0.69 V. As with HDZ, the hydrazone oxidations also produced some fouling of the electrode surface. Electrochemical investigations at lower pH values were not attempted for the metabolites because of potential uncertainties resulting from the relatively rapid acid hydrolysis of both compounds back to hydralazine.

The CV data acquired conventionally as above indicated that ED at comparatively modest values of applied potential might provide a useful approach for drug quantitation. It has been previously reported, however, that simple electrochemical pretreatment procedures often improve the response of glassy carbon electrodes toward numerous analytes (including hydrazines) by shifting the compounds' oxidations to lower potentials [7, 13]. Accordingly, the capability of similarly pretreated electrodes to enhance hydralazine oxidation was also investigated. Fig. 2 also shows the CV response of HDZ at such an electrode pretreated as described under Experimental. As expected, the oxidation wave was shifted to a somewhat lower potential, i.e. +0.05 V vs. Ag/AgCl at pH 7. Upon electrode pretreatment, similar enhancements were also observed for the hydrazone compounds. For HAH, the oxidation wave at +0.63 V was shifted cathodically to +0.51 V while the oxidation at +0.05 V increased slightly in magnitude but remained at essentially the same potential as before. For HPH, two lower potential oxidations were observed at +0.52 V and +0.60 V versus Ag/AgCl, respectively.

Chromatography

The chromatographic approach selected for use in LC—ED was reversed-phase chromatography with an octadecylsilane column. Experience has shown that, using this approach, high-quality LC—ED determinations can be performed in physiological samples without extensive sample preparation provided the compounds of interest can be retained on the column for a minimum of approximately 10 min. Thus, our goal was to achieve a minimum retention time of 10 min for the most rapidly eluting hydralazine species. Initial attempts to perform the chromatography on a C_{18} column with a pH 7 buffer as mobile phase produced peaks which were unacceptably broad and which the addition of organic modifiers such as tetrahydrofuran, methanol, and acetonitrile failed to improve significantly. However, acceptable results were obtained by decreasing the pH of the mobile phase, with optimum performance given by a pH 4 mobile phase containing 0.5 *M* $(NH_4)_2HPO_4$ and 7—20% acetonitrile. A 7% acetonitrile mobile phase content at 1 ml/min produced the desired retention for HDZ. However, these conditions resulted in an unacceptably long retention for the pyruvate and acetone hydrazones (32 and 48 min, respectively) with concomitant broad peaks. Thus, in order to reduce the analysis time, a flow programming sequence was employed with acetonitrile—pH 4 buffer (12.5:87.5) mobile phase to give a successful separation of the three compounds with a total analysis time of 25 min. The initial mobile phase flow-rate was 0.5 ml/min. After 12 min (i.e. after elution of HDZ), the flow-rate was increased to 1.6 ml/min. The retention times obtained for HDZ, HPH, and HAH were 10, 18, and 22 min, respectively. Fig. 3 shows the chromatogram obtained in this manner for a test mixture; amperometric detec-

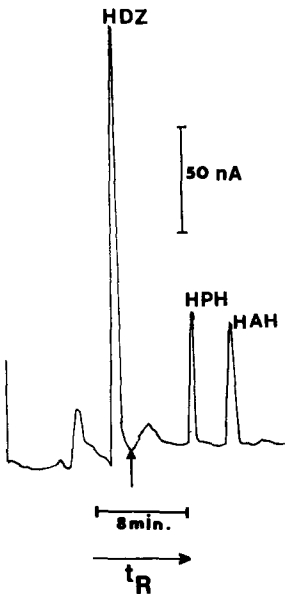

Fig. 3. Chromatogram of HDZ (4 ppm), HPH (14 ppm), and HAH (20 ppm). Mobile phase: acetonitrile—ammonium phosphate buffer, pH 4 (12.5:87.5); *E* = +0.90 V vs. Ag/AgCl. The arrow indicates the point at which the flow-rate was stepped up from 0.5 ml/min to 1.6 ml/min.

tion was carried out at an untreated electrode maintained at +0.90 V vs. Ag/AgCl.

LC—ED of hydralazine and metabolites

In order to determine the applied potential required for optimum LC—ED response, hydrodynamic voltammograms consisting of plots of applied potential versus peak current were obtained for each of the three sample species. (See Fig. 4 where hydrodynamic voltammograms are shown for detection at both untreated and electrochemically pretreated electrode surfaces.) From these measurements, it was apparent that use of a freshly polished, but untreated electrode necessitated operation of the detector at at least +0.9 V vs. Ag/AgCl in order to detect all three compounds. This is in general agreement with the voltammetric data described above. Upon subjecting the electrode to pretreatment, the hydrodynamic voltammograms for all the three compounds were shifted to lower potentials by about 150 mV. The change was most important in the case of pyruvate hydrazone whose oxidation at the untreated surface required such high potentials that no plateau region was seen within the practically accessible potential range. At a pretreated electrode, however, it became possible to detect all three compounds at +0.75 V with close to maximum response. The response for HDZ at this potential was linear in the range 1—100 ng injected; least-squares analysis of the calibration curve yielded the following characteristics: slope = 39.7 nA/ng, y intercept = 1.5 nA, correlation coefficient = 0.99. For the metabolites, response was linear from 5 to 280 ng injected for HPH (slope = 0.21 nA/ng, y intercept = 0.3 nA, correlation coefficient = 0.98) and between 5 and 400 ng for HAH (slope = 0.20 nA/ng, y intercept = 0.6 nA, correlation coefficient = 0.98). In all cases, peak current reproducibility as evidenced by the relative standard deviation for three or more replicate measurements was 2—3%. Nearly comparable LC—ED response was obtained for the three compounds when untreated electrodes were employed; however, an applied potential of +0.90 V or higher had to be

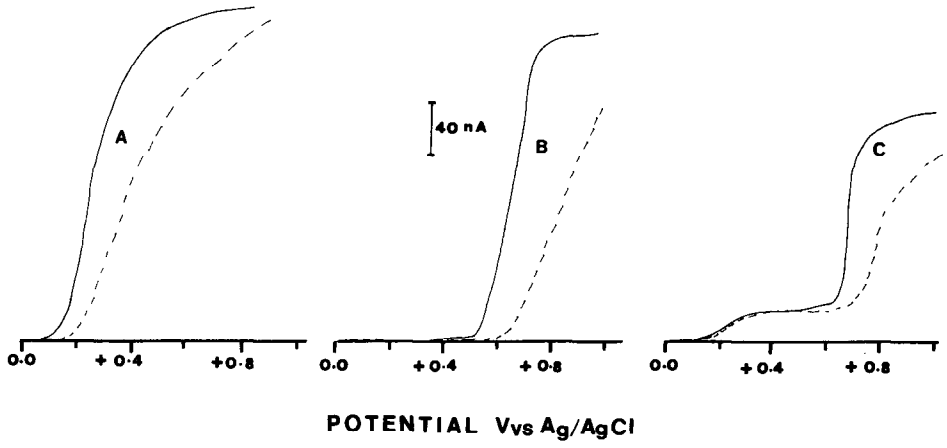

Fig. 4. Hydrodynamic voltammograms for (A) HDZ (8 ppm), (B) HPH (14 ppm), and (C) HAH (20 ppm). Mobile phase: acetonitrile—ammonium phosphate buffer, pH 4 (12.5:87.5); flow-rate: 1.0 ml/min. (- - -) Before pretreatment; (——) after pretreatment.

TABLE I

COMPARISON OF LC—ED DETECTION OF HDZ, HPH, AND HAH AT UNTREATED AND ELECTROCHEMICALLY PRETREATED ELECTRODES

Analyte	$E_{plateau}$ (V vs. Ag/AgCl)		Detection limit* (ng injected)	
	Untreated	Treated	Untreated	Treated
Hydralazine	+0.80	+0.60	1	1
Pyruvate hydrazone	>1.0	+0.80	5	3
Acetone hydrazone	+1.0	+0.80	8	5

*$E_{applied}$ for the untreated electrodes was +0.90 V vs. Ag/AgCl; $E_{applied}$ for the treated electrodes was +0.75 V. Detection limits represented the quantity of each analyte required to give a signal-to-noise ratio of 2.5.

utilized. These results are summarized for both electrode surfaces in Table I. Unlike the CV experiments where fouling of the electrode surface rapidly occurred upon oxidation of any of the three hydralazine species, no deterioration in electrode performance occurred in LC—ED. Electrodes were routinely polished and electrochemically pretreated at the start of each day's work. Even for a full day of continuous chromatography on urine samples, no decrease in response attributable to fouling of the glassy carbon surface by hydralazine oxidation products or by adsorbing sample components was observed.

The most useful aspect of electrochemical detection described here is the fact that no chemical derivatization steps were required prior to analysis. In comparison, the most sensitive determination methods reported previously required that HDZ be converted to the tetraazolo derivative and the metabolites to triazolo forms prior to fluorescence detection [4]. As pharmacokinetic studies of hydralazine metabolism necessarily involve the determination of these compounds in sample matrices such as urine or blood serum, the extension of previously reported absorption or fluorescence methods in practice also required elaborate sample treatment procedures involving multiple extraction steps, evaporative preconcentration, and a final reconstitution prior to injection [4]. It is important to note that the LC—ED assays reported here even for hydralazine-containing urine samples were direct analysis procedures involving no sample treatment other than particulate removal by filtration.

With ED, the selectivity of the analysis depends directly on the magnitude of the applied potential required to initiate the electrode process. In previous work, we have demonstrated that the lowering of the detector potential resulting from electrode pretreatment can be extremely useful in this regard. Therefore, this possibility was investigated while demonstrating the determination of HDZ and its metabolites in urine samples, Fig. 5A shows the chromatogram obtained at conventional (i.e., freshly polished but untreated) glassy carbon electrodes for blank urine from a healthy male volunteer. By comparison, UV monitoring of the same sample at 254 nm was impractical because of the continued elution of intensely absorbing compounds even at long times. Fig. 5B shows the chromatogram obtained at +0.90 V vs. Ag/AgCl for urine that had been spiked with HDZ and its metabolites. It can be seen that the compounds are resolved not only from one another but also nearly

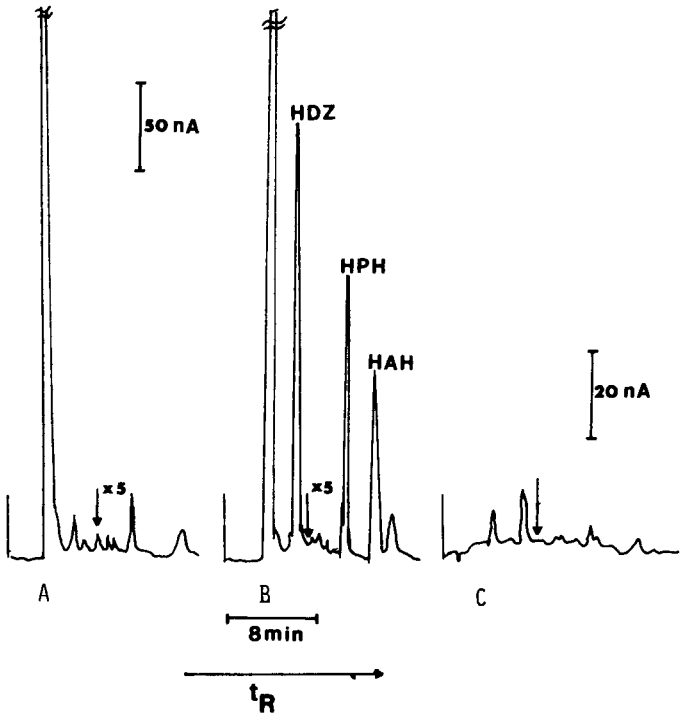

Fig. 5. Chromatograms obtained with untreated glassy carbon electrode for (A) urine blank at +0.90 V vs. Ag/AgCl, (B) urine spiked with 8 ppm HDZ, 20 ppm HPH, and 22 ppm HAH at +0.90 V, and (C) spiked urine at +0.30 V. Chromatographic conditions were the same as in Fig. 3; arrows indicate the time at which the flow-rate was stepped up from 0.5 to 1.6 ml/min; for A and B the sensitivity was also increased five-fold at this point.

completely from the electroactive urine background. Fig. 6 shows the analogous chromatograms of blank and spiked urine samples at +0.75 V at a pretreated glassy carbon electrode. As can be seen by comparison to Fig. 5B, there was no loss of sensitivity toward HDZ as a result of lowering the detector potential from +0.90 V to +0.75 V. However, the peak heights for the metabolites were greater than those obtained at an untreated electrode at the higher potential.

It is well known that in LC—ED the selectivity is determined largely by the detector potential and that lowering the detector potential can lead to improved selectivity and reduced baseline noise. In order to see whether we could improve the HDZ assay still further, the use of a much lower detector potential (+0.30 V versus Ag/AgCl) was attempted. Fig. 5C shows the chromatogram obtained with an untreated electrode for spiked urine at +0.30 V, and Fig. 6C shows the same chromatogram for a pretreated electrode. Although no readily useful information was obtained in the former case, considerable response toward HDZ was still obtained at the pretreated surface. No response was observed for HPH or HAH at the lower potential in either case. It appears that an extremely selective determination of HDZ is obtained by use of this very modest potential.

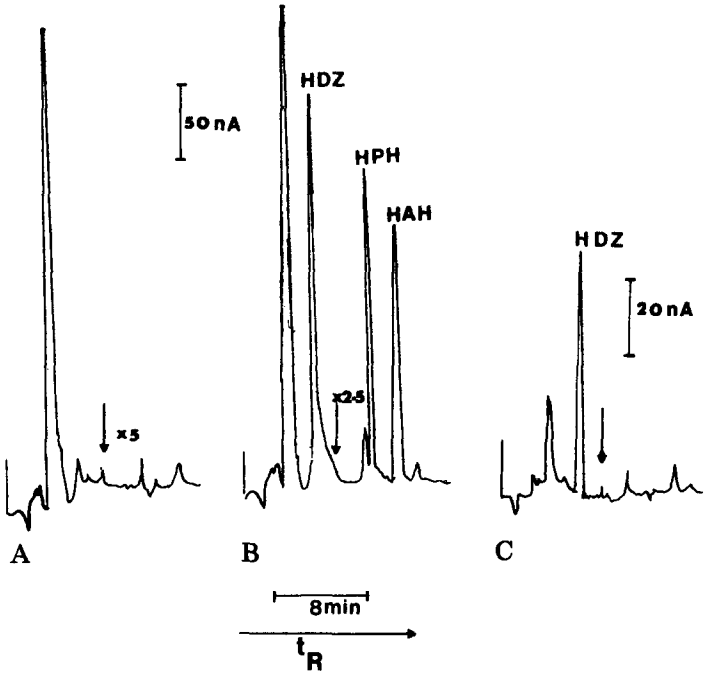

Fig. 6. Chromatograms obtained with electrochemically pretreated glassy carbon electrode for (A) urine blank at +0.75 V vs. Ag/AgCl, (B) urine spiked with 8 ppm HDZ, 20 ppm HPH, and 22 ppm HAH at +0.75 V, and (C) spiked urine at +0.30 V. Chromatographic conditions were the same as in Fig. 3; arrows indicate the time at which flow-rate and sensitivity settings were changed.

CONCLUSION

The electrochemistry of hydralazine and its hydrazone metabolites was investigated in order to evaluate the potential of LC—ED based assays for these compounds. The detection of the compounds was feasible via LC—ED with the detection limits comparable to those of earlier reported assays. The advantage of LC—ED was that no sample treatment or derivatization was required prior to the analysis. The selectivity for all three compounds and the sensitivity for HPH and HAH can be improved by operation at the lower potentials made possible by electrochemical pretreatment of the glassy carbon working electrodes.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of W.H. Pierce in recording the mass spectra of HAH and HPH. This work was supported by grants from the Commission on Academic Excellence and the College of Arts and Sciences, University of Louisville.

REFERENCES

1 T.M. Ludden, A.M. Shepherd, J.L. McNay and M.S. Lin, Clin. Pharmacol. Ther., 28 (1980) 736.
2 D.B. Jack, S. Bruchbuhler, P.H. Degen, P. Zbinden and W. Reiss, J. Chromatogr., 115 (1975) 87.
3 W.J. Proveaux, J.P. O'Donnell and J.K. Ma, J. Chromatogr., 176 (1980) 480.
4 P.A. Reece, I. Cozamanis and R. Zacest, J. Chromatogr., 181 (1980) 427.
5 T.M. Ludden, L.K. Goggin, J.L. McNay, K.D. Haegele and A.M. Shepherd, J. Pharm. Sci., 68 (1979) 1423.
6 T.M. Ludden, L.K. Ludden, K.E. Wade and S.R. Allerhailigen, J. Pharm. Sci., 72 (1983) 693.
7 K. Ravichandran and R.P. Baldwin, Anal. Chem., 55 (1983) 1782.
8 M.H. Shah and J.T. Stewart, J. Pharm. Sci., 72 (1984) 989.
9 K.D. Haegele, H.B. Skrdlant, N.W. Robie, D. Lalka and J.L. McNay, Jr., J. Chromatogr., 126 (1976) 517.
10 K.D. Haegele, H.B. Skrdlant, T. Talseth, J.L. McNay, Jr., A.M. Shepherd and W.A. Clementi, J. Chromatogr., 187 (1980) 171.
11 R.C. Engstrom, Anal. Chem., 54 (1982) 2310.
12 J. Zak and T. Kuwana, J. Amer. Chem. Soc., 104 (1982) 5514.
13 K. Ravichandran and R.P. Baldwin, J. Liquid Chromatogr., 7 (1984) 2031.