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DIFFERENTIAL PULSE AMPEROMETRIC DETECTION OF DRUGS IN PLASMA USING A DROPPING MERCURY ELECTRODE AS A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTOR

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SUMMARY

High-performance liquid chromatographic separation prior to reductive electrochemical determination at the dropping mercury electrode imparts specificity and sensitivity not attainable by conventional polarographic analysis of drugs and their metabolites. The utility of this novel approach is demonstrated by the analysis of chlordiazepoxide and its N-desmethyl metabolite in plasma which previously required thin-layer chromatographic separation prior to polarographic measurement. A mobile phase of methanol—isopropanol—0.0075 *M* acetate buffer, pH 3.5 (53:5:42), is used with the detector operated in the differential pulse mode at E_p = —0.820 V vs. Ag/AgCl. The response was linear (r = 0.998) in the concentration range of 0.05—2.0 μg/ml plasma for each component. The minimum detectability for each component under these conditions is 5.0 ng injected at a current range of 0.5 μA full scale. Techniques for oxygen removal and hydrodynamic considerations for the pumping system are presented.

INTRODUCTION

The utility of highly sensitive and specific high-performance liquid chromatographic (HPLC) assays using UV and fluorescence detection for the assay of drugs in body fluids is well documented [1—3]. Although spectrophotometric (UV) detectors are used in the majority of examples, electrochemical detectors are often required for improved sensitivity and specificity against endogenous substances and/or co-administered drugs. Electrochemical detectors operated in the oxidative mode using either glassy carbon or carbon paste electrodes have been used primarily for the analysis of phenolic and aromatic amine compounds of pharmaceutical and biological importance [4—6]. Recent publications have described the use of HPLC with oxidative amperometric (OA) detection for the analysis of perphenazine and fluphenazine [7], β-cetotetrine [8], 8-hydroxycarteolol [9], acetaminophen [10, 11], theophylline [12, 13],

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methyldopa [14], tocopherols [15], morphine [16], methyltetrahydrofolic acid [17], and mepindolol [18] in biological fluids. HPLC assays using reductive amperometric (RA) determination for drugs in biological fluids have been described for penicillamine using a mercury pool electrode [19] and for ubiquinones and phylloquinone using a glassy carbon electrode [15].

The use of a dropping mercury electrode (DME) as a RA detector for the analysis of drugs in body fluids has not yet been reported. The DME has the distinct advantage of presenting a continuously renewable fresh surface during chromatographic analysis and as such is not subject to "poisoning" as are the solid electrodes. The facile electrochemical reduction of the 4,5-azomethine group of the 1,4-benzodiazepines makes these compounds ideal for the evaluation of a DME—RA detector for the HPLC analysis of drugs in body fluids [20]. The HPLC—RA measurement of the 1,4-benzodiazepines in solution at a mercury pool [21] and at a mercury amalgamated gold disc electrode [22] has been described.

The present work describes a HPLC assay for chlordiazepoxide and its N-desmethyl metabolite in plasma which demonstrates the feasibility of using a DME as a RA detector for the assay of drugs in body fluids. Techniques for oxygen removal and hydrodynamic consideration of the pumping system are described.

EXPERIMENTAL

Column

The column used was a 30 cm × 3.9 mm I.D. stainless-steel prepacked reversed-phase column containing 10-μm μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.).

Instrumentation

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a Model 440 UV absorbance detector operated at 254 nm at an attenuation of 0.01 a.u.f.s. (Waters Assoc.). In series with the UV detector was a Model 310 liquid chromatographic detector controlled by a Model 174 polarographic analyzer [EG & G Princeton Applied Research Corp. (PARC), Princeton, NJ, U.S.A.]. The electrochemical detector was operated in the differential pulse mode at −0.820 V vs. Ag/AgCl (filling solution containing saturated AgCl and KCl) using a 100-mV pulse, large drop size (0.5 mg), 1 drop/sec rate, 1 sec low pass filter time constant and a current range of 1 μA. A stainless-steel tube 15 cm × 4.6 mm I.D. partially filled with mobile phase was placed between the UV and RA detector to act as a "pulse dampener". A Rheodyne Model 70-30 switching valve was placed after the pulse dampener to allow flushing of the HPLC column with methanol without contamination of the supporting electrolyte vessel of the EG & G-PARC Model 310. Chromatographic recording was made on a Model 626 dual-pen strip chart recorder with dual variable imputs (Leeds and Northrup, North Wales, PA, U.S.A.). The chart speed was 30 in./h using 10 mV for the UV detector and 5 V for the polarographic detector (effective current range of 0.5 μA) (see Fig. 1 for the instrumental schematic). Polarographic scans were performed using the EG & G-

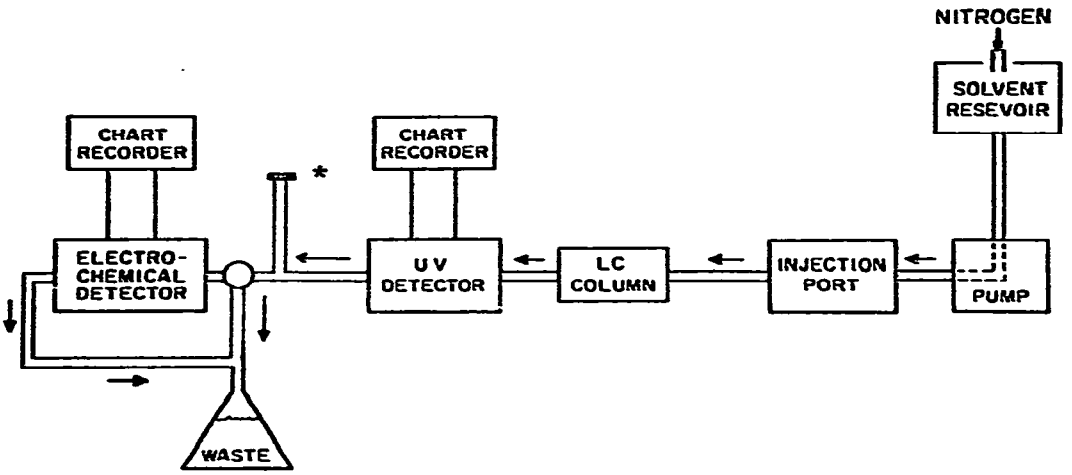

Fig. 1. Equipment used for HPLC (UV and RA) analysis. Asterisk indicates pulse dampener (see text).

PARC Model 174 with a Model 2200-3-3 Omnigraphic X-Y recorder (Houston Instruments, Bellaire, TX, U.S.A.). The X-axis was set at 100 mV/in. and the Y-axis at 1 V/in.

Chromatographic conditions

The isocratic mobile phase was a modification of the one described by Strojny et al. [23] and consisted of a mixture of methanol—isopropanol—0.0075 *M* acetate buffer pH 3.5 (53:5:42). The column head pressure was 1,000 p.s.i. (6.9 MPa) at a flow-rate of 0.9 ml/min. Under these conditions the retention time of N-desmethylchlordiazepoxide, chlordiazepoxide, and medazepam (the internal standard) were 9.8 (k' = 1.8), 12.3 (k' = 2.5), and 15.6 (k' = 3.5) min, respectively. Under the above assay conditions, 100 ng of chlordiazepoxide injected gave peaks which were 60% and 90% of full scale by UV and RA detection, respectively.

Standard solutions

Ten mg each of [I] chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{16}H_{14}N_3OCl$, mol. wt. = 299.71, m.p. = 236—236.5°C), [II] N-desmethylchlordiazepoxide (2-amino-7-chloro-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{15}H_{12}N_3OCl$, mol. wt. = 285.73, m.p. = 255—256°C), and [III] medazepam (7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine, $C_{16}H_{15}N_2Cl$, mol. wt. = 270.76, m.p. = 95—97°C, used as an internal standard), were weighed into separate amberized 10-ml volumetric flasks. All solutions were made in methanol. These stock solutions (containing 1 mg/ml) are used to prepare six 10-ml working solutions (Nos. 1—6) containing 0, 0.5, 1.0, 2.0, 5.0, or 10 μg of [I] and [II] and 1.5 μg of [III] per ml of isopropanol. Aliquots (10 μl) of these working solutions (equivalent to 0, 5, 10, 20, 50, and 100 ng of [I] and [II] and 150 ng of [III]) are injected as the external standards for establishing the HPLC parameters using both UV and RA detection.

Reagents

All reagents were of analytical reagent grade purity and all inorganic reagents were made up in distilled deionized water. Solvents were purchased from Fisher Scientific (Springfield, NJ, U.S.A.) with the exception of diethyl ether (anhydrous, reagent grade) which was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), opened fresh and placed in a 1-l amberized glass-stoppered reagent bottle to which was added approximately 1 g of granular zinc. This prevented peroxide formation which would otherwise decompose low concentrations of [I] and [II] [23].

Assay and calculations

The assay is identical to that described by Strojny et al. [23] with the exception that a single diethyl ether extraction was used and that the residue for HPLC was reconstituted in isopropanol. Along with the samples, a 1.0-ml specimen of control plasma and six 1.0-ml specimens of control plasma containing 100 μl of the working solutions Nos. 1—6 (equivalent to 10, 50, 100, 200, 500, and 1000 ng of [I] and [II] and 1500 ng of [III] per ml of plasma) are processed. These standards are used to establish a linear least squares regression equation ($Y = mX + b$) for the quantitation of the unknowns using the peak height ratio of [I]/[III] or [II]/[III] vs. concentration of [I] or [II], respectively (see Tables I and II). The peak heights of [I] and [II] in these samples are used to calculate the percent recovery of the assay.

TABLE I

UV INTRA-ASSAY STATISTICS

Drug	Amount added (μg/ml)	Amount found (μg/ml) ± S.D.	N	% R.S.D.
Chlordiazepoxide	0.050	0.045 ± 0.001	2	2.22
	0.100	0.104 ± 0.005	3	4.81
	0.200	0.194 ± 0.005	2	2.58
	0.500	0.505 ± 0.003	3	5.94
	1.00	1.00 ± 0.005	3	0.50
	$Y = 0.7104\,X - 0.004743$;		$r = 0.9994$	
N-Desmethyl-chlordiazepoxide	0.050	0.056 ± 0.001	2	1.78
	0.100	0.100 ± 0.004	3	4.00
	0.200	0.199 ± 0.000	2	0.00
	0.500	0.494 ± 0.003	3	0.61
	1.00	1.00 ± 0.004	3	1.40
	$Y = 0.7206\,X - 0.008293$;		$r = 0.9998$	

RESULTS AND DISCUSSION

Selective HPLC detectors have the distinct advantage of being able to measure drugs and metabolites in body fluids in the presence of co-administered drugs and/or endogenous substances. This fact is well documented with exam-

TABLE II

RA INTRA-ASSAY STATISTICS

Drug	Amount added (μg/ml)	Amount found (μg/ml) ± S.D.	N	% R.S.D.
Chlordiazepoxide	0.050	0.048 ± 0.002	2	4.17
	0.100	0.103 ± 0.006	3	5.83
	0.200	0.192 ± 0.006	2	3.13
	0.500	0.505 ± 0.027	3	5.35
	1.00	1.00 ± 0.034	3	3.40
	$Y = 2.198\ X + 0.006028$;		$r = 0.9988$	
N-Desmethyl-chlordiazepoxide	0.050	0.052 ± 0.002	2	3.85
	0.100	0.102 ± 0.003	3	2.91
	0.200	0.198 ± 0.000	2	0.00
	0.500	0.496 ± 0.012	3	2.42
	1.00	1.00 ± 0.027	3	2.70
	$Y = 2.101\ X - 0.002681$;		$r = 0.9994$	

ples of selective detection using fluorescence [3] and OA detection [4–6]. The OA detectors which use either glassy carbon or carbon paste electrodes can typically be utilized in the range of +1.0 to −0.4 V vs. Ag/AgCl. In the negative region (0 to −0.4 V vs. Ag/AgCl) it is also possible to perform analysis on very easily reducible nitroso- and some nitro-containing compounds. However to extend reductions to the region of −1.0 V vs. Ag/AgCl, a mercury electrode is essential due to its high overpotential. The DME is the electrode of choice in the negative potential region because of its ability to present a fresh renewable surface for analysis. Contamination ("poisoning") of the electrode surface with corresponding decrease in sensitivity as it is seen with solid carbon or mercury amalgamated electrodes is eliminated with the DME. In addition, the long equilibration times (from minutes to hours, depending upon the value of the applied potential and purity of the solvent) to minimize background currents with the solid electrodes are also eliminated. The main objections to the use of a DME as a HPLC detector have been its awkwardness and the large dead volume in the measurement cell. The EG & G-PARC Model 310 is a compact device, which delivers the HPLC column effluent directly to the mercury drop, hence has an effective dead volume of less than 1 μl at the point of measurement [24].

In order to utilize the DME as a HPLC–RA detector for high sensitivity measurements, parameters such as the suitability of the mobile phase as a supporting electrolyte, deaeration, pump noise, stability of the reference electrode, and mode of amperometric measurement were examined in detail. In order to monitor the performance of the HPLC system (injector, pump, and column) a UV detector was placed prior to the RA detector in the system (see Fig. 1).

System design

The absolute sensitivity obtainable by RA detection for HPLC is limited by the background currents which result from the reducible impurities in the mobile phase, electrolytic decomposition of the mobile phase and the presence of oxygen. Although reagent grade chemicals are usually sufficiently pure for RA detection it was essential in this study to replace the phosphate buffer reported for HPLC with UV detection for [I] and [II] [23] with 0.0075 *M* acetate buffer to obtain a stable baseline. The applied potential (—0.820 V vs. Ag/AgCl) and the pH (3.5) were selected to minimize electrolytic decomposition of the mobile phase [25]. The removal of dissolved oxygen was problematic in that purging for 5 min with helium or nitrogen as required for UV detection was insufficient for RA detection. The reduction of baseline current due to oxygen removal required exhaustive deaeration of the mobile phase in the reservoir with continued vigorous infusion of an inert atmosphere of nitrogen or helium. In addition, all standard Teflon fluoroethylene polymer (FEP) lines leading from the reservoir to the pumping system were replaced with BEV-A-LINE V-HT tubing (Thermoplastic Scientific, Warren, NJ, U.S.A.) to prevent reabsorption of oxygen through these lines. This tubing, which is impervious to oxygen, consists of a cross-linked ethyl—vinyl acetate copolymer jacket which is lined with cross-linked polyethylene. Although the lining is not as chemically inert as Teflon FEP it is more than adequate for the solvents used in reversed-phase HPLC analysis. Glass or stainless-steel lines were found to be too rigid and PVC tubing non-chemically resistant to be used as transfer lines. With these precautions for oxygen removal, the background currents were reduced to allow measurement at 0.5 μA full scale. At this sensitivity, however, it was apparent that a low frequency cyclic noise of approximately 0.5 Hz was superimposed on the RA baseline (Fig. 2A). This noise, presumably caused by distortions of the double layer surrounding the mercury drop, results from pressure fluctuations (30—40 p.s.i.) during "piston-crossover" in the dual reciprocating pump. These pulsations could not be eliminated by use of the high sensitivity noise filter on the chromatographic pump. A low pass filter with a time constant of 3 sec on the polarograph was very effective in removing the short term noise from the RA signal (Fig. 2B). A dampening device, constructed from a 15-cm length of 4.6-mm stainless-steel tubing capped at one end and filled approximately half-way with either mobile phase or water placed in front of the RA detector (Fig. 1) was very effective in removing the long term cycling from the pumping system (Fig. 2C and D). The optimum conditions for analysis were found to be a 1-sec time constant and the dampener device just prior to the RA detector (Fig. 2E).

Mode of amperometric detection

The direct current (DC), pulse and differential pulse (DP) modes of amperometric detection were evaluated using the system described above with chlordiazepoxide as a test compound. The applied potentials were selected from the DC and differential pulse polarograms on the plateau region (E = —0.950 V vs. Ag/AgCl) and at the peak potential (E_p = —0.820 V vs. Ag/AgCl), respectively, for the reduction of the 4,5-azomethine in the mobile phase [26]. The DC mode of HPLC—RA detection was found to be approximately

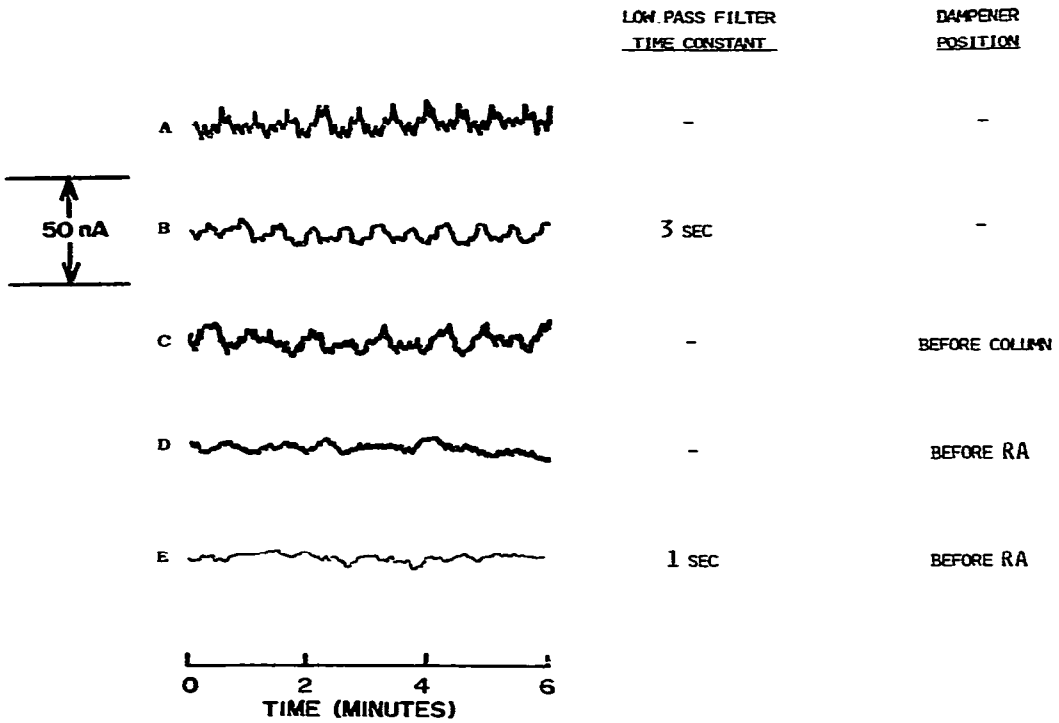

Fig. 2. Effect of filtering and dampening on baseline noise.

two orders of magnitude less sensitive than the DP mode. This result is in fair agreement with comparisons reported for the reduction of the 7-nitro functional group of nitrazepam, a 1,4-benzodiazepin-2-one, using the mercury pool HPLC—RA detector [21]. The sensitivity of 5 ng using DP—RA detection with a DME obtained in this study far exceeds the 300-ng level reported using glassy carbon, carbon paste or the mercury pool electrode for the HPLC—RA measurement of chlordiazepoxide using either the DC or DP amperometric modes [21]. A recent report utilizing HPLC—RA has demonstrated a DC amperometric sensitivity of less than 20 ng chlordiazepoxide using an amalgamated gold disc electrode [22]. The pulse amperometric mode was found to be unsuitable due to high backgrounds and large signal-to-noise ratios which is in agreement with work using the mercury pool electrode for HPLC—RA detection [21].

The use of the DP amperometric mode for high sensitivity determinations places stringent requirements upon the stability of the reference electrode. The RA detector utilized is designed to deliver the HPLC column effluent directly to the mercury drop with minimal band spread prior to mixing with the supporting electrolyte in a vessel which contains the auxiliary and reference electrodes. This vessel is drained via an overflow port and thus allows continuous flow of column effluent. The reference electrode consists of a Ag/AgCl wire in a solution of saturated KCl/AgCl contained in a glass jacketed sleeve with a Vycor frit (attached via heat shrinkable Teflon tubing). With this

experimental set-up repeated injections of chlordiazepoxide over a period of several hours yielded diminished peak heights. This lack of reproducibility can not be attributed to poisoning of the working electrode surface as noted with solid electrodes, since a clean working mercury electrode surface was exposed every second. Experimentation demonstrated that this loss in sensitivity was caused by a drift in the reference electrode potential and a consequent detuning of the selected applied DP potential. This drift appears to result from an exchange of chloride ions between the filling solution in the reference electrode and the supporting electrolyte through the Vycor frit causing a change in reference potential of 10—15 mV and a loss of 25—50% of the reduction current signal. The initial approach to this problem was to maintain a very small ionic concentration of chloride (0.01 *M*) in the mobile phase to prevent this exchange. This worked quite well, but was later abandoned (under advice of the HPLC manufacturer) due to possible corrosive effects of the chloride ion on the stainless-steel tubing throughout the instrument. A second approach which also worked well was to replace the KCl/AgCl filling solution with saturated potassium nitrate and use 0.01 *M* potassium nitrate in the mobile phase. [This change in reference electrode filling solution shifts the peak for the reduction of the 4,5-azomethine for chlordiazepoxide approximately 80 mV more negative, i.e. from —0.820 V vs. Ag/AgCl to —0.900 V vs. Ag/AgCl (saturated KNO_3).] Finally, it was found that removal of the Vycor frit from the reference electrode jacket and its replacement with the asbestos fiber tip (using waterproof epoxy) of a Ag/AgCl micro reference electrode (Microelectrode, Londonderry, NH, U.S.A.), and the use of a filling solution of saturated AgCl and KCl completely eliminated the ionic exchange and yielded a constant reference potential.

Application

Based upon the reported HPLC—UV assay for chlordiazepoxide, its N-desmethyl, demoxepam and nordiazepam metabolites [23], this study aimed to compare the sensitivity and specificity of the UV and RA HPLC detectors. However, upon examination of the HPLC—DP—RA behavior of 500 ng (20-μl injections) of each of these compounds (see Fig. 3), it was noted that the response of the molecules with the 1,4-benzodiazepin-2-one structure (demoxepam, nordiazepam, and diazepam) was considerably reduced when compared to the 1,4-benzodiazepine structure (chlordiazepoxide and N-desmethylchlordiazepoxide) throughout the entire range of potential applied, viz. —0.775 to —0.875 V vs. Ag/AgCl. This is in direct contrast to the DP polarographic activity which shows approximately equal sensitivity at the respective E_p value for the five compounds [20, 26]. This behavior appears to be a kinetic phenomenon which is peculiar to hydrodynamic voltammetry (amperometry in a flowing system) and will be a subject of future investigations. Due to the loss of sensitivity for demoxepam and N-desmethyldiazepam (approximately one order of magnitude), these compounds cannot be measured in plasma samples following therapeutic drug administration. The internal standard medazepam, a 1,4-benzodiazepine, showed equivalent sensitivity to chlordiazepoxide over this potential range. Chromatograms of control plasma and control plasma containing 100 ng/ml chlordiazepoxide and its N-desmethyl metabolite using

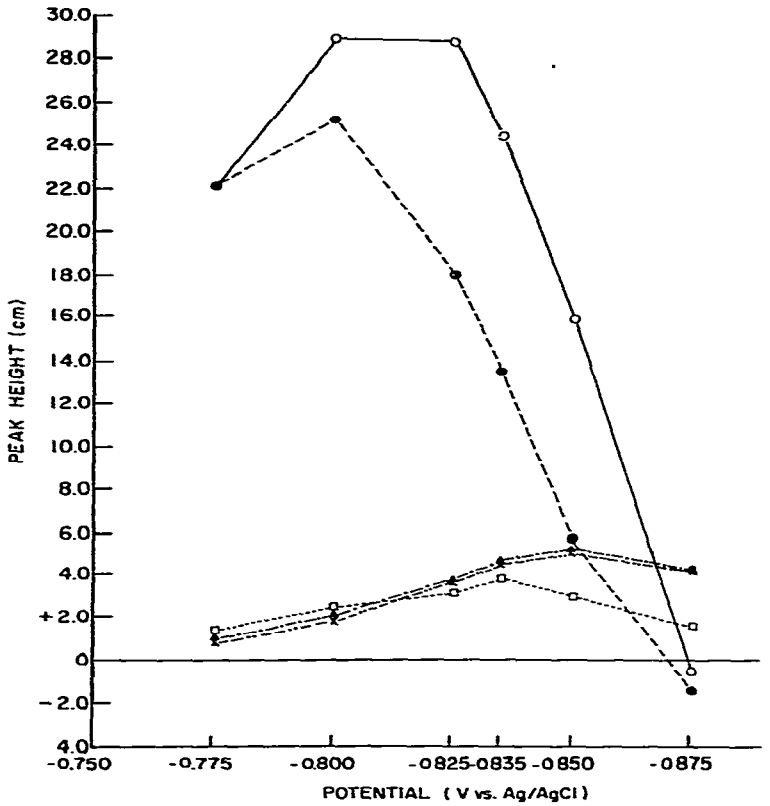

Fig. 3. Amperometric responses of 500 ng (20 μl injected) of 1,4-benzodiazepines and 1,4-benzodiazepin-2-ones in the mobile phase. ○, Chlordiazepoxide; ●, N-desmethylchlordiazepoxide; ×, demoxepam; ▲, nordiazepam; □, diazepam. Current range: 5 μA full scale.

HPLC with UV and RA detection are shown in Figs. 4 and 5, respectively. The presence of a large negative peak ($t_R \approx 7.5$ min) is due to the injection of a small amount of oxygen and is always noted at this high attenuation. Deaeration of the sample does not effectively remove this HPLC peak which has also been reported using the mercury amalgamated gold electrode [22].

The HPLC assay was statistically evaluated over the concentration range of 0.05—2.0 μg/ml for [I] and [II] using both UV and RA detection and showed excellent linearity [correlation coefficient (r) $\geqslant$ 0.99] and precision [average percent relative standard deviation (% R.S.D.) $\approx$ 2—4%], for both techniques (see Tables I and II). The assay can be performed in less than one-half the analysis time required using thin-layer chromatographic separation, elution, and polarographic quantitation of the respective compounds [26].

The clinical utility of the HPLC—RA assay was demonstrated by the analysis of [I] and [II] in a series of plasma samples (0.5—60 h) taken from a subject who received Limbitrol® (product containing 10 mg of chlordiazepoxide and 25 mg of amitriptyline) twice daily. Equivalent data were obtained for these samples using UV and RA detectors in series (r = 0.999 and 0.988 for [I] and

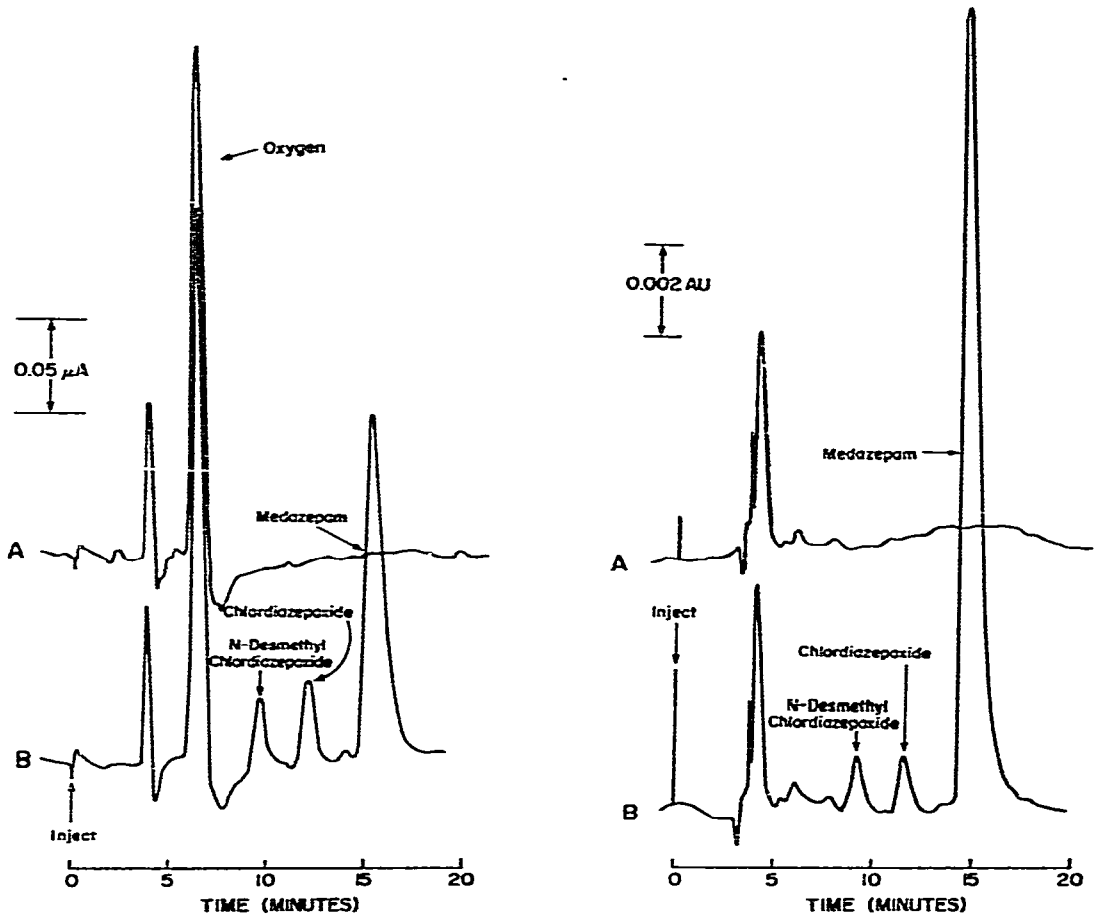

Fig. 4. HPLC chromatogram with RA detection (E_p = —0.820 V vs. Ag/AgCl). A, Control plasma; B, control plasma containing 100 ng/ml chlordiazepoxide and N-desmethylchlordiazepoxide with 1.5 μg/ml medazepam (internal standard).

Fig. 5. HPLC chromatogram with UV detection (λ = 254 nm). A, Control plasma; B, control plasma containing 100 ng/ml chlordiazepoxide and N-desmethylchlordiazepoxide with 1.5 μg/ml medazepam (internal standard).

[II], respectively), see Table III. The sensitivity limit for both detectors is 50 ng/ml for [I] and [II]. Neither the UV nor RA detector showed interference from amitriptyline or its N-desmethyl metabolite which were below the limit of detection for both detectors.

CONCLUSIONS

Reductive electrochemical detection using DP amperometry at the DME with HPLC is a useful technique for the determination of electroactive drugs and their metabolites in biological fluids.

HPLC system requirements for high sensitivity RA detection such as oxygen

TABLE III

PLASMA CONCENTRATIONS OF CHLORDIAZEPOXIDE AND N-DESMETHYLCHLORDIAZEPOXIDE IN MAN FOLLOWING ORAL ADMINISTRATION OF LIMBITROL* TWICE DAILY AS DETERMINED BY BOTH UV AND RA ASSAYS

Time (h)	Chlordiazepoxide		N-Desmethylchlordiazepoxide	
	UV	RA	UV	RA
0.5	1.30	1.30	0.046	0.056
1	1.04	1.04	0.065	0.120
1.5	1.01	1.00	0.055	0.049
2	1.10	1.11	0.074	0.086
3	1.05	1.07	0.090	0.097
4	0.92	0.94	0.14	0.12
6	0.55	0.59	0.43	0.45
8	0.63	0.63	0.17	0.17
10	0.53	0.56	0.21	0.21
12	0.53	0.49	0.23	0.23
16	0.36	0.39	0.31	0.30
24	0.28	0.31	0.36	0.39
30	0.17	0.19	0.36	0.37
36	0.091	0.11	0.30	0.33
48	0.040	0.053	0.17	0.21
60	N.M.**	N.M.	0.11	0.10
	$r = 0.9989$		$r = 0.9877$	

*See text.

**N.M. = < 0.050 μg/ml for RA and < 0.040 μg/ml for UV.

removal, a stable reference electrode and pulse dampening were optimized for the performance of this detector.

Applications of RA detection with simultaneous UV analysis showed both techniques to be of equal sensitivity and precision with excellent correlation for the analysis of plasma samples containing chlordiazepoxide and its N-desmethyl metabolite.

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