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DETERMINATION OF CHLORPROTHIXENE AND ITS SULFOXIDE METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET AND AMPEROMETRIC DETECTION

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SUMMARY

This communication describes a rapid, sensitive and selective method for the assay of chlorprothixene and its sulfoxide metabolite in human plasma, using reversed-phase high-performance liquid chromatography. Alkalinized plasma was extracted with heptane—isoamyl alcohol (99:1), after addition of thioridazine as the internal standard. The residue obtained after evaporation of this extract was chromatographed on a cyano column, using acetonitrile—0.02 *M* potassium dihydrogen phosphate pH 4.5 (60:40) as the mobile phase with ultraviolet (229 nm) detection. Quantitation was based on peak height ratios over the concentration range of 5.0—50.0 ng/ml for both compounds with 85% and 90% recovery for chlorprothixene and its sulfoxide metabolite, respectively, using a 1.0-ml plasma sample. The assay chromatographically resolves chlorprothixene and the sulfoxide metabolite from the N-desmethyl metabolite, which can only be semi-quantitated owing to low and variable recoveries.

The method was used to obtain plasma concentration versus time profiles in two subjects after oral administration of 100 mg of chlorprothixene suspension and in two additional subjects following overdosages of chlorprothixene estimated to exceed several hundred milligrams. These analyses demonstrated that the sulfoxide metabolite is the predominant plasma component following therapeutic administration and overdosages.

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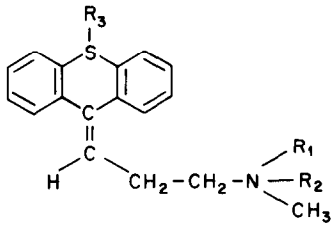
High-performance liquid chromatography with oxidative amperometric detection with the glassy carbon electrode was also evaluated. Although this procedure demonstrated comparable sensitivity and precision to ultraviolet detection for the analysis of chlorprothixene and N-desmethyl chlorprothixene, the sulfoxide metabolite could not be measured with high sensitivity (< 100 ng/ml) owing to endogenous interferences. Hence the utility of this alternative assay technique is limited.

INTRODUCTION

Chlorprothixene (I, 2-chloro-N,N-dimethylthioxanthene-$\Delta^{9,\gamma}$-propylamine) (Fig. 1) is presently marketed as the antipsychotic agent Taractan®. The biotransformation of I in man via oxidation and N-demethylation to chlorprothixene sulfoxide (II) [1—3], N-desmethyl chlorprothixene (III) [3, 4], N-desmethylchlorprothixene sulfoxide (IV) [2, 3] and chlorprothixene sulfoxide-N-oxide (V) [2] has been reported (Fig. 1).

Specificity for the determination of I in the presence of its metabolites requires either selective extraction [1, 5—8] or thin-layer chromatographic (TLC) separation [4, 8]. Selective extraction procedures have been described for the simultaneous determination of I and its major metabolite (II) in blood plasma and urine. These assays require extraction of I and II into heptane or heptane—isoamyl alcohol mixtures from the biological sample adjusted to an alkaline pH. Selectivity against II is assured by back-extraction of II from the organic phase into a pH 5.6 buffer [9]. Intact I is then extracted into dilute acid from the organic phase. Compound I is determined by spectrofluorometry in strong acid as either the intact drug (I) [1, 5, 7, 8] or after potassium permaganate oxidation to 2-chlorothioxanthen-9-one [6, 10]. The S-oxide metabolite (II) is reduced back to I and then determined by spectrofluorometry.



Compound	R_1	R_2	R_3
Chlorprothixene [I]	$-CH_3$	—	—
Chlorprothixene Sulfoxide [II]	$-CH_3$	—	→O
N-Desmethyl Chlorprothixene [III]	—H	—	—
N-Desmethyl Chlorprothixene Sulfoxide [IV]	—H	—	→O
Chlorprothixene Sulfoxide-N-Oxide [V]	$-CH_3$	→O	→O

Fig. 1. Biotransformation of chlorprothixene [1—4].

Toxicological investigations using spectrofluorometry with TLC separation to determine I—IV [4] or selective extraction to determine I and II [8] in post-mortem samples have been reported. In addition, the presence of 2-chlorothioxanthen-9-one was recently identified in the gastric aspirate following a severe chlorprothixene poisoning [11]. Only limited therapeutic drug monitoring data for I are reported [7, 12, 13] which are difficult to correlate owing to the lack of information pertaining to the actual dosage form ingested. Following a single oral dose of 200 mg of I in adults, blood concentrations of I and II ranging from non-measureable (< 10 ng/ml) to 60 ng/ml and from 30 to 140 ng/ml, respectively, were determined 1—8 h post administration [12]. Following single oral 30-mg doses of chlorprothixene peak blood concentrations of I of 12—17 ng/ml were determined 3—4 h post administration [7, 13]. The half-life of the drug following intravenous administration was reported to be in the order of 8—12 h [7].

Separation of tricyclic antidepressants and their metabolites by high-performance liquid chromatography (HPLC) allows for their specific determination in biological fluids without the time-consuming selective extraction or TLC procedures [14]. HPLC procedures have been described for the separation of the *cis*- and *trans*-isomers of I [15] and for the determination of the stability of I under a variety of conditions of photo-irradiation [16]. Amperometric detection coupled with the HPLC separation further increases the specificity of the determination as has been reported for the analysis of phenothiazines, thioxanthene and butyrophenones [17—23] in biological fluids.

The present work describes a reversed-phase HPLC assay with ultraviolet (UV) (cadmium source; 229 nm) detection for the determination of chlorprothixene and its principal plasma metabolite, chlorprothixene sulfoxide (II) using thioridazine (VI) as the internal standard. The assay chromatographically resolves I and II from the N-desmethyl metabolite (III), which can be only semi-quantitated owing to poor and variable recovery. The HPLC—UV assay was utilized to measure concentrations of I, II and III in man following single oral (100 mg) and overdosages exceeding several hundred milligrams of chlorprothixene.

HPLC with oxidative amperometric detection with the glassy carbon electrode was also evaluated and yielded comparable sensitivity and precision for the analysis of I and the semi-quantitative analysis of III. The sulfoxide metabolite (II) could not be measured with high sensitivity (< 100 ng/ml) due to endogenous interferences which limited the utility of this assay technique.

EXPERIMENTAL

Column

The column used was a 25 cm × 4.6 mm I.D. stainless-steel column containing 5-μm LC-PCN (cyano) packing (Supelco, Bellefonte, PA, U.S.A.).

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a Model 440 UV detector (with an extended wavelength module and a 229-nm kit) (Waters Assoc., Milford, MA, U.S.A.). The mobile phase for isocratic reversed-phase HPLC was composed of acetonitrile—0.02 *M*

potassium dihydrogen phosphate pH 4.5 (60:40), prepared by mixing 16.0 ml of 1 *M* potassium dihydrogen phosphate pH 4.5 with 784.0 ml distilled water and diluting to 2 l with acetonitrile. The mobile phase was degassed prior to use and kept under constant sparging with helium (99.995%). The column was maintained at a temperature of 40°C (Goldenfoil™ Column Temperature control system; Systec, Minneapolis, MN, U.S.A.), with a flow-rate of 2.0 ml/min and a pressure of 9.7 MPa (1400 p.s.i.). Under the above conditions, the retention times of compounds I, II, III, and VI were 7.6 min (k' = 9.55), 4.8 min (k' = 5.66), 6.9 min (k' = 8.55), and 8.5 min (k' = 10.82), respectively (Fig. 2A).

Amounts of 50 ng of I, II, and III and 150 ng of VI, yielded peaks of approximately full scale deflection at a UV detection sensitivity setting of $5 \cdot 10^{-3}$ a.u.f.s.

The chart speed on a 10-mV recorder was 15 in./h (0.25 in./min) (Hewlett-Packard, Model 713A, Avondale, PA, U.S.A.). Upon completion of a day's analysis, the phosphate buffer and any endogenous materials were flushed from the column with acetonitrile—water (60:40).

Standard solutions

Weigh out 10.0 mg of chlorprothixene (I, $C_{18}H_{18}ClNS$, mol. wt. 315.85,

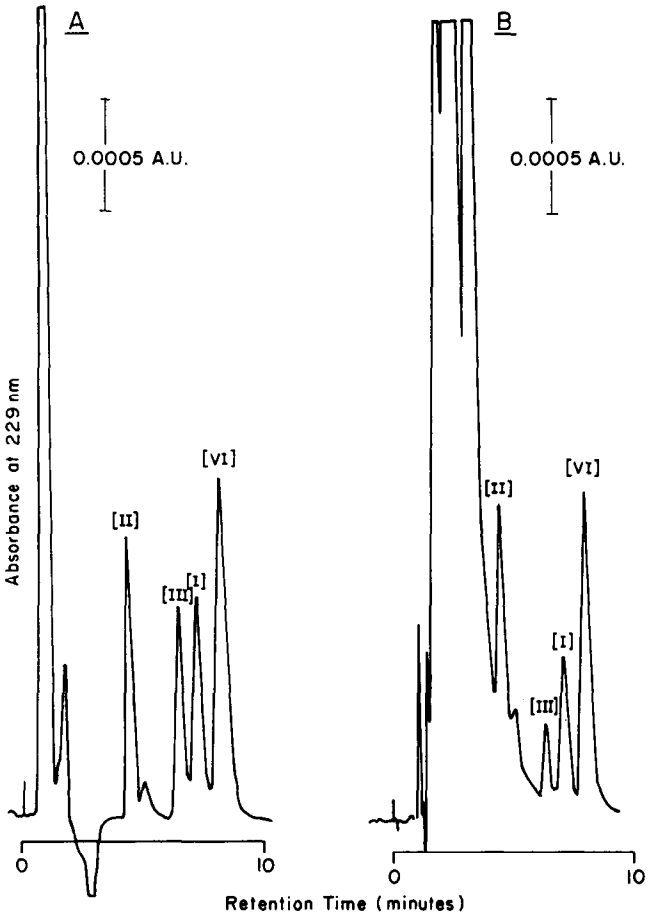


Fig. 2.

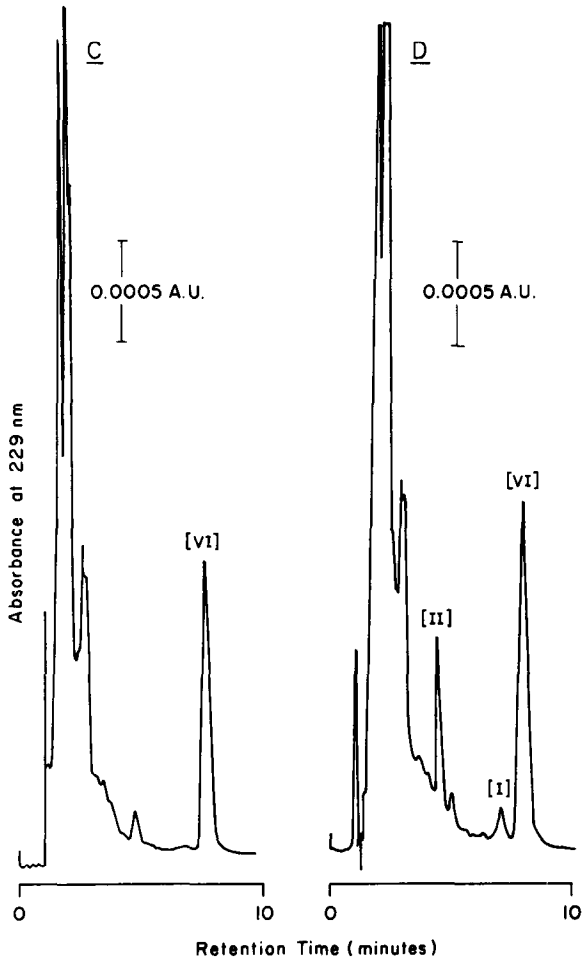


Fig.2. HPLC—UV Chromatograms of (A) external standard injection of 10.0 ng I—III and 50.0 ng of VI; (B) plasma recovered standard supplemented with 40 ng I—III and 250 ng VI; (C) patient plasma control supplemented with 250 ng VI per ml; (D) patient plasma sample (4 h post administration) concentration of I and II were 8.8 and 29.8 ng/ml, respectively.

m.p. = 97—98°C) into a 100-ml volumetric flask, dilute to volume with methanol. This stock solution (A) contains 100 μg/ml I.

In a similar manner, prepare stock solution B of chlorprothixene sulfoxide (II, $C_{18}H_{18}ClNOS$, mol. wt. 331.365, m.p. = 94—95°C), stock solution C of N-desmethylchlorprothixene (III, $C_{17}H_{16}ClNS \cdot HCl$, mol.wt. 338.294, m.p. = 202—204°C), and stock solution D of thioridazine (VI, $C_{21}H_{26}N_2S_2 \cdot HCl$, mol. wt. 407.01, m.p. = 158—160°C).

A series of mixed working standard solutions of I, II and III are prepared by transferring aliquots of 0.010, 0.020, 0.040, 0.060, 0.080 and 0.100 ml of A, B, and C each into 10-ml volumetric flasks and diluting to volume with methanol. These solutions 1—6 contain 5, 10, 20, 30, 40 and 50 ng per 50 μl of solution, respectively, I, II and III.

Aliquots (10 μl) of solutions 1—6 are injected to verify the performance of the HPLC system and to calculate percent recovery from plasma.

A working standard solution of VI, the internal standard, containing 250 ng per 50 μl (solution 7) is prepared by transferring 0.5 ml of D into a 10-ml volumetric flask and diluting to volume.

Reagents

Reagent-grade chemicals were used to prepare 1 *M* potassium dihydrogen phosphate and 2 *M* sodium hydroxide in deionized distilled water.

Other reagents include methanol, acetonitrile (UV) and heptane (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), isoamyl alcohol (Mallinckrodt, St. Louis, MO, U.S.A.) and thioridazine, the internal standard (Sandoz Pharmaceuticals, Hanover, NJ, U.S.A.).

Assay in plasma

Into a 10-ml borosilicate disposable centrifuge tube add 1.0 ml of unknown plasma, 0.050 ml of internal standard (solution 7), 2.0 ml of distilled water and 2.0 ml of 2 *M* sodium hydroxide. Mix well and extract with 10.0 ml of 1% isoamyl alcohol in heptane by slowly shaking on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min.

Centrifuge the sample in a refrigerated centrifuge (Model PRJ with a No. 253 rotor, Damon, IEC Corporation, Needham, MA, U.S.A.) at 5—10°C for 5 min at 2200 rpm (1207 *g*). Transfer 8.5 ml of supernatant into a 10-ml borosilicate disposable centrifuge tube. Evaporate to dryness under a stream of nitrogen in a water bath at 60°C using a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.). Dissolve the residue in 250 μl of acetonitrile—water (60:40). Inject a 50-μl aliquot for HPLC analysis.

A specimen of 1.0 ml of control plasma and six 1.0-ml aliquots of control plasma containing 0.050 ml of solutions 1—6 (equivalent to 5, 10, 20, 30, 40 and 50 ng of I, II and III per ml plasma) and 0.050 ml of internal standard (solution 7) are processed with the samples as the recovered standards. The peak height ratios of I, II and III to VI versus concentration of the respective compound are used to perform a weighted linear regression analysis to establish the calibration curves for the determination of drug and metabolites concentrations in the unknown samples.

RESULTS

Analytical parameters

Inter-assay linearity and precision of the HPLC—UV method were determined over the plasma concentration range of 5.0—50.0 ng/ml for I and II (Table I). The data were accumulated from five separate analytical experiments, in which two runs included triplicate analysis at each concentration. The average coefficients of variation for I and II over this concentration range were 6.4% and 10.0%, respectively. The recoveries of I and II were approximately 85% and 90%, respectively. The sensitivity limit for III was 10 ng/ml with a recovery of 25% and an average coefficient of variation of 20%. Due to the poor and variable recovery of III and the resultant lack of precision, the assay can only be considered semi-quantitative for III.

TABLE I

INTER-ASSAY STATISTICAL EVALUATION (HPLC—UV METHOD)

Concentration added (ng/ml)	n*	Mean concentration found (ng/ml)	Coefficient of variation (%)
Chlorprothixene (I)			
5.0	9	4.9 ± 0.4	7.3
10.0	9	10.1 ± 0.7	6.5
20.0	9	20.5 ± 1.7	8.1
30.0	9	29.8 ± 1.8	6.1
40.0	9	39.9 ± 2.2	5.5
50.0	9	50.2 ± 2.6	5.1
		Average =	6.4
Chlorprothixene sulfoxide (II)			
5.0	8	5.0 ± 0.6	11.5
10.0	8	10.0 ± 0.8	8.0
20.0	9	21.3 ± 2.4	11.2
30.0	9	31.6 ± 3.3	10.6
40.0	8	40.1 ± 4.3	10.7
50.0	9	49.8 ± 3.9	7.7
		Average =	10.0

*Data accumulated in five separate analytical experiments; two runs included triplicate analysis of each concentration.

The selectivity of the HPLC—UV assay for the N-desmethylchlorprothixene sulfoxide (IV) and chlorprothixene sulfoxide-N-oxide (V) was not evaluated due to the unavailability of authentic standards. However, the two compounds are expected to be more polar than II and should therefore elute in the solvent front in the chromatograms. Significant interferences by other neuroleptics in the retention areas of I—IV were noted under the specified HPLC conditions. Thus, in toxicological analysis the presence of I—IV will require confirmation with a second technique, i.e. gas chromatography—mass spectrophotometry.

The stability of I upon collection and handling was determined in that no loss of compound was detected for a period of 4 h at both 25° C and 37° C. The stability of I under extended periods of storage was not evaluated. However, the clinical samples from patients receiving 100 mg Taractan® (described below) were stored at −70° C prior to analysis.

Studies in man

Clinical study. The HPLC—UV analysis of I, II and III was applied to the measurement of chlorprothixene and its metabolite in two healthy subjects following a single oral administration of 100 mg. Serial blood pressure recordings were taken with the subject tilted in a 60° upright position to study the pharmacodynamic effects of the drug.

After fasting overnight two male volunteers received 5 ml of Taractan concentrate (containing 100 mg chlorprothixene per 5 ml) with 250 ml of water. Blood samples (12 ml) were drawn by glass syringe at 0, 1, 2, 3, 4, 5, 6,

8, 12, 16, 24, 32 and 48 h post dosing and immediately transferred into oxalated tubes. The samples were immediately spun down to separate the plasma, which was transferred to another tube and stored at −70°C until analysis. The subjects received water ad libitum until 4 h after drug administration when lunch was served. Standard meals were served for 24 h while the volunteers were confined to the study site. The volunteers reported back to the study site for the 32- and 48-h blood collection. Plasma concentrations of I were measurable in subject 1 at 5 h (5.3 ng/ml) and in subject 2 from 1 to 6 h. A maximum plasma concentration of I of 8.8 ng/ml was measured at 4 h for subject 2. These measurable concentrations of I correlated well with clinical response in that subject 1 displayed an orthostatic response at 6 h, and that subject 2 could not be tilted passively from 2 to 7 h because of frank fainting. Concentrations of II were measurable from 1 to 32 h and 1 to 48 h, in subjects 1 and 2, respectively. Maximum concentrations of II at 4 h of 39.9 and 29.8 ng/ml were measured, which declined to 9.8 ng/ml at 32 h and 7.7 ng/ml at 48 h in subjects 1 and 2, respectively (Table II, Fig. 3). Concentrations of III were non-measurable (< 10 ng/ml) throughout the entire collection period.

Overdose study. The assay was also applied to the analysis of blood samples from two patients suspected of ingesting overdosages of chlorprothixene exceeding several hundred milligrams. Subject 3 was a 2.5-year-old female suspected of ingestion of an unknown quantity of 50-mg Taractan tablets. The blood sample was drawn approximately 24 h following the suspected ingestion. Subject 4 was a 25-year-old female suspected of ingestion of an unknown amount of 25-mg Taractan tablets. The blood sample was drawn

TABLE II

CONCENTRATION OF I AND II IN TWO SUBJECTS FOLLOWING A SINGLE ORAL DOSE OF 100 mg CHLORPROTHIXENE (SUSPENSION OF 100 mg/5 ml OF TARACTAN)

Time post administration (h)	Concentration (ng/ml)			
	Subject 1		Subject 2	
	I	II	I	II
0	n.d.*	n.d.	n.d.	n.d.
1	n.d.	8.4	4.9	6.5
2	n.d.	22.4	7.7	16.7
3	n.d.	28.9	6.1	22.0
4	n.d.	39.9	8.8	29.8
5	5.3	34.6	8.1	22.3
6	n.d.	33.4	6.9	26.0
8	n.d.	23.7	n.d.	21.5
12	n.d.	15.3	n.d.	20.0
16	n.d.	14.9	n.d.	12.5
24	n.d.	11.1	n.d.	11.5
32	n.d.	9.8	n.d.	n.s.**
48	n.d.	n.d.	n.d.	7.7

*n.d., Non-detectable (≤ 5.0 ng/ml).
**n.s., No sample.

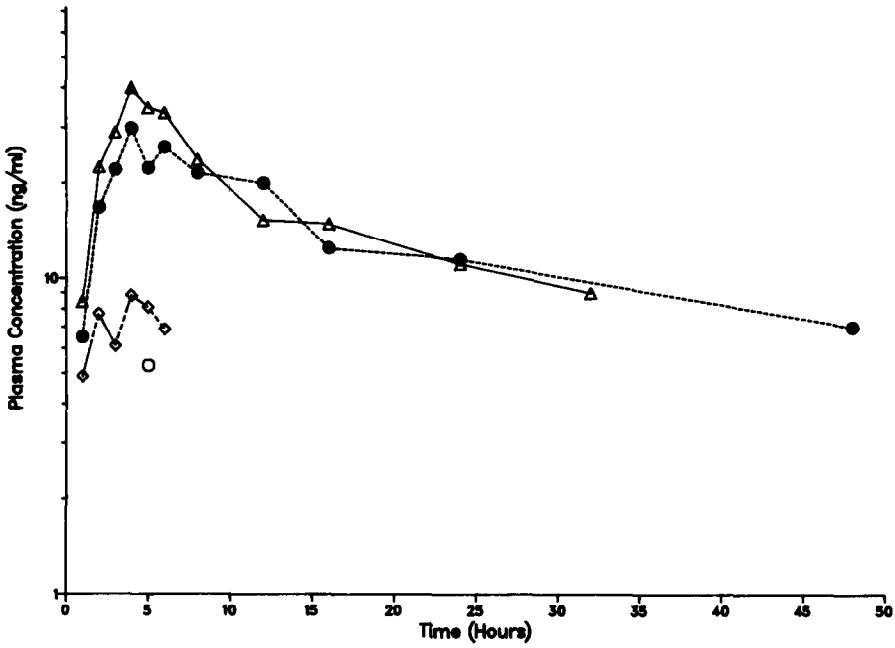


Fig. 3. Plasma concentration versus time profit for chlorprothixene (I) and chlorprothixene sulfoxide (II) in two healthy human subjects following single oral 100-mg doses of Taractan concentrate. (○) Chlorprothixene, subject 1; (△) chlorprothixene sulfoxide, subject 1; (◇) chlorprothixene, subject 2; (●) chlorprothixene sulfoxide, subject 2.

approximately 30 h following the suspected ingestion. Blood samples collected at the respective hospitals were frozen (−17°C) and sent to Hoffmann-La Roche for analysis. Plasma could not be separated from blood for these samples due to freezing, and hence whole blood was used for analysis.

The assay was performed as described in the experimental section with the exception that a CN-AQ cyano column (Varian Assoc., Palo Alto, CA, U.S.A.) was used and maintained at room temperature. A Spectromonitor III detector (LDC Corporation, Riviera Beach, FL, U.S.A.) monitored the column effluent at 230 nm. Dilution of the blood sample or final injection solution was made to bring the concentration of the unknowns within the calibration range. The data (Table III, Fig. 4A) demonstrate that the sulfoxide metabolite (II) was the major blood component and that much lower concentrations of I and III were present.

TABLE III

ANALYSIS OF I–III IN BLOOD FOLLOWING OVERDOSAGE OF TARACTAN

Subject	Concentration (μg/ml)					
	I		II		III	
	UV	Amperometric	UV	Amperometric	UV	Amperometric
3	0.13	0.13	1.0	0.92	0.08	0.06
4	0.25	0.24	0.72	0.61	0.26	0.22

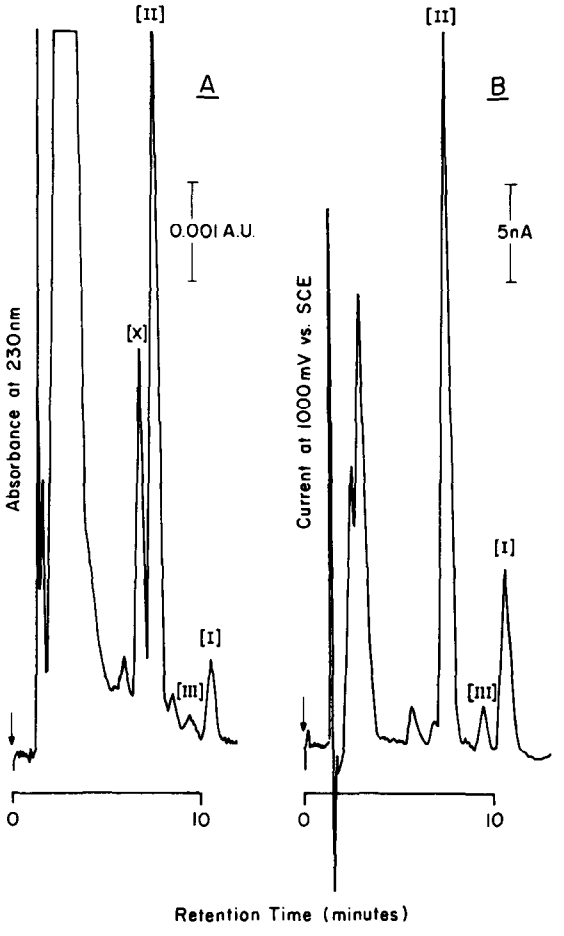


Fig. 4. HPLC analysis with (A) UV and (B) amperometric detection of a blood sample taken from subject No. 3 (see Table III) following an overdosage of Taractan.

Amperometric detection

The goal of the present study was to develop an HPLC assay with simultaneous UV and amperometric detection for I, II and III in both blood and plasma samples following therapeutic drug administration and for toxicological investigations.

Initial investigations with amperometric detection (Model 230; IBM Instruments, Danbury, CT, U.S.A.) monitored changes in oxidation current (50 nA full scale) at the glassy carbon electrode (Model 3892, Kel-F; IBM) with repeated injections and successive increases of 50 mV applied potential (Fig. 5). The data indicated that for injections of 10 ng per 10 μl the sulfoxide metabolite (II) gave only 30—40% of the response of I and III over the potential range examined. This suggested that the product of oxidation of the three compounds may be the sulfone and that the lower current associated with the sulfoxide being converted to the sulfone is due to the lesser number of electrons involved in that oxidation process. The choice of 1000 mV versus saturated calomel electrode (SCE) as the applied potential for the analytical

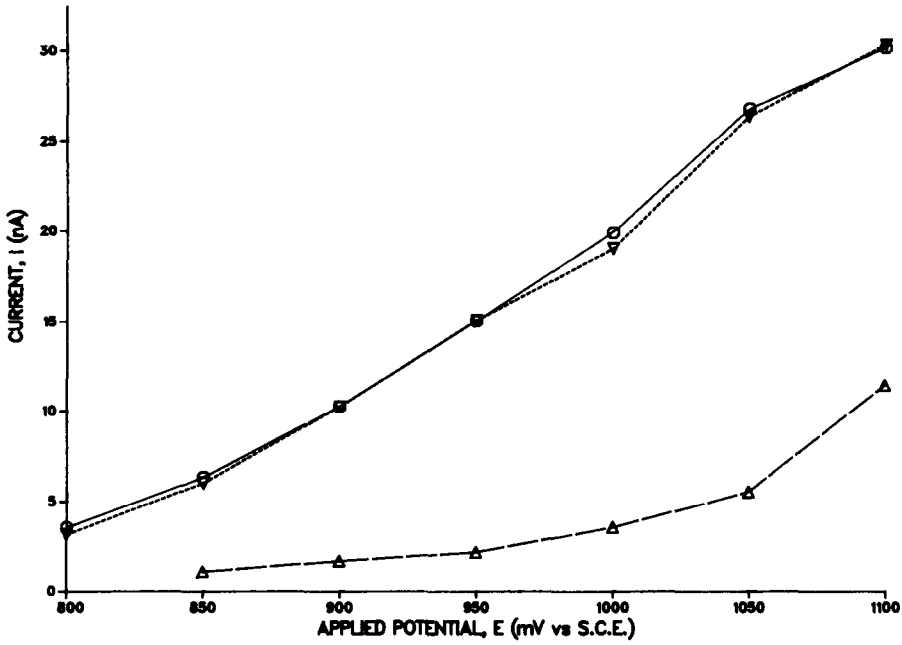


Fig. 5. Hydrodynamic voltammograms for the HPLC analysis of compounds I—III. (○—○) Chlorprothixene; (△— —△) chlorprothixene sulfoxide; (▽- - - - -▽) N-desmethylchlorprothixene.

determination was a compromise between the need for high sensitivity with a reasonably stable baseline. A concentration of 20 ng each of I, II and III yielded peaks of approximately 50%, 20% and 50% of full scale deflection, respectively, at an amperometric detection sensitivity setting of 100 nA with an applied potential of 1000 mV versus SCE.

Statistical validation for the HPLC—amperometric method for I over the concentration range of 10.0—50.0 ng/ml showed approximately equal precision and linearity to the HPLC—UV method. The decreased oxidation current for II along with an insufficiently clean extract for amperometric detection limited the sensitivity of the assay for this compound to < 100 ng/ml. These detection limits are insufficient to determine I or II following the administration of a single therapeutic dose, [7, 12, 13]; hence amperometric detection is recommended only for monitoring the administration of multiple therapeutic doses and for toxicological investigations (see below).

The HPLC—amperometric method was also utilized in the analysis of the samples taken from the two overdosage patients. The UV and amperometric detectors were placed in series with the electrochemical detector downstream of the UV detector. Good agreement was obtained between the two modes of detection (Table III). A typical chromatogram for the toxicological investigations is shown in Fig. 4. A large unknown peak (X) which eluted just prior to II was noted in the UV, but not in the amperometric tracing; hence it does not appear to be related to the ingestion of chlorprothixene.

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