Isocratic High-Performance Liquid Chromatographic Method for the Determination of Tricyclic Antidepressants and Metabolites in Plasma

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Abstract □ An isocratic high-performance liquid chromatographic method for the determination of six tricyclic antidepressants and their major metabolites is presented. Hexane containing 0.5% diethylamine was used as an extraction solvent to minimize adsorption onto glass. A reversed-phase cyanopropylsilane column was used with a mobile phase consisting of 70% acetonitrile and 30% 0.03 *M* acetate buffer, pH 7.0. Good identification and quantitation were obtained by the use of both UV detection at 245 nm and spectrofluorometric detection with an excitation wavelength of 276 nm and an emission filter with a 370-nm cutoff. A minimum detectable limit of <5 ng/ml of plasma is possible with this system. The reproducibility and precision of the method are shown from the analysis of samples containing 20–400 ng/ml in plasma.

Keyphrases □ Antidepressants—tricyclic, isocratic high-performance liquid chromatographic method for the determination of metabolites in plasma □ Metabolites—isocratic high-performance liquid chromatographic method for the determination of tricyclic antidepressants in plasma □ High-performance liquid chromatography—isocratic determination of tricyclic antidepressants and metabolites in plasma

Tricyclic antidepressants have been used extensively in the treatment of psychiatric patients suffering from depression. The relationship between the plasma concentration of these drugs and their clinical effect on depressive symptoms is controversial (1), but monitoring therapeutic levels is important, since the side effects of these drugs are quite common and mainly dose related (2). The major metabolic pathways are demethylation and hydroxylation (3).

In a recent two-part review article (4, 5), both the methodology and the pharmacokinetics of tricyclics were reviewed. The methodology review lists several methods for the determination of tricyclics and their metabolites. Many of the methods, including UV spectrometry (6, 7), fluorimetry (8), and TLC (9), are nonspecific, since the compounds are difficult to separate and the major metabolites have spectral characteristics similar to the parent drugs. Radioimmunoassay has been used, although many antisera show substantial cross reactivity (10).

GLC methods require either derivatization of the compounds or a selective detector (11–14). Quantitation of tricyclic antidepressants and their metabolites have also been performed by high-performance liquid chromatography (HPLC) (15–19), but most of the reported methods did not include the hydroxy metabolites which have been shown to be pharmacologically active (20). Most methods also involve an elaborate multistep extraction procedure.

The present report describes the separation and determination of the most commonly prescribed tricyclic antidepressants. The method involves one extraction step followed by HPLC using a reversed-phase cyanopropylsilane column and dual UV and fluorescence detection. The method is applicable to the direct determination of plasma levels in the presence of one or more of several drugs which might be prescribed concurrently. The applicability of the method has been demonstrated by the analysis of plasma from patients receiving tricyclic antidepressants. The determination of amitriptyline includes the simultaneous analysis of its most important metabolites in plasma.

EXPERIMENTAL

Instrumentation—A high-performance liquid chromatograph[1] was equipped with a fluorometric detector[2] and a cyanopropylsilane[3] column (250 mm long, 4.6-mm i.d.). The degassed mobile phase was pumped through the column at 3.0 ml/min (20–22 mPa). The column compart-

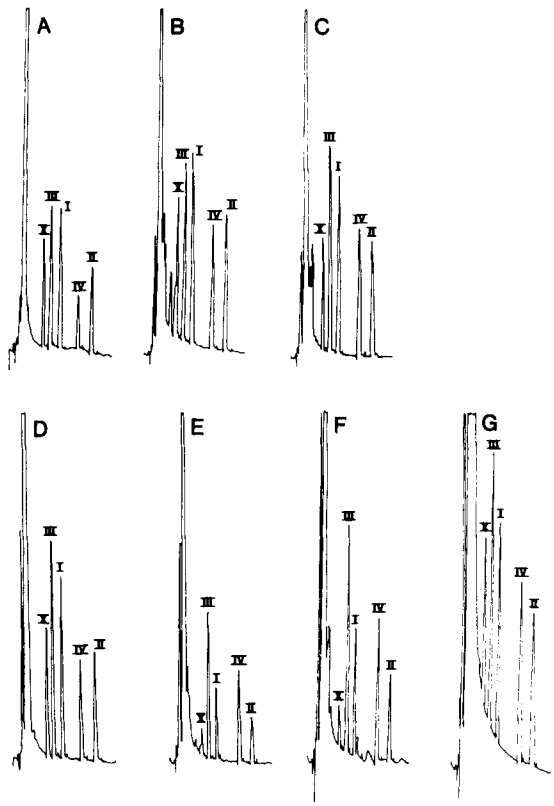

Figure 1—*Chromatograms of the extracts of 1.0 ml of plasma containing 200 ng/ml each of I, II, III, and IV. The extracts were reconstituted with 100 μl of methanol, injection volume 30 μl, absorbance at 245 nm. Key: (A) hexane; (B) hexane plus 2% isoamyl alcohol; (C) heptane plus 4% isobutyl alcohol; (D) hexane plus 0.5% diethylamine; (E) methylene chloride; (F) methylene chloride plus 0.5% diethylamine; (G) ethyl acetate.*

[1] Model 1084B chromatograph with variable wavelength UV detector and autoinjector, Hewlett-Packard Co., Avondale, Pa.
[2] FS-970, Schoeffel, Westwood, N.J.
[3] Ultrasphere Cyano, Beckman Instruments, Inc., Berkeley, Calif.

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Table I—Precision of Tricyclic Antidepressant Assay

Amount Added, ng/ml	Amount Found, ng/ml[a,b] I	II	III	IV	V	VI	VII	VIII	IX
20	19.6 ± 1.1	14.6 ± 1.4	19.3 ± 2.5	17.6 ± 2.6	37.4 ± 3.1	30.5 ± 3.3	19.3 ± 2.7	17.7 ± 1.5	14.2 ± 1.5
50	42.1 ± 3.9	38.3 ± 2.4	48.2 ± 1.4	40.8 ± 6.0	62.6 ± 4.4	58.1 ± 4.9	50.1 ± 15.2	47.6 ± 6.7	47.7 ± 11.9
100	105.5 ± 2.3	96.9 ± 10.0	95.0 ± 5.0	87.5 ± 10.4	116.2 ± 6.8	101.8 ± 6.2	101.6 ± 11.9	95.8 ± 6.0	102.2 ± 2.2
150	150.4 ± 4.8	148.4 ± 5.9	134.2 ± 11.4	129.0 ± 8.3	182.1 ± 19.7	175.5 ± 25.5	159.4 ± 10.7	154.2 ± 14.6	149.3 ± 8.1
200	200.2 ± 8.9	183.4 ± 9.4	198.0 ± 10.6	184.7 ± 24.5	224.6 ± 11.7	211.3 ± 2.5	219.9 ± 12.9	203.2 ± 6.6	225.2 ± 13.0
300	289.2 ± 27.1	285.9 ± 29.1	310.1 ± 15.8	306.0 ± 31.4	318.9 ± 21.8	317.8 ± 37.0	304.7 ± 34.4	289.6 ± 27.2	300.1 ± 4.9
400	403.3 ± 21.0	402.5 ± 21.4	400.8 ± 26.5	404.1 ± 25.7	407.5 ± 26.7	394.2 ± 38.7	393.1 ± 16.4	402.0 ± 15.0	403.7 ± 19.3
Correlation coefficient	0.994	0.993	0.995	0.991	0.993	0.984	0.991	0.995	0.995
Total n	32	31	35	33	30	32	32	32	29
R^2	0.989	0.987	0.989	0.983	0.985	0.968	0.990	0.990	0.990

[a] Absorbance at 245 nm. [b] For each compound values are the mean ±*SD*.

ment was maintained at 45°. The UV was monitored at 245 nm and the fluorometer was set at an excitation wavelength of 276 nm. An emission cutoff filter (type 370 nm) was used.

Chemicals and Reagents—Sodium acetate, sodium phosphate, acetic acid, phosphoric acid, diethylamine, sodium hydroxide, isoamyl alcohol, and isobutyl alcohol were reagent grade. HPLC grade methanol, acetonitrile, hexane, heptane, ethyl acetate, and methylene chloride were used. Amitriptyline hydrochloride (I)[4], nortriptyline hydrochloride (II)[5], 10-hydroxyamitriptyline (III)[4], 10-hydroxynortriptyline hydrogen maleate (IV)[4], imipramine hydrochloride (V)[6], desipramine hydrochloride (VI)[7], doxepin hydrochloride (VII)[8], demethyldoxepin hydrochloride (VIII)[8], protriptyline hydrochloride (IX)[4], and trimipramine hydrochloride (X)[9] were obtained commercially.

Mobile Phase—Sodium acetate solution (0.03 *M*) was prepared in deionized distilled water, and the pH was adjusted to 7 by the gradual addition of acetic acid. The mobile phase consisted of 70% acetonitrile and 30% buffer.

Solution Preparation—*Drug Stock Solutions*—Separate solutions of each drug and metabolite were made containing 5 mg of free base/10 ml of methanol.

Working Drug Solutions—Dilutions to working concentrations (5 ng/μl) were made with methanol for drug–metabolite groups (I–IV; V–VI; VII–VIII) or for a single drug (IX).

Extraction Solution—The internal standard stock solution (X) was diluted with hexane containing 0.5% diethylamine to a concentration of 20 ng/ml.

Sample Preparation—An aliquot (4–80 μl) of the drug working solution containing 20–400 ng, 0.5 ml of 0.1 *M* NaOH, and 10 ml of extraction solution were added to 1.0 ml of heparinized plasma in a 15-ml screwcapped centrifuge tube. The tubes were vortexed for 10 sec and centrifuged for 5 min at 900×*g*. A 9-ml volume of the organic phase was transferred to special concentration tubes[10] and evaporated to dryness at 30° under a gentle stream of nitrogen.

HPLC Separation and Quantitation—The residue was dissolved in 100 μl of methanol and transferred to a polypropylene micro-vial[11] before injection of 30 μl into the HPLC. A standard curve was constructed utilizing a minimum of four replicate plasma extractions simulating concentrations of drugs and metabolites from 20 to 400 ng/ml. The chromatograms were recorded at a chart speed of 5 mm/min. The peak heights were measured, and the ratios (drug/internal standard and metabolite/internal standard) were calculated and plotted *versus* concentration expressed as nanograms per milliliter of plasma.

Interferences—Possible interferences from normal plasma constituents, as well as other drugs and metabolites, were studied.

Factors Affecting Extraction Efficiency—The amounts of drugs and their metabolites extracted at pH 12 were studied using hexane, methylene chloride, ethyl acetate, hexane containing 0.5% diethylamine, hexane containing 2% isoamyl alcohol, methylene chloride containing 0.5% diethylamine, and heptane containing 4% isobutyl alcohol. Extractions were carried out at three different pH values (7.4, 9.5, and 12) with the following solvents: hexane, hexane containing 0.5% diethylamine, and methylene chloride.

Adsorption onto Glass—The effect of several agents on the adsorption of tricyclics and their metabolites onto glass during extraction and evaporation was studied. Diethylamine, isoamyl alcohol, isobutyl alcohol, and silanization of glass were tried.

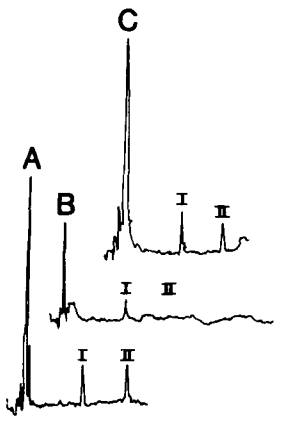

Figure 2—*Chromatograms showing the effect of solvents on adsorption of I and II onto glass. Injection volume 20 μl of 0.2 ng/μl, absorbance at 245 nm. Key: (A) methanol; (B) hexane; (c) hexane plus 0.5% diethylamine.*

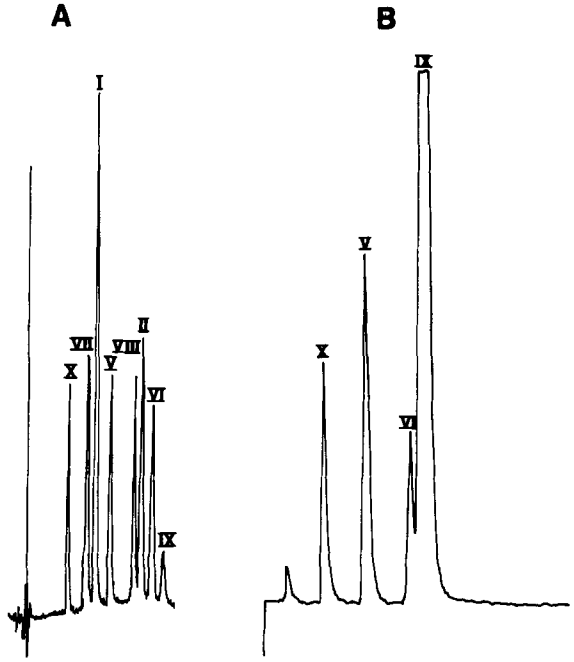

Figure 3—*Chromatograms of a mixture of 40 ng each of I, II, V–X. Key: (A) absorbance at 245 nm; (B) fluorescence at 276-nm excitation and 370-nm emission cutoff filter.*

[4] Merck, Sharp & Dohme Research Laboratory, Rahway, N.J.
[5] Eli Lilly and Co., Indianapolis, Ind.
[6] CIBA Pharmaceutical Co., Summit, N.J.
[7] Merrell-National Laboratories, Cincinnati, Ohio.
[8] Pfizer, Inc., New York, N.Y.
[9] Ives Laboratories, Inc., New York, N.Y.
[10] Concentratubes, Laboratory Research Co., Los Angeles, Calif.
[11] Micro-volume sample flask, P. Weidmann & Co., Romanshorn, Switzerland.

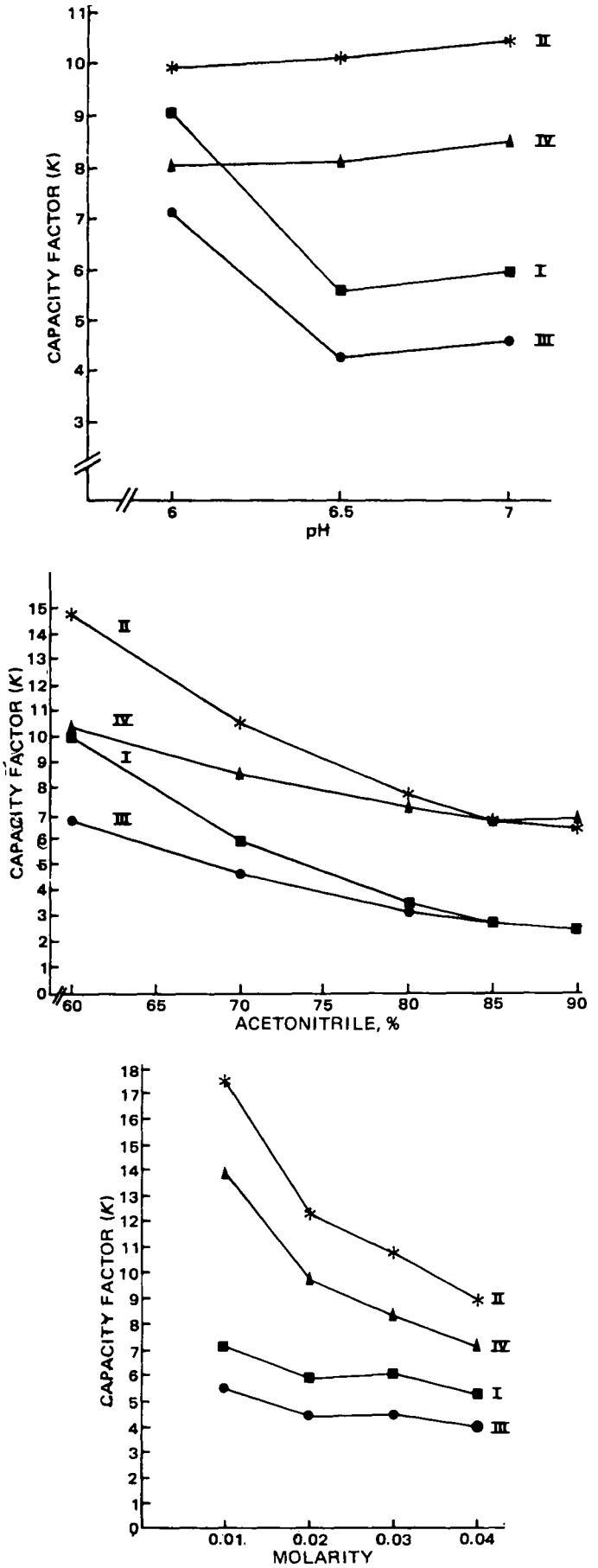

Figure 4—*Effect of pH, percent acetonitrile, and molarity of the mobile phase on capacity factor (K) of I–IV.*

Table II—Drugs Tested for Possible Interference

Drug	Retention Time, min	Absorbance[a]	Fluorescence[b]
Diazepam	SF[c]	+	–
Dextromethorphan	SF	+	+
Iminodibenzyl (Metabolite of Desipramine)	SF	+	+
Lidocaine	SF	+	–
Loxapine	1.90	+	–
Fluphenazine	2.05	+	+
Perphenazine	2.21	+	–
Haloperidol	2.29	+	–
Amoxapine	2.82	+	–
Trimipramine (X)	3.75	+	+
Thiothixene	3.90	+	+
Chlorprothixene	4.02	+	+
Quinidine	4.11	+	+
Trifluoperazine	4.55	+	+
10-Hydroxyamitriptyline (III)	4.66	+	–
Propranolol	4.82	–	+
Doxepin (VII)	5.08	+	–
Chlorpromazine	5.10	+	–
N-Acetylprocainamide	5.15	+	+
Amitriptyline (I)	5.22	+	–
Clomipramine	5.70	+	–
Procainamide	5.80	+	+
Imipramine (V)	6.20	+	+
10-Hydroxynortriptyline (IV)	6.41	+	–
Disopyramide	6.66	+	–
Thioridazine	6.88	+	+
Nortriptyline (II)	7.08	+	–
Demethyldoxepin (VIII)	7.12	+	–
Maprotiline	7.51	+	–
Desipramine (VI)	7.65	+	+
Protriptyline (IX)	7.90	+	+
Mesoridazine	8.75	+	+

[a] Absorbance at 245 nm. [b] Fluorescence at 276/370 nm. [c] SF is the solvent front.

Patient Samples—Heparinized plasma samples from patients receiving oral tricyclic antidepressant therapy were extracted in duplicate using the same procedure. The amounts of drug and metabolites in patient samples were calculated by comparison with a standard curve prepared daily.

RESULTS AND DISCUSSION

The choice of extraction conditions is based on a compromise between extraction yield and selectivity of extraction. Extraction with hexane afforded a cleaner extract than several other solvents (Fig. 1). The addition of 0.5% diethylamine to the hexane minimized the adsorption of the tricyclics onto the glass surface (Fig. 2). This observation confirms that reported previously (21). Evaporation under nitrogen at 30° minimized volatilization of drugs and metabolites and yet is sufficient for ready evaporation of diethylamine and hexane. In addition, the use of an organic phase–aqueous phase ratio in extraction of 10:1.5 made it possible to have a high yield in one extraction step. A slightly higher yield was obtained at pH 12 than pH 7.4 or 9.5. Reconstitution in methanol was chosen over the mobile phase because glass adsorption from acetonitrile considerably reduced the peak heights and prevented accurate quantitation. The effect was the greatest for the metabolites.

The use of a cyanopropylsilane column in a reversed-phase mode with a mobile phase of 70% acetonitrile and 30% acetate buffer (0.03 *M*, pH 7) produced good separation of several tricyclics (Fig. 3) and between each drug and its respective major metabolites. The separation of I–IV depends on the pH and molarity of the buffer as well as the percent acetonitrile in the mobile phase. This is illustrated by the change of *K* values (Fig. 4). The pH, molarity, and percent acetonitrile of the mobile phase may be varied slightly to obtain separation on cyanopropylsilane columns of different manufacturers or on a column after a period of use. The use of fluorescence detection is not necessary for the analysis, since all of the drugs and metabolites absorb at 245 nm and only V, VI, and IX fluoresce. Dual detection using both absorbance and fluorescence is helpful, however, to afford exact identification and good quantitation. The drugs may be monitored at 254 nm with only a slight loss of sensitivity. Monitoring at 288 nm or by fluorescence will enhance the sensitivity for IX. Trimipramine was chosen as the internal standard because it has almost equal absorbance and fluorescence under the experimental conditions and is well separated from all the drugs and metabolites.

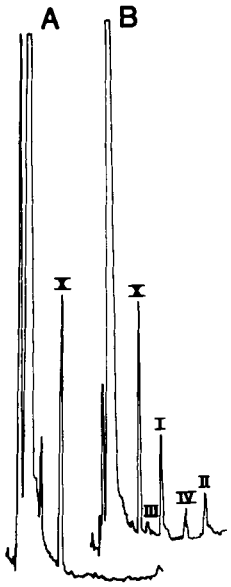

Figure 5—*Typical chromatograms of extracts of 1 ml of plasma, injection volume 30 μl, absorbance at 245 nm. Key: (A) drug free; (B) patient receiving I orally. Concentrations of compounds expressed as ng/ml of plasma are I = 87, II = 50, III = 7, and IV = 38.*

The ratios of the peak heights of the drugs and metabolites to the peak height of the internal standard were calculated. Statistical analysis of the data (Table I) by linear regression indicated linearity and reproducibility in the 20–400-ng/ml range of plasma. This range includes the therapeutic range of the drugs. Absolute recovery of the drugs and metabolites ranged from 55 to 80% of the theoretical amounts. This low recovery may be responsible for the variation in the amount found at low concentrations (Table I). The minimum detectable limit for the compounds is <5 ng/ml of plasma.

No interference from normal plasma constituents was observed (Fig. 5A). Also, several drugs which might be prescribed simultaneously with tricyclics were chromatographed. The retention times are listed in Table II.

The method has been applied to many patient samples and is being used routinely in the laboratory for monitoring therapeutic levels (Fig. 5B). Major advantages of the method are its simplicity, rapidity, and high sensitivity. All of the drugs and metabolites, including the 10-hydroxy metabolites of amitriptyline and nortriptyline, are determined using a single procedure. Adsorbance of the drugs onto glass has been minimized.

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Chelation of Mercury by Polymercaptal Microspheres: New Potential Antidote for Mercury Poisoning

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Received February 18, 1981, from the *Department of Plastics Research, The Weizmann Institute of Science, Rehovot, Israel.* Accepted for publication November 17, 1981.

Abstract □ Newly synthesized polymercaptal microspheres of 0.8 ± 0.02 μm were shown to have a specific and fast intake of mercury compounds over a whole range of pH while maintaining low toxicity. The microspheres bind easily with mercury compounds which are already bound to the biological mercury binders, albumin or cysteine. Mercury was recovered completely from the microspheres by using a solution of thiourea in hydrochloric acid. Due to their high surface area, low toxicity, and strong affinity toward mercury compounds, the microspheres have a potential use as a new oral drug for treatment in cases of mercury poisoning.

Keyphrases □ Microspheres—chelation of mercury, polymercaptal, new potential antidote for mercury poisoning □ Mercury—chelation by polymercaptal microspheres, new potential antidote for poisoning □ Chelation—mercury, polymercaptal microspheres, new potential antidote for mercury poisoning

Mercury compounds, both organic and inorganic, constitute an environmental and agricultural hazard (1, 2). Severe poisoning is known to cause brain damage, fetal disabilities, and death (2, 3). The therapy for mercury poisoning includes intravenous administration of the chelating drugs dimercaprol and/or penicillamine (4).

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