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Liquid Chromatographic Analysis of Promethazine and Its Major Metabolites in Human Postmortem Material

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ABSTRACT: A method is described for the determination of promethazine and some of its major metabolites in postmortem specimens by enzymic digestion followed by high-pressure liquid chromatography of the extracts using a cyano-bonded (μ-Bondapak®-CN) silica column packing and a mobile phase consisting of methanol, water, and *n*-octylamine (adjusted to pH 8). The system will separate and quantitate promethazine sulphoxide, desmonomethyl promethazine, didesmethyl promethazine, and promethazine. Pericyazine (2-cyano-10-[3-(4-hydroxypiperidino)propyl] phenothiazine) was used as internal standard. The parent drug and metabolites were extracted from a enzyme digested tissue homogenates with ethyl acetate using a simple, single micro-extraction procedure. The method was applied to four cases of fatal poisoning involving promethazine ingestion.

KEYWORDS: toxicology, promethazine, chromatographic analysis, major metabolites, micro-extraction technique, high-pressure liquid chromatography, postmortem levels

Promethazine is an effective antihistamine and antiemetic with a prolonged antihistamine action and pronounced sedative effects. The drug is usually administered in daily oral doses of 20 to 50 mg in tablet form or as an elixir [*1*].

Overdosage with promethazine preparations is not very common, with fatal poisonings generally resulting from the additive effects of the drug in the presence of other central nervous system (CNS) depressants. Bonnichsen et al [*2*] described two fatal cases of promethazine poisoning which were analysed by fluorometry, where the concentrations averaged 115 mg/Kg (range 50 to 180) in liver and a blood concentration was 8 mg/L.

A number of techniques have been used to determine promethazine in biological material including spectrophotometry [*3*], gas chromatography [*3-6*], and high-pressure liquid chromatography [*7-11*]. The spectrophotometric methods provide a useful initial screen but have the disadvantage of measuring the total amount of phenothiazine drug plus its metabolites [*2*]. Gas chromatographic analysis is both rapid and sensitive for the parent drug but difficulties in the use of gas liquid chromatography (GLC) for the analysis of promethazine metabolites may arise because of the thermal stress to which the compounds are subjected resulting in decomposition [*4, 12, 13*].

Recently a number of high-pressure liquid chromatographic procedures for promethazine

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have been reported [*8–11*]. However, all of these methods were applied to the analysis of blood or blood plasma, and either require prior derivatization [*8*] or the use of an electrochemical detector [*9*].

The extraction of promethazine and its metabolites presents difficulties since phenothiazine drugs are tightly bound to body proteins [*14,15*] and are labile [*16–19*]. Conventional extraction techniques are based on the chemical behavior of phenothiazine drugs as basic substances and generally involve rendering the blood alkaline (in the case of tissues this step is preceded by an acid hydrolysis/deproteination step) before solvent extraction [*5–11,20,21*]. However extremes of pH result in in vitro oxidation of the parent drug [*18*].

Osselton et al [*22–24*] introduced a procedure involving the use of the proteolytic enzyme Subtilisin (Carlsberg) to release protein-bound drugs from human tissue specimens. Analysis after enzyme digestion showed greatly improved recoveries compared to procedures involving conventional acid hydrolysis [*23–25*].

In this paper a method is described using high-pressure liquid chromatography (HPLC) to determine promethazine and its major metabolites in postmortem specimens after enzymic digestion and extraction with ethyl acetate using a simple, single micro-extraction method.

Case Reports

Case 1

A 29-year-old man was found dead on his bed by his wife. In his room was found a quantity of mixed medication which included Pan Quil® tablets (a proprietary preparation containing promethazine and paracetamol) and Tryptanol® tablets. The deceased was being treated for severe depression which had persisted since the death of his mother two years earlier. He had attempted suicide on four previous occasions, mainly by drug overdose.

Autopsy revealed marked edema of the lungs. Examination of the cardiovascular system showed the heart to be of normal size with no other abnormalities other than slight congestion of the myocardium.

Case 2

A 34-year-old man consumed a number of Pan Quil tablets on returning home at 9:30 p.m. from a beer drinking session with some friends at several clubs. He was found dead by his wife the following morning, lying face down on the bedroom floor. The deceased had suffered from bronchitis since childhood and had had an attack about one week earlier.

Autopsy revealed the direct cause of death to be attributed to cardiorespiratory depression.

Case 3

A 37-year-old woman was found dead by her daughter, lying on the floor beside her bed with her legs under her in a kneeling position. Found beside the bed was a large quantity of medication which included boxes of promethazine ampoules, a number of Amytal® (amylobarbitone) capsules, Tryptanol (amitriptyline) tablets, and Valium® (diazepam) tablets and ampoules. The deceased had been treated for chronic asthma for a number of years.

Autopsy revealed edema and congestion of the lungs.

Case 4

A 62-year-old woman was found dead by a neighbor, lying on her back on the floor in the doorway of the loungeroom of her home. The deceased was well known to local police and had a

past history of drug abuse and mental illness. A number of empty tablet cartons were found throughout the house.

Autopsy revealed edema and congestion of the lungs. The bronchi and trachea contained a copious amount of mucopurulent secretion. The trachea and bronchial mucosa were congested.

Liver, stomach plus contents, blood, urine, and bile specimens, when available, from each case were collected for toxicological analysis.

Experimental Procedure

Standards and Reagents

Promethazine (PMZ) and metabolite standards: promethazine sulphoxide (PMZ SO), desmonomethyl promethazine (Nor_1PMZ), desmonomethyl promethazine sulphoxide (Nor_1PMZ SO), and didesmethyl promethazine (Nor_2PMZ) were obtained from Rhône-Poulenc, Paris, France. Their structures and abbreviations are shown in Fig. 1.

Subtilisin (Carlsberg) (Protease Type VIII) was obtained from Novo Industries, Enzymes Division, Copenhagen, Denmark.

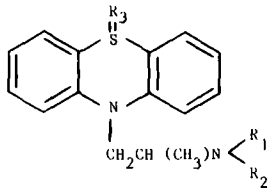
Pericyazine hydrochloride was obtained from Rhône-Poulenc, Paris, France.

Tris base 2-amino-2-(hydroxymethyl)propane-1,3-diol and *n*-octylamine (Fluka chemicals, Buchs, Switzerland) were obtained from Bio Scientific, Gymea, Australia. Tris buffer was prepared by dissolving 121 g in distilled water (1000 mL) and adjusting to pH 10.5 with 1*M* sodium hydroxide.

Ethyl acetate was chromatographic solvent grade obtained from Mallinckrodt Chemical Co., St. Louis, MO. Methanol was liquid chromatographic solvent grade from Waters Associates, Sydney, Australia.

In all experiments double glass distilled water was used. Reagents and other solvents used were analytical grade.

Thin-layer plates coated with silica gel (0.25 mm) and a fluorescent indicator (Merck, Darmstadt) were obtained from Lab Supply, Sydney, Australia.



Compound:	R_1	R_2	R_3
Promethazine (PMZ)	CH_3	CH_3	-
Promethazine sulphoxide (PMZSO)	CH_3	CH_3	O
Desmethyl promethazine (Nor_1PMZ)	H	CH_3	-
Desmethyl promethazine sulphoxide (Nor_1PMZSO)	H	CH_3	O
Didesmethyl promethazine (Nor_2PMZ)	H	H	-

FIG. 1—*Structures of promethazine and its major metabolites.*

Instrumentation

The liquid chromatographic system consisted of a Waters Associates (Milford, MA) Model 6000A solvent delivery system equipped with a Waters Model U6K Universal liquid chromatographic injector and a Waters Model 440 fixed wavelength (254 nm) absorbance detector. A Waters Data Module integrating recorder was used for the collection of data. The chart speed of the module recorder was set at 0.5 cm/min.

Chromatography

Chromatography was performed on a Waters μ-Bondapak®-CN column (30-cm by 4-mm inner diameter) at ambient temperature. A pre-column containing 30- to 38-μm cyano-amino bonded CO : PELL : PAC (Whatman Inc., Clifton, NJ) was fitted to the column to remove any residual particulate matter from the mobile phase and sample and so extend column life. The mobile phase consisted of methanol-water (50:50) containing 0.01*M* *n*-octylamine (pH 8.0, adjusted with phosphoric acid), which was vacuum filtered through a Durapore HVLP 04700 filter (Millipore Corp., Bedford, MA) which served to remove any particulate matter and also degas the solvent. The flow rate on the pump was set at 2.0 mL/min.

The efficiency of the column was tested before use with a mixture of uracil and acenaphthalene and acetonitrile-water (40:60) as mobile phase. At a flow rate of 2.5 mL/min the column efficiency for the acenaphthalene peak was about 3000 plates (N 5σ method).

A master stock solution of pericyazine (1.5 mg/mL, free-base) was prepared in methanol to which was added 1% pH 7.4 phosphate buffer to stabilize the composite promethazine and metabolites standard and stored in the dark at 4°C. A working stock solution of 0.015 mg/mL was used in subsequent preparations. A working standard of 0.01 mg/mL of promethazine and each metabolite plus internal standard (0.015 mg/mL) was used in subsequent analysis. Aliquots of 15 to 20 μL were injected into the HPLC.

Extraction Procedure

Tissue samples (liver and stomach plus contents) obtained from autopsy were finely minced in a Waring blender. Portions of the tissues 10 $\pm$ 0.1 g (10 mL of blood, urine, and bile) were blended with 1*M* Tris buffer (30 mL, pH 10.5) in 150-mL conical flasks. Crystalline subtilisin was added to the blended tissue samples (2 mg of enzyme per gram of sample) with gentle swirling before incubation at 55 to 60°C for 1 h in a reciprocating shaking water bath (Model RW 1812, Paton Industries, Stepney, South Australia). The homogenates were cooled to room temperature and diluted to 50 mL with 1*M* Tris buffer solution. Aliquots of 5.0 mL of the homogenate solution were transferred to 15-mL capped polypropylene centrifuge tubes (Edwards Instrument Co., Narellan, Australia) followed by 5.0 mL of ethyl acetate. The tubes were capped and placed on a rocking table (Luckman Pty Ltd., London, England) for 15 min. The upper organic layer was separated by centrifugation at 2000 rpm for 5 min, 3.0-mL aliquots were drawn off using a graduated glass syringe and transferred into glass centrifuge tubes (5-mL capacity), and evaporated under a gentle stream of nitrogen at 60°C. Pericyazine internal standard solution, 200 to 2000 μL (depending upon concentration as determined by initial screening thin-layer chromatography [TLC]) was added to the dried sample extracts. The tubes were stoppered, placed in an ultrasonic bath for about 30 s, and mixed briefly on a Vortex mixer. The tubes plus contents were then centrifuged at 2000 to 2500 rpm for 5 min to separate any lipid/particulate matter prior to injection into the HPLC.

Thin-Layer Chromatography

Portions of liver, blood, and stomach contents extracts from each case together with promethazine and metabolite standards in methanol, were spotted (5 to 10 μL) onto TLC plates

and developed in methanol:ammonia, 100:1.5. The plates were dried and sprayed with 5*M* sulfuric acid followed by gentle heating (pink spots) and oversprayed with iodoplatinate spray to determine the presence of any other additional basic drugs [*20*].

Recovery Study

The recoveries and reproducibility of the extraction procedure were determined by spiking blank (drug-free) liver samples with known amounts of promethazine and its major metabolites (5 to 20 mg/kg). The macerated spiked livers were allowed to stand for 30 min and then were treated as described previously.

Results and Discussion

Promethazine and its metabolites in each case were identified by TLC, by GLC retention times (Kovats Index) [*26,27*], and by HPLC retention times (Table 1).

A number of stationary phases have been used for the analysis of promethazine and its metabolites, with varying success ranging from silica (high polarity) [*7,11*] to ODS reverse-phase (low polarity) [*9,12*]. The intermediate polarity and reverse-phase characteristics of the cyano-bonded column made it a suitable choice for the separation of drug metabolites of varying polarities [*28*]. A μ-Bondapak-CN column was selected for the separation of promethazine and its metabolites. The addition of water to the mobile phase was adjusted to optimize resolution of the eluting compounds [*29*]. The best separation was achieved at about 50% v/v water.

Typical HPLC chromatograms of standard solutions of promethazine and its metabolites and an ethyl acetate extract of liver and blood from a promethazine overdosage case are shown in Figs. 2, 3, and 4, respectively. All the major metabolites tested eluted at retention times different from those of promethazine and the internal standard, pericyazine (Table 1). It was found, however, that the minor metabolite didesmethyl promethazine was not resolved from pericyazine necessitating the use of promazine as an alternative internal standard, if this metabolite was found to be present (as indicated by initial screening). No extraneous peaks in the area of interest were observed in the chromatogram of a blank liver extract.

The concentration of promethazine and its metabolites injected versus peak area was linear over the concentration range 5 to 50 μg/mL examined (Fig. 5).

The mean ($n = 3$) recoveries for promethazine and its major metabolites were as follows: promethazine, 90%; promethazine sulphoxide, 93.6%; and desmonomethyl promethazine, 96.7%. Method precision was determined by calculating the relative standard deviation of re-

TABLE 1—*Chromatography data for promethazine and its major metabolites.*

Compound	λ max,[a] nm (Methanol)	Rf[b] (×100)	Kovats Index[c]	R_t^d (min) (±3%)
Promethazine sulphoxide (PMZ SO)	236	38	2648	4.66
Pericyazine (internal standard)	268	...	3230	6.83
Didesmethyl promethazine (Nor_2PMZ)	252	52	2199	7.20
Desmethyl promethazine sulphoxide (Nor_1PMZ SO)	232	40	2592	9.40
Desmethyl promethazine (Nor_1PMZ)	252	46	2224	10.56
Promethazine (PMZ)	252	44	2284	12.63

[a] λ = wavelength.

[b] Thin-layer chromatographic system: methanol/water, 100:1.5.

[c] Linear temperature programmed N-P gas-liquid chromatograph [*26*]. The desmethyl metabolites were poorly resolved from the parent drug.

[d] Retention time; HPLC system: methanol/water (0.01*M* octylamine, pH 8.0), 50%:50%; fixed wavelength detector (254 nm) 0.04 attenuation.

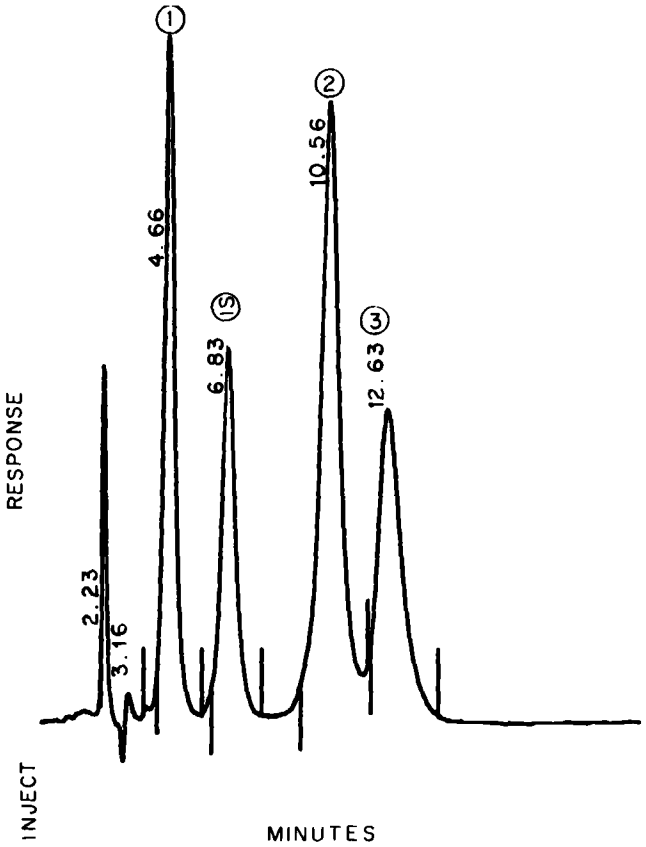


FIG. 2—*Typical chromatograph of standard promethazine and its major metabolites. The retention times were 4.66 min for ① PMZSO (32.2 μg/mL); 6.83 min for Ⓢ pericyazine (internal standard); 10.56 min for ② Nor$_1$-PMZ (10 μg/mL); and 12.63 min for ③ PMZ (10 μg/mL). Nonsystematic abbreviations used: ① promethazine sulphoxide, PMZSO; ② desmonomethyl promethazine, PMZ; ③ promethazine, Nor$_1$-PMZ.*

peated determinations of promethazine and its major metabolites in a liver sample from a fatal case involving promethazine ingestion (Table 2).

Table 3 lists the relative retention times of some basic drugs that may be found in cases of "poly-drug" poisonings involving promethazine and these were tested for interference with the present promethazine and metabolites assay. Generally drugs with retention times differing by much less than 10% can interfere. The drugs chlorpromazine, trimipramine, and doxepin were found to have retention times close to that of promethazine. However, the drugs encountered in the cases, namely: paracetamol, amitriptyline, diazepam, oxazepam, and their respective metabolites were well separated.

The analytical results for promethazine and its major metabolites in four cases are shown in Table 4.

From Table 4, it may be seen that desmonomethyl promethazine (Nor$_1$-PMZ) was found in all the liver specimens examined and in two of the blood samples (Cases 2 and 4). No blood was available for analysis in Cases 1 and 3. Both the unchanged drug and oxidized and demethylated metabolites were found in specimens of bile and urine. No bile was available from Case 1.

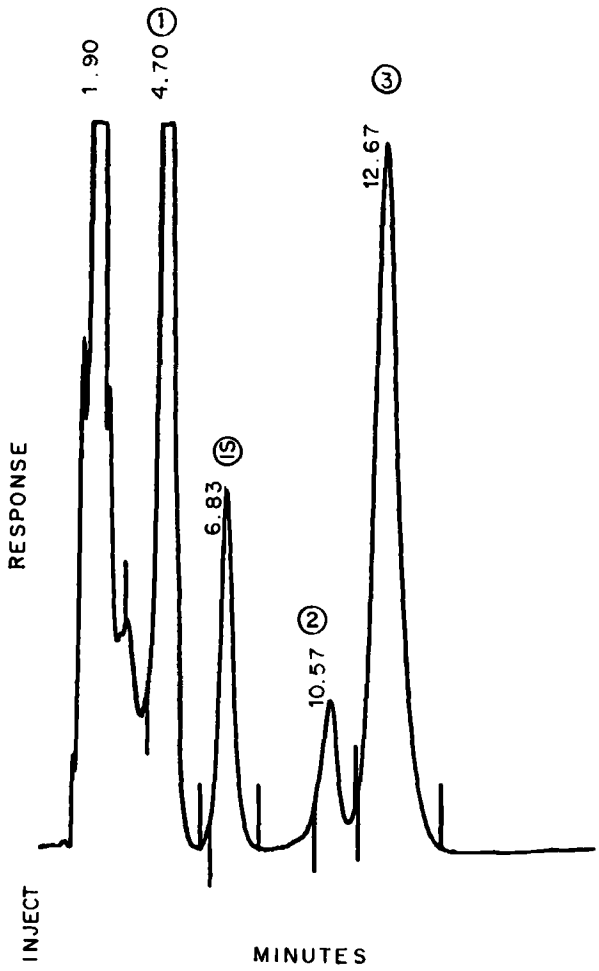


FIG. 3—*Chromatograph obtained for a liver extract from a case of promethazine (PMZ) overdosage.*

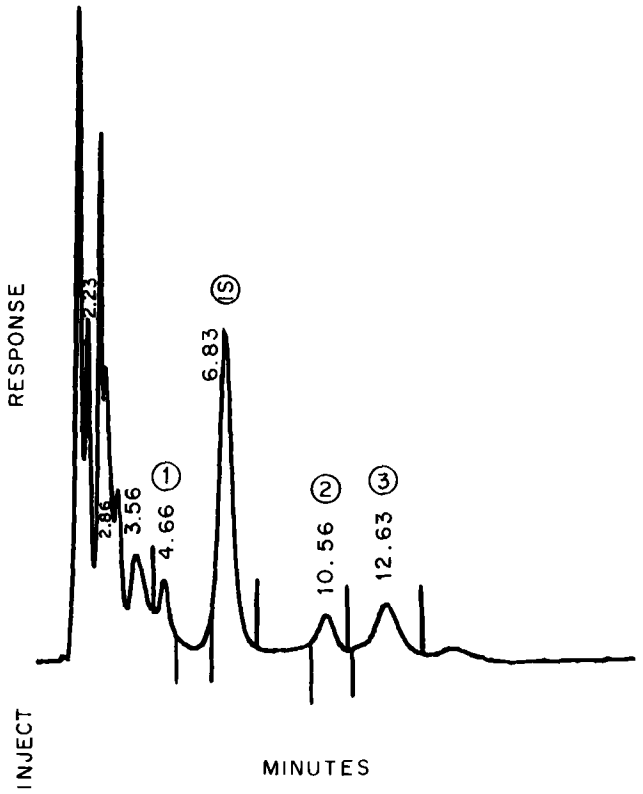


FIG. 4—*Chromatograph obtained for blood extract from a case of PMZ overdosage.*

TABLE 2—*Precision of the analysis for promethazine and some major metabolites in a case of overdosage.*

Compound	Amount Found	Mean, x (n = 4)	Standard Derivation	Coefficient of Variation, %
PMZ	30.4, 31.0, 30.0, 30.8	30.6	0.38	9.7
PMZSO	8.1, 8.7, 8.2, 8.6	8.4	0.25	6.4
Nor_1PMZ	6.1, 6.5, 6.2, 6.4	6.3	0.16	3.9

All of the cases examined were found to involve other drugs; three of these cases (1, 3, and 4) had amitriptyline present.

The highest concentrations of promethazine (PMZ) and its major metabolites, promethazine sulphoxide (PMZSO) and desmonomethyl promethazine (Nor_1-PMZ) were found in the bile and liver. In the urine the total metabolite concentration greatly exceeded that of the parent drug but in two cases (1 and 3) none of the parent drug was detected. In all instances,

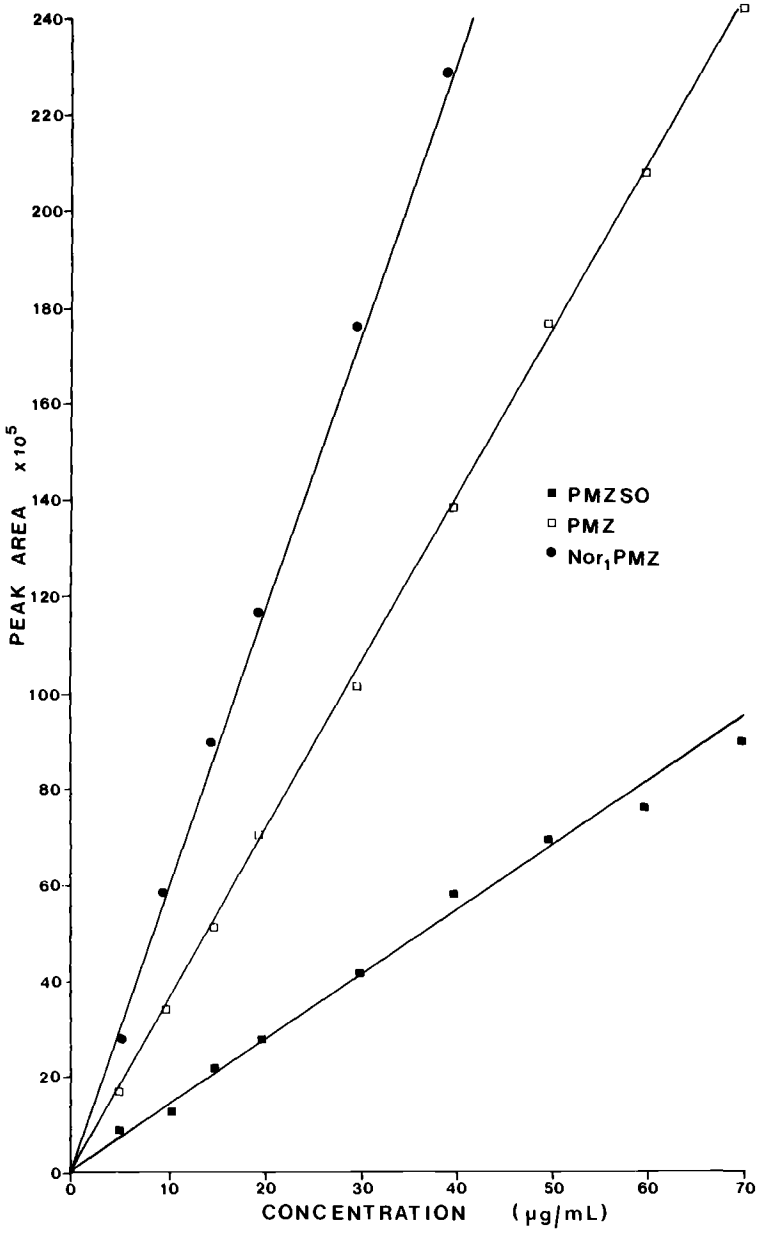


FIG. 5—*Linearity data for promethazine and its major metabolites.*

TABLE 3—*HPLC retention times, relative to promethazine, of various drugs.*

Drug	Relative Retention Times, ±5%[a]
Nortriptyline	1.94
Amitriptyline	1.23
Thioridazine	1.12
Chlorpromazine	1.02
Trimipramine	0.96
Doxepin	0.94
Pethidine	0.77
Quinine	0.74
Quinidine	0.71
Propranolol	0.58
Pericyazine	0.54
Diazepam	0.23
Oxazepam	0.22
Methaqualone	0.22
Caffeine	0.20
Paracetamol	0.20

[a]Promethazine = 1.0. Void volume = 0.18.

promethazine sulphoxide was the major metabolite found in the urine along with a small amount of the monodemethylated metabolites.

Generally low levels of desmonomethyl promethazine were found in the postmortem tissues, which is in accord with the results of recent work by Taylor and Houston [*11*] that showed concentrations of desmonomethyl promethazine were consistently lower than promethazine and promethazine sulphoxide levels following low oral doses of promethazine. In two cases (1 and 4) desmonomethyl promethazine was present also in the stomach.

Promethazine sulphoxide, which was the major metabolite in most of the tissue specimens, was also found in all of the stomach samples possibly as the result of the metabolism of promethazine in the gut wall [*30*]. The differences in promethazine sulphoxide concentrations may be due to a residual base level still remaining from earlier medication. In three of the four cases examined the drug amitriptyline was present. In Case 1, most of the promethazine remained in the stomach probably as a result of a shorter survival period caused by a combination of amitriptyline and alcohol poisoning.

Conclusion

The use of the proteolytic enzyme Subtilisin Carlsberg (Protease Type VIII) followed by extraction with ethyl acetate, was found to be efficient in releasing and extracting the protein-bound promethazine and its major metabolites from human postmortem specimens.

Analysis by HPLC of the extracts combined the advantages of other bioanalytical methods in providing rapid separation and quantitation, but at ambient temperature. Thermal stress (for example, by GLC) of the metabolites (with possible in vitro conversion was avoided).

The analytical method described was applied to four fatal cases involving promethazine ingestion.

Acknowledgment

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TABLE 4—*Promethazine and its major metabolites in body fluids and tissues from four fatal cases.*

Subject No.	Age	Sex	Drug/Metabolite[a]	Blood, μg/mL	Bile, μg/mL	Urine, μg/mL	Liver, μg/g	Stomach Plus Contents, mg	Other Drugs
1	29	M	PMZ	...	...	ND[b]	3.4	208	amitriptyline[c]
			PMZ-SO	...	...	1.0	2.4	6	paracetamol
			Nor_1-PMZ	...	...	0.2	2.6	1.8	alcohol: 3.2 g/L
2	34	M	PMZ	5.2	82.6	6	30.6	23	paracetamol
			PMZ-SO	3.1	23.5	26	8.4	14	alcohol: 1.65 g/L
			Nor_1-PMZ	0.8	14.5	1.0	6.3	ND	
3	37	F	PMZ	...	0.9	ND	2.9	10	polydrug
			PMZ-SO	...	3.3	2.0	1.2	5	overdose:[c]
			Nor_1-PMZ	...	0.8	0.3	2.1	ND	amylobarbitone diazepam amitriptyline
4	62	F	PMZ	2.4	11.6	14.0	23.2	28.6	amitriptyline
			PMZ-SO	0.8	27.0	42.0	5.4	20.6	oxazepam
			Nor_1-PMZ	1.2	3.0	3.0	16.2	5.4	

[a] PMZ = promethazine, PMZ-SO = promethazine sulphoxide, and Nor_1-PMZ = desmonomethyl promethazine.
[b] ND = below limit of detection (that is, <0.01 μg/mL).
[c] Significant in respect of the cause of death.

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