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Determination of *p*-methylthiobenzamide and *p*-methylthiobenzamide-S-oxide from rat plasma using solid-phase extraction and high-performance liquid chromatography

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ABSTRACT

p-Methylthiobenzamide (PMTB) is a thiocarbonyl compound exhibiting marked hepatotoxicity and nephrotoxicity. We describe a high-performance liquid chromatographic method for analyzing PMTB and a metabolite, *p*-methylthiobenzamide-S-oxide (PMTBSO), from rat plasma using a solid-phase extraction technique. In this way, PMTB and PMTBSO can be extracted from 0.5 ml of plasma and separation achieved by an ODS analytical column in as little as 9 min. The mobile phase used was methanol–water (55:45, v/v) and the wavelength for detection was 290 nm. The limits of detection in plasma were 15 ng/ml for PMTB and 33 ng/ml for PMTBSO; the absolute recovery from spiked plasma samples was greater than 84.4% for both compounds and the internal standard. The method was linear throughout the range used with correlation coefficients greater than 0.969. The intra-day accuracy ranged from 1.52 to 15.23% relative error for the PMTB concentration range 151–3025 ng/ml; accuracy of 4.97% or less was obtained for PMTBSO concentrations of 1672–20 068 ng/ml. The intra-day precision (coefficient of variation) of the procedure was found to be no greater than 5.28% for PMTB and 7.9% for PMTBSO. Inter-day accuracy and precision measurements were similar.

INTRODUCTION

Compounds containing a thiocarbonyl group (C = S) find use as pesticides and drugs and are known to cause a variety of toxic effects in experimental animals and man. Thiobenzamide derivatives have been useful model compounds for investigating toxic properties of chemicals containing the group. Thiobenzamide and certain of its ring-substituted derivatives are hepatotoxic [1–3] and nephrotoxic [4] in the rat. The N-methyl derivative, N-methylthiobenzamide (NMTB), is a potent lung toxin in mice and rats [5]. Evidence has been presented that the metabolism of the thiocarbonyl group via two sequential oxidations is required for the expression of hepatic [6] and pulmonary [7] injury.

Radiometric methods have been employed to investigate the hepatic and pulmonary metabolism of toxic thiobenzamide derivatives *in vitro* [6,7]. The publish-

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ed assays, while very sensitive, have the disadvantage of requiring the synthesis and handling of radioactive samples.

We have developed a procedure for the simultaneous measurement of a toxic ring-substituted thiobenzamide, *p*-methylthiobenzamide (PMTB) and its S-oxide metabolite, *p*-methylthiobenzamide-S-oxide (PMTBSO) in rat plasma samples. The method is based on solid-phase extraction followed by high-performance liquid chromatography (HPLC) and UV detection. The technique is sensitive and reproducible. We have applied it to the determination of PMTB and PMTBSO in plasma of rats dosed parenterally with PMTB.

EXPERIMENTAL

Chemicals and reagents

PMTB and NMTB were synthesized in our laboratory according to the method of Fairfull *et al.* [8], evaluated by the appropriate analytical and spectral methods and found to be greater than 99% pure. PMTBSO was synthesized as described previously [4] by dissolving PMTB in glacial acetic acid followed by the dropwise addition of 30% hydrogen peroxide; purification was achieved by chloroform extraction and PMTBSO obtained following rotary evaporation and crystallization following the addition of hexane. Identification and purity were confirmed by UV and high-resolution mass spectrometry (calculated molecular mass, 167.0405; found, 167.0412) and thin-layer chromatography (TLC; support, silica gel GHLF; solvent system, methanol–chloroform, 3:97, v/v; R_F, 0.14). Methanol used for mobile phase was HPLC grade and obtained from Fisher Scientific (St. Louis, MO, U.S.A.). TLC plates were from Analtech, (Newark, DE, U.S.A.). All other reagents were at least reagent grade.

Stock solutions of internal standard (NMTB), PMTB and PMTBSO were prepared daily in methanol–water (55:45, v/v). Dilutions from this stock were made in 55% methanol–water to provide the various concentrations of PMTB and PMTBSO necessary in plasma. Aliquots of fresh, pooled plasma were spiked just prior to extraction and processed immediately.

Animals

Male Sprague–Dawley rats were obtained from a locally maintained Sasco derived breeding colony; animals were caged in groups of two or three and housed in a temperature-controlled room with a 12-h light–dark cycle; they had continuous access to Purina lab chow and tap water.

Chromatography system

Analyses were completed using a Beckman Model 421 liquid chromatography system controller, Beckman Model 110A solvent metering pump (Beckman Instruments, Berkeley, CA, U.S.A.), Shimadzu SPD-6A variable-wavelength UV detector (Shimadzu, Kyoto, Japan) and a Beckman Model 210 sample injector

fitted with a 50-μl loop. Chromatograms were integrated and recorded using a Microtech computer (Microtech Computers, Lawrence, KS, U.S.A.) fitted with a chromatography data acquisition control program (Instrument Design Laboratory, University of Kansas, Lawrence, KS, U.S.A.).

Separations were carried out using a 250 mm × 4.6 mm I.D. column packed with octadecylsilane (Ultrasphere, 5 μm particle size) and a direct-connect guard column (30 mm × 2.1 mm I.D.) packed with pellicular C_{18} silica, 37–44 μm particle size (Alltech, Waukegan, IL, U.S.A.). The mobile phase used was methanol water (55:45, v/v). The column was operated at room temperature (22 ± 1°C). A flow-rate of 1 ml/min was utilized and the wavelength for detection was 290 nm, the absorbance maximum for PMTB and NMTB. The absorbance maximum for PMTBSO is slightly higher than 290 nm, at 334 nm, but it absorbs strongly enough at 290 nm as to allow satisfactory detection.

Preparation of spiked samples and samples from treated animals

Rats were anesthetized with pentobarbital, 60 mg/kg intraperitoneally, and whole blood was collected into heparin-rinsed syringes via aortic puncture. Plasma was obtained following centrifugation of whole blood for 10 min at 0°C and 10 420 *g*. Aliquots of plasma (500 μl) were placed in PTFE-lined plastic tubes and spiked with a known concentration of PMTB and internal standard (NMTB) contained within 10 μl and prepared in methanol–water (55:45, v/v).

For collection of serial arterial blood samples, the femoral artery was catheterized and the catheteter externalized according to the procedure and apparatus described by Yourick and Tessel [9]. The rats were allowed to recover for one day and then dosed with 1.2 mmol/kg PMTB intraperitoneally as a fine suspension in corn oil (injection volume, 2.5 ml/kg). Serial blood samples were collected 15 and 30 min, and 1, 3, 4, and 6 h later, plasma was obtained, spiked with a known concentration of internal standard and processed immediately as described below.

Extraction procedure

A single solid-phase extractor (2.8 ml capacity, 200 mg sorbent, octadecyl bonded silica; Analytichem International, Harbor City, CA, U.S.A.) was used for each plasma sample; following conditioning with 2 ml of 100% methanol and a wash with 9 ml of water, the spiked plasma sample was applied and vacuum-filtered to dryness. The sorbent was then washed with 4 ml of methanol–water (10:90, v/v) and the compound of interest eluted with methanol into a collecting tube. A 200–300 μl aliquot of eluate was placed in a 1 ml bottle with PTFE-lined screw cap lid and evaporated under a gentle stream of nitrogen; the residue was then reconstituted with an equivalent volume of methanol water (55:45, v/v) and chromatographed.

Chromatographic procedure

Once stable chromatographic conditions had been established, duplicate samples were injected. Calculations were completed from plasma standard curves using the ratio of peak areas of PMTB or PMTBSO to internal standard from the integrated chromatogram.

RESULTS AND DISCUSSION

Chromatographic separation

Resolution of PMTB and PMTBSO from substances endogenous to plasma was achieved within 9 min. Retention times for the internal standard (NMTB), PMTB and PMTBSO were 6.0, 6.9 and 7.8 min, respectively. Fig 1A shows a chromatogram of processed blank plasma. No peaks appear after 1.84 min in the chromatogram. Also shown are chromatograms of plasma spiked with internal standard, parent compound and metabolite (B) and that obtained from the plasma of a rat dosed with PMTB and spiked with internal standard (C). The chromatogram of spiked plasma resembles that of the animal which had been dosed with PMTB.

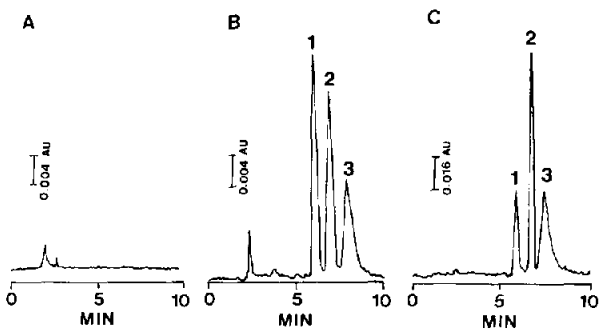

Fig. 1. (A) Chromatogram of blank plasma following solid-phase extraction and reconstitution in 55% methanol–water. (B) Chromatogram of plasma spiked with 3025 ng/ml NMTB (peak 1), 3025 ng/ml PMTB (peak 2) and 13 378 ng/ml PMTBSO (peak 3) following solid-phase extraction and reconstitution in 55% methanol–water. (C) Chromatogram of plasma obtained from a rat which had been treated with 1.2 mmol/kg PMTB. The concentrations of NMTB, PMTB and PMTBSO were 3025 ng/ml, 8394 ng/ml and 46 396 ng/ml, respectively.

Linearity and sensitivity

The linearity and sensitivity of the procedure were investigated by spiking plasma samples with 15–9070 ng/ml PMTB plus internal standard and 284 - 80 270 ng/ml PMTBSO plus internal standard. Replicate determinations of ten different concentrations of PMTB or PMTBSO were used to construct the curves. The relationships between peak-area ratios (PMTB/NMTB and PMTBSO/NMTB) to the concentrations of PMTB or PMTBSO were linear as judged by

correlation coefficients of 0.984 for PMTB and 0.969 for PMTBSO. A slope of 0.0268 and a *y*-intercept of 3.573 were obtained from the regression equation of the plasma curve of PMTB. The slope and *y*-intercept of the equation of PMTBSO's curve were 0.0022 and 5.2474, respectively. The minimum concentration detectable at a signal-to-noise ratio of 5:1 in plasma was 15 ng/ml PMTB and 33 ng/ml PMTBSO; 7.5 ng/ml PMTB was detectable in plasma but quantitation was not established.

Recovery, accuracy and precision of extraction

The recovery of compounds following extraction was quantified by spiking aliquots of plasma obtained from a previously untreated animal with a single concentration of PMTB and PMTBSO and internal standard (3025 ng/ml PMTB, 3025 ng/ml NMTB and 13 380 ng/ml PMTBSO). This was compared to the preparation of all three compounds in mobile phase and injected directly onto the analytical column. The spiked plasma was processed and the resultant reconstituted extract chromatographed. The results are shown in Table I. Greater than 96% of the internal standard can be recovered from plasma following the indicated extraction and as much as 94% of the PMTB and 84.4% of the metabolite could be extracted.

TABLE I

RECOVERY OF PMTB AND PMTBSO FROM PLASMA

Values in parentheses are coefficients of variation (%); $n = 3$–5.

Analyte	Concentration (ng/ml)	Mean area		Recovery (%)
		Direct injection	Plasma extract	
NMTB	3025	1381 (7.75)	1335 (7.91)	96.9 (11.97)
PMTB	3025	1533 (2.70)	1429 (7.86)	93.9 (8.27)
PMTBSO	13378	1188 (10.22)	1000 (9.87)	84.4 (6.97)

Table II depicts the intra-day accuracy and precision of the method from a set of plasma samples spiked with 151–3025 ng/ml PMTB and 1672–20 068 ng/ml PMTBSO. Accuracy is expressed as relative error and precision as coefficient of variation (C.V.). From 1.52 to 15.23% error was incurred in quantifying PMTB while 0.24 to 4.97% relative error was obtained when measuring PMTBSO. Precision (C.V.) of the assay ranged from 1.31 to 5.28% for PMTB and from 0.8 to 7.9% for PMTBSO. The inter-day accuracy and precision of the method is shown in Table III. With respect to accuracy, estimated concentrations were within 12% or less of the concentrations added to plasma. The C.V.'s of the two compounds ranged from 0.17 to 14.8%.

TABLE II

INTRA-DAY PRECISION AND ACCURACY OF PMTB AND PMTBSO EXTRACTION FROM PLASMA ($n = 3$)

Spiked concentration (ng/ml)		Mean assayed concentration (ng/ml)		Coefficient of variation (%)		Relative error[a] (%)	
PMTB	PMTBSO	PMTB	PMTBSO	PMTB	PMTBSO	PMTB	PMTBSO
151	1672	147	1655	5.28	3.13	2.65	1.02
302	2006	256	1908	3.57	4.84	15.23	4.89
756	13378	724	12713	2.03	7.90	4.23	4.97
1512	16723	1535	16763	1.31	0.80	1.52	0.24
3025	20068	2943	19710	2.40	6.86	2.71	1.78

[a] Relative error = [(mean spiked concentration − mean assayed concentration)/mean spiked concentration] × 100%.

Application of the method

The plasma concentration time profile for PMTB and PMTBSO is described in Fig. 2 as an application of the assay. Blood samples were drawn from arterially cannulated rats at 15 and 30 min and at 1, 3, 4 and 6 hours following intraperitoneal administration of PMTB. PMTBSO may be detected in plasma as early as 15 min after dosing. Peak levels of PMTB as high as 7.1 μg/ml at 1 h were observed

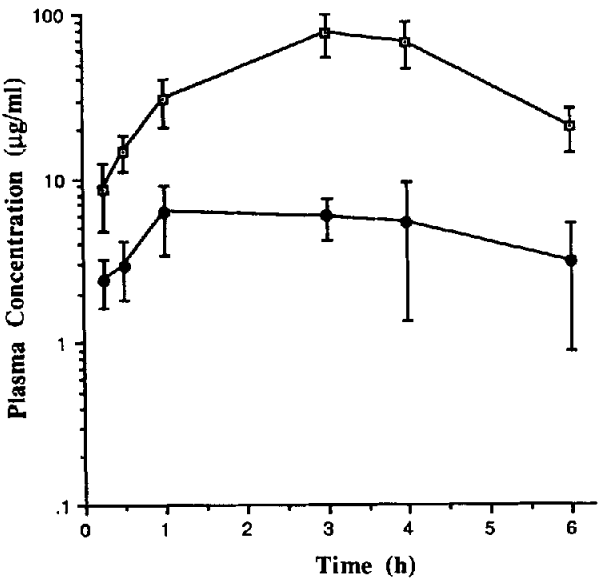

Fig. 2. Plasma levels of PMTB (●) and PMTBSO (□) in rats. Rats were dosed with 1.2 mmol/kg PMTB as described in the Experimental section. Each point represents the mean of four or five rats. Vertical lines equal ± S.E.

TABLE III

INTER-DAY PRECISION AND ACCURACY OF PMTB AND PMTBSO FROM PLASMA

$n = 3$. The values were obtained by analyzing replicate spiked plasma samples on three different days over a period of eight weeks.

Spiked concentration (ng/ml)		Mean assayed concentration (ng/ml)		Coefficient of variation (%)		Relative error[a] (%)	
PMTB	PMTBSO	PMTB	PMTBSO	PMTB	PMTBSO	PMTB	PMTBSO
30	368	27	368	7.61	0.17	10.00	0.00
756	13378	769	15024	5.68	14.84	1.72	12.30
1512	16723	1658	16494	10.37	7.57	9.66	1.37
3025	20068	2959	20210	11.03	5.54	2.18	0.71

[a] Relative error = [(mean spiked concentration − mean assayed concentration)/mean spiked concentration)] × 100%.

while levels of metabolite were highest at 81.5 μg/ml by 3 h. By 6 h, levels of both had dropped considerably. Our data provides the first *in vivo* demonstration of the metabolic conversion of PMTB to the S-oxide. Coadministration of a metabolic inhibitor in combination with PMTB eliminated the nephrotoxicity observed when PMTB was administered alone [4]. This method will provide a powerful tool for correlating plasma levels of metabolite with resultant toxicity following PMTB administration.

In conclusion, a sensitive, accurate and precise method was described here utilizing solid-phase extraction, reconstitution and HPLC. Adequate and selective recovery of parent, metabolite and internal standard could be easily obtained. The advantages of using a solid-phase extraction procedure followed by a reconstitution step are evident; the solid-phase extractor effectively removes interfering substances endogenous to plasma, is readily adaptable to reversed-phase or normal-phase chromatography depending on the reconstitution solvent, and is relatively cheap. Alternatively, to minimize costs, extraction columns may be regenerated and recycled by washing with methanol as described by Kabra *et al.* [10]. The presence of a concentrating step following extraction allows detection when levels of parent or metabolite fall below the detection limits.

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