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Short Communication

Determination of *N*-methylthiobenzamide in rat plasma by high-performance liquid chromatography

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Introduction

Several animal models exist which allow investigators to examine pulmonary edema that results from an increase in lung microvascular permeability. *N*-methylthiobenzamide (NMTB, Fig. 1a) is a compound which, when administered systemically to rodents, causes this type of pulmonary edema, with accompanying hydrothorax and pleural effusions [1]. The mechanism by which NMTB damages the lung is thought to be mediated through metabolism. Specifically, a two-step oxidation of the compound's sulphur atom must occur, followed by covalent binding of the resultant intermediate to tissue macromolecules [2]. The primary lines of evidence which support this hypothesis have been obtained by incubating radiolabelled NMTB with lung microsomes [3]. After incubation, metabolites of NMTB were separated and quantified using thin layer chromatography and liquid scintillation spectrometry, respectively. Covalent binding (CVB) of the radiolabelled metabolites to lung tissue was also measured, and a positive correlation was made between the metabolism and CVB of NMTB and the toxic response induced by NMTB in rodents.

There have been no attempts to characterize the *in vivo* metabolism of NMTB, or other pneumotoxins purported to act via metabolism, such as alpha-naphthylthiourea (ANTU) [4]. Described here is the development of methodology to extract from plasma and measure by high-performance liquid chromatography (HPLC) small quantities of NMTB.

Experimental

Chemicals

NMTB was synthesized as previously described [2]. Thiobenzamide (TB, Fig. 1b) and tertiary-butyl methyl ether (TBME) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Water was deionized and doubly distilled. Chloroform and isopropanol were obtained from Fisher Scientific, Inc. (St. Louis, MO). All reagents used were of chromatography grade.

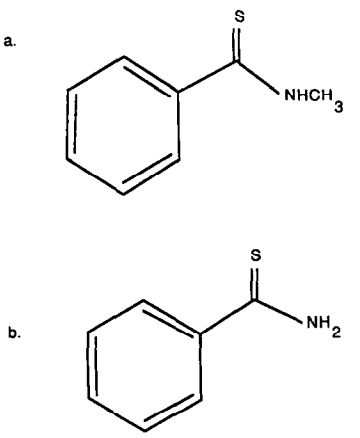

Figure 1
Structure of (a) *N*-methylthiobenzamide and (b) thiobenzamide.

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HPLC system

Separation of NMTB and the internal standard TB was afforded using a normal-phase HPLC system. The apparatus included a Beckman model 110-A pump which delivered an isopropanol–chloroform (0.5:99.5%, v/v) mobile phase to the column at a flow rate of 1.0 ml min^{-1} at ambient temperature. The analytical column was a 250 × 4.6 mm Econosil column packed with silica (5 μm particle size) (Alltech, Waukegan, IL, USA), and was fitted with a silica-packed Direct-Connect Guard Column (also Alltech). An Altex injector was used that was fitted with a 50-μl loop. Compounds were detected with a Shimadzu SPD-6A UV Spectrophotometric Detector (Shimadzu Scientific Instruments, Inc., Kyoto, Japan), which was set at a wavelength of 290 nm. Data were collected and analysed using Chromatography Data Acquisition and Control Software (Instrument Design Laboratory, University of Kansas, KS, USA) and a Microtech computer. After use the chromatography system was flushed with isopropanol–chloroform (30:70%, v/v).

Animals and compound administration

Male Sprague-Dawley rats (200–250 g) were obtained from Sasco Inc. (Omaha, NE, USA) and caged in groups of two or three. All rats were housed in a temperature-controlled, 12-h light/dark cycle environment and allowed access to water and Purina Lab Chow *ad libitum*. NMTB was injected i.p. at a dose of 0.38 mmol kg^{-1} as a solution in corn oil, and at a volume of 2 ml kg^{-1}.

Determination of NMTB in rat plasma

At 8, 15, 60, 120 or 180 min after receiving NMTB, rats were anaesthetized with either methoxyflurane (inhalation) or sodium pentobarbital (50 mg kg^{-1}, i.p.) and maintained under anaesthesia with methoxyflurane. Approximately 5 ml of blood was obtained from the abdominal aorta with a heparinized syringe, and plasma was obtained by centrifuging the blood at 650 ***g*** for 8 min at room temperature. A 1-ml aliquot of plasma was added to 2 ml of TBME and 10 μl of an appropriate concentration of TB (internal standard) in isopropanol–chloroform (0.5: 99.5%, v/v). The mixtures were mixed vigorously for 1 min, and then centrifuged at 500 ***g*** for 5 min at room temperature. An 800-μl aliquot of the resultant supernatant was transferred to a small screw-cap bottle and evaporated under a gentle stream of nitrogen. The remaining residue was resolubilized with 400 μl of mobile phase and mixed vigorously for about 30 s. Fifty microlitres were chromatographed on the HPLC system.

Results and Discussion

To assess the sensitivity of the HPLC system described in the Experimental section, a range of concentrations of NMTB dissolved in mobile phase were injected onto the system at various detector sensitivities (AUFS). A measurable signal was generated by as little as 0.7 ng of NMTB, which had a signal to noise ratio of 10:1 and a retention time of 5.5 min. The behaviour of TB on this HPLC system was also assessed. We felt that structural similarities shared by TB and NMTB (Fig. 1) as well as their similar UV spectral properties could allow for the use of TB as a suitable internal standard in any extraction procedure developed. Thiobenzamide had a retention time of 9.0 min, and a signal was generated by as little as 1.4 ng at a signal to noise ratio of 13:1. Since the two compounds (NMTB and TB) generated similar signals at the same sensitivity and since both were resolved well using the described HPLC system (Fig. 2a), TB was used as an internal standard in developing an extraction procedure.

Both NMTB and TB could be extracted from plasma using TBME, and no interfering peaks resulted from the extraction of plasma alone (Fig. 2a,b). The absolute recovery of the extraction procedure was determined by comparing peak areas of the thioamides extracted from spiked plasma (0.756 and 7.56 μg ml^{-1}) with the peak areas of the thioamides dissolved at the same concentration in mobile phase. The absolute recoveries of extraction ranged between 70.1–81.9% (Table 1). It therefore appeared that a single extraction with 2 vol TBME allowed for the extraction of a substantial fraction of both NMTB and TB.

The final concentration of NMTB in each sample would be estimated by calculating the ratio of the peak area of NMTB to the peak area of TB. This ratio would then be used in conjunction with a standard curve generated using peak height ratios of the added concentration of TB and several concentrations of NMTB. A general description of this method for quantitation can be found in Snyder and

Table 1
Absolute recoveries of the extraction of thioamide compounds*

Analyte	Concentration ($\mu g\ ml^{-1}$) added	Recovery (%)†	RSD (%)‡
Thiobenzamide	0.756	81.9	4.44
	7.56	73.5	4.44
N-methylthiobenzamide	0.756	77.9	13.8
	7.56	70.1	14.01

*Details of the extraction procedure from plasma are described in the Experimental section.
†Peak area of the compound extracted from plasma compared with that of the thioamide in mobile phase, $N = 3$.
‡Relative standard deviation, $N = 3$.

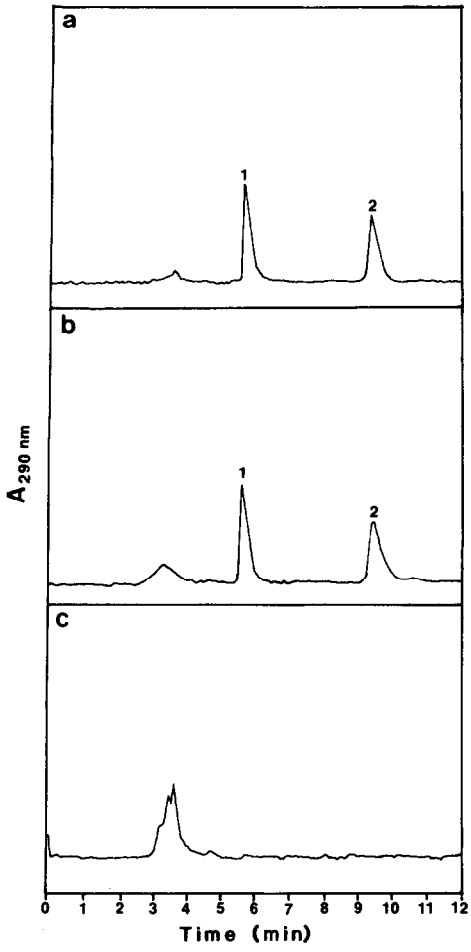

Figure 2
Chromatograms of: (a) NMTB (peak 1) and TB (peak 2) dissolved in mobile phase. Amounts detected here: NMTB, 28 ng; TB, 24 ng. (b) resolubilized extract of plasma spiked with NMTB and TB. (c) resolubilized plasma blank extract.

Kirkland [5]. Pilot experiments indicated that NMTB plasma levels encompassed a range of $100 \pm 0.10\ \mu M$ ($15.12 \pm 0.015\ \mu g\ ml^{-1}$) when examined over a period of 3 h. Since the integration of peaks required that the concentration of TB be similar to the concentration of NMTB in each sample, a series of standard curves were generated at three different detector sensitivities (0.32, 0.04 and 0.002 AUFS) to allow for the on-scale detection of all samples. The response of the system at each of the three sensitivities was linear as judged by correlation coefficients of the standard curves each equalling 0.999.

The recovery and precision of the method were also assessed. The results are shown in Table 2. Using the peak area ratio calculation with the internal standard, the amount of NMTB recovered from each sample relative to the amount added was about 95% with no significant dependence on concentration.

This assay was used for the quantitation of plasma NMTB in rats. Intraperitoneal administration of a pneumotoxic dose of NMTB to rats resulted in rapid absorption of the compound and a steady clearance over time (Fig. 3). This assay will be used in trying to ascertain whether certain drug pretreatments have any effect on NMTB metabolism. A recent publication from this laboratory suggests that the attenuation of NMTB-induced pulmonary edema by reserpine may be mediated by reserpine's capacity to alter the biotransformation of NMTB [6]. This assay will be employed to investigate this issue.

Table 2
Recovery and precision for the determination of *N*-methylthiobenzamide (NMTB) in plasma

Concentration ($\mu g\ ml^{-1}$) added	Concentration ($\mu g\ ml^{-1}$) found	Recovery* (%) ± RSD†
0.756	0.717	98.4 ± 8.7
7.56	7.19	95.1 ± 10.6

*Concentration found divided by concentration added expressed as a percentage.
†Relative standard deviation, $N = 3$.

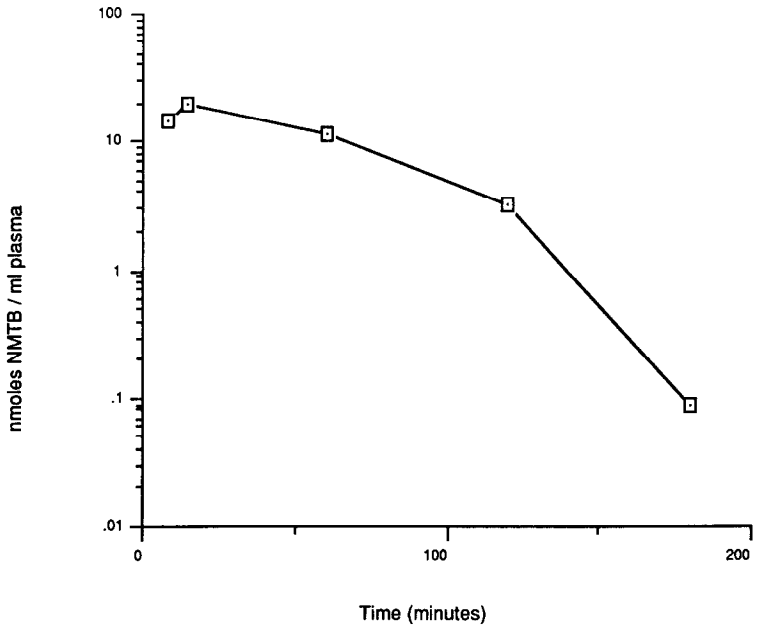

Figure 3
Plasma disappearance of NMTB. Dosing and sampling procedures are described in the Experimental section.

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