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RAPID AND SENSITIVE METHOD FOR THE MICROASSAY OF NITROSOBENZENE PLUS PHENYLHYDROXYLAMINE IN BLOOD

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SUMMARY

An assay method has been developed for the determination of the combined concentration of nitrosobenzene plus phenylhydroxylamine (as nitrosobenzene) in small volumes of blood. The initial step in the procedure consisted of the simultaneous oxidation of phenylhydroxylamine to nitrosobenzene and of ferrous hemoglobin to methemoglobin by ferricyanide. Nitrosobenzene in the ferricyanide-treated blood samples was then extracted into ethyl acetate, and separated and quantitated by reversed-phase high-performance liquid chromatography with electrochemical detection. The sensitivity limit for nitrosobenzene in blood was in the pmol/ml concentration range, less than 100 μl of blood was required for assay, and the procedure was convenient for routine multisample use. In comparison with previous assays, this method was more sensitive, had a lower coefficient of variation, and required 25—40 fold smaller blood sample volumes. The method was combined with the orbital sinus bleeding technique in order to follow the nitrosobenzene time course in vivo using small serial blood samples from rats treated with intraperitoneal injections of phenylhydroxylamine or aniline.

INTRODUCTION

A variety of aromatic amine and nitro compounds, including drugs such as dapsone and chloramphenicol, industrial chemicals such as aniline, and environmental chemicals such as 4-aminobiphenyl and 2-napthylamine, undergo in vivo conversion to N-hydroxy and nitroso derivatives during their metabolic clearance. These metabolic intermediates have been suggested to be mediators of the toxic effects of the parent compounds, which include methemoglobinemia, blood dyscrasias, mutagenicity, and carcinogenicity [1—5]. In most cases, only a small percentage of a dose of the parent compound is converted to N-hydroxy and nitroso metabolites, and these compounds are usually relatively unstable in biological systems. Accordingly, detection and quantita-

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tion of these important metabolites has been difficult because: (1) their concentrations in blood and tissues are low, requiring detection methods with high sensitivity, and (2) loss of the unstable compounds is likely to occur during extraction and separation procedures.

An additional factor complicating the in vivo quantitation of aromatic N-hydroxy and nitroso compounds has been demonstrated in studies of the formation of phenylhydroxylamine and nitrosobenzene during aniline clearance in vivo. These experiments showed that phenylhydroxylamine and nitrosobenzene are interconverted in blood via an enzyme-mediated redox cycle [3]. Thus, in order to evaluate the extent of aniline N-oxidation in vivo, it is necessary to measure the sum of the phenylhydroxylamine and nitrosobenzene concentrations in blood. The assay procedure used in the above experiments continues to be perhaps the most widely used method for quantitation of the sum of nitrosobenzene plus phenylhydroxylamine in blood [6]. It is a colorimetric method that is sensitive in the nanomolar range, but requires relatively large volumes of blood (2—3 ml) and has a high sample-to-sample variability. In addition, several extraction and derivatization steps are required, making the method inconvenient for routine assay of large numbers of samples.

Recently, sensitive techniques utilizing high-performance liquid chromatography (HPLC) and electrochemical detection have been introduced to determine nitrosobenzene or phenylhydroxylamine [7—10]. These methods, designed to measure either nitrosobenzene or phenylhydroxylamine alone in aqueous solutions or in tissue homogenates, have not been adapted for the quantitation of aniline N-oxidation products in blood. This communication presents a simple and rapid procedure for the combined assay of nanomolar concentrations of the major aniline N-oxidation products (phenylhydroxylamine plus nitrosobenzene) in small volumes of blood. The procedure is significantly more sensitive than the previous colorimetric assay, allowing the blood sample volumes required for routine analysis to be reduced to less than 100 μl. Loss of phenylhydroxylamine, which is unstable in aqueous solution [8], is avoided by conversion of phenylhydroxylamine to nitrosobenzene prior to extraction and chromatography; interference by biological materials is avoided by a single extraction step and HPLC separation; and quantitation of nitrosobenzene is accomplished by electrochemical reduction as the column effluent passes across a glassy carbon electrode. This method is suitable for routine analysis of multiple small blood samples serially obtained from laboratory rodents.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 200—250 g were obtained from Camm Research (Wayne, NJ, U.S.A.) and maintained in hanging stainless-steel cages on standard lab chow (Wayne Lab Blox, Allied Mills, Chicago, IL, U.S.A.) and water ad lib.

Chemicals

Phenylhydroxylamine was prepared by zinc-catalyzed reduction of nitro-

benzene in the presence of aqueous ammonium chloride [11] and recrystallized three times from benzene—hexane. Nitrosobenzene (Aldrich, Milwaukee, WI, U.S.A.) was purified by vacuum sublimation. Aniline · HCl (Aldrich) was dissolved in water and purified by extraction into ethyl acetate at neutral pH, precipitation with hydrogen chloride gas, and recrystallization two times from ethanol–ethyl acetate. These compounds were stored in the dark at −20°C under nitrogen. Other chemicals were reagent grade (Aldrich) and were used without further purification. Solvents were distilled-in-glass grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

HPLC system

Chromatography was performed on a system consisting of a Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne No. 7125 injector port, a Whatman guard column, a Partisil PXS 5/25 ODS column (Whatman, Clifton, NJ, U.S.A.), and an LC-3 amperometric detector with an electrochemical cell containing a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A working electrode potential of −200 mV versus a Ag/AgCl reference electrode was used throughout, and the system was operated at a flow-rate of 1.2 ml/min.

Analytical procedure

Standard solutions of nitrosobenzene and phenylhydroxylamine in aqueous 1% methanol were prepared by addition of the compounds, dissolved in 25 μl methanol, to 2.5-ml aliquots of water. Standard solutions of nitrosobenzene in rat blood were prepared using blood obtained by cardiac puncture and divided into 2.5-ml aliquots. Nitrosobenzene was added to the blood samples in 25 μl methanol to yield the desired final concentrations. The blood samples were then mixed by gentle swirling for 1 min and 75-μl aliquots were assayed for nitrosobenzene.

For in vivo experiments, groups of rats received intraperitoneal injections of phenylhydroxylamine dissolved in 5% Tween 80 in 0.9% saline, or aniline · HCl dissolved in 0.9% saline. Serial blood samples were subsequently obtained from the orbital sinus [12, 13] of each animal in 75-μl heparinized capillary tubes and assayed for nitrosobenzene as described below.

The 75-μl blood samples were immediately hemolyzed in 175 μl of 0.005 *M* phosphate buffer, pH 7.0. Fifty μl of 20% potassium ferricyanide in water and 50 μl of 10% potassium cyanide in water were added with brief mixing, followed by 350 μl water-saturated ethyl acetate. The samples were extracted by rapid mixing with a vortex mixer for 2 min, then briefly centrifuged and stored over ice for up to 1 h.

Samples of the organic phases were injected into the HPLC system using a 20-μl sample loop attached to the injector port. Nitrosobenzene in the sample was quantitated by comparing the height of the nitrosobenzene peak to a standard curve of nitrosobenzene peak height versus concentration in rat blood.

RESULTS

HPLC of standard solutions of nitrosobenzene in ethyl acetate yielded a

single peak (retention volume = 8.3 ml) by electrochemical detection in the reducing mode. The response of the detector was linear over a nitrosobenzene concentration range of 20—400 pmol per 20 μl, which in this assay corresponds to a concentration range of 5—100 nmol/ml in the aqueous solutions prior to dilution and extraction. When aqueous solutions of nitrosobenzene (100 nmol/ml) were diluted with phosphate buffer, treated with ferricyanide and cyanide, and extracted with ethyl acetate, as described in Methods, over 90%

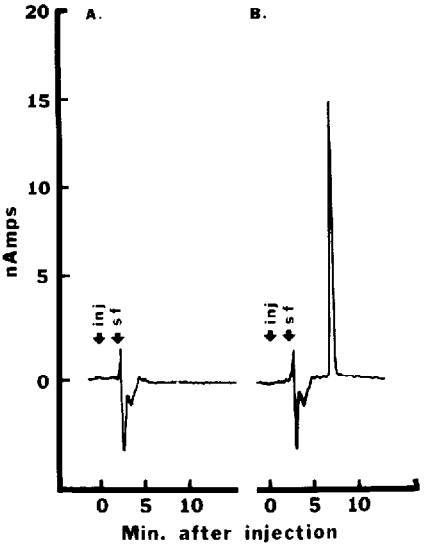

Fig. 1. Electrochemical detection of nitrosobenzene after HPLC of ethyl acetate extracts of rat blood. (A) Chromatogram of 20 μl of the ethyl acetate extract of control blood; (B) chromatogram of 20 μl of the ethyl acetate extract of blood containing 75 nmol/ml nitrosobenzene. inj = injection of the sample into the chromatographic system, sf = solvent front.

TABLE I

DETECTION AND QUANTITATION OF PHENYLHYDROXYLAMINE + NITROSOBENZENE IN ETHYL ACETATE EXTRACTS OF RAT BLOOD

Extraction efficiency of nitrosobenzene from blood*	63 ± 8.1%
Retention volume	8.3 ± 0.04 ml
Sensitivity limit**	0.5 nmol/ml
Coefficient of variation*** (at 5 nmol/ml)	6% (n = 5)
Standard curve linear regression parameters	
Y-intercept	0.15 nA
Slope	0.192 nA/(nmol/ml)
Correlation coefficient	0.996

*Mean recovery of nitrosobenzene at concentrations of 5, 25, 50, 75, and 100 nmol/ml blood.

**The sensitivity limit is taken as the concentration at which the nitrosobenzene peak height is twice the background noise level. This concentration was determined by a linear extrapolation of the standard curve.

***A single nitrosobenzene-spiked blood sample was divided into five aliquots which were extracted and assayed separately. The coefficient of variation was calculated as (S.D.)/(mean nitrosobenzene peak height) for the five aliquots.

of the nitrosobenzene was recovered in the organic phase. When aqueous solutions of phenylhydroxylamine (100 nmol/ml) were similarly treated, over 90% of the phenylhydroxylamine was also recovered in the organic phase as nitrosobenzene. In samples of rat blood spiked with nitrosobenzene (100 nmol/ml), 60% of the added nitrosobenzene was recovered after hemolysis in phosphate buffer, ferricyanide/cyanide treatment, and ethyl acetate extraction. Nitrosobenzene recovery was 8—10% lower in samples not treated with cyanide. An equivalent fraction of phenylhydroxylamine (100 nmol/ml) was recovered as nitrosobenzene from spiked rat blood samples after a similar extraction procedure. Stability experiments indicated that no loss of nitrosobenzene occurred from the organic phase after extraction from blood when the samples were maintained over ice for up to 4 h.

Fig. 1 shows typical chromatograms of ethyl acetate extracts of control and nitrosobenzene-spiked rat blood samples. The only major peak after the solvent front was that of nitrosobenzene, and no endogenous compounds were detected by electrochemical reduction in the vicinity of the nitrosobenzene peak. Total analysis time was approximately 8 min.

Peak height over the concentration range of 0—100 nmol/ml in rat blood was linearly related to concentration; regression analysis of the data yielded a correlation coefficient of 0.996. The limit of sensitivity (signal-to-noise ratio =

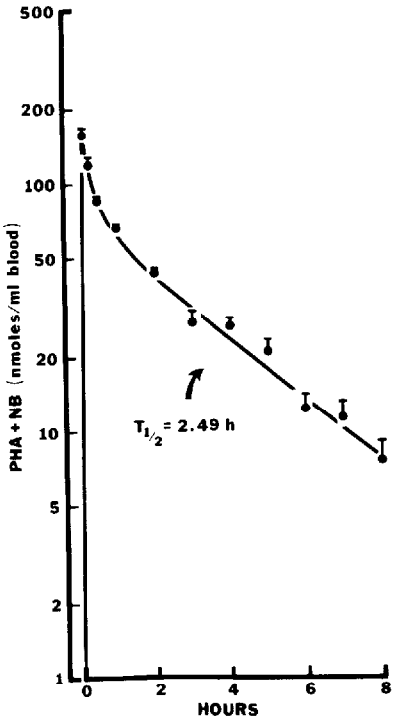

Fig. 2. Semi-logarithmic plot of phenylhydroxylamine + nitrosobenzene concentration in blood after intraperitoneal injection of phenylhydroxylamine (200 μmol/kg). Points and error bars shown are means ± S.D. (n = 4). The half-life of the terminal phase was determined from a regression line fitted to the final seven data points. PHA = phenylhydroxylamine, NB = nitrosobenzene.

2) was 500 pmol/ml blood and the coefficient of variation at 5 nmol/ml was 6%. Extraction efficiency was relatively constant over the range of 5—100 nmol/ml at 63 ± 8.1% (S.D.). These data are presented in Table I.

The use of the assay method in vivo is illustrated by the quantitation of phenylhydroxylamine + nitrosobenzene in serial blood samples from rats after intraperitoneal injection of phenylhydroxylamine or aniline · HCl. Standard solutions of nitrosobenzene were routinely assayed before and after each group of experimental samples, and indicated excellent stability of electrode sensitivity for nitrosobenzene. Fig. 2 shows the blood concentration time course of phenylhydroxylamine + nitrosobenzene after phenylhydroxylamine (200 μmol/kg) administration. Absorption was rapid, with the initial blood sample at 5 min showing the highest concentration of phenylhydroxylamine + nitrosobenzene (155 nmol/ml blood). Two phases of elimination then followed: a relatively rapid phase occurring over the first hour after administration, and a slower terminal phase which was linear on the semi-log plot with a half-life of 2.49 h.

The time course of phenylhydroxylamine + nitrosobenzene in rat blood after aniline · HCl (1.5 mmol/kg, intraperitoneally) was also determined (Fig. 3). Phenylhydroxylamine + nitrosobenzene formation was rapid, with the initial level of 57 nmol/ml at 10 min being the maximum observed. The clearance of phenylhydroxylamine + nitrosobenzene displayed complex kinetics, with a linear initial phase (half-life = 3.52 h) and a terminal phase of more rapid elimination.

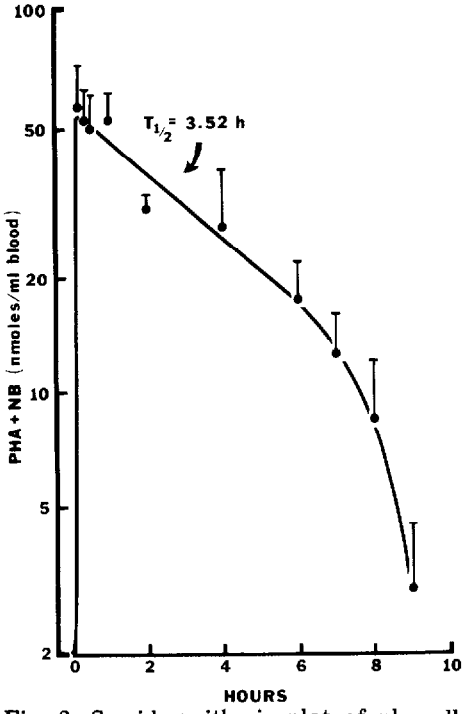

Fig. 3. Semi-logarithmic plot of phenylhydroxylamine + nitrosobenzene concentration in blood after intraperitoneal injection of aniline (1.5 mmol/kg). Points and error bars shown are means ± S.D. (n = 4). The half-life of the initial phase was determined from a regression line fitted to the first seven data points. PHA = phenylhydroxylamine, NB = nitrosobenzene.

DISCUSSION

This paper presents a simple and rapid method for quantitation of the combined amount of phenylhydroxylamine and nitrosobenzene present in small volume blood samples. Like the method of Herr and Kiese [6], the initial step is the addition of ferricyanide to the hemolyzed blood sample. This treatment oxidizes both phenylhydroxylamine to nitrosobenzene and hemoglobin to methemoglobin. Nitrosobenzene is more stable in blood than phenylhydroxylamine [11] and is more easily extracted from aqueous solution. Oxidation of hemoglobin to methemoglobin improves the extraction efficiency of nitrosobenzene from blood by eliminating ferrous heme as a binding site for nitrosobenzene [14]. The addition of cyanide to block methemoglobin by the formation of cyanomethemoglobin results in an additional small increase in the amount of nitrosobenzene extracted, presumably by preventing interaction between nitrosobenzene and ferriheme. Recovery of nitrosobenzene from ferricyanide-treated aqueous solutions of either nitrosobenzene or phenylhydroxylamine was greater than 90%, indicating that ferricyanide quantitatively oxidized phenylhydroxylamine to nitrosobenzene, but did not further oxidize nitrosobenzene to nitrobenzene.

Nitrosobenzene in the oxidized blood samples was extracted into ethyl acetate and assayed directly, after HPLC, by electrochemical reduction at a glassy carbon electrode. Electrochemical detection is a highly sensitive method which has been used with a variety of oxidizable and reducible substances in aqueous solution. When used with HPLC, electrochemical detection provides additional specificity beyond that usually associated with HPLC separations [15]. This is because electrode potentials may be selected which oxidize or reduce compounds of interest, but which do not cause electrochemical reactions in compounds eluting near compounds of interest. With this detection method, an interfering compound not only must co-chromatograph with the compound of interest, it must also show similar electrochemical characteristics. In the present assay, no other easily reducible compounds were found near the nitrosobenzene peak in chromatograms of ethyl acetate extracts of rat blood. Thus background noise from endogenous compounds was very low, which increased the sensitivity of the assay. In addition, a peak with the appropriate retention time could be identified as nitrosobenzene with a high degree of confidence.

The extraction efficiency of ethyl acetate for nitrosobenzene in equal volumes of aqueous solution was greater than 90%, whereas the extraction efficiency against equal volumes of blood was reduced to approximately 63%. This reduction in extraction efficiency was comparable to the 60% extraction efficiency obtained by Herr and Kiese [6] using carbon tetrachloride as the organic phase. Incomplete extraction may result from entrapment of nitrosobenzene in the membrane lipids and denatured protein of the erythrocytes. Although a rapid elimination of nitrosobenzene within the first minute after addition to blood could have resulted in the loss of some of the compound, this is unlikely because no further loss of nitrosobenzene was found when samples were extracted 3 min, rather than 1 min, after nitrosobenzene addition. Recent studies by Eyer and Lierheimer [16] also indicate that

nitrosobenzene is relatively stable in erythrocyte suspensions, with a half-life of approximately 50 min. Although in the present studies the extraction of nitrosobenzene from blood was not complete, extraction efficiency showed excellent reproducibility and was constant over the concentration range of the assay. Therefore, correction for incomplete extraction was straightforward.

The performance of the assay in vivo was demonstrated by determining the blood time course of phenylhydroxylamine + nitrosobenzene in rats after intraperitoneal injection of phenylhydroxylamine. By utilizing the orbital sinus blood sampling technique to collect serial 75-μl blood samples for assay, blood levels of phenylhydroxylamine + nitrosobenzene could be followed over time in individual animals. Phenylhydroxylamine was absorbed rapidly after intraperitoneal injection with maximal blood levels of phenylhydroxylamine + nitrosobenzene occurring within 5 min. The elimination time course showed two phases occurring during the 8 h following administration. Elimination according to an apparent two-compartmental model obviously could result from several mechanisms; however, this finding is consistent with recent data obtained in erythrocyte suspensions indicating the presence of several non-heme reversible intracellular binding sites for nitrosobenzene [16].

As indicated in the Introduction, cyclic interconversion of phenylhydroxylamine and nitrosobenzene within erythrocytes concomitant with the oxidation of hemoglobin to methemoglobin was demonstrated in the 1950's [17—19]. From these and other data, Kiese [3] postulated that phenylhydroxylamine-induced methemoglobin formation is mediated by a cyclic redox system including phenylhydroxylamine, nitrosobenzene, oxyhemoglobin, and methemoglobin. In this system, the blood levels of phenylhydroxylamine and nitrosobenzene would be governed by the position of the redox equilibrium as well as by the total amount of N-oxidized compound present. Since the relative concentrations of the hydroxylamine and nitroso forms are not known under varying experimental conditions, appropriate evaluation of the total amount of methemoglobin-forming compound present in blood must include quantitation of both phenylhydroxylamine and nitrosobenzene. An advantage of the assay procedure reported here for these types of studies is that it allows rapid and convenient measurement in a single procedure of the total amount of putative toxic compounds present (that is, both the hydroxylamine and nitroso forms).

Kiese [20] also demonstrated the presence of phenylhydroxylamine + nitrosobenzene in the blood of a dog during methemoglobinemia induced by aniline given intravenously. Subsequent studies showed that intravenous infusion of low levels of phenylhydroxylamine into a dog was associated with extensive methemoglobinemia [21]. From these results, Kiese [21] proposed that phenylhydroxylamine was a toxic metabolite derived from aniline responsible for the methemoglobinemia observed after aniline in vivo.

Data presented here show that phenylhydroxylamine + nitrosobenzene was also detectable in rat blood in significant levels after intraperitoneal injections of aniline · HCl. Maximal levels are reached within 10 min after injection. Elimination over the subsequent 7 h approximates first-order kinetics, and the half-life of this elimination phase is longer than that seen after phenylhydroxyl-

amine treatment. This lengthened half-life may reflect the elimination rate of the parent compound, aniline [22].

The terminal phase of phenylhydroxylamine + nitrosobenzene elimination after aniline treatment appears to deviate from linearity. This deviation was not seen at equivalent blood levels of phenylhydroxylamine + nitrosobenzene following intraperitoneal injections of phenylhydroxylamine. Several possible explanations exist for the observed elimination pattern. A concentration-dependent change in the metabolic disposition of aniline when blood levels of aniline are low could lead to a relative decrease in the proportion of aniline undergoing N-oxidation with respect to other clearance pathways during the terminal phase of elimination. Other alternatives include non-linear elimination kinetics of aniline, with phenylhydroxylamine + nitrosobenzene levels reflecting the kinetics of the parent compound; or an increase in the activity or availability of elimination pathways in the erythrocyte for nitrosobenzene and/or phenylhydroxylamine as the level of these compounds, or of methemoglobin, in the erythrocytes declines.

These preliminary results indicate the usefulness of the method described here for the investigation of the formation and elimination kinetics of phenylhydroxylamine + nitrosobenzene. The combined amount of these two putative toxic intermediates was measured in blood samples 25—40-fold smaller than required by previous methods, with no loss in sensitivity, and a decreased error. This assay, when combined with appropriate blood sampling technique, will greatly decrease the difficulty of studying the kinetics and toxicology of aniline N-oxidation in vivo by permitting blood time course data to be obtained from individual small laboratory animals. In addition, it is anticipated that this method may be easily modified for analysis of other aromatic hydroxylamine and/or nitroso compounds in biological systems.

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