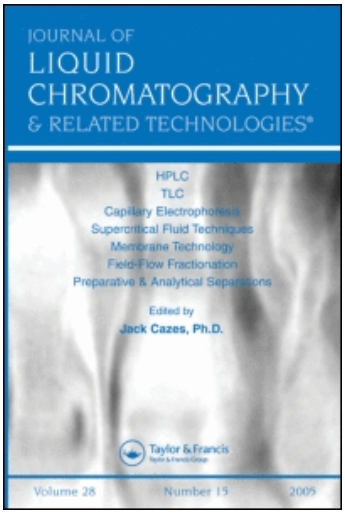

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Ion-Pairing Systems for Separation of *N*-Nitrosodimethylamine and its Metabolites in Reversed-Phase High-Performance Liquid Chromatography

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ION-PAIRING SYSTEMS FOR SEPARATION OF *N*-NITROSODIMETHYLAMINE AND ITS METABOLITES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Separation of *N*-nitrosodimethylamine and certain of its metabolites on a C_{18} column is made possible by the inclusion of ion-pairing reagent into a mobile phase consisting of 7 mM ammonium phosphate, pH 3.0. The use of alkanesulfonates (5 mM) of varying alkyl chain length to afford retention of the putative metabolites, monomethylamine and dimethylamine, is discussed. The incorporation of sodium 1-heptane- or 1-octanesulfonate into the mobile phase appears to provide optimal separation under conditions in which the parent nitrosamine is present in the samples at relatively low or high concentrations, respectively.

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INTRODUCTION

Metabolism of the potent carcinogen, *N*-nitrosodimethylamine (NDMA), *in vitro* (1-5) and *in vivo* (6-9) has been shown to proceed along two distinct pathways (Figure 1). The activating pathway, *N*-demethylation, results in the formation of formaldehyde (FORM), dinitrogen gas, and the highly reactive methylating species, methyldiazonium ion, which subsequently reacts with cellular nucleophiles (the predominant target being water, reaction with which leads to the formation of methanol [MEOH]). An alternative pathway, accounting for 10-20% or more of total metabolism *in vivo* (6,8,9) is metabolic denitrosation. This pathway results in the formation of formaldehyde, monomethylamine (MA), and a nitrogen-containing species (nitric oxide, which is rapidly oxidized in the presence of oxygen to nitrite or nitrate) (10-12). Dimethylamine (DMA) has also been reported to be a minor metabolite resulting from the denitrosative pathway in pigs (7).

In the present study, we describe reversed-phase high-performance liquid chromatographic (HPLC) assays which allow the determination of ^{14}C-radiolabeled parent nitrosamine, as well as metabolically formed carbon-containing products. The retention of the amine-containing products, which have relatively high pK_a values (MA, 10.66; DMA, 10.73) and which are therefore cationic at the pH (3.0) of the mobile phase, is afforded by incorporation of ion-pairing reagents into the mobile phase. The optimized assay conditions should allow the extents of metabolism occurring *via* the two competing pathways to be differentiated by allowing the quantitation of the amine products, which are unique to the denitrosative pathway.

MATERIALS AND METHODS

Materials

[^{14}C]Formaldehyde (15 mCi/mmol), [^{14}C]methanol (59 mCi/mmol) and [^{14}C]dimethylamine hydrochloride (58 mCi/mmol) were purchased from Amersham

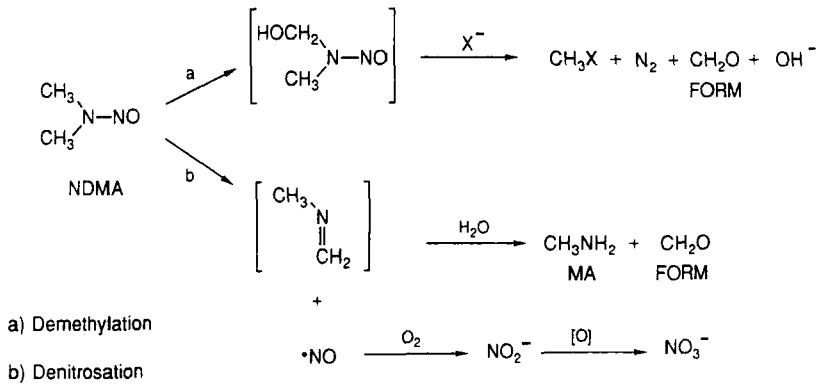

FIGURE 1. Pathways for the *in vitro* and *in vivo* metabolism of NDMA.

(Arlington Heights, IL). [^{14}C]Monomethylamine hydrochloride (7.2 mCi/mmol) and [^{14}C]NDMA (49.6 mCi/mmol) were from New England Nuclear (Boston, MA). Non-radiolabeled NDMA and sodium 1-pentanesulfonate were obtained from Sigma Chemical Co. (St. Louis, MO), while the sodium salts of 1-butane-, 1-octane-, 1-decane- and 1-dodecanesulfonic acids were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium 1-hexanesulfonate and sodium 1-heptanesulfonate were obtained from Fisher Scientific (New Lawn, NJ) and Eastman Kodak Co. (Rochester, NY), respectively.

Chromatographic Conditions

The HPLC system used consisted of a Model 510 pump (Waters Associates, Milford, MA), a Waters Model U6K injector, a Waters Model 481 LC spectrophotometric detector, and a Model IC Flo-one\β radioactive flow-through detector (Radiomatic Instruments, Tampa, FL) arranged in series. A 4.6 x 250 mm Spherisorb column (PhaseSep, Inc., Norwalk, CT) packed with 5 μm C_{18} particles was used at ambient temperature (~25 °C). The column was eluted at 1.0 ml/min with a mobile phase consisting either of 7 mM ammonium phosphate, pH 3.0, alone or of the same buffer containing one of the ion-pairing reagents at a final

concentration of 5 mM. Mobile phases were degassed under vacuum for one hour prior to use, and the column was allowed to equilibrate with mobile phase (~25 °C) for one hour prior to the first injection.

Each of the analytes of interest was injected in triplicate for each of the eight mobile phases. The four known or putative metabolites (FORM, MEOH, MA, and DMA), being [^{14}C]-radiolabeled, were detected *via* the flow-through radioactivity detector. The integrated peak areas so obtained were approximately equivalent to the cpm measured for equivalent volumes of standards quantified by liquid scintillation counting (Beckman Model LS 9000), with Aquassure as scintillant (New England Nuclear). Because it possesses a strong chromophore, NDMA was coinjected with the MA standard and was detected using the spectrophotometric detector, operated at a wavelength of 230 nm. This coinjection was done primarily to save time in performing the assays.

RESULTS AND DISCUSSION

While a variety of analytic methodologies are available for the detection of nitrosamines such as NDMA, including gas chromato-graphy (e.g., 13) and HPLC (e.g., 14), methodologies for quantifying NDMA *and* its putative metabolites simultaneously are less well developed. Often, in measuring total metabolism of this nitrosamine, investigators have employed separate assays for detection of the aldehyde and nitrogen-containing products (1-6,11,12). While the literature concerning the denitrosative pathway of NDMA metabolism *in vitro* was in large part based upon the detection of nitrite as an indicator of denitrosation, the measurement of denitrosation *in vivo* depends upon the detection of ^{14}C- or ^{15}N-labeled amine-containing products. This is due to the fact that, while detection of non-radiolabeled nitrite/nitrate or amine products is possible, these compounds are normal physiological constituents of blood and urine. Also, nitrite is metabolized very rapidly *in vivo*. In the past, detection of the amine products has involved the use of isotope dilution techniques (6) or HPLC coupled with radiometric detection (8,15,16). Ion-exchange columns have been utilized in the latter studies to provide

$$CH_3\text{-}(CH_2)_n\text{-}SO_3^- \quad Na^+$$

Alkanesulfonate	*n*	Number of carbons
1-Butanesulfonate	3	4
1-Pentanesulfonate	4	5
1-Hexanesulfonate	5	6
1-Heptanesulfonate	6	7
1-Octanesulfonate	7	8
1-Decanesulfonate	9	10
1-Dodecanesulfonate	11	12

FIGURE 2. Structure and nomenclature of the ion-pairing reagents employed.

retention of the cationic amines (8,15,16); however, a major disadvantage of these systems has been the inadequate separation of MA and DMA. Therefore, it has in the past been necessary to employ a second HPLC system to separate these putative amine-containing metabolites (8,16).

In the present work we have attempted, therefore, to develop an HPLC system capable of quantitating NDMA and its putative amine-containing metabolites in a single chromatographic run. The methodology makes use of the concept of ion-pairing (17, and references cited therein) to afford retention of the cationic analytes. For the purposes of optimizing the separation of the analytes of interest, alkanesulfonates of varying carbon chain length (Figure 2) were incorporated into an aqueous mobile phase. The parent nitrosamine (NDMA), being uncharged and relatively lipophilic, was retained on the reversed-phase column, either in the absence or presence of ion-pairing reagent (Figure 3). The decreased elution times observed for NDMA in the presence of alkanesulfonates of greater carbon chain length most likely resulted from the increasingly nonpolar

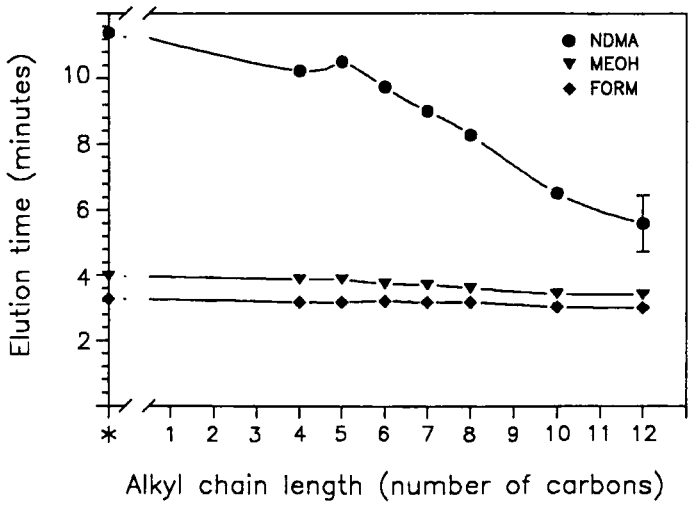

FIGURE 3. Effect of varying alkyl chain length on elution times for the neutral species, *N*-nitrosodimethylamine (NDMA), methanol (MEOH) and formaldehyde (FORM), in the absence (indicated by an asterisk) or presence of a series of alkanesulfonates. Each point represents the mean ± SD for three injections.

nature of the mobile phase caused by the presence of the hydrophobic alkyl groups of these ion-pairing reagents. The neutral metabolites, FORM and MEOH, being relatively polar, eluted near the solvent front (~2.4 min). In the case of these analytes, incorporation of alkanesulfonates of increasing carbon chain length had little effect on elution times. The cationic amine-containing metabolites, MA and DMA, eluted near the solvent front in the absence of ion-pairing reagent (Figures 4 and 5). However, the incorporation of alkanesulfonates of increasing carbon chain length into the mobile phase resulted in exponentially increasing elution times for these charged metabolites on the reversed-phase column. Considerable peak broadening was observed for MA and DMA in the presence of decane- and dodecanesulfonate. This fact, as well as the prohibitively long elution times observed under these conditions, severely limited the usefulness of these ion-pairing reagents for the present application.

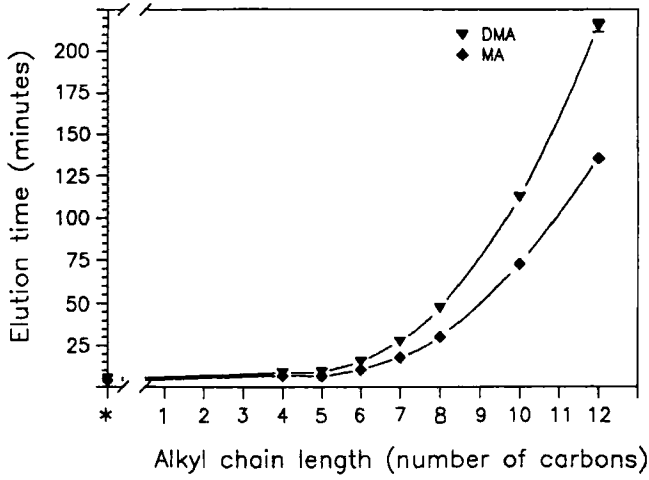

FIGURE 4. Effect of varying alkyl chain length on elution times for the cationic species, monomethylamine (MA) and dimethylamine (DMA), in the absence (indicated by an asterisk) or presence of a series of alkanesulfonates. Each point represents the mean ± SD for three injections.

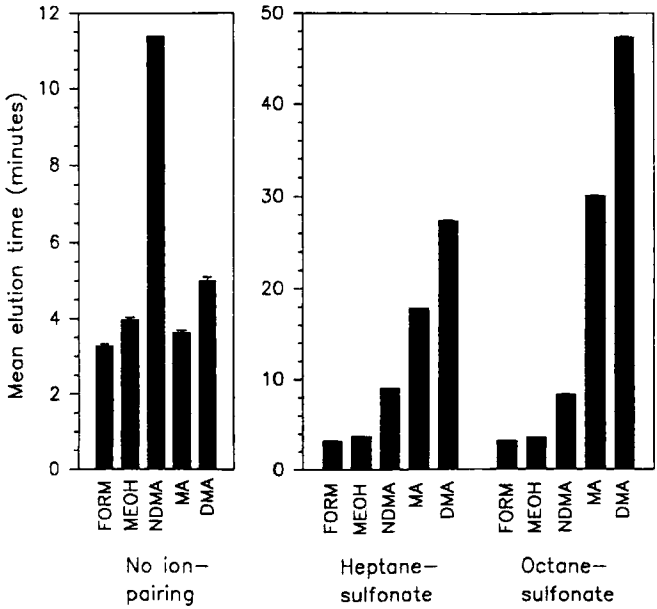

Figure 5. Elution times for formaldehyde (FORM), methanol (MEOH), *N*-nitrosodimethylamine (NDMA), monomethylamine (MA) and dimethylamine (DMA) in the absence of ion-pairing and in the presence of the indicated optimal ion-pairing agents. Note change of scale in ordinate. Each bar represents the mean ± SD for three injections of the individual standards.

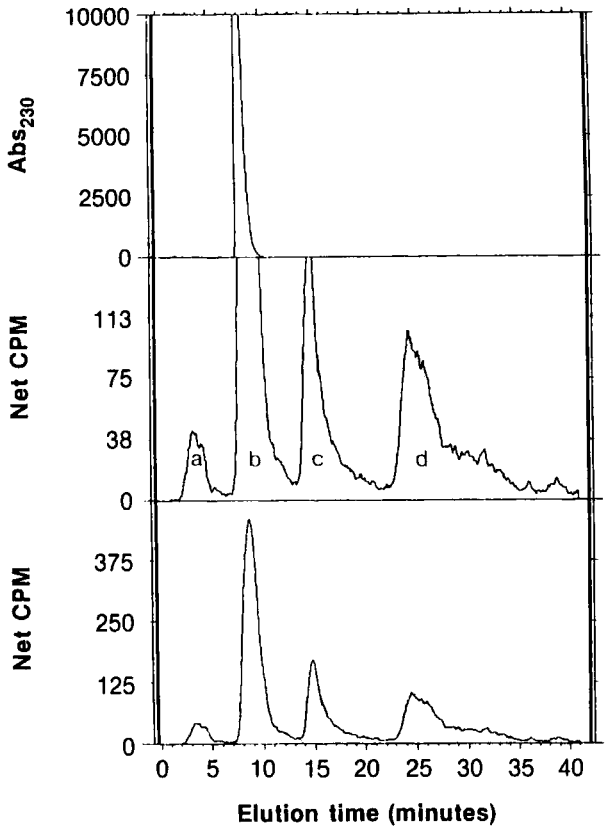

Figure 6. Chromatogram resulting from the coinjection of standards in the HPLC system with heptanesulfonate (5 mM) as ion-pairing reagent: (**a**) formaldehyde and methanol; (**b**) *N*-nitrosodimethylamine; (**c**) monomethylamine; (**d**) dimethylamine. Upper trace, UV absorbance at 230 nm; lower traces, radioactive counts per minute, shown for two different ordinate scales.

In studies in which only total metabolism is being examined, the use of non-modified aqueous mobile phase is probably sufficient to afford separation of the polar products from the parent nitrosamine (Figure 5). Under these conditions metabolism may be quantified either by monitoring the formation of polar metabolites or by observing the consumption of the nitrosamine. However, when

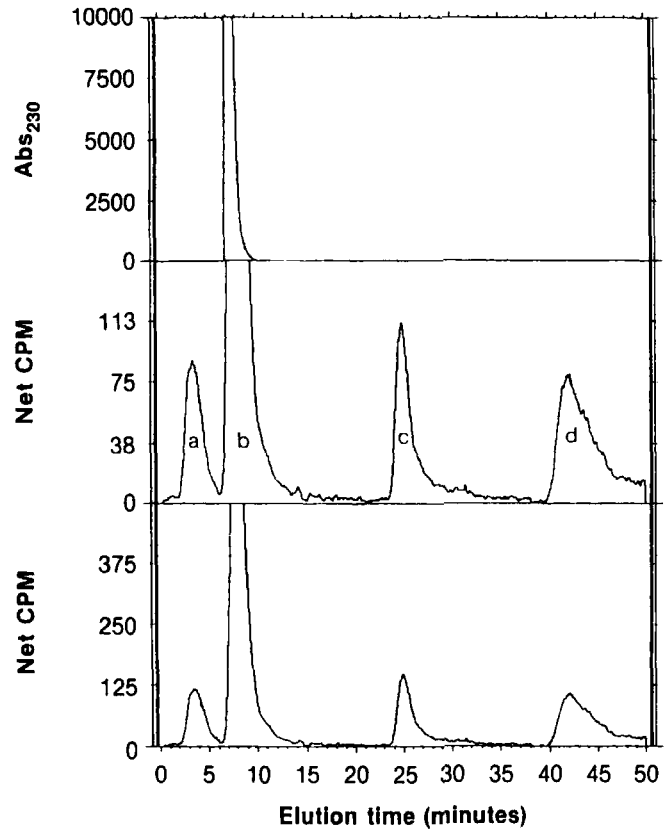

Figure 7. Chromatogram resulting from the coinjection of standards in the HPLC system with octanesulfonate (5 mM) as ion-pairing reagent: (**a**) formaldehyde and methanol; (**b**) *N*-nitrosodimethylamine; (**c**) monomethylamine; (**d**) dimethylamine. Upper trace, UV absorbance at 230 nm; lower traces, radioactive counts per minute, shown for two different ordinate scales.

the extent of formation of the amine-containing metabolites is under investigation, two of the ion-pairing systems would appear to be of great utility. The major difference between the two systems, one incorporating 5 mM heptanesulfonate and the other incorporating 5 mM octanesulfonate into the aqueous mobile phase, is the degree to which the amine-containing metabolites, MA and DMA, are

separated from the nitrosamine. This difference may dictate which system is preferable. For example, under conditions in which the amounts of nitrosamine in the sample are relatively low, less separation between the nitrosamine and amine peaks is necessary, and the heptanesulfonate system is preferable. Such a situation is encountered in the analysis of blood or urine samples from animals administered ^{14}C-NDMA (8). These samples typically contain relatively small amounts of NDMA, and in this case the heptane-sulfonate system provides adequate separation and reduced assay times (Figures 5 and 6). However, in the case of *in vitro* studies, where the fraction of the available nitrosamine which is metabolized may be relatively low, it is often necessary to assay samples in which relatively large NDMA peaks are present. In this case, the retention of the amine-containing metabolites must be prolonged in order to avoid the elution of these analytes in the tail of the NDMA peak. This increased retention of MA and DMA may be achieved through the use of the octanesulfonate system (Figures 5 and 7).

The reproducibility in elution times for each of the analytes examined in these ion-pairing systems appears to be quite good, and while the run times are relatively long, the isocratic nature of the mobile phase delivery allows sequential sample injection without a lengthy reequilibration period.

In conclusion, two ion-pairing systems appear to provide optimal separation of NDMA and its neutral and amine-containing metabolites. The incorporation of heptanesulfonate or octanesulfonate into the aqueous mobile phase at 5 mM allows the separation and detection of the amine-containing metabolites under conditions in which the relative amounts of NDMA are relatively low, or high, respectively.

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