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FLUORESCENCE DETECTION OF SOME NITROSOAMINES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER POST-COLUMN REACTION

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SUMMARY

A selective fluorescence detection method for the determination of some N-nitrosoamines after post-column reaction has been developed for reversed-phase liquid chromatography. The N-nitroso compounds are analyzed by allowing their hydrolysis products to react with the oxidizing species Ce^{4+} to produce the fluorescent ion Ce^{3+}. The detection limit for this method is at the ppb (the American billion, 10^9) level with a linear dynamic range of 2–3 orders of magnitude.

INTRODUCTION

The suspected carcinogenicity of nitrosoamines, combined with the apparent ease of formation with which they are formed in air, water, soil, and even in man, when the appropriate amine and nitrite precursors are present, makes the analytical determination of these compounds important. For a particular analytical technique to be useful for trace analysis of N-nitrosoamines in environmental and biological systems it must be fast, sensitive, and specific, while providing a high degree of accuracy and precision. One specific detection method presently being used for the assay of N-nitrosoamines is a spectrophotometric method which uses a modified Griess reagent for measuring the amount of nitrite formed[1,2] after sample irradiation with UV light. Two disadvantages of that method are: the lengthy irradiation time (*ca.* 30 min) and the time consuming clean-up procedure for eliminating interfering compounds. N-nitroso compounds can be analyzed by thin-layer (TLC)[3,4] and gas chromatography (GC)[5,6].

TLC has been used for both qualitative and quantitative determination of nitrosoamines, but has a low precision. This is probably due to adsorption problems. GC is not generally applicable to the direct analysis of most N-nitrosoamines, such

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as N-nitrosourea, and N-nitrosoguanidines, due to low volatility and/or thermal instability.

In order to avoid some of the problems mentioned above, many analysts are using high-performance liquid chromatography (HPLC) for this assay. For example, since Röper and Heyns[6] reported the analysis of N-nitrosourea and N-nitrosourethanes by reversed-phase HPLC, this technique[7,8] has become widely used for the quantitative detection of nitrosoamines. However, the published techniques lack both the sensitivity and selectivity necessary for trace analysis of environmental samples. In an attempt to address the selectivity problem, Singer *et al.*[9] developed a specific method in which a post-column reaction detection system is used for HPLC. His reaction detector is useful for those compounds which can be hydrolyzed in a dilute acidic solution to give the nitrite ion. This procedure involves the use of the Griess reagent in the post-column reactor for the production of chromophores from the N-nitrosoamines. The detection limit for this method is reported to be 0.5 nmol and it is specific for all N-nitroso compounds cleaved by dilute hydrochloric acid. However, due to slow reaction kinetics of some nitroso compounds, this technique requires both an air segmentation system and an elevated temperature reactor.

Here we report a new, selective and sensitive method for the detection of nitrosoamines. Our technique involves the use of HPLC with fluorescence detection after post-column reaction. The nitrosoamines eluted from the column are first hydrolyzed to produce the nitrite anion, which is then oxidized with Ce^{4+} to give the fluorescent cation Ce^{3+}. A simple tubular coil is used in the post-column reactor, which requires a heating bath but no air segmentation. The chromatographic separation is accomplished by reversed-phase HPLC. Our detection method gives greater sensitivity and precision than the Singer method[10].

EXPERIMENTAL

Apparatus

Two different post-column reactor systems were studied in this work. System A consists of the following items placed in series after the analytical column: a reagent mixing tee (insertion point for reagent from a peristaltic pump), a heated tubular reactor, a cooling coil, and a fluorescence detector. The tubular reactor is made from a piece of PTFE tubing (17 m × 0.5 mm) that is coiled and placed in a heated water bath at 80°C. The cooling coil is PTFE tubing (2 m × 0.5 mm) emersed in an ice bath. System B is the same as A except a second reagent mixing tee (insertion point for a second reagent from another channel of a peristaltic pump) and a packed bed reactor are inserted between the cooling coil and the fluorescence detector. This packed bed reactor is constructed of a PTFE tube (20 cm × 6 mm) and filled with PTFE beads (100/120 mesh, Alltech Assoc., Deerfield, IL, U.S.A.). HPLC PTFE frits (Alltech) with an average pore size of 5 μm were used to retain the PTFE beads in the reactor column. A peristaltic pump (Harvard Apparatus, South Natick, MA, U.S.A.) with dual pumping channels was used to introduce post-column reagents.

Liquid chromatography and detection

Two Waters Model 6000 LC pumps (Waters Assoc., Milford, MA, U.S.A.) controlled by a Waters Model 660 solvent programmer were used to both pump and

control the composition of the mobile phase. The chromatographic column employed was a 30 cm × ¼ in. O.D. micropak MCH-10 (Varian, Walnut Creek, CA, U.S.A.). The optimal chromatographic conditions for the separation of the five nitrosoamines involved a step gradient elution program: beginning at 15% aqueous solution of acetonitrile for 4 min and finishing with 50% acetonitrile for 12 min. The optimum flow-rates for mobile phase and Ce^{4+} reagent solutions were 1.2 and 0.7 ml/min, respectively. Isocratic elution with 20% aqueous acetonitrile gave resolution significantly inferior to that obtained by solvent programming.

A Perkin-Elmer (Norwalk, CT, U.S.A.) fluorescence spectrophotometer Model 650-10s with an 18-μl horizontally illuminated microflow cell was used as the chromatographic detector. A back pressure coil (6 m × 0.8 mm I.D.) was placed after the flowcell to provide the back pressure to the reactor and the flowcell, thus, preventing bubble formation. Injections were accomplished by means of a liquid sampling valve (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl sample loop.

Materials and reagents

N-Nitroso-N-ethylurea (NEU), N-nitroso-N-methylurea (NMU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), obtained from Sigma (St. Louis, MO, U.S.A.) are potential carcinogens. These compounds were kept in their original bottles and were dissolved by injecting through the syringe cap an appropriate volume of acetonitrile as recommended by the supplier. The resulting solutions were serially diluted with 50% (v/v) acetonitrile in distilled water prior to injection. All solutions were prepared in a hood with special care in handling the toxic nitroso compounds. N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG) (Aldrich, Milwaukee, WI, U.S.A.) and N-propyl-N'-nitrosoguanidine (PNNG) (Sigma), also potential carcinogens, were dissolved in acetonitrile and then serially diluted as above.

Acetonitrile (Burdick & Jackson, McGaw Park, IL, U.S.A.) was used as the strong solvent for the HPLC separation of the nitrosoamines. A solution of 20% acetonitrile in distilled water was used for isocratic elution and 15% and 50% acetonitrile for stepwise elution. Cerium(IV) sulfate (reagent-grade, G. Frederick Smith Chemical Co., Columbus, OH, U.S.A.) solutions ($1 \cdot 10^{-4}$ *M*) were prepared as follows: 200 mg of sodium bismuthate was added to the Ce^{4+} solution in 1 l of 1 *N* sulfuric acid and heated to boiling for several minutes. The boiled reagent solution was cooled in an ice bath to below 5°C. The cold solution was transferred to a brown bottle reservoir which was connected to a peristaltic pump via silicon tubing (1.52 mm I.D.; Rainin Instrument, Woburn, MA, U.S.A.). This solution was mixed with the column effluent at a simple post-column tee fitting.

A sulfanilamide reagent solution was prepared by dissolving 1 g of sulfanilamide (Aldrich) in a mixture of 90 ml of distilled water and 10 ml of concentrated hydrochloric acid. A 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride solution was also prepared in this mixture. All the other reagent solutions were prepared as reported in our previous paper[10]. All reagent solutions were prepared fresh daily.

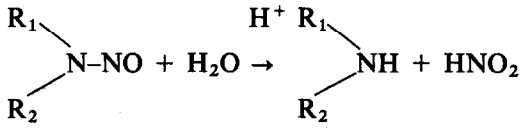
Preliminary kinetics study

The hydrolysis reaction kinetics of the five nitrosoamines were explored in a semi-quantitative manner. Such experiments were carried out as follows: 20 ml of a 20% acetonitrile solution was mixed with 10 ml 1 *N* sulfuric acid in a test tube, a

nitrosoamine was injected into the test tube and placed in a water bath at 80°C. At time intervals an aliquot was removed and cooled in an ice bath. The chilled sample was then analyzed by a modified Griess reagent method[11] uising sulfanilamide and N-naphthylethylenediamine reagent solutions, and by the post-column cerium fluorescence detector. A tubular coil reactor was used in a heated water bath to provide the constant reaction temperature for the post-column reactor. The aliquot of reaction products removed from the test tube were quickly cooled in the cooling coil before entering the fluorescence flowcell.

RESULTS AND DISCUSSION

The N-nitroso compounds are known to undergo cleavage at the N–NO bond in the presence of aqueous mineral acids, resulting in the formation of a corresponding amine and the liberation of nitrous acid according to the following reaction[12]:



where R_1 and R_2 are organic moieties.

A cerium fluorescence detection system with a tubular coil reactor can be used as a selective detector for all compounds which are hydrolyzed by a dilute acid solution (hydrochloric and sulfuric acid) to produce nitrite. The reactivity of the nitrosoamines under the conditions of acid-catalyzed hydrolysis is dependent upon their chemical structure. For example, the N-nitrosoamines with a double bond on the neighboring group are more reactive than simple alkyl nitrosoamines (*e.g.*, N-nitrosodimethylamine). The bond strength of the N–NO bonds in the N-nitrosoamines can be weakened by a proton attack on the double bond (C=O or C=N) of the neighboring group. Thus, the reaction rates for the hydrolysis of nitrosoamines such as N-nitrosoguanidines and ureas are much faster than the simple aliphatic or aromatic nitrosoamines.

The kinetics of the hydrolysis reactions (in 1 *N* sulfuric acid at 80°C) of the nitrosoamines used in this paper was examined in a semi-quantitative manner. The results showed that the amount of nitrite formed reached a maximum within 4–5 min, and then decreased to zero as the reaction time increased. This behavior may be explained by the oxidation of nitrite to nitrate under the sulfuric acid conditions[10].

The chromatographic column chosen for the separation of the nitrosoamines studied was a reversed-phase C_{18} column (MCH-10). This column was particularly well suited for this separation because the analytes are polar and water soluble. Acetonitrile was selected as the mobile phase for this work because acetonitrile–water mixtures do not interfere with the Ce^{4+} oxidation reaction. This is not true for some mobile phases previously used by others *e.g.*, Singer's HPLC method[9] using *n*-propanol, because solvents like *n*-propanol produce high background noise from the reaction of *n*-propanol with Ce^{4+}. A back pressure restrictor coil was attached after the fluorescence flow cell to keep the acetonitrile from vaporizing in the heated reactor at 80°C. This is significant since it eliminates the need for the air segmented

TABLE I

CHROMATOGRAPHIC PROPERTIES OF NITROSOAMINES ON REVERSED-PHASE LC IN THE POST-COLUMN SYSTEM

Solution: 10^{-4} *M* Ce^{4+} in 1 *N* sulfuric acid. t_R = Retention time; *N* = plate-number.

Compound	*Isocratic**			*Gradient**		
	t_R *(min)*	*k'*	*N*	t_R *(min)*	*k'*	*N*
NMU	5.40	0.60	2000	5.70	0.69	1800
MNNG	6.60	0.96	2700	7.13	1.11	2400
NEU	7.05	1.09	2300	7.95	1.36	2700
ENNG	10.10	1.98	3400	10.8	2.20	6500
PNNG	18.0	4.33	5000	14.3	3.24	7400

* See text for details.

reactor used by others. When the back pressure coil was removed from the post-column detector, gas bubbles were continuously created in the reaction coil.

Chromatographic performance data are summarized in Table I. As shown in Fig. 1, MNNG and NEU are not resolved in the isocratic elution with 20% acetonitrile in water (resolution, R_s = 0.9). Further resolution of these two peaks was achieved using gradient elution. Finally, a systematic study resulted in optimum sep-

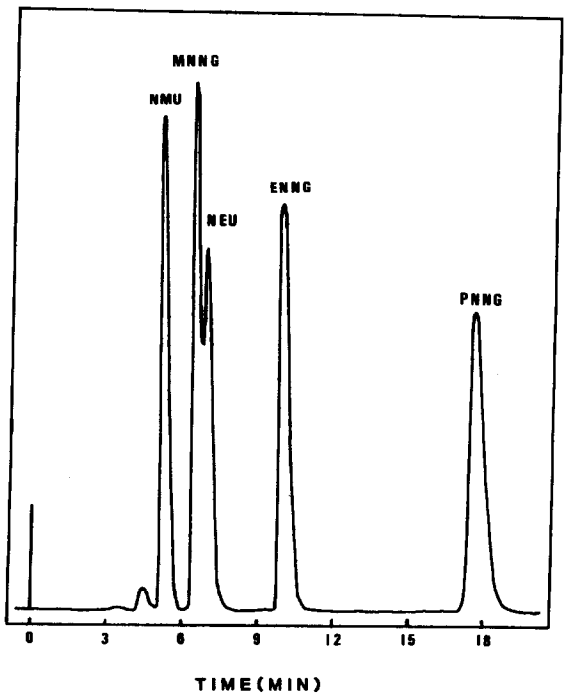


Fig. 1. Chromatogram of N-nitrosoamines with isocratic elution. Isocratic conditions and abbreviations

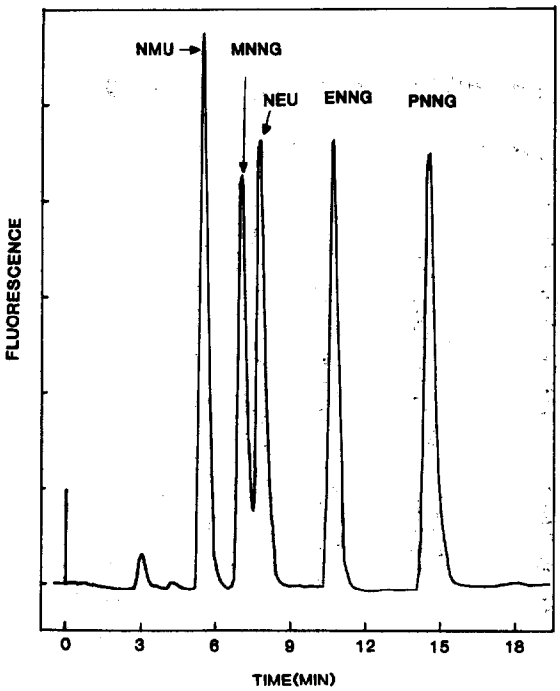


Fig. 2. Chromatogram of N-nitrosoamines with gradient elution. Amount injected is *ca.* 10–20 ng for each component. See text for gradient elution condition.

aration conditions where the starting eluent was 15% acetonitrile for 4 min and the finishing 50% acetonitrile for 12 min. The chromatogram obtained from the step gradient elution is shown in Fig. 2, which presents R_s = 1.3 between MNNG and NEU. In the gradient elution, the retention time of PNNG becomes shorter, but it should be pointed out that additional time for each injection is required compared to the isocratic elution because of column equilibration. This gradient elution, unlike the results of the work of Wolkoff and Larose[13], shows no baseline drift as long as pure acetonitrile (HPLC-grade) is used as the gradient solvent. The results obtained from the step gradient elution mode demonstrate improvement in the resolution between the two peaks (MNNG and NEU). In addition, the separation of the two peaks (ENNG and PNNG) was greatly enhanced as can be seen in Fig. 2.

Two different reactor designs were employed for the post-column reaction system. The first reactor, system A, consists of a single tubular reactor for the introduction of the Ce^{4+} reagent dissolved in dilute sulfuric acid. The acid hydrolysis of the N-nitrosoamines and subsequent oxidation of the nitrite produced occurs in the same reactor to create fluorescent Ce^{3+}. The second reactor system B consists of two different reactors (tubular and packed bed): one for the hydrolysis reaction and the other for the oxidation reaction of nitrite with Ce^{4+}. Both reaction/detection systems gave acceptable results for the detection of the nitrosoamines. Since the lengthy residence time in the tubular reactor produces severe band broadening of the chromatographic peaks, it was necessary to optimize the residence time of the analytes within

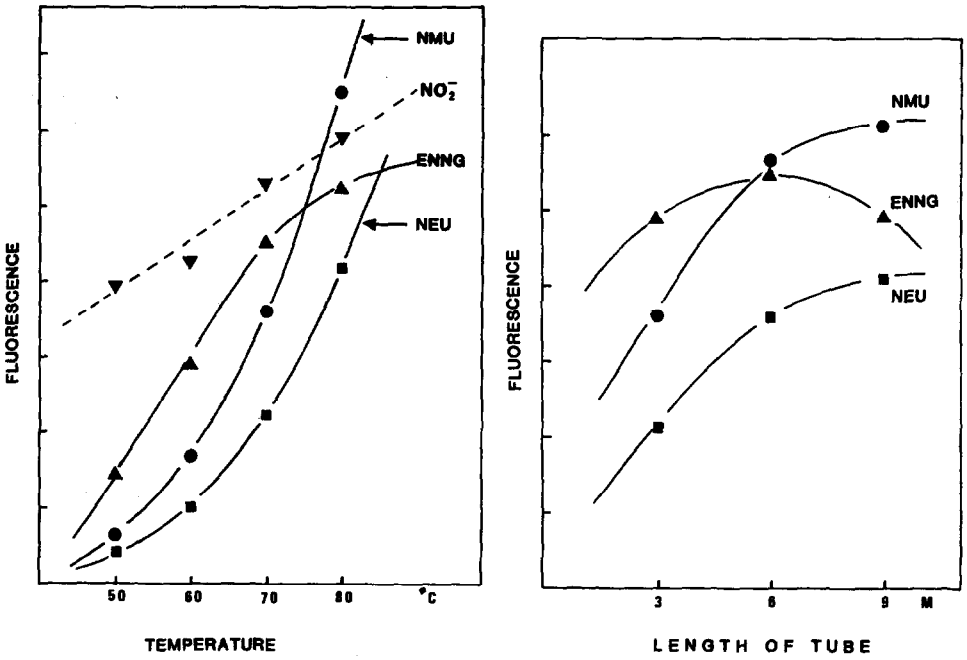


Fig. 3. Effect of temperature in the tubular reactor on fluorescence response of N-nitrosoamines. All compounds are labeled the same as in Fig. 1.

Fig. 4. Effect of the length of the tubular coil reactor on fluorescence response of N-nitrosoamines at 80°C.

the reactor. In addition, the reaction time for hydrolysis should be maximized by optimizing such kinetic parameters as the reaction temperature and reactant composition. The results of the kinetic study indicate that the residence time of analytes in the post-column reactor should be limited to less than 4 min. In this case, the packed bed reactor is more desirable. However, one problem which arose with this reactor was a high fluorescence background due to the vigorous reactivity of Ce^{4+} with organic impurities which may be released from PTFE frits and/or the PTFE bed reactor at 80°C. Thus, the tubular coil was predominantly used as the post-column reactor. The following study was carried out using reaction system A.

Fig. 3 shows that the fluorescence response of the compounds (NMU and NEU) increases exponentially with temperature. The slope of the ENNG response increases linearly and then declines as temperature increases. No fluorescence response for the nitrosoamines appeared at all when the reactor temperature was set at room temperature. These results indicate that the reaction rate of the nitrosoamine increases with the temperature of the reactor and the reactivity of ENNG is greater than that of NMU or NEU in this system. The reaction temperature is limited to a maximum of 80°C, just below the boiling point of acetonitrile, even though the reaction yield for the hydrolysis increases with temperature to give a higher fluorescence response. A reaction temperature of 80°C was used throughout the work.

As is shown in Fig. 4, the fluorescence intensity of NMU and NEU increases with the length of the tubular reactor (0.8 mm I.D.), but the intensity of ENNG

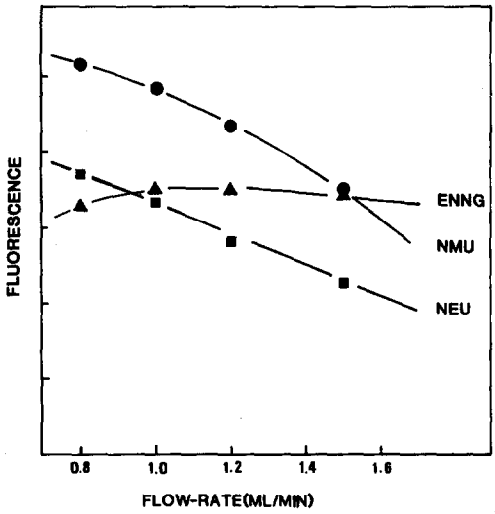


Fig. 5. Effect of flow-rate of the mobile phase on fluorescence response of N-nitrosoamines.

reached a maximum at a length of 6 m (1.6 min reaction time). This suggests an optimum reaction time of about 1.6 min. It can be concluded from the reaction times of the nitrosoamines (see Fig. 5) that an increase in the flow-rate of the mobile phase decreases the relative fluorescence sensitivity, especially for NMU and NEU due to slow reaction kinetics, while the fluorescence yield of ENNG is not affected. Note that the background fluorescence increases with decreasing mobile phase flow-rate due to a decrease in the back pressure of the flowcell. A compromise flow-rate of 1.2

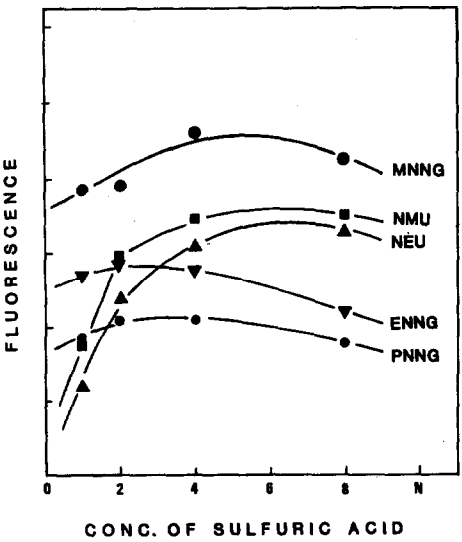


Fig. 6. Effect of concentration of sulfuric acid used in the reagent solution on fluorescence response of N-nitrosoamines.

ml/min was selected for the mobile phase as it provided the optimal signal-to-noise ratio for the compounds of interest.

There are some limitations in the selection of a tubular reactor. A large diameter and/or long length for the reactor results in severe band broadening[14,15]. Furthermore, a small diameter tube for this reactor produces back pressure on the peristaltic pump which has a limited working pressure (< 70 p.s.i.). In view of these limitations, a tubular coil was selected using the dimensions (17 m × 0.5 mm I.D.) for the tubular coil.

The concentration of sulfuric acid influences the hydrolysis reaction kinetics of the N-nitrosoamines in a catalytic fashion (see Fig. 6). The reaction rates of NMU and NEU doubled when the concentration of sulfuric acid was increased from 1 to 8 *N*. N-Nitrosoguanidines (MNNG, ENNG, and PNNG) showed little effect from the change in sulfuric acid concentration. Therefore, the reaction kinetics of the nitrosoamines, especially for NMU and NEU can be increased to a maximum by increasing the concentration of sulfuric acid in the reagent. Note that it is not necessary for the reaction to be complete or well defined as long as it is reproducible in the post-column detection system. This tubular reactor with a 10^{-4} *M* Ce^{4+} reagent in 4 *N* sulfuric acid solution serves well as a post-column reactor.

In system B, the hydrolysis of the nitrosoamines and the oxidation of the nitrite formed with Ce^{4+} are separated. The tubular coil reactor in a heated bath was used for the hydrolysis reaction of the nitrosoamines while a second packed-bed reactor was used for the oxidation of the nitrite produced. This reaction system improves selectivity for the nitrosoamines due to the decrease in reactivity of Ce^{4+} at ambient temperature compared to the reactivity at the temperature of the heating bath (80°C).

Also in the second system, the concentration of the hydrochloric acid is important as a catalyst, since it produces an efficient hydrolysis reaction for the N-nitroso compounds present in the column effluent. It can be seen from Fig. 7 that

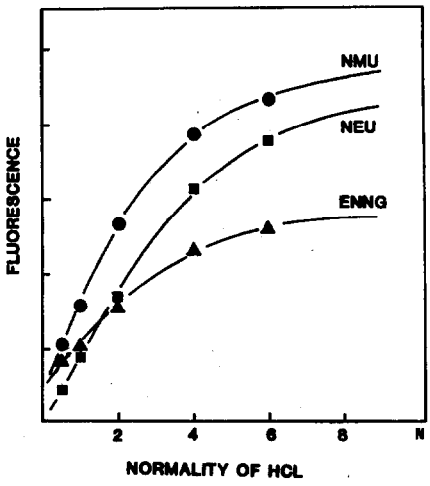


Fig. 7. Effect of concentration of hydrochloric acid used in the reagent solution on fluorescence response of N-nitrosoamines.

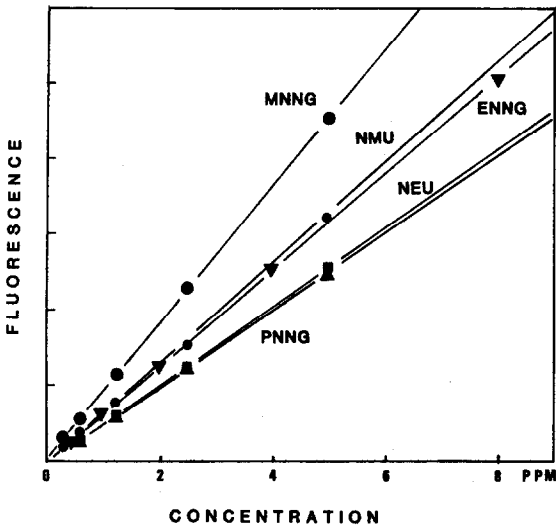


Fig. 8. Linear relationship of fluorescence response to concentration for N-nitrosoamines. Gradient elution and a post-column reagent solution of 4 *N* sulfuric acid were used.

the yield of nitrite from the hydrolysis of N-nitroso compounds increases as the concentration of hydrochloric acid increases. However, the relative sensitivity for the nitrosoamines in this system involving 6 *N* hydrochloric acid was lower than that in the simple oxidation system using 4 *N* sulfuric acid due to the extra column effect (*i.e.*, peak band broadening). The use of 1 *N* sulfuric acid reagent solution instead of 1 *M* hydrochloric acid as the post-column reaction medium decreases the relative detection sensitivity. Therefore, the hydrochloric acid solution becomes the preferred reagent for this system.

Linearity of the calibration curves over two orders of magnitude of concentration of the nitrosoamines was demonstrated. The quantitative determination of the N-nitroso compounds is possible in both isocratic and gradient elution with a relative standard deviation of $< 5.0\%$. When the fluorescence sensitivity of the nitrosoamines in the isocratic elution mode (1 *N* sulfuric acid reagent solution) is compared with that in the gradient elution, ENNG and PNNG show an increase in their fluorescence sensitivity while NMU and NEU remain the same due to the effect of changes in retention times[16]. In addition, a higher concentration of sulfuric acid in the Ce^{4+} reagent solution is required to increase the reaction rate of hydrolysis, especially for the N-nitrosoamines compounds, whereas use of a high concentration of sulfuric acid slightly decreases the fluorescence intensities of the N-nitrosoguanidine compounds due to the acid-suppressing effect on the nitrate reaction with Ce^{4+} (ref. 10). Therefore, an optimum concentration of 4 *N* sulfuric acid was chosen for this detection system. Fig. 8 shows the calibration curves for nitrosoamines using 10^{-4} *M* Ce^{4+} solution in 4 *N* sulfuric acid for the gradient elution mode. In this case, the fluorescence sensitivities of NMU and NEU increase significantly compared to that of the nitrosoguanidines as the sulfuric acid concentration is increased.

TABLE II

DETECTION LIMITS OF NITROSOAMINES BY Ce^{4+} FLUORESCENCE DETECTION

Solution: $1 \cdot 10^{-4}$ *M* Ce^{4+} in 1 or 4 *N* sulfuric acid.

Compound	*Isocratic**	*Gradient**	
	1 N sulfuric acid	*1 N sulfuric acid*	*4 N sulfuric acid*
NMU	24 ppb 2.4 ng 23 pmol	21 ppb 2.1 ng 20 pmol	12 ppb 1.2 ng 12 pmol
MNNG	11 ppb 1.1 ng 7.5 pmol	10 ppb 1.0 ng 6.8 pmol	8.0 ppb 0.8 ng 5.4 pmol
NEU	35 ppb 3.5 ng 30 pmol	33 ppb 3.3 ng 28 pmol	14 ppb 1.4 ng 12 pmol
ENNG	23 ppb 2.3 ng 14 pmol	17 ppb 1.7 ng 11 pmol	11 ppb 1.1 ng 7.0 pmol
PNNG	42 ppb 4.2 ng 24 pmol	23 ppb 2.3 ng 13 pmol	16 ppb 1.6 ng 9.1 pmol

* See text for details.

Each data point on the calibration curves is an average of at least three injections. The data for all the calibrations were treated by a least squares analysis. Their correlation coefficients were > 0.999 over two orders of magnitude in concentration, indicating good linearity over the concentration range examined, *viz.*, 0.1–10 ppm or 10–1000 ng injected. However, all the intercepts for these calibration curves had negative values due to the oxidation of nitrite to nitrate under acidic conditions as described in our previous work[10].

The detection limits determined for the various nitrosoamines analyzed by the post-column cerium fluorescence method are given in Table II. These detection limits obtained range from 8 to 16 ppb*, depending on the compound, and represent an order of magnitude improvement in sensitivity over the technique of Singer[9].

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