Journal of Chromatography, 621 (1993) 157–163
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7106

High-performance liquid chromatographic determination of N-nitroso-N-alkylureas by pre-column fluorescence derivatization and application to blood analysis

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(First received July 1st, 1993; revised manuscript received September 1st, 1993)

ABSTRACT

A method for the derivatization and separation of N-nitroso-N-alkylureas [alkyl = methyl (NMU), ethyl (NEU), and *n*-butyl (NBU)] has been developed. Fluorescent derivatives were formed with sodium sulphide, taurine and *o*-phthalaldehyde and separated by reversed-phase high-performance liquid chromatography. The limits of detection of standard NMU, NEU and NBU were 0.25, 0.8 and 1.5 pmol/200 μl, respectively. The method was applied to the determination of NMU in blood after extraction with acetonitrile in the presence of calcium chloride. NBU was used as the internal standard. The recovery of NMU from blood was *ca.* 95%, and the limit of detection was 10 pmol/400 μl blood. NMU levels in rabbit blood following a single oral administration were also measured.

INTRODUCTION

N-Nitrosamides, such as N-nitroso-N-alkylamides, N-nitroso-N-alkylureas and N-nitroso-N-alkylguanidines, are mostly direct-acting carcinogens. Their intragastric formation from dietary secondary amides and related compounds is suspected to be an etiological factor of human gastric cancer [1,2]. To elucidate the mechanism of intragastric formation of N-nitrosamides and their absorption into blood or biotransformation, N-nitroso-N-alkylureas have been used most frequently as model compounds [3–5]. Therefore, a simple, sensitive and accurate method for the determination of N-nitroso-N-alkylureas in biological fluids is needed, and chromatographic methods are suitable [6]. Gas chromatography (GC) with thermal energy analysis (TEA) [7,8] and several high-performance liquid chromatography (HPLC) methods based on TEA [7,9] and post-column [10–12] or pre-column [13] derivatization reactions using various chromogenic [10,11,13] and fluorogenic reagents [12] have been reported. Their applicability to routine biological analysis, however, is often limited because of the requirement of expensive equipment [7–9] or the lack of simplicity and/or sensitivity [10–13].

Previously, we have reported [14] a fluorogenic reaction of N-nitroso-N-alkylureas [alkyl = methyl (NMU), ethyl (NEU), and *n*-butyl (NBU)] based on S-alkylation with sodium sulphide and subsequent condensation with *o*-phthalaldehyde (OPA) and taurine to form fluorescent isoindole derivatives (Fig. 1). This reaction seems to be promising as a method for pre-column derivatization of N-nitroso-N-alkylureas in their analysis by HPLC. Similar procedures have been successfully used for the HPLC assay

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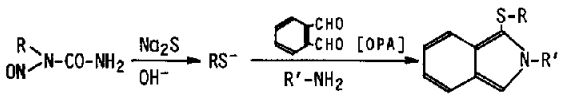
0378-4347/93/$06.00



N-Nitroso-N-alkylurea (abbreviation)	R
N-Nitroso-N-methylurea (NMU)	CH_3
N-Nitroso-N-ethylurea (NEU)	C_2H_5
N-Nitroso-N-n-butylurea (NBU)	n-C_4H_9

Fig. 1. Fluorogenic reaction of N-nitroso-N-alkylureas.

of the antitumour agent triethylenethiophosphoramide (ThioTEPA) and its metabolite [15], and of chiral epoxides [16].

This paper reports optimized HPLC conditions for the derivatized nitrosoureas, and a suitable clean-up procedure for the determination of NMU in blood samples.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical-reagent grade, unless stated otherwise. Water was purified on a Milli RO-Milli Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol and tetrahydrofuran (THF) were used, and acetonitrile was a special-grade reagent for fluorimetry (Kanto Chemical, Tokyo, Japan). Anhydrous calcium chloride, NMU, NEU and NBU were purchased from Nacalai Tesque (Kyoto, Japan).

Stock standard solutions of the nitrosoureas were prepared at 10 m*M* concentration in acetonitrile; working standard solutions were prepared by appropriate dilution of the stock solutions with the same solvent. The nitrosoureas are potent animal carcinogens and should be handled with extreme care.

A borate–citrate buffer (pH 5.5) was prepared by mixing 250 ml of a 0.05 *M* sodium borate solution and 350 ml of a 0.1 *M* potassium dihydrogencitrate solution. A borate–phosphate buffer (pH 8.0) was prepared by mixing 535 ml of a 0.05 *M* sodium borate solution and 465 ml of a 0.1 *M* potassium dihydrogenphosphate solution.

Sodium sulphide nonahydrate was dissolved in the borate–citrate buffer to give a 30 m*M* solution. The final pH of the solution was 8.5. This solution was prepared fresh daily. OPA and taurine (Nacalai Tesque) were used as 0.3 m*M* solutions in the borate–phosphate buffer.

Fluorescence derivatization of nitrosoureas

To 200 μl of nitrosourea standard solution or sample solution in a 1.5-ml brown test-tube with a glass stopper, were added 20 μl of 30 m*M* sodium sulphide solution. After 10 min at room temperature, the reaction mixture was placed in an ice-water bath. To the mixture were added 400 μl each of 0.3 m*M* taurine and 0.3 m*M* OPA solutions, and after 5–10 min at room temperature, a 20-μl aliquot of the reaction mixture was injected into the HPLC system.

HPLC conditions

The HPLC system consisted of a Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), a Rheodyne Model 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, CA, USA) and a 5-μm Cosmosil 5C18-AR column (150 mm × 4.6 mm I.D.) (Nacalai Tesque) with a Cosmosil 5C18-AR guard column (10 mm × 4.6 mm I.D.) (Nacalai Tesque). Two solvent systems were used as the mobile phases: the mobile phase I, methanol–0.1 *M* potassium phosphate buffer (pH 6.8) (58:42, v/v), was used for the determination of the standard nitrosoureas; mobile phase II, methanol–THF–0.1 *M* potassium phosphate buffer (pH 6.8) (53:5:42, v/v/v), was used for the determination of NMU in blood. The column temperature was ambient, and the flow-rate was 1.0 ml/min. Detection was carried out with a Shimadzu RF-535 fluorescence HPLC monitor equipped with a flow-cell (12 μl) and a xenon lamp (Shimadzu), operated at 340 nm excitation and 445 nm emission.

Determination of NMU in blood

Blood was taken by venipuncture into a heparinized tube, and then a 400-μl aliquot of the blood was mixed immediately with 100 μl of 0.1 *M* potassium citrate buffer (pH 3.5) followed by addition of 2 ml of acetonitrile containing 1250 pmol of NBU as the internal standard (I.S.). To

prevent the degradation of NMU, the acidified blood sample must be kept at 0°C until the addition of acetonitrile. Anhydrous calcium chloride (1 g) was added to the mixture and, after vigorous vortex-mixing, the mixture was centrifuged at 1000 *g* for 10 min at room temeprature. A 200-μl aliquot of the upper acetonitrile layer was then submitted to the derivatization reaction followed by HPLC analysis as described above. NMU standard solutions (2, 20 and 40 pmol/200 μl) containing 125 pmol of the I.S. were also treated in the same manner. The NMU concentration was ascertained from the peak-height ratio of standard NMU and the I.S.

RESULTS AND DISCUSSION

Derivatization

The method used here for the fluorescence derivatization of nitrosoureas was essentially the same as that reported previously for the manual determination of NMU [14]. However, to avoid the problems arising from poor stability of the isoindole fluorophores, after S-alkylation with nitrosoureas, the reaction mixtures were stored in an ice-water bath and the addition of taurine and OPA reagents was carried out *ca.* 5–10 min prior to the injection of the sample into the HPLC system. This is based on the good stability of the S-alkylated intermediates, a stability similar to that reported for the HPLC determination of ThioTEPA and its metabolite using a pre-column derivatization with the same reagents [15].

Separation

Separation of the derivatized NMU, NEU and NBU was attempted on a reversed-phase column using isocratic systems consisting of aqueous organic solvents containing phosphate, acetate, or citrate buffer, which had been employed for the separation of various isoindole derivatives [15–18]. As shown in Table I, it was found that the nature of the organic modifiers and ionic media did not have a major affect on the resolution of the derivatives, except for THF as an organic modifier. Therefore, methanol–0.1 *M* phosphate buffer (pH 6.8) (58:42) was used as the mobile phase I for the HPLC analysis of standard N-nitroso-N-alkylureas. A typical chromatogram is shown in Fig. 2. The nitrosoureas were detected

TABLE I

CAPACITY FACTORS OF DERIVATIZED N-NITROSO-N-ALKYLUREAS IN VARIOUS ELUTION SYSTEMS

Elution system[a]	Capacity factor (k')[b]		
	NMU	NEU	NBU
I	1.75	2.75	9.25
II	1.25	1.88	5.50
III	2.00	3.25	10.75
IV	1.88	3.13	10.50
V	2.00	3.25	10.88
VI	2.00	3.00	9.50

[a] Elution system: I = methanol–0.1 *M* phosphate buffer (pH 6.8) (58:42); II = methanol–THF–0.1 *M* phosphate buffer (pH 6.8) (53:5:42); III = methanol–0.1 *M* phosphate buffer (pH 6.0) (58:42); IV = methanol–0.1 *M* acetate buffer (pH 6.0) (58:42); V = methanol–0.1 *M* citrate buffer (pH 6.0) (58:42); VI = acetonitrile–0.1 *M* phosphate buffer (pH 6.8) (32:68). Flow-rate: 1.0 ml/min.

[b] $k' = t - t_0/t_0$; t_0 = 96 s.

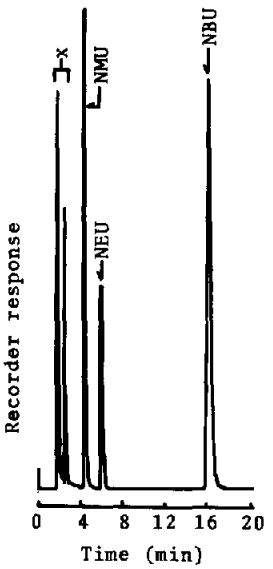


Fig. 2. Separation of the derivatized NMU, NEU and NBU. A 200-μl aliquot of the standard solution in acetonitrile, containing NMU (0.05 nmol), NEU (0.1 nmol) and NBU (0.5 nmol), was derivatized and then chromatographed using mobile phase I. The symbol *x* represents the reagent blank.

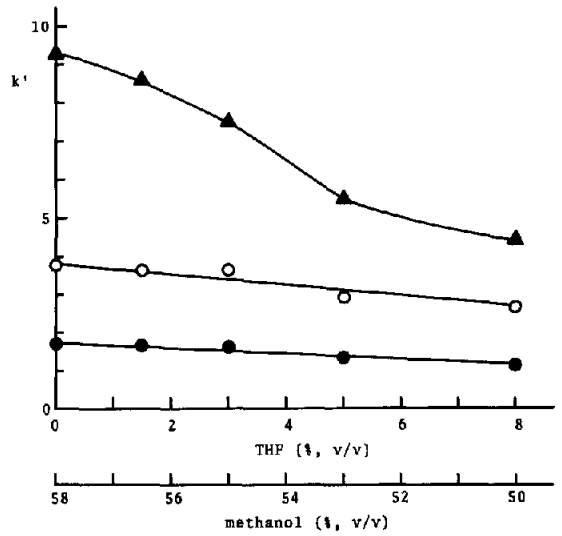


Fig. 3. Effects of THF and methanol concentrations on the capacity factors of derivatized NMU (●), NEU (○) and NBU (▲). The mobile phase contained various proportions of THF and methanol in 0.1 *M* phosphate buffer (pH 6.8).

as sharp peaks, and were separated efficiently from the peaks found in the reagent blank.

As shown in Fig. 3, addition of THF to the methanol–phosphate buffer mobile phase made it possible to decrease selectively the capacity factor (k') of NBU. Hence, methanol–THF–0.1 *M* phosphate buffer (pH 6.8) (53:5:42, v/v/v) was conveniently adopted as the mobile phase II, for the determination of NMU in blood using NBU as the I.S.

Calibration graphs for standard NMU, NEU and NBU

Calibration graphs for standard NMU, NEU and NBU, constructed by using mobile phase I and defined by a least-squares linear regression of standard concentrations (x) *versus* the peak height (y), were linear over the ranges 0.00025–10 nmol/200 μl ($y = 16.25x - 1.01$, $r = 0.9994$), 0.0008–10 nmol/200 μl ($y = 4.15x - 0.09$, $r = 0.9995$) and 0.0015–10 nmol/200 μl in sample solutions ($y = 1.55x + 0.03$, $r = 0.9998$), respectively. The detection limits were estimated to be 0.25 pmol/200 μl for NMU, 0.8 pmol/200 μl for NEU and 1.5 pmol/200 μl for NBU, at a signal-to-noise ratio of 3, which corresponded to 4.9, 15.7 and 29.4 fmol per injection, respectively. Relative standard deviations (R.S.D.) of peak heights at the concentration levels of 0.01 and 1.0 nmol/200 μl were 3.5% and 1.9% for NMU, 3.3% and 3.5% for NEU, and 4.4% and 4.2% for NBU ($n = 5$ in each case).

Several chromatographic methods have been reported for the determination of N-nitroso-N-alkylureas, and some of them are very sensitive. The detection limits obtained from standard NMU using GC–TEA [7], HPLC–TEA [7] and HPLC with fluorimetric [12] and photometric [13] detections were 1, 6, 12 and 80 pmol per injection, respectively. The HPLC method described here is more sensitive than the methods listed above. The reproducibility is also acceptable. In addition, most of the techniques previously used for quantitation of N-nitrosoureas require expensive apparatus [7] or lack simplicity [12,13].

Clean-up of NMU in blood samples

For a study of the clean-up of NMU in blood, venous blood from healthy volunteers was used. NBU was employed as the I.S., and mobile phase II was adopted for the HPLC of derivatized NMU and the I.S.

The stability of NMU in blood was examined first, because NMU is known to be unstable in biological fluids but most stable at pH 3.5–4.0 [13]. For this purpose, human blood spiked with a known amount of NMU was treated by four different procedures (see Fig. 4), and the residual NMU was determined at definite time intervals up to 2 or 6 h. The time-courses of the residual NMU are shown in Fig. 4. The results demonstrate that when 0.1 *M* citrate buffer (pH 3.5) (100 μl) was immediately added to the blood samples (400 μl) [13], and the mixture was kept in an ice-water bath, NMU could be stored for at least 6 h with losses of no more than 10%. When either addition of the buffer or cooling of the mixtures in an ice-water bath was omitted, NMU in blood disappeared gradually. Therefore, after collection of blood, the specimens must be treated immediately with the citrate buffer and then kept

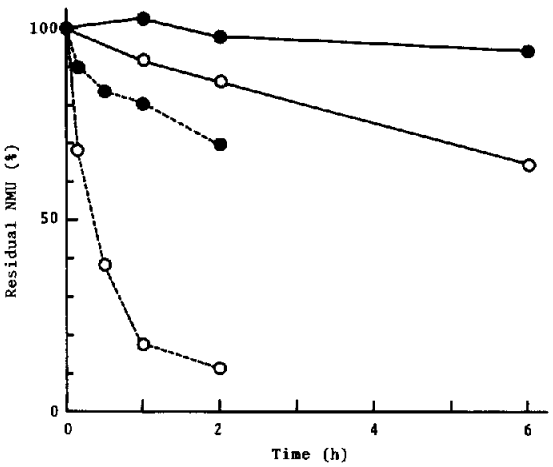


Fig. 4. Stability of NMU in blood. Aliquots (400 μl) of human blood spiked with NMU (250 pmol) were treated and then stored as follows: (–●–) mixed with 100 μl of 0.1 *M* citrate buffer (pH 3.5) and stored at 0°C; (–○–) mixed with the same buffer (100 μl) and stored at room temperature; (--●--) stored at 0°C without addition of the citrate buffer; (--○--) stored at room temperature without addition of the buffer. At specific time intervals, the mixtures were analysed by the method described in Experimental.

at 0°C. These results also imply that plasma and serum are inadequate as samples, because degradation of NMU is unavoidable during their preparation.

Next, a procedure for the purification of NMU in blood was studied. The utility of Extrelut columns for the clean-up of NMU in biological fluids and food samples has been demonstrated [13,14,19]. Such a procedure, however, proved to be inapplicable for our purposes because significant losses of NMU and the I.S. occurred during the evaporation of eluate from the column. On the other hand, Alric *et al.* [20] reported that drugs in biological fluids could directly be extracted into water-miscible organic solvents, when the extraction was carried out in the presence of some inorganic salt to separate the organic layer. The method was referred to as a "solvent demixing" extraction method, and considered to be suitable for the present purpose. Therefore, direct extraction of NMU from blood into acetonitrile containing the I.S., the solvent used for their derivatization, through such a salting-out technique was examined.

Calcium chloride was selected as the salt because it was superior, in the absolute recoveries of NMU and the I.S., to the other salts, such as sodium chloride, potassium chloride and sodium sulphate. When acidified blood samples (500 μl, *i.e.* 400 μl of blood plus 100 μl of citrate buffer) containing 250 pmol of NMU were treated with more than 1.5 ml of acetonitrile (containing 1250 pmol of the I.S.) and 1 g of calcium chloride, the absolute recoveries of NMU and the I.S. reached a maximum (*ca.* 75–80%), which corresponded to more than 90% recovery of NMU based on the peak-height ratio method. Therefore, the amounts of acetonitrile and calcium chloride were fixed at 2 ml and 1 g, respectively.

Fig. 5 shows typical chromatograms from control human blood and blood treated with NMU (250 pmol/400 μl blood). The chromatograms indicate that the clean-up method described is effective for the removal of endogenous substances, which may interfere with the derivatization and the chromatographic separation.

The calibration curve for NMU added to blood was linear up to at least 2000 pmol/400 μl

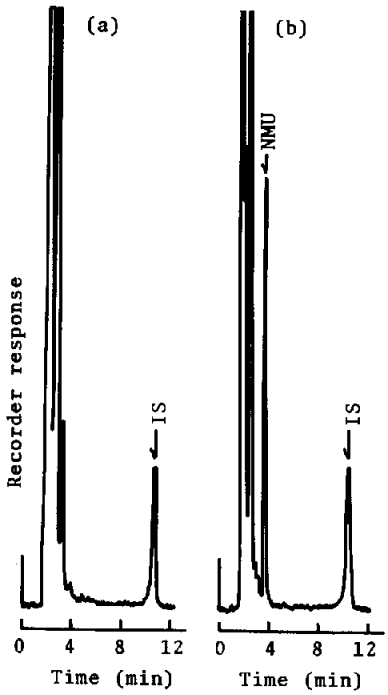


Fig. 5. Chromatograms of the extracts of human blood: (a) control blood; (b) control blood with NMU (250 pmol) added.

blood, with a lower limit of determination of 10 pmol/400 μl blood (*ca.* 2.5 ppb), which is comparable with that obtained for NMU in food samples by using GC–TEA (1 ppb) [19]. The detectability of the present method would presumably be improved when the fluorescence derivatization reaction is performed after appropriate concentration of the acetonitrile extract.

The reliability of the method was tested by a series of recovery tests. The recoveries of NMU added to blood at the concentration levels of 50, 250 and 1000 pmol/400 μl were 96.0 ± 9.6%, 92.5 ± 5.2% and 95.1 ± 9.4% (mean ± S.D., n = 5 in each case), respectively. The recovery and its S.D. were satisfactory for routine use of the method.

If a blood sample contains endogenous methanethiol, a positive error occurs with the present method. This problem, however, can easily be overcome by the following procedure. The derivatization is carried out both in the presence and absence of sodium sulphide, because methanethiol gives the fluorophore even in the absence of the sulphide reagent.

Determination of NMU in rabbit blood

The proposed method was applied to the determination of NMU in rabbit blood after an aqueous NMU solution (10 μmol/ml) had been administered orally to two male rabbits (3.8 kg and 2.2 kg; 5 μmol/kg). Blood specimens were taken by venipuncture into heparinized tubes just before and up to 3 h after administration. A typical chromatogram and time-courses of blood levels of NMU are shown in Fig. 6 (not corrected for recovery). The NMU concentration reached a maximum within 30 min and then declined rapidly. This observation is different from that reported by Yamamoto *et al.* [5], who used guinea-pigs as experimental animals, and found no marked tendency for any change in the blood level of NMU with time after oral administration of NMU. This suggests differences between animal species in the absorption and disappearance of NMU from the stomach.

The HPLC method reported here permits the accurate and sensitive determination of NMU in blood by a simple procedure using a small amount of blood (400 μl), and seems to be useful

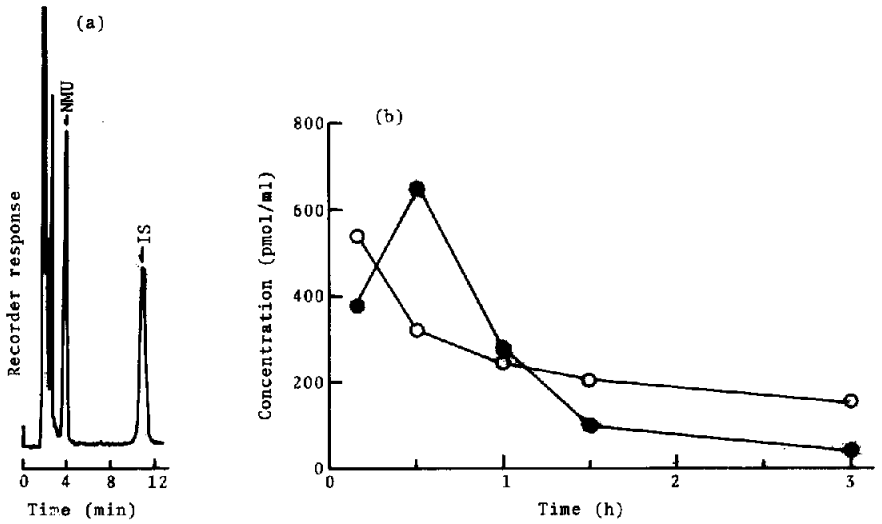


Fig. 6. (a) A typical chromatogram of NMU in blood from a rabbit (containing 656 pmol/ml NMU) 30 min after oral administration of NMU; (b) blood concentration of NMU in two rabbits (○, 3.8 kg; ●, 2.2 kg) following a single oral administration of 5 μmol/kg of NMU.

for biological studies of the intragastric formation and biotransformation of N-nitroso-N-alkylureas using experimental animals [3–5]. In addition, the method may also be applicable to study the formation of nitrosoureas from foodstuffs treated with nitrite under acidic conditions [19].

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