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Note

High-performance liquid chromatography of N-nitrosoproline in urine

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While there have been extensive surveys of the occurrence of pre-formed carcinogenic N-nitrosamines in the environment[1], the contribution to human exposure which arises as a result of the formation of nitrosamines from amine and nitrite precursors in the body has not been adequately investigated[2,3]. Among the reasons for the lack of information on *in vivo* nitrosamine generation probably the most important are the substantial experimental difficulties involved in detecting the formation of most nitrosamines at the extremely low levels likely to be significant in human cancer aetiology[4].

One promising, but indirect, approach has been the determination of the nitrosation of a marker amino acid, which is thought likely to occur under conditions analogous to those which will lead to the formation of carcinogenic nitrosamines[5]. The non-carcinogenic[6] product N-nitrosoproline (NPRO) is excreted without biodegradation and may be determined[5] in the urine[7,8].

We required a rapid high-performance liquid chromatographic (HPLC) technique for determining [^{14}C]NPRO in urine for application to experiments involving the oral administration of [^{14}C]proline to experimental animals. To achieve this objective required the development of an HPLC method for separating NPRO from co-extracting urinary components and the choice of a suitable internal standard to compensate for variations in the recovery of NPRO during sample preparation. In addition the risk of nitrosation during sample collection and extraction[9] necessitates the inclusion of a suitable nitrite trapping agent whose nitrosation can be subsequently quantified. This paper describes the HPLC separation of five nitrosoamino acids of which three proved suitable for the objectives described (Fig. 1).

EXPERIMENTAL AND RESULTS

Material

Azetidine-2-carboxylic acid, pipecolinic acid, isonipecotic acid (Aldrich, Gillingham, Great Britain) nipecotic acid and L-proline (Sigma, Poole, Great Britain) were converted to the corresponding N-nitrosoamino acids by treatment of each compound (0.01 mole) with sodium nitrite (0.05 mole) in a solution of water–glacial acetic acid–conc. hydrochloric acid (14:6:1, v/v/v) at 37°C for 1 h. The reaction solution was then acidified to pH 1 with hydrochloric acid and the products extracted

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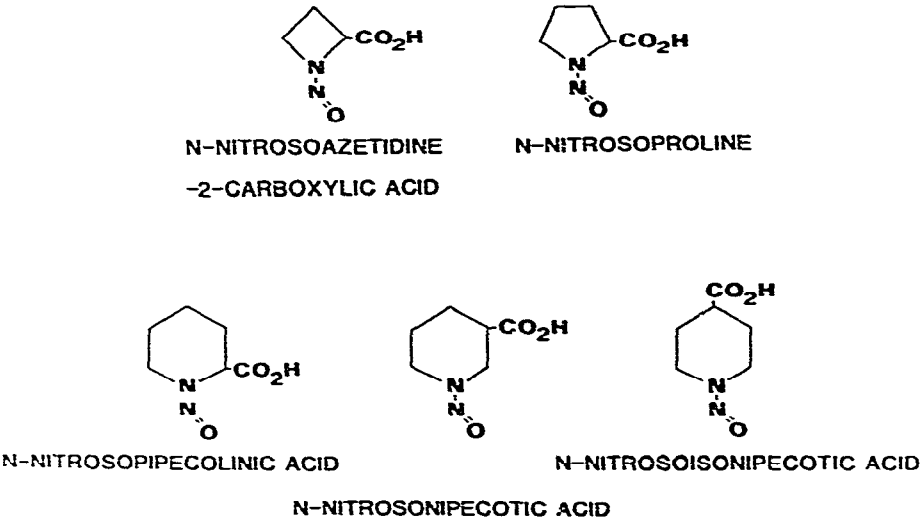

Fig. 1. Structures of N-nitrosoamino acids.

into ethyl acetate, dried (sodium sulphate), rotary evaporated and recrystallised from ethyl acetate–light petroleum (b.p. 40–60°C) (2:1, v/v). The crystalline products were characterized by mass spectrometry.

HPLC-grade methanol, tetrahydrofuran and Grade S acetonitrile were supplied by Rathburn (Walkerburn, Great Britain) and water was glass distilled.

Equipment

A Varian Vista 5040 HPLC (Varian Assoc., Palo Alto, CA, U.S.A.) fitted with a 250 × 4.6 mm I.D. column self-packed with Partisil-10 ODS (II) and a Co:PELL:ODS precolumn (Whatman, Maidstone, Great Britain) was used for all separations. Detection was by UV absorbance at 238 nm with a Varian UV 50 variable-wavelength detector and by liquid scintillation counting of collected effluent fractions in Tritosol scintillant[10].

Development of HPLC separation

Several attempts to separate the five nitrosoamino acids in methanol–water or tetrahydrofuran–water systems modified with sulphuric acid were unsuccessful. The use of acetonitrile as the organic component of the mobile phase, however yielded improved separation. Satisfactory results were obtained with isocratic elution conditions using water–acetonitrile–1 % (v/v) aqueous sulphuric acid (91:8:1, v/v/v) as shown in Fig. 2.

Extraction of urine samples

Urine samples (pH approx. 6.5) were extracted with ethyl acetate (2 volumes) and the organic phase discarded. The aqueous layer was adjusted to pH 1 with sulphuric acid and extracted again with ethyl acetate (2 × 2 volumes). The combined extracts were dried in a stream of nitrogen and redissolved in water (0.2 volumes).

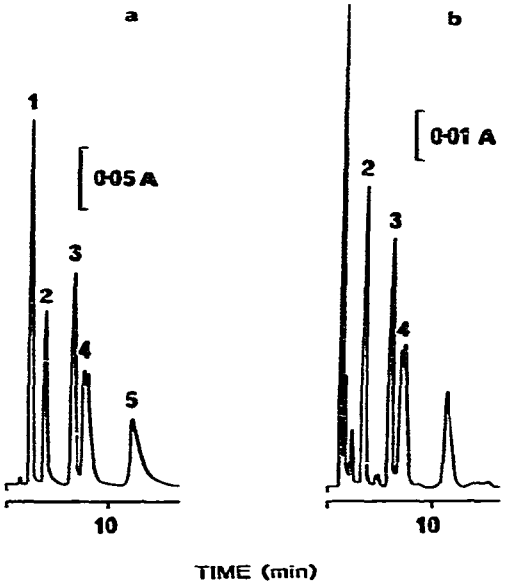

Fig. 2. a, Separation of N-nitrosoamino acids (for conditions see text). Peaks: 1 = NAZET; 2 = NPRO; 3 = NISONIP; 4 = NNIP; 5 = NPIPEC. 4 μg each. b, Urine sample spiked with 100 μg NPRO, NISONIP and NNIP.

Selection of internal standard and nitrite trapping reagent

When extracts of rat urine were spiked with the five nitrosoamino acids and subjected to HPLC separation it became clear that neither N-nitrosopipecolinic acid (NPIPEC), which co-chromatographed with a urinary component, nor N-nitrosoazetidine-2-carboxylic acid (NAZET) which was poorly resolved from unretained material, were suitable internal standards. N-nitrosoisonipecotic acid (NISONIP) eluted as a sharp single peak clear of interfering urinary UV absorbing peaks and was chosen as internal standard. N-Nitrosonipecotic acid (NNIP) eluted as a double peak, presumably resulting from conformational isomerism[11] but was also clear of interfering peaks. Thus nipecotic acid, which is likely to show similar nitrosation behaviour to proline[12], is a suitable nitrite trapping agent.

Analysis of urine samples

A linear response over the range 10–2500 μg/ml was obtained when urine extracts were spiked with NPRO, NNIP and NISONIP and analysed against a standard aqueous solution. Consistent extraction ratios (approximately 1.0) were obtained on analysis of urine samples spiked with these nitrosamines.

The data system was then calibrated for internal standard quantitation with the appropriate response factors for NPRO and NNIP modified to allow for their recoveries on extraction relative to NISONIP. Urine samples from rats which had received [^{14}C]proline were collected into dry ice and stored frozen until analysed. Immediately upon thawing, NISONIP (50 μg/ml) and nipecotic acid (436 μg/ml) were added and the sample was extracted and analysed with reference to an external standard solution of the three nitrosoamino acids. The NPRO peak was collected and the NPRO concentration and specific activity obtained from the UV absorbance and radioactivity data.

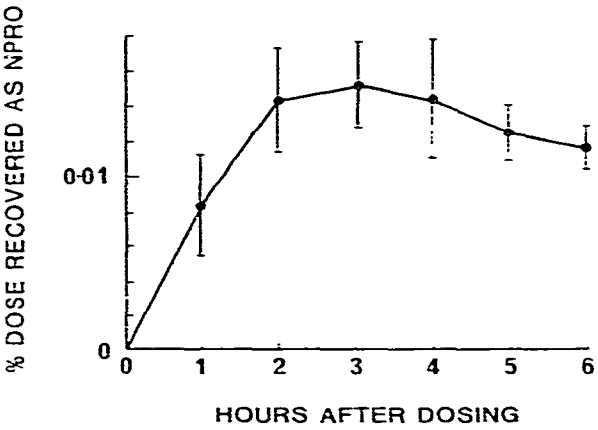

Fig. 3. Excretion of [^{14}C]NPRO in the urine of rats given [^{14}C]PRO (100 mg/kg body weight) and sodium nitrate (1500 mg/kg body weight) orally. Points represent the mean ± S.E.M. for 5 animals.

The concentration of NNIP found in the analysis gave an estimation of the degree of nitrosation occurring during work up of the urine sample and the calculated NPRO concentrations were corrected for this (<3%). Fig. 3 shows the time course from a typical experiment involving the administration of [^{14}C]proline and nitrate to rats. N-nitrosoproline is presumably formed as a consequence of the reaction between proline and nitrite arising from microbial reduction of nitrate in the animals[13].

It is of interest that the specific activity of the [^{14}C]NPRO excreted was measurably lower than that of the [^{14}C]PRO administered. This observation suggests that an appreciable contribution to the formation of NPRO may be made by the endogenous proline pools.

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