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Note

A method for the determination of S-methylcysteine by high-performance liquid chromatography: application to the study of carcinogenic methylating agents

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S-Methyl-L-cysteine (SMC) can be formed in proteins by reaction of cysteine residues with methylating agents[1]. There is also some recent evidence that it is a naturally occuring, although very minor residue, in the haemoglobin of many species[2]. A number of methods exist for the analysis of SMC[3–5] but none of these were practicable for a large number of samples. We were interested in the methylation of cysteine by a number of different methylating agents under standard conditions and required a rapid method of analysis for SMC in aqueous solutions.

The analysis of amino acids as their N-(2,4-dinitrophenyl) derivatives has had wide application since its original inception[6] but it appears that this procedure has not been applied to SMC despite its potential for ease of derivatisation, applicability to high-performance liquid chromatographic (HPLC) analysis and sensitivity. In this note, a simple procedure for the analysis of SMC in aqueous solution is described as well as its use in an *in vitro* methylation study.

EXPERIMENTAL

Chemicals

1-Fluoro-2,4-dinitrobenzene, S,N-di-(2,4-dinitrophenyl)-cysteine, N,N-di-(2,4-dinitrophenyl)-cystine, 2,4-dinitrophenol and N-methyl-N-nitrosourea were obtained from Sigma (St. Louis, MO, U.S.A.). Methyl iodide, methyl methanesulphonate and N-methyl-N-nitroso-N′-nitroguanidine were obtained from Aldrich (Milwaukee, WI, U.S.A.). N-Methyl-N-nitrosoacetamide was a gift from Dr. L. K. Shuker (New England Institute for Life Sciences, Waltham, MA, U.S.A.). All other chemicals were laboratory reagents and used as supplied.

N-(2,4-Dinitrophenyl)-S-methyl-L-cysteine (DNP-SMC)

A stirred solution of SMC (0.2 g) in water (4 ml) was treated with sodium bicarbonate (0.4 g) followed by N,N-dimethylformamide (DMF, 1.0 ml). 1-Fluoro-2,4-dinitrobenzene (FDNB, 0.4 ml) was added and the resulting mixture stirred at

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room temperature, in the dark, for 15 min. The mixture was washed with diethyl ether (3 × 10 ml), acidified (concentrated hydrochloric acid) to pH 2 and extracted with chloroform (3 × 25 ml). The combined extracts were dried (sodium sulphate) and evaporated to give a yellow oil which crystallised on standing. The crystalline residue was extracted with hot diethyl ether (3 × 10 ml). The diethyl ether extract was cooled overnight at 0°C to give yellow crystals which were filtered and washed with a small amount of cold diethyl ether to give DNP-SMC as a 1:1 DMF complex (0.21 g, 47%), m.p. 82–84.5°C; NMR ($CHCl_3$) 2.19 (3H,s,S-CH_3) 2.90, 3.00 [6H,d, $-N(CH_3)_2$] 3.19 (2H,d,J,=6Hz, $-CH_2-$) 4.58 (1H,m,J,=6Hz, –CH) 6.90 (1H,d,J_2=9Hz,6-*H*) 8.05 (1H,s,C*H*O) 8.3 (1H,dd,J_2=9Hz, J_3=3Hz,5-*H*) 9.15 (1H,d,J_3=3Hz,3-*H*) 9.15 (1H,exch,N*H*) 10.8 (1H,s,exch, $-CO_2H$); *m/z* (EI, 70 eV), 301 (M^+, 0.8%) 255 (5%) 193 (16%) 191 (9.6%) 177 (100%) 176 (22%) 131 (26%) 130 (24%) 118 (9.6%) 103 (7.4%) 90 (12.5%) 75 (11.5%); (CI, NH_3) 302 (MH^+). $C_{10}H_{11}N_3O_6S \cdot C_3H_7NO$ requires C, 41.80; H, 4.81; N, 14.95; S, 8.55%; found C, 41.67; H, 4.95; N, 15.00; S, 8.38%.

Derivatisation

Aqueous solutions of SMC (1 ml) were adjusted to pH 8 by addition of solid sodium bicarbonate and treated with excess FDNB (2 μl) and stirred, in the dark, at 37°C for 70 min. Unreacted FDNB was removed by extraction with ether (3 × 1 ml).

Aliquots of the resulting aqueous solution were analysed by HPLC.

HPLC

Samples of DNP-SMC solutions were analysed by reversed-phase HPLC on a 5-μm Lichrosorb RP-18 column (12.5 cm × 4 mm I.D.) with a mobile phase consisting of ammonium dihydrogen phosphate (30 m*M*, adjusted to pH 6)–acetonitrile (4:1 v/v) at a flow-rate of 1 ml/min. The column eluate was monitored at 420 nm.

Calibration

Standard solutions of synthetic DNP-SMC were prepared in aqueous bicarbonate buffer. A calibration curve was prepared by injecting 10-μl aliquots of the standards. The corresponding peak heights were plotted against the amounts of DNP-SMC injected.

Determination of DNP-SMC

Aliquots (10 μl) of derivatised reaction solutions were injected onto the HPLC system and the resulting peak heights measured. The amount of SMC was calculated from the calibration curve.

RESULTS AND DISCUSSION

DNP-SMC had good chromatographic properties on reversed-phase HPLC (Fig. 1). Side-products (DNP, S,N-di-DNP-cysteine and N,N'-di-DNP-cysteine) did not interfere with the analysis. The identity of the derivative of SMC was established unambiguously by synthesis using a standard method[7] and characterised by spectroscopic methods.

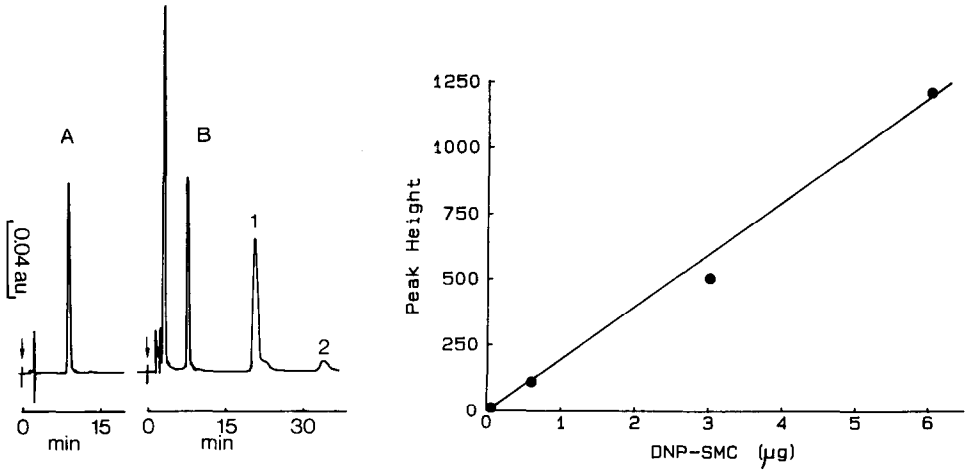

Fig. 1. HPLC chromatograms of (A) DNP-SMC standard (3.01 μg injected) and (B) a typical reaction solution after derivatisation with FDNB. The late eluting peaks correspond to N,N'-di-DNP-cystine (1) and N,S-di-DNP-cysteine (2).

Fig. 2. A typical calibration curve for the determination of DNP-SMC. The peak height scale is in arbitrary units.

The derivatisation of SMC by FDNB was quantitative as determined by derivatising a series of standard solutions of SMC and comparing the resulting DNP-SMC peaks with equimolar solutions of synthetic DNP-SMC analysed under identical conditions.

The peak shape was such that a calibration curve was constructed of peak height (arbitrary units) *versus* concentration and was linear over the range 0.06–6.0 μg (Fig. 2). The concentration of SMC was then determined by using the unknown peak height and reading the result directly off the calibration curve.

The method was applied to the analysis of SMC produced from a range of methylating agents (CH_3–*X*) and cysteine under a standard set of conditions.

Five methylating agents [methyl iodide (MeI), methyl methanesulphonate

TABLE I

YIELDS OF S-METHYL-L-CYSTEINE FROM CH_3–*X* AND L-CYSTEINE

Reaction conditions: CH_3–*X* (2 m*M*) in sodium borate buffers (50 m*M*, pH 9) for 24 h at 37°C in the dark. SMC determined as N-DNP derivative (see text). The results are the average of several determinations.

CH_3–X	*SMC (mM)*	
	L-*Cysteine (4 mM)*	L-*Cysteine (20 mM)*
MeI	1.50	1.60
MMS	1.00	1.50
MNNG	0.15	0.52
MNA	0.16	0.71
MNU	0.17	0.43

(MMS), N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG), N-methyl-N-nitroso-acetamide (MNA), and N-methyl-N-nitrosourea (MNU); Table I] were incubated with excess cysteine in borate buffer for 24 h at 37°C. The yields of SMC were then determined by HPLC following derivatisation with FDNB and the results are summarised in Table I.

The results fall into two groups. MeI and MMS give greater than 50% yields of SMC with only 2-fold molar excess of L-cysteine and these are only slightly increased with a 10-fold molar excess of thiol. In contrast the three N-methyl-N-nitroso compounds yielded less than 10% SMC with 2-fold molar excess L-cysteine but showed a 2–4-fold increase with 10-fold molar excess of thiol.

The reaction between MeI and MMS and L-cysteine would be expected to follow a classical S_N2 reaction pathway[8] with the strongly nucleophilic thiol, or thiolate, competing very effectively with the aqueous solvents for the methyl group. In contrast, the N-nitroso-N-methyl compounds resulted in significantly lower levels of S-methylation, there being little difference in S-methylation between MNU, MNA and MNNG. This is of interest since the decomposition of MNU is not catalyzed by thiols[9], in contrast to the strong catalytic effect on MNNG[10] and MNA[11]. Thus the ratio of products must be determined by the relative rates of reaction of the common intermediate, methyldiazotate, with the sulfhydryl group and water. The final product of these compounds is presumably the methyl diazonium ion which is a highly reactive species[12] which can undergo a reaction with a nucleophile resulting in the displacement of molecular nitrogen[13]. The methyl diazonium ion would be expected to show less selectivity between competing nucleophiles (in this case, thiols and water)[12] resulting in the observed lowering in the yield of S-methylation.

The method of analysis described above has also been applied to the detection of SMC residues in albumin derived from rat hepatocyte cultures following exposure to radiolabelled dimethylnitrosamine[14].

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