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Note

Chromatographic studies on chemical degradation of carcinostatic nitrosoureas

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The compounds 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride (ACNU; NSC-D 245382), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; NSC-79037) and 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (Me-CCNU; NSC-95441) are currently being evaluated as drugs for the treatment of, *e.g.*, Hodgkin's disease and cancer of the brain, lung and digestive organs

The main chemotherapeutic mechanism of these nitrosoureas is alkylation of the nucleosides in the tumour cell, which is correlated with formation of active carbonium ions via a 2-chloroethyldiazene hydroxide intermediate[1]. We have isolated[2] the two active cations $^{+}CH=CH_2$ and $^{+}CH_2CH_2Cl$ as coloured derivatives of *p*-nitrobenzylpyridine[3]. However, these cations react with OH^- ions more rapidly than with the acceptor, producing 2-chloroethanol and acetaldehyde in physiological aqueous medium. Thus, in order to study the alkylating activities of nitrosoureas *in vitro*, the amounts of acetaldehyde and 2-chloroethanol must be determined.

The 2-chloroethanol formed in the buffer solution can be easily determined by direct gas chromatography (GC). Quantitative micro-scale analysis for aldehydes and ketones, has also been carried out by GC[4] and, more recently, by high-performance liquid chromatography (HPLC)[5] after conversion of the compounds into their 2,4-dinitrophenylhydrazones (DNPHs). However, these methods require many operations.

In this paper, we describe a simple HPLC method, without solvent extraction, for determining acetaldehyde (as its DPNH) formed in aqueous solution; a reversed-phase column is used. A series of C_1 to C_{10} aldehyde and/or ketone DNPHs was also analysed to ascertain the efficiency of the separation system, and further possible applications of the system are discussed.

EXPERIMENTAL

Chemicals

ACNU[6], BCNU[7], CCNU[8] and Me-CCNU[8], were synthesised according to methods described in the literature. 2,4-Dinitrophenylhydrazine (DNP), 2-chloroethanol and acetaldehyde were obtained from Tokyo Chemical Industry (Tokyo,

Japan), DNPHs of C_1 to C_{10} carbonyl compounds were prepared by a standard method[9], and acetaldehyde DNPH was used for calibration purposes; these DNPHs were purified by recrystallisation from ethanol.

Gas chromatographic determination of 2-chloroethanol

Each nitrosourea (2.2 nmoles) was dissolved in 200 μl of ethanol, and the solution was added to 2 ml of 40 m*M* phosphate buffer solution of pH 7.4. The reaction mixtures were prepared in 10-ml Reacti-Vials®, each equipped with a PTFE-backed rubber septum. After 3 h at 37°, the vessels were cooled in ice, and 2 μl of each solution were removed, via the septum, for GC. A Hewlett-Packard 7610A instrument equipped with a dual-flame detector, and a recording integrator (3380A), were used. A glass column (1.2 m × 3 mm I.D.) packed with 10% of Carbowax 20M on Diaport S (60–80 mesh) was used; the oven temperature was set at 85°, and the flow-rate of He was 40 ml/min. The calibration graph was established by using a standard solution of 2-chloroethanol and the absolute-peak-area method.

Preparation of DNPH derivatives

The DNP solution was prepared by dissolving 34 mg of DNP in 100 ml of 2 *M* hydrochloric acid. To the residual reaction solution after the determination of 2-chloroethanol were added 4 ml of the DNP solution, through the septum, by means of a 5-ml gas syringe (Precision Sampling Co.). The reaction vessels were allowed to stand for 1 h at room temperature, with occasional shaking, then 2 ml of 4 *M* potassium hydroxide solution in methanol were added, changing the pH from 2 to 4. After precipitating the potassium chloride and unreacted DNP, 10 μl of the remaining solution were injected into the HPLC system.

HPLC method for DNPH determination

A Waters ALC 202/401 liquid chromatograph equipped with a μBondapak C_{18} column (30 cm × 4 mm I.D.) was used to carry out the separations, with methanol–12.5 m*M* phosphate buffer solution of pH 7.0 (3:1) as mobile phase (1 ml/min); the eluate was monitored by means of a 440 UV detector at 340 nm (the detector was operated at a sensitivity of 0.02 a.u.f.s.). Peak-height measurements were used for determining acetaldehyde DNPH, and the calibration graph (0–100 ng per injection) was obtained by using standard DNPH solution.

A mixture of C_1 to C_{10} DNPHs was analysed to estimate the efficiency of this reversed-phase column; the mobile phase used for this purpose was methanol–water (4:1), and the other conditions were the same as those used with acetaldehyde DNPH. Gradient elution, in which the methanol concentration was increased from an initial 80% to 100% during 5 min, was also applied to effect the separation; a Model 660 solvent programmer (operated in mode "4") was used for this purpose.

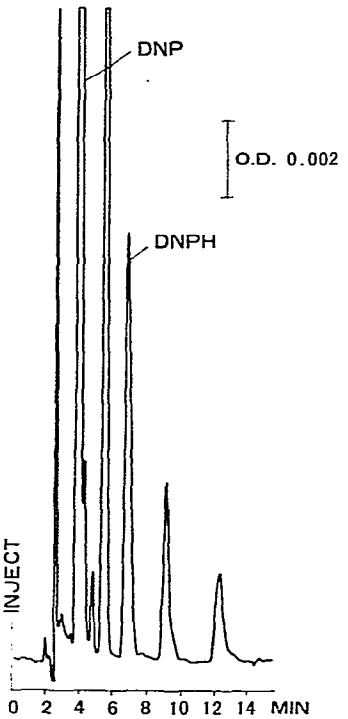
Chloride ion determination

A 2.2-ml portion of each buffered nitrosourea solution, prepared as described above, was placed in a 500-ml Schöniger flask fitted with a stopper. After 3 h at 37°, 100 ml of ethanol were added, and the alcoholic solution was acidified with 0.05 ml of 2.5% nitric acid, then titrated with 0.02 *N* mercuric nitrate solution, diphenylcarbazone being used as indicator[10].

RESULTS AND DISCUSSION

A typical HPLC chromatogram of the acetaldehyde DNPH and a gas chromatogram of the 2-chloroethanol formed from nitrosourea are shown in Figs. 1 and 2, respectively. The amounts of acetaldehyde and 2-chloroethanol in the phosphate buffer solution (pH 7.4) of nitrosoureas at 37° during 3 h are listed in Table I.

For ACNU and BCNU, some 11 to 14% of the nitrosourea was converted into acetaldehyde and about 50% into 2-chloroethanol. With CCNU and Me-CCNU,



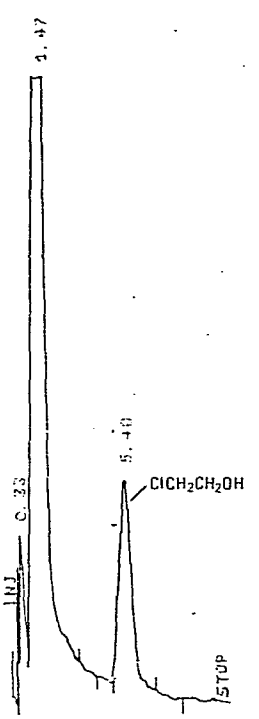


Fig. 1. Typical HPLC chromatogram of acetaldehyde DNPH derived from nitrosourea.

Fig. 2. Typical gas chromatogram of 2-chloroethanol derived from nitrosourea.

TABLE I

FORMATION OF 2-CHLOROETHANOL, CHLORIDE IONS AND ACETALDEHYDE FROM NITROSOUREAS DURING 3 h IN BUFFER (pH 7.4) AT 37°

Nitrosourea	*Half-life (min)*	*Percentage of*				
		Residue	*$ClCH_2CH_2OH$*	*Cl^-*	*Sum of residue, Cl^- and $ClCH_2CH_2OH$*	*CH_3CHO*
ACNU	28	3	48	42	93	14
BCNU	38	4	50	50	104	11
CCNU	49	8	35	65	108	19
Me-CCNU	46	6	34	65	105	24

however, the amount of acetaldehyde formed was about twice that formed from ACNU and BCNU, but formation of 2-chloroethanol only about 66% of that derived from ACNU and BCNU. The residual amounts of the nitrosoureas and their half-lives in the medium were determined by a colorimetric method based on the Griess reaction[11]; the decomposition of each nitrosourea under these conditions appeared to be a first-order reaction, and the sum of the contents of 2-chloroethanol, free chloride ions and residual parent compound totalled nearly 100% in all instances. This suggests that formation of other fragments containing chlorine (such as 1,2-dichloroethane or vinyl chloride) is negligible.

These differences among the nitrosoureas were also observed in thin-layer densitometry, with Koenig's reagent [*p*-nitrobenzylpyridine (NBP)] as cation acceptor (NBP–CH_2CH_2Cl, NBP–$CH{=}CH_2$, and NBP–CH_2CH_2OH had been identified by Asami *et al.*[2] by mass spectrometry of the coloured derivatives).

We conclude that the precursors of acetaldehyde and 2-chloroethanol are a vinyl and chloroethyl cation, respectively (see Fig. 3). BCNU and CCNU, after the 2-chloroethyldiazene hydroxide stage, degrade to the corresponding amine or dialkylurea via an isocyanate intermediate having carbamoylating activity[1,12]. With ACNU, compound 1 (Fig. 3) has been identified as a result of intramolecular aminolysis[13], and it is formed quantitatively in physiological aqueous medium. This reaction might be correlated with the fact that, of the compounds studied, ACNU had the shortest lifetime. It would be worthwhile to investigate possible correlation between *in vitro* alkylation and carbamoylation activities and carcinostatic effects among these nitrosoureas.

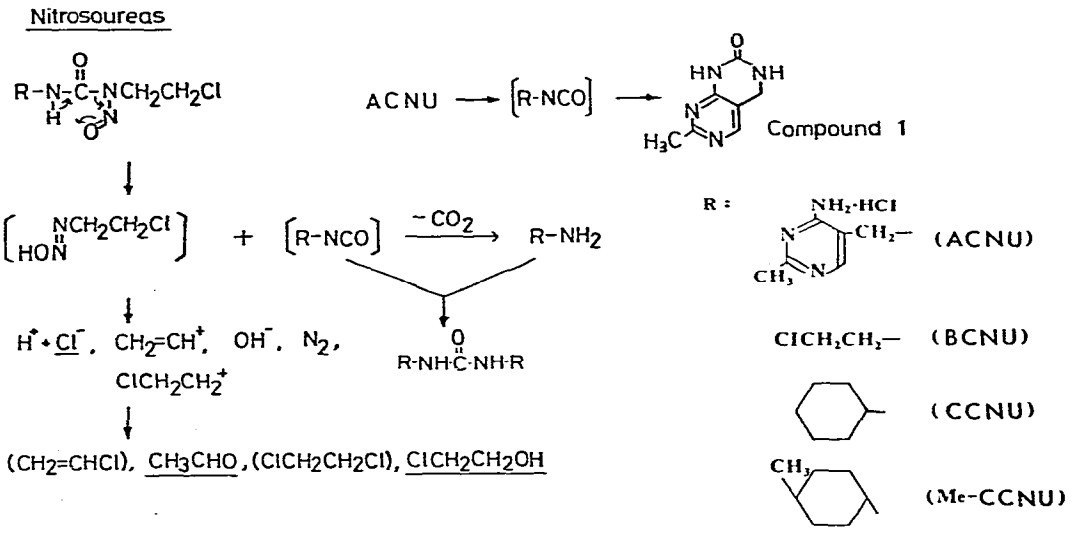


Fig. 3. Chemical degradation scheme proposed for nitrosoureas.

In this work, we found that a simple HPLC method, without solvent extraction, could be used for the acetaldehyde assay. Reversed-phase HPLC was also found to be useful for analysis of various aldehyde and ketone DNPHs, including the derivatives of C_1 to C_{10} compounds (Fig. 4). The rectilinear relationship between the

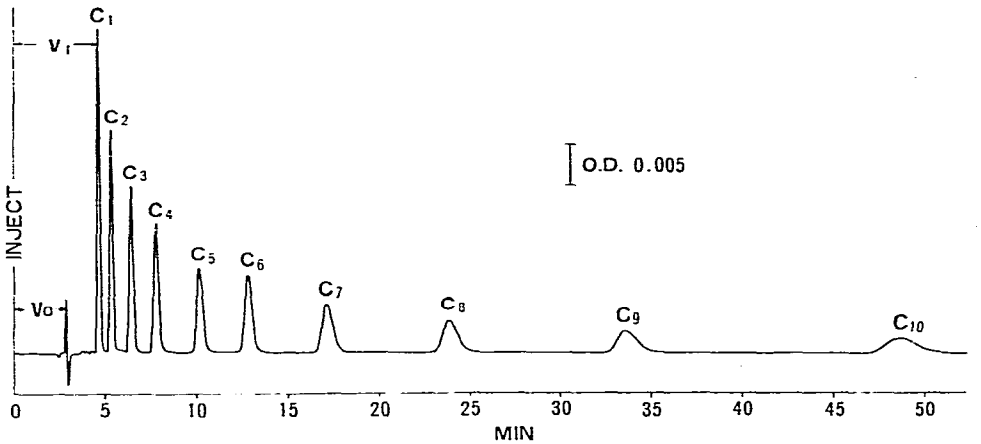
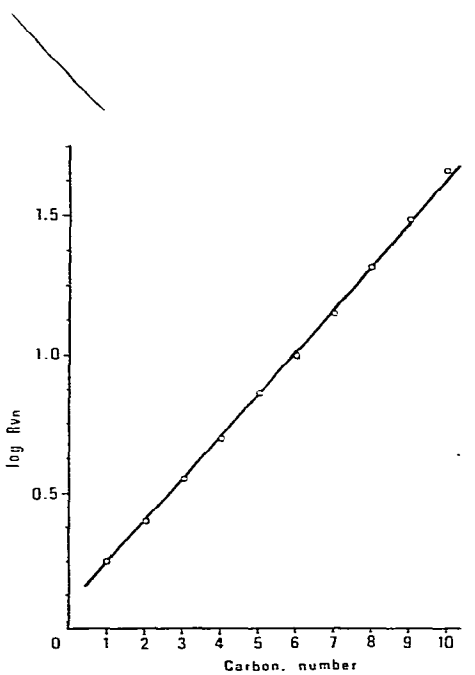


Fig. 4. HPLC chromatogram of C_1 to C_{10} DNPHs (100 ng/injection).

logarithm of the retention volume and the carbon number for these compounds is shown in Fig. 5. These assays, however, can be better effected by using gradient elution (see Fig. 6), the analysis time being thereby shortened to only 11 min. The systems described should be suitable for the micro-determination of aldehydes and ketones in a variety of other applications.



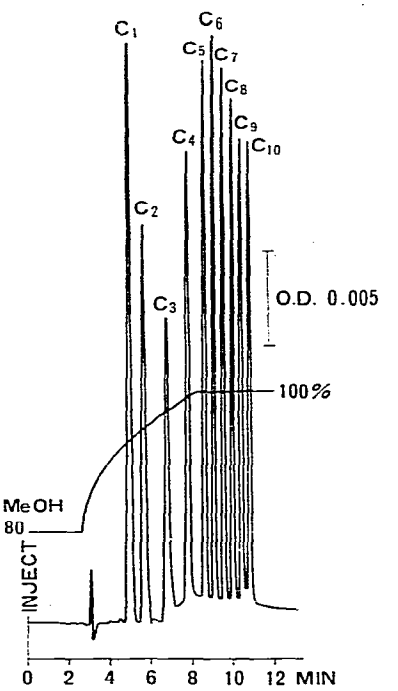


Fig. 5. Relationship between carbon number of aldehyde or ketone and retention volume (Rv_n); $Rv_n = V_n - V_0$ where V_0 is the void volume (ml) and V_n is the elution volume (ml).

Fig. 6. HPLC Chromatogram of C_1 to C_{10} DNPHs under a methanol gradient as eluent (100 ng/injection).

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