Journal of Chromatography, 505 (1990) 402–407
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 276

Note

Analysis of decomposition products of alkyl nitronic acids by reversed-phase high-performance liquid chromatography

A. J. SHAW and A. GESCHER*

Mechanisms of Drug Toxicity Research Group, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET (U.K.)

(First received November 3rd, 1989; revised manuscript received January 11th, 1990)

Nitroalkanes (I), such as 2-nitropropane, are important industrial solvents with hepatotoxic and mutagenic properties[1]. The interpretation of their pharmacological and biochemical properties is complicated by the fact that they exist in tautomeric equilibrium with their respective nitronates (II) and nitronic acids (III).

$$\underset{\text{I}}{R^1R^2CHNO_2} + B \rightleftharpoons \underset{\text{II}}{R^1R^2C{=}NO_2^-} + BH^+ \rightleftharpoons \underset{\text{III}}{R^1R^2C{=}NOOH} + B \qquad (1)$$

The kinetics of equilibrium 1 have been extensively studied [for a review, see ref. 2]. Nitronic acids (III) resemble carboxylic acids in strength (pK_a 2–6). Their isomerisation to the nitroalkane tautomer proceeds essentially to completion for most simple nitronic acids, because of the relatively weaker acidity of a nitroalkane compared to its corresponding nitronic acid. The mechanism of the reaction III→II requires hydrogen abstraction and reaction II→I involves C-protonation on the intermediate anion II. Consequently, the tautomerisation rate is accelerated in neutral to slightly basic solution and inhibited in acidic solution[3]. Strong acid usually favours decomposition of III by hydrolysis, the so called Nef reaction (reaction 2). The Nef reaction is a convenient preparative route to the carbonyl compound IV, aldehydes in the case of primary nitronic acids or ketones in the case of secondary nitronic acids. Two further reactions 3 and 4 invariably accompany the Nef reaction. The redox reaction 3 yields the alkyl oxime V and the nitrosation reaction 4 leads to the pseudonitrole VI:

$$R^1R^2C{=}NOOH + H_3O^+ \rightarrow \underset{\text{IV}}{R^1R^2C{=}O} + H^+ + HNO + H_2O \qquad (2)$$

$$R^1R^2C{=}NOOH + HNO \rightarrow \underset{\text{V}}{R^1R^2C{=}NOH} + HNO_2 \qquad (3)$$

$$R^1R^2C{=}NO_2^- + HNO_2 + H^+ \rightarrow \underset{\text{VI}}{R^1R^2C(NO)NO_2} + H_2O \qquad (4)$$

0021-9673/90/$03.50

The blue colouration that often accompanies these reactions is due to the formation of nitronitroso (VI) and hydroxynitroso [$R^1R^2C(OH)NO_2$] derivatives[4,5]. In the case of primary nitroalkanes the colouration often disappears rapidly as these derivatives rearrange to form the more stable, colourless nitrooximes $RC=NOH(NO_2)$ (VII)[5]. Ultimately the structure of the nitronic acid determines its rate of tautomerisation and decomposition. For example, highly ionised nitronic acids, *e.g.* $XCH=NO_2H$ (X = Cl, Br), tautomerise rapidly even at low pH without undergoing the Nef reaction[5]. Conversely branching at the nitronate α-carbon can greatly hinder both tautomerisation and the Nef reaction[6,7].

As part of an investigation into the role of metabolism in the toxicity of nitroalkanes and their anionic tautomers we have developed a simple reversed-phase high-performance liquid chromatography (RP-HPLC) programme to detect and quantitate potential metabolites with a range of hydrophilic and hydrophobic properties. We show here how this procedure can be used to determine the extent of equilibriation between nitro and nitronate forms and to quantitate the decomposition products of nitronic acids.

EXPERIMENTAL

Materials

All chemicals were purchased from Aldrich (Gillingham, U.K.) with the following exceptions; 1-nitropropane and 2-nitropropane were obtained from Fluka (Glossop, U.K.) and were redistilled until they were > 98% pure. Glutathione and N-acetylcysteine were purchased from Sigma (Poole, U.K.). Nitronates were prepared by dissolving nitroalkanes in 1 *M* aqueous potassium hydroxide in equimolar proportions[8]. These solutions were adjusted to the desired pH and used within 2 h of preparation. 2-Nitro-2-nitrosopropane (propylpseudonitrole) was synthesised by reacting propyl-2-nitronate slowly on ice with concentrated hydrochloric acid according to Nygaard[9]. The cream-coloured precipitate produced, the dimeric form of the compound, was reverted to the blue monomeric state by dissolution in acetic acid. S-Nitrosoglutathione was synthesised by reacting HNO_2 with reduced glutathione as described by Hart[10]. S-Nitroso-N-acetylcysteine was prepared by reacting HNO_2 with N-acetylcysteine in a similar fashion.

RP-HPLC

The chromatographic equipment consisted of a Waters (Milford, MA, U.S.A.) 600E multi solvent delivery system equipped with a Waters 480 UV detector. The detector signals were recorded on a Waters 745B data handling system capable of baseline subtraction. The column was a Waters reversed-phase Nova-Pak C_{18} radial column (4 μm particle size; 10 cm × 5 mm) contained within a Waters RCM compression module. Injections (10 μl) were made using a Waters 700 Satellite automatic sample processor. Mobile phase: solvent A was 0.15% acetic acid (FSA Laboratory Supplies, Loughborough, U.K.) in HPLC-grade water (BDH, Poole, U.K.); solvent B was HPLC-grade methanol (BDH). Both solvents were passed through a 0.2-μm Millipore filter and degassed before use. Solvents were sparged with helium during use. The solvent programme commenced with 95% solvent A in B for 5 min followed by a linear transition to 20% A in B over 10 min. The solvent mixture

was reversed (convex curve) to its original proportions over a 5-min period and left to establish the initial conditions for 15 min. The flow-rate was 1.0 ml/min and the column effluent was monitored at 220 nm.

RESULTS AND DISCUSSION

The RP-HPLC retention times of the simple nitroalkanes studied increased with chain length, and, predictably, they were greater for primary nitroalkanes than for the respective isomeric secondary ones (Table I). Calibration graphs for the nitroalkanes in the concentration range 1–25 mM showed good linearity with correlation coefficients $\geqslant 0.998$. All nitroalkanes under study except the tertiary derivative 2-methyl-2-nitropropane tautomerised completely to the nitronates on addition of equimolar sodium hydroxide. The resolution of the neutral and ionic tautomers was good except in the case of the highly hydrophilic (nitromethane) or hydrophobic (1-nitropentane and 1-nitrohexane) nitroalkanes (Table I and Fig. 1A and B). Regeneration of the nitroalkane from the nitronate was >99% when the pH was gradually adjusted to pH 5.5 using Tris–HCl buffer. Calibration graphs of the well-resolved nitronates within the concentration range 0.5–10 mM displayed good linearity with correlation coefficients $\geqslant 0.997$. The limit of detection for nitroalkanes and nitronates was 50 μM using 220 nm as detection wavelength. In studies of the equilibrium I→III or the enzymatic oxidation of II nitronates have hitherto been quantified spectrophotometrically[8] using their UV absorption between 220 and 235 nm. Compared to the UV-spectrophotometric analysis the HPLC method described here should allow greater versatility and better specificity.

Addition of strong acid, *e.g.* hydrochloric acid, to nitronates generates the

TABLE I

RETENTION TIMES OF NITROALKANES, THEIR NITRONATES AND PRODUCTS OF NITRONIC ACID DEGRADATION

Retention times are the mean of five determinations made on separate days. The standard deviation was <2% of the mean in all cases.

Nitroalkane	*Retention times (min)*[a]				
	Nitro form (I)	*Nitronate (II)*	*Alkyl oxime (V)*	*Nitrooxime (VII)*	*Pseudonitrole (VI)*
Nitromethane	2.0	1.9	–	–	–
Nitroethane	3.8	1.9	2.4	2.0	–
1-Nitropropane	10.6	3.6	5.1	–	–
1-Nitropentane	19.7	19.6	18.4	17.8	–
1-Nitrohexane	21.1	21.0	20.1	19.7	–
2-Nitropropane	10.4	3.0	4.8	–	16.0
Nitrocyclopentane	18.0	9.0	14.5	–	19.5
Nitrocyclohexane	19.8	15.7	17.2	–	20.9
2-Methyl-2-nitropropane	16.9	–	–	–	–

[a] Assignment of retention times by comparison with reference compounds in the case of V and VI, speculative for VII.

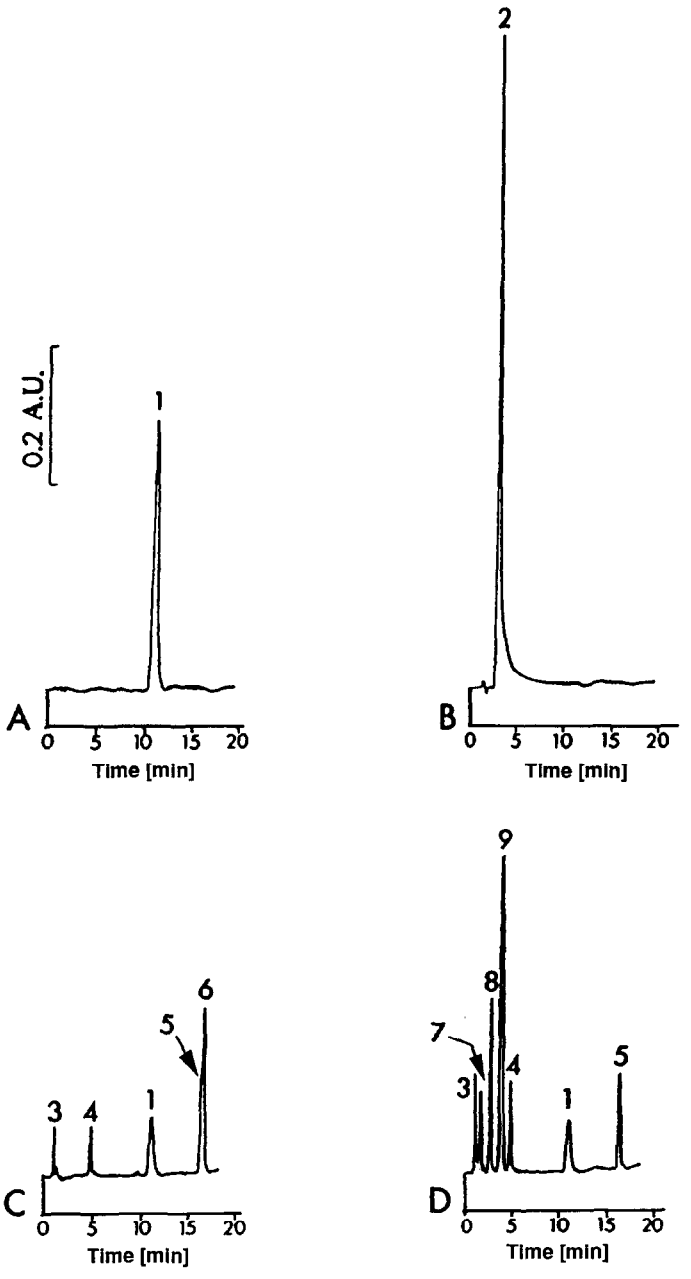

Fig. 1. RP-HPLC chromatograms of (A) 2-nitropropane (25 m*M*) in water, (B) propyl-2-nitronate (10 m*M*), (C) products of tautomerisation and degradation of propyl-2-nitronate 20 min after rapid adjustment to pH 3 using hydrochloric acid, and (D) products of propyl-2-nitronate 20 min after rapid adjustment to pH 3 and 10 min after addition of reduced glutathione (10 m*M*). For chromatographic conditions see Experimental. The eluent was monitored at 220 nm. The chromatograms were obtained after subtraction of the background recorded in solvent controls. Peaks (tentatively assigned by comparison with reference compounds, or by speculation in the case of peak 5): 1 = 2-nitropropane; 2 = propyl-2-nitronate; 3 = nitrous acid; 4 = acetone oxime; 5 = 2-hydroxy-2-nitrosopropane; 6 = propylpseudonitrole; 7 = glutathione (GSH); 8 = glutathione disulfide (GSSG); 9 = S-nitrosoglutathione.

corresponding nitronic acids which, due to the acidic environment, undergo rapid hydrolysis and further reactions (reactions 2–4, Fig. 1C). Aldehydes and ketones generated by the Nef reaction were undetectable by RP-HPLC. Other products were tentatively identified by cochromatography with authentic material as alkyl oximes (V) and pseudonitroles (VI). These decomposition products were well resolved as they were eluted before and after the precursor nitroalkanes, respectively (Table I). Calibration graphs of the oximes at 220 nm and of the pseudonitroles at either 220 or 290 nm showed good linearity with correlation coefficients $\geqslant 0.997$.

We have utilised the RP-HPLC programme described here to investigate the reaction of nitroalkane derivatives with biological thiols such as glutathione and N-acetylcysteine. The blue pseudonitroles VI generated from secondary nitroalkanes, for example propyl-2-pseudonitrole (VI with $R^1,R^2=CH_3$), reacted with glutathione to produce a red coloured conjugate with a retention time of 3.8 min (Fig. 1D). The conjugate was identified as S-nitrosoglutathione (VIII, R = glutathionyl, see reaction 5) by fast atom bombardment mass spectroscopy and high-field ^1H NMR spectroscopy[11].

$$\underset{}{RSH} + \underset{VI}{R^1R^2C(NO)NO_2} \rightarrow \underset{VIII}{RSNO} + \underset{III}{R^1R^2C{=}NO_2H} \qquad (5)$$

S-Nitrosglutathione decomposed slowly to form glutathione disulfide (retention time: 2.8 min). Similarly N-acetylcysteine reacted with secondary alkyl pseudonitroles to form S-nitroso-N-acetylcysteine (retention time: 5.4 min) which degraded slowly to N-acetylcystine (retention time: 4.5 min). Production of S-nitroso conjugates, monitored at 220 or 336 nm, was accompanied by further generation of alkyl oxime V. In the light of reaction 5 V is presumably generated by acidic decomposition of the nitronic acid III formed via denitrosation of VI.

CONCLUSIONS

The RP-HPLC method described here can be used to (i) determine the concentration of nitroalkanes in aqueous and non-aqueous solutions, (ii) detect and measure the nitronate tautomers of nitroalkanes and (iii) detect products of the decomposition of nitronic acids. The procedure should be applicable to most nitroalkanes except those which are strongly hydrophobic. A disadvantage of the technique is its inability to detect aldehydes or ketones as nitronic acid degradation products, their measurement requires different chromatographic conditions.

ACKNOWLEDGEMENT

This research was supported by a generous grant from the Health and Safety Executive of Great Britain.

REFERENCES

1 IARC (International Agency for Research on Cancer), in *Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 29, WHO, Lyon, 1982, p. 331.
2 A. T. Nielson, in H. Feuer (Editor), *The Chemistry of the Nitro and Nitroso Groups*, Part 1, Interscience, New York, 1969, p. 349.

3 H. Feuer and A. T. Nielson, *Tetrahedron*, 19 (Suppl. 1) (1963) 65.
4 M. F. Hawthorne, *J Am. Chem. Soc.*, 79 (1957) 2510.
5 J. Armand, *Bull. Soc. Chim. Fr.*, (1965) 3246.
6 E. E. Van Tamelen and R. J. Thiede, *J. Am. Chem. Soc.*, 74 (1952) 2615.
7 R. A. Smiley and W. A. Pritchett, *J. Chem. Eng. Data*, 11 (1966) 617.
8 D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, 258 (1983) 9913.
9 E. M. Nygaard, *U.S. Pat.*, 2 401 267 (1946); *C.A.* 40 (1946) 6092.
10 T. W. Hart, *Tetrahedron Lett.*, 26 (1985) 2013.
11 A. J. Shaw, A. Gescher, D. Tooth, I. Linhart and P. B. Farmer, *Chem. Res. Toxicol.*, in press.