Journal of Chromatography, 243 (1982) 362–367
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,871

Note

Capillary gas chromatographic analysis of mercapturic acids

Decomposition of mercapturic acids according to a "retro-Michael" reaction

W. ONKENHOUT*, G. J. GUIJT, H. J. DE JONG and N. P. E. VERMEULEN
Department of Analytical Chemistry and Pharmaceutical Analysis, University of Leiden, P.O. Box 9502, 2300 RA Leiden (The Netherlands)
(Received March 4th, 1982)

Mercapturic acids, S-substituted N-acetyl-L-cysteine derivatives, are end-products of an important pathway in the metabolism of xenobiotics, *i.e.*, conjugation to glutathione. This type of conjugation, generally catalysed by glutathione transferases, is of special importance in the detoxification of reactive electrophiles which may be formed in the biotransformation of xenobiotics[1,2]. Consequently, the measurement of thioether excretion in urine[3], or more specifically mercapturic acid excretion in urine, has evoked interest as an indication of exposure to potentially harmful compounds and also as a reflection of the relative importance and the underlying mechanisms of glutathione conjugation *in vivo*[4,5]. In addition to the determination of mercapturic acids with the aid of the so-called thioether test[6], the selective determination

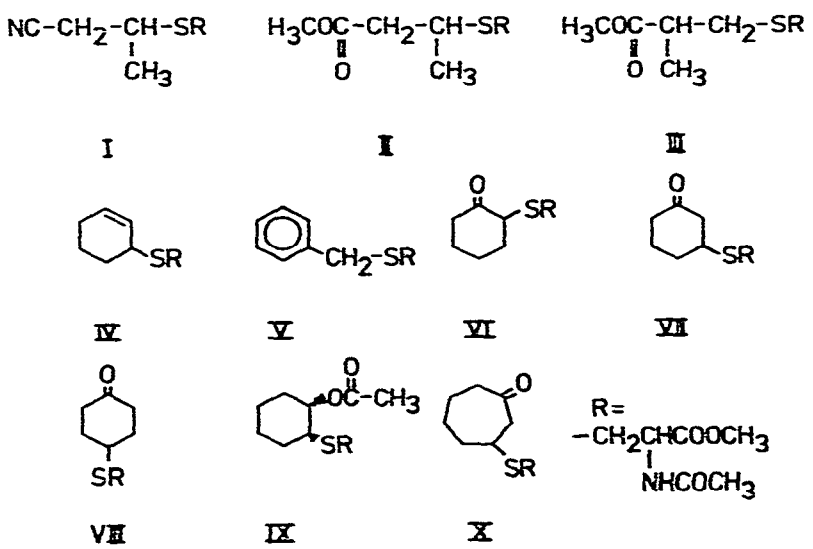

Fig. 1. Structures of ten synthetically prepared mercapturic acids (for names see Table I).

0021-9673/82/0000–0000/$02.75

of specific mercapturic acids has been performed by paper chromatography[7], high-performance liquid chromatography (HPLC)[8–10], gas–liquid chromatography (GLC)[11,12] and by gas chromatography–mass spectrometry (GC–MS)[13–17].

In this paper the separation of a mixture of ten synthetically prepared mercapturic acids (Fig. 1) by capillary support-coated open tubular (SCOT) column GLC is described. The mercapturic acids differ in volatility and polarity and therefore they were considered as a test mixture for GC analysis. As was shown by GC–MS, a number of these compounds decomposed thermally in the injection port of the gas chromatograph according to a "retro-Michael" reaction.

EXPERIMENTAL

Synthesis of reference compounds

Compounds I, II and III were a generous gift from Dr. L. P. C. Delbressine (University of Nijmegen, The Netherlands)[15,16].

The mercapturic acids IV, V, VI and VII were prepared according to procedures described previously[18].

Synthesis of VIII. To a solution of 1.66 g (14.6 mmol) 4-hydroxycyclohexanone in 9 ml of pyridine was added in 1 h a solution of 4.02 g (15.0 mmol) of tosyl chloride in 9 ml of chloroform at 5–8°C. After stirring for 2 h at room temperature the reaction mixture was poured into 250 ml of 1 *N* hydrochloric acid. The organic layer was separated, washed with 125 ml of 1 *N* hydrochloric acid, dried (magnesium sulphate) and the solvent was evaporated to give 2.0 g (7.5 mmol, 50%) of cyclohexanone-4-tosylate as light brown crystals. A solution of 6.7 g (25 mmol) of this compound and 4.4 g (25 mmol) of N-acetyl-L-cysteine methyl ester[18] in 70 ml of methanol was added to 25 mmol of sodium methoxylate in 100 ml of methanol. After refluxing for 1.5 h the solvent was evaporated. To the residue were added chloroform and water, the organic layer was separated and the aqueous layer was washed once with chloroform. The collected organic layers were dried (magnesium sulphate), filtered and evaporated to give an orange oil, which was purified by column chromatography (silica gel, 0.05–0.2 mm, eluents chloroform–acetone, 1:1). VIII was obtained as a yellow solid in 10% yield (0.7 g, 2.5 mmol).

^{1}H Nuclear magnetic resonance (NMR) data: δ 1.40–2.60 ppm (m, 8H), 2.04 ppm (s, 3H), 2.90–3.24 ppm (m, 3H), 3.76 ppm (s, 3H), 4.66–4.88 ppm (m, 1H), 6.50–6.78 ppm (m, 1H).

Synthesis of IX. *trans*-S-(2-Hydroxycyclohexyl)-N-acetyl-L-cysteine methyl ester was synthesized according to Van Bladeren *et al.*[18]. Acetylation (acetic anhydride, pyridine) gave the product in 75% yield.

^{1}H NMR data: δ 1.12–1.80 ppm (m, 8H), 2.07 ppm (s, 3H), 2.10 ppm (s, 3H), 2.44–2.80 ppm (m, 1H), 3.05 ppm (d, 2H), 3.76 ppm (s, 3H), 4.44–4.82 ppm (m, 2H), 6.40–6.64 ppm (m, 1H).

Synthesis of X. To a solution of 0.53 g (3.0 mmol) of N-acetyl-L-cysteine methyl ester[18] in 5 ml of dry toluene was added 0.35 ml (3.1 mmol) of 2-cyclohepten-1-one and 0.15 ml of triethylamine. The solution was stirred magnetically under nitrogen for 24 h. The mixture was diluted with 20 ml of toluene, washed with 5 ml of 0.1 *N* hydrochloric acid, dried (magnesium sulphate) and filtered. Subsequent evaporation of the solvent *in vacuo* gave 0.70 g (81%) of the product as an orange oil.

^{1}H NMR data: δ 1.32–2.80 ppm (m, 10H), 2.02 ppm (s, 3H), 2.84–3.16 ppm (m, 3H), 3.72 ppm (s, 3H), 4.66–4.92 ppm (m, 1H), 6.48–6.84 ppm (m, 1H).

Apparatus

The gas chromatograms were obtained on a Packard 428 gas chromatograph equipped with a glass splitter injection system, a flame-ionization detector and a capillary SCOT column with OV-101 as the stationary phase (Chrompack, Middelburg, The Netherlands) on Tullanox 500 (Alltech, Eke, Belgium).

The capillary SCOT column (Pyrex glass, 19.0 m × 0.45 mm I.D.) was prepared as follows. The empty column was leached with 20% (v/v) hydrochloric acid in water at room temperature, rinsed thoroughly with water and dried. Subsequently the column was coated dynamically three times with a suspension of 0.5% (w/v) Tullanox in dichloromethane. After each coating step the column was dried and the direction of coating was reversed. For deactivation the column was dynamically coated with a solution of 2.5% (w/v) OV-101 in dichloromethane, dried, sealed under nitrogen and deactivated according to the PSD method of Schomburg and co-workers[19,20] (temperature increased at 5 C min up to 350 C; the column was held for 8 h at this temperature). The column was subsequently rinsed with acetone, dried and coated with a solution of 5% (w/v) OV-101 in dichloromethane. Finally, the column was dried and conditioned under a helium flow of 4 ml/min and a temperature rise of 1 C min from 20 to 250 C. The latter temperature was maintained for 2 h.

Mass spectra were taken on an LKB 2091-2130 gas chromatograph–mass spectrometer equipped with a Digital PDP 11/05 computer. The column was as specified above. Injection was performed with a ball-valve solid injector. Helium was used as the carrier gas at a flow-rate of 3 ml/min. At the end of the column an additional flow of helium was used to ensure a total flow-rate in the separator of 18–20 ml/min. The ion source temperature was 200°C, separator temperature 210°C, electron energy 70 eV, trap current 50 μA and accelerating voltage 3.5 kV.

The NMR spectra were recorded on a Jeol PS 100 apparatus. The samples were dissolved in (C^2HCl_3) and tetramethylsilane was used as the internal standard.

RESULTS AND DISCUSSION

The analysis of mercapturic acids in animal and human urine has become increasingly important during the last decade[21]. Depending on the starting points, for this purpose non-specific[3,6] or specific assay techniques such as HPLC and GLC[8–12] have been used. With regard to the applicability of these techniques, HPLC in combination with the most widely used detector, *viz.*, the UV detector, is limited to the analysis of mercapturic acids that possess a chromophoric group. Because of the availability of sensitive and selective detectors, *e.g.*, nitrogen-selective or mass spectrometric, GLC is particularly suitable for the analysis of mercapturic acids in biological fluids.

Until now the GLC analysis of mercapturic acids, being relatively polar compounds, has been performed mostly on medium-polarity or polar stationary phases such as OV-17 or PEG 20M[13–17].

In this work the chromatographic behaviour of a number of synthetically prepared mercapturic acids was studied on a capillary SCOT column coated with the non-polar stationary phase OV-101.

Fig. 2 illustrates the chromatographic behaviour and separating power of such an apolar column. A total of 20,000 theoretical plates was calculated for mercapturic acid V, which is the only diastereomerically pure compound. Owing to a careful deactivation procedure even the polar mercapturic acids VI–X do not show tailing effects resulting from adsorption. In comparison with the medium-polarity and polar SCOT columns previously applied to the analysis of mercapturic acids[13–17], the OV-101 capillary column demonstrated far better temperature stability, *viz.*, up to 350°C. This observation is of special importance in view of its more general applicability to the GLC analysis of mercapturic acids.

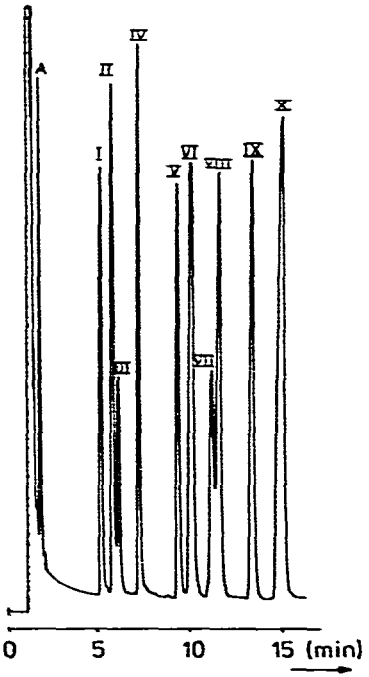

Fig. 2. Gas chromatogram of a mixture of ten mercapturic acids (for structures and names see Fig. 1 and Table I, respectively). Conditions: detector temperature, 270°C, injector temperature, 270°C, column temperature, increased from 180 to 195°C at 1°C/min; split flow-rate, 50 ml/min; splitting ratio, 21:1; amount of the mercapturic acids injected 1.0–4.0 μg in absolute ethanol; attenuation, 16 × 1.

The peaks of mercapturic acids indicated in Fig. 2 (I–X; Fig. 1) were identified by GC–MS. In Table I the most abundant *m/z* values occurring in the mass spectra are listed. Apart from molecular ions ($M^{+\cdot}$), the ions with *m/z* 176 or 177, corresponding to RS^- or $RSH^{-\cdot}$, respectively, were present in most mass spectra. An other important fragmentation reaction is the loss of 59 from the molecular ion ($M^{+\cdot} - CH_3CONH_2$ or $- COOCH_3^{\cdot}$).

Peak A (Fig. 2) corresponded to N-acetyl-L-cysteine methyl ester (RSH), as was derived from its mass spectrum (Table I) and from comparison with authentic material. As the mercapturic acids I, II, III, VII and X were synthesized according to a Michael addition[14,15,22] of RSH to the respective α,β-unsaturated precursors, a ther-

TABLE I

MOST ABUNDANT IONS IN THE MASS SPECTRA OF THE MERCAPTURIC ACIDS I–X AND THE DI COMPOSITION PRODUCTS OF VII AND X

The intensities are given in parentheses as percentage of the base peak.

Peak No.	*Name*	$M^{+\cdot}$	*Most abundant ions*
I	N-Acetyl-S-(1-methyl-2-cyanoethyl)-L-cysteine methyl ester	244	244(27), 185(54), 176(35), 117(100) and 88(86)
II	N-Acetyl-S-(1-methyl-2-carboxymethyl-ethyl)-L-cysteine methyl ester	277	277(17), 218(100), 117(37) and 59(50)
III	N-Acetyl-S-(2-methyl-2-carboxymethyl-ethyl)-L-cysteine methyl ester	277	277(12), 218(100), 186(35) and 117(29)
IV	N-Acetyl-S-(2-cyclohexenyl)-L-cysteine methyl ester	257	257(20), 198(23), 176(29) and 81(100)
V	N-Acetyl-S-benzyl-L-cysteine methyl ester	267	267(12), 208(31), 176(29) and 91(100)
VI	N-Acetyl-S-(2-oxocyclohexyl)-L-cysteine methyl ester	273	273(53), 214(26), 176(100) and 144(83)
VII	N-Acetyl-S-(3-oxocyclohexyl)-L-cysteine methyl ester	273	214(58), 177(19) and 97(100)
VIII	N-Acetyl-S-(4-oxocyclohexyl)-L-cysteine methyl ester	273	214(98), 181(40), 180(31) and 97(100)
IX	N-Acetyl-S-(*trans*-2-acetoxycyclohexyl)-L-cysteine methyl ester	317	257(33), 198(100) and 81(33)
X	N-Acetyl-S-(3-oxocycloheptyl)-L-cysteine methyl ester	287	269(18), 228(12), 177(30) and 111(100)
A	N-Acetyl-L-cysteine methyl ester (RSH)	177	118(21), 88(59) and 60(100)
	2-Cyclohexen-1-one	96	96(5), 68(24) and 28(100)
	2-Cyclohepten-1-one	110	110(30), 81(100) and 67(79)

mal decomposition reaction resulting in the formation of RSH was suspected. This reaction is probably a so-called "retro-Michael" reaction[22], as indicated in Fig. 3.

From a separate GLC analysis of each of these mercapturic acids it became clear that only VII and X decomposed under the GLC conditions according to this type of retro-reaction. At a column temperature of 70°C, in the latter two instances it was also possible to identify by mass spectrometric analysis 2-cyclohexen-1-one and 2-cyclohepten-1-one (Table I). Obviously this observation can be seen as additional support for the suggested mechanism of the decomposition reaction.

The temperature dependence of the decomposition reaction was also investigated. The peak height of RSH in the gas chromatogram of VII (injection port

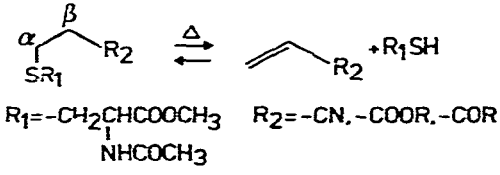

Fig. 3. Mechanism of the thermal "retro-Michael" reaction.

temperature 210°C, column temperature 190°) became at least a factor of 10 higher when the injection port temperature was 400°C, whereas the peak-height ratio of RSH to VII changed from 0.11 to 1.55. When the residence time in the injection port was prolonged by stopping the flow, the intensity of the RSH peak became even higher.

In conclusion, it can be stated that a well deactivated capillary SCOT column coated with the apolar stationary phase OV-101 is fairly well suited to the GLC analysis of mercapturic acids. As far as thermal degradation of mercapturic acids under normal GLC conditions is concerned, the decomposition of mercapturic acids according to a "retro-Michael" reaction should be particularly considered.

REFERENCES

1 I. M. Arias and W. B. Jacoby (Editors), *Glutathione, Metabolism and Function*, Raven Press, New York, 1976.
2 P. J. van Bladeren, *Ph.D. Thesis*, Leiden, 1981.
3 R. van Doorn, Ch.-M. Leijdekkers, R. P. Bos, R. M. E. Brouns and P. Th. Henderson, *Ann. Occup. Hyg.*, 24 (1981) 77.
4 F. Seutter-Berlage, L. P. C. Delbressine, F. L. M. Smeets and H. C. J. Ketelaars, *Xenobiotica*, 8 (1978) 413.
5 R. van Doorn, L. P. C. Delbressine, Ch.-M. Leijdekkers, P. G. Vertin and P. Th. Henderson, *Arch. Toxicol.*, 47 (1981) 51.
6 F. Seutter-Berlage, H. L. van Dorp, H. G. J. Kosse and P. Th. Henderson, *Int. Arch. Occup. Environ. Health*, 39 (1977) 45.
7 H. G. Bray, J. C. Caygill, S. P. James and P. B. Wood, *Biochem. J.*, 90 (1964) 127.
8 L. P. C. Delbressine, P. J. van Bladeren, F. L. M. Smeets and F. Seutter-Berlage, *Xenobiotica*, 11 (1981) 589.
9 B. Yagen, O. Hernandez, J. R. Bend and R. H. Cox, *Chem.-Biol. Interact.*, 34 (1981) 57.
10 B. Yagen, O. Hernandez, J. R. Bend and R. H. Cox, *Bioorg. Chem.*, 10 (1981) 299.
11 S. P. James, R. H. Waring and D. A. White, *J. Chromatogr.*, 26 (1967) 255.
12 C. M. Kaye, J. J. Clapp and L. Young, *Xenobiotica*, 2 (1972) 129.
13 P. J. van Bladeren, D. D. Breimer, C. J. R. Seghers, N. P. E. Vermeulen, A. van der Gen and J. Cauvet, *Drug Metab. Dispos.*, 9 (1981) 207.
14 P. J. van Bladeren, D. D. Breimer, G. M. T. Rotteveel-Smijs, R. A. W. de Jong, W. Buijs, A. van der Gen and G. R. Mohn, *Biochem. Pharmacol.*, 29 (1980) 2975.
15 P. J. van Bladeren, L. P. C. Delbressine, J. J. Hoogeterp, A. H. G. M. Beaumont, D. D. Breimer, F. Seutter-Berlage and A. van der Gen, *Drug Metab. Dispos.*, 9 (1981) 246.
16 L. P. C. Delbressine, F. Seutter-Berlage and E. Seutter, *Xenobiotica*, 11 (1981) 241.
17 N. P. E. Vermeulen, J. Cauvet, W. C. M. M. Luijten and P. J. van Bladeren, *Biomed. Mass Spectrom.*, 7 (1980) 413.
18 P. J. van Bladeren, W. Buijs, D. D. Breimer and A. van der Gen, *Eur. J. Med. Chem.*, 15 (1980) 495.
19 G. Schomburg, H. Husmann and H. Borwitzky, *Chromatographia*, 12 (1979) 651.
20 G. Schomburg, H. Husmann and H. Behlau, *Chromatographia*, 13 (1980) 321.
21 N. P. Vermeulen, P. J. van Bladeren, L. P. C. Delbressine and C. E. M. Zoetemelk, in preparation.
22 H. Krauch and W. Kunz (Editors), *Reaktionen der organischen Chemie*, Hüthig, Heidelberg, 4th ed., 1969, p. 408.