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Derivatization to stabilize some aliphatic primary hydroxylamines for g.l.c. analysis

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By appropriate choice of trimethylsilylating and trifluoroacetylating reagents and organic solvents for extraction, stable derivatives of aliphatic primary hydroxylamines metabolites, *N*-hydroxyphentermine, *N*-hydroxychlorphentermine, *N*-hydroxymexiletene, *N*-hydroxyphenethylamine, *N*-hydroxyamphetamine, and *N*-hydroxy-3,4-dimethoxyamphetamine, were obtained and examined by g.l.c. analysis without decomposition and without interference from the parent drug or other metabolic products.

Some aliphatic primary hydroxylamines, that are metabolites of certain drugs (Beckett, 1971; Beckett & Al-Sarraj, 1972; Cho, Lindeke & Hodshon, 1972; Beckett & Bélanger, 1974), cannot be determined by direct gas liquid chromatography because of their instability (Beckett, 1974). The trimethylsilyl (Lindeke, Cho & others, 1973; Beckett, Haya & others, 1975) and trifluoroacetyl (Gal, Gruenke & Castagnoli, 1975) derivatives of some hydroxylamines give more stable compounds. We have investigated the usefulness of such derivatives for the analysis of the hydroxylamines of phentermine, chlorphentermine, mexiletine, phenethylamine, amphetamine, and 3,4-dimethoxyamphetamine, which decompose under normal g.l.c. conditions. The extraction of these hydroxylamines from aqueous solutions and from different biological fluids before derivatization was also examined. Further, the usefulness of the derivatives for mass spectrometric identification of the hydroxylamines was investigated.

MATERIALS AND METHODS

Reagents and chemicals. *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Company, trifluoroacetic anhydride (TFAA) from R. N. Emanual Limited; hydroxylamines were synthesized in this laboratory; diethyl ether and n-pentane were distilled before use; cofactor solution (each 10 ml of aqueous solution containing 34 mg of nicotinamide-adenine-dinucleotide phosphate 60 mg of glucose-6-phosphate, 2 ml of 0·01 M magnesium chloride and 1 ml of 0·6 M nicotinamide).

Gas liquid chromatography. All compounds were chromatographed on a Perkin-Elmer Model F.11 gas chromatograph equipped with a flame ionization detector. Air and hydrogen pressures were 175 and 140 kNm^{-2}, respectively. Column temperatures (identical with injection port) are specified later. System A: 1 m × 4 mm i.d. glass column, packed with 3% OV 17 on 80–100 mesh Chromosorb G (AW) DMCS; nitrogen, 105 kNm^{-2} (1·66 $cm^3 s^{-1}$). System B: 2 m × 4 mm i.d. glass column, packed with 2% XE 60 on 80–100 mesh Chromosorb G (AW) DMCS; nitrogen, 140 kNm^{-2} (1·4 $cm^3 s^{-1}$).

Combined gas liquid chromatography—mass spectrometry. All mass spectra were obtained on a Perkin-Elmer Model 270 instrument using a 1 m × 4 mm i.d. glass column, packed with 3% OV 17 on 80–100 mesh chromosorb G (AW) DMCS, and the following conditions: ionization potential, 70 eV; source temperature, 180°; manifold temperature, 215°; helium (carrier gas), 140 kNm^{-2}; column and injection port temperatures, as mentioned above.

Methods

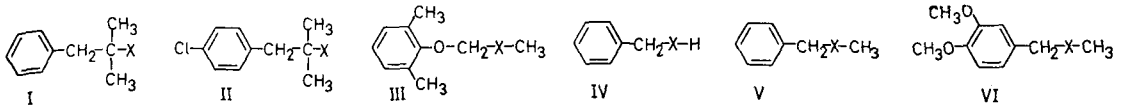
Hydroxylamine free bases were extracted with ether from an aqueous solution of their oxalate salts in phosphate buffer (pH 7·4). The extract was concentrated to about 0·1 ml in a water bath (45°), dried under a stream of nitrogen and submitted to the derivatization procedure.

Trimethylsilyl (TMS) derivatives. The sample (2 mg) was dissolved in acetonitrile (0·2 ml, previously dried over $CaCl_2$). To 50 μl of this solution was added 50 μl of BSTFA and an aliquot examined by g.l.c. analysis (see Table 1).

Trifluoroacetyl (TFA) derivatives. The sample (2·5 mg) in TFAA (0·5 ml) was allowed to stand for 15 min and an aliquot analysed by g.l.c. (see Table 1).

* Correspondence.

Table 1. *Retention times of hydroxylamines and other possible metabolites of arylalkylamines and their derivatives.*



Structures	Treatment	Temp (°C) X =	Retention time (min) a NHOH	b NH_2	c NO	d NO_2		System
I	None	120	BD	2·0	1·8	8·2		A
	TFAA	120	2·2†	3·9	—	—		A
	BSTFA	120	4·7	3·8	—	—		A
II	None	150	BD	2·1	1·9	7·6		A
	TFAA	150	2·1†	3·7	—	—		A
	BSTFA	150	4·3	3·7	—	—		A
		X =	a CHNHOH	b* $CHNH_2$	c CNOH	d CHOH	e CO	
III	None	140	BD	3·6	14·6	—	4·9	B
	TFAA	140	5·7	22·6	BD	—	—	B
	BSTFA	140	5·7	3·6*	4·4	—	—	B
IV	None	100	BD	3·1	9·2	—	—	A
	TFAA	100	3·0	12·3	5·5	—	—	A
	BSTFA	120	4·6	5·8	3·8	—	—	A
V	None	100	BD	3·4	11·1	2·7	4·0	A
	TFAA	100	3·1	10	9·4	1·8	—	A
	BSTFA	130	3·4	1·8	2·8	1·3	—	A
VI	None	150	BD	8·3	21·6	9·2	9·5	A
	TFAA	150	4·9	14·1	2·5	4·4	—	A
	BSTFA	180	5·7	3·8	5·2	2·3	—	A

BD Breaks down to amine and *C*-nitroso compound when there is no H on the α-C-atom, and to amine and oxime when there is at least one H on the α-C-atom.
TFAA Trifluoroacetic anhydride.
BSTFA *N,O*-bis (trimethylsilyl)-trifluoroacetamide.
The TMS derivatives of the hydroxylamines and the corresponding oximes were completely resolved in System B but not in System A.
† A small additional peak also produced (Rt 1·0 min)
* Not silylated with BSTFA under the g.l.c. conditions used.

Extraction of the hydroxylamines from biological fluids

Preparation of solution. Hydroxylamine oxalate (50 μmol) was dissolved in 0·1 M HCl (1–2 ml) and diluted with water so that each ml of the final solution contained 0·2 μmol of the hydroxylamine.

Liver homogenates. 1·0 ml of the above solution was transferred into a glass centrifuge tube. Water (1 ml) in a second tube served as control. Both tubes were treated as follows. Liver homogenates (1 ml), cofactor solution (1 ml), pH 7·4 phosphate buffer (4 ml) and ether (8 ml) were added. The tube was shaken gently (5 min), centrifuged (2 min) to clarify the layers, and the ether layer was withdrawn into an evaporating tube. The extraction was repeated with further (2 × 8 ml) portions of ether and the extracts were combined. The tube was placed in a water bath (45°) and the extract concentrated to about 0·1 ml, after which the tube was washed with ether (1 ml) which was then evaporated with nitrogen. The residue was dissolved in dry acetonitrile (25 μl), BSTFA (25 μl) added from a syringe, mixed, kept on ice, and an aliquot (4 μl) injected onto a column (see Table 1) within 5 min after treatment with the reagent. The extraction of hydroxylamine IIa was carried out using n-pentane instead of ether and saturating the aqueous phase with sodium chloride.

Urine. The procedure was same as above with the following exceptions: the cofactor solution was omitted; 2·5 ml of urine and 1 min of vigorous shaking were employed.

RESULTS AND DISCUSSION

Hydroxylamines of arylalkylamines are unstable under aqueous alkaline conditions (Beckett &

Al-Sarraj, 1973), but can be readily extracted from aqueous solutions at a pH of 7·4, without decomposition, into some organic solvents. With ether, two extractions were sufficient to extract the hydroxylamines (1 μmol). With n-pentane, complete extraction was achieved when the aqueous phase was saturated with sodium chloride and extracted three times. Only the hydroxylamine IIIa decomposed slightly to the corresponding oxime IIIc during extraction at pH 7·4 with both ether and n-pentane.

When hydroxylamines were dissolved in urine from different human subjects and then analysed, the endogenous constituents extracted from urine did not interfere with the analysis. However, the extract of liver homogenates with ether but not with n-pentane when subjected to g.l.c. analysis gave a peak which overlapped with the derivatives of the hydroxylamine IIa. The parent amines or their metabolic products likely to be present did not interfere with the analysis.

TMS and TFA derivatives all produced sharp symmetrical peaks (Figs 1 and 2). The retention times of the derivatives of the hydroxylamines, the parent compounds and some of the likely metabolic products of the arylalkylamines are given in Table 1.

The retention times of a number of hydroxylamines, amines, oximes, and their TMS and TFA derivatives are shown in Fig. 3. For each related hydroxylamine, amine, and oxime, the retention times, using System A, of the derivatives were measured at the temperature shown in Table 1 for the TFA derivatives. The production of single peaks by the TMS derivatives of the hydroxylamines and the ease of preparation and handling of the TMS derivatives, makes BSTFA preferable to TFAA as the derivatizing reagent. The hydroxylamines extracted from the biological fluids were therefore analysed as the TMS derivatives. However, the applicability of the TFA derivatives to hydroxylamines extracted from biological fluids was investigated to ensure that this approach could be used for analysis should interfering peaks be present in extracts treated with BSTFA.

The g.l.c. peaks of the derivatives of the hydroxylamines were identified by combined g.l.c.—mass spectrometry, the TMS derivatives giving characteristic mass fragmentation (see Fig. 4 for TMS-IIIa and -VIa). The (M-15) peak was observed with all the TMS-hydroxylamines, and in some cases the molecular ion peak (0·5%). Metastables were observed between the *m/e* 132 and *m/e* 116 fragments and between the *m/e* 116 and *m/e* 75 fragments, respectively (Lindeke & others, 1973, have also

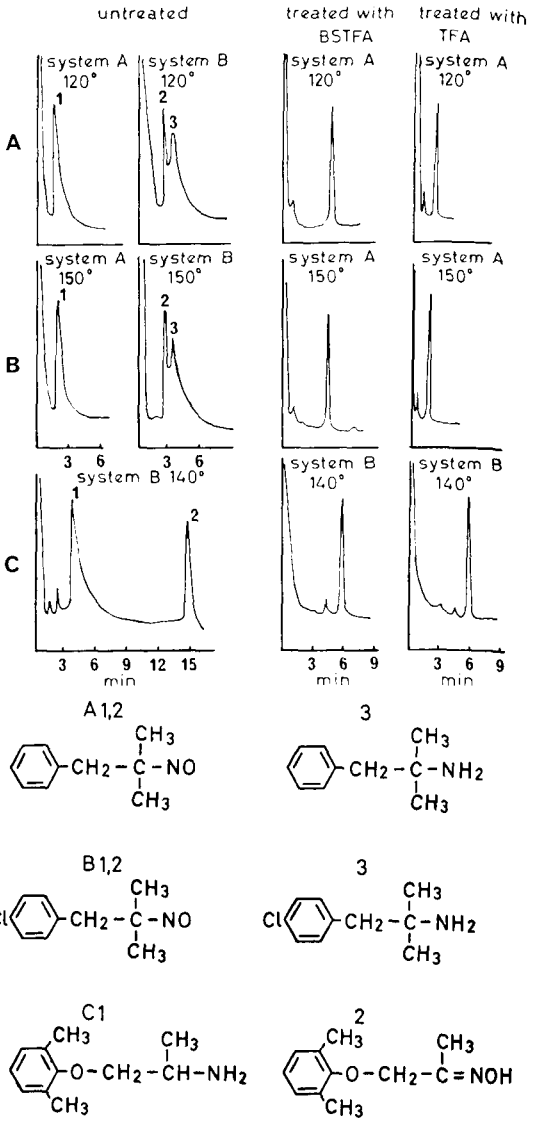


FIG. 1. Chromatography of the free base and the derivatives of (A) *N*-hydroxyphentermine (Ia), (B) *N*-hydroxychlorphentermine (IIa), and (C) *N*-hydroxymexiletine (IIIa).

reported the mass spectra of some TMS-hydroxylamines).

The mass spectra of the TFA derivatives of the hydroxylamines did not give any characteristic peak. The mass spectrum of the TFA derivative of the hydroxylamine IIIa showed the base peak at *m/e* 263 (M-28) which was not present in the acetyl derivative (prepared in the same way as the TFA derivative) or in the TFA derivatives of the other hydroxylamines.

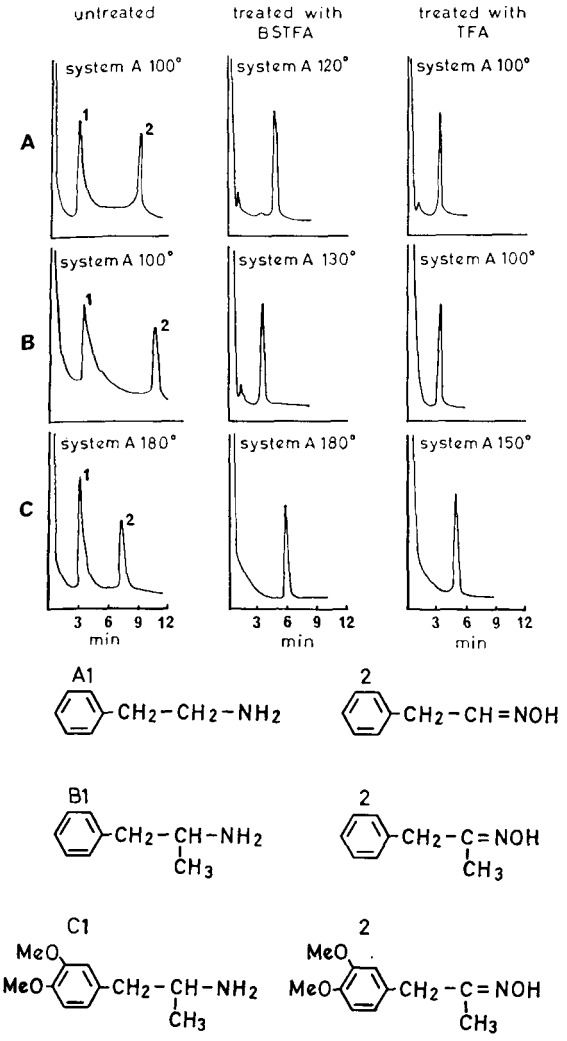


FIG. 2. Chromatography of the free base and the derivatives of (A) *N*-hydroxyphenethylamine (IVa), (B) *N*-hydroxyamphetamine (Va), and (C) *N*-hydroxy-3,4-dimethoxyamphetamine (VIa).

Amphetamine Vb forms a mono-TFA derivative with TFAA (ms and nmr evidence), which gives the base peak at *m/e* 140 corresponding to the ion $CH_3CH = \overset{+}{N}HCOCF_3$ (Cho, Lindeke & others, 1973). Both *N*- and *O*-TFA derivatives of hydroxylamine Va might give a peak at *m/e* 156. If an *N*-TFA derivative was formed, the ion $CH_3CH = \overset{+}{N}OHCOCF_3$ could lose a molecule of water to give a peak at *m/e* 138. If an *O*-TFA derivative was formed, the ion $CH_3CH = \overset{+}{N}HOCOCF_3$ could lose a molecule of acid to give a peak at *m/e* 42. The absence of a peak at *m/e* 138 and the presence

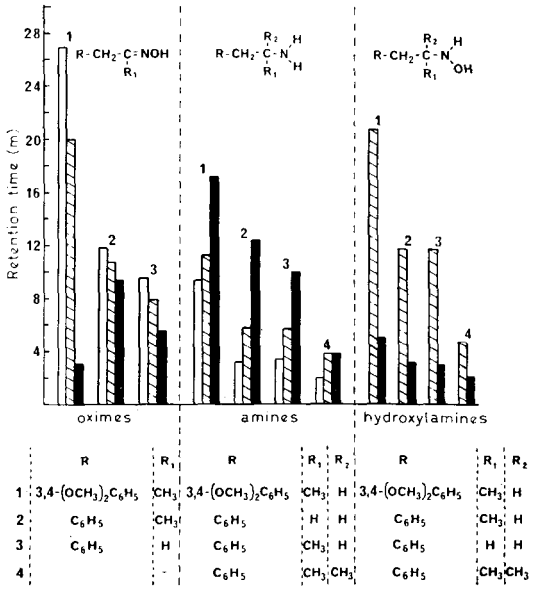


FIG. 3. Change in the retention times of some oximes, amines, and hydroxylamines upon treatment with trimethylsilylating and trifluoroacetylating reagents. Open columns untreated; hatched columns treated with BSTFA; solid columns treated with TFAA.

of one at *m/e* 42 indicates that acylation probably occurred on the oxygen rather than on the nitrogen. This analogy could also be applied to the other hydroxylamines.

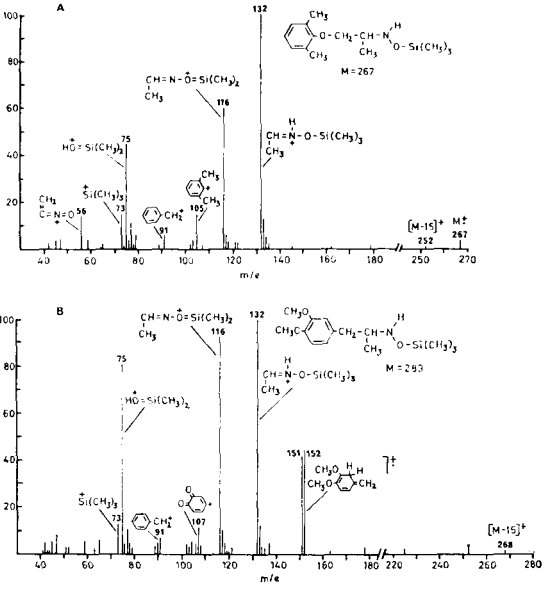
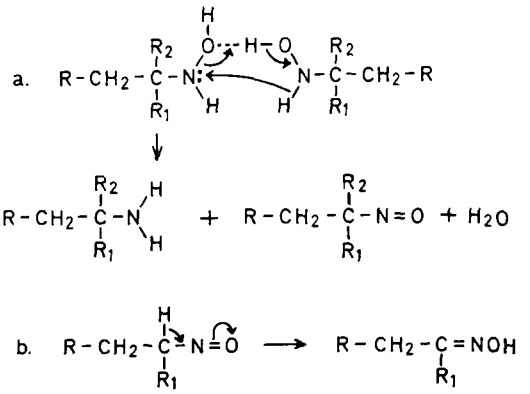


FIG. 4. Mass spectra of the TMS derivatives of (A) *N*-hydroxymexiletine (IIIa) and (B) *N*-hydroxy-3,4-dimethoxyamphetamine (VIa).

The pattern of decomposition of the underivatized hydroxylamines on g.l.c. differed according to their structures about the α-carbon atom. The hydroxylamines Ia and IIa each gave two peaks corresponding to the respective amines and *C*-nitroso compounds in System B (in System A amines and *C*-nitroso compounds were not resolved). In both Systems A and B, the hydroxylamines IIIa–VIa gave two peaks corresponding to the respective amines and oximes as confirmed by g.l.c.-mass spectrometry.

Since hydroxylamines without hydrogen on the α-carbon atom gave approximately equimolar amounts of amines and *C*-nitroso compounds when subjected to g.l.c. analysis (Beckett & Bélanger, unpublished results); on column disproportionation and dehydration according to Scheme 1 is suggested. When $R_1 = H$ or $R_1 = R_2 = H$, approximately equimolar amounts of amine and oxime were obtained, the latter arising from the thermal rearrangement of the *C*-nitroso compound; however, only the equivalent of about 70% of the original hydroxylamine was recovered, possibly due to adsorption in the column followed by slow elution.

The present study shows that the decomposition during g.l.c. of aliphatic primary hydroxylamines with or without substituents on the α-carbon atom or on the ring follows a common pathway. Stable TMS and TFA derivatives of these hydroxylamines are readily formed and are directly amenable to gas chromatography. Since the derivatives are formed by reaction with the active hydrogen, the adsorption on the column is reduced and the sensitivity is increased, so that the analysis of small concentrations of hydroxylamines in metabolic studies is facilitated. Also the derivatives have characteristic mass fragmentation which facilitates the identification of the hydroxylamines.



Scheme 1. Proposed mechanism of the formation from the primary hydroxylamines of (a) amines and *C*-nitroso compounds and (b) oximes from the *C*-nitroso compounds with at least one H on the α-*C*-atom.

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