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The identification, properties and analysis of *N*-hydroxyamphetamine—a metabolite of amphetamine

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N-Hydroxyamphetamine (Ib) can be extracted from aqueous solutions at neutral pH, but is readily decomposed into *syn*- and *anti*-phenylacetone oximes (II) at alkaline pH values. Gas chromatography of Ib is possible, provided the compound does not come into contact with heated metal surfaces. In aqueous media the hydroxylamine can be determined (1) by extraction from the medium with ether and analysis of the ethereal solution by g.l.c.; (2) by separation of the compound on a thin-layer then oxidation of the hydroxylamine spot with ammoniacal silver nitrate to give phenylacetone oximes which may then be determined by g.l.c.; (3) by reduction with lithium aluminium hydride to amphetamine (Ia) followed by g.l.c.; (4) conversion to phenylacetone oximes by shaking at pH 12 in air, extraction of the oximes, followed by g.l.c.; (5) Alternatively, *N*-hydroxyamphetamine can be determined directly in aqueous solution by polarography provided the measurement is made immediately after the supporting electrolyte, sulphite/NaOH, has been added.

N-Hydroxyamphetamine (Ib); has been reported as a metabolite of amphetamine (Beckett & Al-Sarraj, 1972a). Subsequently, the metabolic *N*-oxidation of aliphatic primary and secondary amines to give corresponding hydroxylamines of differing stabilities and routes of breakdown was shown to be a general pathway (Beckett, 1971; Beckett & Al-Sarraj, 1972b). Hydroxylamines as metabolites, have not been reported until recently (see Beckett, 1971) because of their labile nature under the experimental conditions. This applies to the presence of *N*-hydroxyamphetamine as an important metabolite of amphetamine, probably because of:

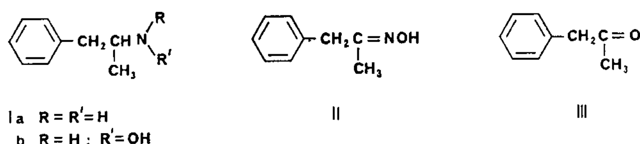
(i) the difficulty of its extraction from biological media and its instability at alkaline pH values; (ii) its decomposition upon evaporation of a solution in certain organic solvents, e.g. in ethyl acetate and in peroxide-containing ether; (iii) breakdown occurring in some analytical techniques, e.g. heated metal surfaces, or metal ions in the gas stream, lead to oxidation to oximes on g.l.c. and mass spectrometry; (iv) its substantial breakdown within a few hours in aqueous solutions even at neutral pH.

N-Hydroxyamphetamine, which readily breaks down to the phenyl acetone ketoximes (II) and phenyl ketone (III), was chosen for detailed examination as a model compound so that the problems of identification and quantitative determination of metabolites of this type and stability could be defined. Some hydroxylamines are more, and others are less, stable.

Because of the inherent instability of *N*-hydroxyamphetamine in dilute aqueous solutions as well as during extraction and analytical techniques, and because some of the products of decomposition, e.g. II and III are also derived from metabolites of

amphetamine other than the hydroxylamine (Ib) (Beckett, 1971), five analytical methods, based on different techniques, have been devised, combinations of which enable this elusive metabolite to be quantitatively estimated with an accuracy of ±15%.

N-Hydroxyamphetamine (Ib), being a much weaker base (pKa 8·25)* than amphetamine (pKa 9·77), unlike the latter, can be extracted quantitatively from aqueous solution at neutral pH with organic solvents, e.g. benzene or freshly-distilled ether.



MATERIALS AND METHODS

Compounds and reagents

N-*Hydroxyamphetamine succinate* was kindly supplied by SKF, USA. *Phenylacetone. Phenylacetone oximes* as the *anti/syn* mixture (2/1) were prepared synthetically (Neber, 1926). *Ethylamphetamine hydrochloride* was from Medial-Riker laboratories. p-*Chloropropiophenone* (Aldrich Chemical Co.) marker solution was prepared by dissolving 30 μmol in a few drops of acetone and then diluting to 100 ml with water. *Analar ether* was freshly distilled.

Liver microsomal preparations were the 10 000 *g* fraction (2 ml) from 0·5 g of liver. Ethereal solutions, freshly prepared, of *N*-hydroxyamphetamine and phenylacetone oximes were obtained by extraction at pH 7·4 of freshly prepared solutions of the compounds added to phosphate buffer, or to urine, or of solutions of the compounds after microsomal incubation.

Gas-liquid chromatography

Instrument. Perkin Elmer F.11, instrument flame ionization detector.

Chromatographic conditions. System A. Glass column, 2 m, ¼″ o.d., 7·5% Carbowax 20 M on DMCS treated Chromosorb W 80–100 mesh; oven temp. 167°, N_2 30, H_2 20, air 20 p.s.i. *System B.* Glass column, 1 m, ¼″ o.d. 10·0% Apiezon on DMCS treated Chromosorb W 80·100 mesh; oven temp 150°; N_2 15, H_2 25, air 20 p.s.i. *System C.* Stainless steel column, 2 m, 1/8″ o.d. 10% Apiezon containing 10% KOH on DMCS treated Chromosorb G 80–100 mesh, oven temp. 155°, N_2 30, H_2 20, air 20 p.s.i. *System D.* Stainless steel column, 2 m, 1/8″ o.d., 7·5% Carbowax 20 M on DMCS treated Chromosorb W 80–100 mesh, oven temp. 140°, N_2 32, H_2 20, air 20 p.s.i.

Method 1

Chromatographic analysis. Calibration curves using system A were constructed separately for *N*-hydroxyamphetamine (0·02–2·0 μmol) and phenylacetone ketoximes

* Determined according to Leffler, Spencer & Burger (1951).

(0·1–1·0 μmol) dissolved in phosphate buffer pH 7·4 to which *p*-chloropropiophenone (1 ml marker solution) had been added; after the pH of the solution had been adjusted to 7·4 with sodium hydroxide solution the compounds were extracted with ether and chromatographed using System A.

For *N*-hydroxyamphetamine, the ratio of the phenylacetone ketoximes to marker was also measured because some oxidation of the hydroxylamine to these oximes occurred on the column.

Method 2

Thin-layer chromatography. Silica gel G (Merck), 20 × 20 cm, 0·5 mm thickness dried for 1 h at 110°. Solvent system methanol–chloroform (1:4). Spray reagents: ammoniacal silver nitrate, triphenyltetrazolium chloride solution (TTC), iodine vapour.

Ethereal solution (0·1 ml containing 0·5 μmol of *N*-hydroxyamphetamine) was spotted onto t.l.c. plates. After development, the spots containing the hydroxylamine were located with the three reagents and treated as follows (before and after spraying):

(*a*) the spot was extracted with ether and the hydroxylamine, phenylacetone and phenylacetone oximes were determined by quantitative g.l.c. on system A (p. 329);

(*b*) the spot was scraped off and eluted with 3·0 ml phosphate buffer pH 7·4 to which 0·5 ml sodium hydroxide solution (5N) was added; after shaking for 12 h, the solution was extracted with ether and the compounds determined as in (*a*);

(*c*) the spots, after spraying (black after silver nitrate; red after TTC; brown after iodine vapour) were scraped off, extracted with ether and the compounds determined as in (*a*).

Method 3

Reduction with lithium aluminium hydride. To ethereal solutions (about 50 μl) containing *N*-hydroxyamphetamine (0·1 to 0·25 μmol) and ethylamphetamine as marker (0·3 μmol), was added dry ether (about 2 ml) and lithium aluminium hydride (about 0·15 g) and the solution shaken occasionally and then left overnight at 20°. Moist ether (2·0 ml) was added followed by water (2 ml) dropwise and then 20% NaOH solution (0·2 ml). The ether layer was separated and the solution further extracted with 2 × 2 ml ether. The combined ethereal extract was concentrated in tapered tubes on a water bath at 42° and then analysed for amphetamine content by g.l.c. system C (cf. Beckett & Tucker, 1966).

Method 4

Oxidation with alkali. *N*-Hydroxyamphetamine (0·01 to 0·25 μmol) in phosphate buffer (3 ml) or buffer (1 ml) plus 10 000 *g* liver microsomal preparations (2 ml) was adjusted to pH 12 by the addition of 20% NaOH (0·5 ml) and shaken for 20 h. *p*-Chloropropiophenone, marker solution (1 ml) was added and the solution extracted with ether (3 × 3 ml). The ethereal extract was concentrated to small bulk and analysed for phenylacetone ketoxime by g.l.c. system A by comparison with the appropriate calibration curve.

Method 5—Polarography

Instrument. Cambridge Polarograph Model C.

Conditions. Polarograms of *N*-hydroxyamphetamine were determined over the range 0·05 to 0·5 μmol in 20 ml solution containing Na_2SO_3 (2%) and NaOH (0·9%) of pH 12·4 at 20° after deoxygenation with oxygen-free N_2 for 15 min; potentials were recorded with reference to a saturated calomel electrode. The dropping mercury electrode had the following characteristics: height of mercury column = 50 cm, $m^{2/3}t^{1/6}$ = 1·70 mg.

Other techniques used in identity tests

Mass spectrometry. The *N*-hydroxyamphetamine and phenylacetone oximes were examined using a Perkin-Elmer model 270 gas chromatography—mass spectrometer system at an electron energy of 70 eV.

Nmr. The spectra of *N*-hydroxyamphetamine (as the salt) and phenylacetone oximes were recorded in DMSO using a Perkin-Elmer R–10 nmr spectrometer plus a Northern scientific 544 CAT with tetramethylsilane as the internal standard.

Infrared. The spectra of *N*-hydroxyamphetamine and phenylacetone oximes were determined in Nujol mulls using a Unicam SP 200 spectrometer.

Quantitative investigations of N-*hydroxyamphetamine*

Experiments involving at least six replicates were made using each of the five methods and these were then applied to the analysis of a solution (Table 1) of *N*-hydroxyamphetamine (Ib).

Table 1. *The analysis by various methods of* N-*hydroxyamphetamine* (Ib) (0·48 μmol ml^{-1}) *in an aqueous solution in phosphate buffer pH 7·4* (see Fig. 1 for analytical scheme outline).

METHOD 1 μmol found as			METHOD 2 μmol found as				METHOD 3 μmol found as	METHOD 4 μmol found as						METHOD 5 μmol found as
Ib	II	Total	II	III	Ib	Total	Ia	II		III		Total		Ib
								A	B	A	B	A	B	
0·44	0·07	0·51	0·39	0·02	0·002	0·41	0·48	0·35	0·44	0·10	nil	0·45	0·44	0·41
0·45	0·08	0·53	0·40	0·05	0·008	0·46	0·46	0·34	0·45	0·14	,,	0·48	0·45	0·40
0·46	0·05	0·51	0·41	0·02	0·03	0·46	0·46	0·28	0·48	0·14	,,	0·42	0·48	0·46
			0·41	0·03	0·003	0·44	0·46	0·32	0·50	0·15	,,	0·47	0·50	
			0·41				0·46	0·36	0·48	0·12	,,	0·48	0·48	
							0·50	0·35	0·46	0·1	,,	0·45	0·46	

Method 1. G.l.c. system A, after ether extraction; approx. 0·05–0·1 μmol injected.
Method 2. T.l.c. of ethereal extract, conversion to oximes by $AgNO_3$ of hydroxylamine spot: 0·50 μmol per spot; analysis of ethereal extract of oximes by g.l.c. system A.
Method 3. Reduction of ethereal extract to yield amphetamine followed by g.l.c. analysis using system C.
Method 4. A; diluting 1 ml with 2 ml of phosphate buffer pH 7·4, B; diluting 1 ml with 2 ml of 10 000 *g* liver microsomal preparations. Solutions A and B were then extracted with ether and the ethereal extracts were analysed by g.l.c. system A.
Method 5. Direct polarography of aqueous solution, concn range used 0·05 to 0·50 μmol.

RESULTS AND DISCUSSION

Identification of the amphetamine metabolite as N-*hydroxyamphetamine*

Synthetic *N*-hydroxyamphetamine and the material extracted from microsomal incubations and from urine of various species were shown to be identical in the tests used.

G.l.c. system A Rt of Ib 8·5 min (phenylacetone oxime 12 min), system B 1·76 min (phenylacetone oxime 1·46 min); there was only slight breakdown of the hydroxylamine to oxime in system A but more in B.

On the metal columns C and D, the hydroxylamine was transformed completely to oximes of Rt 12·3 and Rt 27 min on the respective columns. On t.l.c. on silica gel, the hydroxylamine had R_F 0·8 (amphetamine 0·3, phenylacetone oxime 0·95, and phenylacetone 0·9) and gave a black colour with ammoniacal silver nitrate, a brown colour with iodine vapours and a red colour with TTC. The t.l.c. hydroxylamine spot was oxidized to phenylacetone oxime by ammoniacal silver nitrate or iodine vapours as indicated by g.l.c. analysis of the black and the brown spot respectively; virtually no unchanged hydroxylamine was present.

Reduction of *N*-hydroxyamphetamine in ether with lithium aluminium hydride gave amphetamine. Shaking *N*-hydroxyamphetamine in alkaline solution gave extensive oxidation to phenylacetone ketoxime plus some breakdown to phenylacetone, whereas shaking in dilute acid solution gave mainly phenylacetone with some ketoximes.

N-Hydroxyamphetamine underwent anodic oxidation under the polarographic conditions described, $E\frac{1}{2}$ − 0·47 V. It gave mass spectra identical with that of phenylacetone ketoximes because of its oxidation during g.l.c. mass spectroscopy. The nmr spectrum of the protonated form exhibited a broad three proton singlet at τ 2·18 which was eliminated upon treatment with D_2O and is attributed to the $\overset{+}{N}H_2OH$ group; in the infrared, the OH of this group gave a peak at 3300 cm^{-1}.

Quantitative analysis of N-*hydroxyamphetamine* (*Ib*) *in aqueous solution*

The reactivity of the compound is such that the methods devised require great care and attention to detail if a successful analysis is to be made. Factors affecting the techniques are included under the relevant method.

Method 1. From aqueous solution, *N*-hydroxyamphetamine can be extracted into ether without decomposition at pH 7·4. In analysis by direct g.l.c. a number of difficulties may be encountered because even the best column in system A causes some oxidation (about 10% to oximes II) and the extent of oxidation increases as the amount injected decreases. Sometimes the peak height ratio of hydroxylamine: marker and that of the decomposed product, i.e. oximes, against marker gave straight calibration lines, but sometimes curves were obtained. The location of the end of the glass column relative to the injection port and block, and also the design of the block influence the degree of oxidation of Ib to the oximes II. Calibration of synthetic oximes against marker always gave reproducible straight lines. If it is assumed that the oxidation of hydroxylamine to oximes is quantitative, calculation of the equivalent amount of hydroxylamine for its partial conversion to oximes in g.l.c. and addition of these values to the hydroxylamine curves, gave straight lines against the marker; over one year the slopes for 'corrected hydroxylamine' then varied only by a maximum of ±15%. Thus, g.l.c. may be used for the *approximate* determination of hydroxylamine content provided phenylacetone ketoximes are not present in solutions to be analysed.

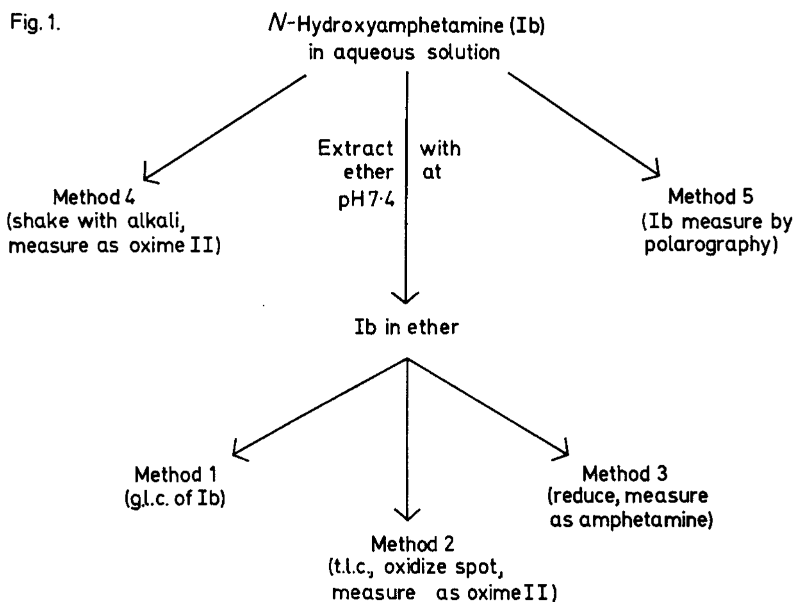
Method 2. The thin-layer chromatographic procedure separates *N*-hydroxyamphetamine from amphetamine (Ia) and the oximes (II); various methods were therefore used to attempt to determine the hydroxylamine content in the spots.

Scraping off the unsprayed spot and extracting with ether and then using g.l.c. system A indicated that there had been some oxidation to oximes and minor breakdown to phenylacetone. Shaking the material scraped from the spot with dilute sodium hydroxide solution gave phenylacetone ketoximes and about 0·5% phenylacetone; no unchanged hydroxylamine was detected. Spraying the spot with TTC followed by extraction indicated that there had been partial conversion of *N*-hydroxyamphetamine to phenylacetone and its ketoximes but mainly the latter. Spraying the spot with ammoniacal silver nitrate solution to give a black spot which was scraped off and extracted with ether and analysed by g.l.c. system A, gave almost quantitative conversion of *N*-hydroxyamphetamine to phenylacetone oximes; less than 10% of phenylacetone resulted from the oxidation of the hydroxylamine by this method.

Method 3. Reduction with lithium aluminium hydride gave a quantitative conversion to amphetamine which could then be extracted and determined by g.l.c. system C as described; the method gave results of $100 \pm 10\%$. However, phenylacetone ketoximes, which may be present in solution after experiments with 'amphetamines', are also reduced to amphetamine by this method and therefore their concentration must be established before this method of analysis for Ib content is employed.

Method 4. When freshly prepared aqueous solutions of *N*-hydroxyamphetamine were made alkaline and shaken in the presence of air, there was *mainly* conversion to oximes but some ketone was also produced; when the phosphate buffer solutions containing 10 000 *g* liver microsomal preparation were used, the conversion of hydroxylamine to oximes was virtually quantitative and no ketone was produced.

Method 5. The direct determination of *N*-hydroxyamphetamine in low concentrations in aqueous solutions was also attempted by polarography. Under the conditions described, the compound underwent anodic oxidation at —0·47 V whereas the oximes gave a reduction step of $E_{\frac{1}{2}}$ of $-1{\cdot}47$; both compounds gave straight line



calibrations between 0·05 and 0·20 μmol, and the accuracy of the method was ±7% in ½ h and ±15% in 1 h after preparing the solutions and adding supporting electrolyte. The determination of *N*-hydroxyamphetamine without interference from its oxidation product, i.e. oximes, in aqueous solutions constitutes the advantage of this analytical method.

The application of the analytical methods is outlined in Fig. 1. The results of applying the methods to the determination of an aqueous solution of synthetic *N*-hydroxyamphetamine, freshly dissolved in freshly prepared phosphate buffer solution at pH 7·4, are shown in Table 1; the concentration of the solution used was similar to that found in solutions from biological experiments involving amphetamines and *N*-alkyl and *N*-dialkyl amphetamines (Beckett & Al-Sarraj, 1972c).

In metabolic studies, some conversion occurs of the metabolite, *N*-hydroxyamphetamine (Ib), to the metabonates phenylacetone ketoxime(II) and phenylacetone(III). Because most of the analytical methods described lead to some inadvertent partial conversion to oximes and ketone, there is need for the application of several of the techniques in the analysis of the metabolic conversion of 'amphetamines' to *N*-hydroxyamphetamine. It is recommended that Method I be used for the analysis of Ib when it has been proved, using pure synthetic Ib in dilute aqueous solution, that the particular g.l.c. column and instrument used leads to only minor and quantitative conversion to II. Method 2 should be used to provide an independent measure of Ib content. Furthermore, after separately determining the content of II in the solution Method 3 can be used with advantage to determine the content of Ib. The average result for Ib content in solution by these methods should be considered as the content of this metabolite when the results by the three methods do not differ by more than ±15%.

Method 4 is useful in the analysis of Ib in the presence of 10 000 *g* hepatic fractions but does not give quantitative results in simple buffer solutions and therefore the quantitative aspects of this method must be separately established for each biological system.

Polarography, e.g. Method 5 constitutes a useful direct method for the determination of Ib in biological fluids provided the analysis is carried out immediately after the supporting electrolyte has been added.

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