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Identification and Quantitative Analysis of Phendimetrazine and Some of its Metabolites in Biological Fluids

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In urine, after oral doses of phendimetrazine to man, were found unchanged drug, the N-demethylated metabolite, phenmetrazine, and the N-oxide of phendimetrazine, but not N-hydroxyphenmetrazine; the metabolites were identified using t.l.c. and g.l.c. The stability of the drug and its metabolites in biological fluids and in ether was studied. A gas chromatographic procedure for the quantitative determination of unchanged drug, its N-oxide and phenmetrazine in urine, plasma and saliva was developed, the N-oxide being reduced before analysis.

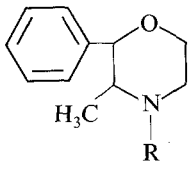
(Keywords: Amphetamins; Anorectics; Antiobesity; CNS stimulants; Phendimetrazine)

Identifizierung und quantitative Analyse von Phendimetrazin und einigen Metaboliten in biologischen Flüssigkeiten

Nach oralen Dosen von Phendimetrazin an Menschen wurde im Harn unverändertes Phendimetrazin, der N-demethylierte Metabolit, Phenmetrazin und das N-Oxid von Phendimetrazin, nicht jedoch N-Hydroxyphenmetrazin gefunden; die Metaboliten wurden mittels TLC und GLC identifiziert. Die Stabilität von Phendimetrazin und dessen Metaboliten wurde in biologischen Flüssigkeiten und in Ether untersucht. Es wurde eine gaschromatographische Methode für die quantitative Bestimmung von Phendimetrazin, seinem N-Oxid und Phenmetrazin in Harn, Plasma und Speichel entwickelt, wobei das N-Oxid vor der Analyse reduziert wurde.

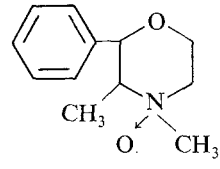
N-oxidation is an important metabolic route *in vivo* and *in vitro* in animals and man[1, 2, 8–10, 16, 18]. Tertiary aliphatic amines yield N-oxides, e.g. propoxyphene[20], imipramine[15], chlorpromazine[5], chlorcyclizine[19], nicotine[11], and methadones[6]. Secondary aliphatic amines yield hydroxylamine e.g. mephentermine[3] and dibenzylamine[4]. N-hydroxyphenme-

trazine (**1 c**) has been reported from the *in vitro* metabolism of phenmetrazine (**1 b**) and phendimetrazine (**1 a**) by liver microsomes of various animal species[7].



1

a Phendimetrazine: $R = CH_3$
b Phenmetrazine: $R = H$
c N-Hydroxyphenmetrazine: $R = OH$



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Phendimetrazine N-oxide

Preliminary work indicated that after administration of phendimetrazine (**1 a**) to man, the N-oxide (**2**) and the dementhylated product (**1 b**) were produced in high yields, but N-hydroxyphenmetrazine (**1 c**) could not be detected. We therefore investigated the properties of **1 a**, **1 b** and **2** and developed a method for their quantitative analysis in biological fluids; the N-oxide (**2**) was reduced before g.l.c. analysis. The synthesis of **2** is described. Also *Hundt* et al.[17] reported a method for the determination of phendimetrazine (**1 a**) in serum using a g.l.c. method.

Materials and Methods

Compounds and Reagents

Phendimetrazine tartrate was obtained from Ayerst Laboratories Limited, N.Y.; phenmetrazine·HCl from Boehringer Ingelheim (Germany); N-hydroxyphenmetrazine was supplied by Prof. *Coutts*[14]; chlorphentermine·HCl by William R. Warner and Co. Limited (Hampshire); titanium trichloride solution, technical grade 30% *w/v* $TiCl_3$ in ca. 24% *w/v* HCl (total), from B.D.H. Limited (Poole, England). The packing materials for gas liquid chromatography were all purchased from Perkin-Elmer Limited (Beaconsfield, England); N,O bis-(trimethylsilyl)-trifluoroacetamide (*BSTFA*) from Pierce Chemical Co. U.S.A. The ether was purified before use, by shaking it in a separating funnel with a 10% aqueous solution of sodium metabisulphite (2 × 100 ml) for about 5 min, then with 20% NaOH (2 × 100 ml) and finally with distilled water (2 × 100 ml). The washed ether was distilled, the first and last 50 ml being rejected.

Synthesis of Phendimetrazine N-Oxide (**2**)

Phendimetrazine base (0.005 mol, 955 mg) in ethanol (20 ml) was treated with hydrogen peroxide 29% (6 ml) for 3 days. The excess of the latter was destroyed with manganese dioxide powder (25 mg). After filtration and concentration under

vacuum, the oily residue was treated with dry ethyl acetate and ether to crystallise the N-oxide (50% yield) as off-white crystals which were washed with dry ether: m.p. 99–100°. The N-oxide (**2**) showed t.l.c. characteristics different from those of the parent amine (Table 1) and unlike the parent amine was highly water soluble and could not be extracted with diethylether under alkaline conditions; upon treatment with $TiCl_3$/HCl it was reduced to phendimetrazine. Found C 63.9, H 8.5, N 6.1. Required for $C_{12}H_{17}NO_2 \cdot H_2O$: C 63.9, H 8.4, N 6.2%. Nmr ($CDCl_3$) δ 1.1 (d, 3H, C—CH_3), 3.2 (s, 3H, N—CH_3), 3.35 (m, 1H, CH—C), where the corresponding δ values for the parent drug were 0.83, 2.34, and 2.2 respectively.

Thin Layer Chromatography (t.l.c.)

Glass plates 20 × 20 cm were spread to a thickness of 0.5 mm with a mixture of silica gel G (Merk) and water (1 : 2). The plates were allowed to dry at room temperature for 10 min and then heated for 1 hour at 110°. The solvent systems used were: A chloroform-methanol (4 : 1), B benzene-methanol-diethylamine (75 : 15 : 10), C methanol-NH_4OH (98.5 : 1.5) and D chloroform-acetone (50 : 50).

The following reagents were used for visualising the spots: iodine vapour, *Draggendorff*'s reagent, iodoplatinate, $AgNO_3$ (ammoniacal) and t.t.c. (1 part 4% triphenyl tetrazolium chloride added to 1 part 1 *N* sodium hydroxide).

Gas-Liquid Chromatography (g.l.c.)

A Perkin-Elmer F11 chromatograph with a flame ionisation detector was used. Chromatographic column A was glass tubing 6 mm o.d. and 2 m long, packed with acid washed, *DMCS* treated Chromosorb G (100–120 mesh) coated with Carbowax 20M (3% *w/w*) and potassium hydroxide (5% *w/w*) and was conditioned at 190° for 24 h before use; oven temperature 150°; injection block temperature setting 4; H_2, 138 kN/m^2; air, 138 kN/m^2 and N_2, 69 kN/m^2. Column B was glass tubing 6 mm o.d. and 1 m long, packed with acid washed, *DMCS* treated Chromosorb G (80–100 mesh) coated with UCW98 (10% *w/w*) and was conditioned at 250° for 24 h before use; operating conditions were as for column A. Column C was glass tubing 6 mm o.d. and 2 m long, packed with acid washed, *DMCS* treated Chromosorb W (80–100 mesh) coated with Carbowax 20M (4% *w/w*) and potassium hydroxide (4% *w/w*) and was conditioned at 190° for 24 h before use; oven temperature 140°, other operating conditions as for column A.

Gas Liquid Chromatography—Mass Spectrometry

The mass spectra were obtained using a Perkin-Elmer model 270 gas chromatograph—mass spectrometer system at an electron energy of 70 eV. One meter 6 mm o.d. glass column packed with acid washed, *DMCS* treated Chromosorb G (100–120 mesh) coated with Carbowax 20M (2% *w/w*) and potassium hydroxide (5% *w/w*) was used; helium, 103 kN/m^2 was the carrier gas, and the oven temperature was 160°.

Proton Magnetic Resonance (p.m.r.)

Spectra in D_2O and $CDCl_3$ were recorded using a Perkin-Elmer R-10 spectrometer plus a Northern Scientific 544 CAT with *TMS* as the internal standard.

In Vivo Oral Administration of Phendimetrazine (**1 a**) *and Phenmetrazine* (**1 b**)

Phendimetrazine tartrate (105 mg) and phenmetrazine HCl (25 mg) were given separately to a number of male subjects (age 27–35) as a single dose either under normal (*pH* 5–8) or acid (*pH* 4.8 ± 0.2) urine conditions on separate occasions. Control urine, blood and saliva samples were collected just before the administration of the drug. Urine samples were collected periodically over a period of 36 h; blood (10 ml) and saliva (collected during 6 min) samples were collected hourly for 10 h. The blood samples in plastic heparinized tubes were immediately centrifuged at 10 000 r.p.m. for 15 min and the plasma was transferred to clean plastic tubes. The cells were resuspended in a small volume of normal saline and recentrifuged twice. All the samples were stored at 4 °C if not analysed immediately.

Determination of **1 a** *and* **1 b** *in Urine*

Urine samples (10 ml) obtained after administration of **1 a** were made alkaline (*pH* 12) with sodium hydroxide (20%) and extracted with freshly distilled diethylether (3 × 10 ml). The bulked ethereal extracts were concentrated to about 0.1 ml in a water bath (42°) and a 2 to 4 μl sample was injected either onto column A or C, a 5 μl sample injected into the g.l.c.-mass spectrometer and the rest was applied to t.l.c. plates. Reference compounds were similarly treated at the same time. The blank urine samples were treated the same way as above. Urine samples obtained after administration of **1 b** were extracted as after using **1 a** and analysed using column C. Compound **1 b** was acetylated with acetic anhydride and the product analysed using column B.

Determination of the N-Oxide (**2**)

Urine samples (50 ml) obtained after administration of **1 a** were extracted at *pH* 12 with diethylether (3 × 30 ml) to remove the amines **1 a** and **1 b**, and then extracted with $CHCl_3$ (3 × 20 ml); the combined chloroform extracts were concentrated under a stream of N_2 in water bath at 42° and the concentrates applied to t.l.c. plates. Reference N-oxide spots were applied near the plate margins and the solvent system A (Table 1) was used for development. The plate margins were sprayed with *Dragendorff*'s reagents to visualise the reference spots and the silica gel between the spots corresponding to the N-oxide was collected and divided into two portions. Each portion was transferred to centrifuge tubes; distilled water (5 ml) and 20% sodium hydroxide (0.5 ml) were added to one tube and the solution extracted with diethylether (3 × 10 ml). The ethereal solution was concentrated to about 50 μl and 3 μl were injected onto column A. To the other portion was added distilled water (5 ml), 6 *N* HCl (0.3 ml) and 30% *w/v* $TiCl_3$ solution (0.2 ml) and after 30 min the solution was adjusted to *pH* 12, then extracted with diethylether and the concentrated extracts analysed on column A as above. Blank urine samples were treated the same way and analysed concurrently.

Investigation for N-Hydroxy-phenmetrazine (**1 c**)

Replicate urine samples (10 ml) obtained after administration of **1 a** were extracted with diethylether at *pH* 10 and 13, the concentrated extracts analysed on the column **B** before and after silanizing with *BSTFA*.

In addition, the samples were hydrolysed at *pH* 4.5 and at 37 °C for 20 h in the presence of sulphatase/β-glucoronidase (suc d'Elix Pomatio 0.1 ml) followed by extraction at *pH* 4, 7.4, and 10 with ether and analysed on column B. Also urine

samples (10 ml) obtained after administration of **1 b** under acid condition were extracted with ether at *pH* 9.5 before and after enzymatic hydrolysis (same as for **1 a**) and analysed (before and after silanizing with *BSTFA*) using column B.

Investigation for Conjugated Phenmetrazine

Replicate urine samples (5–10 ml) obtained after administration of **1 b** were extracted at *pH* 12 with ether (3 × 10 ml). Then the urine was hydrolysed at *pH* 4.5 with a mixture of sulphatase/*β*-glucoronidase as described above for **1 c**.

The solution was made alkaline (*pH* 12) and extracted with ether (3 × 10 ml) and analysed for **1 b** using column C.

Reduction of **2** *by* $TiCl_3$

Samples (5 ml) of phendimetrazine-N-oxide (**2**) solution (30 μg/ml) were reduced in $TiCl_3$/HCl for various periods (3–30 min) and then analysed by the general method (see below).

Stability of **1 a** *to* $TiCl_3$/HCl

Samples (5 ml) of phendimetrazine solution (40 μg/ml) were left in contact with 6 *N* HCl (0.3 ml) and $TiCl_3$ solution (0.2 ml) overnight and then analysed by the general method (see below); the results were compared with those for a standard solution of **1 a** which had not been in contact with $TiCl_3$/HCl.

Stability of Compound **1 a**, **1 b** *and* **2** *in Biological Fluids*

1 ml of solution of the mixture of **1 a**, **1 b**, and **2** (10 and 40 μg of each/ml) were left in contact with acid (*pH* 4.9) and neutral (*pH* 6.8) urine samples (5 ml). The samples were analysed by the general method (see below), after 3 h and after one week and 4 weeks storage (at 4°). Also 1 ml of solution containing 5 μg/ml of **1 a**, **1 b** and **2** was added to plasma and saliva (5 ml); the solutions were analysed by the general method [but using 1 ml chlorphentermine (5 μg/ml) in pentane as the external standard] after 3 h and after one week and 2 weeks storage.

Stability of **1 a**, **1 b** *in Ether*

Ethereal solution of **1 a**, **1 b**, and chlorphentermine (internal standard) were stored at 4° for 10 days and the solutions analysed periodically using column A.

Quantitative Analysis of **1 a**, **1 b**, *and* **2** *in Urine, Plasma and Saliva*

Calibration curves of mixtures of compounds **1 a**, **1 b**, and **2** in blank urine, plasma and saliva were constructed based on the peak height ratio of the compounds to their g.l.c. reference standard. The data were subjected to linear regression analysis to give the appropriate calibration factors.

Ranges for calibration curves: In urine, for compound **1 a**, 1–40 μg/ml, for compounds **1 b** and **2** 1–20 μg/ml; in plasma, for compounds **1 a**, **1 b** and **2** 0.03–0.2 μg/ml, and in saliva, for compounds **1 a** and **1 b** 0.04–0.8 μg/ml.

General Method for Quantitative Analysis of **1 a**, **1 b**, *and* **2**

Urine

To urine (5 ml) containing compounds **1 a**, **1 b** and **2** was added an internal standard (1 ml) containing 50 μg chlorphentermine and 20% NaOH (0.5 ml). The

solution was extracted with ether (10 ml) 3 times. The combined ethereal extracts were concentrated in a finely tapered tube on a water bath at 42° to about 50 μl; 2–4 μl were injected onto column A. The *pH* of the urine was then adjusted to 1–2 with 6 *N* HCl and $TiCl_3$ solution (0.2 ml) was added and the solution allowed to stand in the dark for 30 min (see [6, 13]). The solution was then made alkaline (*pH* 12–13) with 20% NaOH after addition of 1 ml internal standard and assayed for phendimetrazine as above.

Plasma

Plasma (4 ml) was diluted with water (3 ml) and analysed as for urine, using 1 ml chlorphentermine solution (5 μg) as internal standard.

Saliva

Saliva (3 ml) was diluted with water (3 ml) and analysed as for plasma.

Red Blood Cells

Washed red blood cells were haemolysed in specimen tubes with gentle shaking for 5–10 min by resuspension in a volume of water similar to the volume of the red cells, and the solution then extracted and analysed as for plasma above.

Percentage Recovery of **1 a**, **1 b**, *and* **2** *in Urine, Plasma, and Saliva*

1 ml of aqueous solution containing (5 μg each) of compounds **1 a**, **1 b**, and **2** was added to duplicate samples of blank urine (5 ml), saliva (3 ml), plasma (4 ml) and water (5 ml) as reference. The volume of each tube was adjusted to 7 ml with water and analysed by the general method, but 1 ml *n*-pentane solution of chlorphentermine (5 μg) as external marker was added to the ethereal extracts before evaporation.

The peak height ratio of compounds obtained from biological fluids were compared to those obtained from water, and the relative percentage recoveries were calculated.

Results and Discussion

The *Rf* values on t.l.c. plates using different solvent systems and *Rt* onto different columns, of compounds **1 a**, **1 b**, **1 c**, and **2** are shown in Table 1.

Interaction with Spray Reagents on T.L.C.

After developing the t.l.c. plates, various spots were visualised after contact with iodine vapour (brown colour with **1 a**, **1 b**, **1 c**, and **2**) or after being sprayed with *Dragendorff*'s reagent (orange colour with **1 a**, **1 b**, and **2** against yellow background), iodoplatinate reagent (violet colour with **1 a**, **1 b**), ammoniacal silver nitrate solution (blackspot with **1 c**) or triphenyltetrazolium chloride reagent (dark red spot with **1 c**).

Metabolic Products of Phendimetrazine (**1 a**)

After administration of phendimetrazine tartrate to subjects, the N-demethylated product, phenmetrazine (**1 b**), and N-oxide (**2**) were found in high yields in urine, but N-hydroxyphenmetrazine (**1 c**) was not found.

The ethereal extract of urine (*pH* 12) gave two spots on t.l.c.; the *Rf* value of one spot corresponded with that of authentic phendimetrazine (**1 a**) and the other spot to that of authentic phenmetrazine (**1 b**) (Table 1).

Analysis by g.l.c. (column A and C) of the same ethereal extract gave two peaks, one of which corresponded to **1 a** whilst the other with longer retention time corresponded to **1 b** (Table 1). The g.l.c. mass spectrum of the first peak was identical to that obtained from authentic phendimetrazine (**1 a**) and similar to that reported[12]; it gave fragment ions at *m*/e 42

Table 1. *G.l.c. and t.l.c. characteristics of phendimetrazine* (**1 a**) *and its metabolites, phenmetrazine* (**1 b**). *phendimetrazine N-oxide* (**2**), *N-hydroxyphenmetrazine* (**1 c**) *and related derivatives*

Compound	Derivate	G.l.c. *Rt* values (min) (Columns)			T.l.c. *Rf* values in different Solvent Systems[b]			
		A	B	C	A	B	C	D
1 a		7.6	5.6	9	0.64	0.63	0.64	0.22
1 b		10.2	5.0	12.5	0.47	0.53	0.52	0.08
1 c		—	9.4	—	0.70	0.58	[c]	0.50
2		—	—	—	0.15	0.21	0.34	0.00
1 b	*AC*	—	21.5	—				
1 c	*BSTFA*	—	16	—				
CP[a]		5	3.4	6				

[a] *CP* Chlorphentermine used as reference standard (on column A and C).
[b] See Exp. Part.
[c] Not determined, *AC* acetyl derivative.

(52%), 56 (18.3%), 57 (100%), 85 (87%), 86 (5.9%), 117 (2.9%) and 191 (7.9%). The g.l.c. mass spectrum of the second peak was identical to that obtained from authentic phenmetrazine and similar to that reported[14]; it gave fragment ions at *m*/e 42 (40%), 43 (29%), 56 (33%), 71 (100%), 77 (5.2%), 105 (3.6%) and 177 (9.6%). The chloroform extract of urine (after removing the amines **1 a** and **1 b** by ether) gave one spot on t.l.c., the *Rf* value of which corresponded to that of synthetic N-oxide (**2**). The presence of the N-oxide was further confirmed by extraction from the t.l.c. plate followed by $TiCl_3$/HCl reduction to give the parent amine as confirmed by g.l.c. analysis. Analysis by g.l.c. of an ethereal extract of the spots corresponding to the N-oxide in the thin layer chromatogram showed that the parent amine **1 a** was absent before reduction. Ethereal extract could therefore be used to remove unchanged phendimetrazine and its demethylated metabolite phenmetrazine from the urine to leave **2** in the aqueous alkaline solution. Reduction of the N-oxide remaining in

solution by the general method described before produced the parent amine **1a**. The presence of **1c** could not be demonstrated in urine after doses of **1a** and **1b** by analysing the following extracts using column B: the extracts (normal conditions) at *pH* 10 and 13 before and after silanizing with *BSTFA*; the extracts (normal conditions) at *pH* 4, 7.4, and 10 after hydrolysing the urine samples at *pH* 4.5 and 37 °C for 20 h with sulphatase/β-glucoronidase; and the extracts (acid conditions) at *pH* 9.5 before and after enzyme hydrolysis (as above) with or without derivatisation with *BSTFA*.

When the urine samples (obtained after administration of **1b**) were hydrolysed at *pH* 4.5 by enzyme (same as for **1c**) and the ethereal extracts (at *pH* 12) analysed on column C, an additional 3% of **1b** was recovered.

Quantitative Aspects

Reduction of N-oxide (**2**) to the parent amine **1a** with $TiCl_3$/HCl was complete in about 20 min. Consideration of the slopes of replicate calibration curves of **2** and the parent drug **1a** showed that convertion of **2** to **1a** is about 90% using the conditions stated in the experimental section. *Brooks* and *Sternglanz*[13] reported that the yields of a number of amines by reduction of the corresponding N-oxides by $TiCl_3$/HCl were 100% within a few minutes. Similar yield of methadones have been reported[6].

All compounds **1a**, **1b**, and **2** were stable in biological fluids at 4 °C over the period of time stated; also the ethereal solution of compound **1a**, **1b**, and chlorphentermine was stable. The parent amine and its metabolites were not observed with analysis of red blood cells. Analysis using the extract from plasma and specially from red cells decreased the life of the column.

Calibration curves of **1a**, **1b**, and **2** in urine, plasma and saliva were linear in the ranges stated. The correlation coefficients of replicate calibration curves for all 3 compounds from biological fluids were between 0.99–1.00. Therefore a quantitative method for the analysis of unchanged phendimetrazine and its demethylated metabolite and N-oxide was developed as described in the general method. The method involves extraction of the drug and its demethylated metabolite from an alkaline solution into ether and subjecting the extract to quantitative g.l.c. analysis using chlorphentermine as internal standard. The N-oxide remaining in the alkaline solution was reduced with $TiCl_3$ after acidification with HCl to yield the parent amine which was extracted and analysed by g.l.c.

The percentage recoveries of compound **1a**, **1b**, and **2** from urine, saliva and plasma relative to recovery from water were 93 ± 4%, 85 ± 2% and 95 ± 5% respectively (using standard added after extraction, i.e.

external standard). The use of an internal standard gave similar results, indicating no binding of drug, its metabolites or the internal standard in the biological fluids.

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