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SPECIES VARIATIONS IN THE *N*-OXIDATION OF CHLORPHENTERMINE

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Abstract—The metabolism and excretion of orally administered or injected [^{14}C]chlorphentermine has been studied in man, rhesus monkey, marmoset, rabbit, guinea-pig and rat. These species excreted 55–95% of the administered radioactivity in the urine over 5 days. Two metabolites were characterised by thin-layer and paper chromatography, gas–liquid chromatography and g.c.–m.s. and these were *N*-hydroxychlorphentermine and 1-(4′-chlorophenyl)-2-methyl-2-nitropropane. There are marked species differences in the excretion of *N*-oxidation products which were found in the urine of human volunteers, rhesus monkeys, rabbits and guinea-pigs, but not in the urine of marmosets or rats. The rat, rabbit and marmoset also excreted an unidentified unstable acid-labile precursor of chlorphentermine. The results are discussed in relation to the toxicity of the drug and to the metabolism of amphetamines in general.

Chlorphentermine (2-amino-1-[4′-chlorophenyl]-2-methylpropane) is an amphetamine analogue formerly used as an anorectic drug. It is no longer used because it has been associated with the development of pulmonary hypertension [1]. The metabolic fate of amphetamine and several related β-phenylisopropylamines has been studied in detail [2–4], but little is known concerning the metabolism of such amines carrying a substituent on the α-carbon as in the case of phentermine and chlorphentermine. We have therefore extended our investigations to a study of the metabolic fate of chlorphentermine in human subjects and five species of laboratory animal, namely the rat, guinea-pig, rabbit, marmoset and rhesus monkey. It will be shown that the drug undergoes extensive *N*-oxidation in the guinea-pig, rabbit, rhesus monkey and man, but not in the rat or marmoset. The findings are discussed in relation to the toxicity of the drug and to the metabolism of related amphetamines.

MATERIALS AND METHODS

Compounds. Samples of chlorphentermine hydrochloride, m.p. 225°, were gifts from William R. Warner, Ltd., Eastleigh, Hants., England and H. Lundbeck & Co., Copenhagen, Denmark. [^{14}C]Chlorphentermine hydrochloride, labelled in the α-methyl groups and of specific activity 0·07 μCi/mg, m.p. 225°, was the gift of Warner-Lambert Research Institute, Morris Plains, N.J., U.S.A., research affiliate of Warner-Chilcott Laboratories. Authentic samples of *N*-hydroxychlorphentermine oxalate, m.p. 167°, and its nitro analogue, 1-(4′-chlorophenyl)-2-methyl-2-nitropropane, an oil, were gifts from Prof. A. H. Beckett, Department of Pharmacy, Chelsea College, London, S.W.3., and were synthesized in quantity according to the methods given below. Other compounds were purchased and purified where appropriate.

N-Acetylchlorphentermine. A solution of chlorphentermine hydrochloride (1 g) dissolved in water (20 ml) was adjusted to pH 14 with 2 M NaOH and extracted with diethyl ether (3 × 40 ml). The ether extracts were combined and the solvent removed by evaporation on a rotary evaporator. The oily residue was treated with acetic anhydride (5 ml) and 6M NaOH (5 ml) and the reaction mixture left to stand overnight. The material that separated was filtered, recrystallised from aqueous ethanol to give 0·89 g (92% yield) of white needle crystals of *N*-acetylchlorphentermine, m.p. 115°. ($C_{12}H_{16}NOCl$ requires C, 63·85; H, 7·14; N, 6·21%. Found, C, 63·82; H, 7·01; N, 6·34%). Its mass spectrum showed a small molecular ion at *m/e* 225 (relative intensity, 0·5%) with prominent peaks at *m/e* 168, (8·5); 166, (16); 125 (17) and 100 (100). Its infra-red spectrum showed characteristic bands at 3260 cm^{-1} (N—H stretch), 1645 cm^{-1} (amide I band; C=O stretch) and at 1550 cm^{-1} (amide II band; N—H bend) with additional bands at 840 cm^{-1}, 775 cm^{-1} and 715 cm^{-1}.

On mild acid hydrolysis (2M HCl) for 1 hr at 100° the compound was shown by thin-layer chromatography to afford chlorphentermine (R_f 0·36 in solvent B; Table 1).

N-Benzoylchlorphentermine. Chlorphentermine hydrochloride (1 g), dissolved in 20 ml 6M NaOH, was treated with benzoyl chloride (5 ml). The material that separated was filtered and recrystallised from aqueous ethanol to give white needle crystals (1·27 g; yield 81%) m.p. 121°, of N-benzoylchlorphentermine. ($C_{17}H_{18}NOCl$ requires C, 70·95; H, 6·3; N, 4·87%. Found, C, 71·07; H, 6·15; N, 4·87%). Its mass spectrum showed a small molecular ion at *m/e* 287 (relative intensity 0·5) with prominent peaks at *m/e* 166 (6·5), 162 (49·5), 125 (5·5) and 105 (100%). Its infra-red spectrum showed characteristic absorption bands at 3260 cm^{-1} (N—H stretch), 1620 cm^{-1} (amide I band; C=O stretch) and at 1530 cm^{-1} (amide II band; N—H bend) with additional bands at 840 cm^{-1}, 770 cm^{-1} and 715 cm^{-1}. On mild acid hydrolysis (2M HCl at 100° for 1 hr) the compound was shown by thin-layer chromatography to afford chlorphentermine (R_f 0·36 in solvent B, Table 1).

N-Succinylchlorphentermine. Chlorphentermine free base (2 g), dissolved in dry pyridine (40 ml), was treated with succinic anhydride (1·2 g) and the mixture stirred for 3 hr and left at room temperature overnight. The pyridine was removed by evaporation under reduced pressure and the residue treated with dry ether (20 ml). The material that separated was filtered and recrystallised from aqueous methanol to give 2·3 g (72% yield) of white plate crystals of *N*-succinylchlorphentermine (m.p. 156°) ($C_{14}H_{18}NO_3Cl$ requires C, 59·26; H, 6·39; N, 4·94%. Found C, 59·06; H, 6·32; N, 4·83%. Equivalent weight by titration (0·1 M NaOH) was 283; $C_{14}H_{18}NO_3Cl$ requires 283·5). Its infra-red spectrum showed characteristic absorption bands at 3300 cm^{-1} (N—H stretch), 1700 cm^{-1} (C=O stretch of free –COOH), 1635 cm^{-1} (amide I band; C=O stretch) and at 1545 cm^{-1} (amide II band: N—H bend) with additional bands at 830 cm^{-1}, 770 cm^{-1} and 715 cm^{-1}.

1-(4′-Chlorophenyl)-2-methyl-2-nitropropane. This was prepared by the general procedure of Kornblum *et al.* [5] for the conversion of *t*-carbinamines to their respective tertiary nitroparaffins. Potassium permanganate (108 g) was added in small portions over a 2-hr period to a solution of chlorphentermine (20 g) in acetone (250 ml) and water (50 ml) containing 17 g purified dried $MgSO_4$ (B.D.H. Ltd., Poole, Dorset) and this was maintained at a temperature of 40–50° for 16 hr. The reaction mixture was filtered to remove manganese dioxide and the filtrate treated with sufficient H_2O_2 (100 vol.) to remove excess $KMnO_4$. The reaction mixture was filtered once more and the filtrate reduced to 100 ml using a rotary evaporator. The aqueous concentrate was extracted with $CHCl_3$ (400 ml), the latter separated and washed successively with M HCl (2 × 50 ml) and finally dried over anhydrous Na_2SO_4. After filtration the chloroform was removed using a rotary evaporator. The viscous oily residue crystallised on cooling at 4° to give pale green needle crystals of 1-(4′-chlorophenyl)-2-methyl-2-nitropropane (18·5 g; 73% yield). ($C_{10}H_{12}NO_2Cl$ requires C, 56·12; H, 5·61%; N, 6·36%. Found, C, 56·30; H, 5·35; N, 6·07%). Its mass spectrum gave a molecular ion at *m/e* 213 with prominent peaks at *m/e* 167, 125, 115 and 89 (Fig. 1c), and its infra-red spectrum had characteristic absorption bands at 1535 cm^{-1} (asymmetric-NO_2 stretch) and at 1360 cm^{-1} (symmetric-NO_2 stretch) with additional bands at 850 cm^{-1}, 790 cm^{-1}, 735 cm^{-1} and 710 cm^{-1}.

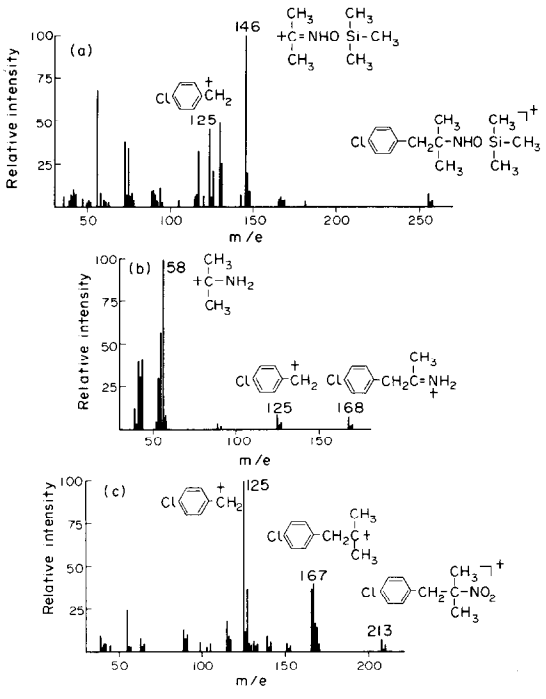

Fig. 1. Mass spectra of chlorphentermine and its metabolites. (a) *N*-Hydroxychlorphentermine trimethylsilyl derivative. (b) Chlorphentermine. (c) 1-(4′-Chlorophenyl)-2-methyl-2-nitropropane. Mass spectra were obtained by subjecting authentic samples of the compounds and extracts of urine (both prepared as described in the text) to g.c.–m.s. analysis using a Varian Aerograph 1700 gas chromatograph coupled to a Varian MAT CH5 mass spectrometer. The g.c. was equipped with a glass column (1·52 m. int. dia. 2 mm) packed with Celite AW-DMCS (100–200 mesh) coated with OV-101 (3% w/w). Injection port temperature was 180°, column 150°, interface 170°, Biemann–Watson-type molecular separator 160°, inlet line 180° and the source 200°. Helium flow was 40 ml/min at 195 kN/m^2 (28 lb/in^2) and electron beam energy was 70 eV. Retention times of these compounds in this system were; *N*-hydroxychlorphentermine trimethylsilyl derivative, 2·6, chlorphentermine, 0·4, 1-(4′-chlorophenyl)-2-methyl-2-nitropropane, 2·2 min respectively. The probable identities of significant ions are shown.

N-Hydroxychlorphentermine. 1-(4′-Chlorophenyl)-2-methyl-2-nitropropane (5 g) dissolved in tetrahydrofuran (25 ml) was added dropwise over a period of 30 min to aluminium amalgam (prepared from 5·4 g aluminium foil [6]) suspended in a mixture of tetrahydrofuran (90 ml) and water (10 ml) the temperature being maintained at 0°. The reaction mixture was filtered, the filtrate dried over anhydrous Na_2SO_4 and after filtering the solvent removed at reduced pressure using a rotary evaporator. The oily residue was dissolved in ether (10 ml) and treated with an excess of an ethereal solution of HCl gas (2·5 M). The ether was reduced to a small bulk and the latter treated with acetone (5 ml) whereby white crystals of *N*-hydroxychlorphentermine hydrochloride (2·48 g; 48% yield) separated, m.p. 145°. The m.p. was not further raised by subsequent recrystallisation from acetone. $C_{10}H_{15}NOCl_2$ requires C, 50·85; H, 6·36; N, 5·93%. Found, C, 50·98; H, 6·35; N, 6·08). The compound was strongly reducing towards triphenyltetrazolium which is consistent with the presence of a reducing hydroxylaminic function. The compound failed to give a satisfactory mass spectrum, and was therefore derivatised for this purpose. A sample (1 mg) was dissolved in bis(trimethylsilyl)acetamide (50 μl) and a portion used for mass-spectrometry by direct inlet. This showed a molecular ion with *m/e* 256 corresponding to the trimethylsilyl derivative of *N*-hydroxychlorphentermine, together with prominent peaks at *m/e* 146, 130, 125, 90 and 75 (Fig. 1a).

Animals and drug treatment. Female Wistar albino rats (200 ± 15 g body wt), female Dutch rabbits (2·3 ± 0·3 kg), female Dunkin–Hartley guinea-pigs (800 ± 50 g), female rhesus monkeys (4 kg) and a male marmoset (500 g) were used. [^{14}C]Chlorphentermine hydrochloride, as an aqueous solution, was administered orally (100 mg/kg; 7 μCi/kg) to rats, guinea-pigs

and rabbits and after sterilisation (ultrafiltration) by intramuscular injection (15 mg/kg; 2 μCi/kg) to the rhesus monkeys and the marmoset. The animals were kept individually in suitable metabolism cages which allowed the separate collection of urine and faeces for up to 5 days after dosing. They were all maintained on an appropriate diet with access to water.

For the human studies two healthy male volunteers, whose informed consent was obtained, aged 22 and 27 yr and body wt 67 and 79 kg respectively, each took a dose (65 mg) of [^{14}C]chlorphentermine hydrochloride (5 μCi) enclosed in a gelatine capsule. Urine samples were collected daily for 5 days after dosing.

Radiochemical techniques. ^{14}C in urine, faeces and on paper and thin-layer chromatograms was determined by scintillation counting (Packard Scintillation Spectrometer Model 3320) as described by Caldwell *et al.* [3]

Reverse isotope dilution for chlorphentermine. Chlorphentermine hydrochloride (0·5 g) was dissolved in urine (10 ml) and the latter adjusted to pH 14 with 10 M NaOH. The mixture was extracted with ether (3 × 50 ml), the ether extracts combined and dried over anhydrous Na_2SO_4. After evaporation of the ether the residue was treated with acetic anhydride (5 ml) and 2·5 M NaOH (10 ml). The *N*-acetylchlorphentermine that separated was filtered and recrystallised from aqueous ethanol to constant specific radioactivity (m.p. 115°). In some experiments the chlorphentermine was converted to its *N*-benzoyl derivative (m.p. 121°) which was similarly recrystallised from aqueous ethanol to constant specific radioactivity.

In some experiments 'total' chlorphentermine was estimated following acid hydrolysis. For this purpose chlorphentermine hydrochloride (0·5 g) was dissolved in urine (5 ml), an equal volume of 10M HCl added and the mixture refluxed for 2 hr. After cooling, the reaction mixture was adjusted to pH 14 with 10M NaOH and the chlorphentermine extracted, converted to its *N*-acetyl derivative and recrystallised to constant specific activity as described above.

Chromatography. The chromatographic properties of chlorphentermine and related compounds are shown in Table 1. Portions (0·01–0·1 ml) of treated (see below) or untreated urine (containing 10^4–10^5 dis/min) were chromatographed (descending technique) on Whatman No. 1 paper or on thin layers (Merck Silica gel GF_{254} 0·2 mm thick on aluminium support) by ascending technique. In some experiments the urine was treated with β-glucuronidase or sulphatase, or acid-hydrolysed prior to chromatography as follows:

(i) Treatment with β-glucuronidase. Portions (2 ml) of urine, adjusted to pH 5 with glacial acetic acid were incubated with 5000 units of β-glucuronidase (Ketodase, Warner-Chilcott, Eastleigh, Hants., U.K.) at 37° for 24 hr. The activity of the β-glucuronidase was checked by including a urine sample adjusted to pH 5 as above and to which had been added phenolphthalein glucuronide (1 μmole). At the end of the experiment the latter was treated with 2M NaOH (0·5 ml).

(ii) Treatment with sulphatase. Portions (2 ml) of urine, adjusted to pH 5 as above and buffered with 0·2 M pH 5 acetate buffer (1 ml) were incubated at 37° for 24 hr with 0·5 ml of a sulphatase preparation (H-2, Sigma Chemical Co.) Saccharo-1,4-lactone (Sigma Chemical Co.) (1×10^{-4} M) was added to inhibit the β-glucuronidase present in the enzyme preparation.

(iii) Acid hydrolysis. Portions (5 ml) of urine were refluxed with an equal volume of 10M HCl for 2h. The hydrolysate was neutralised with $NaHCO_3$ prior to chromatography.

Mass Spectrometry. A Varian MAT CH5 Mass Spectrometer was used together with a Varian Data 620/i computer. Perfluoroalkane 250 was used for calibration.

Concentration of urinary metabolites by extraction on XAD-2 resin for paper and thin-layer chromatography. Amberlite XAD-2 resin (BDH Chemicals Ltd., Poole, U.K.) was washed successively with 4 × 4 bed vol acetone, 3 × 3 vol methanol and 4 × 4 vol distilled water. It was packed as a column (10 × 1 cm) and irrigated with urine (10 ml) obtained from animals dosed with [^{14}C]chlorphentermine, and the effluent collected. The column was washed with water (20 ml) and the metabolites eluted with methanol in 10 × 5 ml portions. The methanol fractions were combined and reduced to 5 ml on a rotary evaporator and portions (0·05 ml) used for chromatography. The methanol eluates contained 98% of the ^{14}C applied to the column.

Gas-liquid chromatography. An F and M model 402 apparatus (Hewlett-Packard Inc., Pasadena, California, U.S.A.) with flame ionisation detection was used. The glass column (1·80 m, int. dia. 3 mm) was packed with AW-DMCS treated Chromosorb G (100–120 mesh) coated with SE-30 (3%, w/w). The conditions used were as follows: injection port temperature 210°, oven temperature 160°, N_2, air and H_2 pressures, 275 165 and 140 kN/m^2 (40, 24 and 20 lb/in^2) respectively, flow rate of N_2, 30 ml/min. The retention times (min) were: chlorphentermine 2·2, *N*-hydroxychlorphentermine trimethylsilyl ether, 5·8, 1-(4′-chlorophenyl)-2-methyl-2-nitropropane, 4·8.

Extraction and derivatisation of metabolites for gas–liquid chromatography and mass spectrometry. Portions (2–5 ml) of urine from animals dosed with [^{14}C]chlorphentermine were buffered with an equal volume of 0·2 M Tris buffer pH 7·0 and extracted with 5 vol ether. The ether extracts were bulked and reduced to dryness under nitrogen. The residue was dissolved in benzene (0·5 ml), treated with bis(trimethylsilyl)acetamide (0·1 ml) and the reaction mixture incubated for 3 hr at 50° under nitrogen. After evaporation of the solvent the residue was re-dissolved in benzene (0·05 ml) and the latter, containing the trimethylsilyl derivative of *N*-hydroxychlorphentermine, namely 1-(4′-chlorophenyl)-2-trimethylsilyloxyaminopropane, used for gas–liquid chromatography and mass spectrometry. The structure and fragmentation pattern of the trimethylsilyl derivative of *N*-hydroxychlorphentermine are shown in Fig. 1a. A blank was prepared in the same way using the urine from untreated animals.

The separation procedure allowed quantitative recovery of both *N*-hydroxychlorphentermine and 1-(4′-chlorophenyl)-2-methyl-2-nitropropane and 50% recovery of chlorphentermine when these compounds

Table 1. Chromatographic properties of chlorphentermine and related compounds

Compound	R_f value in solvent A	B	C	Colour reaction with spray 1	2	3	4	Appearance under u.v. light
Chlorphentermine	0·86	0·36	0·0	blue	orange-red	—	—	quench
N-Hydroxychlorphentermine	0·91	0·79	0·10	—	—	red	—	quench
1-(4′-Chlorophenyl)-2-methyl-2-nitropropane	0·95	0·93	0·74	—	—	pink*	—	dark quench
N-Acetylchlorphentermine	0·91	—	—	—	—	—	purple	quench
N-Succinylchlorphentermine	0·93	—	—	—	red	—	purple	quench

* On heating at 100° for 5 min.

The solvents used were: (A) Butan-1-ol–glacial acetic acid–water, (4:1:5, by vol.); (B) Chloroform–methanol (1:1, by vol.); (C) *n*-hexane–diethyl ether–dichloromethane (4:3:2, by vol.). Solvent A was used for descending paper chromatography (Whatman No. 1 paper) and solvent systems B and C for ascending thin-layer chromatography (Merck Silica Gel GF_{254} 0·2 mm thick, on aluminium support (20 × 5 cm).

The following reagents were used as sprays: (1) 0·1% bromocresol green in ethanol; (2) 0·1% *p*-dimethylaminocinnamaldehyde in ethanol containing 10% v/v 2 M HCl; (3) 0·5% 2, 3, 5-triphenyltetrazolium chloride in a solution containing equal parts of methanol and 6 M NaOH; (4) Chlorine and starch/KI solution [7]. Chromatograms were also viewed beneath ultra-violet light (Hanovia Chromatolite, 254 nm).

(10–100 μg) were added to urine samples (2–5 ml) and extracted as above.

Identification of urinary metabolites. Urine samples from the six species dosed with [^{14}C]chlorphentermine were subjected to treatment with XAD-2 resin as described above and the methanol eluates used for chromatography.

Guinea-pig and rhesus monkey. On paper, in solvent A, two ^{14}C peaks with R_f values 0·71 and 0·86 respectively, were found for both the guinea-pig and rhesus monkey. The peak at R_f 0·86 gave a blue colour with bromocresol green and showed a reducing reaction towards triphenyltetrazolium. The peak at R_f 0·71 gave a blue colour with the naphthoresorcinol reagent. Following treatment of the urine with β-glucuronidase, but not sulphatase, the peak at R_f 0·71 disappeared to give a much enlarged peak at R_f 0·88. This latter peak now gave a stronger red colour with the triphenyltetrazolium reagent, which indicates the presence of a reducing compound. The eluate obtained from urine samples treated with β-glucuronidase on chromatographing on thin-layer in system B showed two ^{14}C peaks, R_f values 0·36 and 0·79 respectively. The first peak, which was strongly reducing towards triphenyltetrazolium, was identical in chromatographic properties with *N*-hydroxychlorphentermine (Table 1). The peak at R_f 0·36 gave a blue colour with bromocresol green and was identical in mobility with chlorphentermine. Gas–liquid chromatography of ether extracts prepared as described above and treated with bis(trimethylsilyl)acetamide showed two peaks of retention times of 2·2 and 5·8 min which corresponded to chlorphentermine (R_t 2·2 min) and the trimethylsilyl ether of *N*-hydroxychlorphentermine (R_t 5·8 min) respectively. The peak size of the latter was markedly increased for both species when the urine has been subjected to a preliminary treatment with β-glucuronidase. The presence of both unchanged chlorphentermine and its *N*-hydroxy derivative were further confirmed by g.c.–m.s. analysis of the ether extracts which afforded mass spectra identical to those obtained with authentic samples of these compounds (Figs. 1a–c). Reverse isotope dilution also confirmed the presence of chlorphentermine.

Man. Radiochromatogram scanning of t.l.c. plates developed in system B showed the presence of four ^{14}C peaks at R_f 0·30, 0·34 (major), 0·80 and 0·91 (minor). The latter three peaks corresponded to chlorphentermine, *N*-hydroxychlorphentermine and 1-(4′-chlorophenyl)-2-methyl-2-nitropropane respectively. Extracts of urine which had been treated with β-glucuronidase showed the presence of three ^{14}C peaks of R_f values 0·34, 0·80 and 0·91. The loss of the peak at R_f 0·30 was accompanied by a large increase in the size of the peak at R_f 0·80 (due to *N*-hydroxychlorphentermine) suggesting that the compound at R_f 0·30 is a glucuronic acid conjugate of the latter.

When similarly prepared extracts of β-glucuronidase treated urine were chromatographed on thin-layer in solvent C three ^{14}C peaks were seen of R_f values 0·00 (chlorphentermine), 0·08 (*N*-hydroxychlorphentermine) and 0·73 (1-[4′-chlorophenyl]-2-methyl-2-nitropropane). The concentrations of the metabolites in the extracts were too low to enable colour tests to be carried out, but further confirmation of identity was obtained by gas chromatography and g.c.–m.s.

Gas–liquid chromatography of ether extracts of human urine samples, derivatised as described before showed three peaks of retention times (min) 2·2 (chlorphentermine), 4·8 (1-[4′-chlorophenyl]-2-methyl-2-nitro-propane) and 5·8 (trimethylsilyl derivative of *N*-hydroxychlorphentermine). G.c.–m.s. gave three mass spectra for these three peaks which corresponded to the identities assigned above. The presence of unchanged chlorphentermine in the urine was also confirmed by reverse isotope dilution.

Rabbit. Paper chromatography in solvent A of extracts of rabbit urine showed two broad ^{14}C peaks the centres of which had R_f values 0·68 and 0·90 respectively. The peak at R_f 0·68 gave a blue colour with the naphthoresorcinol reagent while the peak at R_f 0·90 gave a blue colour with bromocresol green and was also reducing towards triphenyltetrazolium suggesting that this peak was composite.

Chromatography of extracts of urine pretreated with β-glucuronidase showed once again two ^{14}C peaks of R_f values 0·68 and 0·90. However, the size of the former peak had decreased with a corresponding increase in the size of the latter (R_f 0·90). After acid hydrolysis the peak at R_f 0·68 disappeared and this was accompanied by a marked increase in the size of the peak at R_f 0·90. Further chromatography showed that the two original peaks at R_f 0·68 and

0·90 were both composite. The material at R_f 0·90 was eluted from paper chromatograms and the eluate rechromatographed on thin-layer in system B. Radiochromatogram scanning of these plates showed two peaks with R_f values 0·36 and 0·84 respectively. The spot at R_f 0·36 gave a blue colour with bromocresol green, an orange spot with *p*-dimethylaminocinnamaldehyde and corresponded in chromatographic properties with chlorphentermine. The spot at R_f 0·84 was darkly quenching under ultra-violet light and was strongly reducing towards triphenyltetrazolium. This spot was eluted from the plate and rechromatographed on thin layer in system C. Scanning for ^{14}C showed two peaks of radioactivity, one of R_f 0·74 which was dark quenching under ultra-violet light, gave a pink colour with triphenyltetrazolium after heating and corresponded in R_f value to 1-(4′-chlorophenyl)-2-methyl-2-nitropropane, and a second spot at R_f 0·07 which gave a red spot with triphenyltetrazolium and which corresponded to *N*-hydroxychlorphentermine.

The material corresponding to the original peak of R_f 0·68 on paper was eluted with water and incubated with β-glucuronidase at pH 5 overnight. This was rechromatographed on paper in solvent A and on scanning this showed two peaks at R_f 0·68 and 0·91 respectively. The peak at R_f 0·91 was reducing towards triphenyltetrazolium and corresponded in R_f to *N*-hydroxychlorphentermine. This suggests one component of the original peak at R_f 0·68 was the glucuronic acid conjugate of *N*-hydroxychlorphentermine. The other peak (R_f 0·68 after β-glucuronidase treatment) has not been characterised. This compound is unstable and gives chlorphentermine on mild acid hydrolysis; a compound with similar properties is also found in the urine of rats and marmosets dosed with chlorphentermine.

Ether extracts of the urine of rabbits dosed with chlorphentermine and treated with bis(trimethylsilyl)-acetamide on g.l.c. showed three peaks of retention times 2·2, 4·8 and 5·8 min which corresponded to chlorphentermine, 1-(4′-chlorophenyl)-2-methyl-2-nitropropane and the trimethylsilyl derivative of *N*-hydroxychlorphentermine respectively. The presence of these three compounds was further confirmed by g.c.–m.s. of ether extracts of urine. The presence of chlorphentermine in urine was further confirmed by reverse isotope dilution. Unlike guinea-pig, rhesus monkey and human urine, rabbit urine contains an acid-labile precursor of chlorphentermine since on acid hydrolysis (2M HCl for 2 hr) there occurred an increase in the amount of chlorphentermine as determined by reverse isotope dilution.

Rat and marmoset. Paper chromatography in solvent A of extracts of the urine of rats and marmosets dosed with [^{14}C]chlorphentermine showed by radiochromatogram scanning two ^{14}C peaks at R_f 0·70 and 0·86 respectively. The peak at R_f 0·86 gave a blue colour with bromocresol green and an orange spot with *p*-dimethylaminocinnamaldehyde and corresponded in chromatographic properties with chlorphentermine. The peak at R_f 0·70 failed to give any colour reactions. Pretreatment of the urine with β-glucuronidase or sulphatase did not affect this peak but it disappeared following acid hydrolysis of the urine (2M HCl for 2 hr at 100°) and this was accompanied by an increase in the size of the peak at R_f 0·86. Similarly, thin-layer chromatography in solvent B of urine extracts from both species showed two ^{14}C peaks with R_f 0·45 and 0·58 respectively. The latter spot corresponded in colour reactions and mobility to chlorphentermine while acid hydrolysis caused the peak at R_f 0·45 to disappear with a proportional increase in the size of peak at R_f 0·58.

The presence of unchanged chlorphentermine in the urine of both species was further confirmed by reverse isotope dilution. There also occurred an increase in the amount of chlorphentermine, as revealed by isotope dilution, following acid hydrolysis of the urine. Further reverse isotope dilution studies showed that this acid-labile precursor of chlorphentermine was not *N*-acetylchlorphentermine or *N*-succinylchlorphentermine.

RESULTS

The quantitative and qualitative aspects of the excretion of chlorphentermine and its metabolites by the six species studied are shown in Tables 2 and 3.

Rat. This species dosed orally (100 mg/kg) with [^{14}C]chlorphentermine excreted about 56% of the dose in the urine over 5 days with a small amount (0·4%) appearing in the faeces. In separate experiments using bile-duct cannulated rats [8] 2% of an intraperitoneally injected dose (20 mg/kg) of

Table 2. Urinary and faecal excretion of radioactivity following the administration of [^{14}C]chlorphentermine to various species

Species (No., Sex)	% dose of [^{14}C] excreted in urine on day: 1	2	3	4	5	Total	Faeces	Total recovery
Rat (3F)	12 (5·4–17)	15 (12–21)	14(12–18)	9·7 (7·2–13)	5·2(3·9–6·0)	56 (47–66)	0·4 (0·3–0·5)	56 (47–66)
Guinea-pig (3F)	24 (16–28)	20 (13–27)	11(7·5–13)	8·9 (6·6–11)	6·9 (5·4–8·1)	71 (62–77)	—	71 (62–77)
Rabbit (3F)	17 (9–30)	40 (11–51)	26 (1·0–60)	1·0 (0·3–1·6)	0·3 (0·1–0·6)	84 (79–91)	1·0 (0·7–1·2)	85 (80–92)
Rhesus monkey (2F)	83, 89	4, 11	2·1, 2·4	—	—	89, 101	0·9, 0·1	90, 101
Marmoset (1M)	52	12	8	—	—	72	1·9	74
Man (2M)	22, 13	12, 19	10, 12	9, 11	—, 11	53, 66	—	53, 66

Values are means for 3 animals with ranges in parentheses and where only one or two animals were used individual values are given.

[^{14}C]Chlorphentermine was administered as an aqueous solution of the hydrochloride salt, orally to the rat, guinea-pig and rabbit (100 mg/kg; 7 μCi/kg) and by intramuscular injection to the marmoset and rhesus monkey (15 mg/kg; 1·2 μCi/kg). The two human subjects each took a capsule containing 65 mg chlorphentermine (1 mg/kg; 0·05 μCi/kg), for the times shown. Urine and faeces were collected as described in the text.

Table 3. Urinary metabolites of Chlorphentermine in various species

Compound	% of the dose of ^{14}C Rat	Guinea pig	Rabbit	Rhesus monkey	Marmoset	Man
Chlorphentermine	30 (21–43)	28 (17–41)	12 (4·6–20)	20; 24	22	18; 28
Chlorphentermine precursor*	12 (10–15)	n.d.	34 (13–66)	n.d.	30	n.d.
N-Hydroxychlorphentermine (free)	n.d.	14 (7–19)	11 (7·4–15)	6·9; 11	n.d.	10; 5
N-Hydroxychlorphentermine (conjugated)	n.d.	13 (7·0–18)	22 (4·1–41)	61; 47	n.d.	12; 7·4
N-Hydroxychlorphentermine (total)	—	27 (14–37)	33 (12–56)	68; 58	—	22; 12
1-(4′-Chlorophenyl)-2-methyl-2-nitropropane	n.d.	n.d.	2·0 (1·4–2·5)	n.d.	n.d.	3·5; 2·8
Sum of metabolites	42 (30–56)	54 (36–67)	81 (11–90)	88; 82	52	44; 43
Total ^{14}C in urine	41 (30–55)	55 (37–68)	84 (79–81)	89; 83	52	44; 43

* Assayed by difference between free and total (after acid hydrolysis) chlorphentermine.

Details of dosing as in Table 2. In the case of the rat, guinea-pig, rabbit and the two human subjects the values are for the 0–72 hr urine samples; for the rhesus monkey and marmoset values are for the 24 hr urine. Values are means with ranges in parentheses for three animals; where one or two animals were used, individual values are given. The metabolites were determined by quantitative radiochromatography; chlorphentermine was also measured by reverse isotope dilution. n.d. = not detected.

[^{14}C]chlorphentermine appeared in the bile in 24 hr, with 15% in the urine. The major radioactive compound in the urine was unchanged chlorphentermine, which accounted for about 30% of the dose. *N*-Oxidation products, *N*-hydroxychlorphentermine or 1-(4′-chlorophenyl)-2-methyl-2-nitropropane, were not detected in the urine. Besides the unchanged drug, the urine contained an acid-labile precursor of chlorphentermine (12% of dose). The nature of this precursor has not been ascertained but reverse isotope dilution experiments eliminated *N*-acetyl- and *N*-succinyl-chlorphentermine.

Guinea-pig. Guinea-pigs dosed orally with [^{14}C]chlorphentermine excreted about 70% of the dose of radioactivity in the urine in 5 days, most (44%) of the excretion occurring in the first 2 days after dosing. The extent of faecal excretion was not determined in these experiments. The main radioactive compounds in the urine were unchanged chlorphentermine (28% of dose) together with *N*-hydroxy chlorphentermine (27% of dose). The latter was present partly free and partly in a conjugated form which on chromatograms gave a positive reaction with naphthoresorcinol and afforded *N*-hydroxychlorphentermine following treatment with β-glucuronidase. This conjugate is presumably the *O*-glucuronide of *N*-hydroxychlorphentermine. Neither the acid-labile precursor of chlorphentermine nor 1-(4′-chlorophenyl)-2-methyl-2-nitropropane were detected in the urine.

Rabbit. Rabbits dosed orally with [^{14}C]chlorphentermine (100 mg/kg) excreted 80% of the dose of radioactivity in the urine within 3 days with only 1% of the dose in the faeces. The major excretion products were free and conjugated *N*-hydroxychlorphentermine (33% of dose), unchanged chlorphentermine (12%) and the acid-labile precursor of the latter (34%). The urine also contained a small amount (2% of dose) of 1-(4′-chlorophenyl)-2-methyl-2-nitropropane.

Rhesus monkey. The two rhesus monkeys examined excreted 83 and 91% of the dose of [^{14}C]chlorphentermine (15 mg/kg i.m.) respectively in the urine in 24 hr with less than 1% in the faeces. The major excretion product was *N*-hydroxychlorphentermine which accounted for 68 and 58% of the dose respectively for the two animals. Most of this was in a conjugated form with glucuronic acid. The urine also contained unchanged chlorphentermine (20 and 24% of dose) respectively but not the acid-labile precursor found in rat and rabbit urine.

Marmoset. The single marmoset examined excreted 72% of an injected dose (15 mg/kg, intramuscularly) of [^{14}C]chlorphentermine in the urine in 3 days with a further 2% in the faeces. The major excretion products were unchanged chlorphentermine (22% of the dose) and the acid-labile precursor of chlorphentermine (30% of dose). *N*-Oxidation products namely, *N*-hydroxychlorphentermine and the nitro analogue 1-(4′-chlorophenyl)-2-methyl-2-nitropropane were not detected as excretion products.

Man. The two human subjects excreted in the urine about 50% of the radioactivity in 4 days after an oral dose of 65 mg of [^{14}C]chlorphentermine. The major excretion products were free and conjugated *N*-hydroxychlorphentermine (22% and 12% of the dose respectively for the two subjects), unchanged chlorphentermine (18 and 28%) and a small amount (3·5 and 2·8%) of 1-(4′-chlorophenyl)-2-methyl-2-nitropropane. The acid labile conjugate of chlorphentermine was not detected.

DISCUSSION

Major species differences in the pattern of excretion and metabolism of chlorphentermine emerge from this study. The main biochemical reaction of this drug is *N*-oxidation but the extent to which this occurs varies with species, being extensive in the guinea-pig, rabbit, rhesus monkey and the two human volunteers but absent in the rat and marmoset. The main excretory product of the *N*-oxidation of chlorphentermine is its *N*-hydroxy derivative, *N*-hydroxychlorphentermine. The identity of this metabolite was established

by comparison of the paper, thin-layer and gas-chromatographic properties, mass spectrum and colour reactions of the compound isolated from urine of animals treated with chlorphentermine with those given by an authentic sample of the compound. In the guinea-pig, rabbit, rhesus monkey and the two human volunteers the *N*-hydroxy derivative appeared in urine both as such and as a glucuronic acid conjugate. The latter is probably an *O*-glucuronide, the glucuronic acid moiety being attached through the hydroxyl function of the hydroxylaminic group. The conjugate readily afforded *N*-hydroxychlorphentermine on treatment with β-glucuronidase and did not reduce triphenyltetrazolium suggesting that the reducing hydroxylamino group was blocked.

In addition to the *N*-hydroxy derivative, small amounts of the nitro analogue of chlorphentermine, namely, 1-(4′-chlorophenyl)-2-methyl-2-nitropropane, were found as excretion products in rabbit and human urine. This compound was characterised as before by comparison of chromatographic and mass spectral properties with those of an authentic sample.

In two species, the rat and marmoset, no *N*-oxidation products of chlorphentermine were detected. However, the urine of these two species contained, in addition to the unchanged drug, an acid-labile precursor of chlorphentermine, the identity of which has not been determined. This compound is readily broken down to chlorphentermine by warming with 2 M HCl and is unaffected by β-glucuronidase or sulphatase. This compound has also been found in rabbit urine. Dubnick *et al.* [9] also reported the occurrence of such an acid-labile precursor of chlorphentermine in the urine of rats and mice dosed with the drug. They suggested that it was not an *N*-acetyl or *N*-glucosiduronic acid conjugate and that it may be an *N*-conjugate with a dicarboxylic acid. An *N*-succinyl conjugate of a related compound, namely (±)-4′-chloro-2-(ethylamino)-propiophenone has been reported as a urinary metabolite in rats and dogs [10]. However, reverse isotope dilution and chromatography showed that the acid-labile derivative of chlorphentermine was not the *N*-succinyl derivative.

N-Oxidation has been known for many years to be a metabolic reaction of aromatic amines, amides and certain *N*-heterocycles [11]. In recent years evidence has accumulated that primary and secondary aliphatic amines can also undergo *N*-oxidation *in vitro* in liver preparations [12–14]. The products arising from *N*-oxidation of primary aliphatic amines include the nitroso, hydroxylamino, nitro and oxime derivatives of the parent amine. The nitroso and hydroxylamine derivatives probably arise from enzyme catalysed reactions of the parent aliphatic amine, whereas the nitro compound may arise from spontaneous oxidation of the nitroso derivative [16] and the oxime can arise from the instability of the hydroxylamine [13]. The qualitative and quantitative aspects of the urinary excretion of *N*-oxidation products of aliphatic amines have not previously been clearly established although they are well known for several aromatic amines and amides. This is probably due to the instability of the products of *N*-oxidation of aliphatic amines and the further metabolic transformations that these can undergo [13]. In this paper the qualitative and quantitative aspects and species differences in these respects, of the *N*-oxidation of chlorphentermine have been defined.

Chlorphentermine is probably a model compound for exploring the species occurrence of primary aliphatic amine *N*-oxidation because of the relative stability of its *N*-hydroxy derivative. The *N*-hydroxy derivatives of related amines, such as amphetamine, are unstable [22] and are prone to further changes, both chemical and metabolic, which make it difficult to interpret findings. The instability of the *N*-hydroxy derivative of amphetamine and related compounds is probably attributable to the presence of the α-proton which is absent in the chlorphentermine structure. Furthermore, the presence of the 4′-chloro substituent in the molecule of chlorphentermine precludes the alternative metabolic option of 4′-hydroxylation which occurs in the case of phentermine, amphetamine and methamphetamine [2–4].

Numerous species differences in the pattern of metabolism of foreign compounds are known [17]. Many of these differences arise from relative defects in the ability of a species to carry out a particular metabolic reaction. So far most of the described species defects in metabolism are concerned with the metabolic conjugation reactions. The finding that the rat and marmoset are relatively defective in their ability to *N*-oxidise the primary aliphatic amine group of chlorphentermine is probably an example of a species defect in one of the so-called Phase I metabolic reactions. Furthermore, the apparent failure of the rat to effect *N*-oxidation of the aliphatic primary amine group as in chlorphentermine is probably the basis of why this species metabolises the amphetamines in a manner different to that of other species so far examined. In several species (man, rhesus monkey, rabbit and guinea-pig) the amphetamines undergo extensive deamination and side-chain degradation giving benzoic acid and its conjugates, whereas in the rat, amphetamines tend to undergo an alternative reaction, namely, aromatic hydroxylation. The major initial reaction in the deamination of the amphetamines is *N*-oxidation, the products of which are transformed to benzyl methyl ketone [13] which in turn is metabolised to benzoic acid and its conjugates [23]. The extensive *N*-oxidation of chlorphentermine by man, the rhesus monkey, rabbit and guinea-pig, and the relative inability of the rat and marmoset to effect this reaction is consistent with the previously observed species differences in the deamination of the amphetamines [4, 18].

Chronic chlorphentermine administration to rats, guinea-pigs, mice and rabbits results in the marked accumulation of phospholipids in various tissues leading to phospholipidosis [19, 20]. Although *N*-hydroxy metabolites have been implicated in the mechanism of toxicity of several amines [21] it seems unlikely that the *N*-oxidation products of chlorphentermine are responsible for the phospholipidosis induced by the drug, as this readily occurs in the rat, a species which does not *N*-oxidise the compound. It seems likely therefore that this effect is related to the parent compound.

It is noteworthy that compared to amphetamine and other amines of this type, chlorphentermine has an unusually long excretion half-life and this may be of significance in relation to its toxicity. Thus, the

excretion half-lives of chlorphentermine in the rat and guinea-pig are 94 and 64 hr respectively, whereas the corresponding values for both amphetamine and methamphetamine in the two species is 12–16 hr [2, 3].

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