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DISTRIBUTION AND METABOLISM OF CHLORPHENTERMINE-C^{14} IN RATS AND MICE

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Abstract—The distribution and metabolic fate of the anorectic drug, chlorphentermine (*p*-chloro-α,α-dimethylphenethylamine-C^{14}), has been studied in mice and rats. High tissue to plasma ratios were noted. In particular, high levels of unchanged compound in the brain and prolonged retention compared to analogous drugs lacking a *para*-chlorine seem to be characteristic. However, there is no storage of the drug in adipose tissue. Both rats and mice excreted most of the drug in the urine in 24 hr. Rats excreted 70–90 per cent as unchanged drug, presumably due to blockage of both *para*hydroxylation and deamination as metabolic pathways. However, female mice excreted only about 25 per cent unchanged and 60 per cent as an acidic conjugate that could be separated by countercurrent fractionation of the urine. The remaining 15 per cent also separated as a distinct fraction. Hydrolysis of the conjugate gave rise to chlorphentermine-C^{14} as shown by isotope dilution, chromatography and the organic:aqueous distribution ratio. The conjugate is neither *N*-acetylchlorphentermine nor a glucuronide, but discloses, perhaps, a novel metabolic option.

CHLORPHENTERMINE (*p*-chloro-α,α-dimethylphenethylamine)* is a clinically useful anorectic drug. The long duration of the pharmacological effects as well as the prolonged presence of high concentrations in the brain of mice may be attributable to the *para*-chlorine,[1] since this blocks an important metabolic option, *para*hydroxylation. It has been known that *para*hydroxylation is a major pathway for biotransformation of amphetamine[2, 3] and of mephentermine[4] in the dog and rat. Since oxidative deamination as a metabolic pathway is also likely to be hindered in chlorphentermine by the α,α-dimethyl substitution, its conversion by the mouse to material no longer assayable by methyl orange[1] raised the question of alternative pathways for the metabolic disposition of this analog of phenethylamines.

These studies in mice and rats using chlorphentermine-C^{14} describe the tissue distribution of the drug and its excretion, in part, as a metabolite that can be converted to free chlorphentermine by acid hydrolysis.

EXPERIMENTAL

Chlorphentermine-C^{14} hydrochloride (sp. act., 1·06 μc/mg) was synthesized by Mr. E. Merrill of this Institute with labeling in the α-methyl groups. All doses are given as the free base.

* Presate, Warner-Chilcott Laboratories.

Female mice (18–22 g, Swiss, MF-1 strain, Manor Farms, Staatsburg, N.Y.; unfasted) and male rats (200 g, Wistar descendants, Royal Hart Laboratory Animals, Inc., New Hampton, N.Y.; fasted overnight) were used for most of the studies except, as noted, for some cursory experiments in male mice and female rats of the same strain as their opposites for comparison of the metabolic disposition. The animals were housed with 3 mice or 1 rat to a single glass and stainless steel metabolic unit. Pooled mouse tissues or single rat tissues were homogenized in water. Portions of the homogenates were made 1 N with NaOH and extracted with 2·5 vol. benzene. The homogenates were counted directly in a liquid scintillation spectrometer (Packard Tri Carb, model 314X) with the Bray dioxane cocktail; the benzene extract was counted in toluene cocktail. In this way both total C^{14} and "alkaline-extractable C^{14}" were obtained. It has been shown that the base which was extracted with benzene and which was measured by complexing with methyl orange has the chromatographic and distribution (benzene/pH 7·4 phosphate) characteristics of authentic chlorphentermine.[1] Additional ascending, unidimensional paper and thin-layer chromatography on silica gel G of chlorphentermine was carried out with upper phases of solvent systems 1, 2 and 3 for paper and systems 4 and 5 for TLC. R_f values for chlorphentermine in these systems are as follows: 1) *n*-butanol:acetic acid:water (4:1:5), $R_f = 0{\cdot}85$; 2) *n*-butanol:toluene:acetic acid:water (10:10:5:5), $R_f = 0{\cdot}77$; 3) ethyl acetate:water (1:1), $R_f = 0{\cdot}10$; 4) ethanol:benzene:dioxane:ammonia (50:50:40:1), $R_f = 0{\cdot}36$; 5) ethanol:pyridine:dioxane:water (25:10:60:5), $R_f = 0{\cdot}42$. Radioactive chromatograms were scanned on a Nuclear-Chicago model C 100A Actigraph II. Radioactive thin-layer chromatograms were scraped from the plates in sections and counted to determine the R_f values. Nonradioactive chromatograms were sprayed with methyl orange, brom-cresol green or Dragendorff's reagent for visualization.

Urine was kept cold during collection to help preserve any unstable metabolites, either at 0° in a propylene glycol bath or at dry ice temperature. Countercurrent fractionation of the urine (ethyl acetate/pH 3·0 glycine, 0·2 M) was also carried out in the cold at 4°.

Isotope dilution as a criterion for the identity of chlorphentermine-C^{14} in urine was carried out by the synthesis and repeated recrystallization of *N*-acetylchlorphentermine as follows: after carrier chlorphentermine hydrochloride was dissolved in the urine, the solution was adjusted to pH 11 and extracted twice with 2 vol. benzene. The benzene was evaporated, 15 ml of acetic anhydride was added to the residue and the solution was refluxed for 2 hr. The amide so formed was crystallized by pouring the solution over cracked ice. A portion of the filtered amide was dried and counted as the first crystallization. The compound was alternately recrystallized from ethanol–water and benzene–heptane (solution in a minimum amount of hot ethanol or benzene and addition of water or heptane until the first crystals formed). Since the benzene–heptane provided drier and cleaner crystals, only these were counted.

RESULTS

Distribution and excretion of chlorphentermine-C^{14} in the rat. About 70 per cent of an oral dose of chlorphentermine-C^{14} was excreted in the urine in 48 hr, but only a little in the feces (Table 1). In the experiment recorded and in three additional ones, 70–90 per cent of the radioactivity in urine adjusted to pH 11 was extractable into benzene.

All of the radioactivity excreted in the feces was likewise alkaline-extractable. The radioactive material in the benzene extract had a distribution ratio between benzene and pH 7·4 phosphate corresponding to that of authentic chlorphentermine as well as the identifying R_f values upon TLC. An additional amount of the C^{14} in urine, approximately the entire remainder, became alkaline-extractable with benzene upon hydrolysis (2 N H_2SO_4 for 3 hr on the steam bath).

TABLE 1. TISSUE DISTRIBUTION AND EXCRETION OF CHLORPHENTERMINE-C^{14} IN MALE RATS*

Tissue	C^{14} as chlorphentermine (μg/g)					
	30 min	1 hr	2 hr	5 hr	24 hr	48 hr
Brain	16·6	20·0	31·8	24·6	14·3	6·2
Fat	13·3	9·3	17·8	10·6	1·2	1·4
Heart	14·0	8·4	12·9	9·5	5·0	2·3
Kidney	25·8	23·8	33·6	21·6	11·8	6·2
Liver	51·1	32·4	44·5	25·7	9·7	4·9
Lung	40·2	51·4	74·3	54·4	28·8	17·4
Muscle	2·3	3·4	6·2	5·1	2·5	1·3
Plasma	0·6	0·4	0·7	0·5	0·3	0·4
Urine (% of dose)					53·0	68·6
Feces (% of dose)					0·3	2·2
CO_2 (% of dose)					< 0·01	

* The dose of chlorphentermine was 10 mg/kg, p.o. The data given are the average of determinations on 3 individual rats per point. The collection flask for the urine was kept at dry ice temperature during the collection period.

Noteworthy in the distribution of radioactivity shown in Table 1 is the high tissue to plasma ratio. In particular, high levels in the brain and prolonged retention seem to be characteristic. The concentration of chlorphentermine in the lungs is also unusually high. However, there is no storage of the drug in fat. The C^{14} in all of the tissues up to

TABLE 2. TISSUE DISTRIBUTION AND EXCRETION OF AN INTRAVENOUS DOSE OF CHLORPHENTERMINE-C^{14} IN FEMALE MICE

Tissue	C^{14} as chlorphentermine (μg/g)							
	1 hr		2 hr		5 hr		24 hr	
	Total	Base*	Total	Base	Total	Base	Total	Base
Brain	31·0	34·1	21·9	20·3	13·6	12·3	0·6	nil
Fat	3·8	3·0	3·1	1·9	2·5	1·6	nil	nil
Heart	10·1	10·8	7·7	5·7	5·4	3·9	nil	nil
Kidney	32·2	31·6	21·5	19·6	18·4	14·6	1·1	0·8
Liver	24·1	23·1	14·2	14·2	13·3	11·9	0·9	0·5
Lung	33·7	34·8	35·7	36·2	21·2	18·3	1·5	0·7
Muscle							nil	nil
Plasma	5·2	1·0	4·3	0·7	3·6	0·6	0·3	nil
Urine (% of dose)							93·0	13·0
Feces (% of dose)							0·2	0·03
CO_2 (% of dose)							< 0·01	

* Radioactivity extracted by 2·5 vol. benzene from tissue homogenates made 1 N with NaOH; all of standard chlorphentermine added to tissues can be extracted in this way. The dose of chlorphentermine was 10 mg/kg. These data were obtained by using the pooled tissues of 3 mice. The urine was at dry ice temperature during the collection period. Although the 24-hr excretion of C^{14} was over 90 per cent in this experiment, it averaged about 75 per cent in 4 other experiments.

and including the 5-hr point was totally extractable into benzene from pH 11, i.e. apparently chlorphentermine.

Distribution and excretion in the mouse. Previous experiments in mice with a colorimetric (methyl orange) assay showed that high brain levels, prolonged retention in the brain and slow biotransformation were characteristic of chlorphentermine.[1] It was suggested that the high brain level might be attributable to the high lipoid solubility reflected in the high benzene/pH 7·4 phosphate distribution ratio. However, the distribution of chlorphentermine-C^{14} shown in Table 2 (i.v. dosage) and in Table 3 (oral) does not show a particular concentration or retention of chlorphentermine-C^{14} in the fat, while confirming the high brain (and other tissues) to

TABLE 3. TISSUE DISTRIBUTION AND EXCRETION OF AN ORAL DOSE OF CHLORPHENTERMINE-C^{14} IN FEMALE MICE

	C^{14} as chlorphentermine (μg/g)							
	1 hr		2 hr		5 hr		24 hr	
Tissue	Total	Base*	Total	Base	Total	Base	Total	Base
Brain	18·8	19·1	18·1	18·0	11·2	10·9	0·5	nil
Fat	1·5	1·3	1·7	1·4	1·6	1·1	nil	nil
Heart	7·3	6·3	7·4	6·9	2·1	1·7	nil	nil
Kidney	22·3	19·6	20·3	19·0	15·7	13·3	1·4	1·0
Liver	18·7	18·0	16·6	16·0	8·1	7·1	0·9	0·6
Lung	33·6	33·3	37·0	36·0	23·7	23·3	2·0	1·5
Muscle	3·7	3·3	3·3	3·0	2·8	2·1	nil	nil
Plasma	2·2	0·6	2·2	0·6	3·5	0·2	0·4	nil
Urine (% of dose)							87·6	11·4
Feces (% of dose)							2·5	0·13
CO_2 (% of dose)							< 0·01	

* Radioactivity extracted by 2·5 vol. benzene from tissue homogenates made 1 N with NaOH. The dose of chlorphentermine was 10 mg/kg. These data were obtained by using the pooled tissues of 3 mice. The urine was at dry ice temperature during the collection period.

plasma ratio. Except for urinary C^{14}, only in the plasma is there a greater than 2-fold difference between total radioactivity and extractable "base" (presumably chlorphentermine) at 1, 2 and 5 hr. The distribution data obtained from tissues taken 30 sec after dosage (omitted because of the timing inaccuracy) showed all of the C^{14} in all of the tissues to be totally extractable at that time, thus serving as a "standard" for recovery of chlorphentermine from tissues by benzene extraction. The earlier observation[1] that chlorphentermine is converted in the whole mouse to material no longer assayable with methyl orange is supported by the demonstrated excretion of chlorphentermine-C^{14} in a form largely unextractable by benzene from alkalinized urine.

Biotransformation of chlorphentermine: evidence for formation of a conjugate. The initial observations which suggested the possibility that chlorphentermine might be excreted as a conjugate were based on chromatography and the effect of hydrolysis on extractability. Paper chromatography of mouse urine with butanol:acetic acid:water (in which chlorphentermine had an R_f of 0·85) gave a principal radioactive peak at an R_f of 0·69. Upon elution and rechromatography, this radioactive peak had the R_f of chlorphentermine, suggesting that hydrolysis might have taken place. Hydrolysis of mouse urine (1 N H_2SO_4, 24 hr) increased the amount of methyl orange-reactive base

extractable with benzene upon alkalinization. A similar increase in extractable C^{14} could be accomplished and has already been mentioned with respect to rat urine.

More definite data about the excreted C^{14} are given in Table 4. For the experiment summarized in Table 4, 12 mice were dosed with chlorphentermine-C^{14} (10 mg/kg, i.v.). Of the total dose, 57·3 per cent was recovered in the urine overnight at room

TABLE 4. DEMONSTRATION OF "FREE" AND "CONJUGATED" CHLORPHENTERMINE IN MOUSE URINE BY ISOTOPE DILUTION AND RECRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY (CPM/MG)

Ppt. No.	Unhydrolyzed	Hydrolyzed*	Standard†
1	583	1465	1503
2	567	1450	1530
4	576	1440	1522
6	565	1435	1495
8	567	1443	1485
10	555	1398	1475
12	559	1433	1503
Average	568	1438	1502
% of Dose	18·9	47·8	50·0
K (Benz/Phos)‡		1·87	1·53

* On the steam bath for 10 hr in 3 N H_2SO_4; 16 hr of hydrolysis gave no increase in chlorphentermine recovered.

† An amount of chlorphentermine-C^{14} equivalent to 50 per cent of the dose was added to control urine and also subjected to the hydrolysis and subsequent procedures. There was no effect of the hydrolytic procedure on recovery of the standard.

‡ Eleven mg of ppt. No. 6 was boiled in 3 N H_2SO_4 for 3·5 hr and extracted with benzene to remove any unchanged amide. The pH was adjusted to 11 and the C^{14} base was extracted with benzene. Five ml of the second benzene extract was equilibrated with 5 ml of pH 7·4, 0.4 M phosphate.

temperature. Carrier chlorphentermine was added to unhydrolyzed and hydrolyzed portions of the urine. The randomized chlorphentermine was extracted, converted to *N*-acetylchlorphentermine and recrystallized to constant specific activity.

Based on the isotope dilution and on the distribution ratio of the recovered radioactivity, the C^{14} rendered extractable from mouse urine by hydrolysis is chlorphentermine; in this experiment about one-third of the chlorphentermine was excreted unchanged, about one-half was conjugated and about one-sixth was something else. When *N*-acetylchlorphentermine was itself tested as a possible metabolite, by addition of carrier amounts to the benzene extract of alkalinized urine, the radioactivity was lost upon recrystallization. It was concluded that *N*-acetylation of chlorphentermine is not a detectable metabolic pathway in female mice.

Separation of the metabolites of chlorphentermine in mouse urine by countercurrent distribution. The countercurrent distribution (CCD) of urinary metabolites of chlorphentermine-C^{14} between ethyl acetate and pH 3 glycine buffer separated several distinct radioactive peaks (Fig. 1). Peak I (K = 0·05), representing about 25 per cent of the total C^{14}, is unchanged chlorphentermine as determined by running authentic material in the CCD. Peak II (K = 1·33), the major metabolite representing about 60 per cent of the urinary C^{14}, was converted to material with K = 0·05 by 3 hr of hydrolysis of the residue from evaporation of the ethyl acetate in 2 N H_2SO_4 on the

steam bath. Hydrolysis of peak III (K = 5·06), representing about 15 per cent of the C^{14}, did not produce chlorphentermine, but did cause the C^{14} to be distributed with a K = 0·78 instead of 5·06 in the ethyl acetate/pH 3 glycine system. Usually, incubation of urine at 37° produced a fourth peak at K = 19 at the expense of peak II, but this artifact would arise upon incubation of the chlorphentermine standard itself. For this reason, both the urine collection and the CCD were carried out in the cold.

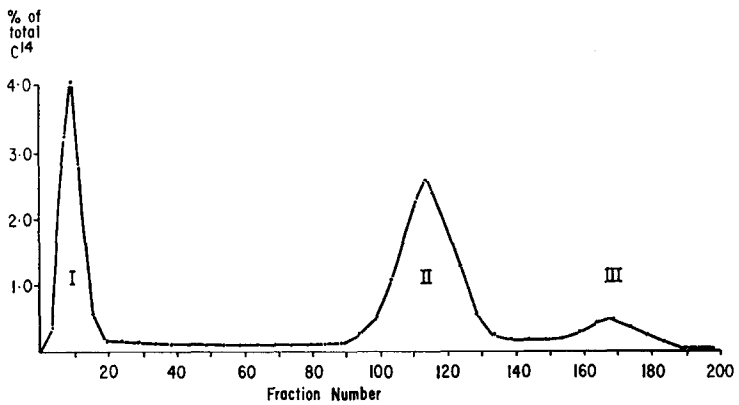

FIG. 1. Countercurrent fractionation of urine from mice treated with chlorphentermine-C^{14}. Peak I (K = 0·05) is chlorphentermine. Peak II (K = 1·33) gives rise to chlorphentermine upon hydrolysis. Peak III (K = 5·06) is uncharacterized. Nine female mice were dosed i.v. (10 mg/kg) with a total of 6·32 μc and the urine was collected for 24 hr; the collection flask was kept at 0°. Countercurrent fractionation of the pooled urines was carried out for 200 transfers in a cold room.

These results, taken together with the isotope dilution data given above, demonstrate the metabolic formation of a conjugate of chlorphentermine in female mice. Presumably, it is the material which is not extracted by benzene from alkaline urine. It is also not extracted by benzene from urine at pH 3 or by ethyl acetate from aqueous solution at pH 7·4; i.e. it is acidic and more polar than chlorphentermine. Countercurrent fractionation of male rat urine in the same manner showed only a small peak at K = 1·1, representing about 10 per cent of the C^{14}. A cursory study of urine collected from male mice and a female rat showed that the C^{14} was largely alkaline-extractable (about 80 per cent), an excretion pattern like that in the male rat. However, the female mice consistently excreted metabolites of chlorphentermine-C^{14} as described above.

DISCUSSION

Distribution and metabolism studies have been reported with chlorinated aralkylamines, including chlorphentermine, which are relevant to our own.[1] The study by Pletscher *et al.* with DL-4-chloro-*N*-methylamphetamine[5] and by Fuller and Hines of DL-4-chloroamphetamine[6] showed that, like chlorphentermine, these compounds also reach higher levels in the brain and are metabolized more slowly than unchlorinated analogs. Thus, blockade of the *para*-position does appear to interfere with an important, usual pathway. This would explain the prolonged anorectic effect of chlorphentermine in rats.[7] Conversely, lipolysis in response to this drug,[1, 8–10] either as a direct effect on adipose tissue or as a consequence of anorexia, may explain

the lack of storage in the fat despite a high lipoid solubility. Opitz and Weischer reported that chlorphentermine is mainly excreted unchanged by rats,[9] a finding which would agree with the results reported here. However, we would like to focus attention on the portion which *is* changed. That portion is a major one in female mice. In studies by Portnoy *et al.*,* the major portion of chlorphentermine-C^{14} was apparently also biotransformed by monkeys, but only 10 per cent was changed when it was given to human subjects.[11] We determined in this laboratory that conjugation might be a significant pathway also in man by showing an increase in methyl orange-reactive material upon hydrolysis of the urine of three male subjects after ingestion of the drug.

Attempts at isolation and identification of the metabolite have so far been unsuccessful. However, a brief review of some of these attempts and the ancillary information obtained thereby may be instructive as to the nature of the compound.

We considered the possibility that it might have been synthesized in the urine by reaction with some constituent. However, no conjugate formed upon incubation of chlorphentermine-C^{14} in mouse urine at room temperature for short (hours) or long (days) periods at acid, neutral or alkaline pH; it may also be noted that it was not dissociated by dilution during countercurrent fractionation or by the addition of carrier chlorphentermine to unhydrolyzed urine for the isotope dilution study. In fact, the metabolite was relatively stable at 60° for 1 hr in urine made 2 N with H_2SO_4, but was hydrolyzed to free chlorphentermine in 3 hr on the steam bath in the 2 N acid. Yet, for reasons not apparent, it was unstable on paper when chromatographed in butanol–acetic acid and was lost upon evaporation of the ethyl acetate when attempts were made to isolate it from mouse urine by extraction with that solvent.

We tried to establish whether or not the conjugate was a glucuronide by methods applied in another study:[12] it was not hydrolyzed by incubation with β-glucuronidase (Ketodase, Warner-Chilcott) or β-glucuronidase/aryl sulfatase (Boehringer,Mannheim) preparations. We were unable to find C^{14} in the CCD fraction containing the metabolite after injection of radioactive glucose *in vivo*, or to find the expected amounts of glucuronic acid in that fraction by direct assay. When mouse liver microsomes were prepared and used as previously described,[12, 13] neither glucuronide formation in the presence of uridine-5′-diphosphoglucosidouronate (UDP-GA) nor any other metabolic conversion of chlorphentermine to material not extractable by benzene occurred. Also, pentobarbital pretreatment of female mice did not influence the rate of chlorphentermine metabolism *in vivo* and chronic treatment with chlorphentermine did not alter either pentobarbital sleeping times or chlorphentermine metabolism (methyl orange assay on whole mice), providing an additional argument against a microsomal site for the biotransformation of chlorphentermine in female mice. As a result of these experiences, we have concluded that the metabolite in question is not an *N*-glucuronide and that chlorphentermine probably is not metabolized in the microsomes.

The formation of a conjugate of chlorphentermine (other than *N*-acetyl) presumably involving the amino group would be an unusual or unrecognized metabolic pathway, since an analogous transformation has not been reported for other phenethylamines to our knowledge. However, not all the metabolites recovered after administration of C^{14}-amphetamine to rats, dogs, monkeys, rabbits and man have been identified, so

* Additional data are on file at Warner-Lambert Research Institute, Morris Plains, N.J.

the possibility has not been eliminated.[3, 14] Although acetylation of chlorphentermine was found not to occur in these studies, and although preliminary attempts to form the metabolite with mitochondria have so far not suceeded, perhaps acylation by some other acid (dicarboxylic?) is the conjugation reaction involved.

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