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Metabolism and excretion of 3-hydroxyphenyltrimethylammonium and neostigmine

M. A. HUSAIN,* J. B. ROBERTS, B. H. THOMAS AND A. WILSON

Department of Pharmacology and General Therapeutics, University of Liverpool

1. Carbon-14 labelled 3-hydroxyphenyltrimethylammonium (3-OH PTMA), an active metabolite of neostigmine, has been given to rats by intramuscular injection and its excretion, distribution and metabolism have been studied.

2. A method is described for the separation and estimation of free and conjugated 3-OH PTMA in urine and liver.

3. In the first hour, about 20% of a dose is excreted in the urine as free 3-OH PTMA and thereafter the rate of excretion of glucuronide conjugate exceeds that of free 3-OH PTMA. In 24 hr 76.8% of the dose is excreted in urine mainly as the conjugate.

4. The peak concentration of radioactivity in blood occurs within 30 min and in liver within 1 hr after administration. More than 90% of the radioactivity in liver occurs as the glucuronide conjugate. Relatively high concentrations of radioactivity were found in liver and heart.

5. In the hen 3-OH PTMA is rapidly excreted by renal tubular secretion.

6. Experiments with carbon-14 labelled neostigmine show that up to 1 hr mainly unchanged neostigmine is excreted in urine; thereafter increasing amounts of free 3-OH PTMA and its glucuronide conjugate are excreted.

7. It is concluded that the duration of action of neostigmine is determined by its rapid renal excretion and by its metabolism to the glucuronide conjugate of 3-OH PTMA.

Studies in the rat and man have indicated that neostigmine is metabolized to 3-hydroxyphenyltrimethylammonium (3-OH PTMA) (Scott, Nowell & Wilson, 1962; Roberts, Thomas & Wilson, 1965b, 1968). This phenolic compound is closely related in structure to edrophonium and its actions on smooth and voluntary muscle resemble those of neostigmine (Cowan, 1938; Randall & Lehmann, 1950; Macfarlane, Pelikan & Unna, 1950; Riker & Wescoe, 1950). Phenolic compounds are often conjugated and excreted as glucuronides or sulphates (Williams, 1959), and it seemed likely that conjugation of 3-OH PTMA may occur. Experiments using ^{14}C-labelled 3-OH PTMA were designed to study its fate after intramuscular administration to rats; in a preliminary communication some of these results have been reported (Roberts, Thomas, Hossain & Wilson, 1967). Similar experiments

* Present address: Institute of Public Health, Mohakhali, Dacca-5, East Pakistan.

were also carried out with ^{14}C-labelled neostigmine with a view to further elucidating the metabolism and elimination of this drug.

Methods

^{14}C-Neostigmine iodide and ^{14}C-3-hydroxyphenyltrimethylammonium iodide (3-OH PTMA) each labelled with ^{14}C in one of the methyl groups of the quaternary nitrogen were supplied by the Radiochemical Centre, Amersham, and had specific activities of 15.2 μc/mg and 36.15 μc/mg respectively. Ketodase, a solution of β-glucuronidase in 0.2 M pH 5 acetate buffer, was obtained from Warner & Co. and a supply of sulphatase from Sigma & Co.

Male rats weighing 150–200 g were allowed food and water *ad libitum* up to the day of the experiment and were then placed in metabolism cages. They were given two doses of warm tap water 5 ml./100 g body weight by stomach tube, with an interval of 1 hr between doses. ^{14}C-neostigmine (25 μg) or ^{14}C-3-OH PTMA (100 μg) were injected intramuscularly into the hind limb, and urine free from faeces was collected at stated time intervals. Faeces and intestinal contents were collected at 24 hr. The methods used for the extraction and estimation of radioactivity in tissues were those described by Roberts, Thomas & Wilson (1965a).

Estimation of the metabolites of 3-OH PTMA and neostigmine

Neostigmine and its metabolites were separated by paper electrophoresis. Samples of urine and extracts of liver were applied as a band to Whatman 542 paper (4, 8 or 19 cm wide). The papers were run in either sodium borate buffer 0.1 M, pH 9.2 or veronal buffer 0.05 M, pH 6.9 for 2 hr 15 min at 300 V.

For quantitative estimation the papers were cut transversely into serially numbered strips 1 cm wide and each strip was counted for radioactivity in a Packard scintillation counter, using scintillation fluid consisting of 2,5-diphenyloxazole (PPO) 4 g and 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) 0.2 g per l. of toluene.

Results

Excretion in urine after administration of 3-OH PTMA

In preliminary experiments the radioactivity was estimated in the urine excreted by rats at hourly intervals up to 5 hr after intramuscular injection of ^{14}C-3-OH PTMA. The urine samples were then subjected to paper electrophoresis at pH 6.9. Two areas of radioactivity were detected ; one at 10–13 cm and the other at 5–9 cm from the origin. The former was considered to be unchanged 3-OH PTMA for concurrently run authentic samples of this compound ran to this position. The latter occurred in increasing proportions in the later samples of urine and was assumed to be a metabolite of 3-OH PTMA.

The possibility that the metabolite was a glucuronide conjugate of 3-OH PTMA was explored by incubating 0.5 ml. of each sample of urine with an equal volume of Ketodase for 24 hr at 37° C. Control samples of urine were incubated with 0.2 M pH 5 acetate buffer. After incubation 3 volumes of ethanol were added and the specimens were centrifuged at 5,000 rev/min for 10 min. The supernatants were then subjected to paper electrophoresis at pH 6.9. Little or no degradation of the metabolite occurred in the controls but the metabolite virtually disappeared

in the samples incubated with β-glucuronidase. Table 1 gives a typical example of the results obtained with urine collected at hourly intervals. The findings support the assumption that the second area of radioactivity described above is almost entirely a glucuronide conjugate of 3-OH PTMA. Similar experiments in which 1 mg (4 units) of sulphatase was used in addition to Ketodase failed to show any further reduction in the amount of metabolite.

For the quantitative study of the excretion and metabolism of 3-OH PTMA, urine was collected from each of three rats at 15 min intervals for 1 hr and then at 2, 4 and 24 hr after intramuscular injection of 3-OH PTMA (100 μg). Samples of each urine were counted for radioactivity and subjected to paper electrophoresis at pH 6.9. The mean values and standard deviations for 3-OH PTMA and glucuronide conjugate at each time interval expressed as a percentage of the dose are shown in Fig. 1. During the first hour 20% of the dose was excreted as unchanged 3-OH

TABLE 1. *Effect of incubating the metabolite of 3-OH PTMA with β-glucuronidase*

	Time (hr)				
	1	2	3	4	5
Before incubation	14·3	55·0	70·2	86·6	82·0
After incubation	2·1	2·7	4·2	5·3	6·0

Figures are the amounts of metabolite, expressed as a percentage of the radioactivity excreted in rat urine at hourly intervals after intramuscular injection of ^{14}C-3-OH PTMA (100 μg).

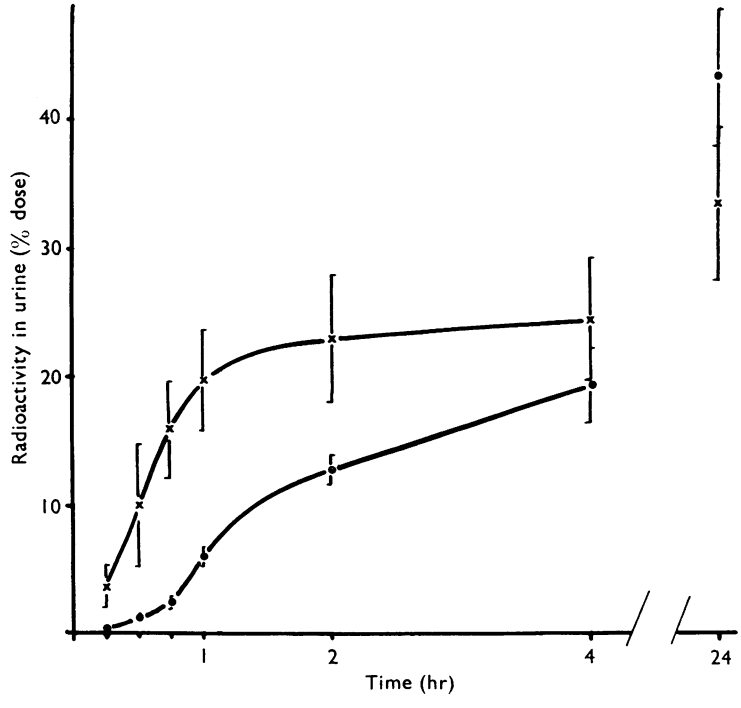

FIG. 1. Excretion of free (×) and conjugated (●) 3-OH PTMA in rat urine after intramuscular injection of ^{14}C-3-OH PTMA (100 μg). Each point is the mean of three experiments. The standard deviations are represented by the vertical lines.

PTMA, thereafter the rate of excretion of the conjugated compound exceeded that of the unchanged drug; by 24 hr 76.8% of the dose had been excreted in the urine. Estimation of the radioactivity in the faeces and intestinal contents collected 24 hr after administration gave a value of $6.5 \pm 3.2\%$ (mean $\pm$ standard deviation). The combined results of these experiments show that 83.3% of the dose was accounted for.

As neostigmine is in part excreted by renal tubular secretion and is also metabolized and excreted as 3-OH PTMA (Roberts *et al.*, 1965a, b), it was of some interest to observe whether 3-OH PTMA is also secreted by the renal tubules.

Using the methods of investigation previously described (Roberts *et al*, 1965a), ^{14}C-3-OH PTMA (250 μg) was injected intramuscularly into the leg of a hen (2.2 kg body weight) and urine was collected separately from each ureter. Estimation of radioactivity showed that in 90 min 58% of the dose was excreted by the ipsilateral and 6.9% by the contralateral kidney. Because the difference between the percentage of the dose excreted by each kidney is 51.1% and greater than can be accounted for by passive diffusion (Sperber, 1948) it is reasonable to conclude that 3-OH PTMA, like neostigmine, is in part excreted by renal tubular secretion.

Distribution in tissues

The distribution of radioactivity in the tissues of two rats 30 min and 60 min after intramuscular injection of 3-OH PTMA, (100 μg) is shown in Table 2. It will be seen that the greatest concentration of radioactivity was found in the liver; relatively high levels were also detected in the heart and kidneys, but most other tissues contained only low concentrations. The amount found in brain was negligible.

In view of the high concentrations of 3-OH PTMA found in the liver and its probable role in the metabolism of this compound, a more detailed study was made of this aspect. Rats were injected intramuscularly with 3-OH PTMA and concurrent samples of blood and liver collected at specified time intervals were estimated for radioactivity.

Table 3 shows the results obtained from rats $\frac{1}{2}$, 1, 2, 3, 5 and 8 hr after administration of 3-OH PTMA. The peak blood level recorded at 30 min had markedly fallen by 1 hr whereas in the liver there was little difference between the concentra-

TABLE 2. *Distribution of radioactivity in rat tissues* 30 *min and* 60 *min after intramuscular injection of* ^{14}*C-3-OH PTMA* (100 μg)

	Concentration (μg/g wet weight of tissue) at			
	30 min		60 min	
	Rat 1	Rat 2	Rat 3	Rat 4
Liver	3·13	3·88	4·46	3·85
Heart	1·23	1·07	0·55	1·00
Kidneys	1·43	0·71	0·61	0·44
Skeletal muscle	0·54	0·92	0·96	0·41
Lungs	0·75	0·72	0·39	0·38
Intestinal wall	0·56	0·46	1·02	0·36
Spleen	0·21	0·26	0·18	0·19
Blood/ml.	0·20	0·12	0·17	0·12
Thymus	0·20	0·23	0·30	0·37
Brain	0·01	0·01	0·02	0·01

tions found at 30 min and 1 hr. It is noteworthy that more than 20% of the dose accumulated rapidly in the liver and that the concentration slowly declined thereafter so that even 2 hr after administration 15% of the dose was still present in this tissue. After incubation of the liver extracts with Ketodase and electrophoresis as previously described, it was found that in all the liver specimens more than 90% of the radioactivity was present as the glucuronide conjugate.

Excretion of 3-OH PTMA and glucuronide after administration of neostigmine

The results described led to the final group of experiments to be reported here. It is known that 3-OH PTMA is a major metabolite of neostigmine, so it was relevant to determine whether the glucuronide conjugate of 3-OH PTMA is also excreted after administration of neostigmine.

Urine from each of three rats was collected at 15 min intervals for 1 hr and then at 2, 4 and 24 hr after intramuscular injection of 25 μg ^{14}C-neostigmine. By subject-

TABLE 3. *Radioactivity in rat liver and blood at different time intervals after intramuscular injection of ^{14}C-3-OH PTMA* (100 μg)

Time after injection (hr)	Liver		Blood concentration (μg/ml.)
	Concentration (μg/g wet weight)	Total content (% dose)	
½	2·58±0·33 (4)	20·82±4·45	0·232±0·104 (4)
1	2·95±0·48 (4)	20·93±1·66	0·145±0·047 (4)
2	2·11±0·52 (4)	14·79±4·29	0·055±0·023 (4)
3	1·34±0·56 (4)	9·52±3·73	0·045±0·034 (4)
5	1·11±0·39 (4)	6·24±0·99	0·030±0·012 (2)
8	0·83±0·17 (4)	4·37±1·19	0·020±0·012 (4)

Radioactivity is expressed as equivalent amounts of ^{14}C-3-OH PTMA. Values are means ± standard deviations. Number of rats in parenthesis.

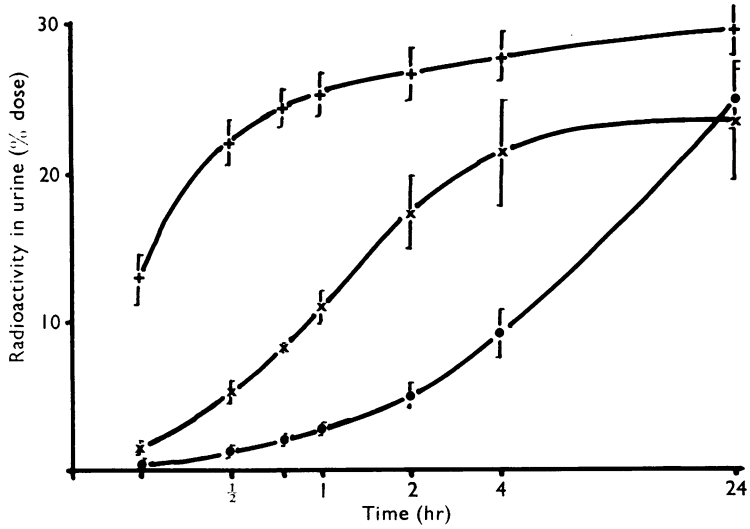

FIG. 2. Excretion of neostigmine (+), 3-OH PTMA (×) and conjugated 3-OH PTMA (●) in rat urine after intramuscular injection of ^{14}C-neostigmine (25 μg). Each point is the mean of three experiments. The standard deviations are represented by the vertical lines.

ing samples of urine to electrophoresis at pH 9.2, neostigmine is found at 10–13 cm from the origin and free and conjugated 3-OH PTMA at 5–9 cm ; at pH 6.9 neostigmine and 3-OH PTMA are found at 10–13 cm, and the glucuronide at 5–9 cm. Neostigmine and glucuronide were estimated directly and by substracting the glucuronide value from that of free and conjugated 3-OH PTMA obtained at pH 9.2, it was possible to determine the amount of free 3-OH PTMA. The presence of glucuronide conjugate was confirmed by incubation with Ketodase.

Figure 2 is a plot of the mean values of unchanged neostigmine, free 3-OH PTMA and its glucuronide conjugate excreted at the stated intervals after administration of neostigmine. After the first hour very little unchanged neostigmine was excreted; after 2 hr the excretion of free 3-OH PTMA began to decline while that of its glucuronide conjugate continued to rise so that by 24 hr about equal proportions of the dose of neostigmine had been excreted as free and conjugated 3-OH PTMA.

3-OH PTMA and glucuronide in liver after administration of neostigmine

It has previously been shown that after intramuscular administration of neostigmine more than 90% of the radioactivity in liver is present as metabolite (Roberts *et al.*, 1965b). In view of the results currently reported, it was considered important to determine to what extent the metabolite in liver is present as free and conjugated 3-OH PTMA. Estimations of total metabolite and of glucuronide conjugate in extracts of liver obtained from three rats 30 min and 4 hr after intramuscular injection of neostigmine showed that 3-OH PTMA glucuronide conjugate accounted for $40.2 \pm 5.3\%$ of the radioactivity in the liver at 30 min, and $93.8 \pm 2.2\%$ at 4 hr.

Discussion

The experiments with 3-OH PTMA have shown that like neostigmine, it is in part excreted by renal tubular secretion, and that only about 6% of the dose is excreted into the intestine. The distribution studies have shown that the liver is the major site of uptake of 3-OH PTMA ; this has also been found after administration of neostigmine (Roberts *et al.*, 1965a). The rapid increase and decline in blood concentration of 3-OH PTMA together with its rapid uptake and retention in the liver is in keeping with the relatively brief action of this compound (Randall & Lehmann, 1950 ; Macfarlane *et al.*, 1950 ; Riker & Wescoe, 1950). The relatively high concentrations found in heart muscle are also noteworthy and closely parallel those found after neostigmine administration (Roberts, Thomas & Wilson unpublished). This evidence serves to underline the potential hazard of severe bradycardia or even cardiac arrest associated with parenteral administration of neostigmine.

Modification of the paper electrophoresis technique previously used, led to the detection of a metabolite of 3-OH PTMA in liver and urine and on further investigation this was shown to be a glucuronide conjugate of 3-OH PTMA. In view of the evidence that after administration of 3-OH PTMA most of the radioactivity in the liver is present as a glucuronide it is logical to conclude that the liver is the likely site of conjugation.

The experiments with neostigmine have added further information about the metabolism and excretion of this drug. The presence of an increasing proportion of the glucuronide conjugate of 3-OH PTMA in the urine after administration of

neostigmine is consistent with evidence obtained from corresponding studies of the concentrations of free and conjugated 3-OH PTMA in the liver, where the proportion of glucuronide conjugate at 4 hr was substantially greater than at 30 min after neostigmine injection. The delay in glucuronide formation and excretion is probably related to the sequence of events involved in the metabolic conversion of neostigmine to 3-OH PTMA. Roberts *et al.* (1968) have shown that *in vitro*, neostigmine is first metabolized to 3-OH PTMA by the liver oxidative microsomal enzyme system. They suggested that the metabolism involves the oxidation of one of the terminal methyl groups of the dimethylcarbamate side chain to the methylol, from which the phenolic compound is then formed. Thus the proportion of free to conjugated 3-OH PTMA found at different intervals after injection of neostigmine probably depends on the relative rates of phenol formation and conjugation.

Although we have no evidence that conjugation with glucuronic acid inactivates 3-OH PTMA it is reasonable to assume that this is so. The rate of glucuronide formation and excretion may thus be an important factor, which in addition to the rapid renal excretion of neostigmine, determines the duration of action of this drug. These findings are relevant to the repeated therapeutic use of neostigmine especially in patients with impairment of kidney or liver function and in premature or neonatal babies where conjugation mechanisms are poorly developed.

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