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DISTRIBUTION AND EXCRETION OF [^{14}C]-NEOSTIGMINE IN THE RAT AND HEN

BY

J. B. ROBERTS, B. H. THOMAS AND A. WILSON

From the Department of Pharmacology and General Therapeutics, University of Liverpool

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Neostigmine has been increasingly used in clinical practice during the past twenty-five years. A number of problems have been encountered in its use as a diagnostic and therapeutic agent for patients with myasthenia gravis and as an antagonist to tubocurarine. For example, the daily requirement of myasthenic patients varies considerably and many can tolerate very large doses without experiencing unpleasant muscarinic effects. Neostigmine resistance or failure to respond to a normal therapeutic dose of neostigmine has been described by physicians in the diagnosis of myasthenia gravis and by anaesthetists in reversing the effects of tubocurarine.

Little information is available about its absorption, metabolism and excretion; an exhaustive search of the literature has shown that only two groups of research workers have studied the fate of neostigmine. Goldstein and his colleagues deduced the fate of the drug by measuring its inhibitory effect on the cholinesterase activity of serum (Krayer, Goldstein & Plachte, 1944; Goldstein, Krayer, Root, Acheson & Doherty, 1949). When they infused neostigmine intravenously into dogs they found that the method of clearance depended on the concentration infused. At low concentrations most of the drug was destroyed by the plasma and a little was excreted by the kidneys; whilst at higher concentrations renal excretion by glomerular filtration was considered to play a predominant role.

Nowell, Scott & Wilson (1962a) developed a method of measuring directly the amount of neostigmine in urine by coupling the drug with the dye bromophenol blue which afforded a colorimetric method of quantitative analysis. They reported that, in patients with myasthenia gravis who were given doses of neostigmine by intramuscular injection, up to 67% of the daily dose was excreted in the urine in 24 hr. By contrast, after oral administration of the drug less than 5% of the daily dose was excreted. They also reported the presence of two metabolic products of neostigmine in extracts of urine from patients who had been given oral neostigmine (Scott, Nowell & Wilson, 1962). Later work by Nowell and his colleagues provided the first evidence that in myasthenic patients up to 50% of an intramuscular dose of neostigmine is excreted in the urine within 2 hr (Scott, 1962). The colorimetric method described by Nowell *et al.* (1962a) cannot be used for the estimation of the two metabolic products described by them since these compounds form a weak colour complex with bromophenol blue. It was decided therefore to use a radioactive form of neostigmine for further investigation of the drug. Since all the evidence regarding the nature of the metabolic products pointed in the direction of hydrolysis of the carbamic

ester group it was clearly desirable to label the drug in one of the methyl groups of the quaternary nitrogen moiety. The work described in this paper is concerned with the excretion and distribution of radioactivity after parenteral administration of [^{14}C]-neostigmine to the rat and the hen. A preliminary report of some of these results has already been published (Roberts, Thomas & Wilson, 1963).

METHODS

[^{14}C]-Neostigmine iodide, supplied by the Radiochemical Centre, Amersham, had a specific activity of 15 μc/mg and was used to examine its excretion in the urine and distribution in the tissues of rats injected intramuscularly with a standard dose of 25 μg in 0.1 ml. of water. The method of administration and doses used in excretion studies in the hen are described later.

Rat

Male rats weighing 150 to 200 g deprived of solid food overnight were placed in metabolism cages (Bollman, 1948). They were hydrated by the administration of 5 ml./100 g of warm tap water by stomach tube. This was repeated 1 hr later. Neostigmine (25 μg) was then injected intramuscularly into a hind-limb, and urine free from faeces was collected at 15-min intervals for 1 hr and then at 2 hr.

In the distribution studies rats were decapitated; blood was collected and the organs were immediately dissected and weighed. In one group of experiments the samples were placed in cellophane bags and the radioactivity in each sample was determined by the Schöniger combustion and liquid-scintillation technique described by Kelly, Peets, Gordon & Buyske (1961). In the experiments concerned with the distribution of neostigmine at different intervals after injection of neostigmine, blood, liver, intestinal contents and faeces were extracted as follows.

Blood. 5 ml. of blood were collected in tubes containing 1 ml. of 4% trisodium citrate. 6 ml. of a 20% solution of trichloracetic acid was added and, after centrifuging, the supernatant fluid was collected. The precipitate was washed three times each with 10 ml. of 10% trichloracetic acid. The combined supernatant fluid and washings were extracted three times each with 25 ml. of diethyl ether. The ether was discarded and the aqueous extract was evaporated on a boiling-water bath to about 1.5 ml.

Liver. After dissection the liver was drained on blotting paper and weighed. The entire organ was homogenized in an Ultraturrax homogenizer with 15 ml. of distilled water. 2.5 g of trichloracetic acid was added and the volume was adjusted to 25 ml. The homogenate was centrifuged at 5,000 revs/min for 15 min, the supernatant fluid was collected and the solid residue was washed twice each with 25 ml. of 10% trichloracetic acid. The combined supernatant fluid and washings were extracted three times each with 75 ml. of ether. The ether layers were discarded and the aqueous layer was evaporated on a boiling-water bath to 10 ml.

Intestinal contents and faeces. Faeces free from urine were collected during 24 hr using the method described by Brittain & Spencer (1963). After the animals had been killed the intestine was ligated at the pyloric sphincter, and the whole of the intestinal tract distal to the ligature was dissected and placed in a beaker. The intestinal contents were washed out with distilled water and mixed with the faeces. The volume was recorded and, after centrifuging, the supernatant fluid was decanted and estimated for radioactivity.

Hen

Sperber (1946, 1949) described a technique for studying the renal tubular secretion of drugs in birds. The technique depends on the peculiar renal vascular system of the hen in which blood returning from the lower limbs is conveyed directly to the peritubular vessels of the kidney without passing through the glomerulus. Thus when a drug is injected intramuscularly or intravenously into the leg it is transported directly to the tubules of the kidney on that side and if it is secreted by the tubules its concentration in the urine collected from the ipselateral kidney will be greater than in that from the contralateral kidney.

Hens weighing 1.5 to 3 kg were anaesthetized by the injection of pentobarbitone sodium (Monoven) into the pectoral muscles. An initial dose of 90 mg was usually sufficient and anaesthesia could be maintained for several hours by hourly injections of 30 to 60 mg. The anaesthetized hen was placed in a V-shaped

wooden trough with its head suspended over the open end of the trough. A polyethylene cannula was inserted into a wing vein through which 0.45% saline was delivered by a Palmer continuous slow-injection apparatus at a rate of 60 ml./hr. The ureters were cannulated by the method described by Sperber (1946, 1949) and urine from each ureter was collected in graduated centrifuge tubes. When the flow of urine from each ureter was approximately 1.0 ml. in 10 min the dose of [^{14}C]-neostigmine was administered. In four experiments it was administered intramuscularly in 0.5 ml. or 1.0 ml. of 0.9% saline into either the right or left leg. In another group of experiments it was injected into the right or left saphenous vein into which a polyethylene cannula had been previously passed. The dose was washed in with 0.5 ml. of 0.9% saline. In other experiments neostigmine was administered as a continuous infusion of a solution in 0.9% saline. Before the estimation of radioactivity each specimen of urine was centrifuged for 10 min at 3,000 revs/min.

Determination of radioactivity

The radioactivity of samples of urine and extracts of tissues were determined by counting scintillations using the scintillation fluid described by Kinard (1957). All results were corrected for quenching using an internal standard.

The basic cyanine dye 1'-ethyl-3,6-dimethyl-2-phenyl-4-pyrimido-2'-cyanine chloride (Cyanine 863) was used as a freshly prepared solution in 0.9% saline.

RESULTS

Excretion in urine

Rat. The radioactivity excreted in the urine of seven individual rats is expressed as a percentage of the dose. Fig. 1 shows the initial rapid excretion of the drug during the first hour when about 43% of the dose was excreted. This rapid excretion is remarkably similar to that observed in man by Nowell and his colleagues (Scott, 1962) and led us to the tentative conclusion that the drug is secreted by the renal tubules. To explore this possibility we then investigated its renal tubular secretion in the hen.

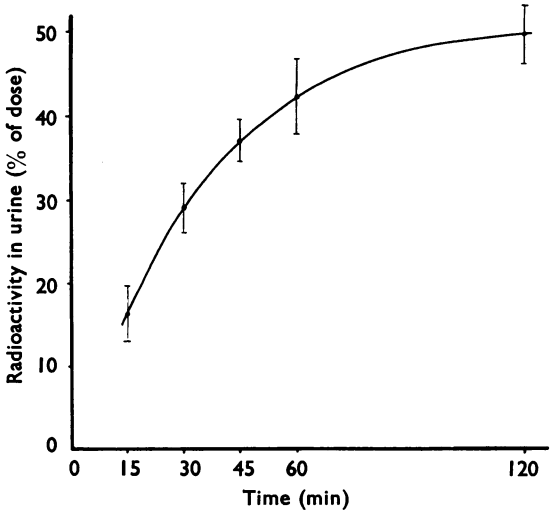

Fig. 1. Radioactivity excreted in the urine of the rat after intramuscular injection of 25 μg of [^{14}C]-neostigmine. Each point is the mean of at least seven experiments. The standard deviations are represented by the vertical lines.

Hen

The results of four experiments with intramuscular injection and ten experiments with intravenous injection of [^{14}C]-neostigmine are summarized in Table 1. It will be seen that in each of the experiments the percentage of the dose excreted by the ipselateral kidney is greater than by the contralateral kidney. This is clear evidence that the drug was secreted by the renal tubules. The possibility that the greater proportion of the dose excreted by the ipselateral kidney might be accounted for by passive diffusion cannot be entertained since the difference between the percentage of the dose excreted by the two kidneys is greater than 10 (Sperber, 1948).

TABLE 1

EXCRETION OF RADIOACTIVITY BY IPSELATERAL AND CONTRALATERAL KIDNEYS OF THE HEN AFTER INJECTION OF [^{14}C]-NEOSTIGMINE

Hen No.	Dose of [^{14}C]-neostigmine (μg)	Duration of urine collection (min)	Radioactivity excreted by kidney (% of dose)		
			Ipselat. (*a*)	Contralat. (*b*)	Difference (*a*−*b*)
	(a) *Intramuscular*				
1	666	110	40·0	5·9	34·1
2	333	210	60·2	16·6	43·6
3	333	210	51·4	7·1	44·3
4	333	195	49·7	6·1	43·6
	(b) *Intravenous*				
5	66·6	60	49·4	2·5	46·9
	66·6	60	60·8	3·5	57·3
6	66·6	30	89·0	5·5	83·5
	66·6	30	84·8	7·1	77·7
	66·6	30	81·0	6·2	74·8
7	66·6	30	51·6	11·6	40·0
8	66·6	30	42·1	3·8	38·3
	66·6	30	39·9	11·7	28·2
9	75·0	30	58·9	3·3	55·6
10	75·0	30	48·2	4·2	44·0

Inhibition of renal tubular secretion

The renal transport of tetraethylammonium and some other quaternary nitrogen compounds has been shown by Rennick, Kandel & Peters (1956) to be inhibited by Cyanine 863. Various experiments were designed to investigate the effects of Cyanine 863 on the excretion of neostigmine.

In an initial experiment 75 μg of [^{14}C]-neostigmine was injected into the saphenous vein of the hen and urine was collected for 30 min. After a further 1 hr, 836 μg of Cyanine 863 was injected into the same vein and after 10 min the same dose of neostigmine was repeated. The difference between the percentage dose of neostigmine excreted by the ipselateral and contralateral kidney before Cyanine 863 was 55.6, and after Cyanine 863 was 9.4.

In three hens neostigmine was infused at a constant rate of 2.2 or 2.5 μg/min throughout the experiment. Urine was collected at intervals of 10 min. At a suitable point in the experiment, usually after 1 hr the syringe containing the neostigmine solution was changed to one containing the same concentration of neostigmine together with a specified molar concentration of Cyanine 863. Urine collection was continued for approximately 1 hr. The mean values for the excretion of radioactivity by each kidney were used to calculate

the apparent tubular excretion factor of neostigmine before and during the infusion of Cyanine 863. This factor was calculated by the method described by Rennick *et al.* (1956) as

$$\frac{\text{Excretion rate (I)} - \text{Excretion rate (C)}}{\text{Infusion rate}}$$

where (I) = ipselateral and (C) = contralateral.

The results, which are summarized in Table 2, show that in each experiment the apparent tubular excretion factor of neostigmine was reduced by cyanine. From this evidence we conclude that neostigmine is excreted by the same tubular transport mechanism as the other quaternary nitrogen compounds referred to above.

TABLE 2

THE EFFECT OF CYANINE 863 ON THE APPARENT TUBULAR EXCRETION FACTOR OF NEOSTIGMINE IN THE HEN

[^{14}C]-Neostigmine was administered by continuous infusion into the saphenous vein (*a*) alone and (*b*) mixed with Cyanine 863. For calculation of apparent tubular excretion factor, see text

Hen No.	Infusion rate of neostigmine (μg/10 min)	Molar ratio cyanine : neostigmine	Duration of urine collection (min)		Apparent tubular excretion factor	
			(*a*) Alone	(*b*) With cyanine	(*a*) Alone	(*b*) With cyanine
11	25·0	2·2 : 1	40	40	32·1	13·8
12	22·2	8·0 : 1	70	70	59·2	21·6
13	22·2	20·0 : 1	60	90	72·2	7·8

The inhibitory effect of Cyanine 863 was also studied in four rats. Cyanine 863 was injected intramuscularly into each rat 1 hr before the intramuscular injection of 25 μg of [^{14}C]-neostigmine. Urine from each animal was then collected at stated intervals for 2 hr and estimated for radioactivity. In two rats the molar ratio of Cyanine 863 to neostigmine was 2 : 1 and in the other two rats it was 4 : 1. The results were calculated as the summated output from each animal and are recorded in Table 3 as the mean output of the four rats. Control observations were made on five rats which had not been injected with Cyanine 863. It will be seen from Table 3 that the mean output of radioactivity from the rats treated with Cyanine 863 was significantly less than from the control animals.

TABLE 3

THE EFFECT OF CYANINE 863 ON THE EXCRETION OF RADIOACTIVITY IN THE URINE OF THE RAT

Cyanine was injected intramuscularly 1 hr before the injection of [^{14}C]-neostigmine (25 μg). Total radioactivity excreted is summated at each time period and expressed as a percentage of the dose of neostigmine. Figures are the means and standard deviations of four Cyanine 863-treated and five control rats. At each time period the difference between means is significant ($P<0·001$)

Time after [^{14}C]-neostigmine (min)	Radioactivity in urine (% of dose) for	
	Cyanine 863-treated	Control
15	8·4±2·6	16·7±1·8
30	16·3±0·9	27·3±1·7
45	22·4±3·6	36·2±2·7
60	27·1±5·0	43·1±5·4
120	36·4±7·0	49·6±3·2

Distribution in rat tissues

In preliminary experiments 25 μg of [^{14}C]-neostigmine was injected intramuscularly into rats. At 30 min after the injection the animals were decapitated and the radioactivity was estimated in duplicate samples of various organs by the Schöniger combustion method. The highest activity was detected in the liver and the intestinal contents. Much lower amounts were detected in the heart, kidney and lung, and little or no activity was found in skeletal muscle and brain.

These results suggested a further study of the concentration of drug in the liver at different times after injection. When a suitable method for extraction of radioactivity from the liver had been devised (see Methods) the concentration was determined at different times after intramuscular injection of a standard dose (25 μg) of [^{14}C]-neostigmine. The results are set out in Table 4 which shows the mean concentration in liver and the mean total activity expressed as a percentage of the dose. In these experiments blood was also collected and estimated for radioactivity, and these results are included in Table 4. It will be seen that the concentration in the liver rapidly reaches its peak in 30 min and thereafter slowly decreases to a negligible level at 24 hr, whereas the peak activity in blood occurs

TABLE 4

RADIOACTIVITY IN RAT LIVER AND BLOOD AT DIFFERENT TIME INTERVALS AFTER INTRAMUSCULAR INJECTION OF 25 μg OF [^{14}C]-NEOSTIGMINE

Values are means and standard deviations. Numbers of rats are in parentheses. Radioactivity is expressed as equivalent amounts of [^{14}C]-neostigmine

Time after injection	[^{14}C]-Neostigmine in Liver: Concentration (μg/g wet weight)	[^{14}C]-Neostigmine in Liver: Total content (% of dose)	[^{14}C]-Neostigmine in Blood: concentration (μg/ml.)
10 min	0·72±0·07 (3)	18·9±2·4	0·06 ±0·027 (4)
30 min	1·03±0·15 (3)	26·3±1·9	0·022±0·007 (3)
60 min	0·82±0·13 (2)	20·0±2·5	0·015±0·007 (2)
3 hr	0·45±0·2 (3)	11·6±5·2	0·01 ±0·01 (3)
24 hr	0·02±0·01 (4)	0·4±0·2	0·003±0·002 (3)

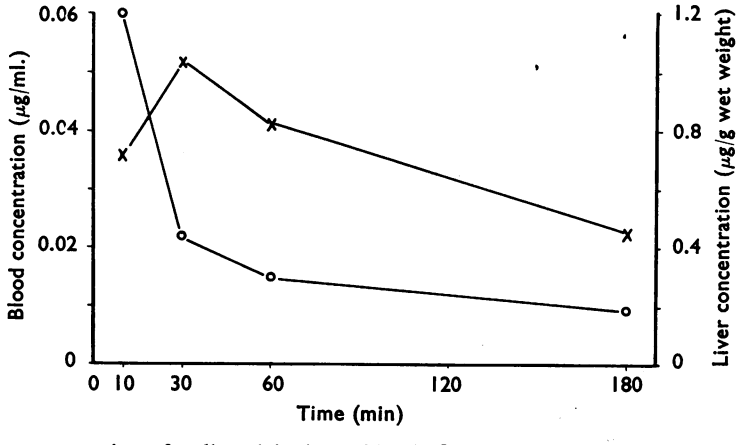

Fig. 2. Mean concentration of radioactivity in rat blood (○) and liver (×) after intramuscular injection of [^{14}C]-neostigmine (25 μg).

10 min after injection and rapidly declines within 30 min. Fig. 2 is a plot of the mean concentrations in liver and blood up to 3 hr after the dose of neostigmine; the standard deviations are given in Table 4. Fig. 2 emphasizes the rapid rise and fall in blood level and the concurrent changes in liver concentration. The relevance of these findings is discussed later.

An opportunity was taken to collect and estimate the radioactivity in the intestinal contents and faeces and urine of the four rats which were killed 24 hr after injection of neostigmine. The outputs (mean and standard deviation) of radioactivity in the urine were $82\pm5.4\%$ and in the faeces $6\pm1.8\%$. This suggests that the main route of excretion is by the kidneys and that biliary secretion plays a minor role in the elimination of the drug.

DISCUSSION

All the results have been expressed in terms of radioactivity rather than of neostigmine because it is probable that part of the dose may have been metabolized. Evidence of the metabolism of neostigmine has already been presented by Scott *et al.* (1962) and Nowell, Scott & Wilson (1962b). Subsequent work, which will be published later, in this laboratory has fully confirmed this in experimental animals.

The results of the excretion experiments in the rat amplify the observation that neostigmine is rapidly excreted in the urine of patients with myasthenia gravis (Scott, 1962). Further evidence provided by the hen experiments has shown that the rapid excretion in the urine is largely due to renal tubular secretion and that this mechanism can be inhibited by Cyanine 863. The fact that prior administration of this compound to rats also inhibits the urinary output of radioactivity strongly suggests a similar mechanism for tubular secretion in the mammal.

There appears to be at least two mechanisms concerned in the elimination of drugs by their rapid transport from peritubular blood across tubular cells into the lumen. These two mechanisms are represented on the one hand by penicillin, the transport of which is blocked by drugs such as *p*-aminohippuric acid and carinamide; on the other hand, the transport of quaternary nitrogen compounds such as *N'*-methyl nicotinamide, tetraethylammonium and choline is not blocked by *p*-aminohippuric acid or carinamide (Beyer, Russo, Gass, Wilhoyte & Pitt, 1950) but is inhibited by Cyanine 863 in the dog and hen (Peters, Fenton, Wolf & Kandel, 1955; Rennick *et al.*, 1956). We conclude therefore that the latter mechanism is responsible for the excretion of neostigmine in the hen and rat.

Although Cyanine 863 is too toxic for clinical use it is possible that some less toxic compound may be synthesized with a similar action which would effectively delay excretion and prolong the action of neostigmine. A new drug of this kind would be a useful therapeutic contribution to the treatment of myasthenia gravis and in reversing the action of tubocurarine.

The distribution studies have emphasized the importance of the liver in the clearance of neostigmine. The rapid accumulation of radioactivity within 10 min after intramuscular injection and its further accumulation to about 25% of the dose within 30 min clearly establishes the importance of the liver as a reservoir. The slow release of the drug from the liver is reflected in the change in pattern of the blood concentration curve shown in Fig. 2. The rapid rise and fall in the first 30 min can be attributed to rapid absorption and renal tubular

secretion, whilst the subsequent slow decline in blood concentration is probably due to the release of radioactivity from the liver. It is probable that neostigmine is metabolized in the liver and that the biological activity of the metabolite is important in maintaining the effect of the drug.

The presence of radioactivity in the faeces and intestinal contents strongly suggests elimination also by biliary secretion. It is interesting to note that Millburn (personal communication, 1964) has detected radioactivity in the bile of the rat after intraperitoneal injection of [^{14}C]-neostigmine.

We conclude from these studies that after intramuscular injection [^{14}C]-neostigmine is rapidly eliminated in the urine mainly by renal tubular secretion and that the liver also plays an important part in the metabolism and excretion of the drug.

SUMMARY

1. Neostigmine labelled with [^{14}C] in one of the methyl groups of the quaternary nitrogen has been used to elucidate the mechanisms involved in the excretion and distribution of neostigmine.

2. The results are expressed in terms of radioactivity rather than of neostigmine because there is evidence that neostigmine is metabolized.

3. In the rat about 43% of an intramuscular dose is excreted in the urine within 1 hr; in the hen it is also rapidly excreted by renal tubular secretion.

4. Prior administration of Cyanine 863 inhibits renal excretion of neostigmine in the hen and the rat.

5. After intramuscular injection the highest concentration in rat tissues occurs in the liver; about 25% of the dose is present at 30 min and thereafter the level slowly decreases over 24 hr. About 6% of the dose can be recovered from the intestinal contents and faeces 24 hr after intramuscular injection.

6. The peak concentration in the blood occurs within 10 min after intramuscular injection and rapidly declines within 30 min.

7. It is concluded that, after intramuscular injection, [^{14}C]-neostigmine is rapidly absorbed and a high proportion of the dose is rapidly cleared by renal tubular secretion. Concentration of radioactivity in the liver suggests that this organ plays an important role in the metabolism of the drug.

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