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DETERMINATION OF NEOSTIGMINE AND PYRIDOSTIGMINE IN THE URINE OF PATIENTS WITH MYASTHENIA GRAVIS

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A method has been described for the estimation of neostigmine and pyridostigmine in urine by ion exchange treatment and colorimetric estimation of the blue complex produced when either of the drugs is made to react with bromophenol blue. Urine containing 2 μg/ml. or more of neostigmine or 3 μg/ml. or more of pyridostigmine can be quantitatively estimated. After intramuscular injection of neostigmine to patients with myasthenia gravis, up to 67% of the drug is excreted, whilst after oral administration less than 5% is excreted. When pyridostigmine is given by mouth, the amount of drug excreted in the urine varies between approximately 2 and 16%. It has been established by chromatographic analysis that the blue complexes formed under these conditions are due only to neostigmine and pyridostigmine respectively and that the quantitative estimation described is a true measure of the amount of these drugs excreted in the urine. The significance of these results is discussed in relation to the absorption and metabolism of the two drugs.

Neostigmine and pyridostigmine are the two drugs most commonly used in the treatment of patients with myasthenia gravis. They are usually administered orally, but neostigmine is sometimes given by intramuscular and occasionally by intravenous injection. There is considerable variation between patients in the doses which are required for the relief of symptoms, this being particularly marked when the drugs are given by mouth, when the daily requirements may range from 30 mg to 1,500 mg of neostigmine and from 120 mg to 3,600 mg of pyridostigmine. There is reason to believe that the varying doses which are required by different patients are not necessarily related to the severity of the disease but may also depend upon differences between patients in the rates of absorption, metabolism and excretion of these drugs.

Little information is available about the metabolism of neostigmine and pyridostigmine, and we have therefore devised a chemical method for estimating their concentration in urine. The method involves extraction of the drug from urine by adsorption on an ion exchange resin from which it can then be eluted. The eluate is assayed colorimetrically by a modification of the method described by Mitchell & Clark (1952), which depends upon the drug reacting with bromophenol blue under alkaline conditions to form a blue chloroform soluble complex. Thereafter the drug can be liberated from the complex by paper chromatography and its *R*

value used to confirm that the results of colorimetric assay are due to the drug under investigation. These latter methods are, with slight modification, similar to those used for the isolation of quaternary nitrogen compounds from extracts of thymus glands (Nowell & Wilson, 1961).

Some preliminary results are reported on the determination of neostigmine and pyridostigmine in the urine of patients with myasthenia gravis.

METHODS

(*a*) *Colorimetric assay of neostigmine and pyridostigmine.* Solutions of each drug in aqueous 0.2 M K_2HPO_4 can be assayed by formation of the respective bromophenol blue complex. The bromophenol blue reagent was prepared by shaking 30 mg 3-3′; 5-5′ tetrabromosulphonphthalein (Eastman-Kodak) in 100 ml. 30% w/v K_2HPO_4 for 30 min and then filtering through a Whatman 52 paper.

To a stoppered centrifuge tube of 35 ml. capacity containing 3 g sodium carbonate (AR) and 10 ml. chloroform (AR), 5 ml. bromophenol blue reagent was added by pipette, taking care to avoid running through the chloroform layer. 5 ml. of the test or standard solution of the drug in 0.2 M K_2HPO_4 was then pipetted carefully into the upper aqueous layer. The tube was then shaken vertically for 30 sec by a machine which was specially constructed for the purpose to give 2,000 shakes per min. Immediately after shaking, the tube was centrifuged at approximately 2,400 r.p.m. for 15 sec and allowed to stand for 8 min. The upper purple aqueous layer was removed by suction and discarded; the lower blue chloroform layer, containing the bromophenol blue complex, was transferred to a 1 cm cell by pipette and the optical density measured at 605 mμ by a Unicam SP500 spectrophotometer. The concentration of neostigmine or pyridostigmine in the 0.2 M K_2HPO_4 solution was then derived by reference to standard calibration curves (Fig. 1).

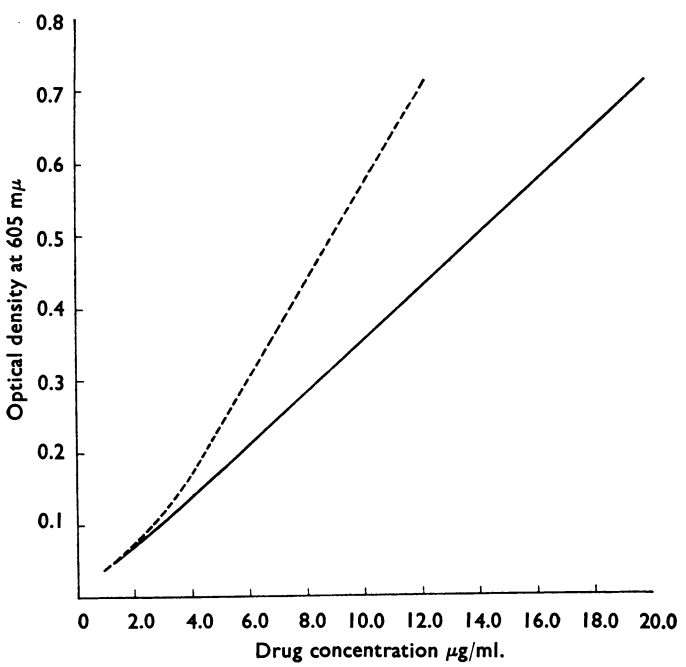

Fig. 1. Calibration curves for neostigmine (– – –) and pyridostigmine (——) showing the relation between colour intensity and concentration of each drug in 0.2 M K_2HPO_4.

Standard calibration curves for neostigmine and pyridostigmine were constructed from the results of estimating the colour intensity obtained with standard solutions containing different concentrations of each drug. They were prepared from Neostigmine methylsulphate (Roche) dissolved in 0.2 M K_2HPO_4; Pyridostigmine bromide ampoules 1 mg/ml. ("Mestinon," Roche) diluted with 0.2 M K_2HPO_4.

(*b*) *Paper chromatography.* The paper chromatographic method was designed to dissociate the bromophenol blue complexes and establish the R_F values of the drug or metabolic products which formed the colour complex. The chloroform solutions of the blue complexes obtained by the assay procedure were too dilute for direct application to the chromatography paper; the chloroform layers were usually taken from a number of assay tubes, combined and then evaporated to dryness in a distillation flask at 37° C under reduced pressure. The residue in the flask was then stored in a silica gel vacuum desiccator.

For paper chromatography the residue was dissolved in a small volume of chloroform to give a concentration equivalent to approximately 100 μg drug/ml. 0.2 ml. of this solution was delivered at a constant rate to the paper using a micrometer syringe driven by pulley from a kymograph motor. The spot size was kept small (0.5 cm) by a current of hot air directed underneath the paper from a conventional hair dryer.

The conditions of chromatography were as follows: Solvents—8 butanol (MFC):2 ethanol: 3 water:0.25 acetic acid (AR); Direction—descending; Paper—Whatman 541; Temperature —room, approximately 20° C; Time—6 hr; Length of run—approximately 40 cm; Paper drying—approximately 12 hr in fume cupboard; Developer—iodine vapour (Brante, 1949), brown spots; iodoplatinate (Munier & Macheboeuf, 1949), purple spots.

Using this method, we have found that when the bromophenol blue complex prepared from a solution of neostigmine in 0.2 M K_2HPO_4 is applied to the paper the R_F value of the spot is the same (R_F 0.5) as that for a solution of neostigmine. Similarly, R_F values for pyridostigmine and for the dissociated blue complex of this drug (R_F 0.38) are identical.

(*c*) *Collection of urine.* The urine excreted during 24 hr was collected from 12 patients with myasthenia gravis who were receiving routine treatment either with neostigmine, given intramuscularly or by mouth, or pyridostigmine by mouth. Immediately after it was voided the urine was transferred to an amber-coloured Winchester bottle containing 5 ml. of chloroform as a preservative. The volume of each cumulative 24 hr specimen was recorded. In most cases at least two 24 hr samples of urine were obtained and duplicate assays performed on each.

(*d*) *Extraction of neostigmine and pyridostigmine from urine.* The procedure described above cannot be used for the direct colorimetric estimation of these drugs in urine because of the presence of other substances which interfere with the assay. Specimens of urine containing the drugs were therefore treated as follows: 5 ml. of filtered urine was run through a 12 cm × 0.7 cm column of Amberlite IRC "50" (100 to 200 mesh B.D.H.) at *p*H 6.86 at approximately 1 ml./3 min (for details of preparation see Nowell, Wilson & Wilson, 1959). The column was washed with distilled water (15 ml.) and then eluted by 0.2 M K_2HPO_4. The first 4 ml. fraction (a) of the eluate was discarded; thereafter five 6 ml. fractions (b to f) were assayed for neostigmine or pyridostigmine by the colorimetric method. The concentration of drug in the urine was calculated from the combined results of assay of all the eluate fractions which gave a blue colour.

RESULTS

A systematic study was made of the relation between the concentration of drug and the optical density of the chloroform solution of blue colour complex in a series of experiments in which concentrations of neostigmine and of pyridostigmine ranging from 1 to 20 μg/ml. in 0.2 M K_2HPO_4 were used. Within this range all the readings were accurately reproducible. It will be evident from Fig. 1, which

summarizes these results and represents the standard calibration curves used in later work, that the sensitivity of the method for estimation of pyridostigmine is slightly less than for neostigmine.

Recovery of drugs when added to urine

Recovery of neostigmine and pyridostigmine when added to urine was investigated in a number of experiments, in which each drug was added to specimens of normal human urine. The samples of urine were then extracted by the ion exchange method described above. The lowest concentrations which could be determined were approximately 2 μg/ml. neostigmine and 3 μg/ml. pyridostigmine. The recovery of the drug when added to urine in concentrations of up to 10 μg/ml. was obtained mainly in fraction (c) of the eluate; the amounts recovered were 90 to 100% of neostigmine and 80 to 90% of pyridostigmine. When higher concentrations were used the drugs were recovered from the eluate in fractions (b), (c) and (d).

Recovery of drugs when administered to patients with myasthenia gravis

Daily excretion. The method was then used to determine if, when administered to patients with myasthenia gravis, these drugs were excreted unchanged in the urine and to verify by chromatographic analysis the identity of the bases forming the blue colour complexes.

TABLE 1

EXCRETION OF NEOSTIGMINE IN THE URINE OF PATIENTS WITH MYASTHENIA GRAVIS TREATED BY (*a*) INTRAMUSCULAR AND (*b*) ORAL ADMINISTRATION OF NEOSTIGMINE

Patient	Sex	Age	Duration of neostigmine treatment	Intramuscular daily dose (mg)	Volume of urine in 24 hr (ml.)	Neostigmine in urine		
						Concn. (μg/ml.)	Total excretion (mg)	% of daily dose
(*a*)	F	55	36 days	3·75	944	2·3	2·2	58·5
	F	55	34	6·25	842	4·5	4·0	63·8
	F	55	35	6·25	426	3·2	1·35	21·6
B.C.	F	55	28	12·5	1,303	6·4	8·4	67·2
	F	55	29	12·5	729	7·4	5·4	43·2
	F	55	30	12·5	967	5·3	5·1	40·8
	F	55	31	12·5	1,595	4·1	6·5	52·0
	F	55	32	12·5	1,330	4·4	5·8	46·4
	F	55	33	12·5	1,465	5·2	7·6	40·8
H.G.	F	55	13	12·5	243	11·1	2·7	21·6
	F	55	14	12·5	913	4·7	4·3	34·4
(*b*)				Oral daily dose (mg)				
B.C.	F	55	14 days	15	661	0	0	0
H.G.	M	38	1 day	45	2,900	0	0	0
B.C.	F	55	19 days	60	2,150	0	0	0
H.G.	M	38	2	75	2,325	0	0	0
H.R.	F	25	5	90	620	1·6	1·0	1·1
	F	25	6	90	665	1·2	0·8	0·9
[illegible].B.	M	45	36 mths	135	1,350	0	0	0
B.C.	F	55	24 days	225	532	5·1	2·7	1·2
	F	55	25	225	1,365	5·9	8·1	3·6
	F	55	26	225	910	3·5	3·2	1·4
	F	55	27	225	1,298	6·5	8·4	3·7
E.J.B.	F	60	18 mths	225	410	27·0	11·1	5·0
H.M.	F	23	3 days	240	734	5·4	3·9	1·6
	F	23	4	240	380	15·7	6·0	2·5
	F	23	25	360	654	4·9	3·2	0·9

TABLE 2

EXCRETION OF PYRIDOSTIGMINE IN THE URINE OF PATIENTS WITH MYASTHENIA GRAVIS TREATED WITH ORAL PYRIDOSTIGMINE

Patient	Sex	Age	Duration of treatment	Oral daily dose (mg)	Volume of urine in 24 hr (ml.)	Pyridostigmine in urine		
						Concn. (μg/ml.)	Total excretion (mg)	% of daily dose
A.W.	F	78	3 mths	180	510	33	17	9
	F	78	3	180	850	12	10	6
H.S.	M	53	6 weeks	720	430	172	74	10
	M	53	6	840	940	110	103	12
J.D.	M	51	6	1,800	1,600	140	224	12
	M	51	6	1,800	1,740	170	296	16
	M	51	6	1,800	1,675	145	243	14
J.F.	F	23	4½ years	2,100	2,178	150	327	16
	F	23	4½	2,100	1,979	144	285	14
	F	23	4½	2,100	1,791	171	307	15
	F	19	6 mths	2,880	1,135	222	252	9
	F	19	6	2,880	1,470	120	176	6
E.S.	F	45	19	3,600	1,120	94	105	3
	F	45	19	3,600	1,425	62	89	2
	F	45	19	3,600	1,125	125	141	4

Specimens of the 24 hr output of each patient were processed and assayed by the methods described above; duplicate assays agreed within 10%. The mean results of these estimations are summarized in Tables 1 and 2.

Excretion of neostigmine

Several estimations were made of the excretion of this drug when given by intramuscular injection to two patients. The results in Table 1 show that by this route of administration the proportion of the daily dose excreted ranged from 21.6 to 67.2%. In one patient (B.C.) who excreted about 50% of the daily dose the variation (21.6 to 67.2%) did not seem to bear any relation to the duration of treatment. In the other case (H.G.) a smaller proportion of the daily dose was excreted (21.6 to 34.4%).

By contrast, after oral administration only a small amount of the drug was excreted. When daily doses of up to 75 mg were given, no drug was detected in the urine, and, with higher doses, the maximum amount excreted was 5% of the daily dose. Since, in all cases, administration of the drug was followed by a typical therapeutic response, it is clear that these differences in excretion cannot be attributed entirely to differences in rate or extent of absorption of the drug. Indeed, from subsequent work which we have done, there is evidence that after oral administration some of the neostigmine is metabolized to its phenolic derivative, a m-hydroxyphenyltrimethylammonium salt, which is not detected by the assay procedure.

Excretion of pyridostigmine

This drug was administered only by mouth in daily doses ranging from 180 to 3,600 mg. As will be evident from Table 2, a greater proportion of the daily dose of pyridostigmine (2 to 16%) was excreted than of oral neostigmine. In general the excretion pattern of each patient is surprisingly constant, but it is clear that there

are considerable differences between patients. Thus one patient (J.D.) who received 1.8 g excreted about twice as much pyridostigmine as another (E.S.) who was given 3.6 g.

Whilst these results might suggest that there is better absorption of pyridostigmine from the alimentary tract than of neostigmine, the possibility should not be overlooked that the drugs may differ in their rate of metabolism, the products of which, especially in the case of neostigmine, are not detected by the assay procedure. Furthermore, the drugs may differ in their rate of excretion. Indeed, there is some evidence that pyridostigmine is relatively slowly excreted. This was demonstrated in two patients whose treatment with this drug was completely stopped. Fig. 2

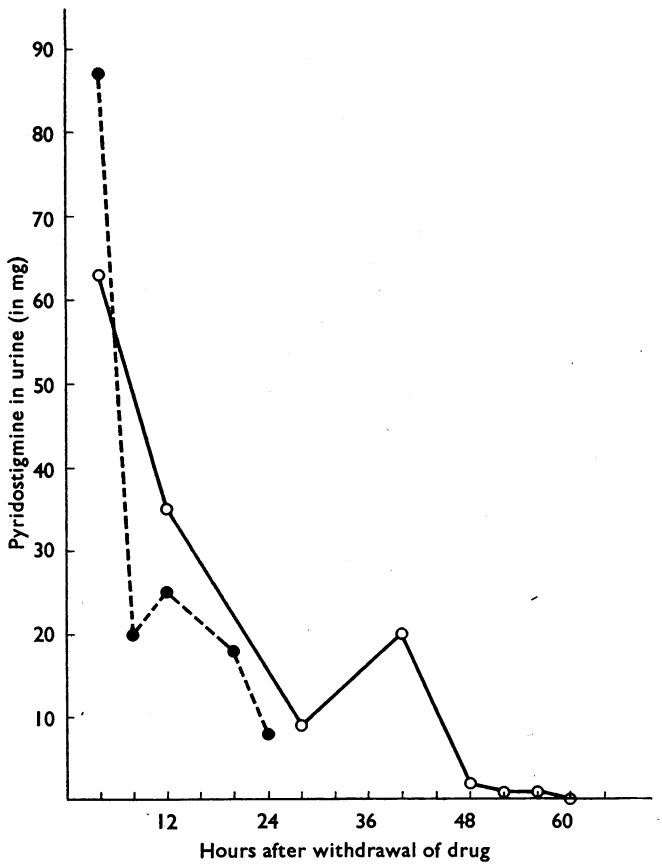

Fig. 2. Continued excretion of pyridostigmine in the urine of two patients (B.C. ○——○; H.G. ●– – –●) after treatment with the drug had been withdrawn. The points on the graph show the amount of drug excreted during respective intervals of time.

shows that excretion of pyridostigmine continued for at least 2 days after administration had ceased. Paper chromatography of the bromophenol blue complex confirmed in each case that the excretion product was pyridostigmine.

Chromatography

When the blue colour complexes used in these quantitative estimations were submitted to chromatographic analysis by the method described above, there was separation in each case of a spot identical in R_F value with that of neostigmine and pyridostigmine respectively (Fig. 3). This, and other evidence to be mentioned later, supports the conclusion that the results of the assays can be attributed to the presence of unchanged drug in the urine.

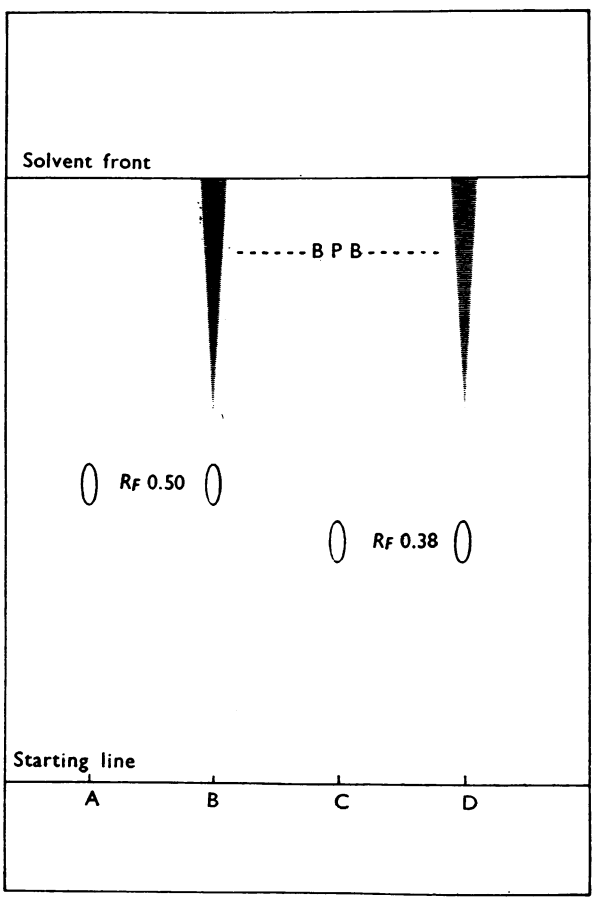

Fig. 3. Chromatogram spots obtained with: (A) neostigmine; (B) bromophenol blue complex from urine of a patient treated with neostigmine; (C) pyridostigmine; (D) bromophenol blue complex from urine of a patient treated with pyridostigmine. BPB—area of bromophenol blue dye dissociated from the complexes.

DISCUSSION

The results of this investigation have shown that it is possible to detect neostigmine and pyridostigmine in the urine of patients with myasthenia gravis receiving treatment with these drugs. The evidence that this method only estimates the amount of

unchanged drug in the urine is supported by the results of paper chromatography. Additional support for this conclusion has been obtained by further studies, to be published later, in which eluted chromatograms were assayed for anticholinesterase activity and by experiments using paper electrophoresis.

One of the most interesting aspects of these results is the observation that, whilst a high proportion of the dose of neostigmine is excreted after intramuscular injection, only a small amount is detected in urine after oral administration. The reason for this is probably that after oral administration neostigmine is metabolized to its m-hydroxyphenyltrimethylammonium salt. This compound cannot be estimated by our method because it does not form a complex with bromophenol blue ; work is at present in progress to investigate this metabolic pathway.

Although it would seem from our results that pyridostigmine is better absorbed than neostigmine from the alimentary tract, there is some evidence that the former is slowly excreted, so that cumulation and delayed excretion of previous doses may complicate the interpretation of the daily output. On this account, and because the method of assay cannot be used to distinguish between neostigmine and pyridostigmine, we have found it important to ensure that, for precise quantitative estimations of the proportion excreted of a dose of either drug, any patient who has been treated with pyridostigmine should have this drug withheld for at least four days prior to the test.

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