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THE DETERMINATION OF PHYSOSTIGMINE BY THIN-LAYER CHROMATOGRAPHY AND ULTRAVIOLET SPECTROPHOTOMETRY*

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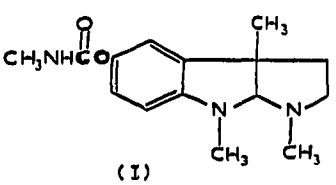
SUMMARY

Physostigmine has been separated from its degradation products by thin-layer chromatography on alumina, with chloroform–acetone (5:4) as the solvent for development. The alkaloid was eluted with methanolic hydrochloric acid and determined by ultraviolet spectrophotometry. Two methods were used for the correction of irrelevant absorbance: a differential method in which absorbance measurements were made at three wavelengths, and a method in which orthogonal functions were applied to absorbance measurements at a set of nine wavelengths.

INTRODUCTION

Physostigmine (I) inhibits the activity of cholinesterase and is used in ophthalmology as a miotic and to decrease intra-ocular pressure in glaucoma; for this purpose it is usually instilled into the eye as an aqueous solution containing up to 1% of physostigmine salicylate or sulphate. In aqueous solution, physostigmine hydrolyses to form a colourless phenolic compound, eseroline (II); this compound is subsequently oxidised to rubreserine (III) and other coloured compounds. The anticholinesterase activity of the drug resides in the methylcarbamate side-chain.



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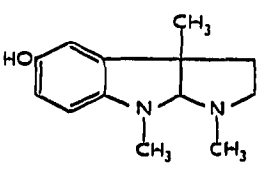

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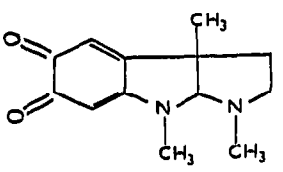

(III)

* This work forms part of a thesis submitted by G. Smith for a Ph.D. degree of the Heriot-Watt University.

Berg[1] separated physostigmine (R_F=0.74) from eseroline (R_F=0.51) and rubreserine (R_F=0.55) by thin-layer chromatography (TLC) on silica gel with chloroform–acetone–33% (w/v) dimethylamine in ethanol (5:4:1) as the solvent for development. The physostigmine was eluted with 0.1 *N* sodium hydroxide and the rubreserine formed by hydrolysis and oxidation was determined colorimetrically at 480 nm. Berg attempted to elute the alkaloid with various organic solvents but the recoveries were low.

The method of Berg was criticised by Smith[2] who reported that rubreserine reacted with dimethylamine within 10 min to form a yellow product with an R_F (0.73) close to that of physostigmine. Smith separated physostigmine (R_F=0.61–0.67) from eseroline (R_F=0.42–0.45) and rubreserine (R_F=0.33–0.36) by TLC on alumina, with chloroform–acetone (5:4) as the solvent for development. At a high or a low relative humidity, the R_F value for rubreserine was less than 0.1 and "tailing" was appreciable. It was found necessary to store the alumina plates over a saturated solution of sodium bromide (relative humidity=58%) for 3 days before use. After separation of physostigmine from its degradation products, Smith determined the alkaloid by direct-reflectance spectrophotometry; the coefficient of variation of 25 spots on 5 plates was 5.81%.

The objective of the present work was to develop an elution technique for the determination of physostigmine from thin-layer chromatograms.

MATERIALS AND EQUIPMENT

Materials

Acetone, B.S. 509; alumina G (Type E), Merck; chloroform, AnalaR; hydrochloric acid, laboratory reagent grade; methanol, spectroscopic grade; physostigmine sulphate, B.P.C.; sodium bromide, laboratory reagent grade. Methanolic hydrochloric acid, B.P.C., 1968, Appendix 7.

Equipment

Centrifuge, Simplex (Martin Christ). Micrometer Syringe, Agla (Burroughs Wellcome). Spectrophotometers, S.P. 500 and S.P. 800 (Pye Unicam), with a matched pair of 1-cm silica cells. Whirlimixer (vortex mixer) (Fisons Scientific Apparatus).

Calculations were made with the aid of a desk-top computer, the Programma 101 (British Olivetti).

EXPERIMENTAL AND RESULTS

Development of the elution method

Alumina was spread in 0.25-mm layers on glass plates (20 × 20 cm) and activated for 1 h at 110°; the plates were stored over a saturated solution of sodium bromide for 3 days. To each plate, three 20-μl samples of a 0.5% aqueous solution of physostigmine sulphate were applied as short streaks, by means of a micrometer syringe. After development with chloroform–acetone (5:4) and location of the spots under screened ultraviolet (UV) radiation at 366 nm, areas (4 × 2 cm) of the alumina containing the physostigmine were removed with a razor-blade and transferred into 10 ml of the solvent in a test-tube; the contents of the tube were mixed for 2 min in a vortex

mixer and clarified by centrifuging (1100 × *g*) for 5 min. The absorbance of the decanted solution was measured from 220 to 360 nm. A spectrophotometric blank solution was prepared by removing a blank area (4 × 2 cm) of the adsorbent at a location corresponding to the R_F value of physostigmine, and treating this blank adsorbent in a similar manner to the adsorbent containing the drug. The difference between the absorbance at 244 nm (maximum) and that at 267 nm (minimum) was taken as a measure of the amount of physostigmine.

In preliminary experiments, four solvents were used to elute physostigmine. Of these solvents, water (recovery 40–64%), 0.1 *N* hydrochloric acid (recovery 58–87%), and methanol (recovery 61–84%) all gave results that were low and erratic. The solvent of choice was methanolic hydrochloric acid for which the recovery of physostigmine was 89–103%.

A possible source of variation in the absorbance of eluted drug, especially at wavelengths lower than 250 nm, was variation in the absorbance of the blank. In an attempt to reduce this irrelevant absorbance, plates were prepared with alumina that had been washed first with chloroform–acetone (5:4), then with methanol, and finally with boiling water to remove traces of the organic solvents. The alumina was then filtered through sintered glass and washed repeatedly with cold water before preparation of the plates. The results of this treatment are given in Table I.

The treatment of the alumina considerably reduced the absorbance of the blank but the results were still variable. In an attempt to further reduce the absorbance of the blank, various membrane filters were used to clarify the solution (Spencer and Beggs[3]) but these filters were unsatisfactory. Millipore GS (cellulose esters), Celotate (cellulose acetate), and Duralon (nylon) were all attacked by the methanolic hydrochloric acid. The pore size of Polyvic (polyvinyl chloride) and Mitef (PTFE) was too large for the removal of the alumina particles. Repeated centrifuging of the eluted solution did not reduce the absorbance of the blank.

Consideration was then given to the possibility that variation in the absorbance of the blanks could be due partly to impurities from the solvent used for development of the plates. The absorbance of blanks at locations corresponding to the R_F of physostigmine was determined for three developed plates and for three undeveloped plates. The mean absorbances at 221, 244 and 267 nm are given in Table II.

From the results in Table II, there was no evidence that the solvent used for

TABLE I

EFFECTS OF WASHING THE ALUMINA ON THE ABSORBANCE OF BLANK SOLUTIONS

Treatment of alumina	*Area on plate*	*Absorbance*		
		221 nm	*244 nm*	*267 nm*
Not washed	1	0.127	0.085	0.052
	2	0.086	0.063	0.044
	3	0.055	0.041	0.033
Washed	1	0.012	0.008	0.006
	2	0.043	0.036	0.025
	3	0.027	0.010	0.007

TABLE II

EFFECTS OF DEVELOPMENT OF THE PLATES ON THE ABSORBANCE OF BLANK SOLUTIONS

Development of plates	*Plate*	*Mean absorbance*		
		221 nm	*244 nm*	*267 nm*
Not developed	1	0.041	0.030	0.011
	2	0.045	0.031	0.008
	3	0.031	0.024	0.006
Developed	1	0.030	0.024	0.007
	2	0.042	0.029	0.008
	3	0.037	0.030	0.010

development affected the absorbance of the blank. If soluble impurities from the solvent or the alumina had affected the absorbance of the blank, the absorbance would have been expected to vary in accordance with the location of the area on the plate (Shellard and Alam[4]); there was no evidence that the absorbance of the blank was affected by the location on the plate.

Since variation in the absorbance of the blank could not be eliminated and did not appear to depend upon the location on the plate, it was decided that in further experiments the results should be calculated by a procedure based upon that of Gänshirt and Morianz[5].

Determination of the accuracy and reproducibility of the elution method

Preparation of plates. Use a magnetic stirrer to stir 50 g of alumina with 100 ml of chloroform and 80 ml of acetone for 15 min. Filter through sintered glass, dry in the air and stir with 200 ml of methanol for 15 min. Boil the alumina with 200 ml of distilled water for 1 h, filter, and wash the residue repeatedly with distilled water. Prepare a slurry by adding 20 ml of distilled water to the washed alumina and spread 20 × 20 cm plates to a thickness of 0.25 mm. Dry the plates in the air and activate by heating at 110° for 1 h. Store the plates for 3 days over a saturated solution of sodium bromide.

Solvent for development. Chloroform–acetone (5:4).

Distance run by solvent. 10 cm.

Method. Apply as short streaks three 20-μl samples of a 0.5% aqueous solution of physostigmine sulphate to each plate. Develop the plate in the solvent in a glass tank lined with filter-paper (Whatman No. 1) saturated with the solvent. After development, dry the plate in the air and locate the spots by examination under screened UV radiation at 366 nm. Remove with a razor-blade areas (4 × 2 cm) of the alumina containing the physostigmine and transfer by means of aluminium foil (6 × 4 cm) into 10 ml of methanolic hydrochloric acid in a test-tube. Mix the contents of the tube for 2 min in a vortex mixer and clarify the solution by centrifuging for 5 min. Measure the absorbance at 221, 244 and 267 nm against a blank of methanolic hydrochloric acid. Since the wavelength of maximum absorbance lies midway between the two wavelengths of minimum absorbance, calculate the absorbance of physostigmine by subtracting half the sum of the absorbance at 221 and 267 nm from the absorbance

at 244 nm. Measure the absorbance at 221, 244 and 267 nm of a 0.001% solution of physostigmine sulphate in methanolic hydrochloric acid, and calculate the recovery of physostigmine for each sample.

This method was applied to three 20-μl samples of a 0.5% solution of physo-

TABLE III

ABSORBANCE OF PHYSOSTIGMINE SULPHATE DETERMINED BY ELUTION FROM THIN-LAYER CHROMATOGRAMS*

Plate	*Area on plate*	*Absorbance of physostigmine sulphate* $A = A_{244\ nm} - 0.5\,(A_{221\ nm} + A_{267\ nm})$	*Recovery of physostigmine* (%)
A	1	0.346	101.8
	2	0.344	101.2
	3	0.345	101.5
B	1	0.332	97.6
	2	0.315	92.6
	3	0.324	95.3
C	1	0.335	98.5
	2	0.342	100.6
	3	0.340	100.0
D	1	0.342	100.6
	2	0.321	94.4
	3	0.355	104.4
E	1	0.332	97.9
	2	0.318	93.5
	3	0.321	94.4
F	1	0.315	92.6
	2	0.310	91.2
	3	0.312	91.8

* Absorbance of 0.001% physostigmine sulphate in methanolic hydrochloric acid = 0.340; mean absorbance of eluted physostigmine = 0.330; mean recovery of eluted physostigmine = 97.2%; coefficient of variation = 4.2% (17 degrees of freedom).

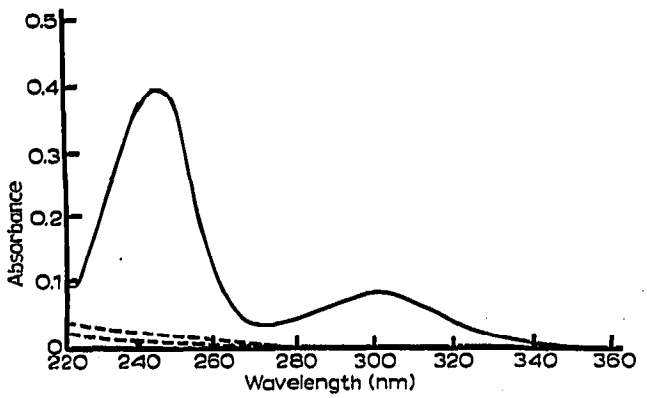

Fig. 1. Plot of absorbance against wavelength for a 0.001% solution of physostigmine sulphate and for two blank solutions. ——, Physostigmine sulphate; – – –, blanks.

stigmine sulphate on each of six plates. The results are given in Table III. The absorbance from 210 to 360 nm of a 0.001% solution of physostigmine sulphate and that of typical blanks are illustrated in Fig. 1.

From these results variation in recovery of the drug was relatively high between plates but lower between areas on the same plate. The elution of similar weights of alumina rather than similar areas also gave a relatively high variation in recovery of the drug from different plates. It was decided that in further work a standard solution should be applied to each plate and the recovery of drug calculated from the absorbance of this standard solution.

Confirmation of the validity of the Lambert-Beer law for the elution method

The elution method was applied to three 20-μl samples of a 0.1% aqueous solution of physostigmine sulphate on one plate, together with one 20-μl sample of a standard solution containing 0.5% of physostigmine sulphate. The experiment was repeated on 0.2, 0.3, 0.4 and 0.5% solutions of physostigmine sulphate together with the standard solution. For each sample solution, the mean absorbance, based on 3 wavelengths, was corrected for recovery of the drug by reference to the absorbance of the standard solution applied on the same plate. The results are given in Table IV.

TABLE IV

ABSORBANCE OF 0.1 TO 0.5% SOLUTIONS OF PHYSOSTIGMINE SULPHATE DETERMINED BY THE ELUTION METHOD

Concentration of physostigmine sulphate (%)	*Mean absorbance*	*Mean absorbance, corrected for recovery on the plate*
0.1	0.053	0.060
0.2	0.100	0.123
0.3	0.188	0.200
0.4	0.246	0.261
0.5	0.327	0.339

By regression analysis, the slope of the calibration graph of absorbance (A) as a function of percentage concentration (c) was calculated to be 0.679. The standard error of the slope was 0.014. The equation for the regression of absorbance upon concentration was represented by:

$$A = 0.679\ c - 0.005$$

In a further experiment, it was shown by two-dimensional TLC that no decrease in content of physostigmine occurred after chromatography in the second dimension.

Correction of irrelevant absorbance by application of orthogonal functions

In the method used in the previous experiment for calculation of the absorbance due to physostigmine it was assumed that the graph of the absorbance of the blank against wavelength was linear. For the blanks examined, the graph was approximately linear but curved very slightly at wavelengths below 240 nm.

As an alternative method of correction for irrelevant absorbance of the blank,

attempts were made to apply orthogonal functions (Glenn[6]). The aim of preliminary experiments was to choose the orthogonal polynomial, number and intervals of wavelengths and the mean wavelength so that the contribution of the absorbance of the physostigmine was a maximum and the contribution of the absorbance of the blank was a minimum.

The absorbance of a 0.001% solution of physostigmine sulphate in water was measured at 1-nm intervals from 215 to 350 nm with a manual spectrophotometer. The absorbances of blank solutions eluted from alumina were also measured.

Choice of polynomial range and number of wavelengths. Since the shapes of the absorbance curves of physostigmine sulphate and of the blank solution were not complex, it was considered that a small number of wavelengths would be adequate for correction of irrelevant absorbance. From the results of preliminary experiments, nine wavelengths over the range 221–267 nm were chosen.

Calculations of the coefficients (p_0, p_1, p_2, p_3, p_4 and p_5) of the orthogonal functions (polynomials P_0, P_1, P_2, P_3, P_4 and P_5) were made for a 0.001% solution of physostigmine sulphate and for two blank solutions. Tables of orthogonal polynomials by Fisher and Yates[7] were used. To illustrate the method of calculation details of the results and calculations are given in Table VI in respect of the coefficients p_0 and p_2 of the physostigmine sulphate solution and one blank solution. The ratios (percentages) of the coefficients (p_0, p_1, p_2, p_3, p_4 and p_5) for the two blank solutions in relation to the coefficients for the solution of physostigmine sulphate are given in Table V.

TABLE V

RATIOS (PERCENTAGES) FOR THE COEFFICIENTS OF ORTHOGONAL FUNCTIONS FOR TWO BLANK SOLUTIONS IN RELATION TO THE COEFFICIENTS FOR A 0.001% SOLUTION OF PHYSOSTIGMINE SULPHATE

Blank solution	$\frac{p_i \text{ blank} \times 100}{p_i \text{ physostigmine sulphate}}$					
	p_0	p_1	p_2	p_3	p_4	p_5
"A"	30.31	26.98	0.04	7.74	0.03	16.31
"B"	28.30	25.92	0.97	9.05	0.99	11.42

From these results, the contribution of the irrelevant absorbance of the blanks is reduced to less than 1% if the coefficient p_2 or p_4 is calculated for the orthogonal function P_2 or P_4. Of these two functions, P_2 was chosen for further work because the calculated coefficient p_2 (6.00) for a 1% solution of the drug was higher than the coefficient p_4 (2.65).

Choice of mean wavelength and intervals of wavelength. The coefficients p_2 for the 0.001% solution of physostigmine sulphate and for a blank solution were calculated for mean wavelengths of 235 to 255 nm, in sets of nine wavelengths at intervals of 5 nm. Convoluted absorbance curves of the p_2 coefficient against mean wavelength are illustrated in Fig. 2.

TABLE VI

CALCULATION OF p_0 AND p_2 COEFFICIENTS OF ORTHOGONAL FUNCTIONS FOR A 0.001% SOLUTION OF PHYSOSTIGMINE SULPHATE AND FOR BLANK SOLUTION "A"

Wavelength (nm)	*Polynomials*		*Physostigmine sulphate, 0.001% solution*			*Blank solution "A"*		
	P_0	P_2	*Absorbance A*	*Products*		*Absorbance A*	*Products*	
				$A \cdot P_0$	$A \cdot P_2$		$A \cdot P_0$	$A \cdot P_2$
226	1	28	0.124	0.124	3.472	0.083	0.083	2.324
231	1	7	0.197	0.197	1.379	0.081	0.081	0.567
236	1	− 8	0.292	0.292	−2.336	0.077	0.077	−0.616
241	1	−17	0.380	0.380	−6.460	0.072	0.072	−1.224
246	1	−20	0.390	0.390	−7.800	0.063	0.063	−1.260
251	1	−17	0.287	0.287	−4.879	0.056	0.056	−0.952
256	1	− 8	0.143	0.143	−1.144	0.050	0.050	−0.400
261	1	7	0.045	0.045	0.315	0.046	0.046	0.322
266	1	28	0.029	0.029	0.812	0.044	0.044	1.232
Sums of products (coefficient)				1.887	−16.641		0.572	−0.007
Normalising factor				1	2772		1	2772
Normalised coefficient				1.887 (p_0)	0.00600 (p_2)		0.572 (p_0)	0.00000253 (p_2)

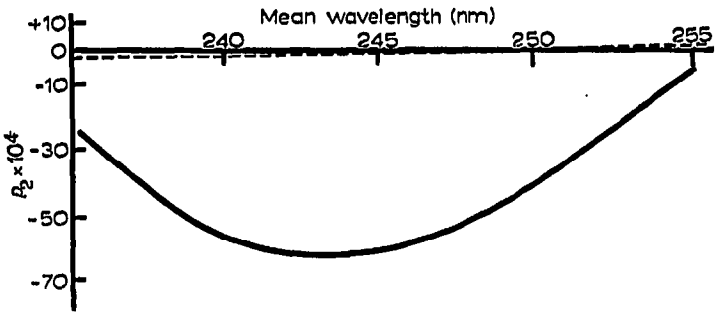

Fig. 2. Plot of p_2 coefficient against mean wavelength of the set of nine wavelengths, for a 0.001% solution of physostigmine sulphate and for a blank solution. ——, Physostigmine sulphate; – – –, blanks.

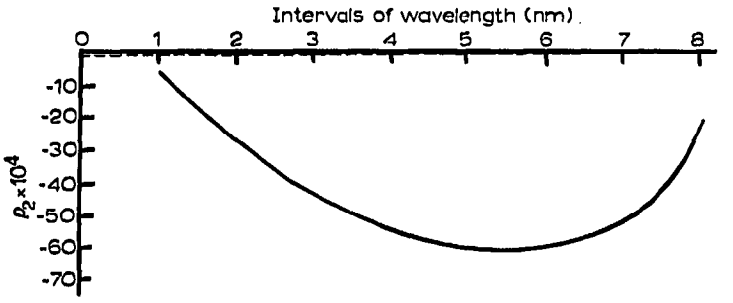

Fig. 3. Plot of p_2 coefficient against intervals of wavelength for the set of nine wavelengths, for a 0.001% solution of physostigmine sulphate. ——, Physostigmine sulphate; – – –, blanks.

Since the contribution of the p_2 coefficient of the blank solution was a minimum for a mean wavelength of 246 nm, that wavelength was chosen for further work. The coefficient p_2 was then calculated for intervals of 1 to 8 nm in sets of nine wavelengths of mean 246 nm. A convoluted absorbance curve of the p_2 coefficient against interval of wavelength is illustrated in Fig. 3.

From these results, 5 nm was chosen as the interval of wavelength since the p_2 coefficient of the solution of physostigmine sulphate was a maximum at that interval of wavelength. For each interval the values of the p_2 coefficients of the two blank solutions were less than 1% of the coefficients of the physostigmine sulphate solution.

Determination of the accuracy and reproducibility of the elution method by application of orthogonal functions

Orthogonal functions (P_2) were applied to the absorbance at 226, 231, 236, 241, 246, 251, 256, 261 and 266 nm of the eluted solutions obtained in the experiment for determination of the accuracy and reproducibility of the elution method (Table III).

The normalised coefficients (p_2) were calculated and the results are given in Table VII.

Confirmation of the validity of the Lambert-Beer law for the elution method by application of orthogonal functions

Orthogonal functions (P_2) were applied to the absorbances at 226, 231, 236, 241,

246, 251, 256, 261 and 266 nm of the eluted solutions obtained in the experiment for confirmation of the validity of the Lambert-Beer law (Table IV).

The normalised coefficients (p_2) were corrected for recovery of the drug on the plate. The results are given in Table VIII.

By regression analysis the slope of the calibration graph of the p_2 coefficient (y)

TABLE VII

COEFFICIENTS OF ORTHOGONAL FUNCTIONS OF PHYSOSTIGMINE SULPHATE DETERMINED BY ELUTION FROM THIN-LAYER CHROMATOGRAMS*

Plate	*Area on plate*	*Normalised p_2 coefficient of physostigmine sulphate $\times 10^4$*	*Recovery of physostigmine* (%)
A	1	−60.14	100.2
	2	−60.50	100.8
	3	−61.22	102.0
B	1	−61.62	102.6
	2	−55.09	91.8
	3	−56.96	94.9
C	1	−58.33	97.2
	2	−59.27	98.7
	3	−60.35	100.5
D	1	−59.74	99.5
	2	−56.85	94.7
	3	−60.75	101.2
E	1	−58.15	96.9
	2	−55.81	93.0
	3	−55.92	93.1
F	1	−55.55	92.5
	2	−55.09	91.8
	3	−54.76	91.2

* $p_2 \times 10^4$ of 0.001% physostigmine sulphate in methanolic hydrochloric acid = −60.02; mean normalised coefficient × 10^4 of eluted physostigmine = −58.10; mean recovery of eluted physostigmine = 96.8%; coefficient of variation = 4.1% (17 degrees of freedom).

TABLE VIII

COEFFICIENTS OF ORTHOGONAL FUNCTIONS FOR 0.1 TO 0.5% SOLUTIONS OF PHYSOSTIGMINE SULPHATE DETERMINED BY THE ELUTION METHOD

Concentration of physostigmine sulphate (%)	*Mean p_2 coefficient $\times 10^4$*	*Mean p_2 coefficient $\times 10^4$, corrected for recovery on the plate*
0.1	− 9.31	−10.14
0.2	−19.41	−23.92
0.3	−33.95	−36.36
0.4	−44.84	−47.98
0.5	−57.83	−60.55

as a function of percentage concentration (c) was calculated to be -124.9. The standard error of the slope was 2.08. The equation for the regression of the p_2 coefficient upon concentration was represented by:

$$y = -124.9c + 1.7$$

DISCUSSION

Orthogonal functions were calculated for the absorbance at nine wavelengths over the range 226–266 nm of the eluted solutions obtained in the experiment for determination of the accuracy and reproducibility of the elution method. Thus the absorbances in Table III can be compared directly with the p_2 coefficients in Table VII. There is no significant difference between the mean results for recovery of physostigmine (97.2% and 96.8%), and the coefficients of variation of the means are of similar magnitude (4.2% and 4.1%).

The reproducibility of the elution method appears to be slightly better than that of the direct-reflectance method[2] (coefficient of variation, 5.81%) but the technique of elution is laborious and slow. Both methods give more reproducible results than the gas chromatographic method of Teare and Borst[8] for physostigmine salicylate (coefficient of variation, 11.5%).

The application of orthogonal functions to the elution method has provided an alternative means for the correction of irrelevant absorbance in the eluted solutions but the results are no better than those obtained by application of the method of Gänshirt and Morianz[5]. Since the graph of the absorbance of the blank against wavelength curves very slightly it might be expected that the method of Gänshirt and Morianz, in which linearity is assumed, would incur greater errors. There are, however, several possible sources of error in the application of orthogonal functions to the determination of physostigmine sulphate.

A common source of error in all spectrophotometric methods is an overall shift in the wavelength-scale of the spectrophotometer. In the application of orthogonal functions for correction of irrelevant absorbance, this error depends upon the slope of the convoluted absorbance curve of the coefficient as a function of the mean wavelength of the set. Examination of Fig. 2 shows that a shift of $+0.5$ nm from the mean wavelength of 246 nm results in a 1.8% decrease in the p_2 coefficient; a shift of -0.5 nm results in a 2.3% increase in the p_2 coefficient.

Errors may also be incurred in setting the wavelength for measurement of absorbance, especially where there is a steep slope in the absorbance curve. Thus errors are more likely to occur in the measurement of the absorbance of physostigmine sulphate at 236 and 256 nm, where the slopes are steep, than at the peak at 246 nm (Fig. 1).

The precision of the coefficient of the orthogonal polynomial may also affect the results. In a contribution by Glenn, quoted by Wahbi[9], the use of "comparative coefficients" is suggested as a rough guide to the precision of coefficients of the orthogonal polynomials. The comparative coefficient, $|q_i|$, is represented by:

$$|q_i| = p_i \cdot N_i^{\ddagger},$$

where N_i is the normalising factor.

The coefficient of variation of the comparative coefficient, c.v. q_i is represented by:

$$\text{c.v. } |q_i| = \frac{100\, s(A)}{|q_i|},$$

where $s(A)$ is the estimated standard deviation of the absorbance values; it is assumed to be constant for the set of wavelengths.

Glenn has suggested a "rule of thumb" that can be used for estimation of the precision of any coefficient for any number of wavelengths. Where the mean wavelength of the set corresponds to a maximum or minimum in the convoluted absorbance curve, $s(A)$ is assumed to be 1.4×10^{-3}. If the coefficient of variation is to be less than 1%, then the comparative coefficient $|q_i|$, must exceed 0.14. Where the mean wavelength of the set does not correspond with a maximum or minimum, $s(A)$ is assumed to be 6.3×10^{-3}. Under these conditions, the comparative coefficient, $|q_i|$, must exceed 0.63 if the coefficient of variation is to be less than 1%.

In the determination of a 0.001% solution of physostigmine sulphate, the comparative coefficient, $|q_2|$, is 0.32. Since the mean wavelength of the set (246 nm) does not correspond with the maximum of the convoluted absorbance curve at 243 nm, $|q_2|$ should exceed 0.63 if the coefficient of variation is to be less than 1%. If it is assumed that $s(A)$ is 6.3×10^{-3}, then the estimated coefficient of variation is about 2%.

In the present work, Glenn's "rule of thumb" appears to provide a realistic estimate of the precision of the coefficient of the orthogonal polynomial.

The apparent complexity of the mathematical theory of orthogonal functions has obscured the simplicity of the calculations used in their application for the correction of irrelevant absorbance. The method merits further trial in the spectrophotometric analysis of drugs eluted from thin-layer chromatograms, especially with the increasing use of calculators and desk computers that enable the calculations to be completed within a few minutes.

REFERENCES

1 B. H. Berg, *Acta Pharm. Suecica*, 3 (1966) 209.
2 G. Smith, *Proc. Soc. Anal. Chem.*, 8 (1971) 66.
3 R. D. Spencer and B. H. Beggs, *J. Chromatogr.*, 21 (1966) 52.
4 E. J. Shellard and M. Z. Alam, *J. Chromatogr.*, 32 (1968) 472.
5 H. Gänshirt and K. Morianz, *Arch. Pharm. (Berlin)*, 293 (1960) 1065.
6 A. L. Glenn, *J. Pharm. Pharmacol.*, 15 (1963) 123T.
7 R. A. Fisher and F. Yates, *Statistical Tables for Biological, Agricultural and Medical Research*, Oliver & Boyd, Edinburgh, 6th ed., 1963.
8 F. W. Teare and S. I. Borst, *J. Pharm. Pharmacol.*, 21 (1969) 277.
9 A. M. Wahbi, *Ph. D. Thesis*, University of London, London, 1967.