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

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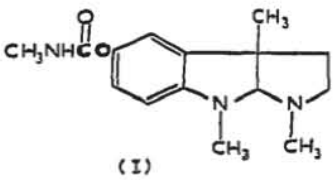
SUMMARY

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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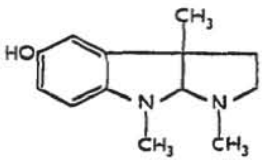

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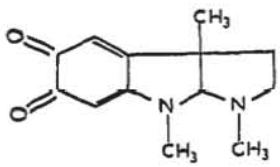

(III)

* 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).

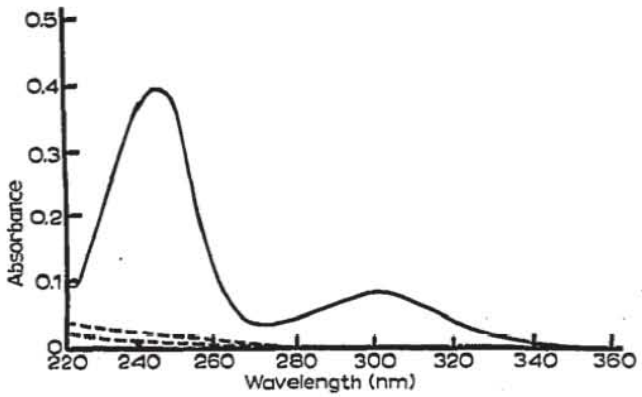

Fig. 1. Plot of absorbance against wavelength for a 0.001% solution of physostigmine sulphate and for two blank solutions. ——, Physostigmine sulphate; – – –, blanks.

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