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Quantitative determination of physostigmine from aqueous solution

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A gas chromatographic method is described for the direct determination of physostigmine base from a solution of physostigmine salicylate. The method involves isolation of physostigmine salicylate from an aqueous solution by a freeze drying process, subsequent conversion to a trimethylsilyl (TMS) derivative and gas liquid chromatography. The trimethylsilyl salt dissociates on injection into the flash vapourizer inlet of a gas chromatograph, and the physostigmine–TMS and salicylic acid–TMS derivatives are eluted separately. Due to the specificity of the silylation reaction it is possible to determine by gas chromatography the alkaloid content in the presence of its degradation products. A modification of the method was necessary for the assay of a commercial formulation containing certain additives.

A gas chromatographic method has been developed to estimate physostigmine from aqueous solutions of physostigmine salicylate. Physostigmine from fresh or degraded solutions can be determined with good sensitivity and with greater specificity than in many other procedures, the most useful of which have been listed (Teare & Taylor, 1967; Taylor, 1967). The methods are not entirely satisfactory because of the lack of specificity for physostigmine in the presence of possible decomposition products (Ellis, 1943) or of salicylate.

The proposed method requires the removal of water from aqueous solutions of physostigmine salicylate by a freeze drying process, and subsequent silylation of the salt residue to the corresponding physostigmine trimethylsilyl (TMS) derivative. The physostigmine–TMS derivative obtained is then gas chromatographed directly without prior liberation of the base. By this procedure it is possible to measure quantitatively intact physostigmine obtained from an aqueous solution of its salicylate salt.

Gas chromatography of alkaloidal salts of pharmaceutical interest, including those of physostigmine has been reported by Brochmann-Hanssen & Baerheim Svendson (1962). Few attempts have been reported for the gas chromatography of complex carbamates or corresponding trimethylsilyl derivatives. Some trimethylsilyl pesticidal carbamates have been examined qualitatively by gas chromatography (Fishbein & Zielinski, 1965).

EXPERIMENTAL

Reagents. Analar pyridine distilled and stored over molecular sieves (Linde Air Products Co., type 4A). Bis(trimethylsilyl)acetamide (BSA) (Pierce Chemical Co., Illinois). Physostigmine salicylate B.P. (B.D.H. Canada). Physostigmine base (Mann Research Laboratories, N.Y.). Physostigmine salicylate—0·25% w/v (Esromiotin) (Crookes-Barnes Laboratories, Inc., Wayne, New Jersey).

Apparatus and operating conditions. Samples were freeze dried using a Vertis Unitrap Automatic Freeze-Dryer.

A Beckmann GC-4 gas chromatograph, with a hydrogen flame ionization detector and a Beckmann 10-inch laboratory potentiometric recorder fitted with a disc chart integrator, Model 236 (Disc Instruments Inc.) were used. The chromatographic columns were glass U-tubes (4 ft × 3 mm, i.d.), packed with silicone gum rubber (3·8% SE-30) on Diatoport S, 80–100 mesh (Hewlett-Packard). The columns were operated under the following conditions: oven temperature 145°; injection flash vapourizer inlet 200°; air 350 ml/min, H_2 50 ml/min and N_2 93 ml/min.

Procedure. Samples of either fresh or partially degraded solutions (having intense red colorations) of about 0·5% w/v physostigmine salicylate were used. A 0·2 ml aliquot of either solution was transferred to a micro tube (1 × 7·5 cm) and the sample freeze dried. Dry pyridine (5 μl) and bis(trimethylsilyl)acetamide (10 μl) were added to the residue and the silylating solution was allowed to stand for 1 h before injecting 1·2 μl into the flash vapourizer inlet of the chromatograph.

Calculation of results

The areas under the peaks due to physostigmine–TMS and salicylic acid–TMS derivatives obtained with assay samples are calculated and compared with the areas obtained by gas chromatography of a known (standard) physostigmine salicylate solution similarly prepared. The weight (W_{up}) in grams of physostigmine in the sample is calculated from the following equation:*

$$\frac{W_{up}}{275{\cdot}35} = \frac{1}{R_1} \cdot \frac{A_{us}}{A_{ks}} \cdot \frac{W_{ks}}{138{\cdot}12} \cdot \frac{1}{k}$$

where: *M* for salicylic acid = 138·12; *M* for physostigmine base = 275·35; A_{us} = peak area of salicylic acid–TMS from physostigmine salicylate of *unknown* concentrations; A_{ks} = peak area of salicylic acid–TMS from physostigmine salicylate of *known* concentration; W_{ks} = known weight in grams of salicylic acid present in a known physostigmine salicylate solution; R_1 = ratio of peak areas of salicylic acid–TMS to physostigmine–TMS from the *unknown* physostigmine salicylate solution; k = ratio of peak areas of physostigmine–TMS to salicylic acid–TMS from the *known* physostigmine salicylate solution (freshly prepared).

RESULTS AND DISCUSSION

Preliminary work on the gas chromatography of 0·5% w/v physostigmine base or salt in a solution of acetone revealed 2 or more peaks, depending on the conditions, which reflected decomposition on the column (Brochmann-Hanssen & Baerheim Svendson, 1962). This decomposition was overcome by conversion of the physostigmine moiety of the salt to the trimethylsilyl (TMS) derivative which chromatographs to give a single peak. Conversion of physostigmine base and the salicylate trimethylsilyl derivatives was attempted with conventional silylating reagents. The method of Fishbein & Zielinski (1965) for the silylation of carbamates was tried but the reaction was incomplete. We found bis-(trimethylsilyl)acetamide (Klebe, Finkbeiner & White, 1966) to react rapidly with physostigmine base in the presence of pyridine. Evidence of a more stable derivative was demonstrated by only one peak in the gas chromatogram. Using the same conditions, physostigmine salicylate was silylated. The physostigmine salicylate–TMS apparently dissociates in the flash heater unit at 200°, and the

* Photo copies of the derivation of this formula are available from the Editorial Department, Journal of Pharmacy and Pharmacology, 17 Bloomsbury Square, London, W.C.1.

physostigmine and salicylic acid moieties were eluted as their TMS derivatives (Fig. 1A). No difference in retention time could be observed whether physostigmine–TMS was prepared from the salt or the free base. Similarly the TMS derivatives of the salicylate moiety of this salt and salicylic acid have identical retention volumes. Good resolution for the TMS derivatives of both moieties of the physostigmine salicylate was achieved with a minimum of tailing (Fig. 1A) on the column used.

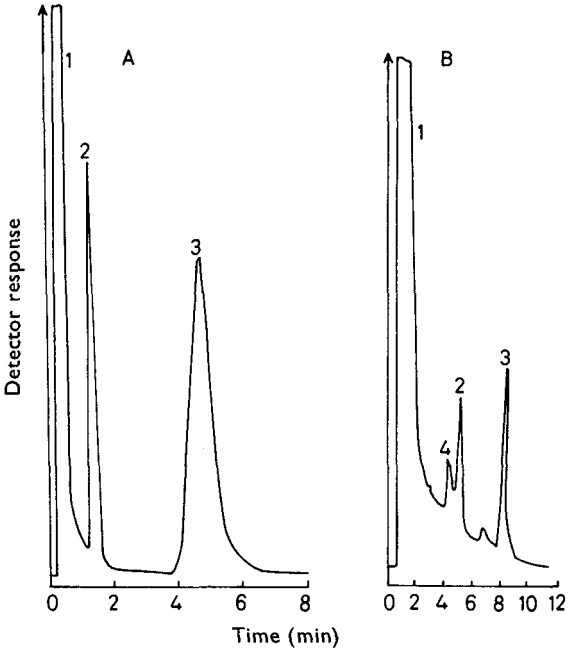

FIG. 1. A. Silylated derivatives of freeze-dried physostigmine salicylate, chromatographed under isothermal conditions. (1) Solvent peak. (2) Salicylic acid–TMS peak (attenuation 2×10^4). (3) Physostigmine–TMS peak (attenuation 2×10^3).

B. Temperature programmed chromatogram of a freeze-dried silylated commercial sample of physostigmine salicylate containing additives.
Conditions: 100° isothermal for 0·6 min, 100° to 150° at 10·3°/min, 154° isothermal for 3·25 min. Other parameters given under earlier isothermal conditions. (1) Solvent peak. (2) Salicylic acid–TMS peak. (3) Physostigmine–TMS peak. (4) Thiomersal peak.

Dissociation of physostigmine salicylate during the silylation reaction would appear not to be possible because pyridine (pK_a 5·12), the solvent for the silylation reaction, will not promote dissociation of the salicylate salt as it is a weaker base than physostigmine (pK_a 6·12 and 12·24). Moreover Brochmann-Hanssen & Baerheim Svendson (1962) reported that dissociation of some alkaloidal salts of organic acids including physostigmine salts always occurs in the flash heater.

In partially degraded aqueous solutions, specificity for physostigmine was satisfactory because the decomposition products, such as rubreserine, eserine blue, eserine brown, will not gas chromatograph, possibly owing to their non-volatility, irreversible adsorption to column material or failure to form TMS derivatives. There was no evidence of the formation of a trimethylsilyl ether of eseroline; this might be expected owing to its rapid oxidation to further decomposition products (Ellis, 1943).

The unknown concentration of the physostigmine moiety in solutions of physostigmine salicylate could be determined by direct comparison of the peak areas of the

unknown and known physostigmine–TMS derivatives. However, the first method of calculating the physostigmine used in this paper takes advantage of the greater reproducibility of the salicylate–TMS derivative peak areas, which enhances the overall precision of the assay.

It must be emphasized that only in freshly prepared solutions of physostigmine salicylate are the salicylic acid and physostigmine moieties on a 1 : 1 molar basis. Whereas the salicylic acid portion remains constant with time in *all* solutions, in the degraded solutions, some physostigmine decomposition product(s) could be also associated with the salicylic acid moiety. However, of these alkaloidal substances *only* the physostigmine forms a TMS derivative which gives a peak on the chromatogram, giving specificity to the method described here.

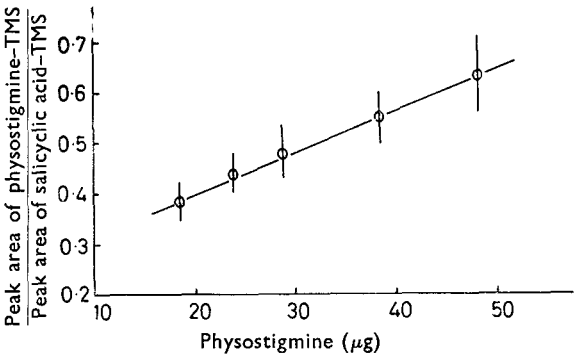

FIG. 2. Linear relation between the ratio of peak areas of physostigmine–TMS and salicylic acid–TMS derivatives and the concentration of physostigmine in known simulated mixtures.

The calibration curve (Fig. 2) is rectilinear for physostigmine–TMS over the range 15–48·4 μg. This calibration curve was constructed by preparing simulated mixtures of salicylic acid and physostigmine base which include those quantities found in the physostigmine salicylate preparations being assayed. The salicylic acid content was kept constant while increasing known amounts of physostigmine base were added to the standards before silylation and subsequent gas chromatography employed for samples of the assay preparation of physostigmine salicylate. The linearity of detector response to increasing amounts of physostigmine salicylate from a freshly prepared solution was demonstrated by the constancy of the ratio of the physostigmine–TMS to salicylic acid–TMS peak areas when increasing volumes of the silylated sample were injected.

Table 1. *Assays of physostigmine solutions I on 5 consecutive days, II on the same day, III on the same day after partial degradation by refluxing for 6 h*

	No. of determinations	Assays made over:	Physostigmine concn (% w/v base ± s.d.) Initial	Found	Found
I	5	5 consecutive days	0·334	0·355 ± 0·041*	0·349 ± 0·045†
II	4	1 day	0·334	0·315 ± 0·031**	0·306 ± 0·038†
III	4	1 day	0·333	0·121 ± 0·009**	0·124 ± 0·008†

* Mean of 6 reference and 6 sample injections.
** Mean of 3 reference and 3 sample injections.
† Results from 1 reference and 1 sample injection.

The results obtained by applying the proposed method to various fresh and degraded solutions are summarized in Table 1. Routine analysis over several days gave a precision of ± 11·5%. Determinations made on the same day show a precision of ± 7 to 10% (Table 1). Assays based on determinations corresponding to single injections of sample and reference show a lower precision. Physostigmine could be detected at about 1·5 μg but quantitative work was not possible in this region. This is most likely due to a combination of factors, irreversible adsorption of the TMS derivative becomes significant at this concentration level, and background noise with baseline drift appears at the high sensitivity settings of the instrument.

Application to stability studies of both salicylic acid and physostigmine present in aqueous solution

Work was initiated to investigate the stability of the salicylic acid moiety of physostigmine salicylate. We hoped to verify the use of the salicylic acid portion of the salt as an internal standard for the assay of the physostigmine moiety in stored solutions of physostigmine salicylate, by showing that the former remained constant throughout the pharmacologically-active life of this salt solution.

The salicylic acid residue of physostigmine salicylate solutions was found by colorimetry and by gas chromatography to remain constant (Table 2) over 5 weeks storage at room temperature. This suggests the salicylic acid portion of the salt could be used as the internal standard for the assay of the physostigmine moiety in the same aqueous solution.

Table 2. *The stability of the salicylic acid moiety of physostigmine salicylate in one solution over the test period*

Physostigmine salicylate stored at 23° (days)	Salicylic acid concentration found in physostigmine salicylate solutions (% w/v) (initial concn 0·169%)		Physostigmine concentration found in physostigmine salicylate solution (% w/v)
	Colorimetric*	GLC†	GLC‡
0	0·170	—	—
2	0·170	0·167	0·326
6	0·172	—	—
9	0·177	0·161	0·333
16	0·178	0·173	0·351
39	0·173	—	—

* Average of 3 determinations from the same sample.
† Average of 6 determinations from the same sample used for the colorimetric assay.
‡ No specific colorimetric or spectrophotometric method is available for the physostigmine moiety in its salts or for the free base.
The GLC method reported for the physostigmine has a precision of about ± 11·5% (see text).

During the 5 week period, aqueous solutions of physostigmine salicylate developed a pink coloration. But such pink solutions do not necessarily reflect significant amounts of decomposition of physostigmine in aqueous solution as estimated by using the salicylic acid moiety as an internal standard. This supports the findings of other workers (Hellberg, 1949).

A modified assay was made on a commercial formulation (Esromiotin-1/4, Crookes-Barnes Laboratories, Inc., Wayne, N.J., U.S.A.) containing physostigmine 0·25% w/v, in boric acid solution with sodium metabisulphite 0·1% and thiomersal 0·01%. Using isothermal conditions, the peak corresponding to the thiomersal overlaps that of

the salicylic acid–TMS derivative. Tailing of the solvent peak is caused by the presence of boric acid which produces significant base line drift and affects the reproducibility of the physostigmine–TMS peak. This base line drift was reduced by temperature programming (Fig. 1B). Using the conditions in Fig. 1B, the physostigmine contents of a commercial preparation was successfully determined. It was calculated first by using the equation previously given in which the ratio of the salicylic acid–TMS peak areas is used, and secondly by considering the ratio of the physostigmine–TMS peak areas of the reference and unknown samples only. The partial overlap of the thiomersal and salicylic acid–TMS peaks (Fig. 1B) appears not to interfere in the estimation of physostigmine content (% w/v) when this is calculated using the ratio of the known and unknown salicyclic acid–TMS peak areas (mean $0{\cdot}160 \pm 0{\cdot}008$, $n = 5$). Calculations based solely on the ratio of the known and unknown peak areas of physostigmine–TMS (mean $0{\cdot}164 \pm 0{\cdot}012$ $n = 5$) also show close agreement with the labelled strength of this commercial preparation.

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