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HPLC DETERMINATION OF PHYSOSTIGMINE IN THE PRESENCE OF DEGRADATION PRODUCTS AND PRESERVATIVES

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ABSTRACT

A reversed phase, ion-pairing liquid chromatographic method was developed for the analyses of physostigmine-containing pharmaceuticals in the presence of its decomposition products, eseroline and rubreserine, and preservatives methyl- and propylparaben. A satisfactory resolution for the peaks for these compounds was obtained with this isocratic method. All peaks were eluted in less than 12 minutes. The capacity factors of these compounds ranged from 1.50 to 9.27. The linearity and repeatability of this method were examined. The CV's of retention times of all compounds ranged from 0.87 to 1.81%, and the CV of physostigmine peak areas was 2.17%.

A gradient-elution, ion-pairing HPLC method was developed for the analyses of pharmaceutical preparations containing physostigmine, eseroline, rubreserine, and benzyl alcohol as an antibacterial agent. The capacity factors of these compounds ranged from 2.75 to 10.51. The CV of retention times ranged from 0.79 to 1.27%. This method is also suitable for the simultaneous quantitation of benzyl alcohol.

INTRODUCTION

Physostigmine is a cholinesterase inhibitor and has been used in the treatment of glaucoma for the reduction of intraocular tension. It is used also as an antidote in the treatment of anticholinergic intoxication. Ophthalmic solutions and injections of physostigmine salts may contain preservatives. Methylparaben and propylparaben were reported to be the preservatives in physostigmine-containing

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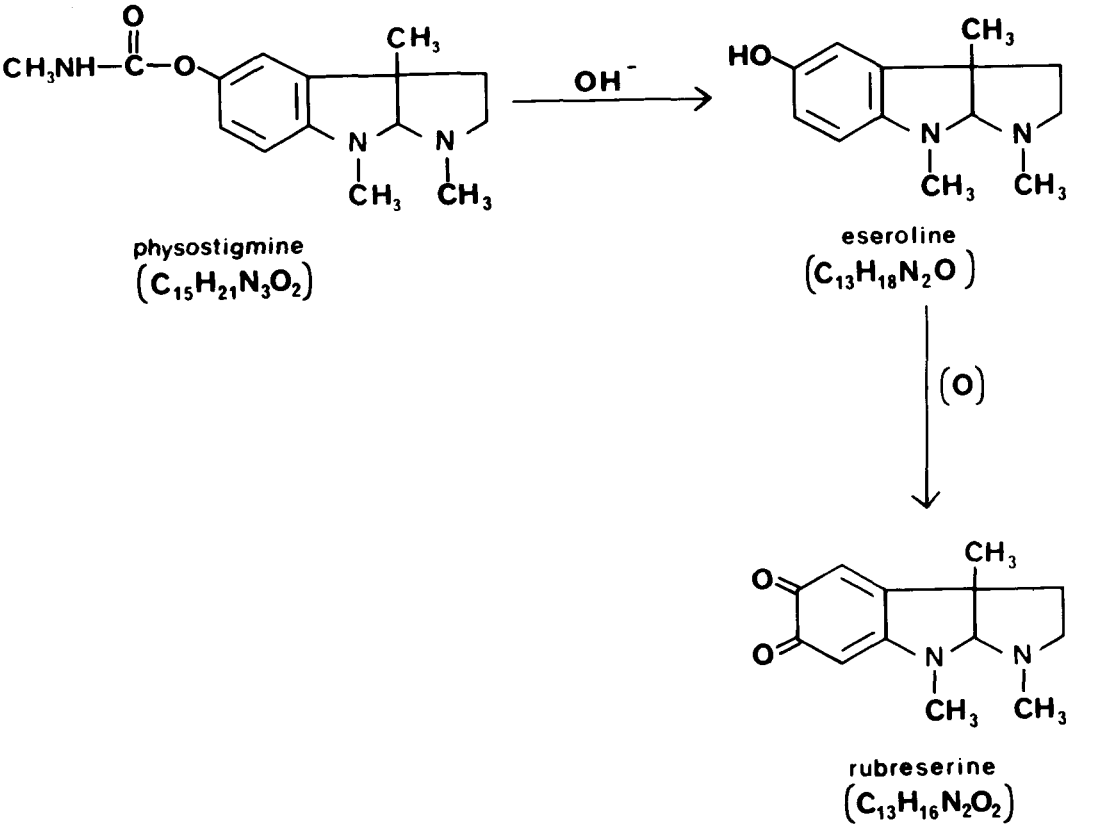

FIGURE 1
Decomposition of physostigmine.

ophthalmic solutions (1). Benzyl alcohol, a preservative often found in parenterals (2), is used in the physostigmine-containing injectables listed in PDR (3).

The stability of physostigmine in solution has been studied by various authors (4-10). Some results obtained by different workers are not in agreement, this may be due to the unreliability or nonspecificity of the analytical methods employed in some of the early studies. An acceptable analytical method should be demonstrated to be stability-indicating, which means separating and detecting the intact drug in the presence of its decomposition products and other components in the preparation. The failure to do so is the most common flaw in stability studies (11).

The decomposition products of physostigmine were reported to be eseroline, rubreserine, shown in Figure 1, and other less known compounds produced through

further oxidation of rubreserine (12-14). Separation and determination of physostigmine in the presence of other interfering compounds by HPLC methods should be simpler and more accurate than most other analytical methods reported. Using HPLC, Yamazoe et al. (15) separated physostigmine from rubreserine and an unknown decomposition compound. Somani et al. (16) separated physostigmine from eseroline. Whelpton (17) separated physostigmine from rubreserine but resolution between physostigmine and eseroline was not good. Yang et al. (18) separated physostigmine from eseroline and rubreserine with adequate resolution. Kneczke (1) separated physostigmine from pilocarpine, rubreserine, methylparaben and propylparaben, but the total elution requires 26 minutes.

The objective of this study is to develop the HPLC procedures for detecting physostigmine in the presence of eseroline, rubreserine, and preservatives in a reasonable time period. Both the isocratic and gradient elution methods were employed.

MATERIALS AND METHODS

Equipment - The liquid chromatograph was a modular system consisting of a Micromeritics (Norcross, GA) Model 750 Solvent Delivery System and a Model 752 Gradient Programmer, a Rheodyne (Cotati, CA) Model 7125 injector, a Varian (Walnut Creek, CA) Model UV-50 detector, a Varian Model CDS 111 data system and a Varian Model 9176 recorder. The injected samples were measured with a 20 μl loop on the injector.

Reagents and Chemicals - Sodium Phosphate Monobasic USP was obtained from Mallinckrodt (St. Louis, MO). Sodium salt of 1-heptanesulfonic acid (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade), ammonium acetate (certified A.C.S.), benzyl alcohol (certified) and benzaldehyde (certified) were purchased from Fisher Scientific (Fairlawn, NJ). Sodium salt of 1-octanesulfonic acid was purchased from Kodak (Rochester, NY). Glacial acetic acid of HPLC grade was obtained from J.T. Baker (Phillipsburg, NJ). Physostigmine Salicylate USP and Propylparaben NF were obtained from Amend (Irvington, NJ). Methylparaben USP was from Ruger (Irvington, NJ). Physostigmine sulfate was obtained from Sigma (St. Louis, MO). Double distilled water was used for the preparation of all aqueous solutions.

Eseroline and rubreserine were prepared in this laboratory according to the methods of Ellis (19) and were confirmed by melting points and elemental (C,H,N) analyses (Atlantic Microlab, Inc., Atlanta, GA). The absorption spectrum for rubreserine coincided with that reported by Ellis (19).

Chromatographic Conditions - A column (250 x 4.1 mm) of reversed phase Versapack C_{18} packing (particle size 10 μm) obtained from Alltech Assoc. (Deerfield, IL), preceded by a guard column (70 x 2.1 mm) containing a similar packing but with 40 μm particle size (Whatman, Clifton, NJ), was used for all separations. The detector was set at 247 nm which is λ_{max} for physostigmine. The flow rate was 2 ml/min and the recorder chart speed was 0.5 cm/min.

Isocratic, ion-pairing elution was employed for the simultaneous separation of physostigmine, eseroline, rubreserine, methylparaben and propylparaben. The mobile phases were prepared by mixing aqueous solutions containing monobasic sodium phosphate and sodium heptanesulfonate of various strengths with methanol at different ratios, and degassed under vacuum. The linearity of the detector response was examined by injecting 20 μl each of solutions of known concentrations of physostigmine. Linear regression was performed on the peak areas counted by the integrator versus the amounts of physostigmine injected. The correlation coefficient thus obtained indicates the closeness of the fit of the points to a straight line. The repeatability of the physostigmine peak areas was determined from 6 replicates of the injections of the same amount of physostigmine. The repeatability of the retention times of all compounds was also examined similarly through replication of the injections and the calculation of coefficient of variability (CV).

The mobile phase for the gradient elution consisted of Solvent A and Solvent B. Solvent A was prepared by mixing an aqueous solution of 0.01M monobasic sodium phosphate and 0.03M sodium heptanesulfonate with acetonitrile (70:30 v/v). Solvent B was prepared by mixing a similar aqueous solution with acetonitrile (85:15 v/v). The aqueous solutions and acetonitrile were degassed under vacuum separately prior to mixing. Solvent A and B were further degassed by sonication and stirring. The initial condition for gradient elution was 100% of Solvent B, the final condition was 100% of Solvent A. The linear gradient was 0-100% of Solvent A in 10 minutes.

RESULTS AND DISCUSSION

Isocratic Elution - The chromatographic conditions reported by Hsieh et al. (20) were employed in early preliminary work but the physostigmine peak shape thus obtained was not satisfactory. Therefore, ion-pairing techniques were included in this study. Such techniques are widely used for the separation of organic ions in reversed-phase procedures (21).

The mobile phase consisting of 0.005M sodium octanesulfonate, 0.008M monobasic sodium phosphate and 1% v/v acetic acid in a mixture of water and methanol (60:40),

as reported by Somani et al. (16), was tested for the separation of physostigmine from its decomposition products and the preservatives. The relatively low pH of such mobile phase (about 3.1) resulted in a peak of salicylic acid from physostigmine salicylate, since salicylate is predominantly non-protonated at this pH. This peak overlaps with the peaks of rubreserine and methylparaben. The sodium salt of heptanesulfonic acid was tested also under the same conditions to determine the effects of chain length of counterion on k′ (capacity factor). The results are shown in Table 1 and Figure 2.

The capacity factor, k′, is calculated as:

$$k' = \frac{t_R - t_0}{t_0} \qquad \text{(Eq. 1)}$$

where t_R is the retention time of the peak of interest and t_0 is the retention time of the peak of uracil, a nonretained compound.

Increasing the chain length of the counterion by one carbon almost doubled the retention times for eseroline and physostigmine because of the increased hydrophobicity of the ion pairs. The other compounds do not form ion-pairs and their k′ values were only slightly affected. Figure 2 indicates that both chromatographic conditions were suitable for determining physostigmine, but unsuitable for simultaneous determination of the preservatives. Our preliminary findings showed that methylparaben did decompose in aqueous solution at ambient temperature. Therefore, it may be important for the assay method to separate and quantify the preservatives. Further investigations in this work were directed at this goal.

The effects of acetic acid (1% v/v) in the mobile phase on the retention of these compounds were examined. Great reductions in k′ were observed for both physostigmine and eseroline in the presence of acetic acid in the mobile phase, and resulted in somewhat less resolution than the same mobile phase without acetic acid. The results are shown in Table 2 and Figure 3.

Adding 1% (v/v) acetic acid into the mobile phase reduced the pH from 4.8 to 3.1. Both eseroline and physostigmine are predominantly protonated at these two pH levels. Changes in their retention times under these conditions cannot be attributed to the pH change of the mobile phase. The reduction in their retention times as indicated by k′ values may be due to the competition of acetate with heptanesulfonate for ion-pair formation with eseroline and physostigmine. Such competitive effects were discussed by Karger et al. (22).

TABLE 1

Effects of Chain Length of Ion-Pairing Agents in Mobile Phase[a] on k′ Values

Compounds	k′	
	octanesulfonate	heptanesulfonate
rubreserine	0.38	0.21
salicylate	0.38	0.36
methylparaben	0.54	0.55
eseroline	1.01	0.49
physostigmine	2.02	1.17
propylparaben	4.18	3.84

[a]0.005M NaH_2PO_4, 0.005M ion-pairing agent and 1% v/v acetic acid in a solvent mixture of water and methanol (60:40 v/v).

TABLE 2

Effects of 1% v/v Acetic Acid in Mobile Phase[a] on k′ Values

Compounds	k′	
	with acetic acid	without acetic acid
rubreserine	0.26	0.31
salicylate	0.49	0
methylparaben	0.65	0.68
eseroline	1.58	1.97
physostigmine	1.31	4.47
propylparaben	4.24	4.35

[a]Mixtures of aqueous solution of 0.005M NaH_2PO_4, 0.005M sodium heptanesulfonate, and methanol (60:40 v/v), with or without 1% v/v acetic.

TABLE 3

Effects of Concentration of Methanol in Mobile Phase[a] on k′ and R_S

Methanol %	k′		R_S
	physostigmine	propylparaben	
45.0	5.13	6.83	1.06
42.5	6.83	8.72	1.18
40.0	8.81	11.45	1.00

[a]The aqueous portion of the mobile phase contained 0.005M NaH_2PO_4 and 0.05M sodium heptanesulfonate.

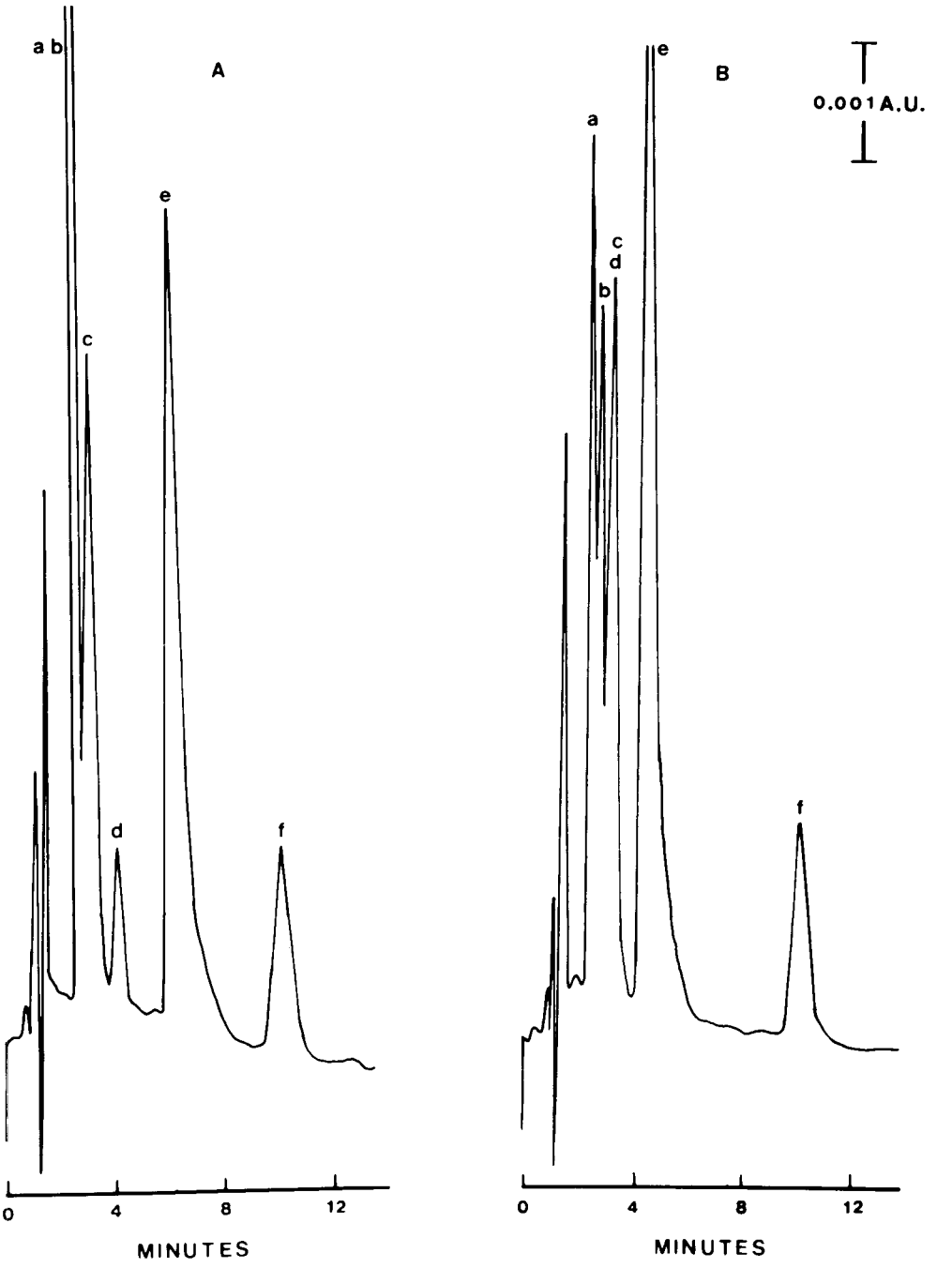

FIGURE 2

A, Sodium salt of octanesulfonic acid as the counterion in the mobile phase; B, sodium salt of heptanesulfonic acid as the counterion in the mobile phase. Peaks: a, rubreserine; b, salicylic acid; c, methylparaben; d, eseroline; e, physostigmine; f, propylparaben.

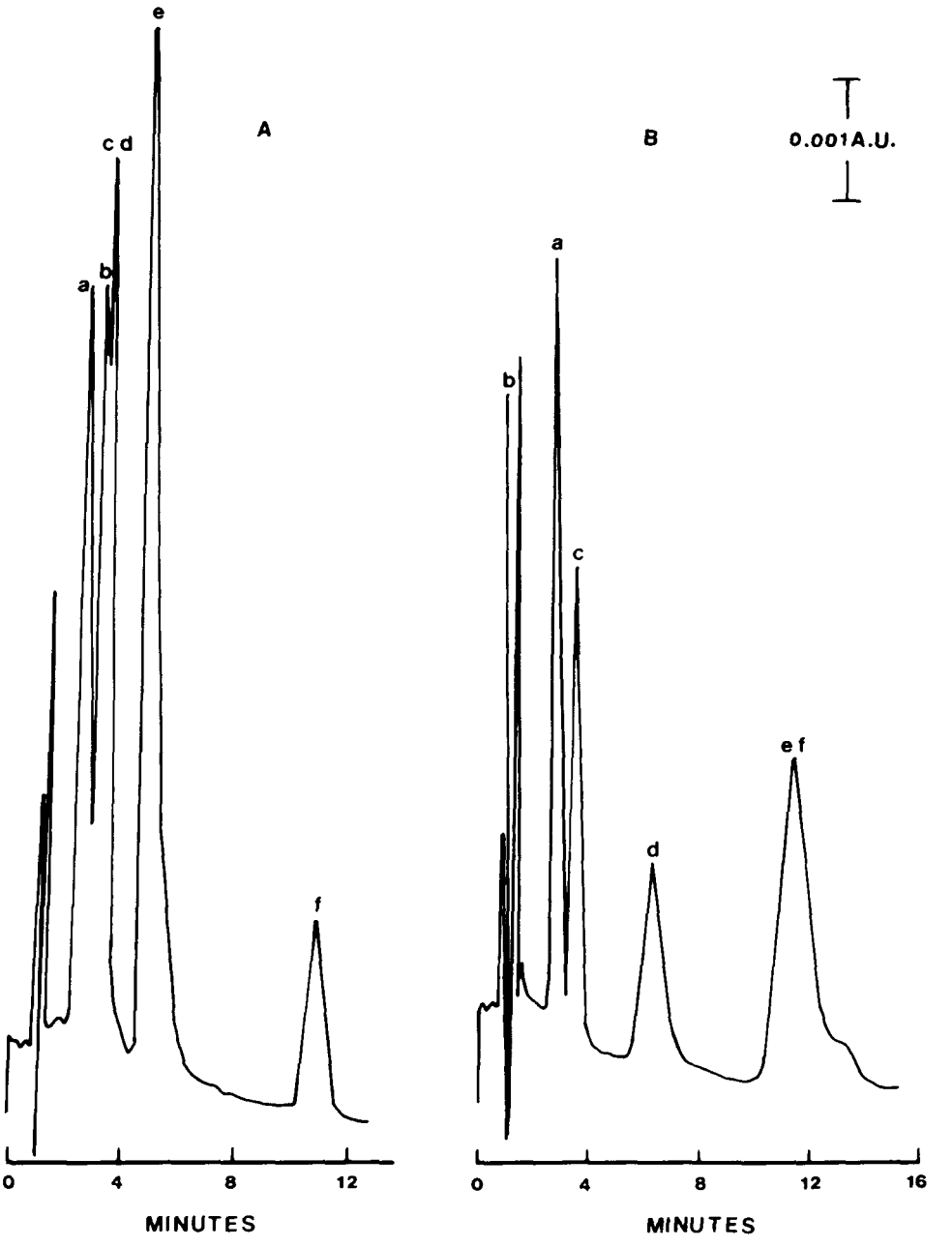

FIGURE 3

A, The mobile phase contained 1% v/v acetic acid; B, a similar mobile phase without acetic acid. Peaks: a, rubreserine; b, salicylic acid; c, methylparaben; d, eseroline; e, physostigmine; f, propylparaben.

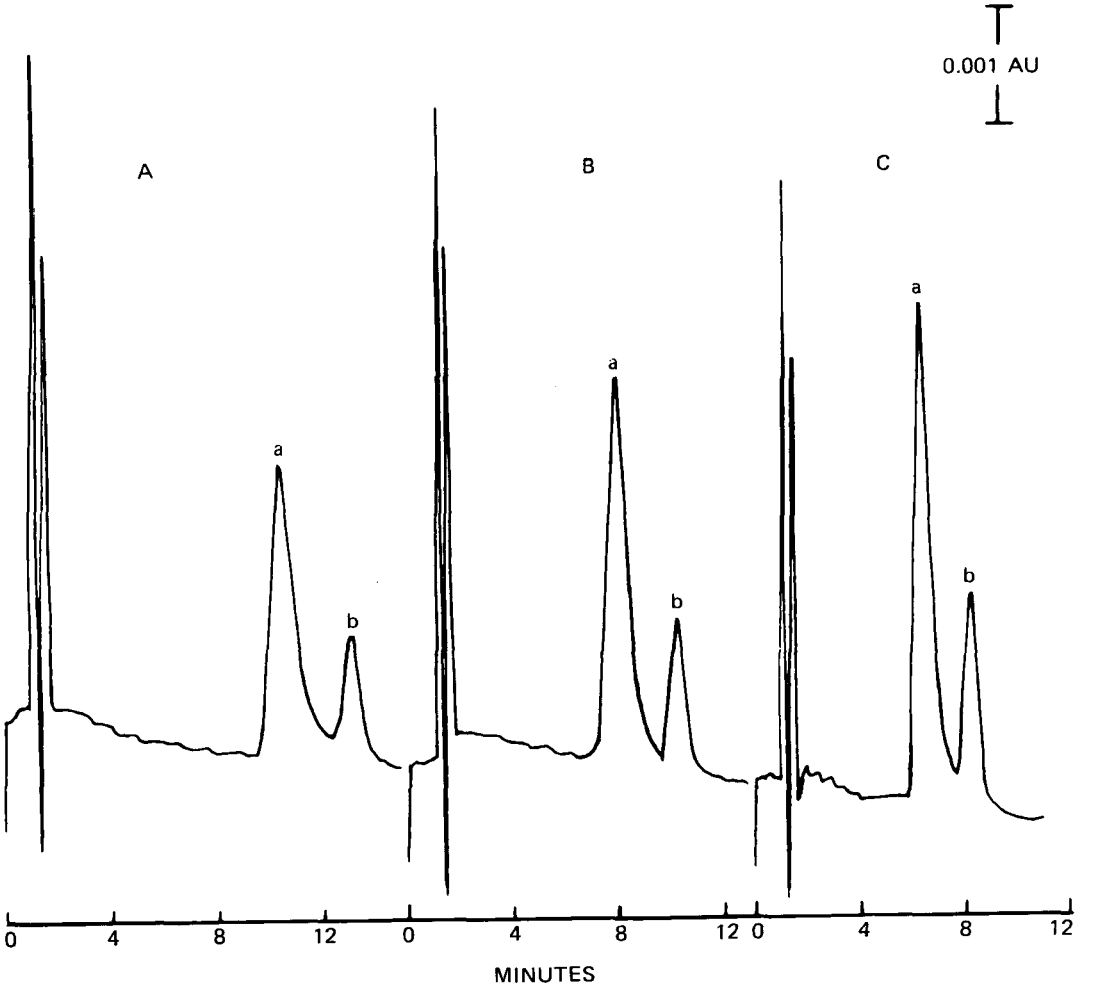

FIGURE 4

A, The mobile phase contained 40% methanol; B, the mobile phase contained 42.5% methanol; C, the mobile phase contained 45% methanol. Peaks: a, physostigmine; b, propylparaben.

As mentioned by Karger et al. (23), manipulation of the concentration and type of organic phase can be a significant control on selectivity. Methanol was chosen because the salts are more soluble in methanol than most other organic solvents. Attempts of optimizing the resolution by adjusting methanol concentrations were made and the results are shown in Table 3 and Figure 4. The resolution (R_S) is calculated as:

$$R_S = \frac{t_2 - t_1}{1/2(t_{w1} + t_{w2})} \qquad \text{(Eq. 2)}$$

where t_1 and t_2 are the retention times for compounds 1 and 2, t_{w1} and t_{w2} are the peak widths at base in terms of time for compounds 1 and 2. In view of these results, a methanol concentration of 42.5% was selected for continued studies.

The effects of counterion concentration on the retention of physostigmine are shown in Table 4. The rapid decrease in retention with increasing counterion concentrations may be due to the formation of micelles or agglomerates from the counterion as suggested by Knox et al. (24). Therefore, it was decided that the counterion concentration not exceed .02M. Further modification of the salt concentrations were conducted in order to improve resolution. The mobile phase consisting of an aqueous solution of 0.015M each of monobasic sodium phosphate and sodium salt of heptanesulfonic acid, and methanol (57.5:42.5 v/v) resulted in the greatest R_S value (1.50). A chromatogram of the simultaneous separation of physostigmine from its degradation compounds, methylparaben and propylparaben, by this mobile phase is shown in Figure 5. The elution of all compounds was completed within 12 minutes.

The presence of rubreserine, in addition to physostigmine, in an aqueous solution of physostigmine sulfate stored at room temperature for five months after its preparation was detected under similar chromatographic conditions. No other peaks were detectable.

The linearity of the detector response to physostigmine was determined by linear regression and a very high correlation coefficient of 0.9994 was obtained. The repeatability of the physostigmine peak area was expressed as CV for 6 replicate injections. CV was calculated as:

$$CV = \frac{S}{Y} \qquad \text{(Eq. 3)}$$

where S is standard deviation and Y is the mean value. The CV for physostigmine peak areas was 2.17%. The k′ and the CV for retention times are shown in Table 5.

Gradient Elution - Attempts at simultaneous separation of physostigmine from eseroline, rubreserine and benzyl alcohol by isocratic elution were not successful because of the wide range of relative retention or the capacity factors (kg values) of these compounds. A mobile phase with low solvent strength is necessary in order to separate the peaks for benzyl alcohol and rubreserine. But such a solvent will result in a prolonged retention time and broadened eluting band for physostigmine.

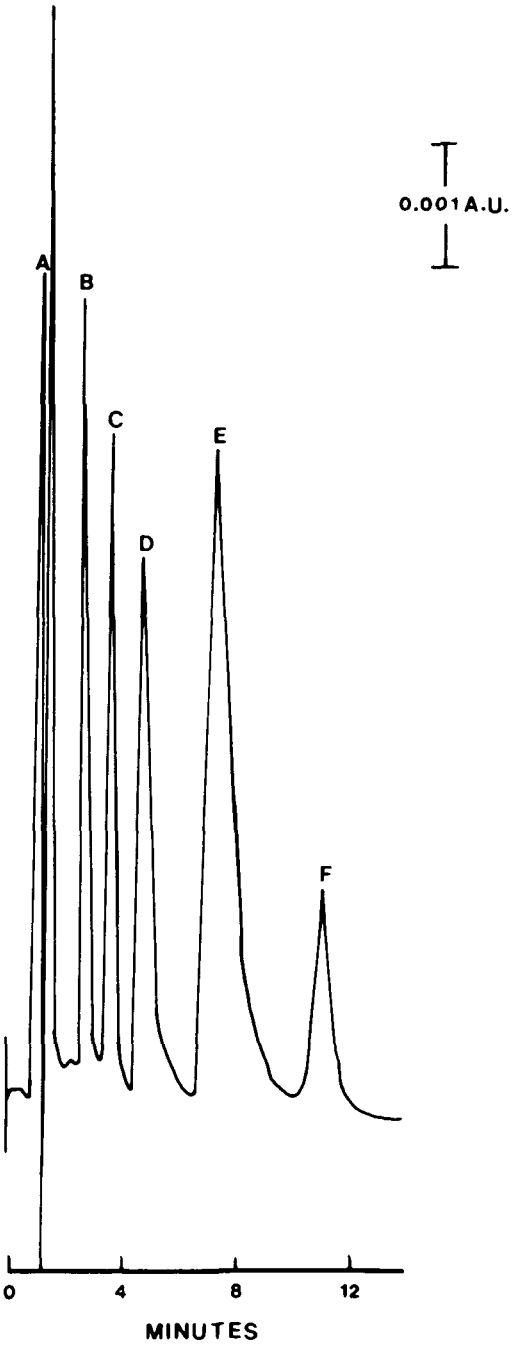

FIGURE 5

Separation of a mixture of physostigmine salicylate 20 μg/ml, rubreserine 20 μg/ml, eseroline 8 μg/ml, methylparaben 0.1 μg/ml and propylparaben 0.1 μg/ml. Detection: UV, 247 nm. Injection volume: 20 μl. Peaks: A, salicylic acid; B, rubreserine; C, methylparaben; D, eseroline; E, physostigmine; F, propylparaben.

TABLE 4

Effects of Concentration of Sodium Heptanesulfonate in Mobile Phase[a] on k'

Sodium heptanesulfonate	k' physostigmine
0.02M	5.23
0.04M	3.43
0.10M	3.40

[a]The aqueous portion contained 0.005M NaH_2PO_4 in addition to heptanesulfonate and was mixed with methanol (50:50 v/v).

TABLE 5

The k' and CV of Retention Times Calculated from Six Chromatographic Determinations

Compounds	k'	CV (%)
rubreserine	1.50	1.03
methylparaben	2.33	0.87
eseroline	3.38	1.48
physostigmine	5.77	1.81
propylparaben	9.27	1.33

Therefore, solvent-programming or gradient elution is a good approach to separate such a mixture. Figure 6 shows the simultaneous separation of a mixture of benzyl alcohol, rubreserine, eseroline and physostigmine under the gradient-elution conditions as described.

The selection of Solvent A and Solvent B was based on their isocratic elution of initial bands and late-eluting bands, respectively. They differ only by 15 % v/v acetonitrile, so the problems generated from inadequate mixing which arises from the use of two solvents with disparate solvent strengths can be avoided.

The CV of the peak areas for physostigmine obtained from 6 replicate injections is 1.1%. Both the resolution, R_S and kg in gradient elution can be defined exactly as for isocratic separation (25). The R_S between the physostigmine peak and the adjacent eseroline peak calculated from these replicates is 3.12. The

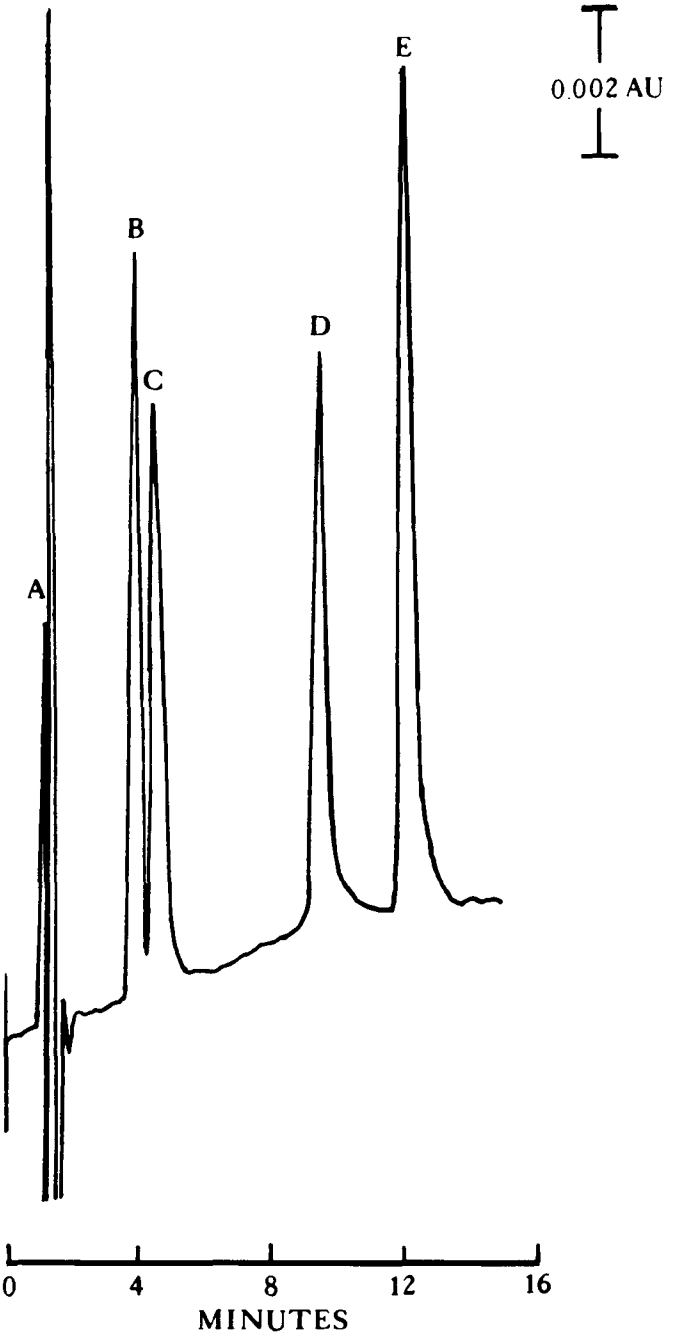

FIGURE 6

Separation of a mixture of physostigmine salicylate 20 μg/ml, rubreserine 40 μg/ml, eseroline 8 μg/ml and benzyl alcohol 200 μg/ml with gradient elution. Detection: 247 nm. Peaks: A, salicylic acid; B, benzyl alcohol; C, rubreserine; D, eseroline; E, physostigmine.

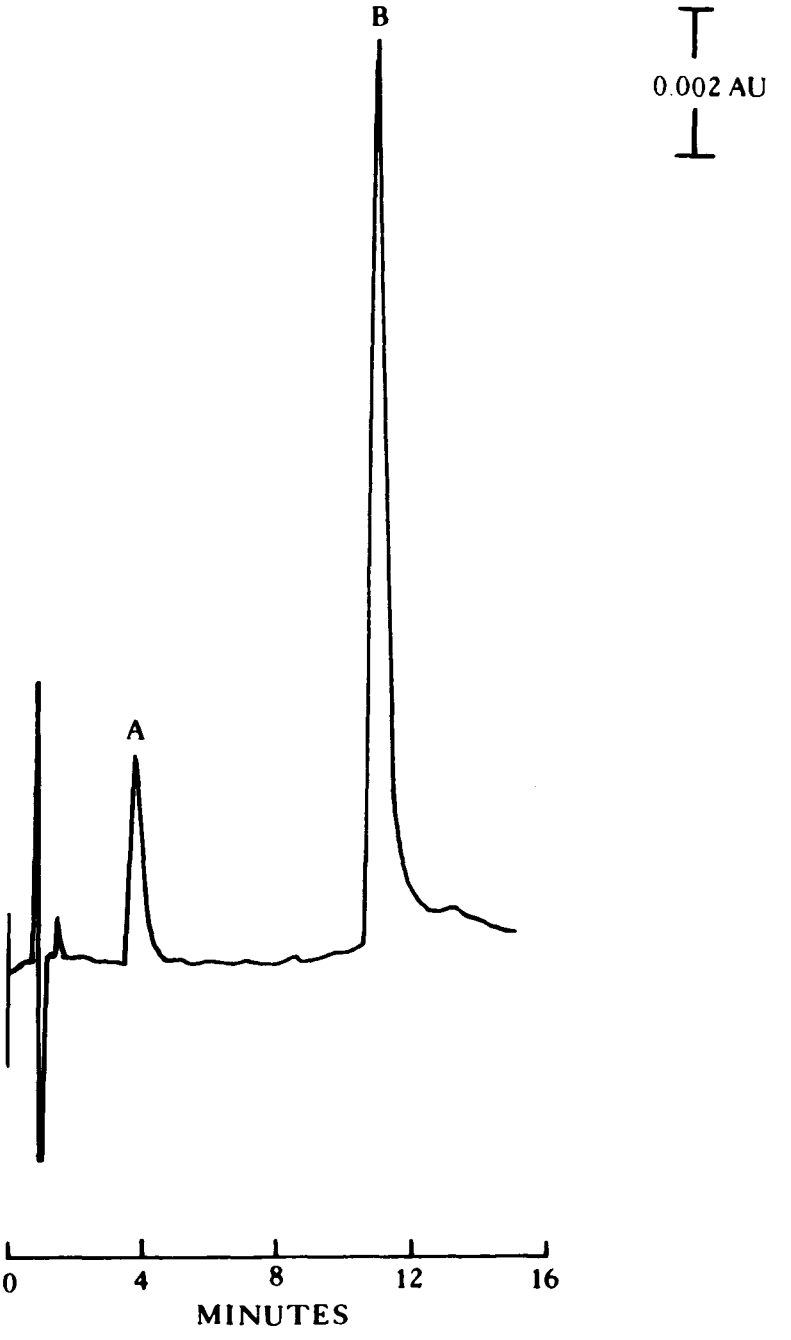

FIGURE 7

Separation of an aged solution of physostigmine sulfate 0.2 μg/ml with gradient elution. Detection: 292 nm. Peaks: A, rubreserine; B, physostigmine.

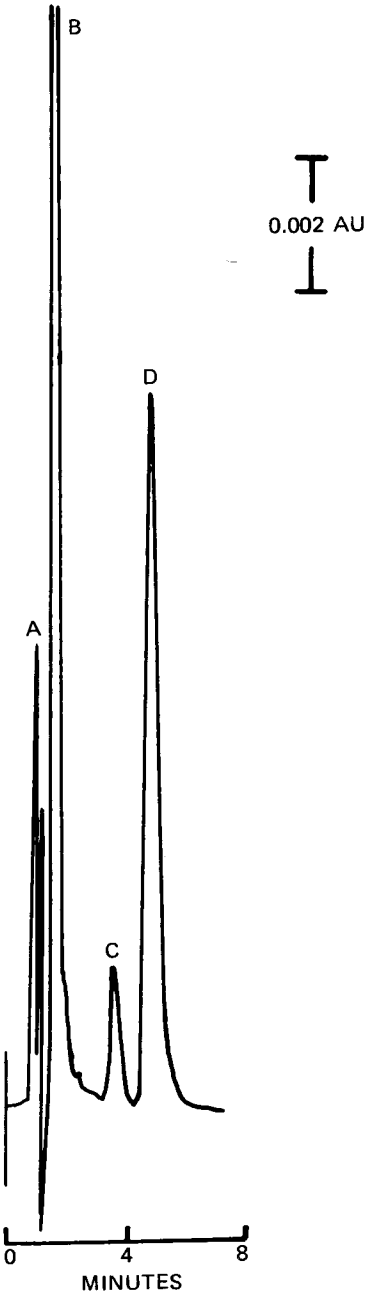

FIGURE 8

Separation of a mixture of physostigmine, rubreserine, eseroline and benzyl alcohol with isocratic elution. Detection: 247 nm. Peaks: A, salicylic acid; B, benzyl alcohol and rubreserine; C, eseroline; D, physostigmine.

TABLE 6

The kg and CV of Retention Times

Compounds	kg	CV (%)
benzyl alcohol	2.75	1.03
rubreserine	3.31	1.13
benzaldehyde	5.61	
eseroline	7.98	0.79
physostigmine	10.51	1.27

kg and CV for the retention times obtained from these replicates are summarized in Table 6. Benzaldehyde, which may be an impurity from benzyl alcohol, is eluted between rubreserine and eseroline, with kg of 5.61. The R_S values are 4.26 and 3.80 between rubreserine and benzaldehyde, and between benzaldehyde and eseroline, respectively. Figure 7 is a chromatogram obtained from an aged solution of physostigmine sulfate, which was stored at 25°C for 7 months, with the detector set at 292 nm which is the λ_{max} for rubreserine.

The chromatographic conditions described in the Second Supplement of USP XXI/NFXVI (26) were tested also for the separation of a mixture of physostigmine, eseroline, rubreserine and benzyl alcohol. The resulting chromatogram is shown in Figure 8. The peaks for benzyl alcohol and rubreserine completely overlap, no improvement in resolution was obtained by adjusting the pH of the aqueous portion of the mobile phase. The degradation of benzyl alcohol was reported (27). If the quantitation of benzyl alcohol in the preparations is also desired, then this official analysis is not appropriate due to the possible presence of rubreserine.

SUMMARY AND CONCLUSIONS

A reversed-phase, ion-pairing HPLC method was developed for assaying physostigmine-containing preparations in which methylparaben and propylparaben are the preservatives. The decomposition products of physostigmine, eseroline and rubreserine, were shown not to interfere with the detection of physostigmine by this method. The complete separation of methylparaben and propylparaben enables the simultaneous determination of these preservatives. Elution was completed in less than 12 minutes.

A gradient-elution, ion-pairing HPLC method was also reported in this work for the assay of physostigmine-containing preparations in the presence of eseroline,

rubreserine and benzyl alcohol. Since an adequate resolution for the peaks of benzyl alcohol and rubreserine was obtained with this method, benzyl alcohol can be quantified simultaneously.

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