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# Liquid chromatographic determination of the pH-dependent degradation of eseroline — hydrolysis product of physostigmine

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**Abstract**: The stability of eseroline, the hydrolysis product of physostigmine under aerobic conditions, was studied by liquid chromatography. A reversed-phase, ion-pair technique was used to separate eseroline from its degradation products. The degradation of eseroline in phosphate buffer solutions of pH 6.91, 7.40, 7.98, 8.41 and 8.94 appears to follow first-order kinetics; the rate constant increased with an increase in pH. The degradation appears to follow specific base catalysis.

**Keywords**: *Eseroline; physostigmine hydrolysis product; reversed-phase ion-pair liquid chromatography; degradation kinetics; pH-dependent degradation.*

## Introduction

Physostigmine, a carbamate-type acetylcholinesterase inhibitor, is used for the reduction of intra-ocular pressure in glaucoma and for antagonizing the central anticholinergic syndrome and the cardiac arrhythmias produced by overdosage or an unusual reaction to anticholinergic drugs [1]. Some clinical investigations [2–7] have suggested that physostigmine may improve the memory of patients with Alzheimer's disease, a deficiency of functional cholinergic neurons in the central nervous system.

The degradation of physostigmine in aqueous solution is reported to be by hydrolysis of the ester linkage to produce a colourless compound, eseroline, which is then oxidized to a red compound, rubreserine, as shown in Fig. 1. Further conversion to other more intensely coloured products is reported to occur [8–10]. The appearance of colour in a liquid preparation of physostigmine as a criterion to evaluate the extent of decomposition [11] is not reliable since the presence of eseroline does not discolour the solution. The use of an antioxidant should prevent the formation of the red compound, rubreserine, but would have no effect on the hydrolysis of the parent compound. Yamazoe *et al.* showed that by high-performance liquid chromatography (HPLC) under anaerobic conditions, physostigmine degraded without discolouration of the solution [12]. Therefore, the presence of eseroline in even a colourless solution of physostigmine

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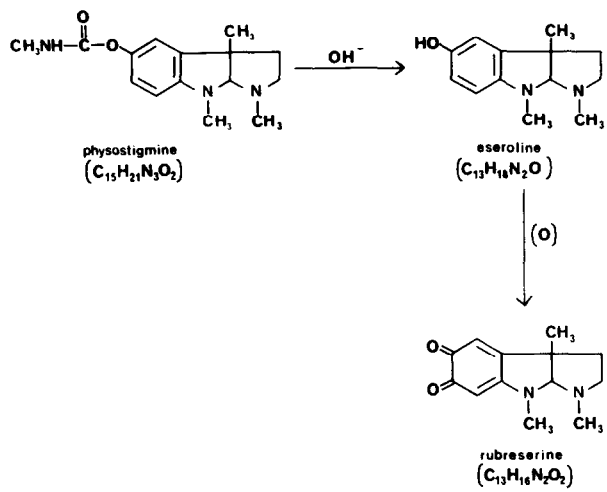


**Figure 1**
Degradation pathway for rubreserine.

is probable if the decomposition progresses according to Fig. 1. This colourless compound, eseroline, may be produced also via enzymatic hydrolysis in a biological system [13].

Since the degradation products are at least one thousand times less active than the parent compound in the inhibition of cholinesterase [14], it is necessary to demonstrate complete separation of physostigmine from both eseroline and rubreserine in order to obtain accurate quantitative results in both stability tests and biological studies on physostigmine. The reported stability studies [15–21] of physostigmine in solution have focused primarily on the disappearance of physostigmine. Very little information is available on the presence of eseroline in an aged solution of physostigmine or in a biological sample containing physostigmine. Somani *et al.* developed an HPLC method which effected good resolution of physostigmine and eseroline; however chromatograms of rat plasma and brain extract after intramuscular doses of physostigmine (650 μg/kg) showed no peaks for eseroline [22]. The extreme instability of eseroline was observed by the authors of the present work during the synthesis, crystallization and recrystallization procedures; such difficulty of purification was also reported by Coyne *et al.* [23]. Preliminary findings by the authors indicated that the rate of disappearance of eseroline in solution appeared to be pH dependent; the rate increased as the pH of the media increased. The objective of the present study was to investigate the degradation rate of eseroline as a function of solution pH under aerobic conditions.

## Experimental

*Equipment*

The liquid chromatograph was a modular system consisting of a Micromeritics (Norcross, GA) Model 750 solvent delivery system, a Rheodyne (Cotati, CA) Model 7125 injector, a Varian (Walnut Creek, CA) Model UV-50 detector, a Varian Model CDS111 data system and a Varian Model 9176 recorder. All pH measurements were made using a Fisher (Pittsburgh, PA) Accumet pH meter Model 620 with a combination electrode; a Mettler (Highstown, NJ) M-5 microbalance was used for weighing all samples of eseroline for the stability tests.

*Reagents and chemicals*

All reagents were used without further purification. Sodium phosphate monobasic U.S.P. (Mallinckrodt, St. Louis, MO), sodium phosphate dibasic purified (Fisher, Fairlawn, NJ) and sodium phosphate tribasic A.C.S. (Matheson Coleman and Bell, Norwood, OH) were used for the preparation of phosphate stock solutions. Physostigmine sulphate (Sigma, St. Louis, MO) and sodium hydroxide, U.S.P. (Amend, Irvington, NJ) were used for the synthesis of eseroline and rubreserine. The sodium salt of 1-heptanesulphonic acid (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ). Ether, benzene and petroleum ether were all A.C.S. grade. All aqueous solutions were prepared in double-distilled water.

*Chromatographic conditions*

A reversed-phase 300 × 4-mm i.d. column (Waters Assoc., Milford, MA), packed with 10-μm Bondapak $C_{18}$ preceded by a Waters guard column packed with a 40-μm Bondapak $C_{18}$ (Whatman, Clifton, NJ), was used for all stability analyses. A reversed-phase 250 × 4.1-mm i.d. column packed with Versapack $C_{18}$ (Altech Assoc., Deerfield, IL), preceded by the same guard column, was used for the simultaneous separation of eseroline, rubreserine and physostigmine.

The chromatographic mobile phase was prepared by mixing an aqueous solution of 0.005 M sodium phosphate monobasic and 0.02 M sodium heptanesulphonate (pH 4.78) with methanol (1:1, v/v). The aqueous portion of the mobile phase was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA) and degassed under vacuum prior to the addition of methanol. The mobile phase used with the Versapack $C_{18}$ column was similar but with a ratio of aqueous phase to methanol of 2:3, v/v.

The detector was set at 247 nm and 0.02 a.u.f.s. for all determinations. This wavelength is the $\lambda_{max}$ for physostigmine sulphate and is near the $\lambda_{max}$ for eseroline, which is 243 nm. All studies were carried out at ambient pressure and room temperature maintained thermostatically at 23 ± 2°C.

*Synthesis of eseroline and rubreserine*

Eseroline and rubreserine were synthesized according to the methods of Ellis [24]. The results from the elemental analyses* are as follows:

| | Rubreserine monohydrate $C_{13}H_{18}N_2O_3$ | | Eseroline $C_{13}H_{18}ON_2$ | |
|---|---|---|---|---|
| | Theory (%) | Found (%) | Theory (%) | Found (%) |
| C | 62.38 | 62.46 | 71.52 | 71.66 |
| H | 7.25 | 7.26 | 8.31 | 8.34 |
| N | 11.19 | 11.18 | 12.83 | 12.76 |

Their melting points were in good agreement with those recorded by Ellis [24]. The UV–visible absorption spectrum for synthesized rubreserine coincided with that reported by Ellis [24]. The UV spectrum for synthesized eseroline is shown in Fig. 2.

*Performed by Atlantic Microlab Inc., Atlanta, GA, USA.

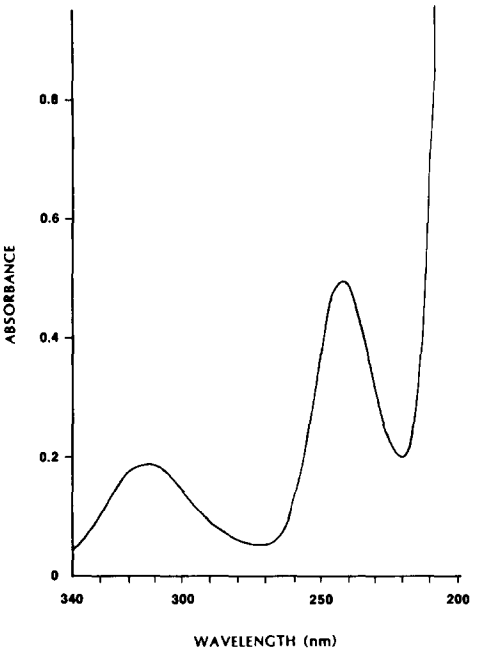


**Figure 2**
UV adsorption spectrum for eseroline.

*Degradation studies*

Three 0.001 M stock solutions of sodium monobasic, dibasic and tribasic phosphate each were prepared. From these stock solutions, five 0.001 M phosphate buffer solutions with pH values ranging from 6.91 to 8.94 at increments of approximately 0.5 pH unit were prepared with the aid of a pH meter. Each stock solution was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA) and degassed under vacuum.

Accurately weighed samples of eseroline (1.000 ± 0.100 mg) were transferred to 100-ml volumetric flasks. The flasks were filled to volume with 0.001 M phosphate buffer solutions of the desired pH, which were prepared from the degassed stock solutions. No further attempts were made to eliminate oxygen from the solutions or the flasks. Quadruplicate samples of 1 mg of eseroline in 100 ml of each of the 0.001 M phosphate buffer solutions (pH 6.91, 7.40, 7.98, 8.41 and 8.94) were prepared and analysed by HPLC. Dissolution was assisted by ultrasonic agitation and manual shaking of the stoppered flasks containing the solutions. The flasks remained stoppered during the entire course of study except momentarily during sample withdrawal.

The time when dissolution appeared to be complete was recorded as zero time. The time recorded for each injection was the interval between zero time and the injection time. This practice was continued until the eseroline peak became unresolved from an adjacent unknown peak. Eseroline is sufficiently stable at the pH of the mobile phase and degradation was essentially terminated when the injection was made. The injected samples were accurately measured with a 20-μl loop on the injector. The mobile phase flow-rate was set at 1 ml/min for all sample solutions except those of pH 8.94, for which the flow-rate was 2 ml/min. Since the rate of degradation of eseroline was very rapid at pH 8.94, a greater flow-rate was necessary in order to obtain at least four chromatograms for each of these solutions before the peak of eseroline became undetectable.

## Results and Discussion

The liquid chromatographic separation of eseroline from rubreserine and other degradation products was achieved using a $C_{18}$ stationary phase and a mobile phase of methanol and phosphate buffer (pH 4.78). The mobile phase was buffered at an acidic pH so that the basic compounds were present as the protonated species; the $pK_a$ of the aliphatic amino-group of physostigmine is 8.08 [25]. The low apparent pH of the mobile phase was also necessary to prevent any on-column degradation of eseroline. Preliminary studies had shown essentially no degradation for eseroline under conditions of similar acidity. Separation of a mixture of eseroline, physostigmine and rubreserine under similar chromatographic conditions is shown in Fig. 3. The mobile phase used in this study was adapted from Hsieh *et al.* [26], modified by the addition of the ion-pairing reagent 1-heptane-sulphonic acid. This resulted in enhanced resolution of physostigmine, eseroline and rubreserine. The capacity factors ($k'$) for these compounds are 1.40, 0.93 and 0.40, respectively. These low capacity factors made the kinetic study for eseroline at high pH possible by eluting the compounds in a short period of time with adequate resolution.

The linearity of the detector response to eseroline was checked for the range of 20–200 ng. The correlation coefficient, 0.9998, indicated a linear relationship between peak area

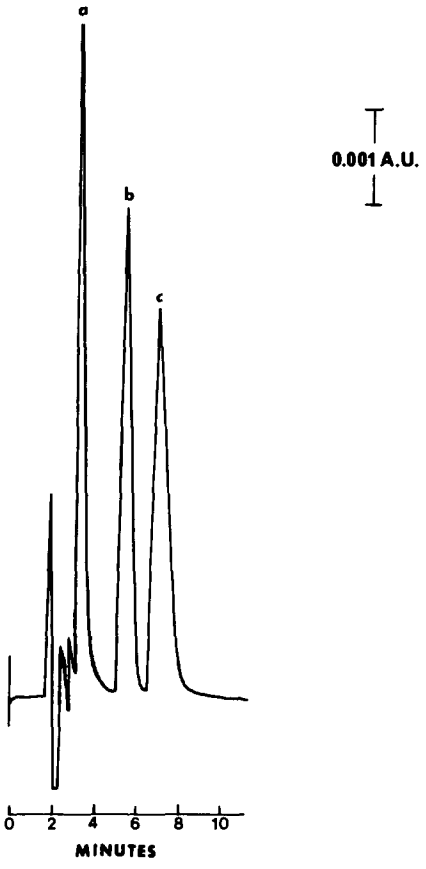


**Figure 3**
Liquid chromatographic separation of a mixture of rubreserine (peak a), eseroline (peak b) and physostigmine (peak c). Flow rate: 1 ml/min.

and amount injected. The precision of the chromatographic procedure was measured on different days; the relative standard deviation (RSD) ranged from 0.07 to 0.25%.

The HPLC separation of eseroline from its degradation products in a test solution of pH 7.98 is shown in Fig. 4 in which peak a has the same retention time as that of the synthetic sample of rubreserine. Other degradation products were not identified. The appearance of several unknown products from the degradation of physostigmine was also described by Yamazoe *et al.* [12]. Peak b (one of the unidentified compounds) was observed at all pH levels. The amount of this compound present in samples appeared to be pH dependent. Thus, in solutions of pH 6.91, peak b was not detected up to 20 h after zero time and then only as a small shoulder on peak a. Similarly, at pH 7.40 and 7.98, peak b was relatively small compared with peak a and remained almost constant in peak height throughout the study. At pH values greater than 8, peak b was much more pronounced than at lower pH values and its height increased rapidly with time as shown in Fig. 5. Peak b was undetectable in the chromatograms obtained from the test solutions of all pH values one month after the degradation study, but peak a (possibly rubreserine) was still detectable. In addition, the presence of some unknown peaks that were eluted prior to the solvent front was observed.

Figures 6 and 7 are the semilogarithmic plots of the percentage of the remaining eseroline as a function of time for the pH values studied. Since the injections of the eseroline solutions at the same pH value were not carried out to exactly the same schedule, only the results from one solution of each pH were plotted in these figures. The reaction appeared to follow first-order kinetics. Such kinetics can be described by equation (1):

$$C = C_0 e^{-kt}. \tag{1}$$

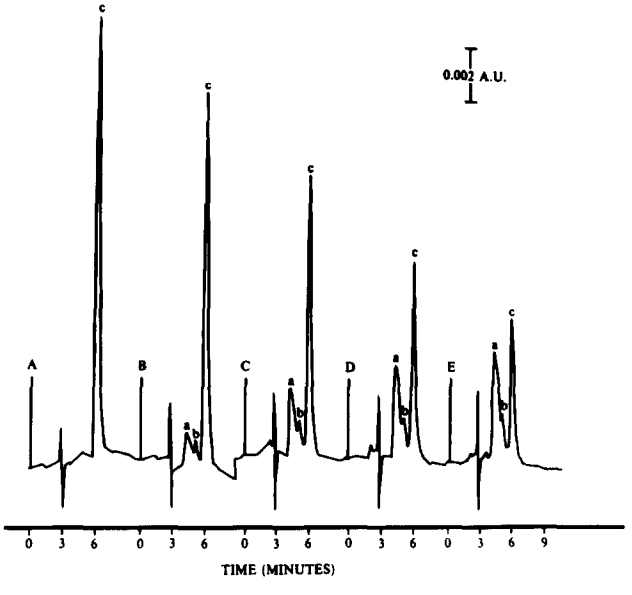


**Figure 4**
Liquid chromatographic analysis of eseroline degradation in a phosphate buffer solution of pH 7.98. Flow rate: 1 ml/min. Reaction times: A, 14.90 min; B, 24.96 min; C, 34.45 min; D, 44.07 min; E, 53.40 min. Peaks: a, rubreserine; b, unknown; and c, eseroline.

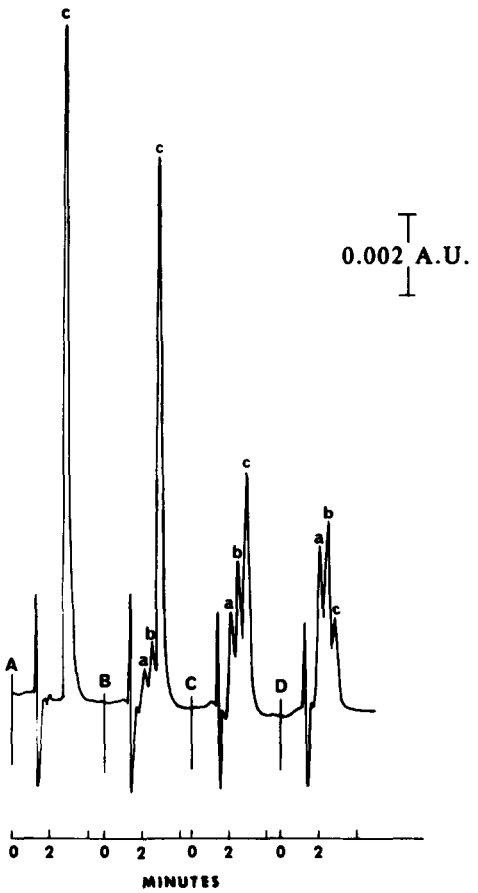


**Figure 5**
Liquid chromatographic analysis of eseroline degradation in a phosphate buffer solution of pH 8.96. Flow rate: 2 ml/min. Reaction times: A, 2.65 min; B, 7.47 min; C, 12.00 min; D, 16.68 min. Peaks: a, rubreserine; b, unknown; and c, eseroline.

In equation (1), $C$ and $C_0$ are the concentrations of eseroline at time $t$ and time zero, respectively, and $k$ is the apparent first-order rate constant.

In Figs 6 and 7, the effects of pH of the buffer solutions on the slopes of the regression lines and, in turn, on the degradation rate constant are clearly shown. The plots for pH 8.41 and 8.94 show only 3 points each but each of the slopes for these lines were obtained from 4 points with an additional point that represented less than 10% eseroline remaining.

Least-squares analysis was performed on the individual plot for each test solution to obtain $k$, the rate constant for the disappearance of eseroline in solution. The mean and standard deviation of the rate constant were then calculated for each pH studied. The results are listed in Table 1.

Figure 8 is the semilogarithmic plot of rate constant versus pH of solution. The regression equation of this plot is $\log k = 1.05\ \text{pH} - 10.04$ with a correlation coefficient of 0.974. The slope of this regression line is 1.05 which indicates that this reaction may be subject to specific base catalysis in which the hydroxide ion is the catalyst [27]. The rate law for such a reaction contains a term that involves the concentration of hydroxide ion in the reaction solution [27]. According to the reaction scheme in Fig. 1, the degradation of eseroline is an aerobic reaction; therefore no special efforts were made to eliminate air from the reaction flasks.

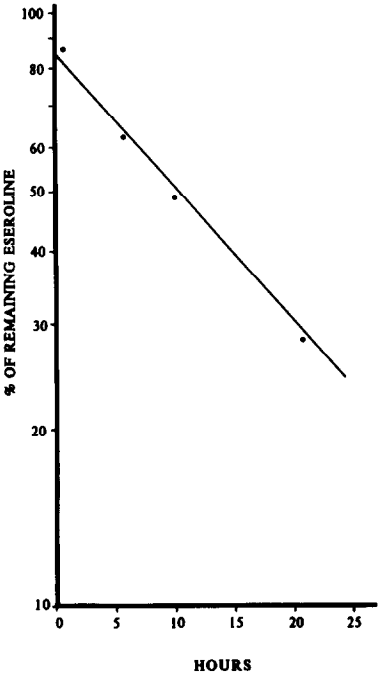


**Figure 6**
Apparent first-order disappearance of eseroline in 0.001 M phosphate buffer solution of pH 6.91 at 23 ± 2°C.

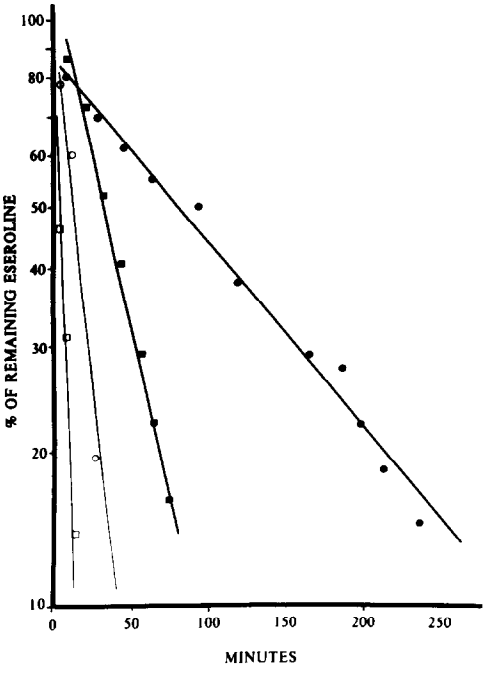


**Figure 7**
Apparent first-order disappearance of eseroline in 0.001 M phosphate buffer solutions of pH 7.40 (●), pH 7.98 (■), pH 8.41 (○) and pH 8.94 (□) at 23 ± 2°C.

**Table 1**
Degradation rate constants of eseroline as a function of pH at 23 ± 2°C

| pH | Rate constant $k$ mean ± SD ($min^{-1}$) | Correlation coefficient mean ± SD |
|---|---|---|
| 6.91 | $9.76 \times 10^{-4} \pm 1.71 \times 10^{-4}$ | 0.993 ± 0.008 |
| 7.40 | $7.90 \times 10^{-3} \pm 0.542 \times 10^{-3}$ | 0.982 ± 0.004 |
| 7.98 | $2.56 \times 10^{-2} \pm 0.272 \times 10^{-2}$ | 0.984 ± 0.018 |
| 8.41 | $8.03 \times 10^{-2} \pm 0.669 \times 10^{-2}$ | 0.990 ± 0.004 |
| 8.94 | $15.3 \times 10^{-2} \pm 1.41 \times 10^{-2}$ | 0.982 ± 0.014 |

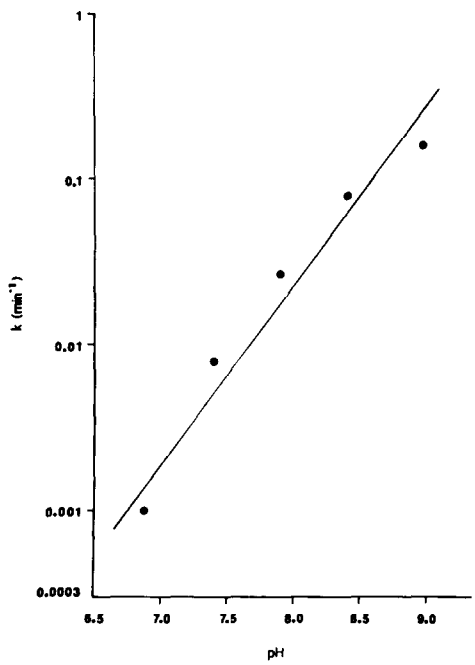


**Figure 8**
Plot of log rate constant versus pH for the disappearance of eseroline at 23 ± 2°C.

The stability of eseroline at pH 7.40 is of special interest since this is also the pH of human plasma. In buffer solutions of low ionic strength the half-life of eseroline is 87.7 min, calculated from its degradation rate constant at pH 7.40. In a biological system, the presence of other factors such as metabolism, can be expected to result in a more rapid degradation. Therefore, this calculated half-life represents a maximum; the half-life may be much shorter in a complex biological system.

The pH values of liquid pharmaceutical preparations of physostigmine are in the acidic range. The half-life of eseroline in 0.001 M phosphate buffer solutions of pH 6.91 is 14.8 h calculated from its degradation rate constant. However, because its half-life is expected to be longer than 14.8 h in more acidic solutions, the stability of eseroline at pH less than 6.91 was not studied. Yamazoe *et al.* [12] found that physostigmine solutions of pH 5.3 degraded to about the same extent under both aerobic and anaerobic conditions at 100°C; anaerobic conditions retarded only the discolouration and not the degradation of physostigmine solutions. Since very small amounts of rubreserine can discolour the

solution (0.5 μg/ml, slight discolouration; 1 μg/ml, distinctly pink [28]), the retardation of discolouration under anaerobic conditions reported by Yamazoe *et al.* suggests that the degradation of physostigmine may stop at eseroline under these conditions. Examination of the UV absorption spectrum of a solution of physostigmine salicylate degraded under anaerobic conditions showed an increase in absorbance near 240 nm [12] ($\lambda_{max}$ of the synthesized eseroline was 243 nm), and an unknown degradation product was detected by HPLC but was not observed in solutions under aerobic conditions [12]. In order to identify by chromatography this unknown degradation product reported by Yamazoe, some preliminary work was carried out in this laboratory. Physostigmine salicylate solution (1 mg/ml) buffered at pH 5.3 was flushed with nitrogen and autoclaved for 15 min, then analysed by HPLC. The resulting chromatogram showed a peak which had the identical retention time as that of synthesized eseroline. This peak was not found in the chromatogram obtained from an unbuffered aqueous solution of physostigmine sulphate stored at room temperature under aerobic conditions for 5 months. Therefore, the presence of eseroline in weakly acidic physostigmine solutions is possible especially under anaerobic conditions or in the presence of antioxidants. A stability-indicating assay for physostigmine in dosage forms should be demonstrated to be capable of separating and determining physostigmine in the presence of both eseroline and rubreserine.

## Conclusions

Eseroline, the hydrolysis product of physostigmine, is unstable in aqueous solution under aerobic conditions. The degradation rate constant in solution is pH-dependent and increases greatly with increasing pH. The reaction appears to follow first-order kinetics and to be subject to specific base catalysis.

Under aerobic conditions, eseroline has a half-life of 87.7 min in 0.001 M buffer solutions of pH 7.4 and is expected to have a much shorter half-life in biological systems owing to the possible combination of metabolism and pH-dependent degradation pathways. Its half-life is 14.8 h in 0.001 M solutions buffered at pH 6.91 and is expected to be longer in more acidic solutions such as ophthalmic or injectable solutions of physostigmine salicylate, especially in the presence of antioxidants. Therefore, the presence of eseroline in biological samples is less likely than in pharmaceutical preparations. In developing a stability-indicating assay for physostigmine, there may be a need to take eseroline into consideration.

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