ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1999) 939–945

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Liquid chromatographic analysis of physostigmine salicylate and its degradation products

Shai Rubnov, Daniel Levy, Hava Schneider *

Medical Corps, Israeli Defence Force, Military POB 02149, Israel

Received 31 December 1997; received in revised form 2 March 1998; accepted 31 March 1998

Abstract

A simple stability-indicating HPLC assay has been developed for physostigmine salicylate, capable of following its degradation. A 250 × 5 mm i.d. column packed with 10 μm Bondapak C_{18} was used, with a mobile phase of acetonitrile–ammonium acetate (pH 6.0; 0.1 M) (50:50, v/v) and flow rate 1.2 ml min^{-1}. All peaks are eluted in < 10 min and the method has good precision. The optimum wavelength for detection of degradation products is 305 nm. Application of the assay for a commercial preparation of physostigmine salicylate for injection is presented.

Keywords: Degradation products; HPLC; Physostigmine; Stability

1. Introduction

Physostigmine salicylate (eserine salicylate) is one of a number of medical drug antidotes against certain psychochemical warfare agents. The decomposition path of physostigmine has been studied [1]. Hydrolysis initially removes the urethane group to produce eseroline, a colorless compound. Subsequent oxidation yields rubreserine, a red compound, which is in turn converted into eserine blue or eserine brown (Fig. 1).

The anticholinesterase activities of physostigmine salicylate and its degradation products have been examined. In vivo, the degradation products are at least 1000 times less active than physostigmine as anticholinesterase agents [1]. Lack of coloration does not indicate full anticholinesterase activity, because eseroline, the colorless product of the hydrolysis, possesses little or no such activity.

Various authors [2–5] have described separation and determination of physostigmine and its degradation products. Somani and Khalique [2] separated physostigmine from eseroline in biological fluids but did not detect other degradation products. Others separated physostigmine from rubreserine but either resolution between physostigmine and eseroline was poor [3], or the total elution time required was very long [4]. Yang and Wilken [5] required gradient elution and a high flow rate in order to achieve good resolution

* Corresponding author. Present address: 8 H'Iris St., Kfar-saba 44278, Israel. Fax: +972 3 5306867; e-mail: srub@cc.huji.ac.il

0731-7085/99/$ - see front matter
PII S0731-7085(98)00099-5

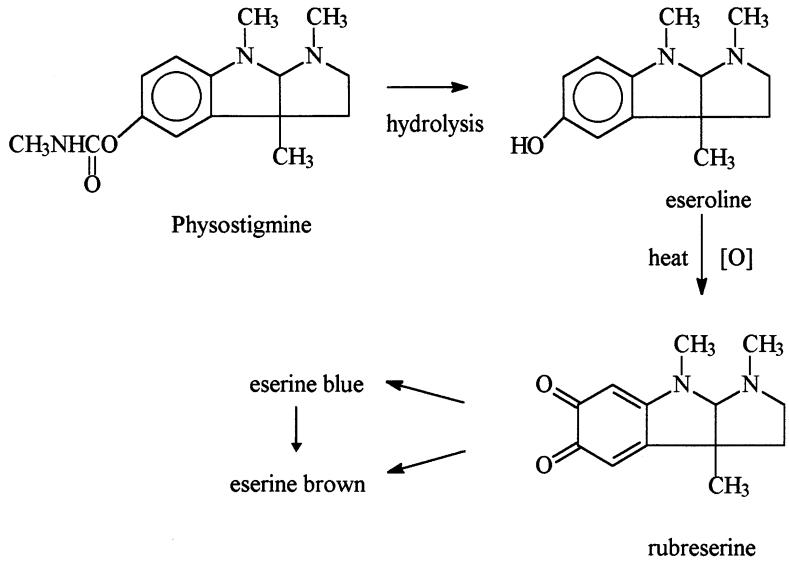

Fig. 1. Decomposition path of physostigmine.

of physostigmine and its degradation products and an acceptable physostigmine peak shape. Quinn and Stewart [6] used a post-column fluorescence ion pair extraction system, which is too complicated to be used as a routine assay. None of these authors studied the rate of appearance of the degradation products. The United States Pharmacopeia assay for physostigmine [7] is not designed to separate physostigmine from its degradation products. We found that the use of benzyl alcohol and benzaldehyde in this method masks peaks of the degradation products.

The objective of this study was to develop a simple stability-indicating HPLC method for the determination of physostigmine in the presence of its degradation products. Using this method, we were able to identify the main degradation products and to follow their rates of appearance during thermal degradation of physostigmine salicylate.

2. Experimental

2.1. Materials

All reagents were of analytical reagent grade and fresh double distilled water was used. Physostigmine base, physostigmine salicylate and salicylic acid were obtained from Sigma, acetonitrile and ammonium acetate were purchased from E. Merck and glacial acetic acid was obtained from Frutarom, Israel. Physostigmine salicylate for injection (40 mg lyophilized) was purchased from Abic Pharmaceuticals, Israel.

2.2. Instrumentation

The liquid chromatograph consisted of a Hewlett-Packard HP 1090 liquid chromatograph equipped with an HP 3396A integrator and a diode array UV detector capable of recording spectra of the chromatographic peaks. The column (250 × 5 mm i.d.) packed with reversed phase 10 μm Bondapak C_{18} was purchased from HPLC Technologies, UK The injector was fitted with a 20 μl loop.

2.3. HPLC conditions

The optimum mobile phase was acetonitrile-ammonium acetate (pH 6.0; 0.1 M) (50:50, v/v), flow rate 1.2 ml min^{-1}. The pH of the aqueous component was adjusted with glacial acetic acid. Where other conditions were used these are specified. Detection wavelengths of 248 and 305 nm were used.

2.4. Thermal degradation of physostigmine salicylate

A solution of 300 μg ml^{-1} physostigmine salicylate in water was placed in a boiling water bath. A sample was taken every 15 min for 2 h, cooled, diluted to 150 μg ml^{-1} with acetonitrile and chromatographed.

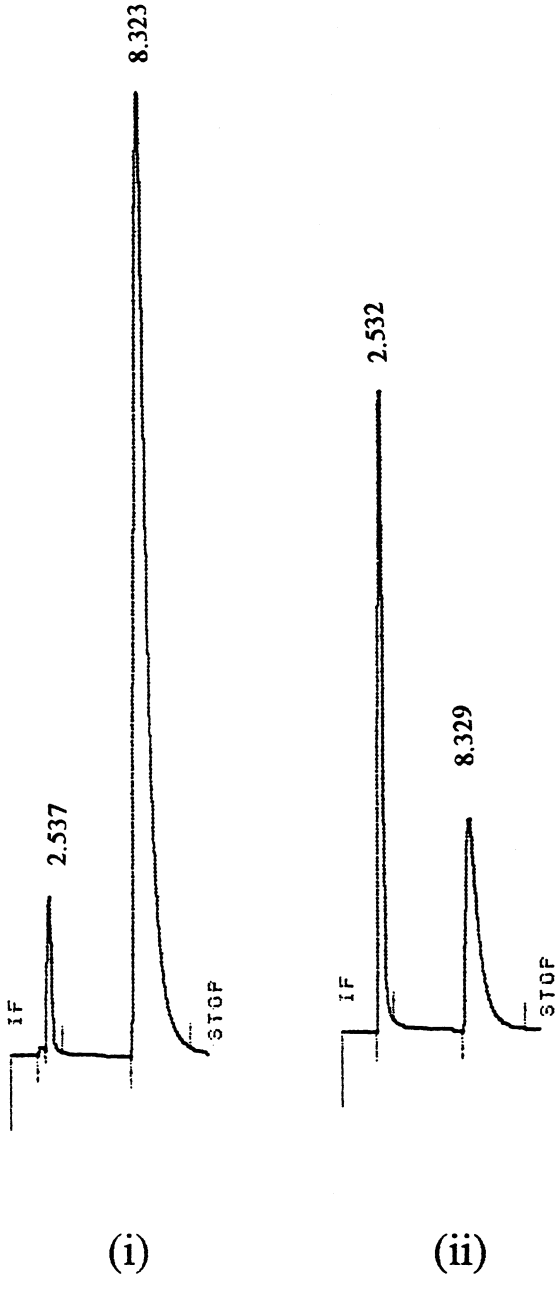

Fig. 2. HPLC separation of physostigmine salicylate, detected at (i) 248 nm and (ii) 305 nm.

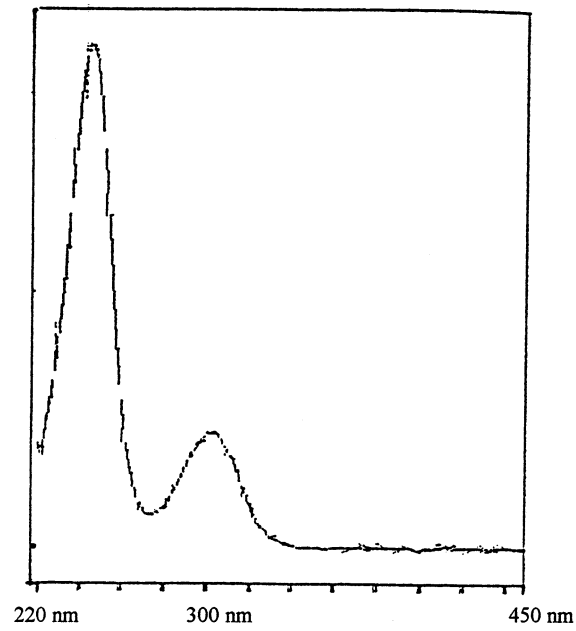

Fig. 3. Spectrum (220–450 nm) of the physostigmine peak, showing maxima at 248 and 303 nm.

2.5. Assay of physostigmine salicylate for injection (40 mg)

The lyophilizate was reconstituted with 10 ml of water and filtered by a syringe filter (0.45 μm) into a 20 ml volumetric flask. The vial, the syringe and the filter were washed with water and brought to volume of 20 ml to give a 2 mg ml^{-1} solution. One milliliter was further diluted in a 10 ml volumetric flask, 4 ml of water were added and the volume made up with acetonitrile to obtain a 200 μg ml^{-1} solution.

A 200 μg ml^{-1} solution of standard physostigmine salicylate was prepared in 50% acetonitrile by dilution of a more concentrated stock solution prepared in acetonitrile.

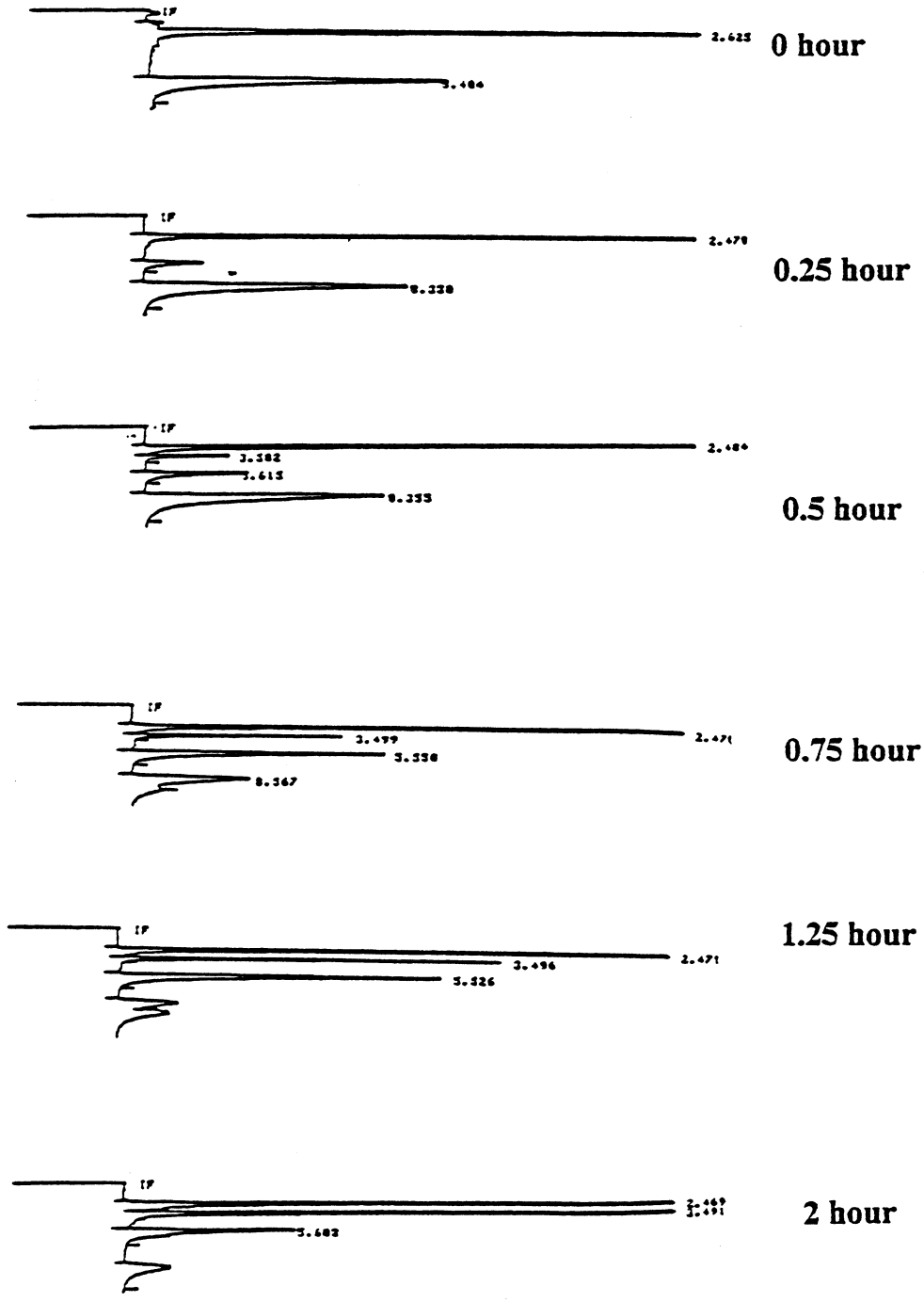

Fig. 4. Thermal degradation of physostigmine salicylate followed at 305 nm.

3. Results and discussion

3.1. Chromatographic separation of physostigmine salicylate

Fig. 2 shows the separation of 150 μg ml^{-1} physostigmine salicylate. By separate injections of salicylic acid and physostigmine base, the peaks were identified as salicylate (2.5 min) and physostigmine (8.3 min). The UV spectrum of the physostigmine peak (Fig. 3) shows maxima at 248 and 303 nm, with no absorbance in the visible range.

3.2. Optimization of chromatographic conditions

The mobile phase of acetonitrile-ammonium acetate (pH 6.0; 0.1 M) (50:50, v/v) with flow rate 1.2 ml min^{-1} provides optimum conditions of retention time and minimum tailing of the physostigmine peak without requiring excessive flow rates. Reduction of the buffer concentration to 0.05 M ammonium acetate increased the retention time of physostigmine and the peak tailed significantly. Increasing the proportion of the organic component from 50 to 60% shortened the retention time of the salicylate peak but had very little effect on the physostigmine peak.

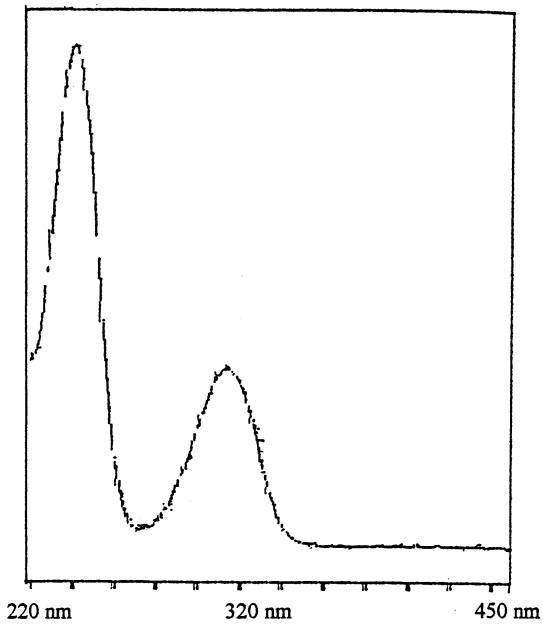

Fig. 5. Spectrum (220–450 nm) of the peak at 5.5 min, identified as eseroline.

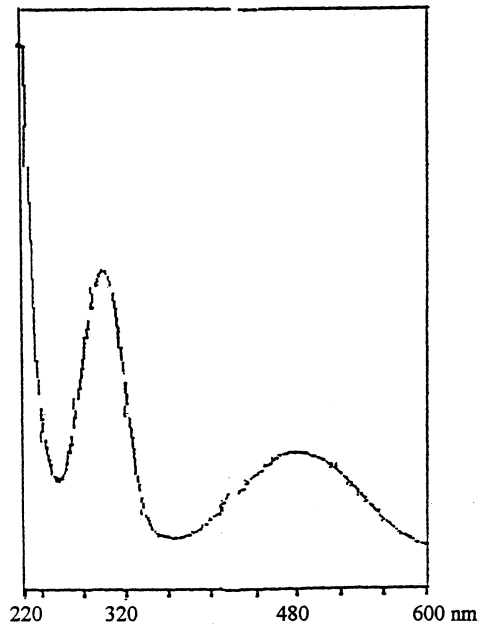

Fig. 6. Spectrum (220–600 nm) of the peak at 3.5 min, identified as rubreserine.

For the assay of physostigmine greatest sensitivity is achieved at 248 nm, the wavelength of maximum absorption (Fig. 2i). During thermal degradation studies, however, it was found that some degradation products have low absorbances at 248 nm. The optimum wavelength for following thermal degradation was 305 nm, because at this wavelength physostigmine and its degradation products were found to have similar specific absorbances. The peak area of physostigmine, however, is considerably reduced at this wavelength (Fig. 2ii).

3.3. Detection of degradation products at 305 nm

The degradation path of physostigmine is shown in Fig. 1. For a stability-indicating assay, the physostigmine peak must not be subject to interference from degradation products and the

latter should be visible. To demonstrate that this method is stability indicating, it was used to follow the thermal degradation of physostigmine salicylate in aqueous solution (Fig. 4). The experiment was performed in a boiling water bath in order to achieve full degradation of physostigmine salicylate within 2 h.

The first degradation product to appear had a retention time of 5.5 min. The peak increased until 1.25 h and then began to decrease. Its spectrum (Fig. 5) shows maxima at 242 and 315 nm, i.e. it is colorless. Hence it might be eseroline, which is the first degradation product and which decreases as it is oxidized to rubreserine.

The second degradation product to appear in the series of chromatograms was the peak at 3.5 min, first visible after 0.5 h and increasing steadily with time. Its spectrum (Fig. 6) shows peaks at 300 and 480 nm. This is compatible with the red color of rubreserine, which was seen to be more intense in successive samples.

A third degradation peak at 9.9 min appeared after 0.75 h as a shoulder to the physostigmine peak. It increased in intensity as the latter decreased until after 2 h it was the only peak of the two. Its spectrum was weak, indicating that it might be only a minor degradation product, with maxima at 305 and 500 nm, i.e. it is colored.

3.4. Identification of the peak at 9.9 min

The possibility that the 9.9 min peak might be eserine blue, the degradation product of rubreserine in the degradation sequence, was checked. A sample of eserine blue was prepared from physostigmine by the method of Ellis [8], dissolved in a small volume of acetonitrile and filtered to give a dark blue solution. With a detection wavelength of 550 nm the blue compound was pinpointed to a retention time of 6.0 min and its spectrum showed a maximum above 600 nm. Hence the 9.9 min peak was not eserine blue and no detectable amount of eserine blue was present in the boiled physostigmine samples. Eserine blue has a UV maximum absorbance at about 312 nm and hence would have been detected at 305 nm if present.

The sample of physostigmine salicylate after boiling for 2 h was boiled for a further 3 h. Its red color assumed a brownish tint, but it did not turn blue. The chromatogram showed a rubreserine peak of similar intensity to the 2-h sample. Eseroline had almost completely disappeared, but the 9.9 min peak was no stronger. From the degradation pathway of physostigmine (Fig. 1) and the color of the solution it can be concluded that this peak is probably eserine brown. Eserine brown does not appear to have been detected in earlier HPLC studies of physostigmine. Further investigation is required to obtain a firm identification.

3.5. Assay of physostigmine salicylate for injection

As a test of this newly developed method we assayed a vial of physostigmine salicylate for injection, manufactured 14 years earlier and stored at room temperature. The vial contained 275 mg of dry lyophilizate, of which 40 mg was physostigmine salicylate.

The sample and standard were each chromatographed five times in order to calculate precision data. The UV detection wavelength was 305 nm.

The results showed an assay of 99.5%, with $RSD < 1.5\%$ for both sample and standard. The chromatograms of sample and standard were almost identical. No trace of degradation products was visible and a 'peak purity' check of the physostigmine peak in the sample showed it to be a single peak.

4. Conclusions

A simple stability-indicating HPLC assay with good precision has been developed for physostigmine salicylate, capable of following its thermal degradation. Physostigmine, salicylate, eseroline, rubreserine and (probably) eserine brown are all separated. 305 nm is the optimum wavelength for detection of degradation products. However, if assay sensitivity is critical, 248 nm is preferable.

A 14-year-old sample of lyophilized physostigmine salicylate was assayed by this method. No chemical degradation was found, showing that dry physostigmine salicylate is stable.

References

[1] B.A. Hemsworth, G.B. West, J. Pharm. Sci. 59 (1970) 118–120.
[2] S.M. Somani, A. Khalique, J. Anal. Toxicol. 9 (1985) 71–75.
[3] R. Whelpton, J. Chromatogr. 272 (1983) 216–220.
[4] M. Kneczke, J. Chromatogr. 198 (1980) 529–533.
[5] S.T. Yang, L.O. Wilken, Drug. Dev. Ind. Pharm. 14 (1988) 1061–1078.
[6] K.D. Quinn, J.T. Stewart, Biomed. Chromatogr. 5 (1991) 8–13.
[7] The United States Pharmacopeia, 23rd edition, USPC, Rockville, MD, 1995, pp. 1223.
[8] S. Ellis, J. Pharmacol. Exp. Ther. 79 (1943) 364–372.