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Note

Determination of pilocarpine, physostigmine, its degradation product rubreserine and preservatives by high-performance liquid chromatography

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Pilocarpine and physostigmine are used in ophthalmology in the treatment of glaucoma. Their effect is to decrease the intra-ocular pressure in the eye. Many studies have been made of their estimations, degradation patterns and kinetics in aqueous solution.

Eye-drops containing both pilocarpine and physostigmine are produced by Swedish pharmacies. Preservatives are often added. No study has been reported of the separation of pilocarpine from physostigmine, its degradation product rubreserine and from preservatives. The analysis of all these components with conventional spectrophotometric methods after extraction is very time-consuming and therefore a high-performance liquid chromatographic (HPLC) method has been developed.

Pilocarpine alone has been determined colorimetrically by the hydroxamic acid method by Brochmann-Hanssen *et al.*[1] and Murray[2], and its hydrolysis and epimerization kinetics in aqueous solution have been investigated by Chung *et al.*[3] and Nunes and Brochmann-Hanssen[4]. HPLC methods have been used by a number of workers[5–8]. Weber[5] also showed that the stability of pilocarpine was good at pH 3.5–5.5. Noordam *et al.*[8] used the same column as in our study. They separated pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid on a reversed-phase column (RP-18) with a water–methanol mixture at pH 2.5. Degradation of pilocarpine solutions has been studied by Baeschlin and co-workers[9–11] and Neville *et al.*[12].

Physostigmine alone has been determined by Hellberg[13–15] by acidimetric or spectrophotometric methods. Christenson[16] has described its kinetics of hydrolysis, and Parrák and co-workers[17,18] showed by oscillographic polarography that the most stable ophthalmic solution had a pH value between 2 and 4.5. The stability of this compound has also been studied by Fletcher and Davies[19] and Rogers and Smith[20,21].

Analysis of pilocarpine and physostigmine in the same solution has been studied by Fagerström[22]. The alkaloids have not been separated but have been determined by two different spectrophotometric methods.

The degradation product rubreserine was made according to Ehrlén[23]. Fig. 1 shows the preparations presently available.

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EYE DROPS

Pilocarpine: 1%, 2%, 4%
Physostigmine: 0.4%
Pilocarpine + Physostigmine: 4+0.4%
2+0.2%
4+0.2%

Preservatives:
Methyl+Propyl p-hydroxybenzoate 0.04+0.02%
Phenethyl Alcohol 0.5%
Phenylmercuric Nitrate 0.001%

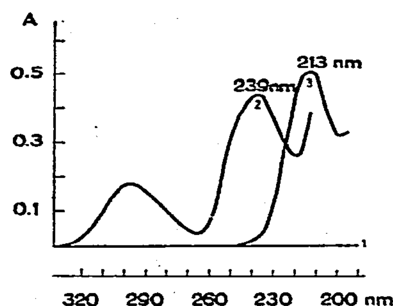
Fig. 1. Different preparations of eye-drops containing pilocarpine, physostigmine and preservatives.

EXPERIMENTAL

Chromatography

A Waters Assoc. pump and injector are used, together with a variable-wavelength detector, LDC Spectromonitor III. The column (30 cm × 3.9 mm) was packed with μBondapak C_{18} (pore size, 10 μm, Waters Assoc.). The mobile phase was 40% methanol with 0.005 *M* heptanesulphonic acid, pH 3.6, filtered through 0.5-μm Celotate®; flow-rate 1 ml/min. Samples of 80 μg pilocarpine and 8 μg physostigimine were injected.

In order to select a suitable wavelength for the HPLC measurements the UV-absorption spectra of the different substances have been recorded (Figs. 2–4). Good results were achieved at 235 nm for the detection of pilocarpine, physostigmine and preservatives. For the detection of the degradation product rubreserine, a wavelength of 292 nm was chosen.



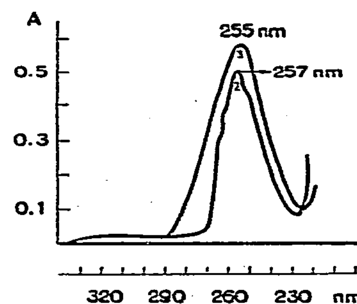


Fig. 2. UV absorption spectra of physostigmine salicylate, 0.001% (2) and pilocarpine hydrochloride, 0.002% (3). 1 = water.

Fig. 3. UV absorption spectra of phenethyl alcohol, 0.3% (2) and methyl + propyl *p*-hydroxybenzoate, 0.004 + 0.002% (3). 1 = water.

As internal standard for unpreserved preparations, methyl *p*-hydroxybenzoate can be used. If the eye-drops are preserved with methyl *p*-hydroxybenzoate for instance, ethyl *p*-hydroxybenzoate can be used.

Fig. 5 shows the chromatographic separation of pilocarpine and phenethyl alcohol. For the detection of phenethyl alcohol the signal from the detector has been amplified ten times.

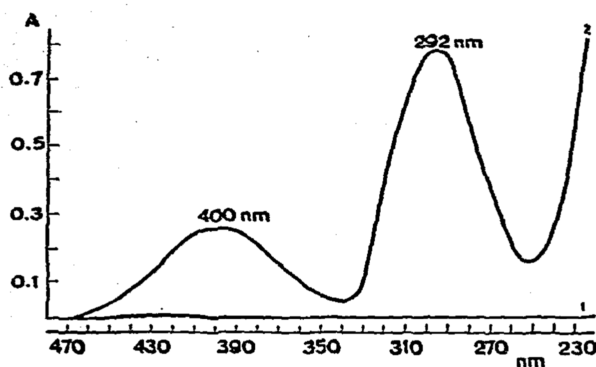


Fig. 4. UV absorption spectrum of rubreserine, 0.17% (2). 1 = water.

Fig. 6 shows the separation of pilocarpine, physostigmine, methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate. The peak from pilocarpine appears about 7 min after injection. Physostigmine salicylate forms two ion pairs and also two peaks, one due to salicylate (2) after 4 min and one due to physostigmine (4) after about 13 min. Methyl *p*-hydroxybenzoate appears after 11 min (3). After *ca.* 15 min the flow is increased to 2 ml/min in order to accelerate the elution of propyl *p*-hydroxybenzoate. The signal from the detector has been amplified twenty times.

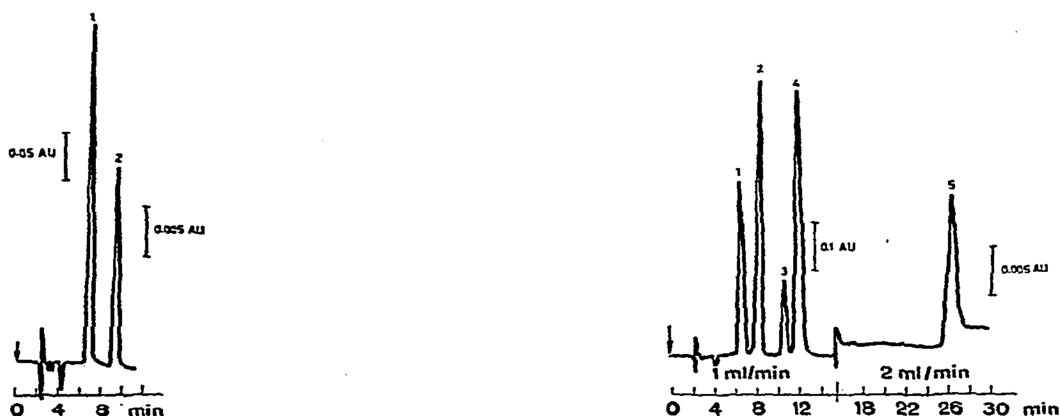


Fig. 5. HPLC of pilocarpine (1) and phenethyl alcohol (2). Detection: UV, 235 nm.

Fig. 6. HPLC of pilocarpine, physostigmine and preservatives. Peaks: 1 = pilocarpine; 2 = salicylate; 3 = methyl *p*-hydroxybenzoate; 4 = physostigmine; 5 = propyl *p*-hydroxybenzoate. Detection: UV, 235 nm.

Stability testing

Degradation of pilocarpine (Fig. 7). Pilocarpine and isopilocarpine have the same retention times and therefore cannot be separated by this method. However, at pH 3–5, which is normally found in the ophthalmic solutions, the stability of pilocarpine is good.

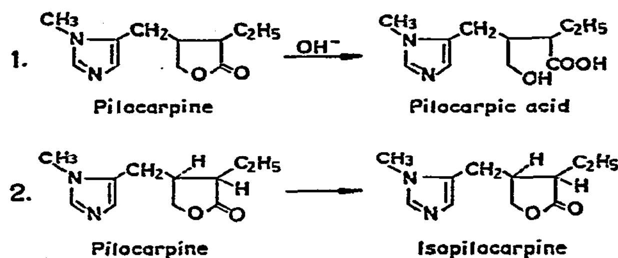


Fig. 7. Degradation of pilocarpine.

Degradation of physostigmine (Fig. 8). Physostigmine is less stable, particularly at pH > 5. During the production of the eye-drops the pH is adjusted to 4.5–5.0.

Physostigmine is hydrolyzed to eseroline, methylamine and carbon dioxide. Eseroline is colourless but is oxidized to rubreserine, which is red. Aged solutions are therefore often red. Fig. 9 shows a chromatogram of an aged physostigmine solution. Rubreserine has been prepared in the laboratory and is used as a reference sample; detection limit, 0.001 μg. A concentration of 0.5 μg/ml rubreserine can be detected by the eye as a very slight discolouration. A sample containing 1 μg/ml is distinctly pink.

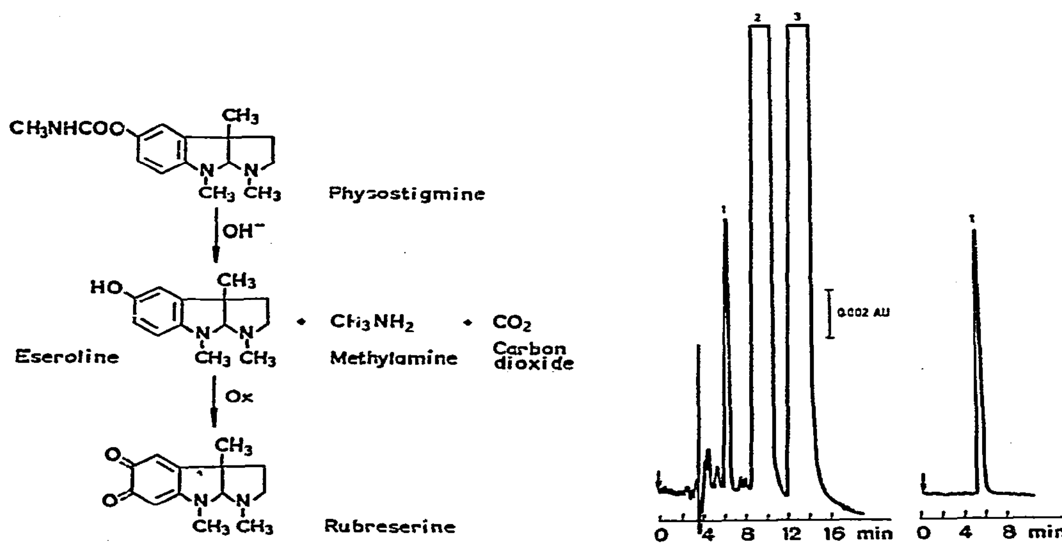


Fig. 8. Degradation of physostigmine.

Fig. 9. HPLC of an aged physostigmine solution (left) and a rubreserine standard solution (right). Peaks: 1 = Rubreserine; 2 = salicylate; 3 = physostigmine. Detection: UV, 292 nm.

RESULTS AND DISCUSSION

Capacity factors, sample concentrations and detection limits have been calculated (Table I). Optimum results are achieved if the capacity factors are between 2

and 6. Rubreserine has the lowest value (1.7) and physostigmine the highest (5.1). The relative standard deviation calculated for the peak height was 1.3% for pilocarpine and 0.3% for physostigmine, and the retention time was for pilocarpine 0.2% and 0.1% for physostigmine.

TABLE I

CAPACITY FACTORS (k'), SAMPLE CONCENTRATIONS AND DETECTION LIMITS

Compound	k'	*Sample conc. (μg)*	*Detection limit (μg)*
Rubreserine	1.7	0.02	0.001
Pilocarpine	2.3	80	0.02
Salicylate	2.9	8	—
Phenethyl alcohol	3.5	10	—
Methyl *p*-hydroxybenzoate	4.2	0.8	—
Physostigmine	5.1	8	0.003

The described method is rapid and accurate. A separation and quantitative analysis of rubreserine, pilocarpine, methyl *p*-hydroxybenzoate and physostigmine is completed in *ca.* 15 min.

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