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Short communication

Development and validation of a stability-indicating high performance liquid chromatographic assay for benoxinate

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Abstract

Benoxinate is a local anaesthetic used for ophthalmic applications. The aim of this study was to develop a rapid and simple stability-indicating method for the determination of benoxinate formulated for ophthalmic use, evaluate its long-term stability and identify its major degradation product. Benoxinate was eluted on a 10 μm Spherisorb phenyl column, 250 × 3.2 mm, with a mobile phase consisting of acetonitrile-buffer (pH 3.5) (35:65, v/v), pumped at 0.8 ml min^{-1} flow rate. The buffer was composed of sodium dihydrogen phosphate (50 mM), sodium hydrogen sulfate (2.5 mM) and 1-heptanesulfonic acid sodium salt (5 mM). The analyte was quantified spectrophotometrically at 308 nm. The chromatograms of benoxinate formulations obtained by this method showed benoxinate ($t = 4.5$ min) well resolved from its degradation product ($t = 2.3$ min), which was separately identified by means of HPLC-MS as 4-amino-3-butoxybenzoic acid. The assay was demonstrated to have high accuracy, precision and linearity. The method was implemented in investigating the long-term stability of benoxinate 0.4% ophthalmic solutions. The method was found to be simple, quick and selective in determining benoxinate concentrations in fresh and aged preparations.

Keywords: Benoxinate; Stability-indicating assay; Reversed phase chromatography; Stability; HPLC-MS; Analytical method validation

1. Introduction

Benoxinate hydrochloride (4-amino-3-butoxy-benzoic acid 2-(diethylamino) ethyl ester, monohydrochloride, Fig. 1), also known as oxybuprocaine hydrochloride, is one of the family of ester type local anaesthetics, which is used in ophthalmology, otology, rhinology and laryngology. It is applied effectively in short ophthalmologic procedures such as minor eye surgery, tonometry, fitting of contact lenses and local analgesia of the injured eye [1,2].

A number of methods have been published for the determination of benoxinate. For example, the

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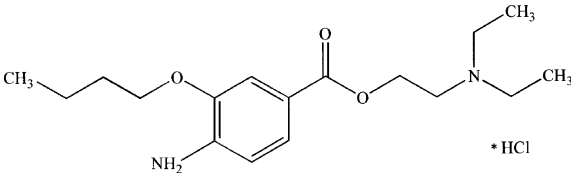

Fig. 1. Chemical structure of benoxinate hydrochloride.

US Pharmacopoeia provides a complicated and time-consuming method, which involves multiple ether extractions and spectrophotometric determination [3]. Chromatographic methods include gas chromatography [4] and high performance liquid chromatography (HPLC) with UV or electrochemical detection [5,6]. However, none of the published studies describes a stability-indicating method designed to investigate degradation products in benoxinate preparations.

Our interest in benoxinate ophthalmic solution arose from the fact that in spite of its wide use, stability data or methods for analysis of the degradation products have not been well documented. In the only study dedicated to the stability of benoxinate that has to our knowledge been published to date, the hydrolysis kinetics were studied as a function of pH and the degradation product was identified as 4-amino-3-butoxybenzoic acid [7].

The current study describes a simple, rapid, stability-indicating method for determination of both the active ingredient and the degradation product of benoxinate hydrochloride in ophthalmic solution. The determination is accomplished by means of HPLC with UV detection. Identification of the degradation product was confirmed by HPLC with mass spectrometric (MS) detection.

2. Experimental

2.1. Chemicals and reagents

Benoxinate hydrochloride, 1-heptanesulfonic acid sodium salt and ammonium acetate (SigmaUltra grade) were from Sigma (St. Louis, MO). Sodium dihydrogen phosphate monohydrate and sodium hydrogen sulfate monohydrate were from Merck (Darmstadt, Germany). Benoxinate 0.4% ophthalmic solutions (Localin®) were from Fischer Pharmaceutical Labs (Bnei Brak, Israel). HPLC-grade acetonitrile was from J.T. Baker (Deventer, Holland). Water was purified with a tandem RiOs (reverse osmosis)/Milli-Q Gradient A-10 system (Millipore, Molsheim, France). All chemicals were of analytical reagent grade.

2.2. Equipment

The chromatographic system consisted of an HPLC model HP 1090L (Hewlett Packard, Palo Alto, CA), including a photodiode-array detector set to 308 nm and interfaced to an HP ChemStation. A 10 μm Spherisorb phenyl column, 250 × 3.2 mm (Phenomenex, Torrance, CA) was used with a 10 μl injector loop.

To identify the major degradation product, degraded benoxinate hydrochloride solutions were analyzed using HPLC with MS detection. The system consisted of a Hewlett Packard model HP 1100 HPLC (Palo Alto, CA) in tandem with a PE-Sciex API 365 Triple Quad mass spectrometer (Concord, Canada). The UV detector was set to 308 nm and the MS conditions were as follows: PPG Q1 Positive, mass range 100–500 amu, step 0.1 amu and dwell time 1 s. A 5 μm Rocket C-18 column, 53 × 4.6 mm (Alltech, Deerfield, IL) was used.

For pH measurements a Metröhm model 744 pH-meter (Herisau, Switzerland) with a combined glass electrode was used.

2.3. Mobile phases

The mobile phase used for the HPLC assay was acetonitrile-buffer (pH 3.5) (35:65, v/v). The buffer was composed of sodium dihydrogen phosphate (50 mM), sodium hydrogen sulfate (2.5 mM) and 1-heptanesulfonic acid sodium salt (5 mM). Flow rate was 0.8 ml min^{-1}. The buffer was prepared by dissolving 6.90 g sodium dihydrogen phosphate monohydrate and 0.35 g sodium hydrogen sulfate

monohydrate in about 950 ml water in a 1-l volumetric flask, adding 1.01 g 1-heptanesulfonic acid sodium salt, adjusting the pH to 3.5 with NaOH (1M) and diluting to volume.

The mobile phase for the HPLC-MS analysis was acetonitrile-ammonium acetate buffer (pH 3.5; 10 mM) (30:70, v/v) pumped at a flow rate of 2.0 ml min^{-1}. The buffer was prepared by dissolving 0.77 g ammonium acetate in water in a 1-l volumetric flask, adjusting the pH with glacial acetic acid and diluting to volume.

The mobile phases were filtered under vacuum through 0.45 μm nylon filters (Millipore, Bedford, MA) and degassed with helium.

2.4. Stock, standard and sample preparation

A stock solution of benoxinate hydrochloride was prepared by dissolving 20.0 mg of the compound in 100 ml water. Appropriate dilutions were made in the mobile phase for the HPLC assay, to final concentrations of 4.8, 6.4, 8.0, 9.6 and 11.2 μg ml^{-1} for the external standard calibration curve.

Forced degradation studies of benoxinate were performed by adding 2 ml of either NaOH (0.1 M), HCl (0.03 M) or water to 5 ml of benoxinate 0.4% ophthalmic solution and incubating the solutions thus obtained at 85 °C for 20 h.

For HPLC-MS analysis an aged, partially degraded benoxinate 0.4% ophthalmic solution was diluted 1:10 in the HPLC-MS mobile phase. A completely degraded sample was prepared by adding 2 ml NaOH (0.1M) to 5 ml of the benoxinate hydrochloride stock solution, incubating at 85 °C for 20 hours and then diluting with an equal volume of the HPLC-MS mobile phase. Retention times' verification was performed by spiking of the partially degraded with the fully degraded sample.

Duplicate samples of benoxinate 0.4% ophthalmic solution were prepared for analysis by diluting 1 ml of the sample with water to 100 ml, 5 ml of which were further diluted to 25 ml with the mobile phase.

3. Results and discussion

3.1. HPLC method development

The benoxinate molecule possesses ionizable amine functions, which prompted the use of reversed-phase HPLC with an anionic ion-pairing agent and an acidic buffer. A mobile phase including phosphate/sulfate buffer (pH 3.5) and acetonitrile was used in order to optimize the chromatographic separation of benoxinate and its degradation product, 4-amino-3-butoxybenzoic acid. Among the different compositions of the mobile phase tested (30–60% acetonitrile), 35% acetonitrile was found to give optimum separation and peak shape, combined with a short analysis time.

The light absorption spectra of benoxinate and 4-amino-3-butoxybenzoic acid are very similar revealing maxima at 308 and 305 nm, respectively (Fig. 2). The detector wavelength was therefore set at 308 nm.

3.2. Separation performances

The elution times of benoxinate and the degradation product separated by the described method were 4.5 and 2.3 min, respectively (Fig. 3). Overall system performances are listed in Table 1. The relative standard deviation (RSD) ($n = 4$) of both the capacity factors (k') and the number of theoretical plates (N) for the components were lower than 1.0% each. The selectivity (α) of benoxinate with respect to the degradant as calculated from the k' values, was found to be 3.14. These values are adequate for the development of a validated analytical method [8].

3.3. Validation of the method

The HPLC assay for benoxinate ophthalmic solutions, as described in Section 2 above using an external standardization method, was validated as in Fig. 3.

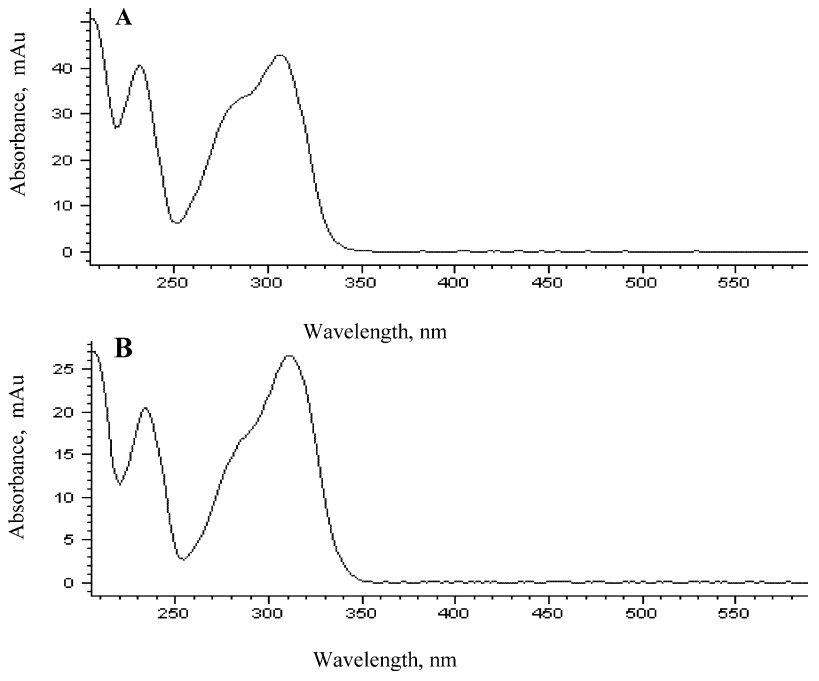

Fig. 2. UV/Vis absorption spectra (205–600 nm) of (A) benoxinate; (B) the degradant, 4-amino-3-butoxybenzoic acid.

3.4. Linearity

Five standard solutions of benoxinate were freshly prepared in the concentration range 4.8–11.2 μg ml^{-1}, corresponding to 60–140% of the nominal concentration of the diluted samples (8.0 μg ml^{-1}). The detector response expressed as the area of the benoxinate peak was found to be linear with concentration. The intercept and slope of the calibration curve were −18.1 mAU s and 35.2 mAU s (μg $ml^{-1})^{-1}$, respectively. The values obtained for the correlation coefficient (r^2) and the quality coefficient [9] were 0.9998 and 0.46%, respectively.

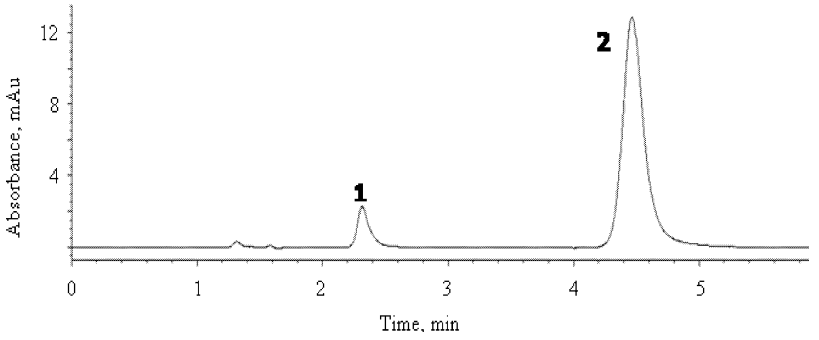

Fig. 3. Chromatogram of an aged benoxinate ophthalmic solution after 12 years of storage at ambient temperature. Peak 1: benoxinate degradation product. Peak 2: benoxinate. Column: 10 μm Spherisorb phenyl, 250 × 3.2 mm. Eluent: 35:65 mixture of acetonitrile and a buffer consisting of 50 mM sodium dihydrogen phosphate, 2.5 mM sodium hydrogen sulfate containing and 5 mM 1-heptanesulfonic acid sodium salt (pH 3.5). Flow rate: 0.8 ml min^{-1}. Injection volume: 10 μl. Detection: 308 nm.

Table 1
The performance of benoxinate and its degradation product separation by the assay (see Fig. 3)[a]

Parameter	Benoxinate	Degradant
k'	2.82	0.90
N	4800	6500
SF	0.48	0.85
TF	1.71	1.28
RSD (%)	0.51	0.77

[a] k' is the capacity factor, N the number of theoretical plates of the column (calculated by the halfwidth method), *SF* the symmetry factor of the peaks, *TF* the tailing factor, and *RSD* (%) the relative standard deviation of peak areas ($n = 4$).

3.5. Repeatability

The repeatability was determined by five consecutive injections of the same sample solution containing 8.0 μg ml^{-1} benoxinate. The recovered amount equalled 7.98 ± 0.03 μg ml^{-1}.

Inter-day variation is summarized in Table 2. The values presented in the table are the mean of four consecutive injections. The mean and standard deviation of all results over the 3 days ($n = 12$) was 7.98 ± 0.03 μg ml^{-1}, demonstrating excellent precision for the method.

3.6. Accuracy

Solutions containing benoxinate at 80, 100 and 120% of the nominal concentration in the benoxinate ophthalmic solution samples (0.4%) were prepared in duplicate by dilution of a 0.8% standard. The solutions were processed according to the sample preparation method and the benoxinate concentrations were determined. The mean recovery values are shown in Table 3 and were in the range 100.0 ± 1.0%.

Table 2
Inter-day assay variation of benoxinate sample (8.0 μg ml^{-1})

	Concentration ($n = 4$) of benoxinate found (μg ml^{-1})
Day 1	7.96 ± 0.16
Day 2	8.01 ± 0.27
Day 3	7.97 ± 0.04

3.7. Selectivity

Selectivity was assessed by the chromatography of standard, aged and stressed benoxinate samples. Under stressed conditions, after incubation of benoxinate in NaOH (0.1 M) at 85 °C for 20 h the benoxinate peak disappeared while the peak of the degradation product increased. The same degradation product was present in a smaller amount (about 4% degradation) after incubation in acidic conditions. The incubation of benoxinate without adding acid or base did not lead to any significant changes in the chromatograms compared to the original solution not subjected to forced degradation. No other peaks were present on the chromatograms of the stressed samples. The peak purity was confirmed by comparing the spectra on the upslope and downslope of both peaks in each of the samples. Hence, this HPLC assay is a stability-indicating method in that both benoxinate and its single observed degradation product are detected and well separated from each other.

3.8. Identification of the degradation product

In order to identify the chemical structure of the degradant, HPLC analysis with MS detection was performed. The separation was achieved using different HPLC conditions and a mobile phase consisting of volatile components as described above. The chromatogram of an aged, partially degraded benoxinate ophthalmic solution is shown in Fig. 4 and demonstrate an adequate separation of benoxinate (Peak B, $t = 5.5$ min) from the degradant peak (Peak A, $t = 4.5$ min). Peak A in Fig. 4 was identified as equivalent to Peak 1 in Fig. 3 by spiking with a fully degraded sample.

A completely degraded benoxinate solution was used for mass spectral identification of the degradation product. The mass spectrum of the degradant peak at $t = 4.5$ min (corresponding to Peak A in Fig. 4) is shown in Fig. 5 and suggested that it was 4-amino-3-butoxybenzoic acid [7]. The molecular ion (M + H) appears at ~210 amu, while the peak at ~419 amu is apparently a dimer (2M + H). The fragment at ~152 amu is probably due to loss of the butyl group. Cleavage of the carbon–

Table 3
Accuracy of assay for benoxinate

Sample solution	Applied concentration (% of nominal)	Mean concentration found ($n = 2$) (% of nominal)	Recovery (%)
1	80.0	80.83	101.0
2	100.0	99.75	99.8
3	120.0	121.21	101.0

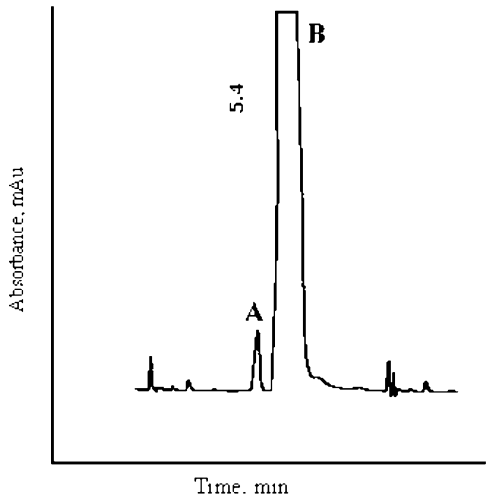

Fig. 4. HPLC chromatogram of an aged benoxinate ophthalmic solution stored for 5 years at ambient temperature obtained by a method suitable for HPLC-MS analysis (See in text). Column: 5 μm Rocket C-18, 53 × 4.6 mm. Mobile phase: ammonium acetate (pH 3.5; 10 mM)-acetonitrile (70:30, v/v). Flow: 2.0 ml min^{-1}. Detection: 308 nm. Peak A: benoxinate degradation product. Peak B: benoxinate.

oxygen bond leading to the formation of this fragment has previously been described in o-methoxyaniline [10] that is expected to have a similar fragmentation pattern.

3.9. Quantitative examination of new and aged benoxinate 0.4% ophthalmic solutions

As described above, the proposed chromatographic assay allows a rapid separation and an accurate analysis of benoxinate samples with the parent compound and its degradation product. Additional significant advantage of the developed method which is particularly intended for stability studies is the straightforward procedure for the sample preparation by a simple dilution. Since this procedure does not involve steps that may potentially lead to a drug loss or degradation (i.e. extraction, heating, etc.) the assay is effective without the need for internal standard addition. The assay therefore uses the more facile external standardization method.

The results of the long-term stability of benoxinate 0.4% ophthalmic solution are summarized in Fig. 6. In this study 32 batches that had been manufactured over a 12-year period and kept at recommended ambient temperature storage conditions were analyzed. The chromatograms of all batches revealed two peaks corresponding to benoxinate and the degradation product. The degradation product, 4-amino-3-butoxybenzoic acid, results from hydrolysis of the parent compound, 4-amino-3-butoxybenzoic acid 2-(diethylamino) ethyl ester.

The reaction is rapid in basic solution but also proceeds slowly under acidic conditions. According to the US Pharmacopoeia [3], the required pH of benoxinate ophthalmic solution is between 3.0 and 6.0. The pH of aged benoxinate solutions analyzed for this study was in the range 4.0–4.3. Hence, under normal storage conditions, it is likely that small amounts of the hydrolysis product would be formed.

The resemblance of the absorption spectra and the close values of the molar absorptions of benoxinate and the degradation product were utilized for quantitation of the latter. In all batches the total observed substance (sum of benoxinate and degradant amounts expressed as a percent of the labeled amount of benoxinate in the formulation) approximated 100% benoxinate formulation content and was not dependent on the storage period (Fig. 6). This indicates that there was no significant degradation by another pathway to additional products that were not detected by the

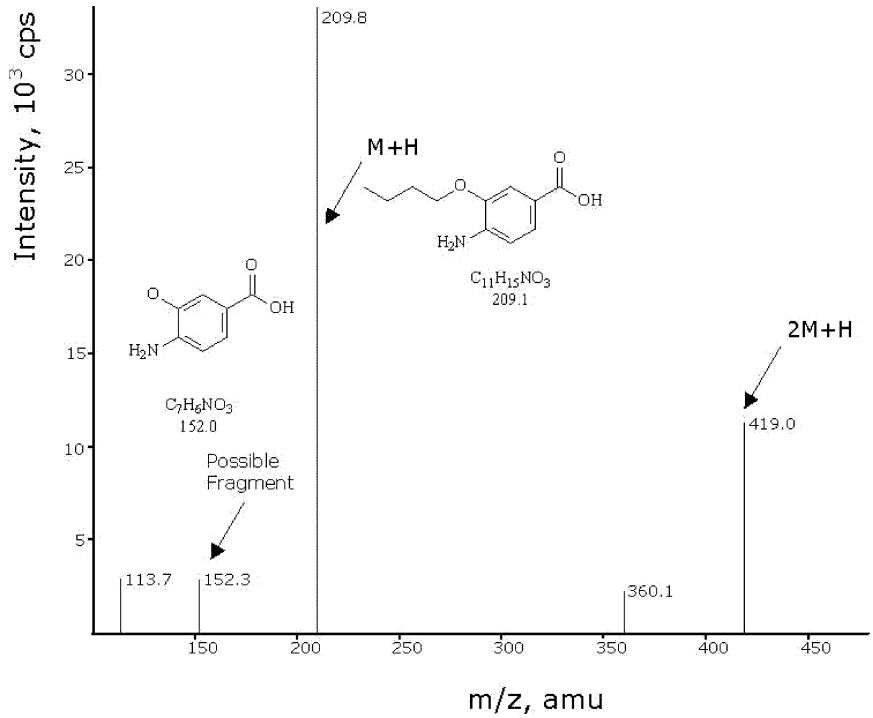

Fig. 5. Q1 Positive mass spectrum of the degradation product of benoxinate hydrochloride.

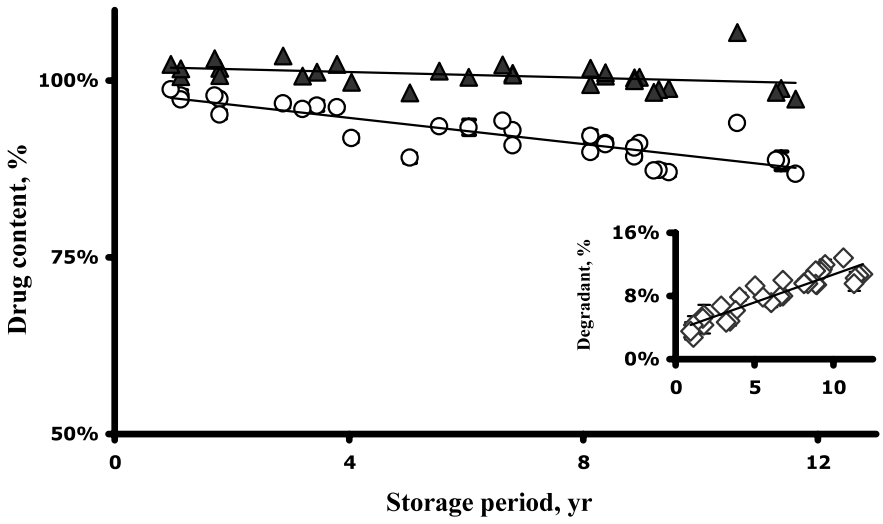

Fig. 6. Stability profile of benoxinate 0.4% ophthalmic solutions. The benoxinate hydrochloride content calculated as % of the labeled amount is presented as a function of storage period. Total amount was calculated as a sum of benoxinate and degradant contents in each batch. Insert: degradant content expressed as % of the labeled amount of benoxinate. Relative standard error between the duplicates was less than 2% for both the drug and degradant in all tested samples. All points are means ± S.D. of four consecutive measurements. Some error bars are too small to be shown. ○, benoxinate HCl; ▲, simulated total; ◇, degradant.

HPLC assay method described here. It also provides evidence for the high accuracy of quantification of the degradant, which could not be estimated directly due to the non-availability of a pure sample of 4-amino-3-butoxybenzoic acid.

A quantifiable level of the degradant was found both in the fresh and aged batches. The concentration of benoxinate in the formulation notably decreased with the storage period (Fig. 6). According to these results, benoxinate ophthalmic solution (0.4%) stored at ambient temperature would be expected to degrade to 95% of its initial content (the lower limit specified by the US Pharmacopoeia) in approximately 5 years.

4. Conclusions

A rapid and simple stability-indicating HPLC assay for benoxinate ophthalmic solutions was developed and validated. The identity of the degradation product was confirmed by HPLC-MS. The assay was applied in a long-term stability study of benoxinate 0.4% ophthalmic solutions providing an estimation of the commercial formulation's shelf life.

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