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First derivative spectrophotometric and LC determination of benoxinate hydrochloride and its degradation products

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Abstract

Two methods are presented for the determination of benoxinate HCl and its acid and alkali-induced degradation products using first derivative (^1D) spectrophotometry with zero-crossing measurements and liquid chromatography. Benoxinate HCl was determined by measurement of its first derivative amplitude in mcllvaine's-citric acid phosphate buffer pH 7.0 at 268.4 and 272.4 nm in the presence of its alkali- and acid-induced degradation products, respectively. The acid- and alkali-induced degradation products were determined by measurement of their first derivative amplitude in the same solvent at 307.5 nm. The LC method depends upon using a μ bondapak CN column with a mobile phase consisting of acetonitrile–water–triethylamine (60:40:0.01, v/v) and adjusted to apparent pH 7. Quantitation was achieved with UV detection at 310 nm based on peak area. The proposed methods were utilized to investigate the kinetics of the acidic and alkaline degradation processes at different temperatures. The pH-rate profile of degradation of benoxinate HCl in Britton–Robinson buffer solutions was studied.

Keywords: Benoxinate HCl; LC; First derivative spectrophotometry; Ophthalmic solution; Kinetics of degradation; pH-Rate profile; Arrhenius plot

1. Introduction

Benoxinate HCl or Oxybuprocaine HCl, 2-(diethylamino) ethyl 4-amino-3-butoxybenzoate hydrochloride, is a surface anaesthetic of the ester type used as a 0.4% solution in short ophthalmological procedures [1]. The official method for determination of benoxinate HCl is the non-aqueous titration using standard perchloric acid as titrant [2,3]. The literature survey reveals a direct UV absorbance measurement after clean-up procedure for determination of benoxinate HCl in ophthalmic solution [2], other spectrophotometric methods through ion pair formation with thiocyanate and molybdenum^{V} or cobalt^{II} [4] and halofluorescein derivatives [5] were applied. Various methods have been reported for the determination of benoxinate and its metabolites includes gas chromatography–mass spectrometry [6], high performance liquid chromatography [7], fluorodensitometry [8], differential calorimetry [9] and

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ion-selective electrodes [10]. However, no method has been reported for the assay of benoxinate HCl in presence of its degradation products. Therefore, it was thought necessary to develop a simple, fast and accurate stability-indicating method for the determination of benoxinate HCl in the presence of its degradation products.

The present work presents two methods for determining benoxinate HCl and its acid- and alkali-induced degradation products using first derivative spectrophotometry and high performance liquid chromatography. Furthermore, the developed methods were used to investigate the kinetics of the acid and alkaline drug degradation at different temperatures. The proposed HPLC method was used for pH-rate profile study of degradation of benoxinate HCl in Britton–Robinson buffer solutions. The two proposed methods were used for determination of benoxinate HCl and its degradation products in fresh and expired commercial ophthalmic solutions without any interference from the excipients normally used in ophthalmic solution formulations.

2. Experimental

2.1. Instrumentation

A double-beam Shimadzu (Japan) UV-Visible spectrophotometer, model UV-1601PC, connected to a promax computer fitted with UVPC personal spectroscopy software version 3.7 (Shimadzu) and a Hewlett Packard DeskJet 600 printer was used. The spectral band width was 2 nm and the wavelength scanning speed was 2800 nm min^{-1}.

The first derivative curves of the spectra of test and reference solutions were recorded in 1-cm quartz cells over the range 350–220 nm with $\Delta\lambda = 4$ nm and scaling factor of 10.

The HPLC (Waters Associates, Milford, MA) instrument was equipped with a model 600 pump, Rheodyne injector with a 20 μl loop and model 996 photodiode array detector. Separation and quantitation were made on a 150 × 3.9 mm (i.d.) Waters μ Bondapak CN Column (10 μm particle size). Detection was made at 310 nm. Peaks data handling was performed with a Waters Millennium 2010 Chromatography Manager software (version 2.15.01) and a Hewlett Packard laser Jet 5 L printer.

Chromatoplates (20 × 20 cm, aluminium plates precoated with 0.25 mm silica gel GF 254) were purchased from E. Merck (Darmstadt, Germany) and used in TLC studies with mobile phase consisted of chloroform–methanol (95:5, v/v). The spots were examined at 254 nm using a UV source for TLC (Desaga, Heidelberg, Germany).

The melting point was recorded on Stuart Scientific melting point apparatus (UK) using open glass capillaries.

The infrared spectrophotometer used was a Shimadzu IR-408.

PMR spectra were recorded on a Varian Gemini 200, using tetramethylsilane as the internal standard.

A Shimadzu mass spectrometer model GCMS-QP 1000 EX was used.

2.2. Materials and reagents

Benoxinate HCl (Orgamol, Switzerland) was kindly supplied by Alexandria Company for Pharmaceutical and Chemical Industries (Egypt), and its purity was certified and analyzed to be 99.80%. The water for HPLC was prepared by double glass distillation and filtration through a 0.45-μm membrane filter. The acetonitrile, chloroform and methanol were HPLC grade (Romil Chem, UK). Triethylamine, sodium hydroxide, disodium monohydrogen phosphate dihydrate, citric, hydrochloric, phosphoric acids were analytical grade.

Mcllvaine's-citric acid phosphate buffer pH 7.0 was prepared by mixing 181.5 ml of 0.1 M citric acid monohydrate and 818.5 ml of 0.2 M disodium monohydrogen phosphate dihydrate.

The commercial ophthalmic solution, Boxinate, was supplied by Alexandria Company for Pharmaceutical and Chemical Industries. Each 100 ml of ophthalmic solution contains 0.4 g benoxinate HCl with phenyl mercuric nitrate, hydroxy propyl methyl cellulose 4000 cp, sodium chloride and boric acid.

2.3. HPLC conditions

The mobile phase was prepared by mixing acetonitrile, water and triethylamine in a ratio of 60:40:0.01 v/v (pH* 10.0), and the apparent pH was adjusted to 7.0 using 0.5 M phosphoric acid. The mobile phase was filtered using a 0.45-μm membrane filter (Millipore, Milford, MA) and degassed by vacuum prior to use. The samples were also filtered using 0.45-μm disposable filters. The flow rate was 2 ml min^{-1}. All determinations were performed at room temperature. The injection volume was 20 μl.

2.4. Preparation of the alkali-induced degradation product

Accurately weighed benoxinate HCl (1 g) was refluxed with 100 ml 0.1 N sodium hydroxide for 2 h. Subsequently the pH of the solution was adjusted to 5.3 using 1 N hydrochloric acid to precipitate the degradation product. The precipitate was filtered, dried under vacuum while protecting from air and light. The melting point was 141°C ($R_f = 0.41$; using the above discussed TLC system).

The stock solution of the alkali-induced degradation product was prepared by dissolving 25 mg of the substance in 25 ml methanol.

2.5. Preparation of the acid-induced degradation products

Accurately weighed benoxinate HCl (1 g) was refluxed with 100 ml 1 N hydrochloric acid for 12 h. Subsequently, the pH of the solution was adjusted to 5.3 using 3 N sodium hydroxide to precipitate the degradation products. The precipitate was filtered, dried under vacuum and protected from air and light. TLC of the dried precipitate indicated the occurrence of two components, namely, 4-amino-3-butoxybenzoic acid with $R_f = 0.41$ and 2-butoxyaniline with $R_f = 0.82$; using the above discussed TLC system.

The stock solution of acid-induced degradation products mixture prepared by dissolving 25 mg of the acid-induced degradation products prepared as above in 25 ml methanol for the ^1D method.

The two components of the acid-induced degradation were separated using glass column (20 × 2 cm) packed with silica gel 60 (0.063–0.200 mm particle size) (E. Merck, Darmstadt, Germany) and mobile phase consisted of chloroform–methanol (98:2, v/v). Each of the two components were separated from the mobile phase, dried under vacuum and protected from air and light.

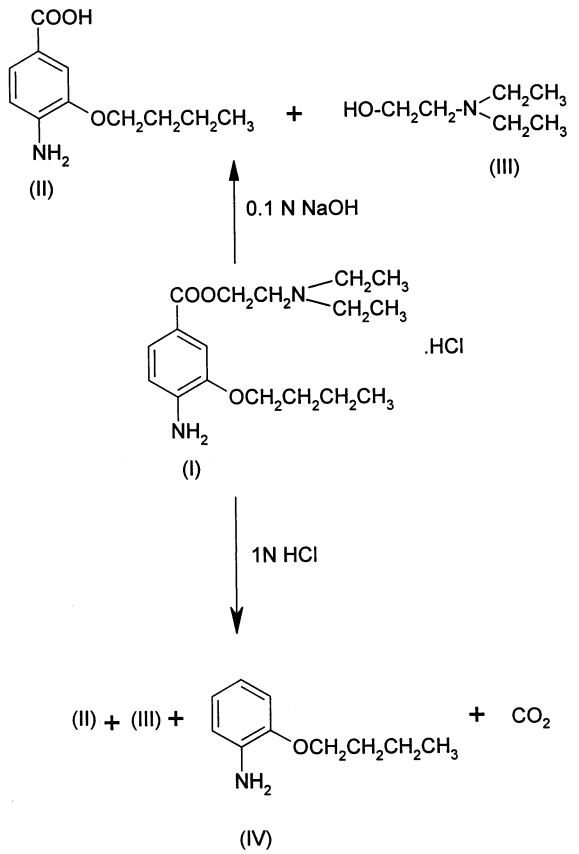
The stock solution of each of 4-amino-3-butoxybenzoic acid and 2-butoxyaniline were prepared by dissolving 25 mg of each in 25 ml methanol for the HPLC method.

2.6. Standard solutions and calibration graphs

2.6.1. First derivative method

Stock solutions were prepared by dissolving benoxinate HCl in water, 4-amino-3-butoxybenzoic acid and acid-induced degradation products in methanol to obtain a concentration of 1000 μg ml^{-1} for each. The standard solutions were prepared by diluting the stock solutions with mcllvaine's-citric acid phosphate buffer pH 7.0 to reach concentration ranges of 10–30, 2–30 and 4–30 μg ml^{-1} for benoxinate HCl, 4-amino-3-butoxybenzoic acid and acid-induced degradation products, respectively. Working standard solutions containing 2, 20 and 30 μg ml^{-1} of 4-amino-3-butoxybenzoic acid and increasing concentrations of benoxinate HCl ranging from 10 to 30 μg ml^{-1}; 4, 20 and 30 μg ml^{-1} of acid-induced degradation products and increasing concentrations of benoxinate HCl ranging from 10 to 30 μg ml^{-1}; 10, 20 and 30 μg ml^{-1} of benoxinate HCl and increasing concentrations of 4-amino-3-butoxybenzoic acid ranging from 2 to 30 μg ml^{-1}; and 10, 20 and 30 μg ml^{-1} of benoxinate HCl and increasing concentrations of acid-induced degradation products ranging from 4 to 30 μg ml^{-1} were prepared from stock solutions by dilution with mcllvaine's-citric acid phosphate buffer pH 7.0.

The first derivative spectra (^1D) of these standard solutions were scanned in the range of 350–220 nm against solvent blank. The values of the ^1D amplitudes at 268.4 (zero-crossing of 4-amino-3-butoxybenzoic acid) and 272.4 nm (zero-crossing of acid-induced degradation products) were



Scheme 1.

measured for the determination of benoxinate HCl in presence of 4-amino-3-butoxybenzoic acid or acid-induced degradation products, respectively. The values of the ^1D amplitudes at 307.5 nm (zero-crossing of benoxinate HCl) were used for the determination of 4-amino-3-butoxybenzoic acid or acid-induced degradation products in presence of benoxinate HCl. The concentrations of each compound versus their absolute first derivative amplitudes were plotted in order to obtain the calibration graph.

2.6.2. HPLC method

Stock solutions were prepared by dissolving benoxinate HCl in water, 4-amino-3-butoxybenzoic acid and 2-butoxyaniline in methanol to obtain concentration of 1000 μg ml^{-1} of each. The standard solutions were prepared by dilution of stock solutions with mobile phase to reach concentration ranges of 10–30, 0.5–15 and 10–100 μg ml^{-1} for benoxinate HCl, 4-amino-3-butoxybenzoic acid and 2-butoxyaniline, respectively. Triplicate 20 μl injections were made for each concentration and chromatographed under the conditions described above. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph for each compound.

Table 1
Assignment of the IR characteristic bands of benoxinate HCl (**I**), 4-amino-3-butoxybenzoic acid (**II**) and 2-butoxyaniline (**IV**)

Wavenumber, cm^{-1}			Assignment
I	**II**	**IV**	
3460 and 3260[a]	3500 and 3350	3300	N–H stretching
2940[a]	2940	2940	Aliphatic and aromatic CH absorptions
2550 and 2460[a]	–	–	–NH stretching
–	2200–3200	–	Carboxylic O–H stretching
1710	–	–	Ester C=O
–	1660	–	Carboxylic C=O stretching
1610	1610	1610	N–H bending
1580 and 1525	1580 and 1525	1505	Aromatic C=C
–	1415 and 1300	–	In-plane C–O–H bending coupled with the C–O stretching of the carboxylic acid
1260	1260	1260	Ether=C–O–C
1220	1220	1220	C–N stretching band
1010	1010	1010	Ether=C–O–C

[a] Splits from a broad band between 2200 and 3500 cm^{-1}.

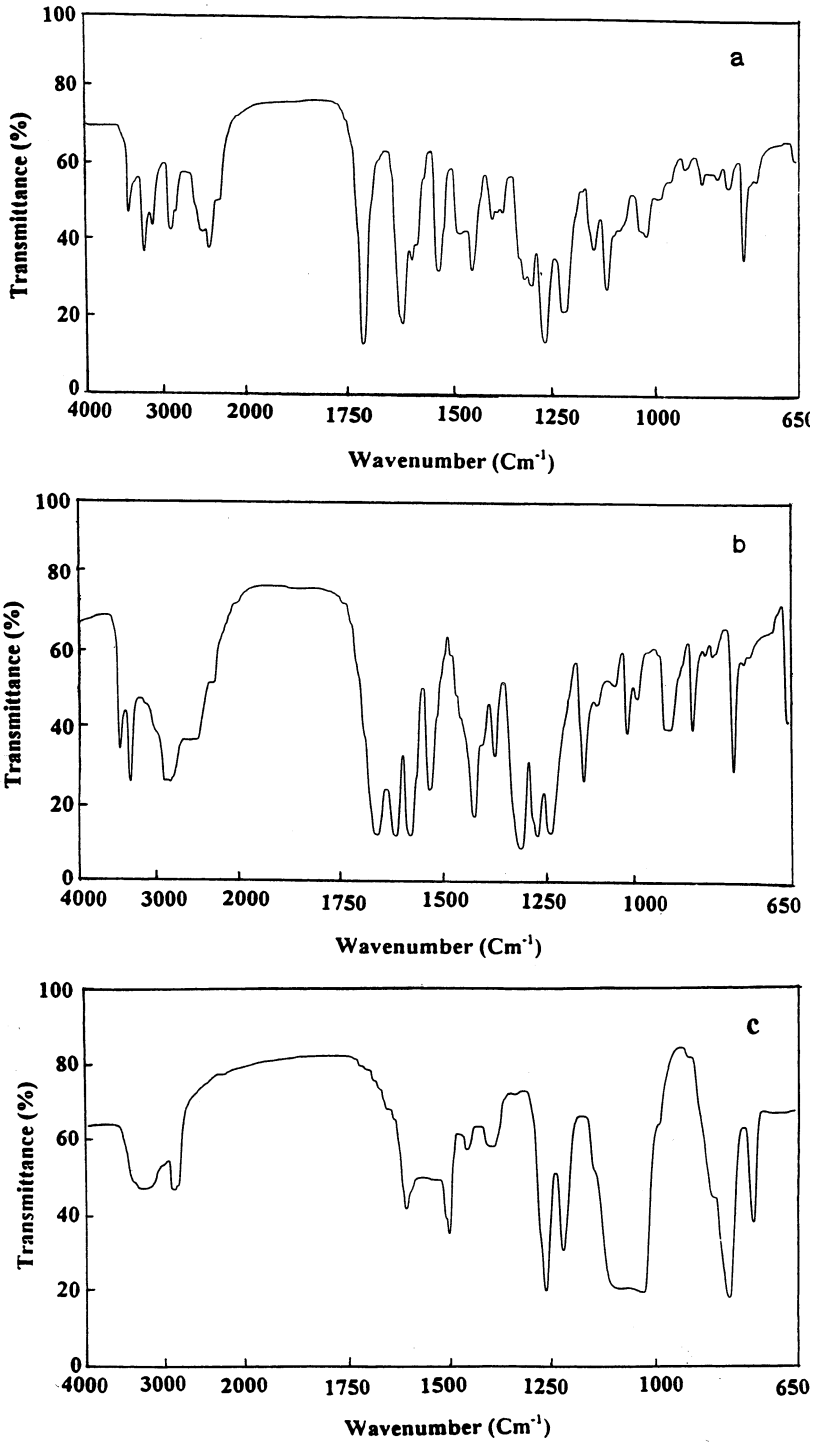


Fig. 1. IR spectrum of (a) benoxinate HCl, (b) 4-amino-3-butoxybenzoic acid and (c) 2-butoxyaniline in KBr.

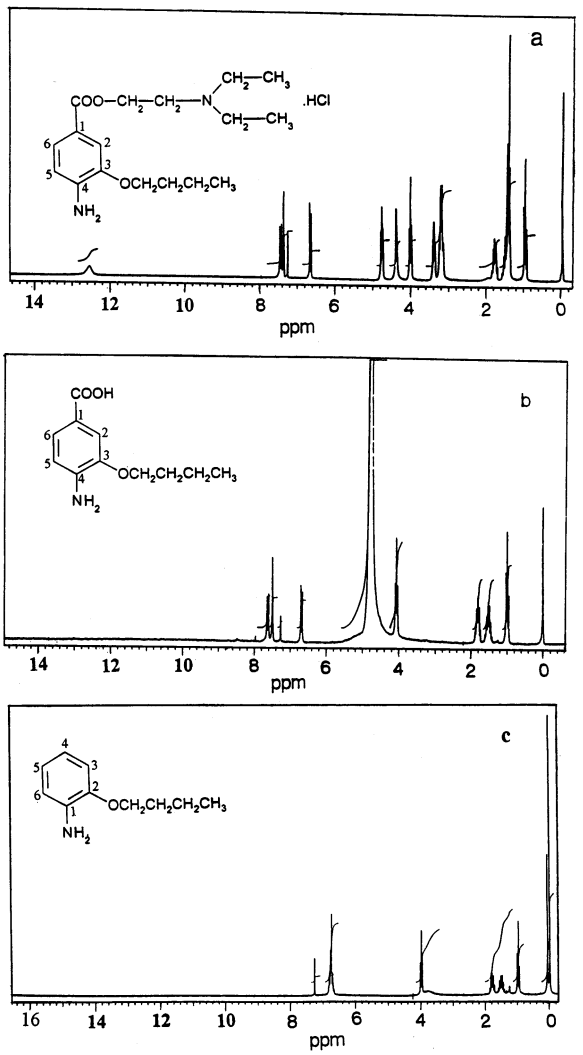


Fig. 2. PMR spectrum of (a) benoxinate HCl, (b) 4-amino-3-butoxybenzoic acid and (c) 2-butoxyaniline in deuterated chloroform.

2.7. Sample preparation

A volume of ophthalmic solution, equivalent to about 20 mg of benoxinate HCl was diluted to 100 ml with mcllvaine's-citric acid phosphate buffer pH 7.0 (for the ^1D method) or mobile phase (for the HPLC method). Further dilution of this solution was carried out with the same solvent to provide a solution of 20 μg ml^{-1}.

2.7.1. For first derivative method

The ^1D amplitude was measured for the sample solution as described under the calibration graph at 268.4 nm for determination of benoxinate HCl and at 307.5 nm for determination of degradation product (4-amino-3-butoxybenzoic acid) and their concentrations in sample were calculated from the regression equations.

2.7.2. For HPLC method

The sample solution was filtered through a 0.45-μm membrane filter. A 20-μl volume was injected into the HPLC, in triplicate, concurrently with the appropriate standard solutions (20, 10 and 10 μg ml^{-1} for benoxinate HCl, 4-amino-3-butoxybenzoic acid and 2-butoxyaniline, respectively) and chromatographed under the conditions described above. The peak area was used for determination of each compound in the sample.

2.8. Percent recovery study

This study was performed by adding different amounts of intact and acid- or alkali-induced degraded benoxinate HCl to a known concentration of the commercial ophthalmic solution (standard addition method). The resulting mixtures were assayed and the results obtained were compared with expected results (Tables 10–12).

2.8.1. Kinetic investigation

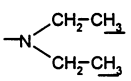
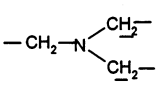
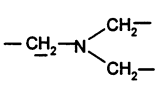
Accurately weighed benoxinate HCl (75 mg) was transferred into a 250-ml volumetric flask and diluted to volume with 1 N hydrochloric acid for acidic degradation or with 0.1 N sodium hydroxide for alkaline degradation. Separate 10-ml aliquots of this solution were transferred into separate stoppered conical flasks. The flasks were placed in a thermostatic oven at different temperatures (90, 80, 70, 65, 60°C for acidic degradation and 80, 70, 60, 50, 40°C for alkaline degradation) for different time intervals. At the specified time interval the contents of the flasks were neutralized to pH 7.0 using predetermined volumes of 1 N sodium hydroxide or 0.1 N hydrochloric acid solutions. The contents of the flasks were transferred into 100-ml volumetric flasks and diluted to volume with mcllvaine's-citric acid phosphate

buffer pH 7.0 (for the ^1D method) or mobile phase (for the HPLC method). For the ^1D method, the ^1D amplitude was measured for the solution directly as described under the calibration graph and the concentration of the remaining benoxinate HCl was calculated at each temperature and time interval. For HPLC method, aliquots of 20 μl of each solution were chromatographed under the conditions described above and the concentration of the remaining benoxinate HCl was calculated at each temperature and at time interval.

2.9. pH-Rate profile

Accurately weighed benoxinate HCl (75 mg) was transferred into 250-ml volumetric flask and

Table 2
PMR spectral assignment for benoxinate hydrochloride (**I**), 4-amino-3-butoxybenzoic acid (**II**) and 2-butoxyaniline (**IV**)

Chemical Shifts δ (ppm)			Multiplicity	No. of protons	Assignment
I	II	IV			
0.96 (J = 7.2 Hz)	0.99 (J = 7.2 Hz)	0.98(J = 7.4 Hz)	Triplet	3	$-OCH_2-CH_2-CH_2-\underline{CH_3}$
1.41 (J = 7.2 Hz)	____	____	Triplet	6	
1.46 – 1.60	1.49-1.60	1.42-1.60	Multiplet	2	$-OCH_2-CH_2-\underline{CH_2}-CH_3$
1.71 – 1.85	1.74-1.88	1.73-1.87	Multiplet	2	$-OCH_2-\underline{CH_2}-CH_2-CH_3$
3.22 (J = 7.2 Hz)	____	____	Quartet	4	
3.39 (J = 5.4 Hz)	____	_____	Triplet	2	
4.02 (J = 6.4 Hz)	4.06(J = 6.4 Hz)	4.00(J = 6.4 Hz)	Triplet	2	$-O\underline{CH_2}-CH_2-CH_2-CH_3$
4.39	4.76*	3.80	Singlet	2	Exchangeable $N\underline{H_2}$
4.76 (J = 5.4 Hz)	____	_____	Triplet	2	$COO\underline{CH_2}-$
		6.68-6.83	Multiplet	4	Aromatic $C\underline{H}$
6.67 (J = 8.4 Hz)	6.66(J = 8.4 Hz)		Doublet	1	Aromatic C_5-H
7.37	7.49		Singlet	1	Aromatic C_2-H
7.46 (J = 8.2 Hz)	7.61(J = 8.0 Hz)		Doublet	1	Aromatic C_6-H
12.55	_____	_____	Broad singlet	1	Exchangeable $N\underline{H}^+$

*Mixed with H_2O

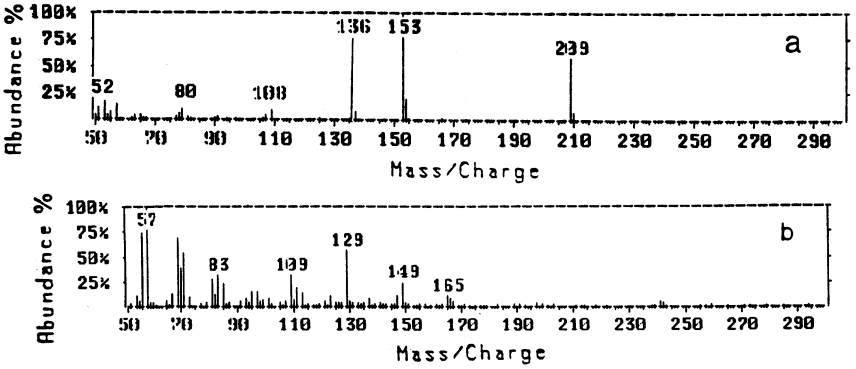


Fig. 3. Mass spectrum of (a) 4-amino-3-butoxybenzoic acid and (b) 2-butoxyaniline.

diluted to volume with Britton–Robinson buffer solutions [11]. The pH values of Britton–Robinson buffer solutions used for measurement of the pH-rate profile of the degradation of benoxinate HCl were as follows; pH 1.8, 2.6, 3.3, 4.6, 5.7, 6.8, 8.0, 9.2 and 11.9. The pH value of these buffer solutions was checked before and after the reaction, and were unchanged. The ionic strength of these buffer solutions was adjusted with sodium chloride. Separate 10-ml aliquots of the buffer solution containing benoxinate HCl (300 μg ml^{-1}) were transferred into separate stoppered conical flasks. The flasks were placed in a thermostatic oven at 80°C for different time intervals. At the specified time interval the contents of the flasks were neutralized to pH 7.0 using 1 N sodium hydroxide or 1 N hydrochloric acid solutions. The contents of the flasks were transferred into 100-ml volumetric flasks and diluted to volume with mobile phase. Aliquots of 20 μl of each solution were chromatographed under the conditions described above and the concentration of the remaining benoxinate HCl was calculated at each pH value and time interval.

3. Results and discussion

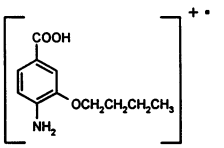
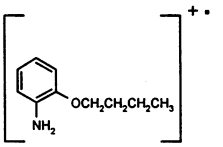
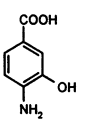
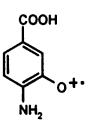
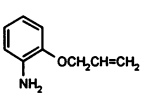
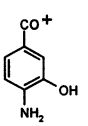
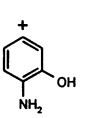
3.1. Identification of the degradation products

When benoxinate HCl was boiled with 0.1 N sodium hydroxide for 2 h, 4-amino-3-butoxybenzoic acid was produced. However, when benoxinate HCl was boiled with 1 N hydrochloric acid for 12 h, 4-amino-3-butoxybenzoic acid was formed along side 2-butoxyaniline. The suggested pathways for the degradation in 0.1 N sodium hydroxide and in 1 N hydrochloric acid are presented in Scheme 1. Using the above mentioned TLC system, the R_f values were found to be 0.20, 0.41 and 0.82 for **I**, **II** and **IV**, respectively.

The assignments of the degradation product **II** and **IV**, as 4-amino-3-butoxybenzoic acid and 2-butoxyaniline, respectively, were based on comparison of the IR and PMR spectral data of the purified specimens, separated from the degradation reaction, with those of the intact compound **I**. The IR spectrum of **II** (KBr; Fig. 1b, Table 1) was characterized by the O–H and C=O stretching and in-plane C–O–H bending coupled with the C–O stretching band of the COOH at 2200–3200, 1660, 1415 and 1300 cm^{-1}, respectively. The spectrum lacked the characteristic ester–C=O band of **I** (Fig. 1a, Table 1). By contrast, the IR spectrum of **IV** (KBr; Fig. 1c, Table 1) lacked the corboxylic acid and ester absorption bands and characterized by the presence of ether=C–O–C band at 1260 and 1010 cm^{-1}.

The PMR spectrum of **II** in deuterated chloroform (Fig. 2b) lacked the N–N diethylaminoethyl proton signals of **I**. The spectral assignment for **II** is shown in Table 2, whereas the PMR spectra of **I** and **IV** in the same solvent are illustrated in Fig. 2a,c and their assignments are given in Table 2. The PMR pattern of the aromatic proton signals

Table 3
Fragmentation pattern of 4-amino-3-butoxybenzoic acid* (**II**) and 2-butoxyaniline (**IV**)

m/z	Fragment	Abundance %	
		II	**IV**
209		58.1	–
165		–	11.0
153		100.0	–
152		13.2	–
149		–	23.9
136		76.9	–
109		8.5	31.2
108		14.0	5.8
57	$CH_3\text{–}CH_2\text{–}CH_2\text{–}CH_2^{\oplus}$	13.3	100.0
55	$CH_3\text{–}CH_2\text{–}CH_2\text{–}CH_2$ -2H	7.6	73.9

* The compound showed M+1 at 210 m/z (7.0%)

Table 4
Statistical analysis of the calibration graphs[a] of benoxinate hydrochloride and its acid- and alkaline-induced degradation products using first derivative spectroscopy for $n = 8$ standard specimens

Compound determined	Concentration of compound determined (μg ml^{-1})	Coexisting compound	Concentration of coexisting compound (μg ml^{-1})	Selected λ (nm)	Calibration graph	Intercept (a)	Standard deviation of intercept S(a)	Slope (b)	Standard deviation of the slope $S(b)$	Correlation coefficient (r)	C.V (%)	Calculated t[b]	b_o
Benoxinate HCl	10–30	–	–	268.4	P_0	8.34×10^{-4}	1.10×10^{-3}	12.14×10^{-3}	5.34×10^{-5}	0.9999	0.39	0.76	12.14×10^{-3}
Benoxinate HCl	10–30	4-amino-3-butoxybenzoic acid	2	268.4	P_1	2.21×10^{-3}	1.78×10^{-3}	12.07×10^{-3}	8.68×10^{-5}	0.9998	0.69	1.24	12.07×10^{-3}
Benoxinate HCl	10–30	4-amino-3-butoxybenzoic acid	20	268.4	P_2	1.77×10^{-3}	1.03×10^{-3}	12.09×10^{-3}	5.03×10^{-5}	0.9999	0.53	1.71	12.08×10^{-3}
Benoxinate HCl	10–30	4-amino-3-butoxybenzoic acid	30	268.4	P_3	-2.27×10^{-5}	1.22×10^{-3}	12.17×10^{-3}	5.96×10^{-5}	0.9999	0.41	0.02	12.18×10^{-3}
Benoxinate HCl	10–30	–	–	272.4	Q_0	-4.40×10^{-4}	1.87×10^{-3}	11.55×10^{-3}	9.10×10^{-5}	0.9998	0.58	0.24	11.55×10^{-3}
Benoxinate HCl	10–30	Acid-induced degradation products	4	272.4	Q_1	6.20×10^{-4}	1.47×10^{-3}	11.56×10^{-3}	7.18×10^{-5}	0.9999	0.47	0.42	11.55×10^{-3}
Benoxinate HCl	10–30	Acid-induced degradation products	20	272.4	Q_2	2.69×10^{-4}	1.35×10^{-3}	11.52×10^{-3}	6.54×10^{-5}	0.9999	0.41	0.20	11.52×10^{-3}
Benoxinate HCl	10–30	Acid-induced degradation products	30	272.4	Q_3	7.20×10^{-4}	1.27×10^{-3}	11.59×10^{-3}	6.16×10^{-5}	0.9999	0.44	0.57	11.58×10^{-3}
4-amino-3-butoxy-benzoic acid	2–30	–	–	307.5	R_0	6.15×10^{-4}	6.21×10^{-4}	16.71×10^{-3}	3.63×10^{-5}	0.9999	0.72	0.99	16.71×10^{-3}
4-amino-3-butoxy-benzoic acid	2–30	Benoxinate HCl	10	307.5	R_1	-9.82×10^{-5}	5.28×10^{-4}	16.71×10^{-3}	3.09×10^{-5}	0.9999	0.58	0.19	16.71×10^{-3}
4-amino-3-butoxy-benzoic acid	2–30	Benoxinate HCl	20	307.5	R_2	4.88×10^{-4}	8.13×10^{-4}	16.69×10^{-3}	4.75×10^{-5}	0.9999	0.78	0.60	16.69×10^{-3}
4-amino-3-butoxy-benzoic acid	2–30	Benoxinate HCl	30	307.5	R_3	-1.73×10^{-5}	4.61×10^{-4}	16.68×10^{-3}	2.69×10^{-5}	0.9999	0.48	0.04	16.67×10^{-3}
Acid-induced degradation products	4–30	–	–	307.5	S_0	-1.16×10^{-5}	3.85×10^{-4}	7.30×10^{-3}	2.25×10^{-5}	0.9999	0.62	0.03	7.30×10^{-3}
Acid-induced degradation products	4–30	Benoxinate HCl	10	307.5	S_1	-1.25×10^{-4}	2.63×10^{-4}	7.31×10^{-3}	1.54×10^{-5}	0.9999	0.67	0.48	7.31×10^{-3}
Acid-induced degradation products	4–30	Benoxinate HCl	20	307.5	S_2	-8.73×10^{-6}	2.48×10^{-4}	7.31×10^{-3}	1.44×10^{-5}	0.9999	0.66	0.04	7.31×10^{-3}
Acid-induced degradation products	4–30	Benoxinate HCl	30	307.5	S_3	-1.46×10^{-4}	2.40×10^{-4}	7.29×10^{-3}	1.40×10^{-5}	0.9999	0.66	0.06	7.29×10^{-3}

[a] Regression equation, $^1D = a + bC$ where C is the concentration in μg ml^{-1} and ^1D is thew first derivative amplitude at the selected wavelengths.
[b] Theoretical value of t at the 95% level of confidence is 2.45

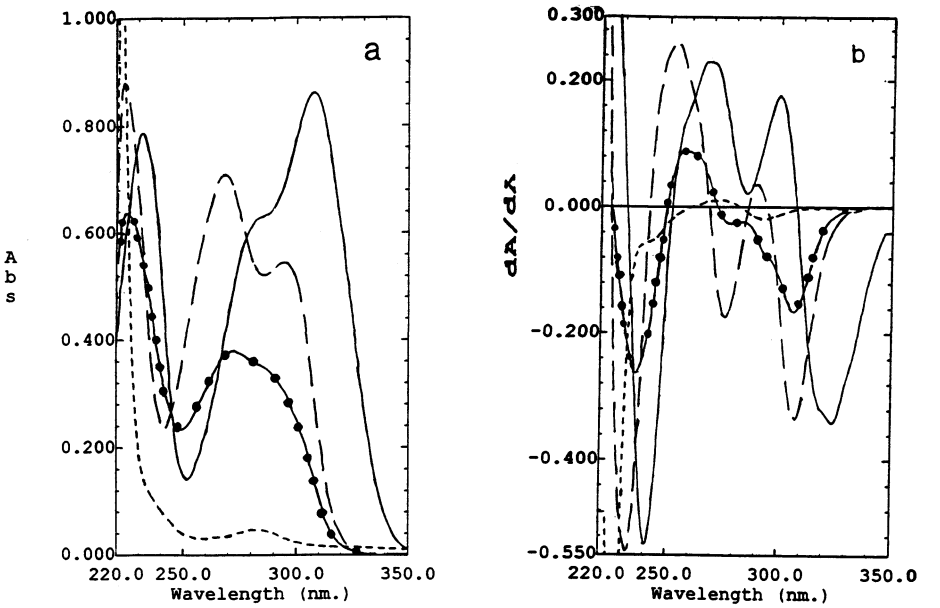


Fig. 4. (a) Zero order absorption spectrum and (b) first derivative spectrum of 20 μg ml^{-1} benoxinate HCl (—), 20 μg ml^{-1} 4-amino-3-butoxybenzoic acid (— –), 5 μg ml^{-1} 2-butoxyaniline (-----) and 20 μg ml^{-1} acid-induced degradation products of benoxinate HCl (●–●–) in mcllvaine buffer pH 7.

of **IV** indicated that it is an ortho-substituted aniline.

The mass spectra of 4-amino-3-butoxybenzoic acid (**II**) and 2-butoxyaniline (**IV**) are shown in Fig. 3a,b. The possible major fragments and their mass/charge ratios are given in Table 3. The spectra of **II** and **IV** were characterized by their molecular ion peaks at m/z 209 (58.1%) and 165 (11.0%) for 4-amino-3-butoxybenzoic acid and 2-butoxyaniline, respectively.

3.2. Assay parameters

3.2.1. First derivative UV spectrophotometric method

The UV absorption spectra of benoxinate HCl, its alkaline-induced degradation product (**II**) and acid-induced degradation products (**II** and **IV**) in mcllvaine's-citric acid phosphate buffer pH 7.0 are overlapped (Fig. 4a), while their first derivative spectra (Fig. 4b) showed significant differences in some areas that permits their determination. Benoxinate HCl was determined by measurement of its first derivative amplitude at the zero-crossing point of alkali-induced degradation product (**II**, at 268.4 nm) or at the zero crossing point of acid-induced degradation products (**II** and **IV**, at 272.4 nm), where the positive first derivative value contribution of **IV** was cancelled by the negative first derivative value contribution of **II**. The ratio of **II** to **IV** is constant at all times and temperatures used in the acid degradation process. Therefore, this ratio does not affect the accuracy of the results. The alkali-in

Table 5
Maxima ratios in first derivative UV spectrum of benoxinate HCl in mcllvaine's-citric acid phosphate buffer pH 7.0 (for $n = 5$ standard specimens)

Selected amplitude	Ratio	RSD (%)
$^1D_{241.3}/^1D_{270}$	2.248	0.83
$^1D_{241.3}/^1D_{324}$	1.565	0.35
$^1D_{324}/^1D_{270}$	1.435	0.78
$^1D_{324}/^1D_{299.7}$	1.951	0.66
$^1D_{270}/^1D_{299.7}$	1.358	0.51

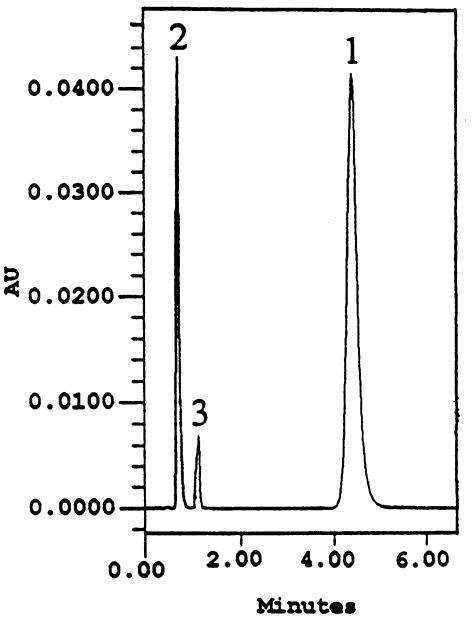


Fig. 5. A typical chromatogram of 20 μl injection of synthetic mixture of (1) 20 μg ml^{-1} benoxinate HCl, (2) 10 μg ml^{-1} 4-amino-3-butoxybenzoic acid and (3) 10 μg ml^{-1} 2 – butoxyaniline.

duced degradation product (**II**) or acid-induced degradation products mixture (**II** and **IV**) were determined by measurement of their first derivative amplitude at the zero-crossing point of benoxinate HCl (at 307.5 nm). The measurement of the absolute value of the total derivative spectrum taken at the above mentioned wavelengths afforded the best linear response of the analyte to concentration.

In order to test the mutual independence of the analytical signals for each compound, the following experiments were performed. For each compound, four calibration graphs were constructed from the first derivative signals for the standard of each compound in absence and in presence of three different concentrations of the other compound. Four calibration graphs were constructed from the ^1D signals at 268.4 nm for standard samples containing between 10 and 30μg ml^{-1} of benoxinate HCl, in absence (P_0) and in presence of 2 (P_1), 20 (P_2) and 30 (P_3) μg ml^{-1} of 4-amino-3-butoxybenzoic acid. Similarly, four calibration graphs were prepared from the ^1D signals at 272.4 nm for standard samples containing between 10 and 30 μg ml^{-1} of benoxinate HCl, in absence (Q_0) and in presence of 4 (Q_1), 20 (Q_2) and 30 (Q_3) μg ml^{-1} of acid-induced degradation products. Also, four calibration graphs were constructed from the ^1D signals at 307.5 nm for standard samples containing between 2 and 30 μg ml^{-1} of 4-amino-3-butoxybenzoic acid, in absence (R_0) and in presence of 10 (R_1), 20 (R_2) and 30 (R_3) μg ml^{-1} of benoxinate HCl. In addition, four calibration graphs were prepared from the ^1D signals at 307.5 nm for standard samples containing between 4 and 30 μg ml^{-1} of acid-induced degradation products, in absence (S_0) and in presence of 10 (S_1), 20 (S_2) and 30 (S_3) μg ml^{-1} of benoxinate HCl. The experiments showed that the amplitudes height of ^1D signals at 268.4, 272.4, 307.5 and 307.5 nm were proportional to concentrations of benoxinate HCl in presence of its alkaline-induced degradation product, benoxinate HCl in presence of its acid-induced degradation products, 4-amino-3-butoxybenzoic acid as alkaline-induced degradation product or acid-induced degradation products, respectively. Table 4 summarizes the statistical analysis of the experimental data. The regression equations calculated from the calibration graphs, along with the standard deviations of the slopes and the intercepts on the ordinates. From Table 4, it can be seen that the slopes of the calibration graphs of each compound were virtually independent of the other compound concentration. Therefore, it can be deduced that amplitudes of the first derivative signals, measured at the selected wavelengths were functionally of the compound under determination. The linearity of the calibration graphs, the adherence of the system to Beer's law and the negligible scatter of the experimental points were validated by the values of the correlation coefficients of the regression equations and the values of the intercepts on the ordinates, which were close to zero (Table 4). At the same time, if the intercept on the ordinate for the regression lines calculated by the least-squares method is negligible, it is necessary to perform the fitting of the data again according to a function whose intercept on the ordinate is

Table 6
Analytical data and regression characteristic of benoxinate hydrochloride, 4-amino-3-butoxybenzoic acid and 2-butoxyaniline using HPLC method

Parameters	Compounds		
	Benoxinate hydrochloride	4-amino-3-butoxybenzoic acid	2-butoxyaniline
Linearity range (μg ml^{-1})	10–30	0.5–15	10–100
Detection limit (μg ml^{-1})	0.02	0.04	0.23
Regression equation (Y)[a]: slope (b)	32.50×10^3	18.59×10^3	43.73×10^2
S.D. of the slope (S_b)	17.45	22.14	4.94
Variance of the slope	3.04×10^2	4.90×10^2	24.38
Relative standard deviation of the slope (%)	0.05	0.12	0.11
Confidence limit of the slope (95% confidence limit)	32.46×10^3–32.54×10^3	18.54×10^3–18.65×10^3	43.61×10^2–43.85×10^2
Intercept (a)	2.39×10^2	-1.40×10^2	-5.87×10^2
Standard deviation of the intercept	3.56×10^2	1.91×10^2	3.19×10^2
Variance of the intercept	12.69×10^4	36.36×10^3	10.15×10^4
Confidence limit of the intercept (95% confidence limit)	(-6.33×10^2)–11.12×10^2	(-6.07×10^2)–3.27×10^2	(-13.67×10^2)–1.94×10^2
Correlation coefficient (r)	0.9999	0.9999	0.9999
Relative standard deviation (%)[b]	0.40	0.38	0.28

[a] $Y=a+bC$ where C is the concentration in μg ml^{-1} and Y is the peak area.
[b] Eight replicate samples of pure compound.

zero and therefore the value of the slope (b_o) was calculated. The results of this study for all the calibration graphs were reported in Table 4. In order to verify if the intercepts, a, of the lines of regression were not significantly different from the theoretically expected value ($a=0$), the Student's t-test at 95% confidence level and six degrees of freedom was applied. The calculated t-values do not exceed theoretical values and hence the intercept on the ordinate is negligible in all instances

Table 7
Determination of synthetic mixtures of benoxinate HCl (**I**) and 4-amino-3-butoxybenzoic acid (**II**)

Mixture no.	Mixture composition (μg ml^{-1})		% Recovery of			
	I	**II**	**I**		**II**	
			^1D	HPLC	^1D	HPLC
1	20	2	100.4	100.0	100.1	100.3
2	20	10	100.2	100.3	100.7	100.1
3	20	15	100.9	100.6	99.6	99.7
4	15	5	99.2	99.8	99.1	99.5
5	30	10	100.7	99.4	100.6	100.3
6	25	15	99.2	100.0	99.0	99.4
7	10	15	99.9	99.9	100.2	100.6
		Mean	100.07	100.0	99.90	99.99
		RSD (%)	0.68	0.38	0.68	0.46

Table 8
Determination of synthetic mixtures of benoxinate HCl and its acid-induced degradation products

Mixture no.	Mixture composition (μg ml^{-1})		% Recovery of	
	Benoxinate HCl	Acid-induced degradation products	Benoxinate HCl (^1D)	Acid-induced degradation products (^1D)
1	20	4	100.0	99.3
2	20	20	99.6	100.2
3	20	30	100.7	100.0
4	15	20	100.4	100.5
5	30	10	99.7	99.6
6	25	15	100.0	99.5
7	10	20	100.3	99.8
		Mean	100.10	99.84
		RSD(%)	0.39	0.42

(Table 4) and the intercepts are not significantly different from zero.

In general, the characteristic profiles of the derivative spectra may constitute a specific fingerprint useful for the drug identification; in particular, the ratios between the amplitudes at selected wavelength can be regarded suitable parameters useful to confirm the drug identity, purity and stability [12,13]. The maxima ratios in the first derivative spectrum of benoxinate HCl in mcllvaine's-citric acid phosphate buffer pH 7.0 (Fig. 4b) are reported in Table 5, which are affected by the acid- and alkaline-induced degradation products of benoxinate HCl. Moreover, these maxima ratios can be used for the identification of benoxinate HCl in freshly prepared Boxinate ophthalmic solution without further extraction procedure.

3.2.2. HPLC method

The developed HPLC method has been applied to the simultaneous determination of benoxinate HCl (**I**) and its degradation products: 4-amino-3-butoxybenzoic acid (**II**) and 2-butoxyaniline (**IV**). To optimize the HPLC assay parameters, the effect of acetonitrile composition and apparent pH of the mobile phase on the capacity factor (k') were studied. A satisfactory separation was ob-

Table 9
Determination of synthetic mixtures of benoxinate HCl (**I**), 4-amino-3-butoxybenzoic acid (**II**) and 2-butoxyaniline (**IV**)

Mixture no.	Mixture composition (μg ml^{-1})			% Recovery of		
	I	**II**	**IV**	**I** (HPLC)	**II** (HPLC)	**IV** (HPLC)
1	20	10	10	99.7	99.8	99.1
2	20	2	10	100.7	100.2	100.8
3	20	15	15	99.6	99.5	99.4
4	15	15	10	100.4	100.1	100.1
5	30	10	15	99.9	100.4	100.7
6	25	5	20	100.0	100.8	99.8
7	10	15	25	99.7	99.5	99.3
			Mean	100.00	100.04	99.89
			RSD(%)	0.41	0.48	0.68

tained with a mobile phase consisting of acetonitrile–water mixture (60:40 v/v). Increasing acetonitrile concentration to $>75\%$ led to inadequate separation of 4-amino-3-butoxybenzoic acid and 2-butoxyaniline. At lower acetonitrile concentration, separation occurred but with excessive tailing and increased retention time for benoxinate HCl peak. Variation of apparent pH of the mobile phase resulted in maximum k' value at apparent pH 8, with loss of peak symmetry for benoxinate HCl peak. At lower apparent pH values (5–5.5) bad resolution was observed for 4-amino-3-butoxybenzoic acid and 2-butoxyaniline peaks. At apparent pH 7, optimum resolution with reasonable retention time was affected. The addition of 0.1 ml of triethylamine per liter of the combined mobile phase was essential to improve the sharpness of the benoxinate HCl peak.

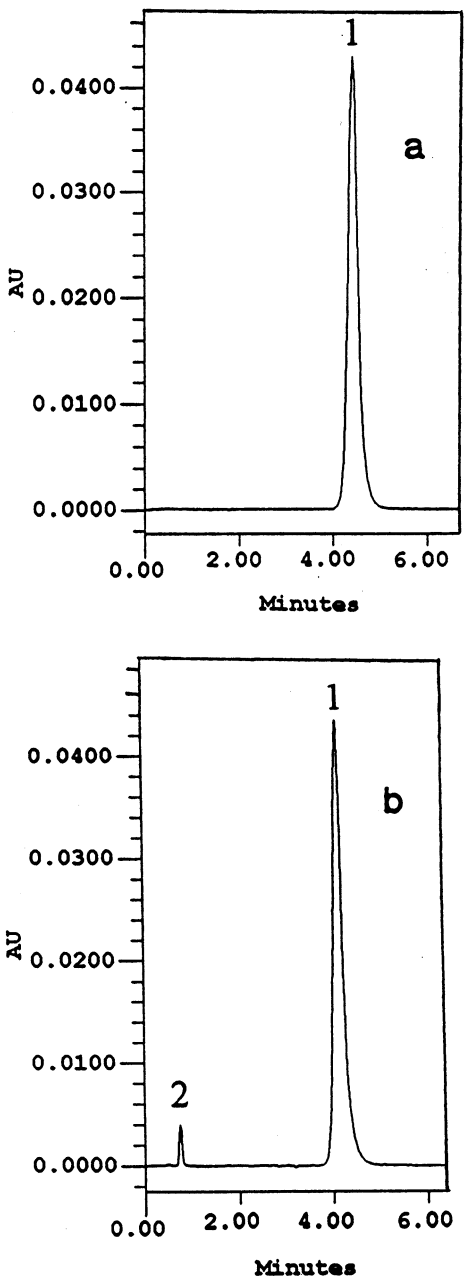


Fig. 6. Chromatogram of (a) freshly prepared and (b) expired Boxinate ophthalmic solution containing (1) benoxinate HCl and (2) 4-amino-3-butoxybenzoic acid as a degradation product.

The specificity of the HPLC method is illustrated in Fig. 5 where complete separation of the three compounds was noticed. The average retention time $\pm$ S.D. for benoxinate HCl and its degradation products **II** and **IV** were found to be 4.40 ± 0.008, 0.66 ± 0.003 and 1.10 ± 0.004 min, respectively, for ten replicates. The peaks obtained were sharp and have clear baseline separation.

To determine the linearity of the HPLC detector response, calibration standard solutions of benoxinate HCl, 4-amino-3-butoxybenzoic acid and 2-butoxyaniline were prepared as described in the text. Linear correlation was obtained between peak area versus concentration for each compound. Each measurement represented the average of three replicates.

For the HPLC method, linearity range, detection limit [14], regression equation and correlation coefficient obtained by least-squares treatment of these results are given in Table 6. The intercept value was not statistically ($P < 0.05$) different from zero for each compound.

3.3. Accuracy and selectivity of the proposed methods

The two proposed methods are highly selective towards benoxinate HCl (**I**), 4-amino-3-butoxybenzoic acid (**II**) and 2-butoxyaniline (**IV**). Regarding 2-diethylaminoethanol (**III**), it is assumed that its ultraviolet absorption characteristics are relatively low to be detected by the assay conditions used in this work.

Table 10
Recovery of benoxinate HCl (**I**), 4-amino-3-butoxybenzoic acid (**II**) added to commercial ophthalmic solution

Experiment no.	Concentration in sample ($\mu g\ ml^{-1}$)	Concentration added ($\mu g\ ml^{-1}$)		% Recovery of added			
	I	**I**	**II**	**I**		**II**	
				^1D	HPLC	^1D	HPLC
1	12	8	2	100.5	100.2	100.4	100.5
2	8	12	10	99.4	99.6	99.5	99.7
3	12	13	8	100.6	100.5	99.6	99.8
4	8	14	6	100.4	100.0	100.2	100.3
5	12	10	12	99.8	99.5	100.0	100.5
6	10	10	5	100.3	100.4	100.2	99.8
			Mean	100.17	100.03	99.98	100.10
			RSD (%)	0.47	0.41	0.36	0.37

Table 11
Recovery of benoxinate HCl and its acid-induced degradation products added to commercial ophthalmic solution

Experiment no.	Concentration of benoxinate HCl in sample ($\mu g\ ml^{-1}$)	Concentration added ($\mu g\ ml^{-1}$)		% Recovery of added	
		Benoxinate HCl	Acid-induced degradation products	Benoxinate HCl (^1D)	Acid-induced degradation products (^1D)
1	12	8	4	99.8	100.7
2	8	12	20	100.4	100.3
3	12	13	30	100.7	99.2
4	8	14	10	99.3	99.5
5	12	10	15	99.6	100.4
6	10	10	20	100.1	100.8
			Mean	99.98	100.15
			RSD (%)	0.52	0.65

The accuracy of the two proposed methods were checked by analyzing seven laboratory-prepared mixtures of benoxinate HCl and its alkali-induced degradation product (Table 7) or acid-induced degradation products (Tables 8 and 9) at various concentration ratios. Satisfactory recoveries with small relative standard deviations (RSD) were obtained, which indicated the high repeatability and accuracy of the two methods.

The ^1D and HPLC methods for determination of benoxinate HCl were compared. The calibration graph of the HPLC method was found to be more linear than that of the ^1D method, based on relative standard deviation of the slope. Relative sensitivity, based on detection limit, was calculated. The HPLC method was found to be more sensitive than the ^1D method.

3.4. Ophthalmic solution analysis

The two proposed methods were applied to the determination of benoxinate HCl in freshly prepared commercial ophthalmic solution. The mean percentage $\pm$ S.D. ($n = 6$) were found to be 99.94 ± 0.69 and 100.00 ± 0.67, determined by ^1D and HPLC methods, respectively. The HPLC

Table 12
Recovery of benoxinate HCl (**I**), 4-amino-3-butoxybenzoic acid (**II**) and 2-butoxyaniline (**IV**) added to commercial ophthalmic solution

Experiment no.	Concentration in sample (μg ml^{-1})	Concentration added (μg ml^{-1})			% Recovery of added		
	I	**I**	**II**	**IV**	**I** (HPLC)	**II** (HPLC)	**IV** (HPLC)
1	12	8	12	12	100.6	99.6	100.4
2	8	12	10	20	100.3	100.6	100.1
3	12	13	8	25	99.4	100.1	99.4
4	8	14	10	15	100.1	99.4	99.8
5	12	10	12	20	99.5	99.8	100.7
6	10	10	5	10	99.8	100.4	100.3
				Mean	99.95	99.98	100.11
				RSD (%)	0.47	0.47	0.46

chromatogram of freshly prepared commercial ophthalmic solution show no any degradation product peak of benoxinate HCl (Fig. 6a). Moreover, to check the validity of the proposed methods, the standard addition method was applied by adding different amounts of intact benoxinate HCl and its alkali-induced degradation product (Table 10) or acid-induced degradation products (Tables 11 and 12) to the previously analyzed ophthalmic solution. The recovery of the added compounds were calculated by comparing the concentration obtained from the spiked mixtures with those of the pure compounds. The results of analysis of the commercial ophthalmic solution and the recovery study (standard addition method) suggested that there is no interference from any excipients which are normally present in ophthalmic solution.

Expired batch of Boxinate ophthalmic solution stored at ambient temperature under normal conditions were analysed by the proposed HPLC method, the 4-amino-3-butoxybenzoic acid as only degradation product of benoxinate HCl was clearly found (Fig. 6b). The mean percentage of benoxinate HCl $\pm$ S.D. and the mean concentration of 4-amino-3-butoxybenzoic acid as a degradation product $\pm$ S.D. ($n = 6$) were found to be $95.41 \pm 0.76\%$ and 132.10 ± 0.91 μg ml^{-1}, determined by ^{1}D method and $95.15 \pm 0.59\%$ and 131.8 ± 0.64 μg ml^{-1}, determined by HPLC method, respectively.

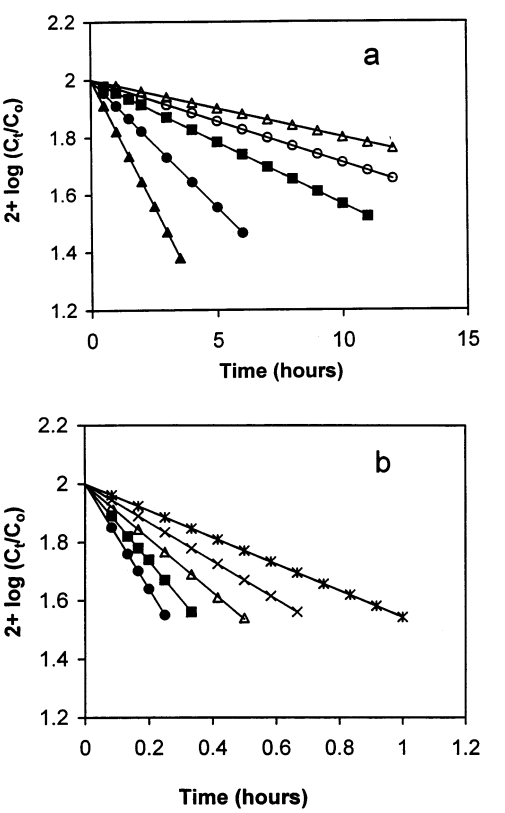


Fig. 7. Pseudo first-order plots for the degradation of benoxinate HCl in (a) 1 N hydrochloric acid and (b) 0.1 N sodium hydroxide at various temperatures using HPLC method. Key: 90 (▲); 80 (●); 70 (■); 65 (○); 60 (Δ);50 (×);and 40°C (★); C_t, concentration at time t, and C_o, concentration at time zero.

Table 13
Degradation rate constant (K_{obs}) and half-life ($t_{1/2}$) for benoxinate HCl in 1 N hydrochloric acid

Temperature (°C)	K_{obs} (h^{-1}) determined by		$t_{1/2}$ (h) determined by	
	^1D	HPLC	^1D	HPLC
90	0.400	0.407	1.734	1.702
80	0.205	0.205	3.384	3.383
70	0.099	0.100	7.004	6.956
65	0.067	0.066	10.412	10.474
60	0.046	0.045	15.128	15.167

Table 14
Degradation rate constant (K_{obs}) and half-life ($t_{\frac{1}{2}}$) for benoxinate HCl in 0.1 N sodium hydroxide

Temperature (°C)	K_{obs} (h^{-1}) determined by		$t_{\frac{1}{2}}$ (h) determined by	
	^1D	HPLC	^1D	HPLC
80	4.139	4.145	0.167	0.167
70	3.044	3.031	0.228	0.229
60	2.117	2.139	0.327	0.324
50	1.502	1.515	0.461	0.457
40	1.040	1.050	0.666	0.660

The results of determination of benoxinate HCl and 4-amino-3-butoxybenzoic acid in ophthalmic solution obtained from ^1D method were compared with the HPLC method. Statistical comparison of the results was performed with regard to accuracy and precision using Student's t-test and the F-ratio test at 95% confidence level. The calculated Student's t-test and F-ratio values were found to be 0.66 and 1.65 for benoxinate HCl, 0.66 and 2.03 for 4-amino-3-butoxybenzoic acid ($n = 6$), while the theoretical values are 2.23 and 5.05, respectively. It is clear that there is no significant difference between the two proposed methods with regard to accuracy and precision.

3.5. Kinetic investigation

To assess the specificity and selectivity of the two proposed methods for the assay of intact benoxinate HCl without interference from its degradation products, acidic and alkaline degradation of benoxinate HCl were carried out under the previously described experimental conditions. The kinetic of acidic degradation of the benoxinate HCl was investigated in 1 N hydrochloric acid, since the decomposition rates of benoxinate HCl at lower strengths of hydrochloric acid were too slow to obtain reliable kinetic data, while alkaline degradation was carried out in 0.1 N sodium hydroxide. A regular decrease in the concentration of intact benoxinate HCl with increasing time intervals was observed for each degradation process. The influences of tempera-

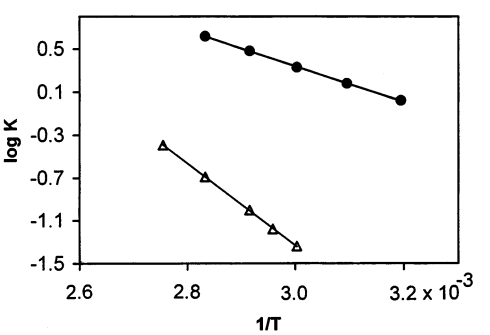


Fig. 8. Arrhenius plots for the degradation of benoxinate HCl in 1 N hydrochloric acid (Δ) and 0.1 N sodium hydroxide (●) using HPLC method.

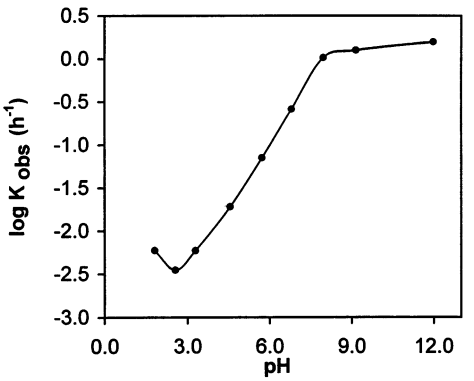


Fig. 9. pH-Rate profile for the decomposition of benoxinate HCl at constant ionic strength and 80°C.

Table 15
Degradation rate constant (K_{obs}) and half-life ($t_{\frac{1}{2}}$) for benoxinate HCl in Britton–Robinson buffer at different pH values and a temperature of 80°C

pH	K_{obs} (h^{-1})	$t_{\frac{1}{2}}$ (h)
1.8	5.942×10^{-3}	116.632
2.6	3.501×10^{-3}	197.960
3.3	5.888×10^{-3}	117.688
4.6	19.115×10^{-3}	36.254
5.7	70.881×10^{-3}	9.776
6.8	26.052×10^{-2}	2.660
8.0	1.035	0.670
9.2	1.258	0.551
11.9	1.575	0.440

ture on the degradation process in 1 N hydrochloric acid and 0.1 N sodium hydroxide are shown in Fig. 7. At the selected temperatures, the acidic and alkaline degradation processes followed pseudo first order kinetics. From the slopes of the straight lines it was possible to calculate the apparent first order degradation rate constant and the half-life at each temperature for both acidic and alkaline degradation processes determined by ^1D and HPLC methods (Tables 13 and 14). Plotting $\log K_{obs}$ values versus $1/T$, the Arrhenius Plots (Fig. 8) were obtained, which were found to be linear in the temperature range 60–90°C for acidic degradation and 40–80°C for alkaline degradation of benoxinate HCl. The activation energy was calculated to be 17.42 and 17.57 kcal mol^{-1} for acidic degradation, 7.62 and 7.55 kcal mol^{-1} for alkaline degradation, determined by the ^1D and HPLC methods, respectively.

The pH-rate profile of degradation of benoxinate HCl in Britton–Robinson buffer solutions was studied at 80°C using HPLC method (Fig. 9). Britton–Robinson buffer solutions were used throughout the entire pH range in order to avoid possible effects of different buffer species. The apparent first order degradation rate constant and the half-life were calculated for each pH value (Table 15). Benoxinate HCl was found to be most stable at a pH of ≈ 2.6.

4. Conclusions

The proposed ^1D and HPLC methods provides simple, accurate and reproducible quantitative analysis for the assay of benoxinate HCl and its degradation product in Pharmaceutical ophthalmic solutions. The HPLC method was found to be more specific and sensitive than the ^1D method. While the ^1D method has the advantages of the low cost and speed.

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