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Short Communication

Analysis of 3- and 4-monopivaloylepinephrine, degradation products in dipivefrin hydrochloride drug substance and ophthalmic formulations

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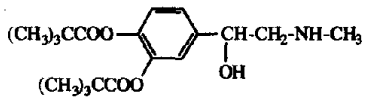
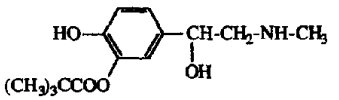
Introduction

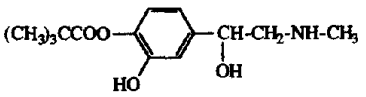
Dipivefrin hydrochloride, **1** (Fig. 1), is a prodrug of epinephrine [1–5] used for the treatment of elevated intraocular pressure in patients with chronic open-angle glaucoma [6]. Stability studies on dipivefrin HCl drug substance and dipivefrin-containing formulations revealed degradation of dipivefrin to a pair of unknown compounds. A search of the literature revealed no information regarding the stability of dipivefrin, other than its probable hydrolysis to epinephrine [2] so the identity of the unknown degradation products of dipivefrin was investigated. This paper reports the identification of a pair of monopivaloylepinephrine degradation products of dipivefrin and a routine LC method by which dipivefrin and these degradation products can be simultaneously monitored in ophthalmic solution or drug substance (raw material).



Dipivefrin, **1**



3-Pivaloylepinephrine, **2**



4-Pivaloylepinephrine, **3**

Figure 1
Chemical structures of dipivefrin, **1**, and degradation products, **2** and **3**.

Experimental

Chemicals and reagents

All chemicals were of analytical reagent grade. Mobile phase solvents were HPLC grade: acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA) and purified water, USP, was obtained from an in-house source. Glacial acetic acid and 50% NaOH were obtained from J.T. Baker, sodium dodecyl sulphate from Mallinkrodt (St Louis, MO, USA), and ammonium acetate from Aldrich Chemical Co. (Milwaukee, WI, USA). Mobile phases were filtered through a 0.45 μm Nylon-66 filter (Rainin Instrument Co., Woburn, MA, USA) prior to use. Dipivefrin hydrochloride was

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obtained from Pharm-Eco, Inc. (Simi Valley, CA, USA). A commercial preparation of dipivefrin ophthalmic solution, USP, 0.1%, was obtained from a local pharmaceutical warehouse. A dipivefrin ophthalmic solution, 0.1%, vehicle was prepared in-house which contained benzalkonium chloride, 0.005%; edetate disodium, 0.138%; NaCl, 0.138%; and purified water, q.s. to 100%.

Degradation standard (or resolution standard) of dipivefrin HCl. A degraded sample was prepared by dissolving dipivefrin HCl in 0.0015 N HCl (1 mg ml^{-1}) followed by the addition of 100 µl of 50% NaOH. Addition of base to the dipivefrin hydrochloride solution caused the clear, colourless solution to become clear and pink-, then brown-coloured. The solution was then stabilized by the addition of 300 µl of concentrated HCl. This solution was stable for several months.

Instruments and conditions

High-pressure liquid chromatography. Analytical separations were performed on several HPLC systems. A typical system consisted of a Waters Associates (Millipore Waters, Milford, MA, USA) 600E pump, WISP 712 autoinjector, either a Lambda-Max 481 UV detector or a Model 991 Photodiode Array detector, and a Spectra-Physics (Spectra-Physics, San Jose, CA, USA) 4270 integrating recorder. Two different column–mobile phase systems were used, designated 'HPLC System 1' and 'HPLC System 2'. HPLC System 1, adapted from the USP XXII monograph HPLC assay for dipivefrin hydrochloride [7], consisted of a Waters Associates µBondapak column (3.9 × 300 mm, 10 µ octadecylsilane packing) with a mobile phase of acetonitrile–1% sodium dodecylsulphate–glacial acetic acid (51:46:3%, v/v/v), a flow rate of 2 ml min^{-1}, and UV detection at 254 nm (attenuation, AUFS: 0.05). HPLC System 2 consisted of a Whatman Partisphere (Whatman LabSales, Hillsboro, OR, USA) or Phenomenex (Phenomenex, Torrance, CA, USA) Spherisorb C8 column (5 µ, octylsilane packing), a mobile phase of 0.1 M ammonium acetate–methanol (1:1, v/v), a flow rate of 2 ml min^{-1}, with UV detection at 254 nm (attenuation, AUFS: 0.05).

Preparative thin-layer chromatography. Whatman preparative silica gel plates (silica gel, with 150 Å pore size, 1000 µm silica gel thickness, 20 × 20 cm) and a mobile phase of chloroform–methanol–formic acid (73:25:2%, v/v/v) were used. Preparative plates were presaturated with mobile phase and air-dried prior to spotting (failure to prewash the plates resulted in loss of degradation spots).

Mass spectrometry. Two mass spectrometer systems were employed. For solid-probe MS analysis, a Finnegan MAT TSQ46 GC/MS/MS with data system (Finnegan Corp., Cincinnati, OH, USA) was used in the chemical ionization mode with isobutane. For HPLC–MS work, a thermospray HPLC–MS system was used which consisted of a Waters Associates 600 MS pump interfaced to a Vestec Model 201A spectrometer system (Vestec Corp., Houston, TX, USA) employing HPLC System 2 with a flow rate of 1 ml min^{-1}, a tip temperature of 227°C, a block temperature of 194°C, and the filament off.

Results and Discussion

Isolation attempts by semi-preparative HPLC and TLC

A degraded standard of dipivefrin HCl was analysed by HPLC System 1 which demonstrated the appearance of a pair of new peaks (1 and 2) at a retention time of about 3 min (Fig. 2). A fraction corresponding to peak 1 on HPLC System 1 was manually collected and re-injected on the same HPLC system giving, surprisingly, both peaks 1 and 2. Likewise, a fraction corresponding to peak 2 on HPLC System 1 was collected and it also gave rise to both peaks upon re-injection. Furthermore, another fraction corresponding to peak 2 was collected in a test tube that was kept cold in an ice bath. Analysis of this fraction gave both peak 1 and peak 2 in a 3:4 ratio. Allowing the cold sample to warm to room temperature prior to injection produced an increase in the size of peak 1, giving a ratio of peak 1 to peak 2 of approximately 1:1, suggestive of a temperature-dependent isomerization process. An attempt to identify the contents of the collected fractions for peaks 1 and 2 was performed by evaporating the fractions to dryness and analysing by solid-probe mass spectrometry. Unfortunately, no reasonable mass values were obtained.

Further attempts were made to isolate and identify the two new compounds by prep-

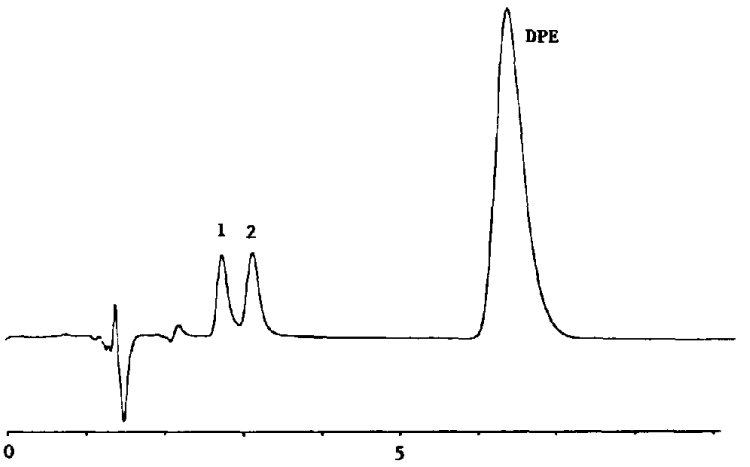

Figure 2
HPLC chromatogram (HPLC System 1) of dipivefrin (DPE) degraded standard (20 μl of a 1 mg ml^{-1} solution injected) showing degradation peaks 1 and 2, corresponding to monopivaloylepinephrine degradation products.

arative TLC. Ten plates were spotted, each with approximately 50 ml of a dipivefrin HCl degraded standard, and developed. Bands corresponding to dipivefrin and the degradation products were well separated (R_f 0.34 and 0.22, respectively, as determined by HPLC). A band corresponding to the degradation product(s) was scraped and the silica was washed with methanol which was collected in a round-bottomed flask. The resulting solution (a total quantity of about 200 ml) was evaporated by a rotovapour apparatus to dryness. The residue was analysed by mass spectrometry, but again, no reasonable mass values were obtained.

Identification of degradation products by MS and HPLC–MS

An alternate HPLC system was investigated which would be compatible with HPLC–MS, i.e. an ammonium acetate–methanol mobile phase (containing no buffer salts which would plug the thermospray capillary). Using this type of system, HPLC System 2 (without ion-pairing), one peak was observed for dipivefrin (retention time, 9 min) and only one degradation product peak was observed (retention time, 1.6–1.7 min) (Fig. 3). Despite various alterations in mobile phase or column type (C8 or C18), only one degradation peak was observed. In order to determine if the two degradation peaks (peaks 1 and 2) observed using HPLC System 1 (Fig. 2) were indeed equivalent to the one degradation peak observed on HPLC System 2 (Fig. 3), collection experiments were again employed. Peaks 1 and 2 were collected using HPLC System 1. Injection of each of these fractions onto HPLC System 2, indeed, gave only one peak (retention time 1.7 min). Conversely, the single degradation peak at 1.7 min on HPLC System 2 was collected and injected onto HPLC System 1, yielding two peaks (peaks 1 and 2 as in Fig. 2), as expected. From these experiments, therefore, it was determined that peaks 1 and 2 observed on HPLC System 1 were equivalent to the single degradation peak observed on HPLC System 2. Again, an attempt was made to identify an appropriate fraction collected from HPLC System 2. The fraction was collected, dried and analysed by mass spectrometry, but again, with no reasonable mass values obtained.

Speculating that the drying process for the preparative TLC and preparative HPLC fractions might have caused further degradation of the isolated compounds, the latter procedure was repeated, omitting the drying step. Using HPLC System 2, a total of 20 fractions were collected, affording a total of 15 ml of solution. Analysis of this solution by HPLC (System 2) demonstrated a purity in excess of 99% for the degradation product(s). A drop of this solution was placed directly on the mass spectrometer probe which was immediately inserted into the mass spectrometer unit. The resulting chemical ionization (isobutane) mass spectrum pattern (Fig. 4) was consistent with either structure, **2** or **3** [*m/z* (assignment, relative abundance): 268 (M + 1, 65%); 250 (loss of H_2O, 12%); 121 ($[C_7H_5O_2]$ + 1, 100%); 103 ($[C_8H_6]$ + 1, 84%)]. To confirm these results, LC–MS was

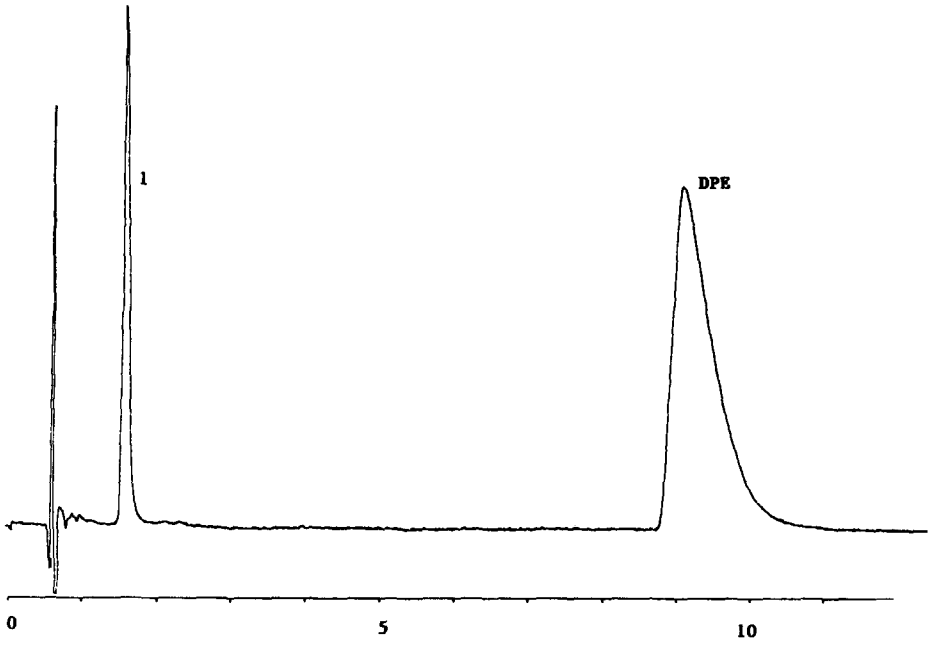

Figure 3
HPLC chromatogram (HPLC System 2) of dipivefrin (DPE) degraded standard (20 μl of a 1 mg ml^{-1} solution) showing DPE and both monopivaloylepinephrine degradation products eluting as peak 1.

employed using HPLC System 2 at a flow rate of 1 ml min^{-1}. Two peaks were observed in the reconstructed ion chromatogram: a peak for dipivefrin was observed at 12.5 min [*m/z* (assignment, relative abundance): 352 (M + 1, 100%)] and the degradation peak was observed at 2.8 min which exhibited a spectral pattern [*m/z* (assignment, relative abundance): 268 (M + 1, 100%); 250 (loss of H_2O, 5%), Fig. 4] consistent with previous MS data (HPLC–MS with ammonium acetate–methanol often gives only M + 1 spectra similar to chemical ionization). Therefore, the unknown degradation products observed by HPLC were identified as a pair of rapidly-interconverting, positional isomeric degradation products of dipivefrin, 3- and 4-monopivaloylepinephrine, **2** and **3** (Fig. 1). It could not be determined which peak on HPLC System 1 corresponded to which isomer, **2** or **3**.

Ultraviolet spectra of 3- and 4-monopivaloylepinephrine

Ultraviolet spectrophotometric analysis of **2** and **3** were performed using a photodiode array detector, collecting data from 400 to 200 nm at 3.47 s intervals. The λ_{max} values found for both the degradation products, **2** and **3**, were 232 and 275 nm, respectively, similar to the λ_{max} values for epinephrine [8], 235 and 281. The λ_{max} values for dipivefrin were 234, 264 and 269. Ultraviolet spectra for dipivefrin, 3- and 4-monopivaloylepinephrine are presented in Fig. 5.

Routine HPLC method for simultaneous monitoring of 3- and 4-monopivaloylepinephrine and dipivefrin HCl in ophthalmic solution or drug substance

System 1 (HPLC) was found to be suitable for the simultaneous analysis of dipivefrin and 3- and 4-monopivaloylepinephrine in either ophthalmic solution or the raw material (drug substance) by comparison of the HPLC peak areas of all compounds to that of a dipivefrin HCl reference standard at 254 nm (since reference standards of the degradation compounds were not available). A resolution standard (degraded dipivefrin HCl standard) was employed to ensure that the HPLC system could resolve the two degradation products. Since no reference standards were available for the degradation products, all linearity, precision and recovery measurements were performed on dipivefrin HCl only.

Samples were prepared in the following manner. For dipivefrin HCl reference standard and/or raw material samples, 25 mg of dipivefrin HCl was diluted to 25.0 ml with 0.0015 N HCl to obtain final dipivefrin concentrations of

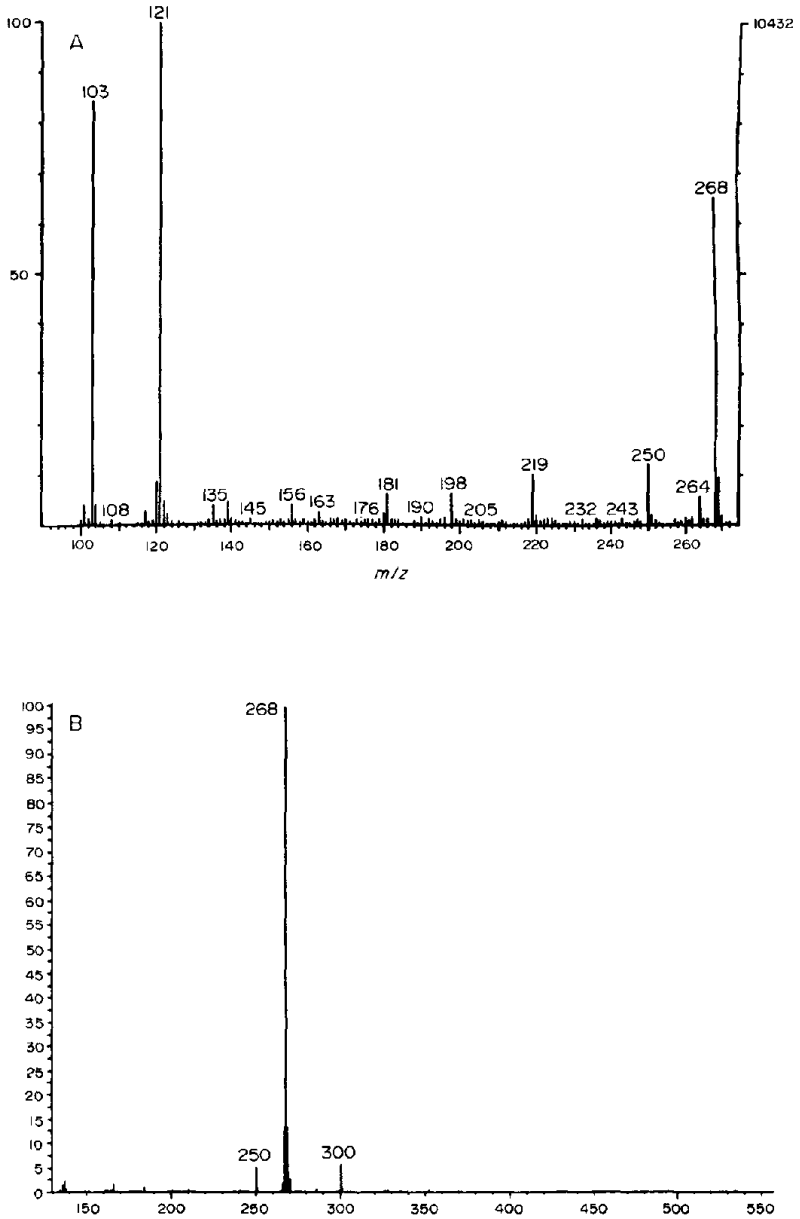

Figure 4
Comparison of mass spectrometric analyses of dipivefrin degradation products. (A) Solid-probe CI–MS of isolated preparative HPLC fraction (peak 1 from HPLC System 2) showing a 268 M + 1 peak consistent with **2** and **3**. (B) Direct thermospray HPLC–MS analysis (peak 1 from HPLC System 2) showing a 268 M + 1 peak also consistent with **2** and **3**.

about 1 mg ml^{-1}. For dipivefrin ophthalmic solution, 0.1%, no dilution was necessary. Samples were injected as is. The resolution standard was prepared as described in the experimental section.

System suitability criteria were established: a relative standard deviation for three standard injections less than 2.0%; greater than 500 theoretical plates per column; a resolution factor between the two degradation product peaks of at least 1.5; and a tailing factor at 5% of peak height of not more than 2.0. The calculation for the quantitation of dipivefrin and/or 3- and 4-monopivaloyepinephrine was as follows:

$$\text{conc. of analyte} = \frac{\text{peak area of analyte} \times \text{conc. of dipivefrin standard}}{\text{peak area of standard}}, \quad (1)$$

where concentrations are in mg ml^{-1}.

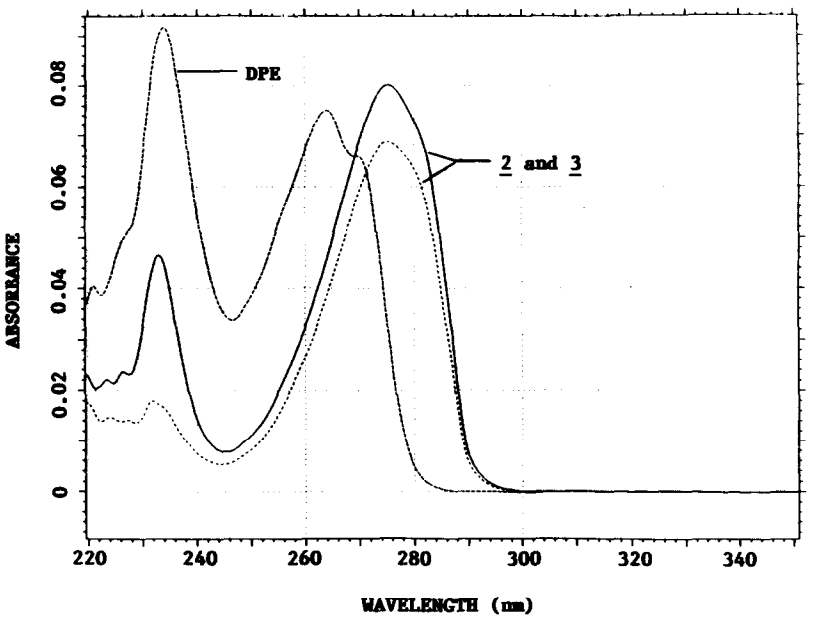

Figure 5
Ultraviolet spectra of dipivefrin, **1**, and degradation products, **2** and **3**, in mobile phase as determined with a photodiode array detector.

The linearity, precision and recovery was determined for dipivefrin HCl. A three-point dipivefrin HCl standard curve (dipivefrin HCl in aqueous solution at concentrations of 0.5, 1.0 and 1.5 mg ml^{-1}) was assayed by this procedure. The method was found to be linear over the concentration range of 0.5–1.5 mg ml^{-1} by regression analysis ($y = 147076x + 2516$; $r^2 = 0.9998$). The relative y-intercept of the standard curve was 1.69% at the mid-point. Also, a three-point vehicle standard curve (dipivefrin-spiked vehicle at concentrations of 0.5, 1.0 and 1.5 mg ml^{-1}) was obtained using this procedure. Again, the method was linear over the concentration range of 0.5–1.5 mg ml^{-1} by regression analysis ($y = 146115x + 1440$; $r^2 = 0.9993$). The relative y-intercept of the vehicle standard curve was 0.97% at the mid-point. The mean recovery for the vehicle standard curve was 99.1% + 1.74%. The standard curve and vehicle standard curves thus obtained were linear, passed through the origin and, therefore, allowed the use of a single point standard for this assay.

A set of six vehicle replicates (dipivefrin HCl-spiked vehicle at concentrations of 1 mg ml^{-1}) was analysed by this procedure. The precision was satisfactory with a RSD of 0.28%. Typical chromatograms of dipivefrin ophthalmic solution, 0.1%, and dipivefrin HCl drug substance showing the two monopivaloylepinephrine degradation products are presented in Fig. 6.

A commercial preparation of dipivefrin ophthalmic solutions, USP, 0.1%, was analysed for the presence of degradation products using the routine HPLC method described herein (HPLC System 1). Concentrations found were as follows: **1**, 1 mg ml^{-1}; 3- and 4-monopivalylepinephrine degradation products, about 0.1 mg ml^{-1} each.

Conclusions

In conclusion, base hydrolysis of dipivefrin in aqueous solutions produced a pair of isomeric degradation products identified by mass spectrometry as 3- and 4-monopivaloylepinephrine. It was not determined, though, which HPLC peak corresponded to which isomer. Each of the 3- and 4-monopivaloyepinephrines, when isolated by preparative HPLC, isomerized to a 1:1 mixture of the two. The USP method for the assay of dipivefrin hydrochloride by HPLC was modified for the simultaneous routine assay of dipivefrin and the 3- and 4-monopivaloylepinephrines in raw material or ophthalmic solution. These degradation products were observed at low levels in long-term stability samples of dipivefrin HCl, a commercial preparation of dipivefrin ophthalmic solution, 0.1%, USP, and other

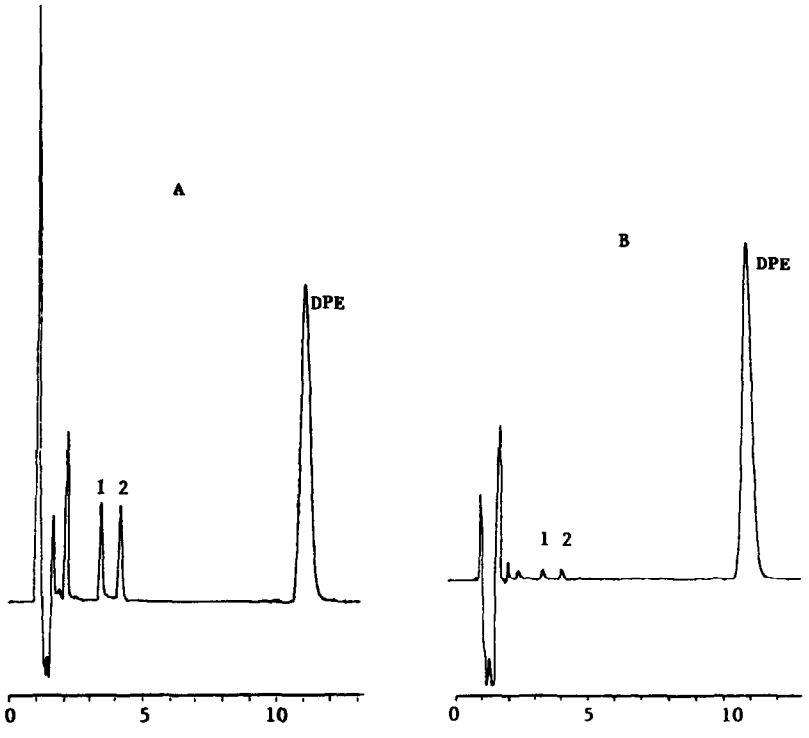

Figure 6
Typical HPLC chromatograms (HPLC System 1) of dipivefrin (DPE) and degradation peaks 1 and 2. (A) A formulation sample of 0.1% dipivefrin ophthalmic solution containing 0.95 mg ml^{-1} DPE and each degradation product at levels of 0.1 mg ml^{-1}. (B) A raw material sample of 1 mg ml^{-1} dipivefrin HCl and both degradation products at concentrations of 1 μg ml^{-1}.

dipivefrin-containing formulations. Therefore, the monopivaloylepinephrines appear to be common, hydrolytic degradation products of the dipivefrin and should be considered when evaluating the stability of the drug substance or active ingredient.

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