International Journal of Pharmaceutics, 82 (1992) 179–183

IJP 02720

Simultaneous determination of bendazac and underivatized lysine in ophthalmic preparations by reversed-phase ion-pair high-performance liquid chromatography

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(Received 6 September 1991)
(Accepted 4 November 1991)

Key words: Bendazac lysine; Aqueous ophthalmic solution, determination; Reversed-phase ion-pair HPLC

Summary

An isocratic reversed-phase high-performance liquid chromatographic procedure was developed for the simultaneous assay of bendazac and free lysine in ophthalmic formulations without any pretreatment or derivatization. An ODS Hypersil column with a time-programmable UV detector and acetonitrile : phosphate buffer (15 : 85, v/v) eluent containing 1-octanesulfonate as the pairing ion were used. The liquid dosage form was diluted with the mobile phase and then injected onto the HPLC column without further treatment. The method is rapid, specific, accurate and reproducible.

Introduction

Bendazac ([(1-benzyl-1H-indazol-3-yl)oxy] acetic acid) is a non-steroidal anti-inflammatory agent used for the treatment of cataracts (Testa et al., 1982; Martindale The Extra Pharmacopoeia, 1989). To reduce side effects associated with oral therapy (Ballesteros et al., 1987), bendazac is administered topically as an aqueous ophthalmic solution. As reported for other drugs (Fabregas and Beneyto, 1980), lysine has been found to enhance the absorption and bioavailability of bendazac (Testa et al., 1982) and consequently the lysine salt of bendazac is advocated in the management of cataracts (Balfour and Clissold, 1990). The extensive use of bendazac lysine (Balfour and Clissold, 1990) and the requirements for certification of sterile ophthalmic solutions necessitated the development of a rapid and precise procedure for the simultaneous determination of both components in their salt for quality control purposes.

The assay of bendazac lysinate in pharmaceutical preparations is performed by the manufacturer using two independent procedures, namely, non-aqueous titration of the α-amino group of lysine (U.S. Pharmacopeia, 1985) and spectropho-

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tometric determination of bendazac. Previous high-performance liquid chromatographic (HPLC) methods (Fabregas and Beneyto, 1980; U.S. Food and Drug Administration, 1984) for the determination of lysine in its salts with medicinal drugs involve a preliminary derivatization step which is time-consuming and can result in incomplete and non-reproducible derivatization reactions. These techniques cannot meet the current quality control requirements of rapidity, simplicity, accuracy and precision.

A rapid HPLC method is described in this study for the simultaneous assay of bendazac and underivatized lysine in ophthalmic solutions. The dosage form was diluted and directly analysed by isocratic reversed-phase HPLC with a mobile phase containing an alkylsulfonate ion-pair reagent. The procedure, without any pretreatment or derivatization, is accurate, precise and labor saving.

Materials and Methods

Reagents

The lysine salt of bendazac, bendazac and 1-benzyl-3-hydroxy-1H-indazole were supplied by Angelini S.p.A. (Ancona, Italy). Lysine hydrochloride and cadaverine were obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and water were from Baker (Phillipsburg, NJ). The sodium salts of 1-hexanesulfonate, 1-heptanesulfonate and 1-octanesulfonate were purchased from Sigma Chemicals (St. Louis, MO). All other chemicals were of analytical grade (Farmitalia Carlo Erba, Milano, Italy).

HPLC

The HPLC apparatus consisted of a Jasco chromatographic system (Model 880-PU pump, Model 880-02 ternary gradient unit and Model 875-UV variable-wavelength UV/Vis detector; Jasco, Tokyo, Japan) linked to an injection valve with a 20 μl sample loop (Rheodyne, Cotati, CA) and a chromatographic data processor (Chromatopac C-R3A, Shimadzu, Kyoto, Japan). Lysine was detected at 210 nm and 0.04 absorbance units full scale (a.u.f.s.) and bendazac at a wavelength of 306 nm and 0.64 a.u.f.s. using the wavelength and sensitivity range time-programming capability. Sample injections were made with a Hamilton Model 802 RN syringe (10 μl; Hamilton, Bonaduz, Switzerland).

Separations were performed on a 3 μm ODS Hypersil column (100 × 4.6 mm i.d.; Shandon Southern Products, Cheshire, U.K.) fitted with a guard column (LiChrospher RP-18, 5 μm, 4 × 4 mm i.d.; Merck) and eluted, isocratically, with 0.02 M sodium phosphate buffer (pH 6.5): acetonitrile (85 : 15, v/v) containing 0.01 M sodium 1-octanesulfonate. The mobile phase was filtered through HVLP-type filters (0.45 μm; Millipore S.A., Molsheim, France) and on-line degassed using a model ERC-3311 solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at ambient temperature, at a flow rate of 1.0 ml/min.

The identity of the separated compounds was assigned by co-chromatography with the authentic substances. Peak areas were used for calculations.

Sample and standard preparation

The ophthalmic formulation was diluted 1 : 1 (v/v) with mobile phase and filtered through a 0.45 μm membrane filter (Millipore S.A.) prior to injection onto the HPLC column.

Standard solutions were prepared by accurately weighing 125 mg of bendazac lysinate reference material into a 50 ml volumetric flask and diluting to volume with water to give a final concentration corresponding to the label claim (0.25%, w/v). The resulting solution was then assayed as outlined above. Placebo preparations containing the ophthalmic solution excipients (i.e., boric acid, sodium borate, potassium chloride, hydroxypropyl methylcellulose and water for injection) were spiked with the appropriate amounts of bendazac lysinate in 50-ml volumetric flasks and subjected to the assay procedure.

Linearity, accuracy and precision

Calibration curves of peak area vs concentration were generated using placebos spiked with known amounts of bendazac lysinate corresponding to 50, 75, 100, 125 and 150% of label.

The recovery of the assay was determined using placebos spiked with the labeled amount of bendazac lysinate. The percentage recovery was calculated by comparing the peak areas of lysine and bendazac from the placebo preparations with those obtained by injections of the standard solution.

The reproducibility of the method was evaluated by replicate assays of a placebo formulation spiked with the labeled amount of bendazac lysinate.

Results and Discussion

Since lysine does not absorb appreciably in the UV region, pre- and post-column derivatization

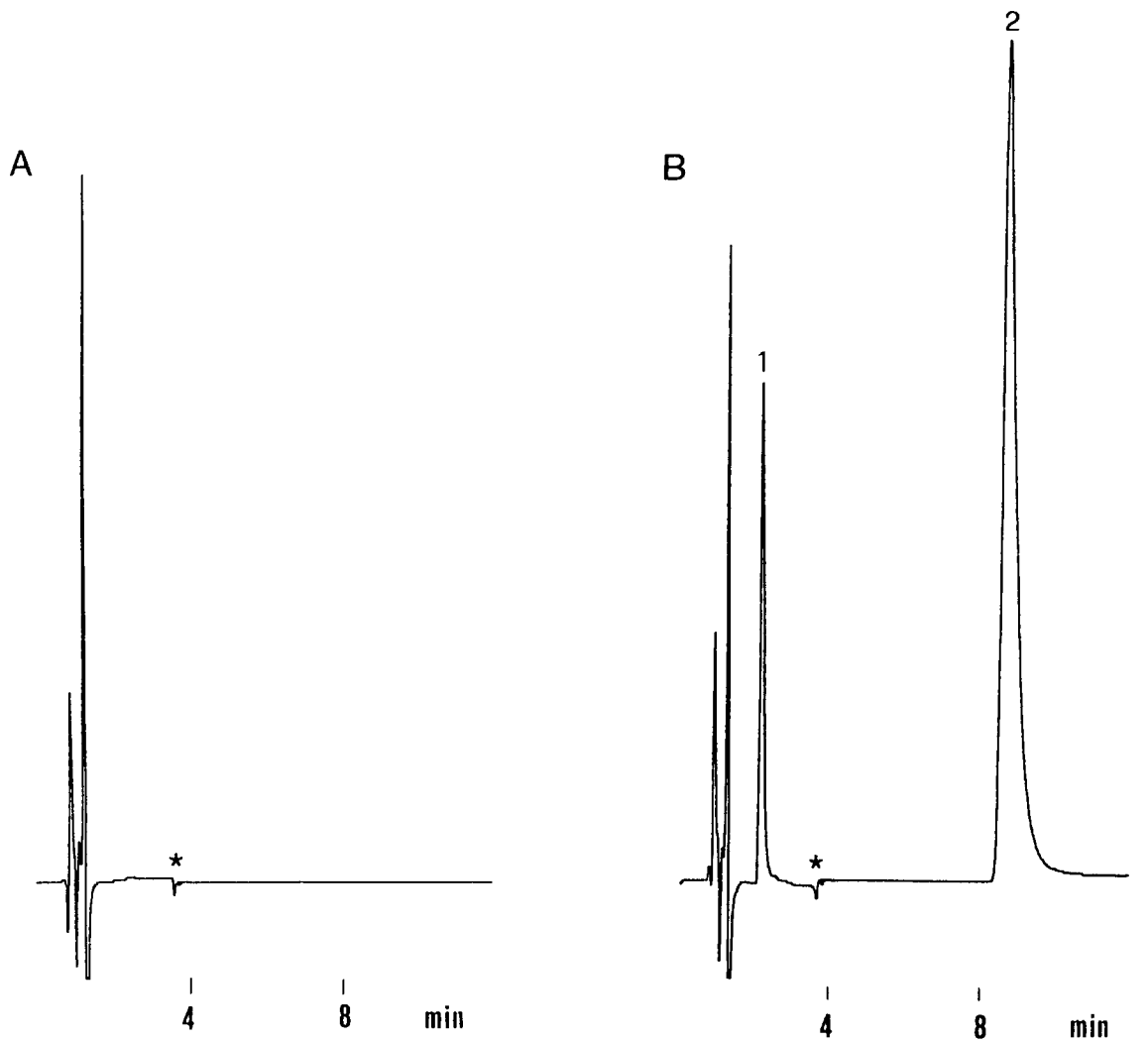

Fig. 1. HPLC chromatograms of: (A) a placebo sample; (B) a commercial ophthalmic preparation containing 0.25% (w/v) of bendazac lysinate. Peaks: 1, lysine; 2, bendazac. Operating conditions: as described in Materials and Methods. Detection: 0 min, 210 nm and 0.04 a.u.f.s.; 3.6 min, 306 nm and 0.64 a.u.f.s. * Time-programmed change of wavelength and sensitivity range.

procedures have been developed for the HPLC analysis of this amino acid in biological materials (Lottspeich and Henschen, 1985) and pharmaceutical preparations (Fabregas and Beneyto, 1980; Muhammad and Bodnar, 1980; U.S. Food and Drug Administration, 1984). However, in this study it was found that due to the high concentrations present in the latter samples, the HPLC assay of lysine in pharmaceuticals can be performed without derivatization at a short UV wavelength (i.e., 210 nm), as previously reported for other substances (i.e., bile acids) lacking a strong chromophore (Scalia et al., 1989). For detection of bendazac, the wavelength of maximum absorptivity (306 nm) was selected automatically during the chromatographic run using the time-programming capability of the detector.

Because of the pronounced difference in polarity between lysine and bendazac, their simultaneous assay by HPLC poses several problems, since gradient elution procedures are not suitable for routine quality control analyses of pharmaceutical products. As reported in earlier investigations (Levin and Grushka, 1985; Walker and Pietrzyk, 1985), underivatized lysine was not retained on the reversed-phase column. In order to enhance the retention of amino acids in RP-HPLC, hydrophobic ion-pair reagents, such as the alkylsulfonates, have been used as mobile phase additives (Knox and Hartwick, 1981; Levin and Grushka, 1985; Walker and Pietrzyk, 1985). In this study, acetonitrile-phosphate buffer eluents containing hexane-, heptane- or octanesulfonate salts were examined for the RP-HPLC analysis of free lysine. The amino acid was appreciably retained when hexanesulfonate was added to the mobile phase (5% by volume acetonitrile in 0.02 M phosphate buffer, pH 6.5) and its capacity factor rose from 0.98 to 1.65 with increasing concentrations of the sulfonate (from 2.5 to 10 mM) due to an increase in the apparent cation-exchange sites on the stationary phase (Walker and Pietrzyk, 1985). Under these conditions, however, bendazac exhibited an excessively long elution time (> 60 min), its retention not being affected by the presence of the pairing ion. In accordance with previous reports (Levin and Grushka, 1985; Walker and Pietrzyk, 1985), the retention of lysine was found to increase markedly with alkyl chain length of the pairing ion. Of the salts studied, octanesulfonate afforded the highest capacity factor for lysine, allowing satisfactory retention of the amino acid at a higher organic modifier concentration (15%, v/v) in the mobile phase. Thus, a reduced elution time (< 10 min) was achieved for bendazac which permitted the simultaneous separation of both compounds above under isocratic conditions (Fig. 1B). Enhanced amino acid retention has been reported (Walker and Pietrzyk, 1985) at lower mobile phase pH ($pH < 4$), because the more acidic mobile phase pH increases the cationic charge of the amino acid enhancing its interaction with the anionic pairing ion and consequently its retention. However, the pH value of 6.5 was selected in this investigation since more acidic pH produced severe peak tailing and increased retention times for bendazac (pK_a, 4.7 in 20% acetonitrile in water) owing to suppression of ionization which enhances the lipophilicity of the molecule, and hence its interaction with the hydrophobic stationary phase.

Representative chromatograms of a placebo sample (Fig. 1A) and of a bendazac lysinate ophthalmic preparation (Fig. 1B) demonstrate that there are no excipient peaks which interfere with the simultaneous HPLC determination of lysine and bendazac. Moreover no interference was observed (chromatogram not shown) from the potential degradation products of lysine and bendazac, namely cadaverine and 1-benzyl-3-hydroxy-1H-indazole, respectively. Under the chromatographic conditions of the assay, cadaverine eluted in the void volume, whereas 1-benzyl-3-hydroxy-1H-indazole was strongly retained (no elution occurred after 90 min). A higher proportion of acetonitrile (60%, v/v) in the mobile phase was required to recover it from the HPLC column.

Sample processing is simply dilution of the liquid dosage form with the mobile phase and filtration before injection onto the HPLC column. This is the simplest possible sample preparation, circumventing extraction or derivatization steps, and an internal standard is not needed (Haefelfinger, 1981; Scalia et al., 1989).

Calibration curves ($n = 6$) were linear in the range 50–150% of the label claim (1.25–3.75 mg/ml). The average correlation coefficients and slopes were, respectively, 0.9996 ± 0.0007, 2.407 ± 0.041 for lysine and 0.9995 ± 0.0004, 0.689 ± 0.019 for bendazac. In no graphs was the intercept on the y-axis significantly different from zero at the 95% confidence interval.

The recovery of the drug from the vehicle matrix was determined using spiked placebos at 100% of the label claim. The average recoveries ($n = 10$) for lysine and bendazac were 100.1% with a relative standard deviation (RSD) of 0.7% and 101.2% with a RSD of 0.8%, respectively.

The reproducibility of the method was found to be 0.4% RSD ($n = 10$) for lysine and 0.7% RSD ($n = 10$) for bendazac.

Two different commercial batches of ophthalmic formulations containing 0.25% (w/v) of bendazac lysinate were assayed using the proposed HPLC procedure and the results are presented in Table 1. The data obtained confirm the precision of the method and demonstrate compliance with the label claim. Moreover, no deterioration in column performance was observed over several weeks of continuous use.

In conclusion, a rapid isocratic RP-HPLC method has been developed for the simultaneous analysis of bendazac and underivatized lysine in mixtures of these two products. Because of the ease of operation, minimal sample preparation, high reproducibility and accuracy the procedure is well suited to quality control assays of ophthalmic solutions containing bendazac lysinate.

TABLE 1

Assay results for bendazac lysinate in ophthalmic preparations

Batch no.	% of label claim (mean ± SD, $n = 10$)	
	Lysine	Bendazac
L 020	99.7 ± 0.6	100.2 ± 0.7
L 028	99.5 ± 0.3	100.3 ± 0.5

The guidelines for the assay development outlined in this study should also be applicable, in principle, to the analysis of other lysine-associated salts of drugs.

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