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Enantiomeric purity assay of moxifloxacin hydrochloride by capillary electrophoresis

Lou Ann Cruz*, Rex Hall

Department of Analytical Chemistry, Alcon Research Limited, 6201 South Freeway, Ft. Worth, TX 76134, USA

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Abstract

A capillary electrophoresis method for determining the enantiomeric purity of moxifloxacin hydrochloride in drug substance and ophthalmic/otic drug products was developed and validated. Because moxifloxacin hydrochloride has two chiral centers, the existence of four different isomers is possible. The method was capable of separating moxifloxacin hydrochloride, which is the *S*,*S*-isomer, from its potential chiral degradation products, which are the *R*,*R*-enantiomer, the *R*,*S*-diastereomer, and the *S*,*R*-diastereomer. The separation was carried out at 20 °C in a 50 μm × 40 cm fused-silica capillary with an applied voltage of −13 kV using a 12.5 mM TEA phosphate buffer (pH 2.5) containing 5% highly-sulfated gamma-cyclodextrin (HS-γ-CD) and 6% acetonitrile. The detection wavelength was 295 nm. The method was validated with respect to its specificity, linearity, range, accuracy, and precision in the expected range of occurrence for the isomeric impurities (0.05–5%). The method was used to investigate whether racemization was a potential degradation pathway for the drug substance, both in the solid state and in solution.

Keywords: Moxifloxacin; Fluoroquinolones; Capillary electrophoresis; Validation; Enantiomeric purity

1. Introduction

Moxifloxacin hydrochloride belongs to the fluoroquinolone class of anti-infective compounds. The compound has a broad antibacterial spectrum against Gram-positive and Gram-negative organisms including anaerobic bacteria and was developed by scientists at Bayer AG for systemic treatment of respiratory tract infections [1]. Alcon Research Limited has licensed the drug from Bayer AG for development as a topical treatment of eye and ear infections.

Moxifloxacin hydrochloride (Fig. 1) possesses two chiral centers [2] and is the *S*,*S*-isomer. Its potential chiral impurities are the *R*,*R*-enantiomer, the *R*,*S*-diastereomer and the *S*,*R*-diastereomer. The FDA's Draft Guidance for Industry on the development of stereoisomeric drugs [3] states that applications for an enantiomeric drug substance or applications for drug products containing an enantiomeric drug substance should include a stereochemically specific identity test and/or a stereochemically selective assay method. However, if it can be demonstrated that stereochemical conversion does not occur during stability testing of the drug substance and drug product, then stereoselective tests may not be needed. Therefore, the aim of the present work was to develop and validate an analytical method capable of separating *S*,*S*-moxifloxacin, the parent drug, from its three potential isomeric impurities, with a quantitation limit of 0.05%, with the intent of using the method to determine if racemization was a potential degradation pathway of the drug substance, both in the solid state and in solution.

For the majority of drug tests that involve quantitation of impurities, HPLC is the preferred analytical method. Direct liquid chromatographic enantiomeric separation of several fluoroquinolones, including gemifloxacin, on a Crownpak CR(+) column has been reported [4]. A normal phase chiral LC method has been described for the separation of two isomers of (±)*cis*-8-benzyl-2,8-diazobicyclo(4.3.0)nonane, an intermediate of moxifloxacin using a Chiralcel OD-H col-

* Corresponding author. Tel.: +1 817 551 8194; fax: +1 817 615 3345.
E-mail address: louann.cruz@alconlabs.com (L.A. Cruz).

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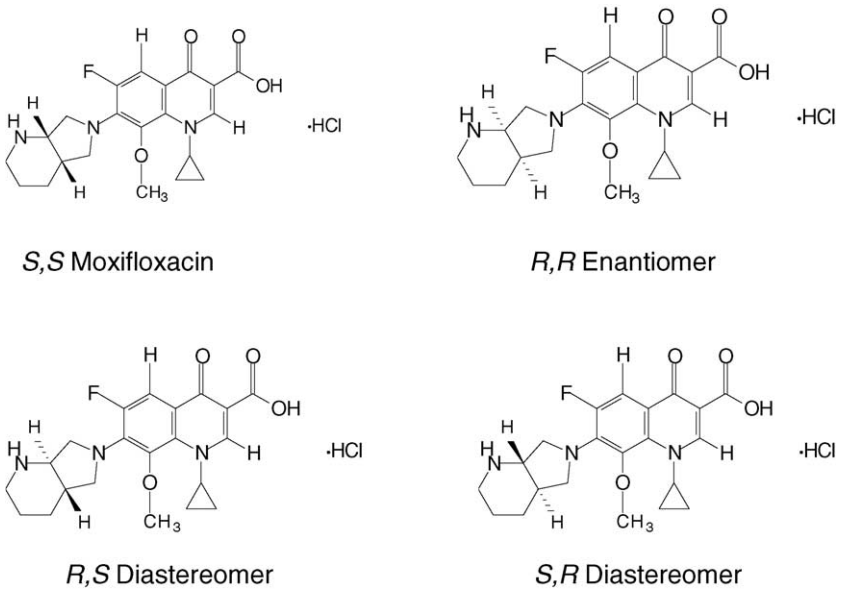

Fig. 1. Chemical structures of *S,S*-moxifloxacin and its potential isomeric impurities.

umn [5]. However, we found these columns unable to separate the moxifloxacin isomers. Twenty-three other HPLC columns were also screened (including four polysaccharide, eight cyclodextrin, three macrocyclic glycopeptide and eight Pirkle-type phases), but none were found to be suitable. We, therefore, investigated the method of capillary electrophoresis (CE) as a practical alternative, because the technique is considered to be complementary to HPLC. Several validated CE methods have been reported for the separation of enantiomeric drugs [6–9] and CE is beginning to gain acceptance within the regulated environment of the pharmaceutical industry. A general test chapter on CE has recently been added to both the US Pharmacopeia [10] and European Pharmacopeia [11]. An enantiomeric separation of the fluoroquinolone drug, ofloxacin, by cyclodextrin-mediated CE has been reported [12]. However, to the best of our knowledge, the work presented here is the first literature report which describes the development and validation of a stereoselective analytical method for moxifloxacin and/or for any fluoroquinolone drug possessing two chiral centers. An unpublished CE method for the separation of the moxifloxacin *S,S*- and *R,R*-enantiomers using heptakis(2,6-di-*O*-methyl)-β-cyclodextrin has been developed and validated by Bayer AG. However, the use of highly-sulfated gamma-cyclodextrin (HS-γ-CD) reported here was able to provide separation of all four moxifloxacin isomers.

2. Experimental

2.1. Materials and reagents

Fused-silica capillaries were purchased from Polymicro Technologies Inc. (Phoenix, AZ). Acetonitrile (HPLC grade), boric acid, citric acid, hydrochloric acid (concentrated), hydrogen peroxide (30%), sodium borate decahydrate, sodium citrate dihydrate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic anhydrous, and sodium hydroxide solution (50%) were purchased from EM Science (Gibbstown, NJ). Sodium hydroxide (0.1N) was purchased from Ricca Chemical Co. (Arlington, TX). Triethylammonium phosphate buffer (50 mM, pH 2.2) and HS-γ-CD (20% (w/v) aqueous solution) were purchased from Beckman Coulter (Fullerton, CA). Moxifloxacin hydrochloride (*S,S*-isomer) and its isomeric impurities (*R,R*-enantiomer, *R,S*-diastereomer, and *S,R*-diastereomer) were kindly provided by Bayer AG (Wuppertal, Germany). The diastereomers were supplied as a racemic mixture. All buffer, standard and sample solutions were prepared using purified water (USP).

2.2. Instrumentation and method conditions

Method development, validation, and degradation pathways studies were carried out on a Beckman P/ACE MDQ capillary electrophoresis instrument equipped with a capillary cartridge cooling system and a photodiode array detector. Data was acquired and processed using Beckman ^{32}Karat software, version 4.01. The capillary was 40 cm long (30 cm effective length) with a 50 μm internal diameter. The applied voltage was 13 kV (0.17 min ramp time) using reverse polarity. The capillary was thermostated at 20 °C. Samples were pressure injected for 15 s at 1 psi followed by a pressure injection of a water plug for 10 s at 0.3 psi. The photodiode array detector was used in single wavelength acquisition mode at 295 nm with a data sampling rate of 4 Hz, a bandwidth of 10 nm, and normal filtering. The capillary was preconditioned each day by flushing with the follow-

ing reagents, in order, for 5 min each at 20 psi: purified water, 0.1N NaOH, purified water, running buffer. The running buffer was prepared by mixing together the appropriate amounts of 50 mM TEA phosphate buffer (pH 2.5), purified water, 20% (w/v) HS-γ-CD, and acetonitrile such that the final composition was 12.5 mM TEA phosphate buffer (pH 2.5), 5% HS-γ-CD and 6% acetonitrile. The running buffer was replenished after approximately every 30 separations.

2.3. Validation standard curves of isomeric impurities

Two, triplicate, low-level, six-point standard curves were prepared of each isomeric impurity in the presence of the *S*,*S*-isomer (100%) and assayed by capillary electrophoresis. One standard curve was conducted in aqueous solution, the other in an ophthalmic/otic drug product formulation. The concentrations of the curve points for the *R*,*R*-enantiomer were 0.05, 0.11, 0.55, 1.10, 2.75, and 5.50 μg/mL corresponding to 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0% of the target moxifloxacin free base concentration of 110 μg/mL. The concentrations of the curve points for each of the diastereomers were 0.05, 0.10, 0.51, 1.02, 2.54, and 5.08 μg/mL corresponding to 0.05, 0.1, 0.5, 0.9, 2.3, and 4.6% of the target moxifloxacin free base concentration of 110 μg/mL. Each point of the aqueous curve was prepared by pipetting the appropriate amounts of each isomeric impurity from stock standard solutions and the appropriate amount of moxifloxacin hydrochloride (*S*,*S*-isomer) from a stock standard solution into volumetric flasks. Each point of the drug product curve was prepared by pipetting the appropriate amounts of each isomeric impurity from stock standard solutions, the appropriate amount of moxifloxacin hydrochloride (*S*,*S*-isomer) from a stock standard solution, and the appropriate amount of the placebo formulation into volumetric flasks. The placebo formulation contained the full formulation of the ophthalmic/otic drug product with the exception of moxifloxacin hydrochloride.

The percent of each isomeric impurity observed in the method was calculated according to Eq. (1):

$$\%\,\text{isomer} = \frac{A_{\text{imp}}}{A_{\text{ss}}} \times \text{CF} \times 100\%, \quad (1)$$

where A_{imp} is the peak area of the isomeric impurity in the standard preparation, A_{ss} is the peak area of the *S*,*S*-isomer in the standard preparation and CF is a correction factor for absorbance differences between the two. The correction factor for all three of the isomeric impurities was found to be 1.2. The necessity of a correction factor was thought to be due to the fact that the *S*,*S*-isomer was purposely mass overloaded onto the capillary in order to detect the impurities at trace levels. The correction factor was determined by comparing the average response per concentration of five injections of a 110 μg/mL standard solution of the *S*,*S*-isomer with the average response per concentration of a 5 μg/mL standard solution of each isomeric impurity.

2.4. System suitability

A resolution solution comprised of 10 μg/mL *S*,*S*-enantiomer and 10 μg/mL *R*,*R*-enantiomer in purified water was prepared and five successive injections were made on the capillary electrophoresis system. Criteria were set as follows: theoretical plates/column (*N*) of the *S*,*S*-enantiomer peak ≥50,000, tailing factor (*T*) of the *S*,*S*-enantiomer peak ≤2.0, resolution (*R*) between the *S*,*S*-enantiomer and *R*,*R*-enantiomer ≥5.5, and system precision of area counts of the *S*,*S*-enantiomer from five successive injections ≤3.0% R.S.D.

2.5. Forcibly degraded aqueous standards and blanks

To demonstrate specificity, aqueous standard solutions of moxifloxacin were prepared and then forcibly degraded using five different stress conditions: heat, acid, base, peroxide, and light. Aliquots of an aqueous stock solution of moxifloxacin free base (2.0 mL of a 2.5 mg/mL solution) were added into each of several 10.0 mL volumetric flasks. Each flask was then treated in one of the following manners: heated for 15 h at 80 °C, added 100 μL of concentrated hydrochloric acid and heated for 15 h at 80 °C, added 50 μL of 50% sodium hydroxide and heated for 15 h at 80 °C, added 10 μL of 30% hydrogen peroxide and heated for 15 h at 80 °C, and placed 10 cm from a 450 W mercury vapor lamp for 15 h. A corresponding blank solution was made for each condition. After removal from the stress condition, all samples were cooled to room temperature, the acid and base samples were neutralized, and all samples were diluted to a final concentration of 110 μg/mL moxifloxacin free base and assayed by capillary electrophoresis.

2.6. Solid state degradation pathways studies

Samples for solid state stress degradation studies were prepared by weighing approximately 0.7 g of moxifloxacin hydrochloride raw material into an appropriate container and spreading to a depth not more than 3 mm. The humidity- and heat-stressed samples were placed into amber glass bottles. The light-stressed sample was placed into a clear glass bottle. The containers of the humidity- and light-stressed samples were left unsealed and the container of the heat-stressed sample was sealed. Samples were stored for 12 weeks under each of the following conditions: 40 °C/75% relative humidity (RH), 60 °C, and in a stability light chamber (provides ICH-required illumination levels with 4-week exposure) at 25 °C/40% relative humidity. After the storage period, an appropriate dilution of the drug substance was prepared in purified water in order to achieve a final concentration of moxifloxacin free base (*S*,*S*-isomer) of about 110 μg/mL and assayed by capillary electrophoresis.

2.7. Solution degradation pathways studies

Solution stress degradation studies were carried out on 1.25 mg/mL moxifloxacin free base (*S*,*S*-isomer) samples

prepared under five conditions in sealed screw-cap test tubes as follows: in 50 mM citrate buffer, pH 4.0; in 50 mM phosphate buffer, pH 7.0; in 50 mM borate buffer, pH 9.0; in water (unbuffered) sparged with O_2 for 20 min; and in water (unbuffered). The acidic, neutral, basic, and oxygenated samples were stored at 50 °C for 12 weeks. The unbuffered sample in water was stored in a stability light chamber at 25 °C/40% relative humidity for 12 weeks. After the storage period, the samples were diluted to a final moxifloxacin free base (*S*,*S*-isomer) concentration of 110 μg/mL and assayed by capillary electrophoresis.

3. Results and discussion

3.1. Specificity

Specificity was demonstrated by the method's ability to resolve each of the four isomers: *S*,*S*, *R*,*R*, *R*,*S* and *S*,*R* (see Fig. 2). For validation purposes, a racemic mixture of the *R*,*S*- and *S*,*R*-diastereomers was used. Because it was not possible to distinguish the configuration of the separated diastereomer peaks as *R*,*S* or *S*,*R*, the earlier-migrating diastereomer was denoted A and the later-migrating diastereomer was denoted B. Specificity was also demonstrated with regard to the forcibly degraded moxifloxacin standards and blanks; the CE method was stability-indicating since degradation of moxifloxacin was observed without interference to the isomer responses. Finally, no evidence of interference with any of the isomer responses was observed from any excipients in the placebo formulation.

3.2. Linearity, accuracy, and repeatability

The responses per concentration obtained of each isomeric impurity for both standard curves are provided in Table 1. Statistical summaries of the data obtained for the standard curves of the isomeric impurities are provided in Table 2. It was decided not to include the lowest (0.05%) level of each curve in the statistics, due to worsened percent recoveries. However, the remaining triplicate, five-point low-level curves were found to have acceptable linearity ($R \geq 0.9925$), accuracy (90–110% recovery range) and repeatability (R.S.D. close to 10%). The assay precision could possibly have been improved through use of an internal standard.

3.3. Standard replicates

The resolution test solution was injected five times in succession to determine system precision. Statistical analysis of the responses revealed good precision, with a relative standard deviation of 1.8% for the *S*,*S*-enantiomer and 1.9% for the *R*,*R*-enantiomer.

3.4. Limit of quantitation

All of the six-level, low concentration impurity standard curves were linear and accurate down to the 0.1% level. Slightly worse percent recoveries and precision were obtained for all of the isomers at the 0.05% level. However, the recovery and precision of the 0.05% points were still considered acceptable for quantitation purposes. The %R.S.D. of the recoveries for the 0.05% points for the *R*,*R*-enantiomer, diastereomer A and diastereomer B curves, respectively, were (7.4%, 7.7%), (9.5%, 2.9%) and (17.2%, 12.2%). Therefore, the limit of quantitation for all impurities was determined to be 0.055 μg/mL which is 0.05% of the target moxifloxacin (*S*,*S*-isomer) concentration of 110 μg/mL.

3.5. Robustness

Parameters investigated were the HS-γ-CD concentration (4.5–6%), acetonitrile concentration (0–10%), phos-

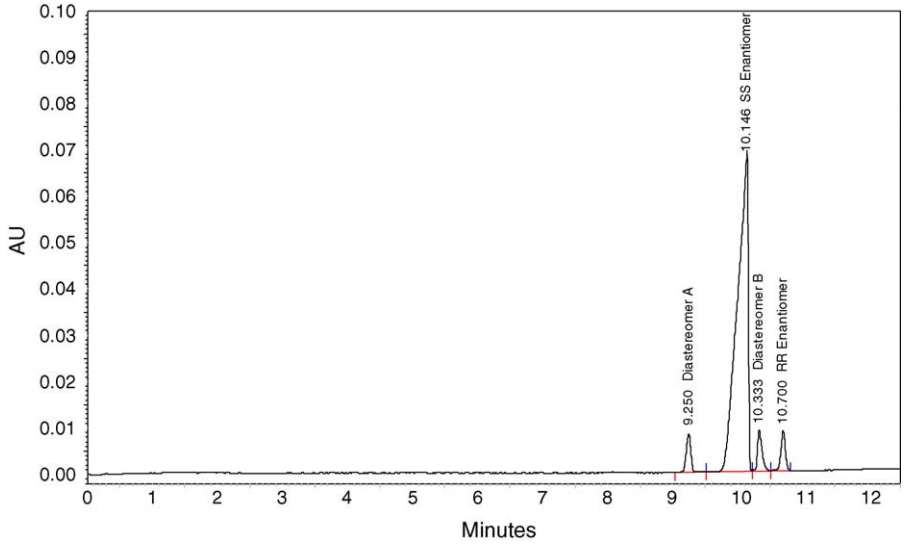

Fig. 2. Electropherogram of *S*,*S*-moxifloxacin and its potential isomeric impurities. Conditions as given in Section 2.

Table 1
Response (area counts) vs. concentration values and percent recoveries of isomeric impurity standard curves

	Aqueous standard curve			Drug product standard curve	
	Concentration (μg/mL)	Response (area counts)	Percent recovery	Response (area counts)	Percent recovery
R,*R*-Enantiomer	0.05	358, 450, 396	101.5, 116.8, 105.0	442, 319, 374	107.8, 93.0, 96.9
	0.11	760, 741, 646	108.5, 107.8, 98.2	809, 814, 728	110.4, 106.2, 103.0
	0.55	2919, 3281, 2937	101.7, 102.5, 102.8	3447, 3046, 3259	101.7, 96.9, 102.0
	1.10	6093, 6259, 6500	101.3, 103.8, 100.8	7269, 7246, 7294	102.2, 101.2, 103.7
	2.75	18825, 17203, 17135	105.4, 104.7, 106.8	20377, 19488, 18747	104.8, 103.6, 102.9
	5.50	29837, 31847, 31122	108.3, 104.9, 105.0	39351, 35019, 40464	105.2, 105.1, 108.4
Diastereomer A	0.05	325, 400, 325	99.8, 112.4, 93.4	344, 305, 333	90.8, 96.3, 93.4
	0.10	563, 583, 597	92.7, 91.8, 92.3	650, 685, 641	96.1, 96.8, 98.2
	0.51	2651, 2881, 2646	100.5, 97.5, 99.8	3045, 2686, 2579	97.3, 92.5, 87.4
	1.02	6073, 5542, 5595	102.0, 99.5, 100.7	6678, 6735, 6492	101.7, 101.9, 100.0
	2.54	15658, 15865, 17062	105.7, 104.5, 103.5	19569, 18171, 17996	109.0, 104.6, 106.9
	5.08	27653, 28808, 26162	101.1, 102.7, 102.8	38230, 31919, 37349	110.7, 103.7, 108.3
Diastereomer B	0.05	362, 369, 275	111.1, 103.7, 79.0	348, 257, 370	91.9, 81.2, 103.8
	0.10	583, 611, 630	96.0, 96.2. 97.4	693, 670, 661	102.4, 94.6, 101.2
	0.51	2509, 2962, 2633	95.1, 100.2, 99.3	3035, 2898, 2755	97.0, 99.8, 93.3
	1.02	6122, 5689, 5745	102.8, 102.1, 103.4	6966, 7013, 6864	106.1, 106.1, 105.7
	2.54	15762, 16091, 17289	106.4, 106.0, 104.9	20074, 18809, 17922	111.8, 108.3, 106.5
	5.08	28390, 29542, 26843	103.8, 105.3, 105.5	39282, 33158, 38232	113.7, 107.7, 110.9

phate buffer concentration (10–50 mM), and temperature (15–25 °C). The applied voltage was adjusted under each condition to minimize Joule heating. The effect that varying each parameter had on the resolution of the four isomers was studied. Concentrations of HS-γ-CD less than 5% resulted in a loss of resolution and concentrations greater than 5% did not improve resolution. Addition of acetonitrile was found to be necessary to separate all four isomers. Concentrations of acetonitrile both less than and greater than 6% resulted in a loss of resolution. A concentration of at least 10 mM phosphate buffer was necessary for sample buffering. Concentrations of phosphate buffer greater than 12.5 mM resulted in improved resolution, but also resulted in a loss of sensitivity. Use of a temperature less than 20 °C resulted in improved resolution, but also resulted in an increased run time. In summary, there is a fine interplay between the parameters in order to achieve the maximum resolution and sensitivity, while minimizing Joule heating and run time. Therefore, only minor adjustments of each parameter (±1%) is recommended.

3.6. Degradation pathways studies

No isomeric impurities ≥0.05% were observed in stressed solid state moxifloxacin hydrochloride samples following storage at 40 °C/75% RH, 60 °C, or in a stability light chamber after 12 weeks. No isomeric impurities ≥0.05% were observed in stressed solutions of moxifloxacin following storage at 50 °C under acidic, neutral, basic, or oxidizing conditions for 12 weeks or in a stability light chamber after 12 weeks. Considering the structure of moxifloxacin, racemization seems an unlikely pathway for degradation since inversion of the chiral centers would require cleavage of non-activated C–C single bonds. Therefore, these studies provide confirmatory evidence that racemization is not a degradation

Table 2
Linearity, accuracy, and repeatability statistics of isomeric impurity standard curves (the 0.05 μg/mL points were not included in the curve statistics)

	Aqueous standard curves			Drug product standard curves		
	R,*R*-Enantiomer	Diastereomer A	Diastereomer B	*R*,*R*-Enantiomer	Diastereomer A	Diastereomer B
r	0.9959	0.9940	0.9947	0.9966	0.9945	0.9952
r^2	0.9918	0.9881	0.9895	0.9933	0.9891	0.9904
y-intercept (area units)	326	366	308	−260	−383	−370
Relative intercept (% of midcurve response)	2.0	2.5	2.1	−1.4	−2.1	−2.0
Slope (area units/μg/mL)	5703	5513	5655	7036	7183	7378
Response sum of squares (area units)^2	1.56×10^7	1.80×10^7	1.68×10^7	1.93×10^7	2.80×10^7	2.60×10^7
Average recovery (%)	104.2	99.8	101.6	103.8	101.0	104.3
Recovery range (%)	98.2–108.5	91.8–105.7	95.1–106.4	96.9–110.4	92.5–110.7	93.3–113.7
Mean response/concentration (area units/μg/mL)	5966	5708	5817	6743	6569	6782
S.D. (area units/μg/mL)	530	433	480	566	750	709
R.S.D. (%)	8.9	7.6	8.2	8.4	11.4	10.5
95% Confidence interval (%)	±17.4	±14.9	±16.2	±16.4	±22.4	±20.5

pathway for moxifloxacin hydrochloride, either in the solid state or in solution.

4. Conclusions

A capillary electrophoresis method for the separation of moxifloxacin hydrochloride (*S*,*S*-isomer) from its potential isomeric impurities (*R*,*R*-enantiomer, *R*,*S*-diastereomer, and *S*,*R*-diastereomer) was developed and validated. The method was validated for quantitation of isomeric impurities at the 0.05% level in both drug substance and in an ophthalmic/otic drug product solution. The method showed suitable performance with regard to selectivity, linearity, accuracy, repeatability, and limit of quantitation. The robustness of the method was also evaluated and appropriate system suitability criteria were set. The method was stability-indicating and was used to confirm that racemization was not a significant degradation pathway for the drug substance, either in the solid state or in solution.

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