Separation of the Two Enantiomers of T-3811ME by Normal-Phase HPLC Using Modified Amylose as Chiral Stationary Phase

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Abstract

A normal-phase enantioselective high-performance liquid chromatographic method is developed for the separation of the undesired enantiomer from T-3811ME and quantitaion of the undesired enantiomer at low levels using a Chiralpak AD-H column (4.6 × 150 mm) packed with modified amylose stationary phase. The 2% water–modified 2-propanol is used for the method development activities, including exploration of various organic modifiers, optimization of additive acid concentration, column screening, and column temperature optimization. The final optimized method separated the undesired enantiomer from T-3811ME and is proven to be robust, sensitive, linear, accurate, and precise.

Introduction

Chiral compounds have been playing more and more important roles in the pharmaceutical industry. Due to the different biological activity of each enantiomer, determination of the enantiopurity and separation of the enantiomers of chiral drugs and their precursors or metabolites became necessary for the development of most today's pharmaceuticals (1). This trend has led to the rapid development of a variety of stereoselective separation technologies (2,3). Direct enantiomeric separation by high-performance liquid chromatography (HPLC) with chiral stationary phases (CSPs) has evolved to be one of the most important techniques for analysis of enantiopurity (3–7), especially for the research and development of chiral drugs in the pharmaceutical industry (6). Among various kinds of CSPs developed for enantioselective HPLC, cellulose- and amylose-derived CSPs are probably the most commonly utilized because of their very wide scope of applications (7). Okamoto et al. and other researchers have introduced many cellulose and amylose derivatives as a coating of polymer on large-pore silica gel (8–10). Commercially available columns made of these CSPs now are extensively used for both analytical and preparative separations of a wide range of enantiomers (11). Separation of enantiomers on chiral stationary phases that are based on cellulose or amylose derivatives is usually conducted under normal-phase chromatography conditions. Mixtures of hexanes and 2-propanol are commonly used as the mobile phase for normal-phase enantioselective HPLC (11–14).

This paper describes our strategy of chiral method development to separate the undesired enantiomer from an active pharmaceutical ingredient (API), T-3811ME, by normal-phase HPLC using modified amylose as CSP. T-3811ME is a new des-fluoro(6)-quinolone, which is active against gram positive and gram negative organisms, including certain quinolone and methicillin-resistant strains (15). The chiral center of T-3811ME, as shown in Figure 1, is located in an environment far away from the bulk of the substituents of the molecule. The closest functional group that is potentially capable of discriminating the two enantiomers is the secondary amine, which is also a relatively weak functional group. Thus, separation of the two enantiomers is extremely challenging.

Major objectives of the method development were to develop a sensitive, rugged, and QC-friendly enantioselective HPLC method which is capable of separating the undesired enantiomer from T-3811ME and quantitating the undesired enantiomer at low levels (~ 0.1% relative to T-3811ME). Individual impurities, including undesired enantiomer(s), in APIs require identification and safety assessment if they are equal to or more than 0.1% (wt/wt) of the API. Therefore, the analytical method should have the capability to quantitate the undesired enantiomer at 0.1% (wt/wt) or lower, which means a quantitation limit of 0.1% (wt/wt) or less for the undesired enantiomer. For a quality con-

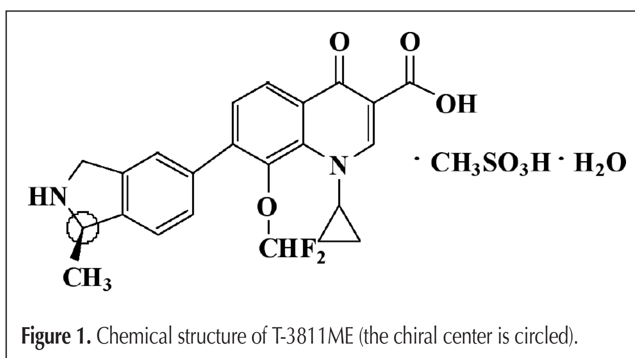

Figure 1. Chemical structure of T-3811ME (the chiral center is circled).

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trol lab, the method should also provide good linearity, accuracy, precision, and robustness.

Experimental

Columns

Preliminary column screening involved π-electron donor/π-electron acceptor chiral columns (Whelk-O 1, ULMO, and DACH-DNB; Regis Technology Inc., Morton Grove, IL), π-electron acceptor chiral columns (Pirkle 1-J, β-Gem 1, α-Burke 2, Phenylglycine, and Leucine; Regis Technology Inc.), macrocyclic glycopeptide based chiral columns (Chirobiotic V, V2, T, T2, R, and TAG; Astec, Whippany, NJ), cyclodextrin based chiral columns (Cyclobond 1 2000, AC, RSP, SN, DMP, and DNP; Astec), modified amylose based chiral columns (Chiralpak AD and AS; Chiral Technologies, West Chester, PA), and modified cellulose column (Chiralcel OD, OJ, OB, OF, OG, and OK; Chiral Technologies).

The column used in the major method development activities was a Chiralpak AD-H (150 × 4.6 mm) column from Chiral Technologies. Six other chiral columns of 150 × 4.6 mm from Chiral Technologies, including Chiralpak IA, Chiralpak QD-AX, Chiralpak QN-AX, Chiralpak IB, Chiralcel OD-H, and Chiralcel OJ-H, were also evaluated for further column screening with mobile phase of hexane–2-propanol–2-ethoxyethanol–water–methanesulfonic acid (75:20:5:0.4:0.05, v/v/v/v/v) at 1.0 mL/min and room temperature. The solution containing the two enantiomers at approximately 1:1 (w_t/w_t) ratio gave a broad peak from all these columns. These two compounds did not elute until 60 min or later for Chiralpak IB, Chiralpak QD-AX, and Chiralpak QN-AX columns.

Chromatography

Chromatography was carried out by using Agilent 1100 systems equipped with DAD (PDA) detectors (Agilent, Santa Clara, CA). A part of the method evaluation was performed on a Waters 2695 Separations Module equipped with a Waters 2487 Dual Wavelength Absorbance Detector (Waters, Milford, MA). The essential components of the mobile phase were hexanes and 2-propanol with methanesulfonic acid. A variety of organic modifiers, most of which were alcoholic solvents, were evaluated at different concentrations. Different column temperatures from 15°C to 40°C were evaluated. For the final developed method, the flow rate was 1.0 mL/min, the column temperature was 35°C, the injection volume was 5 µL, and the detection was via UV absorbance at 280 nm. Methanol was used as the sample diluent, the needle wash, and the seal wash, where applicable.

Chromatographic performance

Retention time (t_R), selectivity factor (α), resolution factor (Rs), and peak efficiency (N) calculations were performed by ChemStation for Agilent systems or Millennium[32] for the Waters system. In some studies, manual integration was involved.

Solvents and chemicals

All solvent and reagent were regent grade or better. They were used as purchased from commercial sources without further purification. Hexanes, 2-propanol, methanol, acetonitrile, 1-butanol, 1-octanol, 2-methoxyethanol, ethylene glycol, sulfuric acid, and acetic acid were from Fisher Scientific (Hampton, NH). 2-Ethoxyethanol was from Acros (Fair Lawn, NJ) or Riedel-de Haën (Seelze, Germany). *t*-Butanol, cyclohexanol, methanesulfonic acid, ethanesulfonic acid, 1-propanesulfonic acid, pentafluoropropionic acid, and trifluoroacetic acid were from Acros. Propylnitrile was from Alfa Aesar (Ward Hill, MA). 3A Ethanol was from Equistar Chemical (Houston, TX). Ethanol (200 proof) was from Pharmaco (Bayonne, NJ). Water was obtained from an in-house Millipore apparatus (Milli-Q system; Millipore, Bellerica, MA).

Reference standards of T-3811ME and the undesired enantiomer were provided by the API vendor for Schering-Plough.

Preparation of solutions and mobile phases

Approximately 20 mg of the T-3811ME reference standard was transferred into a 25-mL volumetric flask and dissolved with methanol. The solution was brought to volume with methanol and mixed well. The standard solutions of undesired enantiomer and the mixture of them were similarly prepared. T-3811ME solutions spiked with low level of undesired enantiomer were prepared by transferring calculated amount of undesired enantiomer standard solution with glass syringe into the T-3811ME standard solution.

The 2% (v/v) water–modified 2-propanol was prepared by mixing calculated amount of water with commercial 2-propanol. The mobile phase was prepared by mixing calculated volumes of components.

Results and Discussion

Preliminary column screening

Initial screening of chiral column was carried out by several chiral HPLC column suppliers. All these columns failed to provide selectivity between T-3811ME peak and the undesired enantiomer peak.

We continued to screen other commercially available chiral columns. A promising condition was identified with a mobile phase of hexane–2-propanol–methanesulfonic acid (70:30:0.1, v/v/v) using a Chiralpak AD-H (250 × 4.6 mm) column. Under these conditions, the two enantiomers were separated (but not baseline separation) and the minor peak (undesired enantiomer) eluted before the major peak (T-3811ME). This gave the advantage for the separation of the undesired enantiomer peak from the main T-3811ME peak. This breakthrough provided a promising starting point for further optimization of the separation and for improvement of the sensitivity of the method.

In the following method development activities, a shorter Chiralpak AD-H column (150 × 4.6 mm) was used to obtain a shorter run time than the 250 mm column.

Effect of trace amount of water in the mobile phase

The effect of trace amount of water in the mobile phase was investigated to determine whether trace amount of water in the

mobile phase has any impact on the reproducibility and ruggedness of the enantioselective HPLC method and on improving the major chromatographic characteristics such as selectivity, resolution, etc. A trace amount of water was deliberately added in the mobile phase: 2% (v/v) water was pre-mixed with 2-propanol, and the 2% water pre-mixed 2-propanol was used to prepare mobile phases for all method development activities. Water constituted approximately 0.5–0.6% (v/v) of the total volume of the mobile phase. The results of the separation were compared to the results that were obtained from the mobile phase prepared using 2-propanol without pre-mixed water. Trace amounts of water in the mobile phase proved to be able to improve the selectivity (α), resolution (Rs), and efficiency (N) of the peaks of T-3811ME and the undesired enantiomer (refer to Table I). Therefore, 2% (v/v) water–modified 2-propanol was used in all subsequent method optimization studies. Details of our investigation on the effect of trace amount of water in the mobile phase in normal-phase enantioselective HPLC chromatography will be published separately.

Organic modifiers

Alcohols such as methanol and ethanol are among the most polar common solvents that are used in the mobile phases of normal-phase enantioselective chromatography. These alcohols can have similar effect on the separation of the two enantiomers of T-3811ME as trace amount of water. These alcohols are also more miscible with the 2-propanol–hexane mixture than water.

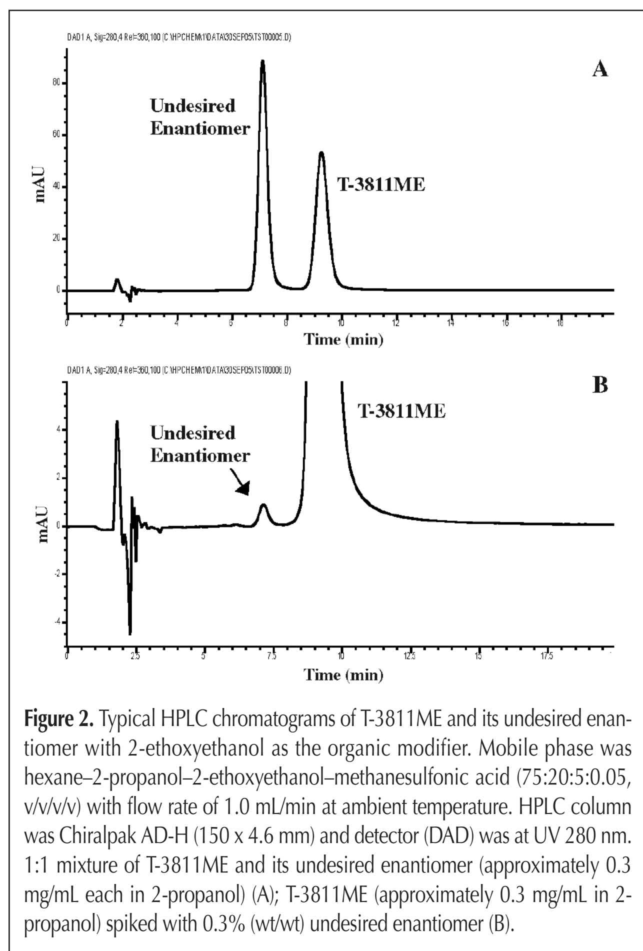

Figure 2. Typical HPLC chromatograms of T-3811ME and its undesired enantiomer with 2-ethoxyethanol as the organic modifier. Mobile phase was hexane–2-propanol–2-ethoxyethanol–methanesulfonic acid (75:20:5:0.05, v/v/v/v) with flow rate of 1.0 mL/min at ambient temperature. HPLC column was Chiralpak AD-H (150 x 4.6 mm) and detector (DAD) was at UV 280 nm. 1:1 mixture of T-3811ME and its undesired enantiomer (approximately 0.3 mg/mL each in 2-propanol) (A); T-3811ME (approximately 0.3 mg/mL in 2-propanol) spiked with 0.3% (wt/wt) undesired enantiomer (B).

Therefore, small amounts of organic modifiers, such as alcohols, were also used in the mobile phase to investigate their impact on the separation of the two enantiomers of T-3811ME.

The organic modifiers assessed were mainly alcohols such as methanol, ethanol (3A and 200 proof), 1-butanol, *t*-butanol, cyclohexanol, 1-octanol, 2-methoxyethanol, 2-ethoxyethanol, and ethylene glycol, as well as acetonitrile and propylnitrile. Different ratios (1–10%, v/v) of these modifiers were investigated with different ratios of hexanes–2-propanol–methanesulfonic acid. The results of resolution factor (R_s) and selectivity factor (α) are summarized in Table I.

In order to further assess the effect of the trace amount of water in mobile phase on the separation of the two enantiomers, in the presence of 1% methanol, 2% methanol, 2% 3A ethanol, or 2% 200 proof ethanol, neat commercial 2-propanol (i.e., without pre-mixed trace amount of water) and 2-propanol with 2% (v/v) water were both used to prepare mobile phases. As shown in Table I, both selectivity and resolution were improved by using 2-propanol that was pre-mixed with 2% water compared to the results obtained from neat commercial 2-propanol. Details of these results will also be presented in the other publication.

All the alcohols that were investigated in this study showed enhancement of resolution between the two enantiomers when they were used in the mobile phase containing hexane–2-propanol. However, acetonitrile or propylnitrile showed insignificant impact on the separation of the two enantiomers.

Elution order of the two isomers was switched when 5% 2-methoxyethanol was used with 10% water–modified 2-propanol, 85% hexane, and 0.05% methanesulfonic acid. The mechanism and reasons for reversion of the elution order of the enantiomers are not clearly established yet.

It was found that addition of 5% 2-ethoxyethanol in hexane–2-propanol–methanesulfonic acid (75:20:0.05, v/v/v/v) resulted in the best chromatographic characteristics (Table I). The separation of an approximately 1:1 (w_t/w_t) mixture solution (in 2-propanol) of the two enantiomers of T-3811ME and a T-3811ME solution (in 2-propanol) spiked with approximately 0.3% (w_t/w_t) undesired enantiomer and is shown in Figure 2.

Polar solvents such as alcohols in the mobile phase can potentially interact with residual metal ions or silanol groups of the stationary phase. These sites then will not be readily available to the analytes for interactions. Therefore, the secondary interactions between analytes and the stationary phase can potentially be minimized or completely suppressed, which can result in improved resolution and/or selectivity. Organic modifier, such as methanol, ethanol, etc., can also interact with the chiral stationary phase (chiral selectors) of the column. When bulky, complex alcohols, such as 2-ethoxyethanol or 2-methoxyethanol, were used as the organic modifier, the alcohol molecules interacting with the column stationary phase (through both residual silanol groups and chiral selectors) can potentially enhance the capability of chiral discrimination between the two enantiomers. Thus, separation of the enantiomers is further improved by using bulky, complex alcohols in the mobile phase.

Additive acid

A trace amount (0.1%, v/v) of methanesulfonic acid was used

in the mobile phase of the initial conditions. The effect of methanesulfonic acid concentration on the separation of T-3811ME and the undesired enantiomer was evaluated by using the Chiralpak AD-H column (150 × 4.6 mm). When no methanesulfonic acid was used in the mobile phase, the two enantiomers eluted together as a broad peak and there was no separation at all. Methanesulfonic acid concentrations of 0.05%, 0.1%, and 0.5% (v/v) was added to the mobile phase of 74:25:1 (v/v/v) hexanes–2-propanol–methanol. Addition of 0.05% methanesulfonic acid gave the best results and was selected for future studies.

The effect of other acids in the mobile phase was also evaluated, including ethanesulfonic acid, 1-propanesulfonic acid, acetic acid, pentafluoropropionic acid, trifluoroacetic acid, and sulfuric acid. All these acids provided either less resolution or no improvement in resolution between the two enantiomers, compared to 0.05% (v/v) methanesulfonic acid in the mobile phase.

Organic acids as mobile phase additives are widely used on polysaccharide-based chiral stationary phases to minimize interactions with residual silanols and to obtain better peak shape and resolution of chiral compounds (16). However, understanding of

Table I. Effect of Trace Amount of Water and Different Percentages of Organic Modifiers in the Mobile Phase on Separation of T-3811ME and its Undesired Enantiomer Using the Chiralpak AD-H Column (150 × 4.6 mm)

Hexane (%)	IPA* (%)	Organic modifier	Water† (%)	MSA* (%)	Retention time (min)		Separation in 1:1 mixture		Separation in 0.2–0.3% spiked sample	
					E*	T*	Rs	α	Rs	α
70	30	–	–	0.1	5.57	7.00	1.87	1.37	–	–
70	30	–	0.6	0.1	5.49	7.07	2.38	1.42	–	–
75	25	–	–	0.1	8.02	10.03	1.70	1.32	–	–
80	20	–	0.4	0.5	7.90	9.44	1.66	1.25	Slightly	Slightly
73	25	2% 3A EtOH*	–	0.1	6.85	8.93	2.52	1.41	–	–
73	25	2% 3A EtOH	0.5	0.1	6.72	8.48	2.54	1.35	–	–
73	25	2% 200 proof EtOH	–	0.1	6.69	8.40	2.15	1.35	–	–
73	25	2% 200 proof EtOH	0.5	0.1	6.66	8.54	2.50	1.38	–	–
74	25	1% MeOH*	–	0.1	7.82	10.04	2.26	1.37	–	–
74	25	1% MeOH	0.5	0.1	7.80	10.28	2.93	1.42	–	–
79	20	1% MeOH	0.4	0.5	6.95	8.28	1.69	1.26	No	No
73	25	2% MeOH	–	0.1	6.92	8.98	2.58	1.40	–	–
73	25	2% MeOH	0.5	0.1	6.72	8.67	2.82	1.39	Slightly	Slightly
78	20	2% MeOH	0.4	0.05	10.11	12.92	2.79	1.34	2.72	1.33
77	20	3% MeOH	0.4	0.05	9.34	11.86	2.72	1.33	2.71	1.32
80	15	5% MeOH	0.3	0.05	11.44	13.92	2.40	1.26	Slightly	Slightly
75	20	5% 1-BuOH*	0.4	0.05	8.59	10.92	2.56	1.34	2.40	1.32
72	25	3% t-BuOH*	0.3	0.05	7.69	9.90	2.59	1.37	2.49	1.35
70	25	5% t-BuOH	0.5	0.05	7.30	9.46	2.19	1.39	2.14	1.36
75	20	5% t-BuOH	0.4	0.05	10.91	14.36	2.81	1.38	2.71	1.26
72	20	8% t-BuOH	0.4	0.05	8.94	11.61	2.67	1.37	2.44	1.35
70	20	10% t-BuOH	0.4	0.05	8.55	11.18	2.47	1.39	2.32	1.36
67	18	15% t-BuOH	0.36	0.05	7.59	9.70	2.21	1.36	2.03	1.33
70	25	5% Cyclohexanol	0.5	0.05	5.84	7.36	2.18	1.38	2.00	1.44
70	25	5% 1-Octanol	0.5	0.05	7.15	8.93	2.14	1.33	1.95	1.30
76	19	5% 2-ME*	0.38	0.05	6.41	7.65	–	–	2.59	1.26
78	17	5% 2-ME	0.34	0.05	7.12	8.30	–	–	2.35	1.22
79	16	5% 2-ME	0.32	0.05	7.19	8.50	–	–	2.52	1.24
80	15	5% 2-ME	0.30	0.05	7.61	8.78	–	–	2.20	1.20
85	10	5% 2-ME	0.20	0.05	9.51	8.48	–	–	No	No
73	25	2% 2-EE*	0.5	0.05	7.27	9.49	2.81	1.41	2.70	1.39
70	25	5% 2-EE	0.5	0.05	5.09	6.54	2.68	1.44	Slightly	Slightly
75	20	5% 2-EE	0.4	0.05	7.10	9.24	2.95	1.40	2.89	1.39
80	10	10% 2-EE	0.2	0.05	7.91	9.99	2.72	1.34	2.73	1.34
74	25	1% EG*	0.50	0.05	6.45	7.68	–	–	2.38	1.26
79	20	1% EG	0.40	0.05	12.16	14.72	–	–	2.64	1.25
74	25	1% ACN*	0.5	0.05	7.76	9.37	2.01	1.27	No	No
74	25	1% PCN*	0.5	0.05	8.29	10.19	2.27	1.29	2.19	1.28

* IPA = Isopropanol; MSA = Methanesulfonic acid; E = Undesired enantiomer; T = T-3811ME; EtOH = Ethanol; MeOH = Methanol; 1-BuOH = 1-Butanol; *t*-BuOH = t-Butanol; 2-ME = 2-Methoxyethanol; 2-EE = 2-Ethoxyethanol; EG = Ethylene glycol; ACN = Acetonitrile; PCN = Propylnitrile.

† Water in the mobile phase was pre-mixed with isopropanol, if any.

the actual mechanism of the effect of additive acid in the mobile phase on the enantioselectivity remains inconclusive. Based on different published reports (16–19), the most probable mechanism/cause is due to the altering of non-specific adsorption (17), due to the additional hydrogen bonding between analytes and the additive acids that are bound to the stationary phase (18), or due to the increased hydrogen bonding via a localized pH effect (19). Establishment of the actual mechanism needs further experiments and investigation.

Column temperature

Well-controlled column temperature is very important for a rugged HPLC method. The column temperature should be at least 10°C above or below the ambient temperature (approximately 25°C) for the thermostated column heater/chiller chamber to provide stable and accurate column temperatures. Elevated column temperature typically decreases the peak broadening phenomenon via more efficient mass transfer and thus improve the peak shape of the analytes. The column temperature of 35°C improved the resolution and peak shape of the two enantiomers and was selected the final column temperature.

Method Evaluation

Based on the data obtained from method development and optimization activities, Chiralpak AD-H (150 × 4.6 mm, 5 µm particle size) column with mobile phase of hexane–2-propanol–2-ethoxyethanol–methanesulfonic acid (76:19:5:0.05, v/v/v/v) (2-propanol is pre-mixed with 2%, v/v, water) was selected for the final method. The flow rate of the final method was 1.0 mL/min with an injection volume of 5 µL and a run time of 20 min. The column temperature was 35°C, and detection wavelength was 280 nm.

The HPLC condition of the final method was evaluated for its robustness, detection limit (DL), quantitation limit (DL), linearity, recovery, and precision.

Robustness study

The method robustness studies were demonstrated by applying column temperature and mobile phase composition variations, from which the results (retention time and resolution) are presented in Table II.

Similar separation of T-3811ME and the undesired enantiomer was achieved at 30°C, 35°C, and 40°C. At 15°C column temperature, the chromatography produced broader peaks with slightly better separation (larger selectivity factor and resolution factor) but reduced sensitivity.

As the ratio between hexane and 2-propanol varied from 74:21 to 80:15 at column temperature of 35°C, the resolution and peak shape remained similar.

Method validation

A chromatographic method that requires validation should not only demonstrate the capability of separating the critical pair, but also be able to accurately quantitate the amount of all analytes. Therefore, the following validation studies were performed to evaluate the estimation of residual amount of the undesired enantiomer of T-3811ME in the presence of T-3811ME. The concentrations of the undesired enantiomer used for these studies is similar to its concentration present in the actual bulk lots of T-3811ME.

DL/QL evaluation

The DL and QL of the undesired enantiomer were studied with T-3811ME standard solutions spiked with different percentages of the undesired enantiomer. The signal-to-noise ratios (S/Ns) of the undesired enantiomer spiked at 0.017%, 0.05%, and 0.1% of T-3811ME standard solutions (~ 0.4 mg/mL in 2-propanol) were approximately 3, 8, and 16, respectively. The DL (S/N ~ 3) and QL (S/N ~ 10) were determined to be approximately 0.02% and 0.06% of T-3811ME concentration (approximately 0.4 mg/mL), respectively. Correspondingly, the concentration of DL and QL are approximately 0.08 and 0.25 µg/mL, respectively, for the PDA detector. Therefore, this method has adequate sensitivity for the detection and estimation of the undesired enantiomer of T-3811ME.

Table II. Effect of Temperature and Mobile Phase Composition on Retention Time and Resolution of the Two Enantiomers

Temperature (°C)	Hexane (%)	IPA* (%)	2-EE* (%)	Water† (%)	MSA* (%)	RT* (min)		Separation	
						E*	T*	Rs	α
35	80	15	5	0.30	0.05	10.81	13.30	2.77	1.27
35	78	17	5	0.34	0.05	9.77	12.21	3.01	1.30
35	77	18	5	0.36	0.05	8.61	10.74	2.89	1.31
35	76	19	5	0.38	0.05	8.03	9.98	2.79	1.31
35	75	20	5	0.40	0.05	6.47	7.85	2.51	1.29
35	74	21	5	0.42	0.05	6.44	7.85	2.67	1.30
40	76	19	5	0.38	0.05	7.79	9.45	2.67	1.27
30	76	19	5	0.38	0.05	8.35	10.74	3.09	1.36
15	76	19	5	0.38	0.05	9.66	13.85	3.57	1.54

* IPA = Isopropanol; MSA = Methanesulfonic acid; E = Undesired enantiomer; T = T-3811ME; EtOH = Ethanol; MeOH = Methanol; 1-BuOH = 1-Butanol; t-BuOH = *t*-Butanol; 2-ME = 2-Methoxyethanol; 2-EE = 2-Ethoxyethanol; EG = Ethylene glycol; ACN = Acetonitrile; PCN = Propylnitrile.
† Water in the mobile phase was pre-mixed with isopropanol.

Linearity

The linearity of the undesired enantiomer was evaluated from 0.1% to 1.0% relative to the T-3811ME concentration (~ 0.4 mg/mL in 2-propanol), which corresponds to 0.45 to 4.50 µg/mL of the undesired enantiomer. The solutions were prepared by spiking the undesired enantiomer stock solution into the T-3811ME solution at calculated ratios. At each level, duplicate injections were performed. The linear regression analysis equation is $y = 14.464853x + 0.2836735$, where x is the concentration of the undesired enantiomer in µg/mL, and y is the corresponding peak area of the undesired enantiomer in mV/sec. The coefficient of determination (r^2) is 0.999844. The method is proven to be linear within the investigated range.

Recovery and precision

Recovery of the undesired enantiomer was studied at the four levels of the enantiomer spiked solutions in the above linearity study, containing 0.45, 0.90, 2.25, and 4.50 µg/mL of the undesired enantiomer, respectively. At each level, duplicate injections were performed. The recovery of the undesired enantiomer ranged from 94% to 98%. The method proved to be accurate in estimating the amount of the undesired enantiomer of T-3811ME between 0.45 and 4.50 µg/mL. The recovery was calculated by the equation:

$$\text{Recovery}(\%) = \frac{PA_{Enantiomer}}{PA_{API}} \times \frac{C_{API}}{C_{Enantiomer}} \times \frac{1}{\text{Spiking level}} \times 100$$

where: $PA_{Enantiomer}$ = observed peak area of undesired enantiomer in spiked solution; PA_{API} = observed peak area of T-3811ME in spiked solution; C_{API} = theoretical concentration of T-3811ME standard solution used for preparing spiked solution; $C_{Enantiomer}$ = theoretical concentration of undesired enantiomer stock solution used for preparing spiked solution.

The relative standard deviation of the recovery at the four concentration levels in the recovery study was calculated to be 1.9%. The method proved to be precise for estimating the amount of the undesired enantiomer of T-3811ME between 0.45 and 4.50 µg/mL.

Conclusions

A new enantioselective HPLC method was successfully developed which is capable of separating the undesired enantiomer of T-3811ME from T-3811ME peak at levels of 0.1% or lower. HPLC conditions were optimized using organic modifiers that are not widely used for enantioselective HPLC method. These atypical organic modifiers, such as 2-ethoxyethanol and to some extent methanesulfonic acid, were critical to obtain the chiral selectivity, and success of the method development goals.

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