Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 153–157

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Simultaneous enantioseparation of a basic active pharmaceutical ingredient compound and its neutral intermediate using reversed phase and normal phase liquid chromatography with a new type of polysaccharide stationary phase

Lili Zhou *, Vincent Antonucci, Mirlinda Biba, Xiaoyi Gong, Zhihong Ge

Merck Research Laboratories, Merck and Co. Inc., P.O. Box 2000, Rahway, NJ 07065, USA

ARTICLE INFO

Article history:
Received 20 August 2009
Received in revised form 25 August 2009
Accepted 31 August 2009
Available online 19 September 2009

Keywords:
Simultaneous enantioseparations
Chiral liquid chromatographic method
Active pharmaceutical ingredient
Polysaccharide stationary phase

ABSTRACT

Simultaneous enantioseparation of a basic API compound, (R)-2-Amino-N-[2-[1,2-dihydro-1-(methylsulfonyl) spiro [3H-indole-3,4′-piperidin]-1′-yl]-2-oxo-1-[(phenylmethyloxy) ethyl]-2-methylpropanamide monomethanesulfonate (compound-A) and its neutral penultimate intermediate, (R)-2-BOC-Amino-N-[2-[1,2-dihydro-1-(methylsulfonyl) spiro [3H-indole-3,4′-piperidin]-1′-yl]-2-oxo-1-[(phenylmethyloxy) ethyl]-2-methylpropanamide monomethanesulfonate (compound-B) was investigated using reversed phase (RPLC) and normal phase liquid chromatography (NPLC). After an initial screening, a Sepapak-4 column, a new type of polysaccharide chiral stationary phase (CSP), was selected for further method development based on hits on separation selectivity for both compounds under RPLC and NPLC. After comparing the *pros and cons*, a method utilizing the Sepapak-4 chiral column (150 mm × 4.6 mm, 3 μm particle size) in RPLC mode was finally developed. Separations were performed in gradient elution mode starting at 50% A (10 mM, NH_4COOH at pH 6.5)/50% B (50/50 EtOH/MeCN) to 25% A (10 mM, NH_4COOH at pH 6.5)/75% B (50/50 EtOH/MeCN). The flow rate was 1.0 mL/min; the column temperature was 50 °C; the UV wavelength was 220 nm and the mass spectrometric detection was APCI in the positive ionization mode. The reaction mixture sample was directly diluted in ethanol. Baseline enantioseparation were achieved for both compound-A and its intermediate simultaneously with resolution greater than 2.0. The method was validated in terms of injection precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and ruggedness. The specificity of the method was further evaluated by spiking a mixture of enantiomers of compound-A and its intermediate into a reaction matrix containing all of the synthetic reagents. No matrix interference was observed across the elution windows of compound-A and its intermediate. Additionally, the peak purity of each enantiomer was evaluated by mass spectra, indicating the specificity of the method.

1. Introduction

It is now very well known that enantiomers of drug compounds may possess quite different pharmacological and toxicological properties [1]. Since the issuance of a guidance document entitled "FDA's Policy Statement for the Development of New Stereoisomeric Drugs" by the US Food and Drug Administration in 1992 [2], the development of enantioselective analytical methods using high-performance liquid chromatography (HPLC), gas chromatography, supercritical fluid chromatography and capillary electrophoresis (CE) has grown rapidly. In comparison to other techniques, LC offers increased simplicity, robustness, limits of detection and method precision. The applications of chiral-LC techniques have been extensively discussed in recent publications [3–8]. Many applications have become routine and straightforward when dealing with only the pure sample.

However, in practice, interfering substances in the sample matrix can often cause problems. This may necessitate the use of two or more methods to completely characterize the sample. Some of chiral stationary phases with new properties hold promise for dealing with this kind of problem. Most notable are newer reversed phase chiral columns, which in addition to offering different selectivity also allow adjustment of chromatographic parameters in such a way as to improve the ability to resolve coeluting components.

For example, as shown in Fig. 1, compound-A (an API) in the present study was synthesized through deprotection of a BOC group from a compound-B (its intermediate) by using methylsulfonic acid. Early attempts to achieve baseline enantioseparation of compound-A with the desired limit of detection using reversed-phase liquid chromatography with various polysaccharide type

* Corresponding author.
E-mail address: lili_zhou@merck.com (L. Zhou).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.08.027

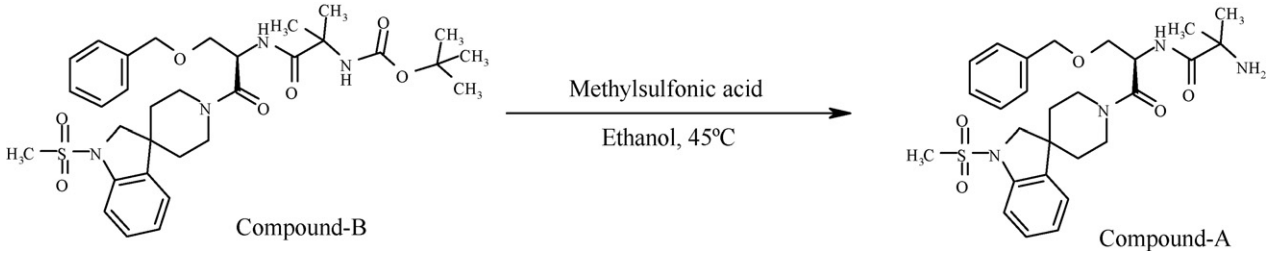

Fig. 1. Reaction scheme for compound-A formation.

columns (Chiralcel OD-RH, Chiralpak AD-RH, Chiralcel OJ-RH, Chiralpak AS-RH), protein type columns (Ultron ES-OVM & Chiral-AGP) and crown ether type columns (CROWNPAK & ChiroSil RCA) as well as normal phase chromatography with various polysaccharide type columns (Chiralcel OD-H, Chiralpak AD-H, Chiralcel OJ-H, Chiralpak AS-H) were unsuccessful. A chiral assay method was developed using capillary electrophoresis with native beta-cyclodextrin [9]. However, compound-B could not be simultaneously separated by the same CE method. The reaction shown in Fig. 1 was initially monitored using two separate methods with two sets of instrumentation.

In this paper, we present an application demonstrating the utility of Sepapak-4 stationary phase to selectively monitor enatiomers of the initial reactant and the final product simultaneously. Both compound-A and compound-B were simultaneously screened on Sepapak-2, Sepapak-3 and Sepapak-4 [6,10–16] under both RPLC and NPLC separation modes and followed by optimization. Utilization of the LC–MS to assure enantiomeric purity of the peaks of interest in the reaction mixture is also demonstrated.

2. Experimental

2.1. Reagents

(R)-2-Amino-N-[2-[1,2-dihydro-1-(methylsulfonyl) spiro [3H-indole-3,4′-piperidin]-1′-yl]-2-oxo-1-[(phenylmethyloxy) ethyl]-2-methylpropanamide monomethanesulfonate (compound-A) and its enantiomer (compound-A′); penultimate intermediate, (R)-2-BOC-Amino-N-[2-[1,2-dihydro-1-(methylsulfonyl) spiro [3H-indole-3,4′-piperidin]-1′-yl]-2-oxo-1-[(phenylmethyloxy) ethyl]-2-methylpropanamide monomethanesulfonate (compound-B) and its enantiomer (compound-B′) were prepared by the Process Research Department of Merck Research Laboratories (Rahway, NJ, USA). All organic solvents used in the studies were HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Phosphoric acid (85%), formic acid, and ammonium acetate were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Water used in the study was deionized water purified through a Millipore deionization device (Milford, MA, USA).

2.2. Instrumentation

An Agilent 1100 system with diode array UV detection and thermal-controller was used. All chromatograms were processed by an Atlas data acquisition system (Version 8.2 Thermo Electron Corporation, PA, USA).

2.3. Chromatographic columns

The chiral columns of Sepapak-2, Sepapak-3 and Sepapak-4 were 15 cm in length and 4.6 mm in ID packed with 3.0 μm particle size and they were purchased from Sepaserve GmbH (Münster, Germany).

2.4. Chromatographic conditions

All LC separations, except where specified, were performed on HP 1100 LCs with UV detectors and controlled column compartments. The column temperature was set at 50 °C. The RPLC mobile phase utilizes a binary gradient while the NPLC was run isocratically. The flow-rate was 1.0 mL/min; the injection volume was 10 μL; UV detection at 220 nm was used in each case. LC–MS experiments were performed on an HP 1100 LC and a quadrupole ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) equipped with an atmospheric pressure chemical ionization (APCI) interface. The capacity factor of each compound was calculated as $k = (t_R - t_0)/t_0$, where t_R and t_0 were the retention times of retained and un-retained compounds, respectively. The Selectivity factors were calculated as $\alpha = k_2/k_1$.

2.5. Preparation of samples

Samples were dissolved in isopropyl alcohol. The concentration of the samples varied from 0.001 mg/mL to 2.0 mg/mL.

3. Results and discussion

3.1. Selection of suitable chiral column

Early attempts to achieve enantioseparation of compound-A and its enantiomer compound-A′ on LC with many chiral station passes were unsuccessful. Demonstrating satisfactory enantioseparation of compound-A and compound-A′ is a key factor to this study. compound-A is basic, initial screens on Sepapak-2, Sepapak-3 and Sepapak-4 were performed using ammonium acetate buffer (pH 8.5) with acetonitrile as mobile phase in RPLC and heptane:ethanol with isobutyl-amine as mobile phase in NPLC. Successful simultaneous enantioseparations of both pairs of enantiomers were achieved only on the Sepapak-4 column under both RPLC and NPLC. Therefore, the Sepapak-4 column was selected for further method development.

3.2. Method optimizations

3.2.1. Effect of chromatographic conditions for NPLC

Method optimization was conducted by varying different parameters, such as polar additives, mobile phase modifiers, mobile phase ratio, sample loading, column temperature etc. Ethanol and IPA are the most suitable mobile phase modifiers for NPLC due to their miscibility with hexane or heptane. In general, IPA often provides higher α values than ethanol since it has less of a tendency to interact through hydrogen bonding with the stationary phase, thus providing higher enantiomeric resolution for compounds that also compete for these active sites. However, in this case, it was somewhat surprising to find that enantioresolution between compound-A and compound-A′ suffered significantly when IPA was used as the polar component of the mobile phase,

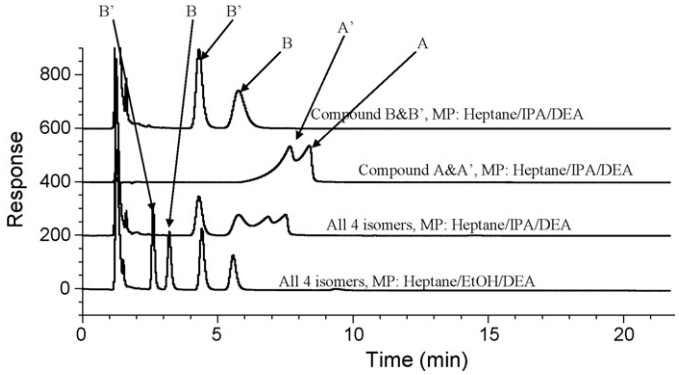

Fig. 2. Effect of mobile phase polar modifiers on NPLC results. Experimental conditions: column: Sepapak-4. Mobile phase for bottom chromatogram: EtOH/Heptane/DEA, ratio: 70/30/0.1 (v/v/v), mobile phase for all other chromatograms: IPA/Heptane/DEA, ratio: 70/30/0.1 (v/v/v). Flow rate: 1.5 mL/min. UV wavelength: 220 nm. Column temperature: 50 °C. Injection volume: 10 μL.

even in the presence of diethyl amine as additive, as shown in Fig. 2. This observation may be attributed to the fact that both species of compound-A and compound-A′ contain a primary amine which is far from the chiral center. Hence the hydrogen bonding interaction between that primary amine functional group and chiral stationary phase is non-enantiospecific. Such a non-specific interaction is reduced through the use of ethanol, which is a well known hydrogen bonding competing agent. Thus, enenatioresolution is enhanced when using ethanol as mobile phase modifier with the addition al benefit of improved peak shapes. The fact that enantioresolution of compound-B and compound-B′ was not affected much by switching from ethanol to IPA is consistent with this explanation since the primary amine group was protected by BOC in compound-B and compound-B′.

Another unique feature of the Sepapak-4 CSP is the maximum allowable column temperature of 50 °C. In many cases, the baseline enantioseparation would increase as the temperature decreased, at the expense of increased run time and broader peaks. However, in this case, the baseline enantioseparation for compound-A and compound-A′ increased as the temperature increased with shorter run time at 50 °C. This can be seen in Fig. 3. More than 500 injections were made at 50 °C and yet no significant change in column behavior was observed. This high column temperature allowed a high flow rate and a high peak efficiency, hence higher detection sensitivity. In this case, we are able to achieve LOD of 0.1% under NPLC mode.

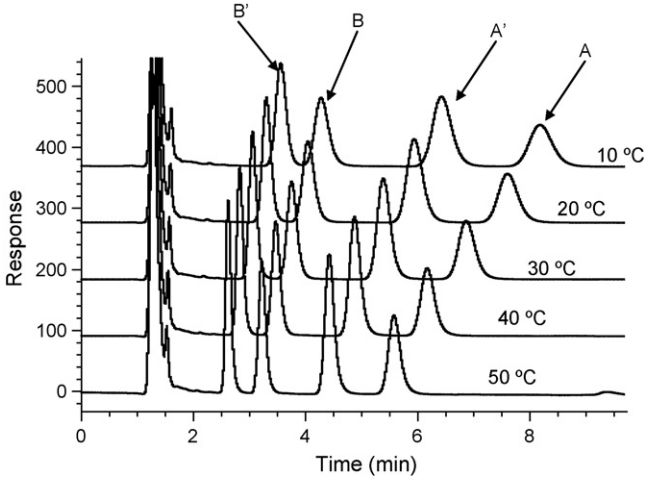

Fig. 3. Effect of column temperature on NPLC results. Experimental conditions: column: Sepapak-4. Mobile phase: EtOH/Heptane/DEA, ratio: 70/30/0.1 (v/v/v). Flow rate: 1.5 mL/min. UV wavelength: 220 nm. Column temperature: varied. Injection volume: 10 μL.

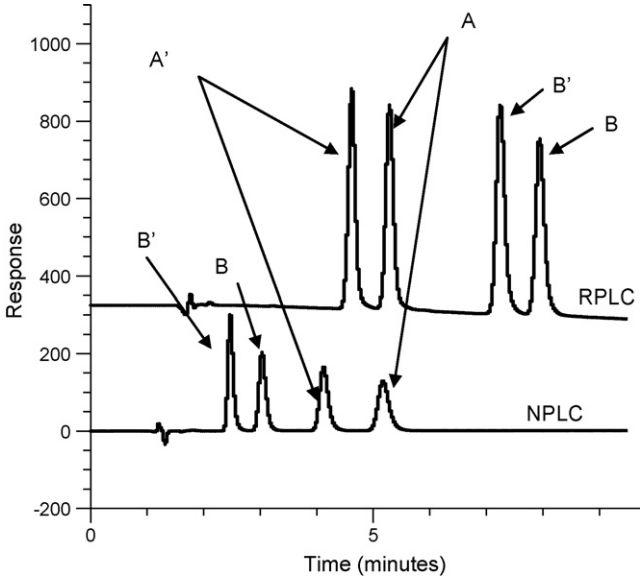

Fig. 4. Comparison of elution order in NPLC and RPLC. Experimental conditions: column: Sepapak-4. Mobile phase for NPLC: EtOH/Heptane/DEA, ratio: 70/30/0.1 (v/v/v). Flow rate for NPLC: 1.5 mL/min. Mobile phase for RPLC: gradient from 50% A (10 mM, NH_4COOH at pH 6.5)/50% B (50/50 EtOH/MeCN) to 25% A (10 mM, NH_4COOH at pH 6.5)/75% B (50/50 EtOH/MeCN). Flow rate: 1.0 ml/min. UV wavelength: 220 nm. Column temperature: varied. Injection volume: 10 μL.

3.2.2. *Effect of chromatographic conditions for RPLC*

Since compound-A and compound-A′ are basic and positively charged under neutral and acidic pH, they interact with CSP less effectively. However, they can be separated more effectively from the neutral intermediates of compound-B and compound-B′ due to their positive charge under such conditions. We varied mobile phase pH from 4.0 to 8.5 and found that pH-6 is an acceptable choice (data not shown).

Another important parameter to optimize is the type of organic modifier. As we reported before [6], for RPLC, phosphate buffers with acetonitrile are the best choice for mobile phase in RPLC for improved efficiency and method sensitivity. Such combinations offer better solute mass transfers and lower UV cut-offs, and thus better LODs. Other organic modifiers may provide better enantioresolution. In this specific case, we used ammonium acetate buffer in order to utilize mass spectrometric detection. Also, we found that a mix of acetonitrile and ethanol at a 1:1 ratio was an optimal choice for organic modifier.

3.2.3. *Comparison between NPLC and RPLC results*

3.2.3.1. Elution order. It is believed that retention in RPLC is due to a partitioning process, whereas retention in NPLC is due to an adsorption process. Therefore, the retention of polar species vs. neutral species is expected to be reversed for the two modes [17]. The chromatograms shown in Fig. 4 are consistent with this prediction. Compound-A and compound-A′ are more polar than compound-B and compound-B′, therefore, they eluted before compound-B and compound-B′ in RPLC mode, but after compound-B and compound-B′ in NPLC mode. Furthermore, the enantiomer elution orders for each pair of enantiomers are the same between two modes.

3.2.3.2. Mobile phase gradient. One of the advantages of using RPLC is the ability to utilize gradient elution. We observed that the gradient elution was superior in terms of peak shapes and reso-

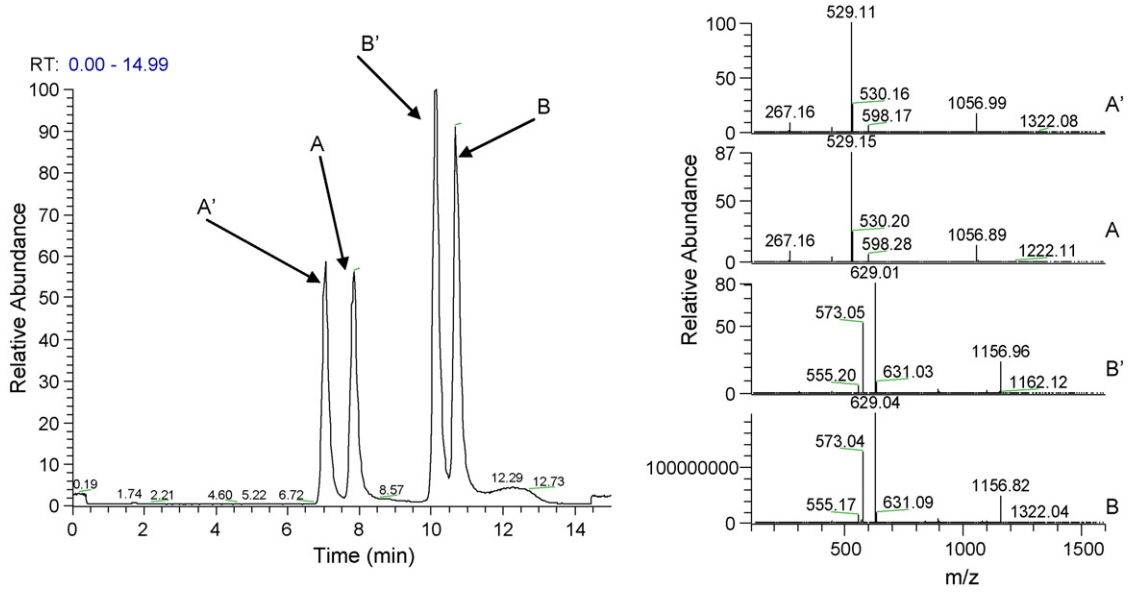

Fig. 5. Typical total ion chromatogram (TIC) and mass spectraypical chromatogram and mass spectra of mixture of 4 stereoisomers. Experimental conditions: column: Sepapak-4. Mobile phase: gradient from 50% A (10 mM, NH_4COOH at pH 6.5)/50% B (50/50 EtOH/MeCN) to 25% A (10 mM, NH_4COOH at pH 6.5)/75% B (50/50 EtOH/MeCN). Flow rate: 1.0 ml/min. Mass detection: APCI, positive. Column temperature: 50 °C. Injection volume: 10 μL.

lution between compound-A (A′) and compound-B (B′). Moreover, since the purpose of this study was to monitor the stereoisomeric changes in a deprotection reaction, using RPLC with gradient elution clearly provides advantages in terms of separating all reaction components from the peaks of interest and eluting any residual reagents present in the matrix during each run.

3.2.3.3. LC–MS utilization. Another advantage of using RPLC is that it is a technique comparable with LC–MS. It is important to make sure that no achiral impurities that co-elute with enantiomers of interest. In this study, we successfully analyzed all four isomeric peaks with mass detection. Fig. 5 shows examples of the LC–MS analysis of all four species of interest.

3.3. Method validation and application

The results discussed above provide an optimal method conditions. These conditions include the use of a Sepapak-4 chiral column (150 mm × 4.6 mm, 3 um particle size) in RPLC mode, the use of a gradient from 50% A (10 mM, NH_4COOH at pH 6.5)/50% B (50/50 EtOH/MeCN) to 25% A (10 mM, NH_4COOH at pH 6.5)/75% B (50/50 EtOH/MeCN) as mobile phase, a flow rate of 1.0 ml/min, column temperature of 50 °C, UV wavelength of 220 nm and APCI positive mass spectrometric detection. Additionally, the reaction mixture sample was directly diluted in ethanol.

The method was validated in terms of injection precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and ruggedness. It was found that the UV response was linear for all stereoisomers from 0.05% to 120% of the 0.8 mg/mL target concentration. Precision was demonstrated by six injections of a mix solution that contained all four isomers. The relative standard deviations for all peak areas were less than 1.0%. The method LOQ and LOD were determined to be 0.1% with signal to noise ratio >3 and 0.3% with signal to noise ratio >10, respectively. The specificity of the method was assessed by LC–MS and further evaluated by spiking individual enantiomers into a reaction matrix containing all of the synthetic reagents.

Following satisfactory method optimization and validation, the RPLC method with both UV and MS detections was applied to monitor the reaction based on a scheme shown in Fig. 1. A mixture of compound-B containing 2% of compound-B′ was reacted with

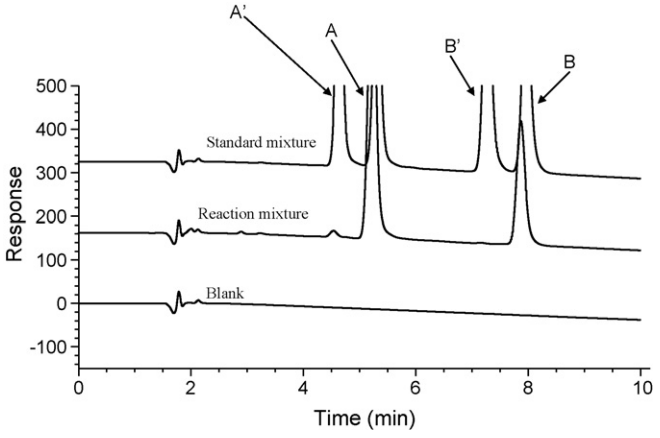

Fig. 6. Chromatogram of reaction mixture at 2-h time point overlaid with chromatogram of standard mixture. Experimental conditions: Experimental conditions: column: Sepapak-4. Mobile phase: gradient from 50% A (10 mM, NH_4COOH at pH 6.5)/50% B (50/50 EtOH/MeCN) to 25% A (10 mM, NH_4COOH at pH 6.5)/75% B (50/50 EtOH/MeCN). Flow rate: 1.0 ml/min. UV wavelength: 220 nm. Column temperature: varied. Injection volume: 10 μL.

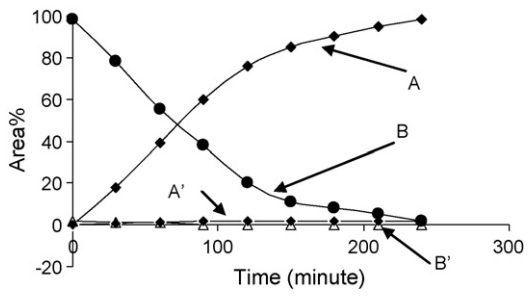

Fig. 7. Reaction profile of compound-A formation. Experimental conditions of reaction: compound-B containing 2% compound-B′ was reacted with methanesulfonic acid in ethanol under nitrogen and heated to 45 °C and aged for approximately 4 h. Reaction profile at 30 min interval was collected. Chromatographic conditions: same as for Fig. 6.

methanesulfonic acid in ethanol under nitrogen, heated to 45 °C and aged for approximately 4 h. Fig. 6 shows a chromatogram of the reaction mixture at 2-h time point overlaid with reference standard mixtures. Reaction profiles were collected at 30 min interval. Based on the data shown in Fig. 7, the reaction was a pseudo first order and 99.5% completed after 2 h. All 2% of compound-B′ was converted to compound-A′ at the 2-hour time point and no racemization occurred during the reaction.

4. Conclusions

A practical, robust, sensitive, specific, accurate and reliable method was developed. Baseline enantioseparations were achieved for an API and its intermediate simultaneously with resolution greater than 2.0. No matrix interference was observed across the elution windows for all peaks of interest. The method has been successfully used to quantify enantiomer excess for monitoring the API synthetic process.

Acknowledgment

The authors wish to thank the Merck Process Research Department for providing the studied compounds.

References

[1] A.G. Mitchell, J. Pharm. Pharm. Sci. 1 (1998) 8–12.
[2] FDA Policy Statement, Chirality 4 (1992) 338–340.
[3] S. Fanali, G. Orazio, K. Lomsadze, B. Chankvetadze, J. Chromatogr. B 875 (2008) 296–303.
[4] J.S. Jin, A.M. Stalcup, M.H. Hyun, J. Chromatogr. A 933 (2001) 83–90.
[5] L. Zhou, B. Mao, Z. Ge, J. Pharm. Biomed. Anal. 46 (2008) 898–906.
[6] L. Zhou, C. Welch, C. Lee, X. Gong, V. Antonucci, Z. Ge, J. Pharm. Biomed. Anal. 49 (2009) 964–969.
[7] N. Byrne, E. Larson, W. Liao, C. Kraml, J. Chromatogr. B 875 (2008) 237–242.
[8] B. Chankvetadze, I. Kartozia, C. Yamamoto, Y. Okamoto, J. Pharm. Biomed. Anal. 27 (2002) 467–478.
[9] L. Zhou, J. Trubig, A. Dovletoglou, D. Locke, J. Chromatogr. A 773 (1997) 311–320.
[10] K.S.S. Dossou, P. Chiap, B. Chankvetadze, A.C. Servais, M. Fillet, J. Crommen, J. Chromatogr. A (2009), doi:10.1016/j.chroma.2009.05.081.
[11] H. Ates, D. Mangelings, Y. Heyden, J. Chromatogr. B 875 (2008) 57–64.
[12] B. Chankvetadze, E. Yashima, Y. Okamoto, J. Chromatogr. A 694 (1995) 101–109.
[13] B. Chankvetadze, E. Yashima, Y. Okamoto, Chem. Lett. 4 (1993) 617–620.
[14] B. Chankvetadze, E. Yashima, Y. Okamoto, J. Chromatogr. A. 670 (1994) 39–49.
[15] B. Chankvetadze, L. Chankvetadze, Sh. Sidamonidze, E. Yashima, Y. Okamoto, J. Pharm. Biomed. Anal. 13 (1995) 695–698.
[16] B. Chankvetadze, L. Chankvetadze, Sh. Sidamonidze, E. Yashima, Y. Okamoto, J. Pharm. Biomed. Anal. 14 (1996) 1295–1303.
[17] L.R. Snyder, J.J. Kirkland, J. Glajch, Practical HPLC Method Development, 2nd ed., Wiley & Sons, New York, 1997.