Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1287–1292
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A feasibility study on direct assay of an aqueous formulation by chiral supercritical fluid chromatography (SFC)

Partha S. Mukherjee [a,*], Steve E. Cook [a]

[a] *Early Development, Pharmaceutical and Analytical R&D, AstraZeneca Pharmaceuticals LP, Wilmington, DE 19850, USA*

Received 17 January 2006; received in revised form 28 March 2006; accepted 29 March 2006
Available online 8 May 2006

Abstract

Supercritical fluid chromatography (SFC) has gained considerable importance in the area of Separation Science in pharmaceutical analysis over the past few years. The synthesis of chiral compounds is of particular significance in the pursuit of new drug entities. SFC is rapidly replacing high performance liquid chromatography (HPLC) in many pharmaceutical and biotechnological companies as the standard screening and method development tool for chiral compounds. Analysis of pharmaceutical formulations of research compounds is an area where SFC is recently being explored as a possible alternate or complementary technique to HPLC in limited scope. A feasibility study was carried out to perform direct assay of a chiral drug compound AZM in 100% aqueous formulations by SFC. The results indicated that this approach has the potential to significantly reduce the typical sample processing time prior to analysis. The method was reproducible, linear over a wide dynamic range, and sensitive enough to detect the minor enantiomeric impurity in the chiral drug compound investigated here. Further application will be pursued for other research compounds in the future to illustrate the broader applicability of this approach.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Supercritical fluid chromatography; Chiral; Direct assay; Formulation; Pharmaceutical analysis

1. Introduction

With the increasing trend of synthesis of chiral compounds and exploration of chiral drug switches, chiral separation has gained significant attention in drug discovery. Historically, HPLC has been the standard technique and first choice for chiral analysis. Capillary electrophoresis and gas chromatography are two alternate techniques also utilized in chiral analysis. HPLC provides the advantage of a wider variety of stationary phases available for chiral analysis. Chiral SFC is, however, rapidly replacing chiral HPLC in many pharmaceutical companies as the standard automated screening and method development tool due to the advantages mentioned above [1–7]. Fast solvent gradients with short 5 cm chiral columns are currently being evaluated by SFC user groups to increase sample throughput in early discovery stage. Packed column SFC has been demonstrated to offer these advantages for analysis of a wide variety of pharmaceutical compounds [8–11].

Assay of pharmaceutical formulations is an area where SFC is lately being considered as a potentially alternate tool to conventional HPLC. Since packed column SFC exhibits chromatographic behavior similar to normal phase, aqueous samples have been a concern. Normal phase systems are typically limited by the long equilibration time and unpredicted retention behavior in the presence of water. Reports have been published on direct achiral assay of emulsions and suspensions by SFC [12–15]. These studies, however, required replacing methanol with 2-propanol in the mobile phase to reduce the water sensitivity of the SFC systems, while maintaining an adequate sample injection volume and acceptable column efficiency.

To the best of our knowledge, the direct chiral/enantioselective assay of drug compounds in 100% aqueous formulations by SFC, without dilution in chromatographic compatible solvents, has been further limited. This is primarily because reversed phase compatible chiral columns (e.g. Chiralpak AD-RH, Chiralcel OD-RH, Cyclobond 2000, etc.) with aqueous buffer/organic mobile phases have been well-established in HPLC and can be used for direct assay of formulations. In the majority of reversed phase chiral HPLC applications, the solvent of choice is 2-propanol or ethanol. Also, the use of a 100% aque-

* Corresponding author. Tel.: +1 302 885 6992; fax: +1 302 886 7470.
E-mail address: Partha.Mukherjee@astrazeneca.com (P.S. Mukherjee).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.03.034

ous buffer as sample solvent for investigational compounds can pose a compatibility issue with the most widely used screening columns in SFC. This has typically required an additive (e.g. 1 mM citrate) in the organic modifier to maintain column integrity, following pure aqueous injection [15].

For analytical support of formulations in early discovery/development stages, the primary requirement is for an assay which can accurately provide the concentration of the research compound as well as be applicable for stability monitoring and other special cases (e.g. enantiomer interconversion). In situations of high sample throughput, the direct assay possibility by SFC will significantly reduce the analytical phase and thus ensure rapid turnaround time to the formulator for subsequent release of the formulation for its intended use.

A feasibility study was conducted using SFC for the assay of a chiral drug compound AZM in aqueous formulations at various pHs. The pH range studied was limited to between 3 and 9.5, as the majority of the formulations are maintained in this range. It is known that chiral SFC typically provides baseline resolution of >90% of the compounds using one of the standard four columns available from Chiral Technologies Inc. (Exton, PA, USA), in the decreasing application order of Chiralpak AD > Chiralcel OD > Chiralcel OJ > Chiralpak AS. The H series of these corresponding columns provide a better chromatographic performance (higher efficiency) due to their smaller particle size (5 μm versus conventional 10 μm). In this application, a Chiralpak AD-H column was, therefore, pursued first. The methodology, instrumentation, and results have been included in this presentation.

2. Experimental

2.1. Materials

The chiral drug compound AZM (*R* enantiomer, initial optical purity 99%) and its minor *S* impurity (initial optical purity 99%) contained one chiral center, and were obtained from AstraZeneca compound management, Wilmingon, DE, USA. Among the various lots of AZM studied, the *S* enantiomer was present at a minimal 1% level. The chiral method was required to be sensitive enough to detect the 1% impurity at the formulation concentration for this compound. HPLC grade methanol and USP grade 200 proof ethanol were obtained from J.T. Baker (Phillipsburg, NJ) and Pharmco (Brookefield, CT), respectively. Isopropylamine (IPA) and dimethylethylamine (DMEA) were used in the SFC method development as additives and purchased from Acros Organics (NJ). The drug compound was a base, and it was therefore necessary to include basic additives in the mobile phase to reduce the possibility of peak tailing. 0.1 M lactic acid (pH adjusted to 3.0) was prepared in-house. 0.05 M potassium phosphate monobasic buffer (pH 7.0) and 40 g/l sodium borate buffer (pH 9.5 adjusted with 0.5N sodium hydroxide) were products of Fisher Scientific (Fairlawn, NJ) and LabChem (Pittsburgh, PA), respectively. The pH of the three buffer solutions were checked prior to use by a Beckman pH meter chiral drug Φ40. SFC grade carbon dioxide was supplied by MC Industries (Malvern, PA).

2.2. Preparation of method development reference solution

For method development and feasibility evaluation of direct injection of aqueous formulation into the SFC system, the following solution was prepared: approximately 5 mg of each enantiomer were weighed out in a 5 ml volumetric flask and brought to volume with 0.1 M lactic acid (pH 3.0) as vehicle. The solution was sonicated for approximately 5 min and further diluted with formulation vehicle to produce a nominal concentration of 0.05 mg/ml of racemic mixture. This solution was used as the SFC method development reference, to ensure baseline resolution of the enantiomers by SFC.

2.3. Preparation of aqueous formulations

For evaluation of direct assay of the chiral drug compound in 100% aqueous solutions, the following solutions were prepared:

(1) Approx. 1 mg/ml of AZM in 1 ml of 0.1 M lactic acid (pH 3.0).
(2) Approx. 1 mg/ml of AZM in 1 ml of 0.05 M potassium phosphate monobasic buffer (pH 7.0).
(3) Approx. 1 mg/ml of AZM in 1 ml of 40 g/l sodium borate buffer (pH 9.5).

The compound was completely soluble in pH 3.0 solution following brief sonication. At pH 7.0, and 9.5, the compound was only sparingly soluble following an extended period of sonication (15–20 min), with solubility lower at higher pH. Ten microliters of each filtered solution was injected onto the column without pretreatment.

2.4. Linearity and limit of quantitation study

For the purpose of linearity evaluation and LOQ study, a 1 mg/ml stock solution at pH 3.0 was progressively diluted with the solvent (0.1 M lactic acid, pH 3.0) and assayed twice at each concentration. The mean determined concentrations were plotted against the corresponding responses to generate the calibration curve.

2.5. Effect of solvent on chromatographic parameters

To determine the effect of solvent on relevant chromatographic properties, the following solutions were prepared:

(1) Approx. 1 mg/ml of racemic mixture in 100% 0.1 M lactic acid buffer.
(2) Approx. 1 mg/ml of racemic mixture in 50/50 0.1 M lactic acid buffer/ethanol + 0.3% DMEA.
(3) Approx. 1 mg/ml of racemic mixture in 50/50 0.1 M lactic acid buffer/methanol + 0.3% IPA.
(4) Approx. 1 mg/ml of racemic mixture in 100% ethanol + 0.3% DMEA.
(5) Approx. 1 mg/ml of racemic mixture in 100% methanol + 0.3% IPA.

Table 1
Analytical chromatography system

Component	Description
SFC system	Berger Analytix
Autosampler	Alcott Chromatography chiral drug 719
Sample injection volume	10 μl
Pump	Berger fluid control module chiral drug FCM 1100
Column compartment	Berger Thermal Control Module TCM 2000
Modifier selection valve	Six port Valco model C22Z
Detector	Agilent HP chiral drug 1050
Detection wavelength	244 nm
Analytical column	Chiralpak AD-H, 4.6 mm × 250 mm, 5 μm
Mobile phase 1	23% methanol with 0.3% IPA/77% CO_2
Mobile phase 2	28% ethanol with 0.3% DMEA/72% CO_2
Flow rate	2.2 ml/min
Temperature	40 °C
Back pressure	100 bar
Data acquisition	BI-SFC Chemstation 3.6.6 and Thermoelectron Atlas

Fifty microliters of each of the solutions (1)–(5) were diluted with 950 μl of the respective solvents to produce 0.05 mg/ml nominal concentration of each enantiomer. Ten microliters of each solution was injected for chromatographic comparison purpose.

2.6. Analytical chromatographic system

Details of the instrument components are provided in Table 1. The instrument for packed column SFC was a Berger analytical system. The injector was electrically actuated and a series 719 from Alcott Chromatography (Norcross, GA, USA). The analytical column was connected into the six-column switching compartment. There have been reports on optimization of SFC method development by varying temperature and pressure [12]. To keep the method development process simple, no attempts were made here to manipulate these two parameters. The two mobile phase polar modifiers listed in Table 1 were used in the method development. A variable wavelength detector HP 1050 was fitted with a high pressure flow cell (maximum pressure rating of 400 bar) suitable for SFC work. Chemstation (Agilent Technologies) was used to control the instrument and Atlas (Thermoelectron Corp.) was used for data acquisition, processing, and obtaining the chromatographic parameters from each sample injection.

3. Results and discussion

3.1. Typical problems with 100% aqueous sample assay by SFC

The direct assay of neat aqueous solutions of compounds by SFC has not been pursued primarily due to the following reasons:

- The aqueous sample would freeze during depressurization in the back pressure regulator.
- Samples in water would precipitate out of solution when encountering the SFC polar organic mobile phase.
- Extremely asymmetric and distorted peaks occur due to the column surface activity generated by the injected water in the chiral column.
- Sensitivity may become an issue and compromised to the extent that minor impurities cannot be detected/quantified in the sample. The necessary sensitivity can only be obtained by further processing of the neat solution (e.g. organic extraction etc.) [15].

In this study, no such problems were encountered primarily due to low injection volume and better instrumentation. The concentration was linear over a wide range, including up to 0.5 mg/ml for AZM. Also, the injection reproducibility was good for aqueous solutions, over the period of the study.

The column performance following repeated injections ($n = 135$) of various pure aqueous samples remained similar in terms of retention time, peak shape, and efficiency. This was also confirmed by using the same column in a separate study following the direct assay experiment.

3.2. Results from method development for aqueous assay

The method was found to be enantioselective, as demonstrated by the lack of interferences across the elution windows of the two enantiomers (Fig. 1). Ethanolic modifier provided better resolution as well as shorter analysis time as compared to methanolic modifier (Fig. 2). Ethanolic modifier was therefore used in the mobile phase for linearity and LOQ study (Fig. 3). The chromatographic peak shape was symmetrical, as determined by the USP tailing factor for all aqueous samples studied. Table 2 lists all chromatographic parameters studied for the racemic mixture in various sample solvents. Both enantiomers eluted earlier in ethanolic mobile phase compared to methanolic mobile phase, irrespective of which solvent was used. The retention times remained unchanged with ethanolic mobile phase for all three sample solvents studied (0.05 mg/ml in 100% water, 50/50 water/mobile phase modifier with base additive, and 100% mobile phase modifier with additive). In methanolic mobile

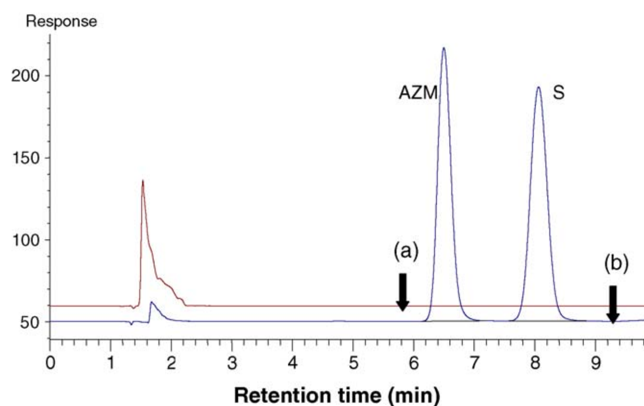

Fig. 1. Demonstration of selectivity of the direct enantioselective assay. Chromatogram (a) represents blank solvent vehicle (0.1 M lactic acid, pH adjusted to 3.0). Chromatogram (b) represents the racemic mixture in the same solvent.

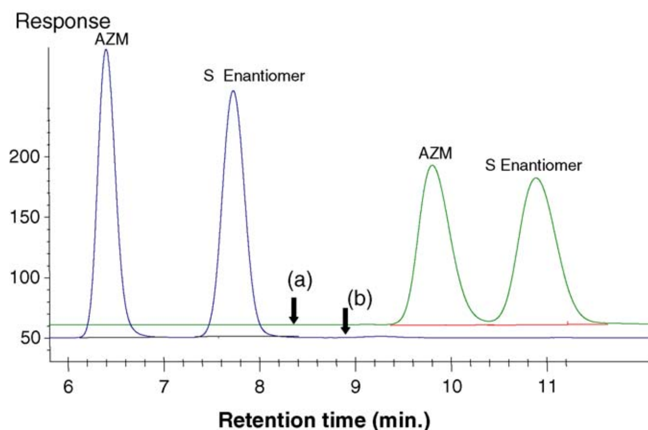

Fig. 2. Chromatograms of a racemic mixture in 0.1 M lactic acid, pH 3.0. (a) The chromatogram obtained with 77% CO_2/23% methanol + 0.3% IPA, 2.2 ml/min, 40 °C, 100 bar. (b) The chromatogram obtained with 72% CO_2/28% ethanol + 0.3% DMEA, 2.2 ml/min, 40 °C, 100 bar.

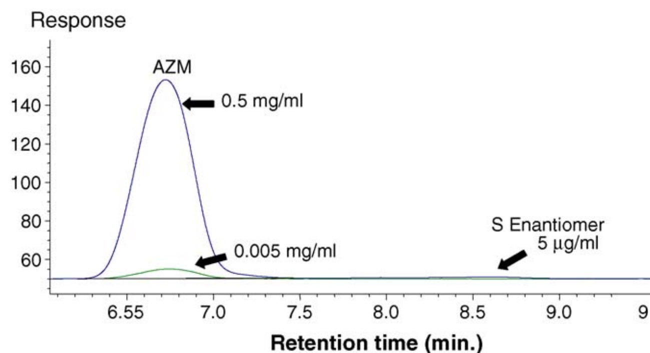

Fig. 3. Chromatograms demonstrating the detection of the *S* enantiomer (impurity) in the 100% aqueous formulation at pH 3.0 without any pretreatment. Initial optical purity of AZM was ∼99%. The assay could detect ∼5 µg/ml of *S* enantiomer.

phase, the retention times of both enantiomers were prolonged as the solvent was changed from 100% mobile phase modifier to 100% water.

The peak widths of both enantiomers increased by changing the sample solvent from 100% modifier to 100% water. This was the case for both ethanol and methanol mobile phase modifiers. This was anticipated based on similar behavior known to occur in HPLC.

The column efficiency was better or higher in methanol mobile phase for all three sample solvents compared to the same in ethanol. This indicated that methanol provided better mass transfer in general.

The USP tailing factors were very similar for all three sample solvents and in both mobile phase modifiers, which indicated that 100% water did not lead to poor peak shape.

Table 2
Chromatographic parameters from direct assay study

Chromatographic parameter	Mobile phase (mp) modifier	Solvent	AZM	S Enantiomer
Retention time, t_r (min)	28% ethanol + 0.3% DMEA	100% mp modifier	6.5	8.1
		50/50 water/mp modifier	6.5	8.0
		100% water	6.4	7.9
	23% methanol + 0.3% IPA	100% mp modifier	7.7	8.6
		50/50 water/mp modifier	9.2	10.3
		100% water	10.5	11.8
Peak width half, $W_{1/2}$ (min)	28% ethanol + 0.3% DMEA	100% mp modifier	0.24	0.31
		50/50 water/mp modifier	0.34	0.42
		100% water	0.35	0.44
	23% methanol + 0.3% IPA	100% mp modifier	0.23	0.25
		50/50 water/mp modifier	0.37	0.44
		100% water	0.43	0.52
Efficiency (N) calculated as $5.54 \times (t_r/W_{1/2})^2$	28% ethanol + 0.3% DMEA	100% mp modifier	4006	3807
		50/50 water/mp modifier	2016	2025
		100% water	1871	1800
	23% methanol + 0.3% IPA	100% mp modifier	6020	6343
		50/50 water/mp modifier	3417	3089
		100% water	3257	2908
Tailing factor	28% ethanol + 0.3% DMEA	100% mp modifier	1.15	1.05
		50/50 water/mp modifier	1.14	0.98
		100% water	1.06	0.93
	23% methanol + 0.3% IPA	100% mp modifier	1.25	1.17
		50/50 water/mp modifier	1.24	1.13
		100% water	1.24	1.12
Resolution	28% ethanol + 0.3% DMEA	100% mp modifier	3.36	na
		50/50 water/mp modifier	2.42	na
		100% water	2.42	na
	23% methanol + 0.3% IPA	100% mp modifier	2.17	na
		50/50 water/mp modifier	1.58	na
		100% water	1.42	na

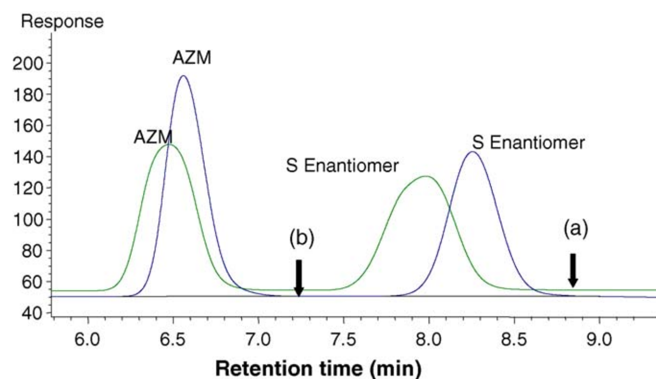

Fig. 4. Solvent effect on chromatographic properties. Chromatogram (a) is obtained when 100% aqueous solution (pH 9.5) is used as solvent for the racemic mixture. Chromatogram (b) is obtained when a 50/50 v/v mixture of 0.1 M lactic acid/ethanol modifier is used to dissolve the same racemic mixture.

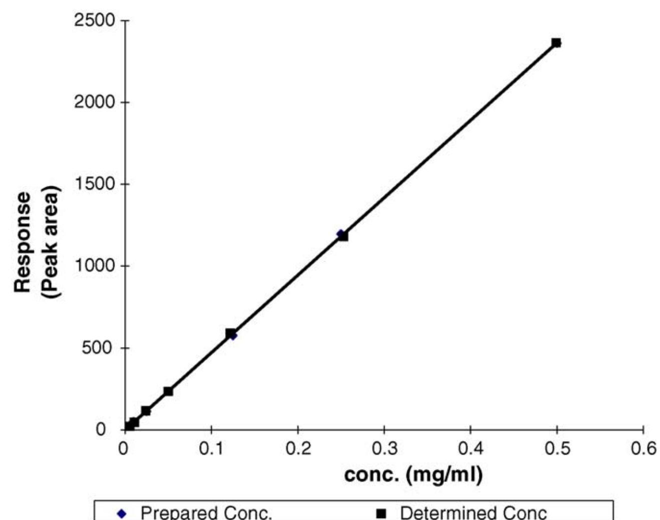

Fig. 5. Linearity of direct assay of chiral drug compound sAZM in 0.1 M lactic acid, pH 3.0.

An exception was for the *S* enantiomer in ethanolic mobile phase.

The best resolution for both enantiomers was observed with ethanolic mobile phase. The resolution decreased when changing the sample solvent from 100% organic to 100% water with both mobile phase modifiers.

The chromatographic peak width can, however, be improved by using at least 50/50 organic solvent/aqueous mixture as solvent compared to 100% aqueous solution (Fig. 4). The assay sensitivity can therefore be enhanced by adding organic solvent to the aqueous sample solvent.

3.3. Selectivity, precision, accuracy, linearity, and sensitivity of direct assay

3.3.1. Selectivity

Comparison of the blank chromatogram (10 μl of 0.1 M lactic acid pH adjusted to 3.0) with the racemic mixture under identical conditions indicated no interfering peaks across the elution windows of both AZM and its *S* enantiomer (Fig. 1). The early perturbation observed in the chromatogram around 1.5 min was due to the water component of the sample solvent. The small intercept value of the calibration curve also confirmed the presence of minimal interference across the elution window of AZM. Also, the baseline resolution of the two enantiomers could be achieved within 10 min, even under not completely optimized chromatographic conditions.

3.3.2. Sensitivity

The assay was also able to detect the <1% *S* impurity in the aqueous formulation both at pH 3.0 (Fig. 4) and pH 7.0. The mean ($n = 5$) area% of the *S* enantiomer was 0.98 and 0.97% at pH 3.0 and 7.0 aqueous buffer, respectively. It has been noted that in order to detect ∼3% chiral impurity in various chiral drug compounds, a 10-fold enhancement in sensitivity was required [15]. This could only be achieved by extracting the compound from a 100% aqueous matrix to a predominantly organic matrix. For the current work, the *S* enantiomeric impurity could be reproducibly detected without pretreatment of the 100% aqueous solvent. At pH 9.5, the peak response was significantly diminished as compared to pH 3.0 and 7.0, due to the low solubility of AZM at higher pH. The sensitivity of the assay to quantitate the *S* enantiomer at the low concentration of pH 9.5 formulation may be achieved by increasing the injection volume. This has yet to be tried.

3.3.3. Linearity

An unweighted least square regression was linear between 0.5 and 0.005 mg/ml at pH 3.0, with a correlation coefficient, slope, and intercept of 0.9999, −1.264, and 4731.9, respectively (Fig. 5). The linear dynamic range extended over two orders of magnitude for this assay.

3.3.4. LOQ and LOD

The assay limit of quantitation (LOQ) for AZM at 10 times the signal to noise ratio was 2.5 μg/ml. The chromatographic peak-to-peak noise was calculated across the expected compound elution window for a blank injection. It may be possible to lower the LOQ by further optimizing the chromatography (e.g. reducing run time to increase peak height, while maintaining baseline resolution of the two isomers). The limit of detection (LOD) at three times the signal to noise ratio was 0.75 μg/ml and the limit of identification (LOI) at six times the signal to noise ratio was 1.5 μg/ml, respectively. The LOD achieved here is lower than that previously reported for a direct aqueous sample assay of a carboxylic acid [15], presumably because of system peak interference differences between the two methods. The LOD for *S* enantiomer as % of AZM was ∼0.08%.

3.3.5. Accuracy

The individual bias (accuracy) was within ±4% of nominal at each of standard concentration of AZM between 0.5 and 0.025 mg/ml and within +12% of nominal at 0.01 and 0.005 mg/ml.

3.3.6. Precision

The injection precision ($n = 3$) based on chromatographic area was found to be 1.4 and 0.6% for AZM and *S* enantiomer, respectively, in a racemic mixture, at a nominal concentration of 0.5 mg/ml of each. The intraday precision ($n = 5$) was based on the area of the enantiomers injected as the racemic mixture throughout the duration of an analysis sequence and determined to be 1.3 and 0.8%, respectively.

4. Conclusions

Chiral SFC has been demonstrated to be useful for assay of neat aqueous solutions of a chiral drug compound AZM. This potentially could reduce the sample processing steps during aqueous formulation assay (e.g. eliminate the necessity for dilution in organic solvent).

In this study, AZM could be analyzed in aqueous solutions at pH 3.0, 7.0, and 9.5. The response was linear between 0.5 and 0.005 mg/ml at pH 3.0, with a correlation coefficient of 0.9999. The assay LOQ was 2.5 μg/ml. It may be possible to further lower the LOQ by optimizing the chromatography (e.g. reducing run time to increase peak height, while maintaining baseline resolution of the two isomers). The assay was also able to detect the 1% chiral impurity (*S* enantiomer) in aqueous formulation both at pH 3.0 and 7.0.

Future work will likely explore this approach in more detail with other research compounds and also with other chiral columns (e.g. Cyclobond, Whelk-O, etc.).

Acknowledgement

The authors would like to thank Dr. Xunxia (Vivian) Bi for providing us the pH 3 lactic acid formulation vehicle for the study.

References

[1] M. Maftouh, C. Granier-Loyaux, E. Chavana, J. Marini, A. Pradines, Y. Heyden, C. Picard, J. Chromatogr. A 1088 (2005) 67–81.
[2] C. White, J. Chromatogr. A 1074 (2005) 163–173.
[3] C. White, J. Burnett, J. Chromatogr. A 1074 (2005) 175–185.
[4] Y. Zhao, G. Woo, S. Thomas, D. Semin, P. Sandra, J. Chromatogr. A 1003 (2003) 157–166.
[5] B. Bolanos, M. Greig, M. Ventura, W. Farrell, C. Aurigemma, H. Li, T. Quenzer, K. Tivel, J. Byland, P. Tran, C. Pham, D. Phillipson, Int. J. Mass Spectrom. 238 (2004) 85–97.
[6] M. Villeneuve, R. Anderegg, J. Chromatogr. A 826 (1998) 217–225.
[7] R. Stringham, K. Lynam, C. Grasso, Anal. Chem. 66 (1994) 1949–1954.
[8] Y. Hsieh, L. Favreau, J. Schwerdt, K. Cheng, J. Pharm. Biomed. Anal. 40 (2006) 799–804.
[9] M. Alasandro, J. Pharm. Biomed. Anal. 14 (1996) 807–814.
[10] K. Williams, L. Sander, S. Wise, J. Pharm. Biomed. Anal. 15 (1997) 1789–1799.
[11] M. Garzotti, M. Hamdan, J. Chromatogr. B 770 (2002) 53–61.
[12] L. Karlsson, O. Gyllenhaal, A. Karlsson, J. Gottfries, J. Chromatogr. A 749 (1996) 193–199.
[13] O. Gyllenhaal, J. Pharm. Biomed. Anal. 40 (2006) 971–974.
[14] O. Gyllenhaal, J. Hulthe, J. Pharm. Biomed. Anal. 29 (2002) 381–386.
[15] O. Gyllenhaal, J. Chromatogr. A 1042 (2004) 173–180.