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Validation of direct assay of an aqueous formulation of a drug compound AZY by chiral supercritical fluid chromatography (SFC)

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Abstract

Supercritical fluid chromatography (SFC) is increasingly being recognized as a powerful technique for analysis of pharmaceutical compounds in various dosage forms. Assay of aqueous formulations of research compounds by SFC is, however, a relatively unexplored area primarily due to the potential problems associated with it. This work describes the development of a direct assay of a chiral drug compound AZY in a 100% aqueous formulation by SFC, and its qualification following ICH and FDA validation guidelines on chromatographic methods. The results indicated that SFC has the potential for assaying aqueous formulations of research compounds with high degree of selectivity, accuracy, precision, robustness, sensitivity, and linearity over a wide range of concentrations. This work also confirmed a previous hypothesis that direct formulation assay by SFC approach is applicable to both acidic and basic pharmaceutical compounds with equal degree of success.

Keywords: Supercritical fluid chromatography; Chirality; Direct assay; Formulation; Analysis; Separation science; Chromatography

1. Introduction

Chiral SFC is rapidly replacing chiral HPLC in many pharmaceutical companies as the standard automated screening and method development tool due to the advantages of faster speed, higher efficiency, less organic solvent usage, and utility of safer and cheaper CO_2, etc. [1–5]. Packed column SFC has been demonstrated to offer these advantages for analysis of a wide variety of therapeutic agents, prepared in different SFC compatible organic solvents [6–9].

Assay of pharmaceutical dosage forms by SFC have been reported by several researchers [10–15]. These works, however, primarily consisted of analysis of solid dosage forms (e.g. tablets and capsules) following dissolution in organic solvents. The success of direct SFC assay of pharmaceutical liquid formulations (e.g. intravenous fluids) has so far been treated with skepticism primarily due to the various limitations associated with this process (e.g. sample freezing, precipitation, and distorted chromatographic peak shape, etc.). Since packed column SFC exhibits chromatographic behavior similar to normal phase, aqueous samples have been a concern. Also, assay sensitivity is often reduced to the extent that minor impurities cannot be detected/quantified in the sample. Limited reports have been published on assay of emulsions and suspensions by SFC [16–19]. These studies, however, required replacing methanol with 2-propanol in the mobile phase and/or inclusion of an additive (e.g. 1 mM citrate) in the organic modifier, or extraction in an organic solvent prior to analysis for better resolution, chromatographic peak shape and assay sensitivity.

To the best of knowledge, the direct enantioselective assay of drug compounds in 100% aqueous formulations by SFC, without any sample pretreatment and/or inclusion of special additive in mobile phase has been extremely limited. In a previous work, the applicability of SFC in neat aqueous formulation assay of a basic drug candidate was demonstrated with great success without any sample pretreatment and with ethanol/CO_2 mobile phase [20]. The goal of the present work was to further extend the application of this direct assay technique for an acidic drug prepared with an aqueous formulation vehicle.

A feasibility study was conducted using SFC for the assay of an acidic drug compound AZY with a chiral center, in aqueous solutions, using FDA and ICH guidelines on analytical validation [21–23]. Due to confidentiality of the on-going project, the structure of AZY could not be further disclosed. It should

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be noted that any specific analytical guideline related to SFC method validation has not been released from FDA/ICH. Some of the guidelines/recommendations for routine HPLC validation in non-biological matrices were, therefore, followed to qualify this SFC methodology. It is known that SFC typically provides baseline resolution of >90% of chiral compounds using one of the standard four columns available from Chiral Technologies Inc. (West Chester, PA, USA), in the approximate 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) owing to their smaller particle size (5 μm versus conventional 10 μm). In this application, a Chiralpak AD–H column was pursued first. The methodology, instrumentation, and results have been included in this work.

2. Experimental

2.1. Materials

The acidic drug compound AZY (of configuration *3S*,*4R*: initial optical purity 99%) and its minor impurity (of configuration *3R*,*4S*: initial optical purity 99%) were obtained from AstraZeneca compound management (MA). USP grade 200 proof ethanol and dimethylethylamine (DMEA) were obtained from Pharmco (Brookefield, CT) and Acros Organics (NJ), respectively. *N*-Methyl-D-glucamine (Meglumine) and D5W USP (5% dextrose–water) were purchased from Acros Orgnics and Abbott Labs (Chicago, IL), respectively. SFC grade 5.0 high purity carbon dioxide was supplied by GTS (Morrisville, PA).

2.2. Preparation of formulation and method development reference solution of AZY

A 0.05 M meglumine solution was prepared in 5% dextrose–water vehicle and the final pH adjusted to 7.4 with 0.1 N HCl. This formulation vehicle was used for preparation of standards, QCs, and in other validation experiments. For method development purpose, a 0.05 mg/ml solution of the racemic mix of AZY and its enantiomer (*3R*,*4S*) was prepared in 0.05 M meglumine. This solution was used as the SFC method development reference, to ensure baseline resolution of the enantiomers by SFC.

2.3. Preparation of calibration standards and quality controls (QCs)

A 1.0 mg/ml stock solution of AZY was serially diluted with formulation vehicle to produce nine calibration standards in the nominal concentration range of 0.75–0.0025 mg/ml. Three QCs were prepared separately from an independent weighing of AZY and diluted with formulation vehicle to produce nominal concentrations of 1.11, 0.11, and 0.011 mg/ml, respectively. Stocks and standards were prepared daily during the 4 days of validation work. The QC stock was refrigerated and used through the entire length of the validation study as it was demonstrated earlier by reversed phase HPLC that refrigerated AZY solution was stable for at least 8 days at a higher concentration (3 mg/ml), in the same formulation vehicle.

Table 1
Analytical chromatography system

Component	Description
SFC system	Berger Analytix
Autosampler	Alcott Chromatography model 719
Sample injection volume	20 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 PDA
Detection wavelength	290 nm
Analytical column	Chiralpak AD–H (Lot ADHθCE–FCθ16), 4.6 mm × 250 mm, 5 μm
Mobile phase	30% ethanol with 0.3% DMEA/70% CO_2
Flow rate	3.0 ml/min
Oven temperature	40 °C
Nozzle temperature	60 °C
Back pressure	100 bar
Data acquisition	BI-SFC Chemstation 3.6.6 and Thermoelectron Atlas

2.4. 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. To keep the method development process simple, no attempts were made here to manipulate temperature and pressure. Following evaluation of different alcohols (e.g. ethanol, methanol, 2-propanol, etc.) as modifiers and additives (e.g. TFA, isopropylamine, DMEA, etc.), ethanol with DMEA provided the best resolution of AZY and its enantiomer for SFC purpose. An Agilent PDA (series 1100) was fitted with a high pressure flow cell (400 bar) suitable for SFC work. Chemstation (Agilent Technologies) was used to control the instrument and Atlas (Thermoelectron Corp.) was used for data acquisition and processing.

2.5. Robustness testing

Of the various parameters that could potentially be tested for ruggedness evaluation of an analytical method, the three most common anticipated variables were evaluated in this study as follows:

(a) Use of a different lot of column on a separate day of analysis. On day 4 of qualification, a different lot (ADHθCE–FFθ77) Chiralpak AD–H column was used along with freshly prepared mobile phase, to demonstrate the robustness of the method. Standards were prepared fresh on day 4 and analyzed with the QC samples refrigerated on day 1.
(b) Variation of mobile phase % composition.

Robustness was tested by slight and deliberate variation of the mobile phase modifier %. The % composition of modifier (ethanol +0.3% DMEA) was changed to 30 ± 2%. The three QCs were analyzed under these modified conditions to determine if the precision and accuracy of the assay could still be maintained in an acceptable and similar level as the original conditions. The analysis was performed on day 1.

(c) Variation of mobile phase flow rate.

The flow rate was also deliberately varied as 3.0 ± 0.15 ml/min at 30% ethanol modifier. The variation was kept at ±5% of intended flow rate as at any higher and lower flow rates; the retention time was too long for SFC and also led to co-elution of the minor impurity with the early eluting AZY, respectively. The three QCs were evaluated in same manner as in 2.6(b). The analysis was preformed on day 1.

2.6. Precision

(a) Analysis repeatability.

The intraday precision of the method was determined for each of the nine calibration standards ($n = 3$) and also for the three QCs ($n = 3$), on each day of validation.

(b) Intermediate precision.

The intermediate precision of direct assay of AZY was calculated from the four separate days of validation, for each of the nine calibration standards as well as for the three QCs.

2.7. Accuracy

The intraday accuracy (bias) was determined at each of the standard and QC concentrations, on each day of validation, based on the differences with respective prepared concentrations. The interday accuracy was determined based on the data pooled from the 4 days of validation, over the entire standard curve range, and also for the three QCs.

2.8. Linearity

Linearity of the assay was evaluated at least in duplicate for each calibration standard by plotting the mean PDA responses versus corresponding nominal concentrations. Appropriate statistical function was applied for best fit of calibration data.

2.9. Detection limit (DL) and quantitation limit (QL)

The DL was calculated based on approach outlined in the ICH guideline for signal to noise (*s*/*n*). The PDA response from a sample with known low concentration of AZY was compared with the baseline noise across the elution window of AZY for blank injection. For QL, a *s*/*n* ratio of 10 was used for QL calculation.

2.10. Recovery

The recovery of AZY from the intended aqueous formulation of meglumine was evaluated by dissolving accurately 5.06 mg in 5 ml of the vehicle and assaying for content in triplicate using the SFC system.

2.11. Sample solution stability

As recommended by FDA [23], a 24 h autosampler stability was carried out for the highest calibration standard (which was similar to the intended preformulation dosage concentration) at approximately 1 mg/ml under laboratory conditions of light, temperature, and humidity. This was studied only at a single concentration to conserve the amount of limited test compound. Also, the underlying assumption was that any degradation, if observed, would be first order and concentration dependent, and therefore the worst case could be observed with the high concentration of 1 mg/ml for AZY.

3. Results and discussion

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

As mentioned earlier in introduction, the direct assay of neat aqueous solutions of compounds by SFC has not been pursued extensively due to the following potential reasons:

- The aqueous sample would freeze during depressurization in the SFC 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.) [19].

In this study, no such problems were encountered primarily due to low injection volume and also different instrumental parameters (e.g. higher nozzle temperature). The concentration was linear over a wide range, including up to 1.0 mg/ml for AZY. Also, the injection reproducibility was excellent for neat aqueous solutions over the length of this entire study, as determined from triplicate assays of a mid-level calibration standard on each day of validation (Table 2).

3.2. Selectivity

Comparison of a blank chromatogram (0.05 M meglumine in D5W) with the racemic mixture under identical conditions indicated no interfering peaks across the elution windows of both AZY and its enantiomer (Fig. 1). The early perturbation

Table 2
Analysis reproducibility

Day	Prepared concentration (mg/ml)	Mean determined concentration (mg/ml)	% RE	Mean % RE	%R.S.D.	Mean % R.S.D.	Mean RT (min)	%R.S.D.	Mean %R.S.D.)
1	0.2525	0.2515	0.4	0.4	0.9	1.6	11.17	0.5	1.1
2	0.2750	0.2723	1.0		2.4		10.99	0.9	
3	0.2650	0.2623	1.0		1.6		10.96	1.4	
4	0.2550	0.2567	−0.7		1.6		10.82	1.6	

observed in the chromatogram around 1.5 min was due to the water component of the sample solvent. The relatively small intercept value of the calibration curve also confirmed the presence of minimal interference across the elution window of AZY. The mean intercept value was not significantly different from zero, as determined by performing a *t*-test with 0.05 level of significance. Also, the baseline resolution of the two enantiomers could be achieved within 13 min, even without completed optimization of the chromatographic conditions.

3.3. System suitability testing

Results of the system suitability testing on the racemic mixture of AZY and its antipode are presented in Table 3. The parameters were calculated following FDA and ICH guidelines on system suitability testing, and all chromatographic parameters met the respective acceptance criteria. This is indicative that the chromatographic system was able to generate reliable results during the validation work.

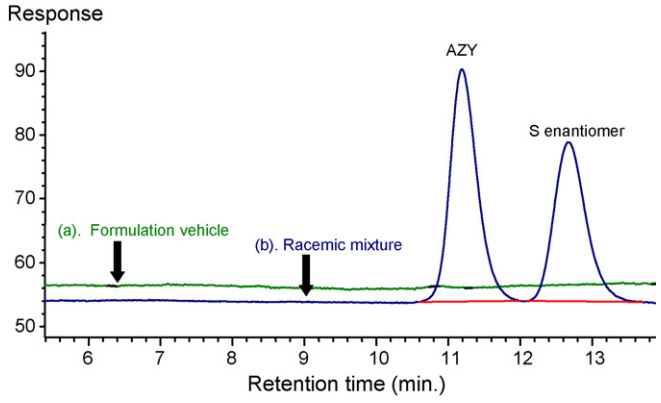

Fig. 1. Demonstration of selectivity of the direct enantioselective assay. Chromatogram (a) represents blank solvent vehicle (0.05 M meglumine, pH adjusted to 7.4). Chromatogram (b) represents the racemic mixture of AZY and its enantiomer in the same solvent, separated using 70% CO_2/30% ethanol +0.3% DMEA, 3 ml/min, 40 °C, 100 bar.

Table 3
System suitability

Analyte	Retention time (min)	k'	Rs	N	T	α
AZY	11.19	10.72	2.01	4267	1.22	1.15
S enantiomer	12.67	12.28		3881	1.23	

k': capacity factor; Rs: resolution; N: number of theoretical plates; T: USP tailing factor; α: enantioelectivity.

3.4. Sensitivity

The assay was able to detect the ∼1% chiral impurity (based on chromatographic area%) in the aqueous formulation of AZY (Fig. 2). 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 [19], which was, however, specific for that particular application. 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 any pretreatment of the 100% aqueous solvent.

3.5. Linearity

An unweighted least square regression was linear between 0.0025 and 1 mg/ml with a mean correlation coefficient of 0.9993. Other relevant regression parameters are reported in Table 4. The linear dynamic range (the difference between the highest and lowest concentrations in the standard curve) was 4×10^2 for this assay. The statistical goodness of fit (a statistical parameter used to determine the degree of fit of a set of data to a particular calibration model) of the calibration curve was determined to be 4.2 based on sum of squares of individual % relative errors and $n - 1$ degrees of freedom (n is the number of

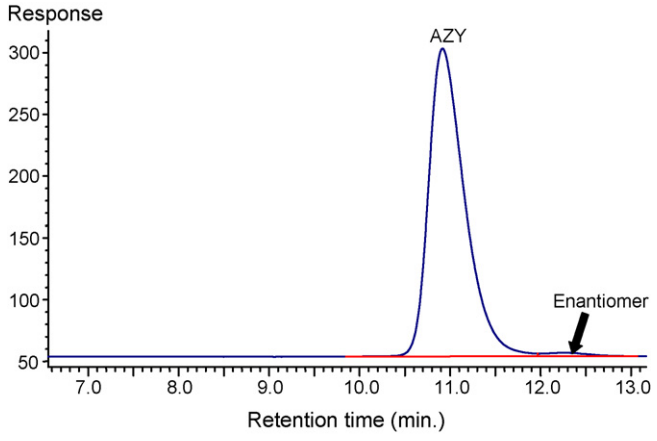

Fig. 2. Chromatogram showing ∼1.3% chiral enantiomeric impurity in 1 mg/ml of AZY formulated in meglumine formulation vehicle. The assay could reproducibly detect the minor impurity in all samples analyzed.

Table 4
Regression analysis parameters

Day	Correlation coefficient	Slope (mV ml/mg)	Intercept	S.E.
1	0.9997	8849.05	−0.8630	65.70
2	0.9995	8330.15	−1.3147	78.15
3	0.9994	8824.21	−2.4831	89.43
4	0.9988	8580.46	−1.6604	115.52
Mean	0.9993	8645.97	−1.5803	87.20
S.D.	0.0003	242.93	0.6847	21.22
−95% CI	0.9983	8019.20	−3.3469	32.45
+95% CI	1.00035	9272.73	0.1863	141.95

total calibration standards) and was also indicative of excellent fit of data to a linear calibration model.

3.6. Accuracy

The intraday individual bias (accuracy) was within ±6% of nominal at each of standard concentration of AZY between 0.0025 and 1 mg/ml (Table 5). The accuracies were within ±9% of nominal at QC concentrations at 0.0111 mg/ml and within ±5% at 0.111 and 1.11 mg/ml, respectively (Table 6). The interday accuracies for the pooled calibration standards results from the 4 days were within ±3% of nominal (Table 5) and within ±2% of nominal for the QCs (Table 6).

3.7. Precision

The analysis repeatability, as observed on each day of validation were within +9% R.S.D. for all calibration standards. The intermediate precision, as determined from the 4 days were all within +4% R.S.D. (Table 7). The intra- and interday precisions for all QCs on the 4 days of analysis were within +9% R.S.D. and +7% R.S.D., respectively (Table 8). It should be noted that the relatively larger values of % R.S.D.s at lower concentrations of standards and QCs were in line with the expectation. The injection repeatability data as obtained by consecutive injections of a mid-level calibration standard is presented in Table 9, and met the criteria of ≤1% R.S.D. as per ICH guideline.

3.8. DL and QL

The QL for AZY based on a *s/n* ratio of 10 was 5 μg/ml (Fig. 3). The chromatographic peak-to-peak noise was calculated across the expected compound elution window in a blank injection. It may be possible to lower the QL by further optimizing the chromatography (e.g. reducing run time/changing additive to increase peak height, while maintaining column selectivity). The DL was determined to be 1.5 μg/ml and the identification limit (IL) at six times the *s/n* ratio was 3.0 μg/ml. The DL and QL achieved here were comparable with those obtained for direct assay of a formulation of a basic compound in a previous work [20]. This demonstrated the direct assay technique offered similar sensitivity for both basic [20] and acidic (present work) compounds in neat aqueous formulations.

Table 5
Intra- and inter-day accuracy for calibration standards of AZY for direct assay

Standard nominal concentration (mg/ml)	Validation day	Intraday mean ($n=3$) % RE	Interday mean ($n=4$) % RE
0.0025	1	3.7	−0.2
	2	−6.2	
	3	3.4	
	4	−1.5	
0.005	1	1.3	−2.7
	2	−3.3	
	3	−8.4	
	4	−0.4	
0.01	1	−2.2	−2.0
	2	−5.8	
	3	0.5	
	4	−0.5	
0.025	1	−1.1	−2.1
	2	−4.0	
	3	−3.2	
	4	0.1	
0.05	1	−1.0	−2.2
	2	−2.4	
	3	−3.7	
	4	−1.6	
0.1	1	0.7	−0.8
	2	−1.6	
	3	−1.2	
	4	−0.9	
0.25	1	−2.0	−0.5
	2	−0.8	
	3	−0.2	
	4	0.8	
0.75	1	−0.7	1.4
	2	0.6	
	3	4.6	
	4	1.3	
1.0	1	−0.5	−0.4
	2	−0.3	
	3	−0.5	
	4	−0.3	

Table 6
Intra- and inter-day accuracy of AZY QCs for direct assay

QC nominal concentration (mg/ml)	Validation day	Intraday mean ($n=3$) % RE	Interday mean ($n=4$) % RE
0.0111	1	−8.8	−2.2
	2	−0.9	
	3	3.7	
	4	−3.0	
0.111	1	−4.5	−0.9
	2	0.8	
	3	0.3	
	4	−0.3	
1.11	1	−2.9	0.6
	2	4.7	
	3	0.0	
	4	0.8	

Table 7
Intra- and interday precisions of AZY calibration standards for direct assay

Standard nominal concentration (mg/ml)	Validation day	Intraday mean ($n=3$) %R.S.D.	Interday mean ($n=4$) %R.S.D.
0.0025	1	2.4	4.1
	2	4.2	
	3	1.5	
	4	8.6	
0.005	1	8.5	4.1
	2	na	
	3	1.0	
	4	2.9	
0.25	1	1.4	1.5
	2	1.3	
	3	1.7	
	4	1.6	
1.0	1	1.1	2.0
	2	1.8	
	3	1.1	
	4	3.7	

Table 8
Intra- and interday precisions of AZY QCs for direct assay

QC nominal concentration (mg/ml)	Validation day	Intraday mean ($n=3$) %R.S.D.	Interday mean ($n=4$) %R.S.D.
0.0111	1	7.8	7.3
	2	8.7	
	3	6.9	
	4	6.0	
0.111	1	0.5	1.4
	2	1.5	
	3	2.4	
	4	1.1	
1.11	1	0.8	1.4
	2	1.6	
	3	2.4	
	4	0.6	

3.9. Robustness testing

Table 10 list the results obtained with the robustness testing for mobile phase flow rate and % composition of the modifier. The method was demonstrated to be robust as the precision could be maintained at <3% R.S.D. for concentration and <1% R.S.D. for retention time (RT) of the high QC despite variations. The robustness testing data of the calibration standards and QCs on day 4 (with different lot of column and freshly prepared mobile phase) were compared with the corresponding mean concentrations of the standards and QCs, as determined on the first 3 days of validation and included in Tables 5–8 for interday precision and accuracy evaluations. A two-tailed *t*-test performed on the mean slope and intercept from 3 days of validation and compared with the slope and intercept obtained on day 4 indicated no significant differences at 0.025 level of significance. This further indicated that the direct assay method was robust and could be potentially applied in high throughput assay situations.

Table 9
Injection repeatability

Parameter	Mean ($n=6$)	S.D.	%R.S.D.	Mean (% RE)
AZY concentration (0.2525 mg/ml)	0.2509	0.0027	1.1	−0.6
AZY peak area (a.u.)	2219358	24007	1.1	na
AZY retention time (min)	11.19	0.06	0.5	na

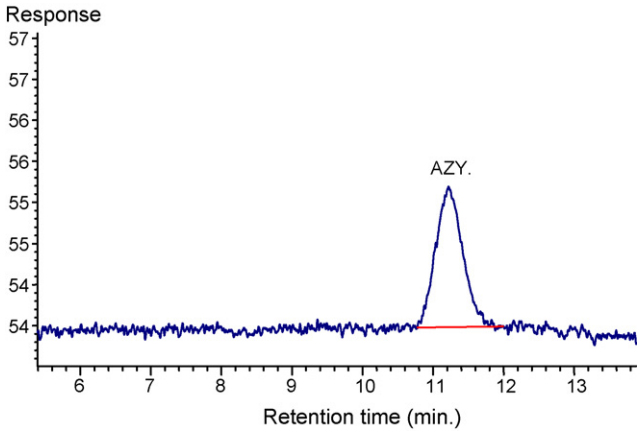

Fig. 3. Chromatogram demonstrating the presence of AZY close to the quantitation limit of 5 μg/ml. The assay could detect ∼1.5 μg/ml of AZY.

3.10. Sample solution stability

The direct assay method was able to monitor the 24 h autosampler stability of AZY formulated in the intended vehicle and the results are included in Table 11. The results thus indicated that the compound was stable for at least 24 h in an autosampler vial sealed with regular vial cap, in the intended formulation vehicle at room temperature.

Table 10
Robustness testing

Condition	Mean determined concentration (mg/ml)	%R.S.D. for concentration	Mean RT (min)	%R.S.D. for RT
30% ethanol, 3 ml/min	0.1060	0.5	11.09	0.9
32% ethanol, 3 ml/min	0.1054	1.6	9.40	0.3
28% ethanol, 3 ml/min	0.1027	2.7	12.02	0.4
30% ethanol, 3.15 ml/min	0.1119	0.9	10.48	0.3
30% ethanol, 2.85 ml/min	0.0992	1.0	11.91	0.1

Table 11
Autosampler stability

Time (h)	Prepared concentration (mg/ml)	Mean determined concentration (mg/ml)	%R.S.D.	Mean % RE	% Target	% of time 0
0	1.01	1.017	3.7	0.7	100.7	100
24	1.01	0.9977	0.7	−1.2	98.8	98.1

4. Conclusions

Chiral SFC has been demonstrated to be very useful for assay of neat aqueous solutions of a chiral drug compound AZY in a formulation vehicle. This work demonstrated that direct assay SFC technique was applicable to acidic drugs with equal degree of success as with basic drugs analyzed in a previous work. This technique potentially could reduce the sample processing steps during aqueous formulation assay. The calibration response was linear between 1.0 and 0.0025 mg/ml, with a mean correlation coefficient of 0.9993, as validated over 4 days. The assay QL was 5 μg/ml and could be further lowered by optimizing the chromatography. This direct assay method for aqueous formulations was demonstrated to be specific, precise, accurate, sensitive, and robust, based on the validation results.

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