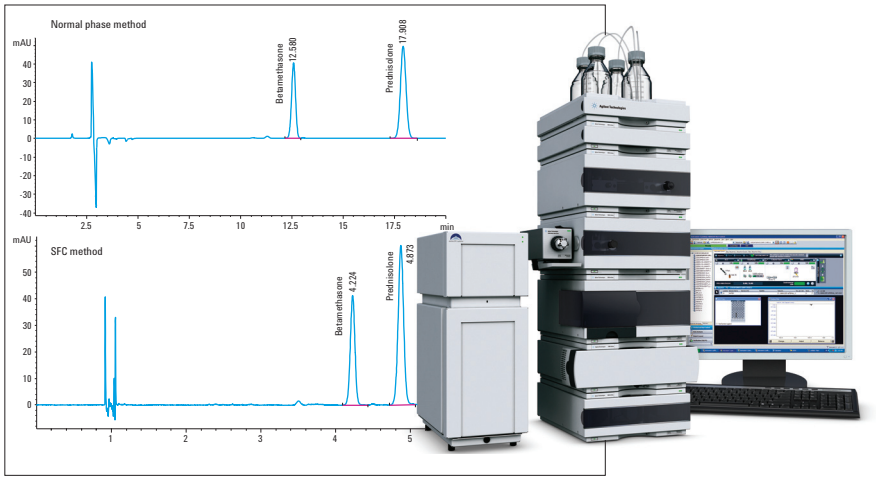
Agilent Application Solution

Transfer of a USP method for prednisolone from normal phase HPLC to SFC using the Agilent 1260 Infinity Hybrid SFC/UHPLC System

Saving time and costs

Application Note

Pharmaceutical QA/QC



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Abstract

Normal phase liquid chromatography (LC) methods often have long run times and involve environmentally toxic/costly solvents. Supercritical fluid chromatography (SFC) methods on the other hand are faster, inexpensive, and eco-friendly. SFC involves the use of low viscosity supercritical carbon dioxide that can be operated at flow rates up to 3x higher than LC without losing separation efficiency and thereby leading to faster analysis. In this Application Note, we describe a method to transfer a United States Pharmacopeia (USP) prednisolone assay normal phase HPLC method to SFC. The Agilent 1260 Infinity Hybrid SFC/UHPLC System was used to perform both normal phase as well as the SFC methods. The results show that the SFC method meets the system suitability criteria, is 4x faster, and results in 17x lower solvent expenses. Robustness tests on the SFC method demonstrate excellent robustness for routine analysis.

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Introduction

Prednisolone is a synthetic adrenal corticosteroid. Corticosteroids have potent anti-inflammatory properties. They are used in a wide variety of inflammatory conditions such as arthritis, asthma, bronchitis, and others. The USP assay method for prednisolone uses a normal phase method that includes chloroform as a sample diluent while 1-chlorobutane (butyl chloride) is used as the mobile phase. Chloroform is a known carcinogen, potentially toxic to analysts, and expensive to dispose. SFC is considered a green technology, because of the use of carbon dioxide (CO_2) as a major component of the mobile phase. In the recent decade, SFC has shown the capability to replace many achiral LC methods. Especially, compared to normal phase methods, SFC methods offer faster separation without losing efficiency, and faster column re-equilibration[1]. In this Application Note we show the development of a SFC method to replace a normal phase prednisolone assay in which betamethasone is used as an internal standard (Figure 1). This method uses methanol as sample diluent instead of chloroform. Linearity, limit of detection (LOD), limit of quantification (LOQ) and robustness[2] of the method was demonstrated.

The Agilent 1260 Infinity SFC/UHPLC Hybrid system[3] was used to perform both the normal phase as well as the SFC method on a single instrument. With this unique hybrid solution, the need to invest in two individual systems is eliminated. This eliminates the need for system-to-system variability and saves significant cost and a laboratory space.

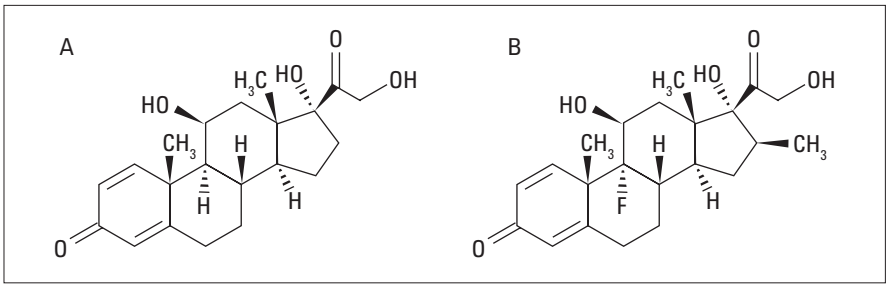

Figure 1
Molecular structures of Prednisolone (A) and Betamethasone (B).

Experimental

Instruments

An Agilent 1260 Infinity Hybrid SFC/UHPLC system (G4309A) consisting of the following modules was used:

- Aurora SFC Fusion A5 module
- Agilent 1260 Infinity Degasser
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity SFC Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode array Detector

Additional components were needed:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1290 Infinity Universal Valve Drive (G1170A)
- Agilent 2-position/10-port valve kit – 600 bar (G4232A)
- Agilent 1260 SFC/UHPLC Hybrid Capillary Kit (G4306A)

Software

- Agilent ChemStation B.04.03

Reagents and materials

All solvents used were HPLC grade. Purified water was used from a Milli-Q water purification system (Millipore, USA). Methanol super gradient were purchased from Lab-Scan. HPLC grade, butyl chloride, tetrahydrofuran, glacial acetic acid, and chloroform were purchased from Sigma-Aldrich (India). Prednisolone (Vetranal, analytical reagent >99%), and betamethasone (USP grade) were also purchased from Sigma-Aldrich (India). For the testing of assay method, another prednisolone standard with a different part number was purchased from Sigma-Aldrich (India).

Chromatographic parameters

The chromatographic parameters for SFC chromatography with the 1260 Infinity Hybrid SFC/UHPLC System are shown in Table 1. The SFC flow rate and back pressure regulator (BPR) was maintained at a low value of 1 mL/min and 90 bar respectively, to keep the system under pressure while switching to SFC after normal phase runs.

Preparation of standards

Preparation of water-saturated chloroform: To 500 mL of chloroform, 300 mL of water was added in a separatory funnel and mixed. After phase separation, the bottom layer (chloroform) was collected.

Normal phase internal standard solution preparation: Betamethasone was accurately weighed out, to which tetrahydrofuran was added to obtain a concentration of 5 mg/mL. This solution was then diluted to 0.5 mg/mL using water saturated chloroform.

Normal phase standard solution: 1 mg prednisolone (USP grade) was added to a 10-mL volumetric flask, followed by 0.5 mL of methanol to dissolve. To this flask, 2 mL of internal standard solution was added, followed by dilution to the 10-mL mark using water saturated chloroform.

SFC internal standard solution: Betamethasone, which was accurately weighed out, was dissolved in 100% methanol to obtain 0.5 mg/mL.

SFC standard solution: First, 1 mg of USP prednisolone was dissolved in 0.5 mL of 100% methanol and then, 2 mL of SFC internal standard solution was added. The solution was filled to the 10-mL mark with 100% methanol.

Linearity and robustness sample preparation: The SFC solution described above was used for linearity and robustness studies (100 ppm of prednisolone and betamethasone).

Sample preparation

Normal phase/SFC assay test solution: Approximately 1 mg of prednisolone (test standard) was transferred to a 10-mL volumetric flask, followed by 0.5 mL methanol to dissolve. 2 mL of normal phase internal standard solution was added, followed by water saturated choloroform to the 10-mL mark.

Parameters	Normal phase method	SFC method
Column	Agilent ZORBAX Rx-SIL 4.6 × 250 mm, 5 µm (p/n 880975-901)	Agilent ZORBAX Rx-SIL 4.6 × 250 mm, 5 µm (p/n 880975-901)
Thermostatted column compartment solvent preheating	25 °C	40 °C
Thermostatted column compartment solvent post conditioning	not controlled	37.5 °C
Detection	254/16 nm (Ref 360/100 nm) 40 Hz acquisition rate	254/16 nm (Ref 360/100 nm) 40 Hz acquisition rate
Flow cell	10 mm path length, 13 µL volume high pressure flow cell	10 mm path length, 13 µL volume high pressure flow cell
Injection volume	5 µL*	5 µL
Injector program	Yes	Yes
BPR	90 bar	150 bar
SFC flow rate	1 mL/min	2.9 mL/min
Normal phase flow rate	1 mL/min	0 mL/min
SFC run	-	15% B isocratic
Normal phase run	100% A isocratic	-
Run time	20 minutes	5.5 minutes
Mobile phase	Mixture of butyl chloride, water-saturated butyl chloride, tetrahydrofuran, methanol, and glacial acetic acid (95:95:14:7:6)	85% supercritical fluid CO_2, 15% methanol

*The injector volume was decreased from 10 µL to 5 µL to fit the 5 µL fixed loop.

Table 1
Chromatographic parameters used in the Agilent 1260 Infinity Hybrid SFC/UHPLC System.

Procedure

The normal phase pump seal (p/n 0905-1420) was used in the 1260 Infinity Binary Pump of the hybrid system. The pump was equilibrated with isopropyl alcohol prior to use normal phase solvents. The 1260 Infinity SFC/UHPLC Hybrid System was operated in normal phase mode by switching the 2-position/10-port valve. The normal phase runs were performed using the "normal phase standard solution" to determine the USP system suitability parameters. The 2-position/10-port valve was then switched to SFC mode to perform the SFC runs to determine the system suitability parameters. In the SFC mode, linearity and robustness studies were also performed.

A solution of 100% methanol (super gradient) was injected as blank, followed by 11 linearity levels in replicate injections. The average of six area and retention time (RT) information for each level was used to calculate the relative standard deviation (RSD) values. The average area of each linearity level in the linearity range was plotted against the concentration to obtain a calibration curve. The LOD and LOQ for prednisolone and betamethasone was established from the lower linearity level injections based on signal-to-noise ratio. The dilutions for the linearity levels were prepared as per Table 2.

Calibration levels	Prednisolone (µg/mL)	Betamethasone (µg/mL)
1	7.2	6.0
2	24.0	20.0
3	50.4	42.0
4	100.8	84.0
5	144.0	120.0
6	192.0	160.0
7	240.0	200.0
8	288.0	240.0
9	360.0	300.0
10	408.0	340.0
11	480.0	400.0

Table 2
Dilution table for prednisolone and betamethasone.

To evaluate the robustness of the method, five method parameters were evaluated:

- Flow rate ± 2%
- Column temperature ± 2.5%
- Injector volume ± 3%
- Absorption wavelength ± 1 nm
- Modifier concentration ± 1%

For each robustness parameter, a SFC standard preparation of 100 ppm solution of prednisolone and betamethasone were injected, six replicates were used to calculate area, RT and resolution of prednisolone compared to betamethasone. The original method was also performed similarly. The percentage deviation (% accuracy) of area/retention time was calculated from the original method.

To determine the amount of prednisolone in the test sample, the normal phase/SFC assay test solution was used. The same sample was run on the normal phase method as well as on the SFC method. The prednisolone peak area was compared against the prednisolone peak area obtained from the "normal phase standard." The quantity of prednisolone was determined in mg using the formula specified in the USP assay method.

Results and discussion

Separation and detection

The system suitability mixture was used to optimize the separation conditions. The separation was initially performed at initial SFC conditions (TCC temperature of 35 °C, back pressure regulator at 150 bar, Agilent ZORBAX Rx-Sil column and flow rate of 3.0 mL/min). The methanol percentage was varied from 20% B (methanol) isocratic by decreasing it systematically to 5% isocratic in different runs. The ideal separation was found to be at 15% B isocratic. Following the mobile phase optimization, flow rate optimization was carried out. The flow rate was changed from 1.5 mL/min to 3.5 mL/min in increments of 0.2 mL/min where area/RT of the peaks were recorded. The ideal flow rate was determined to be 2.9 mL/min. The TCC temperature was also varied from 25 °C to 45 °C where the ideal temperature was found to be at 40 °C.

Figure 2 shows the chromatogram of the SFC method performed at the final optimal condition overlaid with the USP normal phase method. The detector was set at 254 nm as suggested in the USP method. Figure 2, in the SFC method, shows some additional peaks around the column void time (~0.9 minute). These peaks originate from the "super gradient" methanol used to dilute the sample.

The system suitability test was performed using both methods. The SFC method provided acceptable relative retention time values and resolution (Table 3). The area precision for four replicate injections showed better results in the SFC method as compared to the USP normal phase method. The added benefit of SFC is to be able to run the sample at a faster flow rate. It also used methanol as the only modifier. The advantage of the SFC method compared to normal phase method in regards to analysis time and solvent cost (US $) per 100 sample analysis is displayed in Table 4. A 4-fold decrease in analysis time and a 17-fold decrease in cost was achieved with the SFC method for every analytical run. Assuming analysis time to be US $ 80/hr, the overhead cost would decrease to US $ 20/hr.

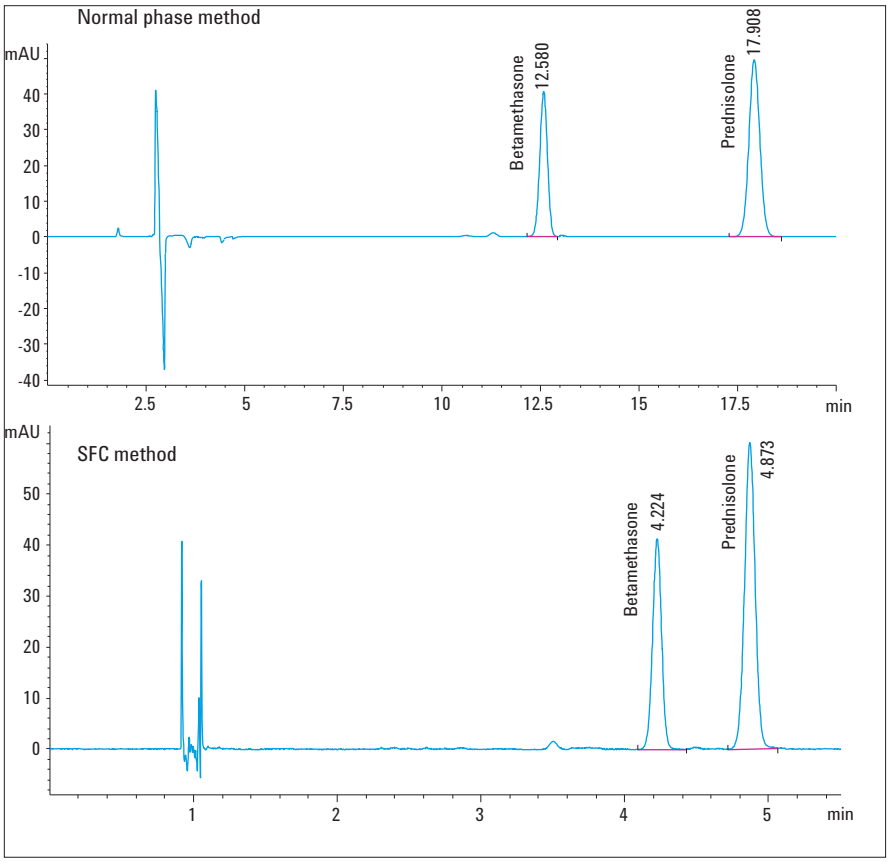

Figure 2
Separation of 100 ppm solution of prednisolone and betamethasone using an Agilent ZORBAX Rx- SIL 4.6 × 250 5 µm column.

Parameter		USP method	USP normal phase method	SFC method
RRT	Prednisolone	1.0	1.0	1.0
	Betamethasone	0.7	0.7	0.9
Resolution		NLT 3.5	11.6	4.1
Std injection (n=4) (Prednisolone)		RSD Area NMT 2.0%	0.5%	0.1%

Table 3
USP prednisolone system suitability acceptable limits compared with USP normal phase method and SFC method. NLT = Not less than, NMT = Not more than.

	USP normal phase method	SFC method	Savings
Analysis time per sample (min)	20	5.5	3.6×
Solvent cost per 100 analysis (US $)	292	17.2	17×

Table 4
Savings in analysis time and solvent cost per 100 sample analysis when using the SFC method compared with the normal phase method.

LOD, LOQ, and linearity using the SFC method

The analyte concentration that provides a signal-to-noise ratio (S/N) was considered as LOD, while the analyte concentration with S/N ratio > 10 was considered as LOQ. LOD, LOQ, and linearity was performed for SFC method only. Table 5 shows that the LOD for prednisolone was found to be at 2.4 µg/mL while the LOQ was found to be at 7.0 µg/mL.

The linearity levels were determined using the SFC method starting from the LOQ level of prednisolone and betamethasone. Figure 3 shows calibration curves for these two compounds. Both calibration curves were found to be linear having correlation coefficient (R^2) values of >0.999. The results show the excellent performance of SFC as a replacement method for normal phase method.

		LOD		LOQ		Linearity range		Number
Sl no	Name	µg/mL	S/N	µg/mL	S/N	µg/mL	R^2 value	of levels
1	Prednisolone	2.4	5.0	7.2	13.2	7.2–480	0.9993	11
2	Betamethasone	2.0	3.9	6.0	10.0	6.0–400	0.9992	11

Table 5
LOD, LOQ, and linearity of prednisolone and betamethasone.

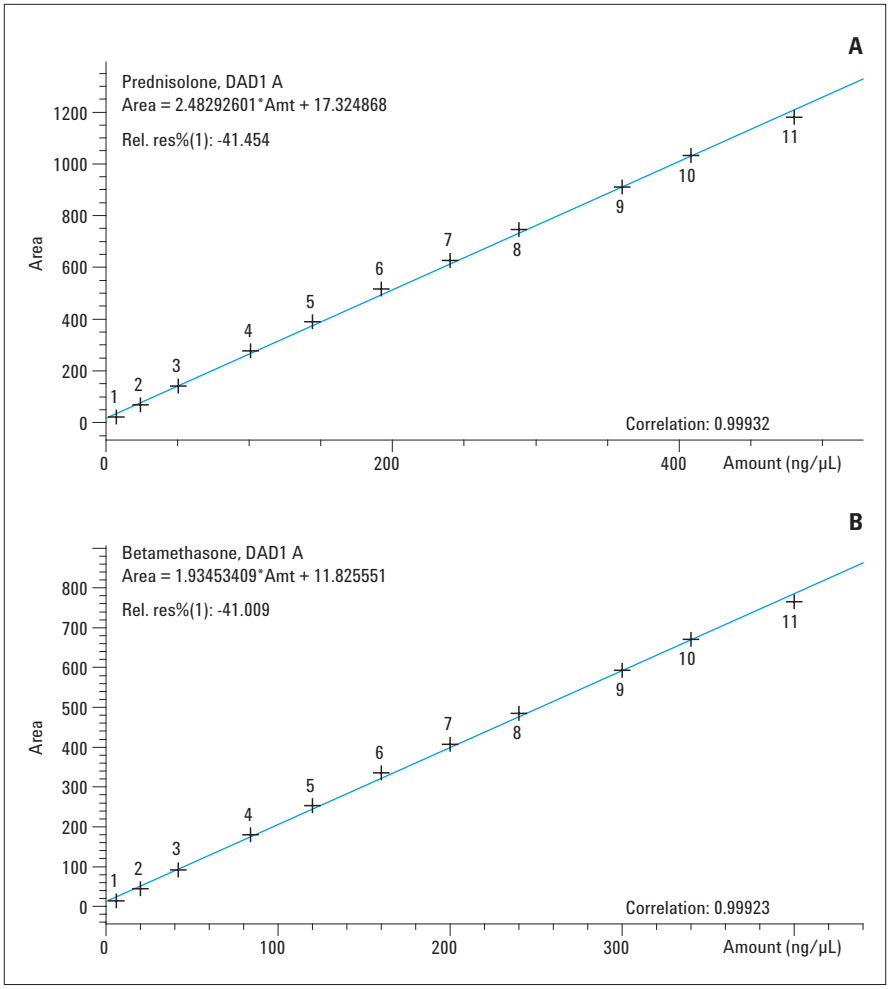

Figure 3
Linearity curves of prednisolone (A) and betamethasone (B).

Precision of retention time and area

The area precision was measured as RSD (%) across the linearity levels with the SFC method. The maximum RSD value of 2.2% and 2.1% for level 1 (L1) were obtained for prednisolone and betamethasone respectively. Similarly, RT precision calculations obtained a maximum RSD value <0.2% for both prednisolone and betamethasone. Graphical representation of area RSD values are displayed in Figure 4.

Robustness

To test the robustness of the method, a standard solution containing 100 ppm of prednisolone and betamethasone was used. Five critical method parameters (flow rate, column temperature, injector volume, absorption wavelength, and modifier concentration) were varied individually. The peak areas from the six replicate injections were compared. The allowed deviation for the area and retention time was set to ± 5% and ± 3% respectively. The results of the robustness tests are summarized in Table 6. The red numbers indicate where the result exceeded the allowed deviation. A change in flow rate and TCC temperature does not vary the method. The injection volume of 15 µL is taken in order to fill 3x the fixed injection loop of 5 µL. A deviation in injection volume of ±3% from 15 µL also does not affect the method. It is recommended to use 15 µL to overfill the 5 µL injection loop. The modifier concentration rise by 1%, changes the RT of the compound but not the area. The lowering of the modifier concentration however does not change the RT. The deviation in area due to wavelength of 254 nm is also very sensitive because of the position of 254 nm in the absorption spectra of both prednisolone and betamethasone.

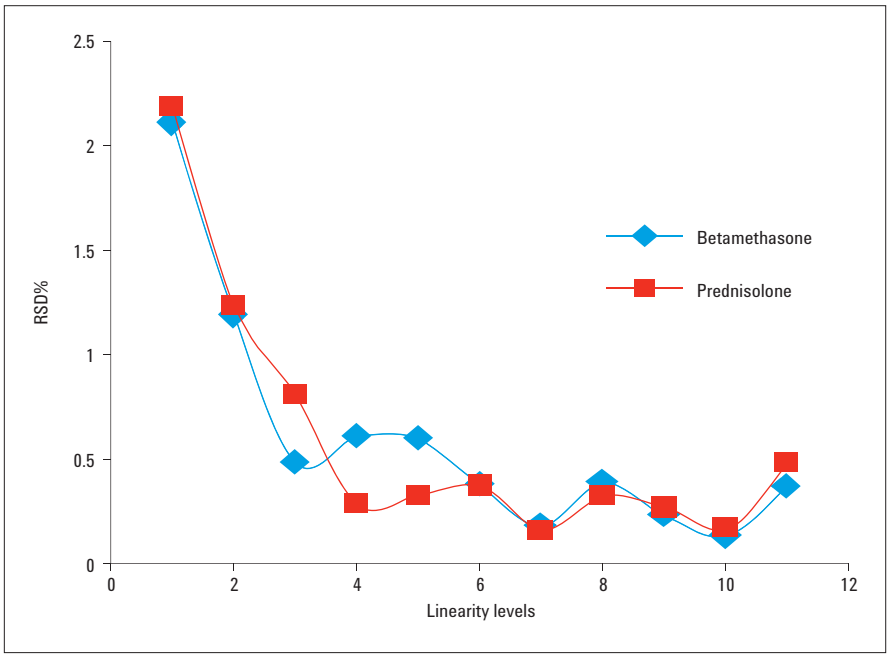

Figure 4
Area precision measured as RSD(%) for six replicates at each concentration level for prednisolone and betamethasone.

		Prednisolone			Betamethasone	
Parameters	**Variations**	**% area**	**% RT**	**Resolution**	**% area**	**% RT**
Flow: 2.9 mL/min ± 2%	High: 2.96 mL/min	-3.5	-2.9	4.1	2.2	-2.8
	Low: 2.84 mL/min	1.9	1.7	4.1	-3.4	1.8
TCC: 40 °C ± 2.5%	High: 41°C	0.1	0.8	4.0	0.1	0.9
	Low: 39°C	-0.4	-1.4	4.0	-0.4	-1.3
Injector: 15 µL ± 3%	High: 15.5 µL	-0.1	-0.6	4.0	-0.2	-0.3
	Low: 14.5 µL	-0.3	-0.7	4.0	-0.2	-0.5
Wavelength: 254 ± 1 nm	255 nm	-4.8	-0.8	4.0	-6.0	-0.6
	253 nm	2.5	-0.8	4.0	3.6	-0.5
Modifier concentration: 15% B ± 1%	High: 15.2 %B	0.2	-3.7	3.9	0.2	-3.3
	Low: 14.8%B	0.0	1.3	4.0	0.0	1.5

Table 6
Results of the robustness test methods compared to the standard method at concentration of 100 ppm. The red values in the table indicate that the deviations exceeding the allowed limits of 5% for area and 3% for retention time.

The absorption wavelength needs to be constant as well as unchanging during the analysis. Alternatively, a different UV region such as 240 nm can be chosen for further studies. The resolution of prednisolone was not found to be changing in any of the robustness testing methods. Robustness results indicate that the method is reliable for normal usage, where, to a great extent, the performance remains unaffected by deliberate changes of the method parameters. However, some parameters, such as the wavelength and percentage modifier concentration are critical, which must be carefully controlled.

Assay results

To test the accuracy of both the methods, the normal phase/SFC assay test solution (see page 3) was tested with both the SFC method and the normal phase method. The analysis was performed according to the USP assay method, which involves comparing the area with that of the system suitability mix. The results from Table 7 show that for the same test sample approximately 1.3 mg of prednisolone was detected in a 10-mL solution, for both of the methods, confirming similar performance.

Method	Assay results (mg)
SFC method	1.278
Normal phase method	1.272

Table 7
Assay results obtained from SFC method and the USP normal phase method.

Conclusion

The Agilent 1260 Infinity Hybrid SFC/UHPLC System was used to develop a novel SFC prednisolone assay method and this method was compared to the original USP normal phase method. While meeting the system suitability requirements, the new SFC method was 4x faster and 17x less expensive than the normal phase method. Additionally, the amount of prednisolone from a test sample delivered similar results with both methods. The linearity and robustness test results were excellent for the SFC method with a LOD value of about 2 ppm for both prednisolone and betamethasone. The SFC method does not require purchase and disposal of expensive environmentally hazardous chemicals. Hence, the newly developed SFC method provides a fast, cost effective, and safe solution.

References

1.
Colin F. Poole, "Progress in Packed Column Supercritical Fluid Chromatography: Materials and Methods," J. Biochem. Biophys. Methods, 43: 3-23, **2000**.

2.
Bieke Dejaegher, Yvan Vander Heyden, "Ruggedness and Robustness Testing," J. Chrom A., 1158: 138-157, **2007**.

3.
Martin Vollmer, Markus Becker, "Agilent 1260 Infinity Hybrid SFC/UHPLC System" Agilent Technical Overview. Pub No. 5990-9514EN, **2012**.

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