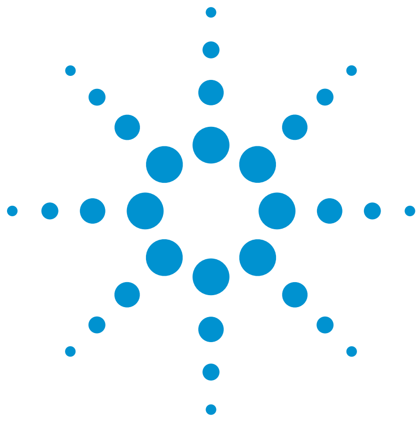

Transfer of USP Cholecalciferol Normal-phase HPLC Method to SFC Using the Agilent 1260 Infinity Hybrid SFC/UHPLC System

Application Note

Pharmaceutical QA/QC

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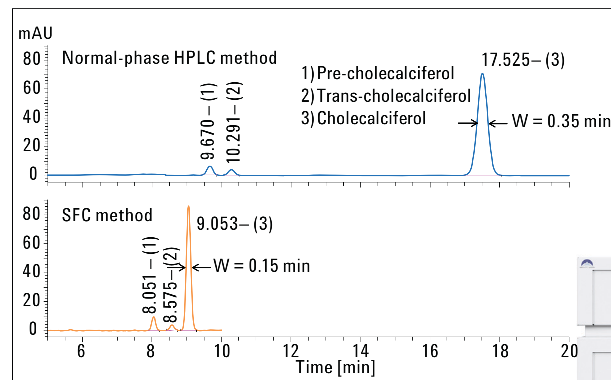

Abstract

The Agilent 1260 Infinity Hybrid SFC/UHPLC System offers a fast, reliable, green, robust, and cost-effective solution by coupling the advantages of HPLC and the benefits of supercritical fluid chromatography (SFC). This Application Note describes the conversion of the United States Pharmacopeia (USP) normal-phase HPLC method for cholecalciferol assay to an SFC method using serially coupled columns. The 1260 Infinity Hybrid SFC/UHPLC System was used to perform both the HPLC as well as the SFC methods. The results show that the SFC method meets the system suitability criteria, is 2x faster, and achieves 26x lower solvent costs compared to the USP method. Robustness tests on the SFC method demonstrated excellent robustness for routine analysis.

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Introduction

Cholecalciferol is a form of vitamin D, which is also called vitamin D3. The USP assay method for cholecalciferol is a normal-phase HPLC method that includes the separation of cholecalciferol and two isomers: pre-cholecalciferol and trans-cholecalciferol (Figure 1).

The USP assay method uses toluene as sample diluent whereas dehydrated hexane is used as the mobile phase. Exposure to toluene concentrations ranging from 10,000 to 30,000 ppm has caused narcosis and death (WHO, 1985). Hexane is included in the list of chemicals on the US Toxic Release Inventory (TRI). In contrast, SFC is considered to be a green technology due to the use of nontoxic solvents and carbon dioxide (CO_2) as a major component of the mobile phase. Compared to normal-phase HPLC methods, SFC methods offer faster separation without loss of efficiency, and faster column equilibration. In SFC, the pressure drop is substantially lower than in HPLC because of the lower viscosity of the mobile phase. This enables coupling of columns to achieve the desired separation. The potential for using serially coupled columns in SFC to obtain higher efficiency and resolution has already been recognized.[1]

This Application Note describes the development of an SFC method using two serially coupled columns each with different selectivity to replace a USP normal-phase HPLC method for cholecalciferol assay. The 1260 Infinity SFC/UHPLC Hybrid System was used to perform both the HPLC and the SFC method on a single instrument. This unique hybrid solution eliminates the need to invest in two individual systems, thus excluding system-to-system variability and saving significant costs and laboratory space.[2]

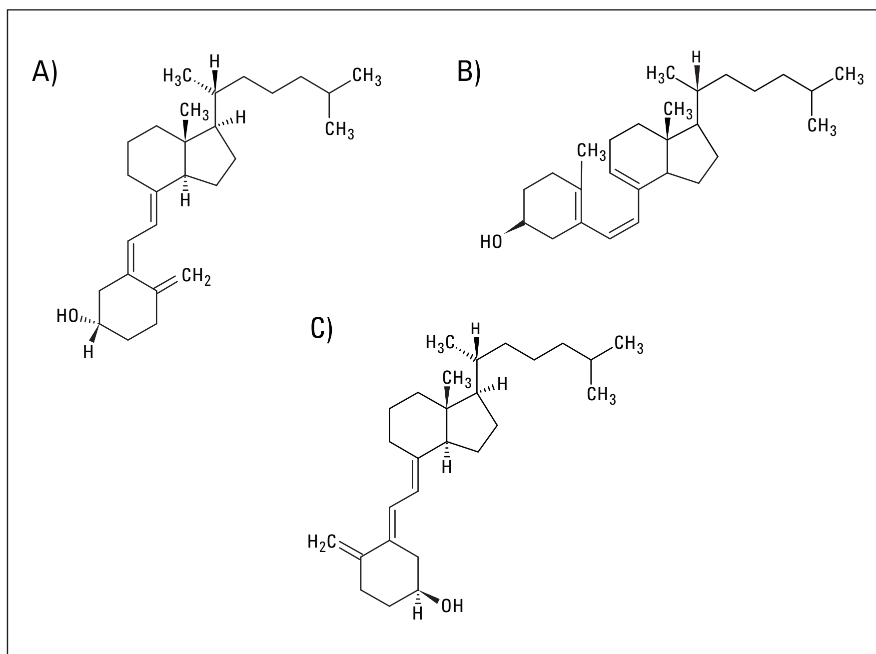

Figure 1
Molecular structures of (A) cholecalciferol, (B) pre-cholecalciferol, and (C) trans-cholecalciferol.

Experimental

Instrumentation

An Agilent 1260 Infinity Hybrid SFC/UHPLC System consisting of the following modules was used for the experiments.

Description	Model number
Agilent 1260 Infinity Analytical SFC System	G4309A
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 OpenLAB CDS ChemStation Edition, Rev. B.04.03.

Reagents and Materials

All solvents used were HPLC grade. Purified water was obtained from a Milli-Q water purification system (Millipore, USA). Methanol super gradient was purchased from Lab-Scan. HPLC grade, *n*-Hexane, toluene, 1-pentanol (*n*-amyl alcohol) and cholecalciferol were purchased from Sigma-Aldrich (India). USP Vitamin D assay system suitability mix was purchased from USP (India). Calcirol, (cholecalciferol, powder) manufactured by CADILA Pharmaceuticals, was used for formulation experiments.

Chromatographic Parameters

Table 1 shows the chromatographic parameters for SFC with the 1260 Infinity Hybrid SFC/UHPLC System. While working with the normal phase method, 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.

Preparation of Standards

System Suitability Solution (Normal-phase HPLC)

A 200 µL amount of USP Vitamin D Assay system suitability was dissolved in 1,200 µL of a mixture of equal volumes of toluene and normal-phase mobile phase solution. The solution was heated under reflux at 90 °C for 45 minutes, and cooled back to room temperature. This solution contained cholecalciferol, pre-cholecalciferol, and *trans*-cholecalciferol.

System Suitability Preparation (SFC)

A 100 µL solution of USP Vitamin D assay system suitability was dissolved in 200 µL of normal phase mobile phase (6/1,000 of 1-pentanol in *n*-Hexane). This solution was mixed with 400 µL of methanol and heated using an Eppendorf Thermomixer Comfort at 90 °C, 300 rpm for approximately 2 hours. This solution contained cholecalciferol, pre-cholecalciferol, and *trans*-cholecalciferol.

SFC Robustness Solution

A 1 mg amount of cholecalciferol was dissolved in 10 mL methanol. The solution was heated using an Eppendorf Thermomixer Comfort at 90 °C, 300 rpm for approximately 2 hours. This solution contained cholecalciferol and pre-cholecalciferol.

Linearity Solution

Cholecalciferol was dissolved in methanol to obtain a stock solution of 600 µg/mL solution. Various calibration levels were prepared by serial dilution from this stock solution.

Standard Solution

Approximately 1.6 mg of cholecalciferol were transferred and dissolved in 2 mL of toluene followed by addition of 8 mL of mobile phase (normal phase).

Parameter	Normal-phase HPLC method	SFC method
Column	Agilent ZORBAX Rx-SIL, 4.6 × 250 mm, 5 µm (p/n 880975-901)	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl, 4.6 × 100 mm, 5 µm (p/n 959996-912) Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm (p/n 959990-902)
Detection	254 nm, 2.5–10 Hz acquisition rate	254 and 262 nm, 2.5–10 Hz acquisition rate
Thermostatted column compartment	40 °C (pre- and post-column solvent heating)	40 °C (pre- and post-column solvent heating)
Flow cell	High pressure flow cell with 10-mm path length and 13-µL volume	High pressure flow cell with 10-mm path length and 13-µL volume
Injection loop	5 µL (injected 3× loop size)	5 µL (injected 3× loop size)
Back pressure regulator	90 bar	120 bar
SFC flow rate	1 mL/min	2.5 mL/min
Normal-phase flow rate	1.5 mL/min	0 mL/min
Mobile phase	6/1,000 mixture of N-amyl alcohol in dehydrated hexane	A: Supercritical fluid CO_2 B: Methanol
Chromatographic run	100% A isocratic	2% B isocratic
Run time	20 minutes	10 minutes

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

Cholecalciferol Formulation Solution
A 1 g amount of cholecalciferol powder (containing 1.6 mg of cholecalciferol) was dissolved in 2 mL of toluene followed by 8 mL of mobile phase (normal phase). After sonication, the supernatant was filtered through an Agilent Econofilter PTFE, 0.20 µm, 25 mm (p/n 5185-5834) and the filtrate was injected.

Procedure

The normal phase pump seal (p/n 0905-1420) was used in the Agilent 1260 Infinity Binary Pump of the hybrid system. The pump was equilibrated with isopropyl alcohol prior to 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 system suitability solution to determine the USP system suitability parameters. The 2-position/10-port valve was then switched to SFC mode for the SFC runs, to determine the system suitability parameters, robustness, and linearity. Robustness studies were performed using the SFC robustness solution and six method parameters were evaluated.

For each robustness parameter, six replicates were used to calculate area and retention time (RT). The percentage deviation (% accuracy) of area and RT was determined compared to the standard SFC method.

Linearity of the method was demonstrated by injecting the linearity solution. The dilutions for the linearity levels were prepared as shown Table 2. Cholecalciferol has an absorption maximum at 262 nm and, hence, was used in linearity studies. A solution of 100 % methanol was injected as blank, followed by nine linearity levels in replicate injections. The average area of each linearity level in the linearity range was plotted against the concentration to obtain a calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) for cholecalciferol were established from the lower linearity level injections based on signal-to-noise ratio.

Linearity level	Cholecalciferol (µg/mL)
1	8.29
2	18.02
3	38.51
4	77.02
5	148.12
6	224.42
7	307.42
8	385.24
9	458.62

Table 2
Dilution table for cholecalciferol.

Results and Discussion

Separation and Detection

The SFC system suitability solution was used to optimize the separation conditions. Efficient and selective separation was determined through serial coupling of a ZORBAX Eclipse Plus Phenyl-Hexyl, 4.6 x 100 mm, 5 µm column followed by a ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm column. The ideal flow rate and temperature were determined to be 2.5 mL/min and 40 °C respectively. Figure 2 shows the chromatogram of the SFC method performed overlaid with the chromatogram of the USP normal phase method. The detector was set at 254 nm for both methods according to the USP method.

The system suitability test was performed using both methods. The SFC method provides better resolution between trans- and pre-cholecalciferol (Rs = 2.3).

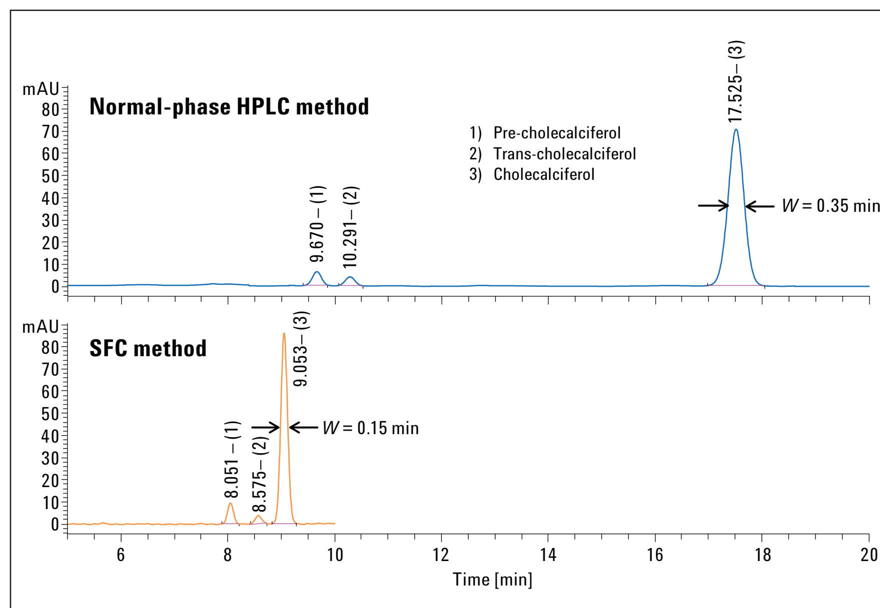

Figure 2
Separation of system suitability mix using the normal-phase HPLC and SFC methods at 254 nm. Narrower peak widths were achieved with SFC.

In SFC method, the resolution between cholecalciferol and trans-cholecalciferol is 2.0. In addition, the area precision for four replicates was within the limit of USP criteria (Table 3).

An added benefit of SFC compared to the USP normal-phase method was a significant cost reduction (Table 4). The analysis time saving was calculated based on the run time for each chromatographic run, whereas the solvent saving was calculated based on material cost calculations. A twofold decrease in analysis time and a 26× decrease in costs were achieved with the SFC method for every 100 analytical runs. Assuming analysis time to be US $ 80/hour, the overhead cost would decrease to US $ 40/hour.

Parameter	USP method	USP normal-phase HPLC method	SFC method
Resolution between trans- and pre-cholecalciferol	Not less than 1	1.9	2.3
Peak area for cholecalciferol (n = 4)	RSD area not more than 2 %	0.2	0.8

Table 3
USP cholecalciferol system suitability limits compared with USP normal-phase HPLC method and SFC method.

	USP normal-phase HPLC method	SFC method	Savings
Analysis time per sample (min)	20	10	2x
Solvent cost per 100 analyses (US $)	226	8.7	26x

Table 4
Savings in analysis time and solvent cost per 100 analyses using SFC.

Robustness

To test the robustness of the method, the SFC robustness solution was used. Six critical method parameters were varied individually (Table 5). The allowed deviations for the area and retention time were set to ± 5% and ± 3% respectively. The underlined numbers indicate where the result exceeded the allowed deviation.

Changes in backpressure and column temperature do not vary the method. The injection volume of 15 µL was used in order to overfill the fixed injection loop of 5 µL three times. A deviation in injection volume of ± 3% from 15 µL does not affect the method. The absorption wavelength in the robustness studies was chosen to be 254 nm according to USP recommendation. Flow rate variation of 2% affected area of pre cholecalciferol significantly and RT of cholecalciferol and pre-cholecalciferol marginally. The resolution of cholecalciferol and pre-cholecalciferol was not changed by any of the robustness testing parameters. Results of robustness studies indicate that the method is reliable for normal usage, whereas, to a great extent, the performance remains unaffected by deliberate changes of the method parameters. However, some parameters such as the flow rate and wavelength are critical and must be carefully controlled.

		Cholecalciferol			Pre-cholecalciferol	
Parameters	Changes	% area	% RT	Resolution	% area	% RT
Flow: 2.5 mL/min ± 2%	High: 2.55 mL/min	0.8	0.8	4.5	-0.2	0.7
	Low: 2.45 mL/min	-4.5	<u>-3.7</u>	4.5	<u>-8.0</u>	<u>-3.8</u>
Column: 40 °C ± 2.5%	High: 41 °C	0	-0.6	4.5	0.6	-0.6
	Low: 39 °C	-0.3	0.8	4.5	3.0	0.7
Injector: 15 µL ± 3%	High: 15.5 µL	-1.0	-0.1	4.5	1.0	-0.1
	Low: 14.5 µL	-0.4	0.2	4.5	-0.2	0.2
Wavelength: 254 ± 1 nm	255 nm	-3.0	-0.4	4.5	0.8	-0.4
	253 nm	0.6	-0.8	4.5	<u>5.7</u>	-0.8
Modifier concentration: 2% B ± 5%	High: 2.1% B	-1.4	1.2	4.4	1.4	0.8
	Low: 1.9% B	-2.0	-2.5	4.6	-1.5	-2.1
Backpressure: 120 ± 2 bar	High: 122	0.1	1.9	4.5	4.3	1.8
	Low: 118	-0.3	-2.1	4.5	1.0	-2.0

Table 5
SFC robustness test results, the underlined numbers in the table indicate that the deviations exceeding the allowed limits of 5 % for area and 3 % for retention time.

LOD, LOQ, and linearity using the SFC method

The analyte concentration that provides a signal-to-noise ratio (S/N) greater than three was considered as LOD, while the analyte concentration with S/N ratio greater than 10 was considered as LOQ. Table 6 shows that for the SFC method, the LOD for cholecalciferol was 3.8 µg/mL while the LOQ was 8.29 µg/mL.

The linearity levels were determined using the SFC method starting from the LOQ level of cholecalciferol. The linear calibration curve showed a correlation coefficient (R^2) values of 0.999 (Figure 3) highlighting the excellent performance of SFC as a replacement method for the normal phase method.

Formulation Analysis Results

The amount of cholecalciferol in the formulation was determined by running the cholecalciferol formulation solution and comparing the detected amount to the standard solution. The same sample was analyzed using normal phase and SFC methods respectively. The amount of cholecalciferol present in each formulation gave similar results for both methods (Table 7) suggesting similar accuracy of both methods.

	LOD		LOQ				
Name	**µg/mL**	**S/N**	**µg/mL**	**S/N**	**Linearity range (µg/mL)**	**R^2 value**	**Number of levels**
Cholecalciferol	3.80	9.2	8.29	14.04	8.3 – 458.6	0.999	9

Table 6
LOD, LOQ, and linearity of cholecalciferol as performed using the SFC method.

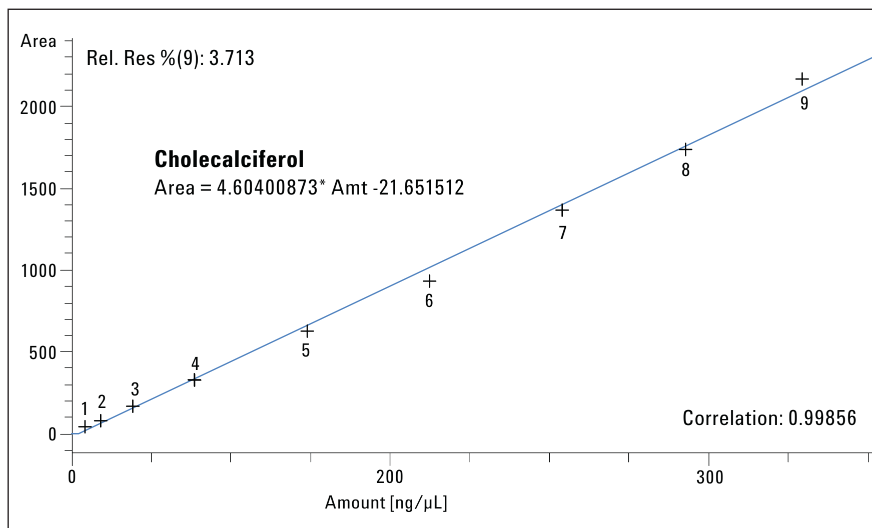

Figure 3
Linearity curve for cholecalciferol.

Method	**Assay results for tablet 1 (mg in 1 g of formulation)**	**Assay results for tablet 2 (mg in 1 g of formulation)**
SFC method	2.0	1.8
Normal-phase HPLC method	2.0	1.9

Table 7
Amounts of cholecalciferol in formulations from SFC method and the USP normal-phase HPLC method.

Conclusion

The Agilent 1260 Infinity Hybrid SFC/UHPLC System was used to develop a novel SFC cholecalciferol assay method and this method was compared to the original USP normal-phase HPLC method. Serially coupled columns yielded efficient separation of cholecalciferol and its two isomers. While meeting the system suitability requirements, the SFC method was 2× faster and 26× less expensive than the normal-phase HPLC method. Additionally, the amount of cholecalciferol from two sets of formulations delivered similar results using both the methods. The linearity and robustness test results were excellent for the SFC method with an LOD value of approximately 4 ppm. In addition, the SFC method does not require the purchase and disposal of expensive and environmentally hazardous chemicals. Hence, the SFC method provides a fast, environmentally friendly, cost effective, and efficient solution.

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