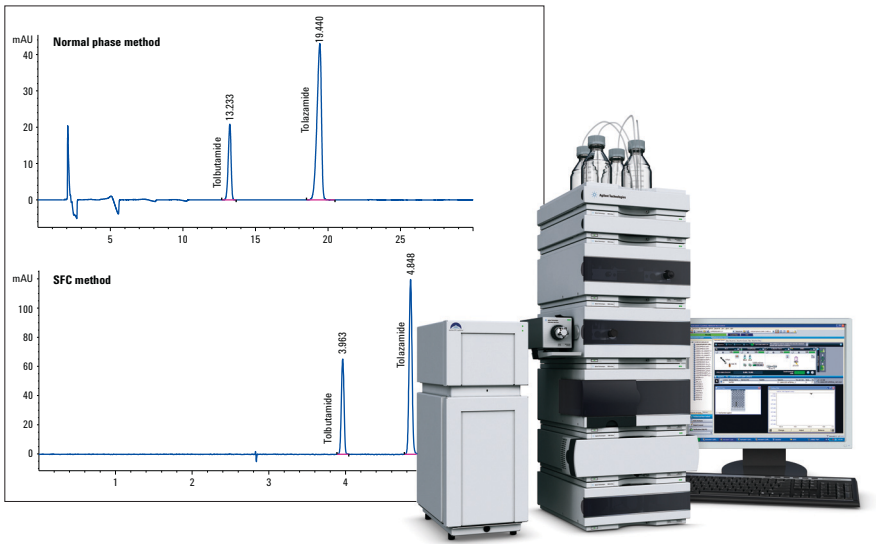
Agilent Application Solution

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

Improving peak shape and providing wider UV selectivity

Application Note

Pharmaceutical QA/QC



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Abstract

Normal phase liquid chromatography 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, which can be operated at flow rates up to three times higher than liquid chromatography (LC) without losing separation efficiency, thereby leading to faster analysis. This Application Note describes a method to transfer a Tolazamide United States Pharmacopeia (USP) normal phase HPLC assay 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 and displays narrower and taller peaks. In addition, the SFC runs were 4× faster and created 19× lower solvent expenses than the normal phase method. Robustness tests demonstrate excellent results for routine analysis with SFC.

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Introduction

Tolazamide is an oral, blood glucose lowering drug of the sulfonylurea class. The USP assay method for tolazamide uses a normal phase method that includes chloroform as a sample diluent, while hexane is used as the mobile phase. Chloroform is a known carcinogen, potentially toxic to analysts and expensive to dispose. Hexane is included in the list of chemicals on the US Toxic Release Inventory (TRI). SFC is considered a green technology with a low risk of flammability, due to the use of carbon dioxide (CO_2) as a major component of the mobile phase. Recently, SFC has demonstrated that it can 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]. This Application Note shows the development of a SFC method to replace a normal phase tolazamide assay in which tolbutamide is used as an internal standard (Figure 1). This method uses methanol as a sample diluent instead of chloroform.

The Agilent 1260 Infinity SFC/UHPLC Hybrid System was used to perform both the normal phase as well as the SFC method on the single instrument[2]. With this unique hybrid solution, there is no need to invest in two individual systems, thus excluding system-to-system variability and saving significant cost and laboratory space.

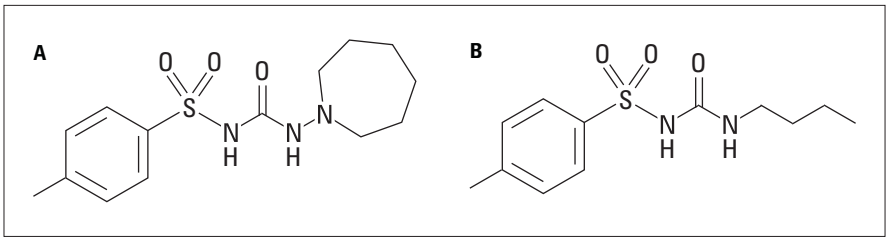

Figure 1
Molecular structures of Tolazamide (A) and Tolbutamide (B).

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

In addition the following 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 gradients were purchased from Lab-Scan. HPLC grade, hexane, tetrahydrofuran, glacial acetic acid, and chloroform were purchased from Sigma-Aldrich (India). Tolazamide and tolbutamide (Vetranal, analytical reagent >99%) were also purchased from Sigma-Aldrich (India).

Chromatographic parameters

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

Preparation of standards

Preparation of water-saturated hexane: Add 1:1 ratio of pure hexane and H_2O in a separating funnel mix well. After phase separation, draw out the water from the bottom and collect the remaining water saturated hexane

Normal phase internal standard solution: Dissolve a suitable quantity of tolbutamide in alcohol-free chloroform to obtain a solution with a known concentration of approximately 1.5 mg/mL.

Normal phase standard solution: Dissolve an accurately weighed quantity of USP Tolazamide RS in normal phase internal standard solution to obtain a solution with a known concentration of about 3 mg/mL.

SFC internal standard solution: Tolbutamide was accurately weighed out, and dissolved in 100% methanol to obtain 1.5 mg/mL.

SFC standard solution: Dissolve an accurately weighed tolazamide in SFC internal standard solution to obtain a concentration of approximately 3 mg/mL.

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:	25 °C	40 °C
Detection:	254/4 nm (Ref 360/100 nm) 10 Hz acquisition rate	254/4 nm (Ref 360/100 nm) 10 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	3 mL/min
Normal phase flow rate:	1.5 mL/min	0 mL/min
SFC run:	-	Time % B 0 0 4.5 12 5.0 12 5.1 0 5.5 0
Normal phase run:	100% A isocratic	-
Run time:	30 minutes	5.5 minutes
Mobile phase:	Hexane, water-saturated hexane, tetrahydrofuran, methanol, and glacial acetic acid (475:475:20:15:9).	A: Supercritical fluid CO_2 B: 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 Agilent 1260 Infinity Binary Pump of the hybrid system used the normal phase pump seal (p/n 0905-1420). 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 standard 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. In the SFC mode, robustness studies were also performed using SFC standard solution.

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

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

For each robustness parameter, a SFC standard preparation of 100 ppm solution of tolazamide and tolbutamide was injected, and six replicates were used to calculate area, RT. The resolution of tolazamide was compared to tolbutamide. The original method (without robustness parameters) was also performed in six replicates. The percentage deviation (% accuracy) of area/retention time (RT) was calculated from the original 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, backpressure 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. However, the separation was found to be good in gradient runs where the mobile phase was changed from 0% B to 12% B in 4.5 minutes.

Flow rate optimization followed mobile phase optimization. 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 was recorded. The ideal flow rate was determined to be 3 mL/min. The TCC temperature was also varied from 25 °C to 45 °C where the ideal temperature was found to be 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. An additional peak appears at 2.9 minutes in the SFC method (Figure 2). This peak originates from the "super gradient" methanol used to dilute the sample.

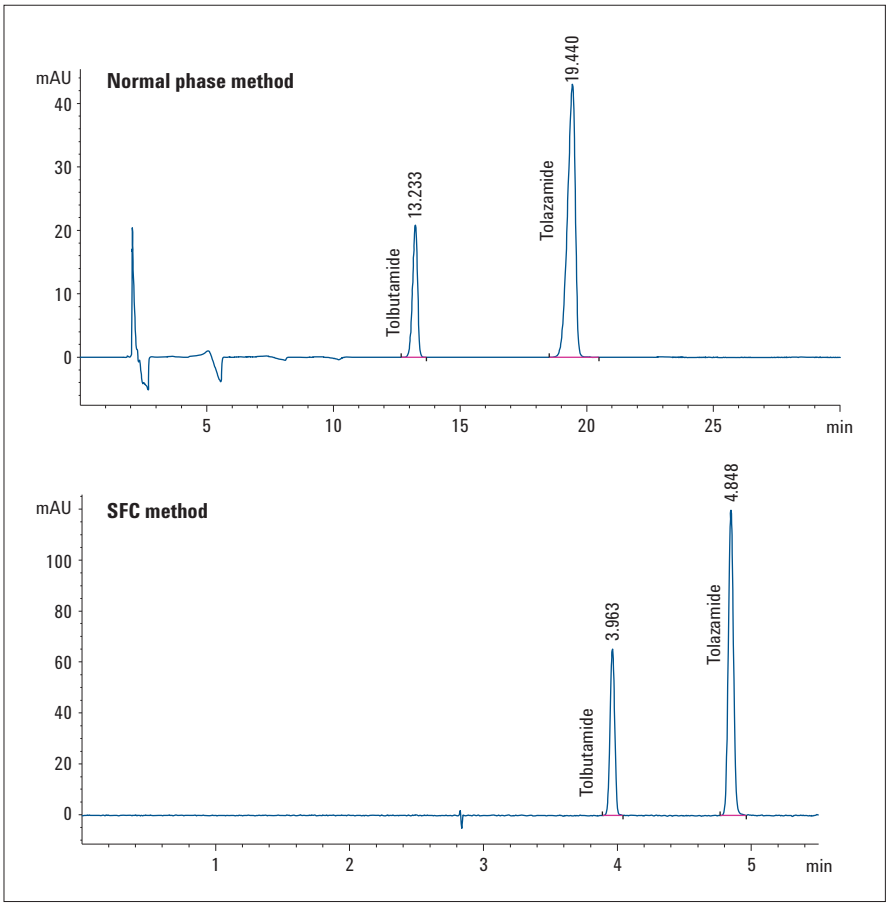

Figure 2
Separation of standard solution of tolazamide and tolbutamide using an Agilent ZORBAX Rx- SIL 4.6 × 250 mm, 5 µm column.

The system suitability test was performed using both methods. The SFC method provided similar relative retention time (RRT) values and resolution (Table 3). The area precision for four replicate injections was performed as per USP method. The results are better in the SFC method than the USP normal phase method. An added benefit of SFC is being able to run the sample at a faster flow rate and use methanol as diluent.

SFC chromatographic runs produced sharper and narrower peaks compared to normal phase methods. The tolazamide peak width at half height was 0.04 minutes, 7× lower compared to the normal phase method (Table 4).

Another advantage of the SFC method versus the USP normal phase method is a significant cost reduction (Table 5). A 4× decrease in analysis time and a 19× decrease in cost were 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.

		System suitability		
Parameter		**USP method**	**USP normal phase method**	**SFC method**
Relative retention time	Tolazamide	1.0	1.0	1.0
	Tolbutamide	0.6	0.7	0.8
Resolution		NLT 2	13	13
Std injection (n=4) (Tolazamide)		RSD area NMT 2%	2%	1%

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

	Peak properties			
Parameter	**Normal phase method**		**SFC method**	
	Tolazamide	**Tolbutamide**	**Tolazamide**	**Tolbutamide**
Peak width at half height	0.3	0.2	0.04	0.04
Peak height	43	21	120	66
Peak symmetry	1.7	1.3	1.0	1.0
Peak tailing	0.7	0.9	1.0	1.0

Table 4
Comparison of peak properties obtained from normal phase and SFC method.

	USP normal phase method	**SFC method**	**Savings**
Analysis time per sample (min)	20	5.5	3.6 x
Cost per sample (US $)	248	13.2	19 x

Table 5
Savings in analysis time and solvent cost per 100 analysis using SFC.

Increased sensitivity in SFC through lower UV cut off

The solvents/modifiers used in the normal phase methods have various UV cut off regions. Table 6 shows the UV cut off of normal phase solvents used in the tolazamide method. Chloroform displaying the highest cut off of 245 nm, restricts the usage of wavelength at a lower region. Tolazamide, with a UV maximum at 228 nm (Figure 3), cannot be used with chloroform. The typical SFC modifier and sample diluent methanol displays a UV cut off of 205 nm, thereby making it ideal for the analysis of tolazamide. A 28×increase of sensitivity was observed when tolazamide was analyzed using 228 nm (see table in Figure 4) .

	UV cut off	
Solvents	**Common normal phase solvents**	**Common SFC method**
Hexane	195	-
THF	212	-
Chloroform	245	-
Methanol	205	205
Super critical fluid CO_2	-	190

Table 6
UV cut off values of solvents used in normal phase and SFC methods[3].

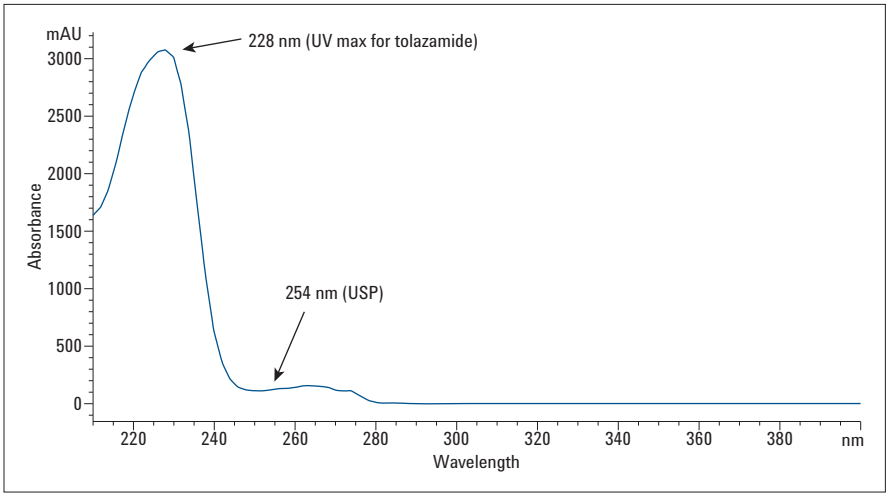

Figure 3
The UV spectra of tolazamide showing 228 nm as UV maximum and 254 nm wavelength recommended as per USP.

Compound name	254 nm	228 nm – Area	254 nm – S/N	228 nm – S/N
Tolazamide	322	9084	250	5934
Tolbutamide	199	6306	150	4377

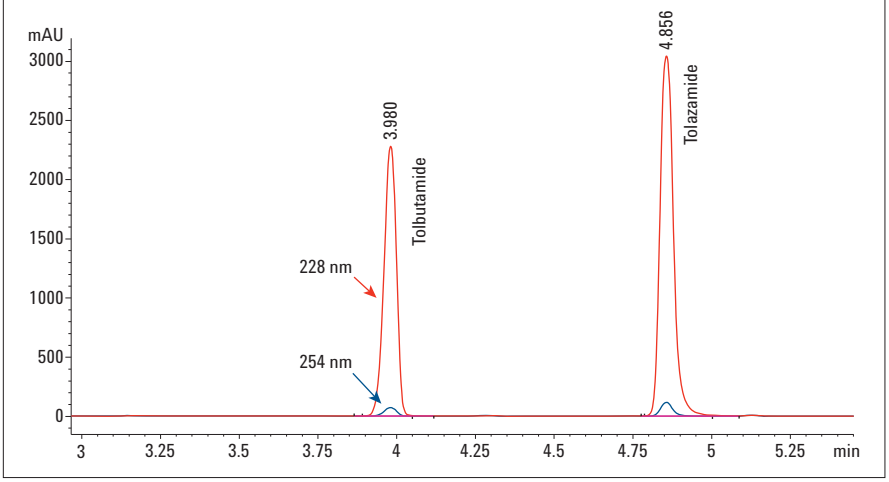

Figure 4
Chromatogram of tolazamide and tolbutamide performed at 228 nm (red) and 254 nm (blue). Insert: peak areas and signal-to-noise ratio (S/N) obtained using two different UV acquisition wavelengths.

Robustness

To test the robustness of the method, the SFC standard solution containing 3 mg/mL of tolazamide and 1.5 mg/mL of tolbutamide was used. Six critical method parameters (flow rate, column temperature, injector volume, absorption wavelength, modifier concentration, and backpressure) were varied individually (Table 7)[4]. The peak areas from the six replicate injections were compared. The allowed deviations for the area and retention time were set to ± 5% and ± 3% respectively. The red numbers indicate where the result exceeded the allowed deviation. Changes in flow rate and TCC temperature do not vary the method. The injection volume of 15 µL is taken 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. It is recommended to use 15 µL to overfill the 5 µL injection loop. The absorption wavelength in robustness studies was chosen to be 254 nm as per USP recommendation. However, for better sensitivity, 228 nm can also be used. A backpressure regulator change of 2 bar affected only the internal standard area. The resolution of tolazamide was not found to be changing at 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 backpressure are critical, and must be carefully controlled.

		Tolazamide			Tolbutamide	
Parameters	**Changes**	**% Area**	**% RT**	**Resolution**	**% Area**	**% RT**
Flow: 3 mL/min ± 2%	High: 3.06 mL/min	0.5	1.0	12.8	4.0	1.2
	Low: 2.94 mL/min	-3.1	-1.1	12.6	-0.8	-1.3
TCC: 40 °C ± 2.5%	High: 41 °C	-0.9	-0.6	12.8	0.9	-0.5
	Low: 39 °C	-1.3	0.3	12.7	0.5	0.2
Injector: 15 µL ± 3%	High: 15.5 µL	-1.2	-0.3	12.8	0.4	0.2
	Low: 14.5 µL	-1.4	-0.3	12.8	0.0	-0.4
Wavelength: 254 ± 1 nm	255 nm	-4.3	-0.4	12.9	-3.2	-0.4
	253 nm	3.8	0.0	12.9	3.3	-0.1
Modifier concentration: 12% B ± 1%	High: 12.2% B	0.5	0.4	12.9	-1.1	0.1
	Low: 11.8% B	0.2	-1.0	12.8	-1.8	-1
Backpressure: 150 ± 2 bar	High : 152	-1.4	0.0	12.8	-6.7	-0.02
	Low : 148	-1.9	-0.7	12.9	-6.7	-0.9

Table 7
SFC robustness test results. The red values in the table indicate that the deviations exceeding the allowed limits of 5% for area and 3% for retention time.

Conclusion

The 1260 Infinity Hybrid SFC/UHPLC System was used to develop a novel SFC tolazamide assay method. This method was then compared to the original USP normal phase method. While meeting the system suitability requirements, the new SFC method was 4× faster and 19× less expensive than the normal phase method. The peaks obtained by SFC method were narrower and taller compared to USP method. The low UV cut off of SFC modifiers and sample diluents allow for greater flexibility to choose appropriate UV regions with the SFC method, which can lead to increased sensitivity as shown in this example. Robustness test results were excellent for the SFC method. Additionally, the SFC method does not require the purchase and disposal of expensive environmentally hazardous chemicals. Hence, the newly developed tolazamide SFC method provides a fast, cost effective, safe, and sensitive solution compared to normal phase method.

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