Mixed-Mode, Weak Anion-Exchange, Solid-Phase Extraction Method for the Extraction of Niflumic Acid from Human Plasma

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Application Note 20789

Key Words
SOLA WAX, Accucore, mixed mode, weak anion exchange, reproducibility, matrix clean up, SPE

Abstract
An HPLC-MS/MS method has been developed for the determination of niflumic acid in human plasma. This application note demonstrates the use of Thermo Scientific™ SOLA™ WAX SPE products, which allow mixed-mode extractions to be carried out with strong acids, giving improved removal of matrix interferences and reproducible results. The use of a Thermo Scientific™ Accucore™ HPLC column provided fast and efficient separation without the need for an ultra high pressure system. MS/MS detection was performed on a Thermo Scientific™ TSQ Vantage™ mass spectrometer.

Introduction

Niflumic acid is a drug used for treatment of joint and muscular pain. It is categorized as an inhibitor of cyclooxygenase-2. In experimental biology, it has been employed to inhibit chloride channels. It has also been reported to act on GABA and NMDA channels and to block T-type calcium channels.

Niflumic acid is a strong acid that remains ionized across the whole pH range. As a result, mixed-mode, strong anion exchange solid-phase extraction (SPE), which is often the first choice for clean reproducible extraction of acidic compounds, can be challenging as it is not possible to disrupt the ion exchange mechanism by pH adjustment. Extractions can be achieved by using salts as a competitive counter ion; however, the high concentration of salts in the elution solvent can be undesirable particularly for LC-MS/MS analysis. This limits options for extraction to reversed-phase SPE, which can be difficult due to the polar nature and therefore weak retention of many strong acids. Alternative approaches to SPE such as protein precipitation can achieve high recovery of the compounds of interest. However, these approaches tend to exhibit poor reproducibility and significant modification of ionization compared to SPE as they do little to remove endogenous interferences and minimize matrix effects.

SOLA WAX is a mixed-mode, polymeric, weak anion-exchange product that introduces additional selectivity into the SOLA SPE range. SOLA WAX polymeric SPE utilizes a hydrophobic backbone functionalized with a primary amine with a pKa of approximately 6. This allows users to control the ionization of the stationary phase by modifying the pH. As a result, it is possible to develop simple and effective SPE methods for strong acids that take advantage of the additional matrix cleanliness mixed-mode extractions can offer and the reproducibility benefits of SOLA SPE. SOLA solid phase extraction products introduce next generation, innovative technological advancements, giving unparalleled performance characteristics compared to conventional SPE, phospholipid, and protein precipitation products.

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These include:

- Higher levels of reproducibility
- Reduced sample and solvent requirements
- Higher levels of extract cleanliness
- Increased sensitivity

SOLA SPE plates or cartridges provide significant advantages when analyzing compounds in complex matrices, particularly in high-throughput bioanalytical and clinical research laboratories where reduced failure rates, higher analysis speed, and lower sample/solvent requirements are critical. SOLA products' superior performance gives higher confidence in analytical results and lowers cost without compromising ease-of-use or requiring complex method development.

Accucore HPLC columns use Core Enhanced Technology™ to facilitate fast and highly efficient separations. The 2.6 µm diameter particles are not totally porous, but instead have a solid core and a porous outer layer. The optimized phase bonding creates a series of high coverage, robust phases. Accucore RP-MS uses an optimized alkyl chain length for more effective coverage of the silica surface. This coverage results in a significant reduction in secondary interactions and yields highly efficient peaks with very low tailing. The tightly controlled 2.6 µm diameter of Accucore particles results in much lower backpressures than typically seen with sub-2 µm materials.

Experimental Details

Consumables	Part Number
Fisher Scientific™ LC/MS grade water	W/011217
Fisher Scientific LC/MS grade methanol	M/4062/17
Fisher Scientific analytical grade formic acid	F/1900/PB08

Sample Handling Equipment	Part Number
Thermo Scientific™ HyperSep™ glass block manifold 24 port	60104-233
Stopcocks for 24 port manifold	60104-244
Vacuum pump	60104-241
Thermo Scientific™ Chromacol™ WebSeal™ UltraVap™ High Speed Sample Concentrator	CLS-229070
Thermo Scientific™ eVol™ Sample Dispensing System	66002-024
Thermo Scientific™ Finnpipette™ F2 pipettor kit	PMP-020-220F

Vials and Closures	Part Number
Wide Open Short Screw Thermo Scientific™ Chromacol™ SureStop™ Vial, Clear with ID Patch, GOLD Grade and Red PTFE/White Silicone Septum, Convenience kit	2-SVWGKST-CPK

Sample Pretreatment
100 µL of human plasma diluted 1:1 with 2% phosphoric acid

Sample Preparation		Part Number
Compound(s):	Niflumic Acid, niflumic acid D3 (IS)	
Matrix:	Human plasma	
SPE:	SOLA WAX 10 mg 1 mL cartridge	60109-005
Conditioning stage:	500 µL methanol	
	500 µL water	
Application stage:	Load sample at 0.5 mL/min	
Washing stage:	500 µL 25 mM ammonium acetate (NH_4+CH_3COO-) buffer in water	
	500 µL methanol	
Elution stage:	500 µL methanol with 2% ammonium hydroxide (NH_4OH) at 0.5 mL/min Dry under nitrogen and reconstitute in 100 µL 2% formic acid in water	

Separation Conditions		Part Number
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 LC system	
Column:	Accucore RP-MS 2.6 µm, 50 × 2.1 mm	17626-052130
Guard column:	Thermo Scientific™ Accucore™ RP-MS Defender™	17626-012105
	Thermo Scientific™ Uniguard™ drop-in guard holder	852-00
Flow rate:	750 µL/min	
Run time:	3 min	
Column temperature:	30 °C	
Injection details:	2 µL full loop injection	
Injection wash solvent 1:	Water	
Injection wash solvent 2:	IPA / acetonitrile / acetone (45:45:10, v/v/v)	
Mobile phase A:	Water + 0.1% formic acid	
Mobile phase B:	Methanol + 0.1% formic acid	
Gradient conditions:	Table 1	

Time (min)	%A	%B
0.0	60	40
2.0	0	100
2.1	60	40
3.0	60	4

Table 1: Gradient conditions

MS Conditions	
Instrumentation:	TSQ Vantage Mass Spectrometer
Ionization conditions:	HESI
Polarity:	Positive
Spray voltage:	3000 V
Vaporiser temperature:	475 °C
Sheath gas pressure:	50 arb
Aux gas pressure:	60 arb
Capillary temp:	300 °C
Collision pressure:	1.5 mTorr
Scan time:	0.02 s
Q1:	0.7 FWHM
Q3:	0.7 FWHM

Compound transition details are provided in Table 2.

Compound	Parent *(m/z)*	S-Lens (V)	Product *(m/z)*	Collision Energy (V)
Niflumic acid	283	78	265.0	21
Niflumic acid D3 (IS)	288	90	270.1	22

Table 2: Compound transition details

Data Processing	
Software:	Thermo Scientific™ LCQUAN™ software version 2.6

Results

Niflumic acid standards, extracted from human plasma, gave a linear calibration curve over the dynamic range of 1 to 1000 ng/mL with an R^2 coefficient of 0.999 (Figure 1 and Table 3). The chromatography of the LCQ sample at 3 ng/mL is shown in Figures 2 and 3. QC samples were analyzed at concentrations of 3, 15, 600, and 800 ng/mL (Table 3). Overspikes (post-extraction fortified blank samples) were analyzed at a concentration of 3 ng/mL and used to calculate the percentage recovery level of niflumic acid at 86.6 % (Table 4).

Replicate extractions (n= 20) were also made at the 3 ng/mL level to assess the reproducibility of the assay. The % CV was calculated at 6.3% for niflumic acid with no internal standard correction and 4.3% when calculated using the response ratio of the internal standard (Table 5). This data shows that the extraction is reproducible even without the addition of an internal standard. The reproducibility of the assay is significantly better than values quoted in literature for a protein precipitation extraction for a 10 ng/mL sample (13.5% n=6).[1]

Matrix effects for niflumic acid were calculated at 7.96% (Table 6) compared to 94% quoted for a protein precipitation method.[1] This highlights the improved interference removal and reduction of matrix effects achieved with a mixed-mode SPE extraction.

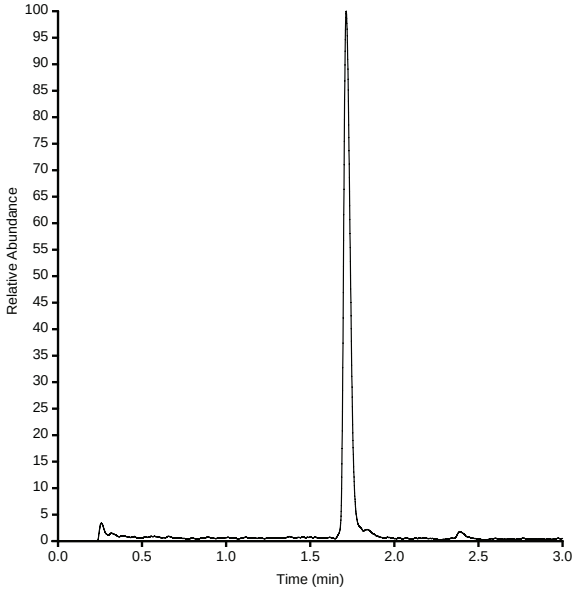

Figure 2. Example chromatogram of 3 ng/mL niflumic acid

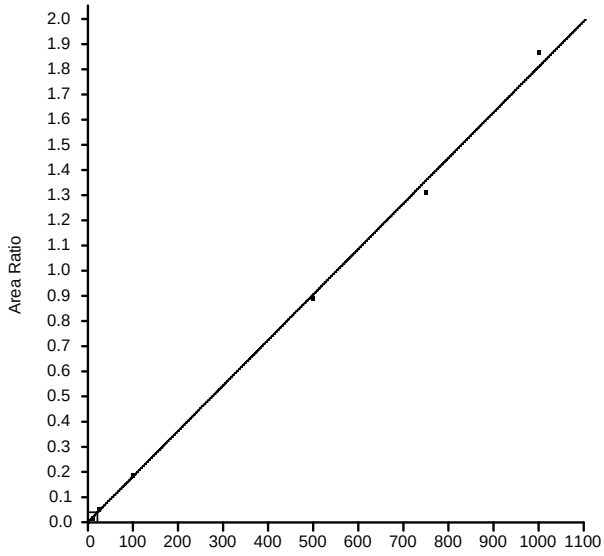

Figure 3: Niflumic acid linearity over the dynamic range 1–1000 ng/mL

Standard	Specified Conc	Calculated Conc	% Diff
S1	1.0	0.940	-6
S2	10.0	9.80	-2
S3	25.0	27.0	8
S5	100.0	102	2
S6	500.0	491	-2
S7	750.0	724	-3
S8	1000.0	1030	3
QC L	15.0	16.2	8
QC M	600.0	609	2
QC H	800.0	826	3

Table 3: Accuracy data for the calibration range 1–1000 ng/mL

Standard	Response	Absolute Recovery
Average area response	199024	86.6%
Overspike area response	229655	

Table 4: Recovery for niflumic acid at 3 ng/mL

Standard	Response	Matrix Effect
Standard area response	212708	7.9%
Overspike area response	229655	

Table 6. Matrix effects at 3 ng/mL

Compound	% CV (n=20)
Niflumic acid	6.3%
Niflumic acid D3	7.4%
Response ratio	4.3%

Table 5: Precision data at 3 ng/mL (n=20)

Conclusion

- SOLA WAX chemistry allows for the fast and easy extraction and quantification of niflumic acid from human plasma.
- Extraction recovery was 86.6%.
- SOLA WAX gave excellent precision for the extraction with % CV (n=20) less than 6.3% even without internal standard correction.
- SOLA WAX achieved low matrix suppression effects at less than 7.9%.
- Accucore RP-MS columns achieved a fast run time of less than 3 minutes.

Reference

[1] Kang, W., Determination of Talniflumate and Niflumic Acid in Human Plasma by Liquid Chromatography–Tandem Mass Spectrometry, *Analytical Science*, **2009**, 2, 571 -574.

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