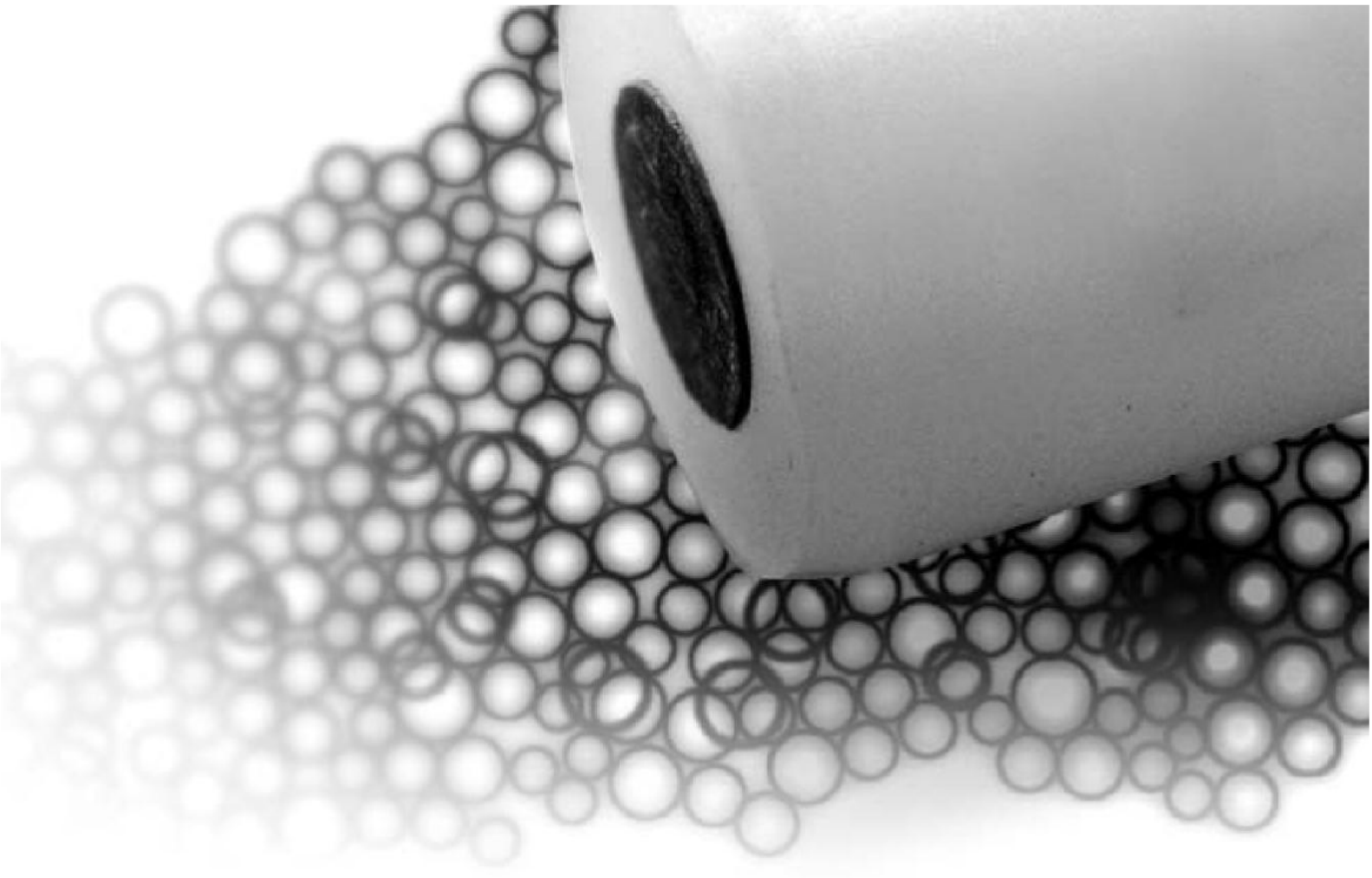


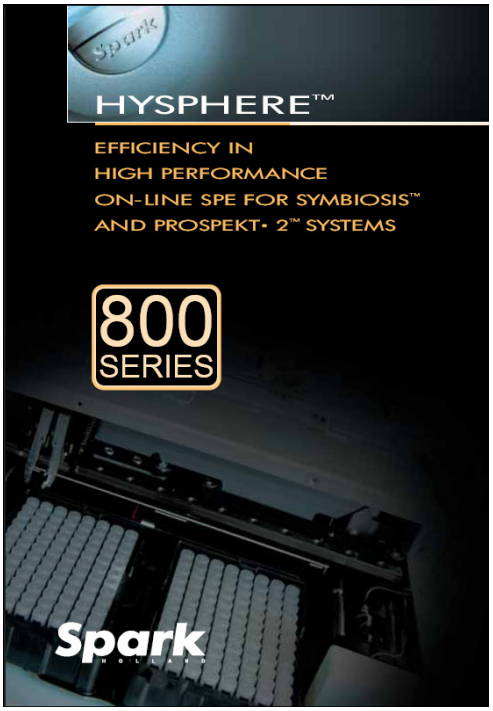
New HySphere™ mixed mode ion exchange Online SPE cartridges

cleaner extracts due to selective washing steps and higher extraction efficiency

Spark Holland, a pioneer in automated online SPE since 1992, is launching new Hysphere™ cartridges based on polymer mixed-mode ion exchangers. These cartridges are the newest members of the Hysphere™ family which starts from silica-based medium polar and non-polar sorbents to polymeric sorbents. Hysphere™ is a brand specially designed for the online SPE with Symbiosis™/ Prospekt-2™ systems that combines small particle sizes with optimized dimensions for high extraction capacity. Compared to traditional SPE sorbents, Hysphere™ sorbents need smaller elution volumes, resulting in higher analyte concentration and, consequently, higher assay sensitivity. Furthermore, the Symbiosis™ System enable a higher sample loading speed, reducing the cycle time.

Fast and highly efficient clean-up of acidic and basic drugs using the new Hysphere™ MM ion exchange cartridges

As the majority of extracts in bioanalytical applications are not clean enough, there is a need for introducing mixed-mode ion exchangers. Silica-based mixed-mode ion exchangers have been established in drug analysis since the late 80s. In general, they are available as strong acidic cation exchangers with sulfonyl groups and strong basic anion exchangers with quaternary amine function, mixed with a hydrophobic C18 or C8 sorbent.
So, what are the advantages of polymer-based mixed-mode exchangers in drug metabolism, ADME, and forensic laboratories in comparison with other sorbents?
Unlike Silica-based mixed-mode ion exchangers, polymer-based mixed-mode exchangers are not blended. This means that recovery and reproducibility problems are minimized. In addition, compared to traditional silica mixed-mode phases, the polymer-based exchangers do not contain free silanols that may complicate the retention mechanism. Furthermore, the analyte capacity is higher.



700 series:
- 0722.612 HySphere™ MM anion
- 0722.613 HySphere™ MM cation

800 Series:
- 0822.612 HySphere™ MM anion
- 0822.613 HySphere™ MM cation

Physical properties:

Cartridge
- Dimension: 2x10 mm
- Packing: 15mg/cartridge
- Maximum pressure: 300 bar

Sorbent media
- Polymer bases: DVB Gel
- Particle size: 10 um
- Pore size: 100 A
- Chemical stability: pH 1-14

HySphere™ MM cation for Basic compounds

For an efficient clean-up of basic drugs, mixed-mode cation exchanger cartridges are the best choice. Under acidic conditions, strong retention of basic compounds will occur. The mixed-mode mechanism also allows the use of different washes at different pH levels and organic concentrations, thus eliminating neutral or acidic impurities from the biological sample.

Typical SPE protocol:

- **Solvation:**
  1mL ACN
- **Equilibration:**
  1mL 20% ACN in 1% FA
- **Sample loading:**
  1mL 20% ACN in 1% FA
- **Wash 1:**
  1mL 20% ACN in 1% FA
- **Wash 2:**
  1mL 30% ACN in 2% NH4OH
- **Wash 3**
  1mL 90% ACN in 1% FA
- **Elute:**
  1mL 50% MeOH in 2% NH4OH

HySphere™ MM anion for Acidic compounds

Strong basic mixed-mode anion exchangers have a unique selectivity for retention of organic acids or biomolecules from biological and aqueous matrices. Here, the same protocol can successfully be applied. By performing a cycle of different strong organic wash steps at selected pH levels, very clean extracts will be achieved.

Typical SPE protocol:

- **Solvation:**
  1mL ACN
- **Equilibration:**
  1mL 20% ACN in 2% NH_4OH
- **Sample loading:**
  1mL 20% ACN in 2% NH_4OH
- **Wash 1:**
  1mL 20% ACN in 2% NH_4OH
- **Wash 2:**
  1mL 90% ACN in 2% NH_4OH
- **Wash 3**
  1mL 20% ACN in 1% FA
- **Elute:**
  1mL 90% MeOH in 1% FA

Take Home Message:

The two new mixed-mode cartridges are specially developed for selective removal of interferences in analytical biological samples and for retaining and concentrating acidic and basic drugs, with recoveries greater than 90%. By using a combination of different wash steps, interferences are removed and thereby optimizing the clean-up procedure.

Determination of Clofibric Acid in Serum by XLC-MS using Symbiosis™ Pharma and the new Mixed-Mode Anion Cartridge

Introduction

Symbiosis™ Pharma is Spark Holland's unique solution for integrated online SPE-LC-MS automation (XLC-MS). The system offers large flexibility in processing different types of samples, by selecting one of the three fully automated operational modes:

- MS (single LC run)
- XLC-MS (with online SPE)
- AMD (Advanced Method Development)

This application note describes the protocol for analyzing Clofibric Acid in serum by using mixed-mode anion exchange cartridges. With reversed phase and ion exchange washing steps, optimum recovery and highest cleaning efficiency have been achieved.

Clofibric Acid is a plant growth regulator and acts as an antiauxin. The official name is 2-(4-chlorophenoxy)-2-methylpropanoic acid. Furthermore, Clofibric Acid is the intermediate to produce clofibrate, an anti-hyperlipidemic agent to reduce elevated serum lipids.

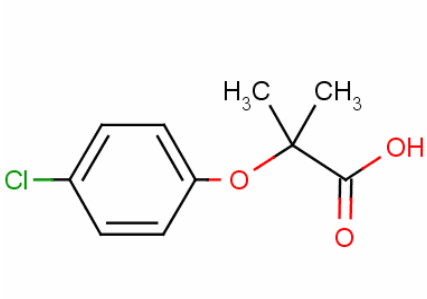


Figure 1: Clofibric Acid

- CAS# 882-09-7
- $C_{10}H_{11}ClO_3$
- Mw 214.65 g/mol
- Physical properties:
  pka dissociation constant: 3.5
  logP (octanol-water): 2.6

Mixed-Mode Anion Exchange Cartridge

Spark's mixed-mode anion exchanger has a strong anion exchange group uniformly bonded on the polymeric surface. The strong basic anion exchanger site provides strong retention of acidic compounds, thereby enabling the use of reversed phase and anionic washing steps for removal of interferences, and improving cleanliness of the extract.
In aqueous solution, Clofibric Acid can have two forms: neutral and charged, depending on the pH of the sample solution. At the pKa both forms are equally represented, i.e. at pH 3.5 Clofibric Acid is 50% free acid and 50% salt. Using an anion exchange cartridge, best recovery and very clean extracts are achieved with a reversed phase wash at low pH and an anion exchange wash step at high pH. SPE must be performed at a pH that is at least 2 pH units from the pKa of the functional group in order to ensure that 99.5 % of the molecules will be in the desired form.

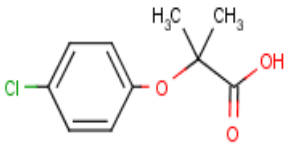


Figure 2a: Neutral form

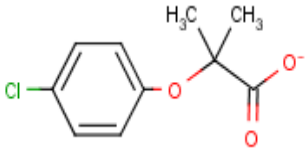


Figure 2b: Deprotonated form

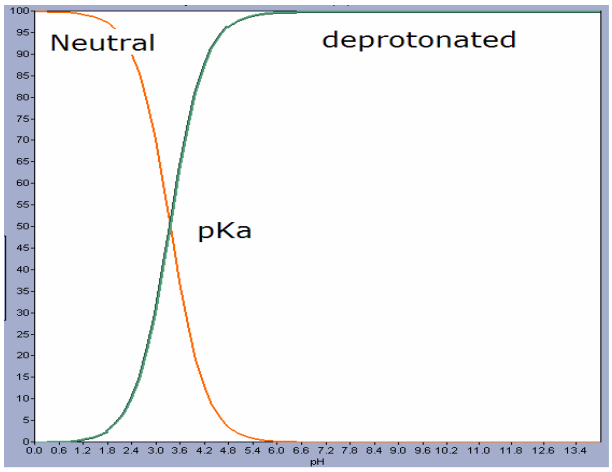


Figure 3: Deprotonated and neutral forms of Clofibric Acid at different pH levels

XLC-MS Protocol

The serum samples are processed with the developed XLC-MS method (as described below) using a Symbiosis™ Pharma and a Sciex API 3000 system (Tables 1-4).

The XLC-MS method contains a protocol for:
- the autosampler (injection and wash routine)
- the online SPE (extraction and clean-up)
- the LC gradient
- MS settings

Figure 4: Symbiosis™ Pharma system

Autosampler Conditions

50 µL of serum is injected using the default autosampler configuration.

Washing is performed with two solvents:
Wash solvent 1: 50% ACN in 0.1% FA
Wash solvent 2: 90 % ACN

Table 1: Wash routine autosampler

Wash solvent	Wash volume
1	700 µL
2	700 µL
1	1500 µL

SPE conditions

Table 2: SPE settings

Cartridge:	10 x 2 mm HySphere™ mixed-mode anion Spark PN 0722.612	
Solvation:	1 mL ACN	5 mL/min
Equilibration:	1 mL 20% ACN in 2 % NH4OH	5 mL/min
Sample Loading:	1 mL 20% ACN in 2 % NH4OH	2 mL/min
Wash 1:	1 mL 20% ACN in 2 % NH4OH	5 mL/min
Wash 2:	1 mL 90% ACN in 2 % NH4OH	5 mL/min
Wash 3:	1 mL 20% ACN in 1 % FA	5 mL/min
Elution:	400 µL 90% MeOH in 1% FA	200 µL/min
Matrix:	Serum	

Elution of the cartridge is performed with an HPD focusing step. More information on HPD focusing can be found in the application note "Determination of Salbutamol in serum by XLC-MS/MS using Symbiosis™ Pharma".

LC conditions

Column:	Waters Xterra MS C18 2.1 mm x 50 mm
Mobile phase A:	0.2% formic acid in water
Mobile phase B:	0.2% formic acid in ACN

Table 3: LC gradient

Time (min:s)	Flow (mL/min)	A (%)	B (%)
00:00:01	0.5	95	5
00:02:01	0.5	95	5
00:02:05	0.6	95	5
00:03:35	0.6	10	90
00:04:00	0.6	10	90
00:04:12	0.6	95	5
00:05:00	0.6	95	5

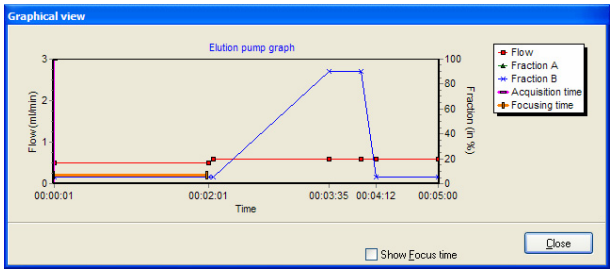


Figure 5: LC pump gradient

A 1 to 2 split ratio is used to allow a 200 µL/min flow entering into the MS.

MS Conditions

A Sciex API 3000 with a Turbo IonSpray in negative mode is used to analyze the samples.

Table 4: MS settings of Clofibric Acid

	Clofibric Acid		
Q1 mass	212.9	CUR	13
Q3 mass	126.8	IS	-4500
Dwell time	150	TEM	450
DP	-20	NEB	11
FP	-100	CAD	8
EP	-10		
CE	-18		
CXP	-7		

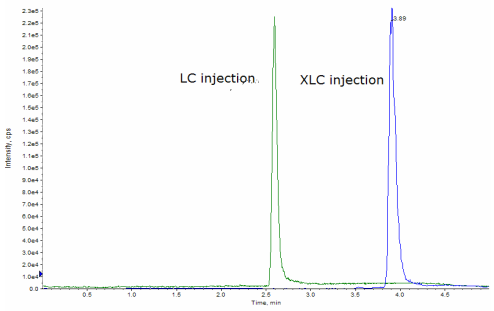


Figure 6: Overlay of different chromatograms: LC run of Clofibric Acid in aqueous solution and XLC run of a spiked serum sample using the mixed-mode anion exchanger.

Results

The mixed-mode anion exchanger offers hydrophobic as well as anionic interactions for the best clean-up of Clofibric Acid: the reversed phase and ion-exchange mechanisms. By simply washing with different organic concentrations at high and low pH, interferences are removed and cleaner extracts achieved.

The following study represents the results of different washings (Figures 7,8).

Load the sample onto the cartridge under basic conditions and wash the cartridge with ACN at low pH (1% formic acid) to investigate the reversed phase trapping mechanism.

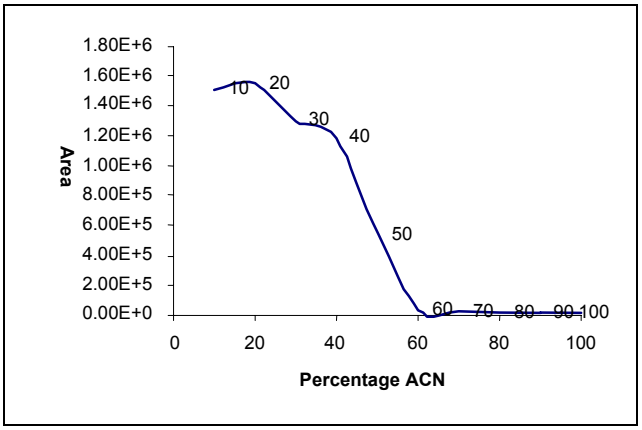


Figure 7: Effect of %ACN at low pH on the extraction recovery. Up to 20% ACN could be applied without significantly decreasing the recovery.

Load the sample onto the cartridge under basic conditions and wash the cartridge with ACN at high pH (2% NH4OH) to investigate the ion exchange trapping mechanism.

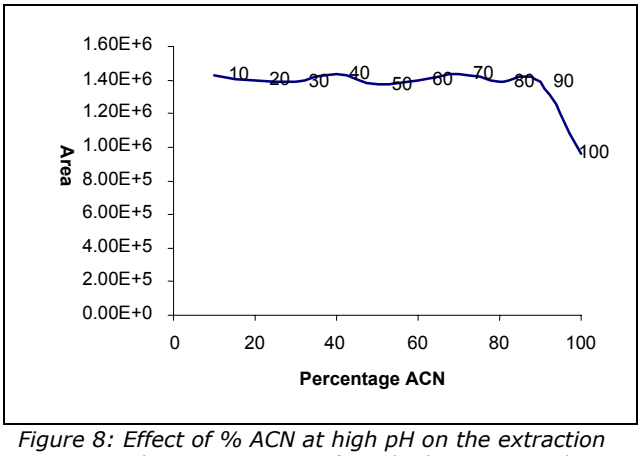


Figure 8: Effect of % ACN at high pH on the extraction recovery. The recovery significantly decreases with a 100% ACN wash step.

In the final method, after the initial wash, the cartridge is washed with 20% ACN in 1% formic acid and with 90% ACN with 2% NH4OH.

Samples

For the quantitative analysis, the following samples have been prepared by spiking standard stock solutions Clofibric Acid in new born calf serum. No protein precipitation has been performed

Figure 9 represents a chromatogram of a spiked sample in the upper limit of the calibration curve.
Calibration standards:1.0; 2.0; 5.0; 10; 20; 50; 100; 200; 500; 1000 ng/mL
QC samples: 1; 50; 800 ng/mL

Chromatograms

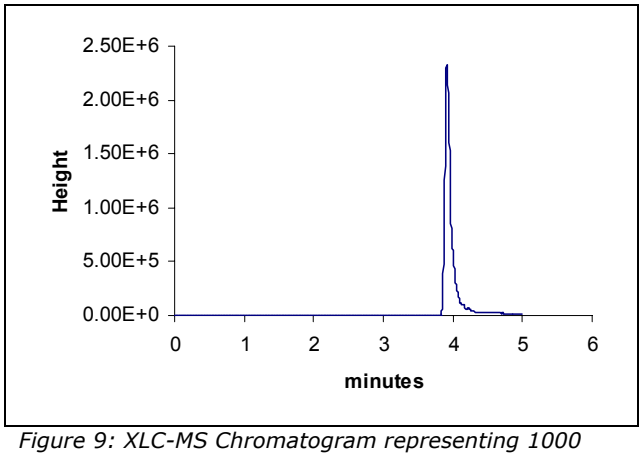


Figure 9: XLC-MS Chromatogram representing 1000 ng/mL Clofibric Acid in serum

In Figure 10, a chromatogram of the blank serum is given immediately after injection of the high standard solution. The carry-over is less than 10 % of LLOQ.

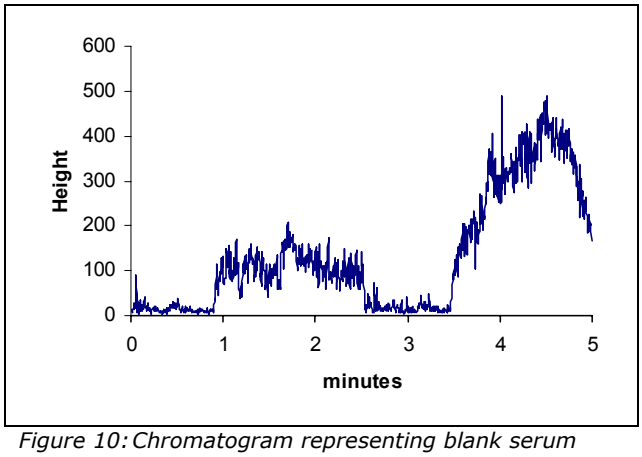


Figure 10: Chromatogram representing blank serum (less than 10% of LLOQ)

Linearity, Accuracy and Precision

A calibration curve was determined by combining the results of 4 repeated injections of a full set of calibration standards. This resulted in a R2 = 0.997 with a 1/X weighting.

Table 5: Accuracy and precision calculated from 4 combined sets of calibration standards

Exp. concentration (ng/mL)	CV (%)	Accuracy (%)
1	10.1	88.0
2	7.31	93.7
5	7.89	97.5
10	7.46	104
20	8.94	93.2
50	7.57	106
100	5.24	102
200	3.39	104
500	1.45	103
1000	5.69	94.6

Table 6: Accuracy and precision calculated from 12 combined sets of QC standards.

Exp. concentration (ng/mL)	CV (%)	Accuracy (%)
QC 1	12.6	85.3
QC 50	8.25	100
QC 800	8.75	97.5

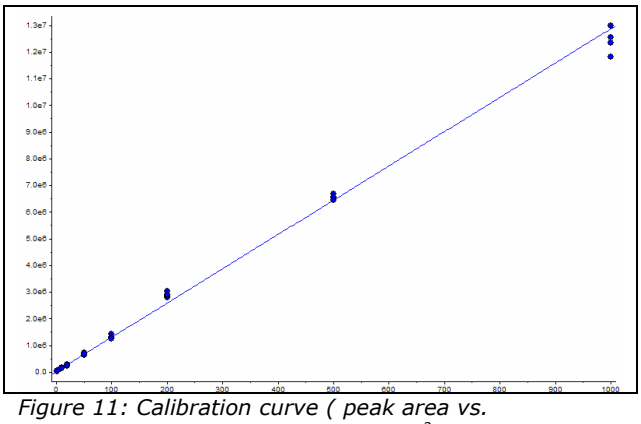


Figure 11: Calibration curve (peak area vs. concentration) of Clofibric Acid with R^2=0.997

Conclusions

From this study, it can be concluded that within a time frame of 2 days it is possible to develop an XLC-MS method with an absolute recovery >90%. Running a set of calibration standards yields a linear range of 1 to 1000 ng/mL (R^2 = 0.997), an accuracy of 88-106% and a precision of < 11%. This is achieved without the use of an internal standard. Carry-over is less than 10% of the LLOQ.
The total XLC-MS time, consisting of the sample preparation time and LC-MS runtime, is 6 minutes.

Spark's mixed-mode anion exchanger has a strong anion exchange group uniformly bonded on the polymeric surface unlike traditional silica-based mixed- mode sorbents. The strong basic anion exchanger site provides strong retention of acidic compounds, thereby enabling the use of reversed phase and anionic washing steps for removal of interferences and improving cleanliness of the extract.

Determination of Atenolol in Serum by XLC-MS using Symbiosis™ Pharma and the new Mixed-Mode Cation Cartridge

Introduction

Symbiosis™ Pharma is Spark Holland's unique solution for integrated online SPE-LC-MS automation (XLC-MS). The system offers large flexibility in processing different types of samples, by selecting one of the three fully automated operational modes:

- LC-MS (single LC run)
- XLC-MS (with online SPE)
- AMD (Advanced Method Development)

This application note describes the procedure for analyzing Atenolol in serum by using a mixed-mode cation exchange cartridge. With reversed phase and ion exchange wash steps, best recovery and highest cleaning efficiency have been achieved.
Within 2 days it was possible to develop an online XLC-MS method that generates good accuracy, precision and linearity over the calibration range.

Atenolol is a beta blocker and belongs to the class II of antiarrhythmic agents. The drug is primarily used for treatment of coronary heart diseases and hypertension.
Like other antihypertensive drugs, Atenolol lowers the systolic and diastolic blood pressure by 15 to 20% in a single drug administration.

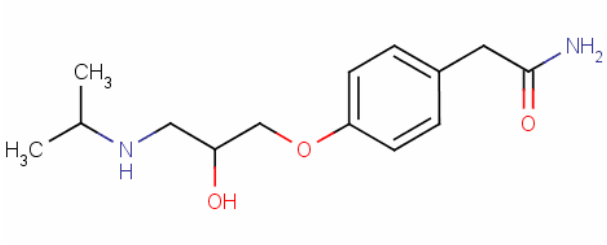


Figure 1: Atenolol

- CAS# 29122-68-7
- $C_{14}H_{22}N_2O_3$
- Mw 266.34 g/mol
- Physical properties:
  Water solubility (37°C): 26.5 g/L
  pKa dissociation constant: 9.9
  Log P (octanol-water): 0.56

Atenolol can also be considered for the therapy of supraventricular and selected cases of ventricular arrhythmias.
It can be combined with diuretics, vasodilators, ACE inhibitors, and other cardiac drugs.

Mixed-mode cation exchange cartridge

In aqueous solution, Atenolol can have two forms: a neutral or a charged form, depending on the pH of the sample solution. At the pKa both forms are equally represented, i.e. at pH 9.9 Atenolol is 50% neutral and 50% protonated. With Spark's new mixed-mode cation exchange cartridge, a reversed phase wash at high pH and a cation ion-exchange wash at low pH are possible, thereby achieving cleaner extracts. SPE must be performed at a pH that is at least 2 pH units from the pKa of the functional group in order to ensure that 99.5 % of the molecules will be in the desired form.

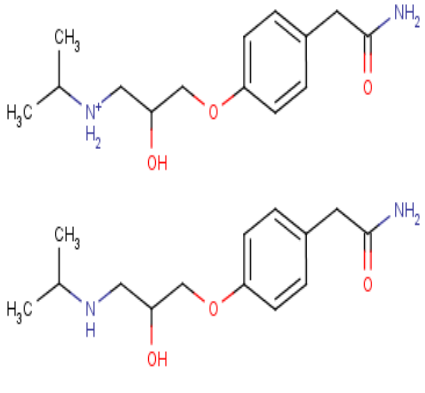


Figure 2: Protonated and neutral forms of Atenolol

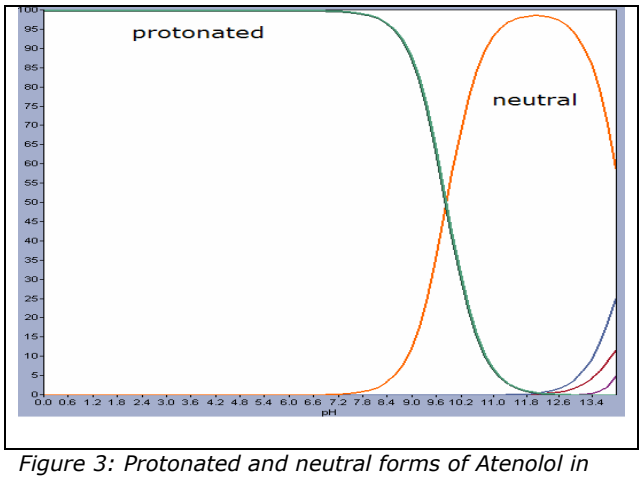


Figure 3: Protonated and neutral forms of Atenolol in dependence on the pH

XLC-MS Protocol

The serum samples are processed with the developed XLC-MS method (as described below) using a Symbiosis Pharma™ and a Sciex API 3000 system *(Table 1-4).*

Figure 4: Symbiosis™ Pharma System

The XLC-MS method contains a protocol for:

- the autosampler
- the online SPE
- the LC gradient
- MS settings

Autosampler Conditions

50 µL of serum is injected using the default autosampler configuration.
Washing is performed with two solvents:
Wash solvent 1: 50% ACN in 0.1% FA
Wash solvent 2: 90 % ACN

Table 1: wash routine autosampler

Wash solvent	Wash volume
1	700 µL
2	700 µL
1	1500 µL

SPE conditions

Table 2: SPE settings

Cartridge:	10 x 2 mm HySphere™ Mixed Mode Cation Spark PN 0722.613	
Solvation:	1 mL ACN	5 mL/min
Equilibration:	1 mL 20% ACN in 1 % FA	5 mL/min
Sample Loading:	1 mL 20% ACN in 1 % FA	2 mL/min
Wash 1:	1 mL 20% ACN in 1 % FA	5 mL/min
Wash 2:	1 mL 30% ACN in 2 % NH4OH	5 mL/min
Wash 3:	1 mL 90% ACN in 1 % FA	5 mL/min
Elution	300 µL 50% MeOH in 2% NH4OH	100 µL/min
Matrix:	Serum	

Elution of the cartridge is performed with an HPD focusing step.

LC Conditions

Column: Waters Xterra MS C18 4.6 mm x 50 mm
Mobile phase A: 0.2% formic acid in water
Mobile phase B: 0.2% formic acid in ACN

Table 3: LC conditions; A 1 to 3 split ratio is used to allow a 250 µL/min flow entering into the MS.

Time (min:s)	Flow (mL/min)	A (%)	B (%)
00:00:01	0.9	100	0
00:03:01	0.9	100	0
00:03:05	1.0	95	5
00:05:05	1.0	95	5
00:07:05	1.0	20	80
00:07:10	1.0	20	80
00:07:30	1.0	100	0
00:09:00	1.0	100	0

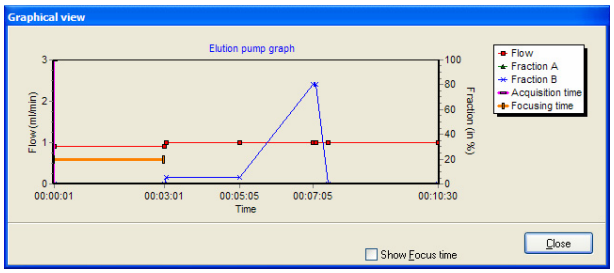


Figure 5: LC pump gradient

MS Conditions

A Sciex API 3000 with a Turbo IonSpray in positive mode is used to analyze the samples.

Table 4: MS settings of Atenolol

	Atenolol		
Q1 mass	267.15	CUR	10
Q3 mass	190.10	IS	5000
Dwell time	150	TEM	400
DP	31	NEB	15
FP	280	CAD	6
EP	10		
CE	27		
CXP	12		

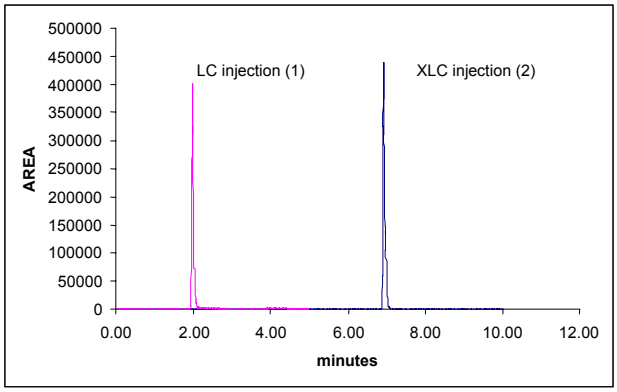


Figure 6: Overlay of different chromatograms:
(1) LC run of Atenolol in aqueous solution
(2) XLC run of a spiked serum sample using the mixed-mode cation exchanger. Recovery is > 90%.

Results

The mixed-mode cation exchanger offers hydrophobic as well as cationic interactions for the best clean-up of Atenolol: the reversed phase and ion-exchange mechanisms. By simply washing with different organic concentrations at high and low pH, interferences are removed and cleaner extracts achieved.

The following protocol represents the results of these washings *(Figures 7-8)*:
After loading the sample onto the cartridge under acidic conditions and washing the cartridge with ACN at high pH (2% NH4OH), the reversed phase trapping mechanism is investigated.

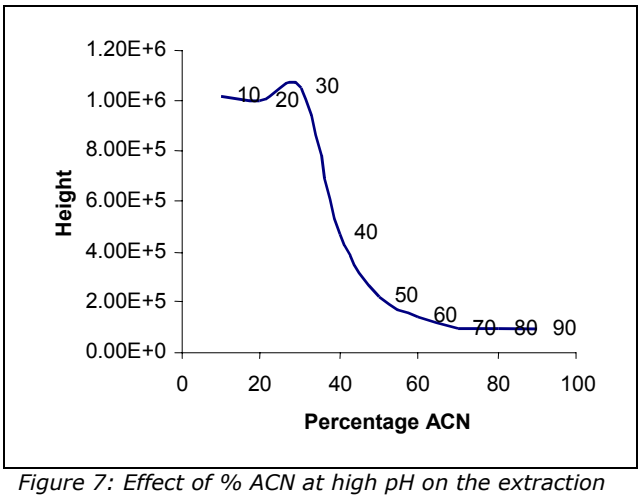


Figure 7: Effect of % ACN at high pH on the extraction recovery. Up to 30% ACN can be applied without significantly decreasing the recovery.

After loading the sample onto the cartridge under acidic conditions and washing the cartridge with ACN at low pH (1% formic acid), the ion exchange trapping mechanism is investigated.

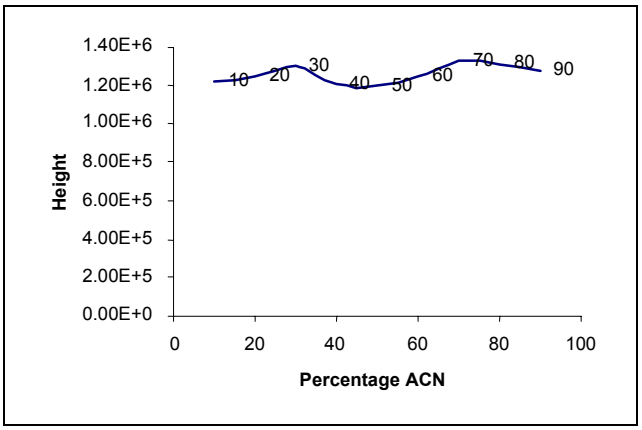


Figure 8: Effect of % ACN at low pH on the extraction recovery. Up to 90% ACN is possible without significantly decreasing the recovery.

In the final method, after the initial wash step, the cartridge is washed with 30% ACN in 2% NH4OH and with 90% ACN with 1% formic acid *(Figure 8).*

Serum interferences

Phospolipids can be used as biomarkers for SPE clean-up efficiency. In order to monitor the efficiency of the combined washing steps, the 496/184 mass of a phospholipid is added to the acquisition method of the MS.

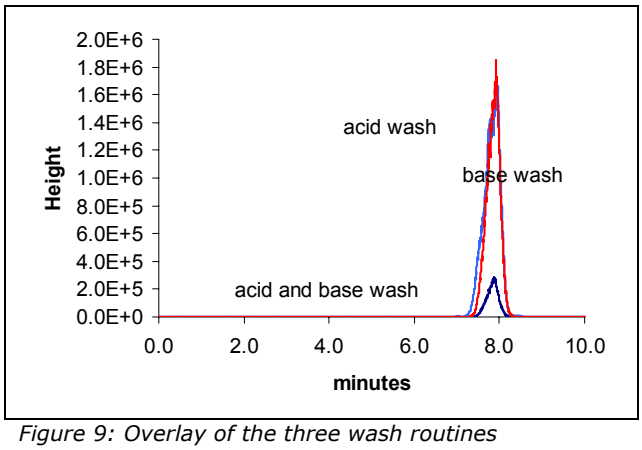


Figure 9: Overlay of the three wash routines

If the two washing steps at different pHs are combined, the amount of phospholipid retained on the cartridge is decreased by a factor of ten.

Samples

For the quantitative analysis, the following samples have been prepared by spiking standard solutions of Atenolol in new born calf serum. Figure 10 represents a chromatogram of a spiked sample in the upper limit of the concentration curve.

- Calibration standards:1.0; 2.0; 5.0; 10; 20; 50; 100; 200; 500; 1000 ng/mL
- QC samples: 1; 50; 800 ng/mL

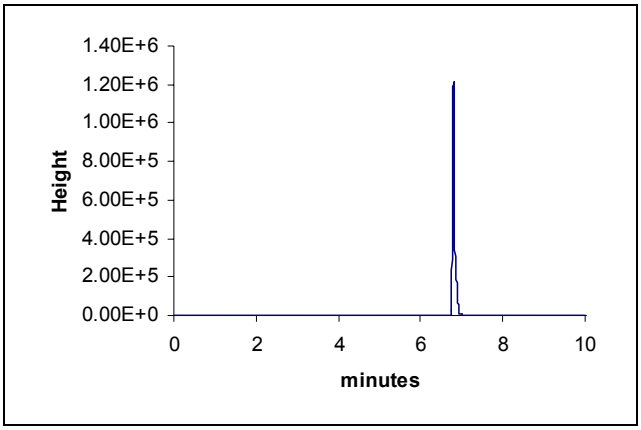


Figure 10: XLC-MS chromatogram representing 1000 ng/mL Atenolol in serum

Figure 11 shows a chromatogram of a blank serum - immediately after injection of the high standard solution. Carry-over is less than 20 % of LLOQ.

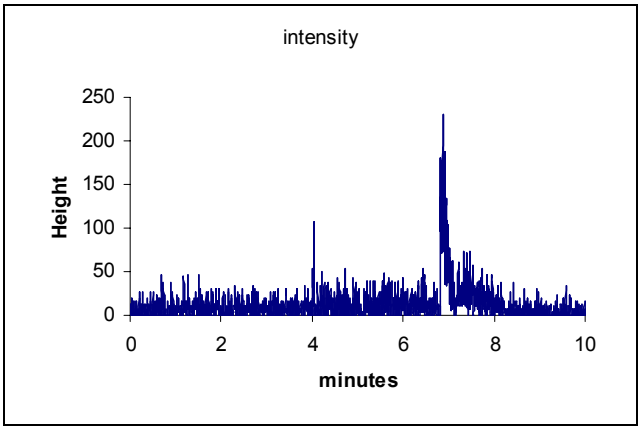


Figure 11:Chromatogram representing blank serum (less than 20% of LLOQ)

Linearity, Accuracy and Precision

A calibration curve was determined by combining the results of 3 repeated injections of a full set of calibration standards. This resulted in a **R^2 = 0.996** with a 1/X weighting.

Table 6: Accuracy and precision calculated from three combined sets of calibration standards

Exp. Conc. (ng/mL)	CV (%)	Accuracy (%)
1.00	10.7	110
2.00	6.91	92.4
5.00	6.97	106
10.00	5.65	106
20.00	0.87	107
50.00	3.41	103
100.0	9.53	108
200.0	1.40	106
500.0	2.18	98.4
1000	5.70	95.9

Table 7: Accuracy and precision calculated from nine combined sets of QC standards

Exp. Conc. (ng/mL)	CV (%)	Accuracy (%)
QC 1	10.7	110
QC 50	8.83	106
QC 800	9.05	98.4

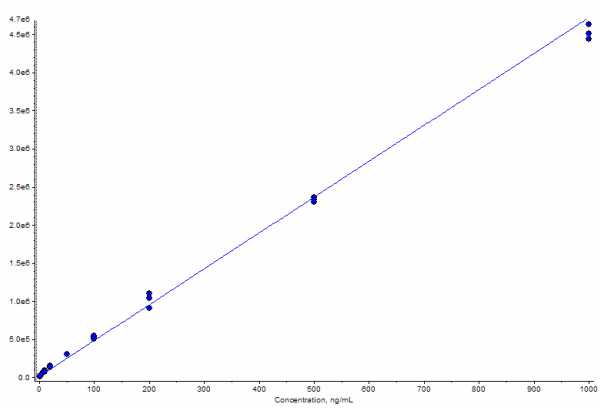


Figure 12: calibration curve (peak area - concentration) of Atenolol with R^2=0.996

Conclusions

From this study it can be concluded that within a time frame of 2 days it is possible to develop an XLC-MS method for Atenolol with an absolute recovery >90%. Running a set of calibration standards yields a linear range of 1 to 1000 ng/mL (R^2 = 0.996), an accuracy of 96-110% and a precision of < 11%. This is achieved without the use of an internal standard. Carry-over is less than 20% of the LLOQ.
The total XLC-MS time, consisting of the sample preparation time and LC-MS runtime, is 9 minutes.

Spark's mixed-mode cation exchanger has a strong cation exchange group uniformly bonded on the polymeric surface. The strong acidic cation exchanger site provides strong retention of basic compound, thereby enabling the use of reversed phase and cationic washing steps in order to improve cleanliness of the extract.

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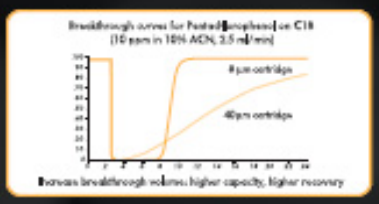
HYSPHERE™ SPE CARTRIDGES HAVE BEEN SPECIFICALLY DESIGNED FOR ON-LINE SOLID PHASE EXTRACTION. THE SORBENTS ARE EXCLUSIVELY DEVELOPED BY SPARK TO ENSURE OPTIMAL EFFICIENCY AND REPRODUCABILITY FOR A WIDE RANGE OF COMPOUNDS.
THE SMALL PARTICLE SIZES (UP TO 7 MICRON) GUARANTEE A SIGNIFICANT HIGHER EFFICIENCY COMPARED TO AVERAGE OFFLINE SORBENT PARTICLES.



SELECTION OF SORBENTS FOR THE HYSPHERE™ RANGE IS BASED ON PROVEN PERFORMANCE QUALITY FOR ON-LINE SPE AND GUARANTEED CONTROL OF PHYSICAL AND CHEMICAL CHARACTERISTICS SUCH AS PARTICLE SIZE DISTRIBUTION, ABSENCE OF FINES, BATCH-TO-BATCH REPRODUCIBILITY ETC. A CERTIFICATE OF ANALYSIS COMES WITH EVERY BOX OF CARTRIDGES; IT KEEPS YOU INFORMED ABOUT BATCH PERFORMANCE, IT KEEPS US COMMITTED TO DELIVER CONSTANT CARTRIDGE QUALITY!

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