APPLICATION INFO

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

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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, 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 how to analyze 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 treatment.
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.

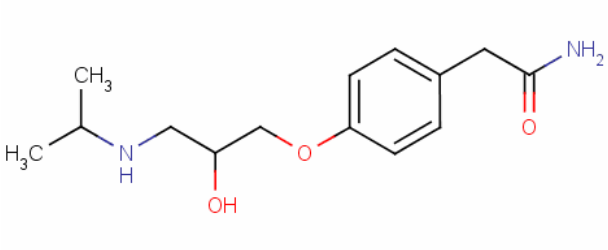
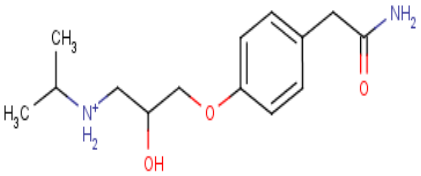


Figure 1: Atenolol

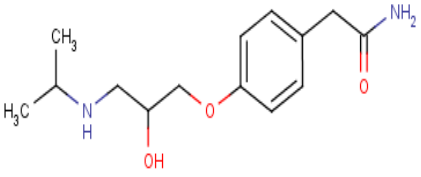
- CAS# 29122-68-7
- $C_{14}H_{22}N_2O_3$
- Mw 266.34 g/mol
- Physical properties:
  Water solubility (37°C): 26.5 g/L
  pKa dissociation constant: 9.9
  Log P (octanol-water): 0.56

Spark's mixed-mode cation exchange cartridge

In solution, Atenolol can have two forms; a neutral and 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.



Protonated form



Neutral form

Figure 2: Protonated and neutral forms of Atenolol

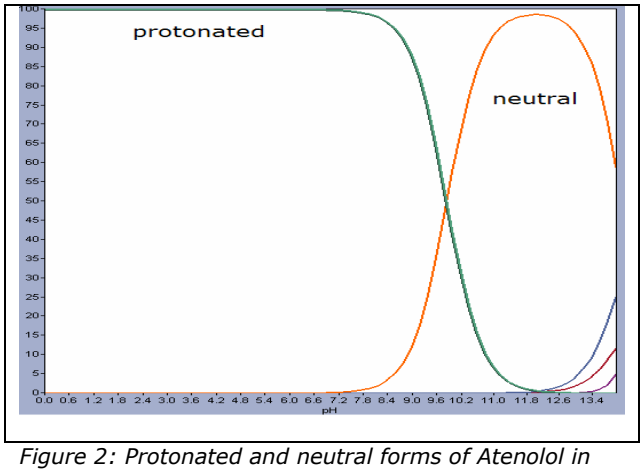


Figure 2: Protonated and neutral forms of Atenolol in dependence on the pH

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 3: Symbiosis™ Pharma System

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

Autosampler Conditions

50 µL of serum is injected using a standard autosampler configuration (which configuration)?.
Washing is performed with two solvents:
Wash solvent 1: 50% ACN in 0.1% formic acid
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)	
Solvation:	1 mL ACN	5 mL/min
Equilibration:	1 mL 20% ACN in 1 % formic acid	5 mL/min
Sample Loading:	1 mL 20% ACN in 1 % formic acid	2 mL/min
Wash 1:	1 mL 20% ACN in 1 % formic acid	5 mL/min
Wash 2:	1 mL 30% ACN in 2 % NH4OH	5 mL/min
Wash 3:	1 mL 90% ACN in 1 % formic acid	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***. More information on HPD focusing can be found in the application note "Determination of Salbutamol in serum by XLC-MS/MS using the Symbiosis Pharma".

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

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

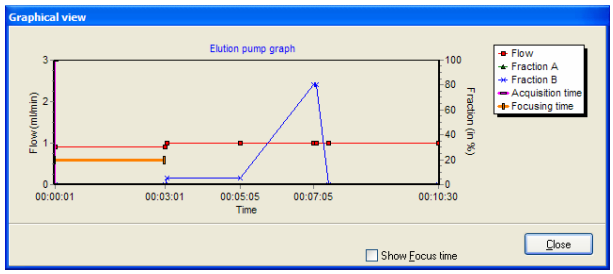


Figure 4: LC pump gradient

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

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		

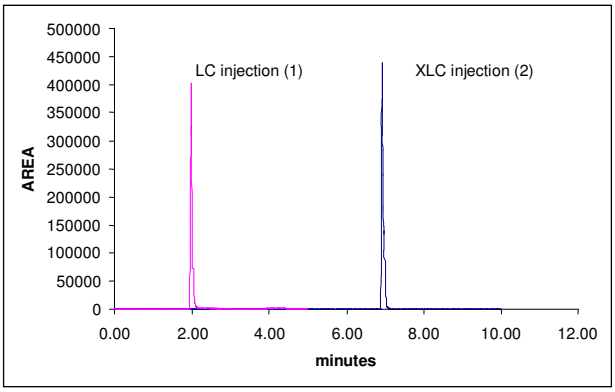


Figure 5: Overlay of different chromatograms:
(1) LC run of Atenolol in aqueous solution
(2) XLC run of a spiked serum sample using the mixed-mode cation exchanger. Recovery is > 90%.

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 *(Fig. 6-7)*:
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.

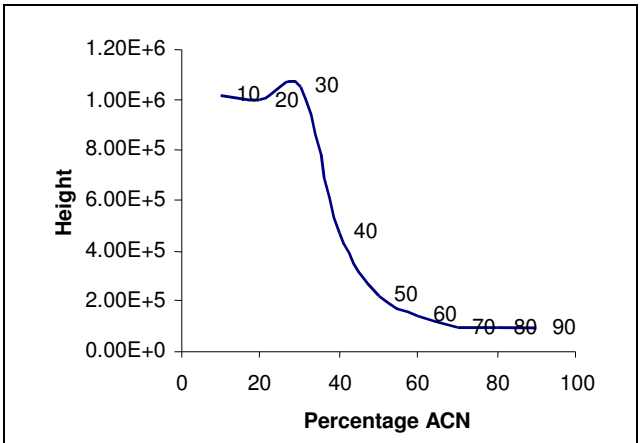


Figure 6: Effect of % ACN at high pH on the extraction recovery. Up to 30% ACN can be applied without significantly decreasing the recovery.

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.

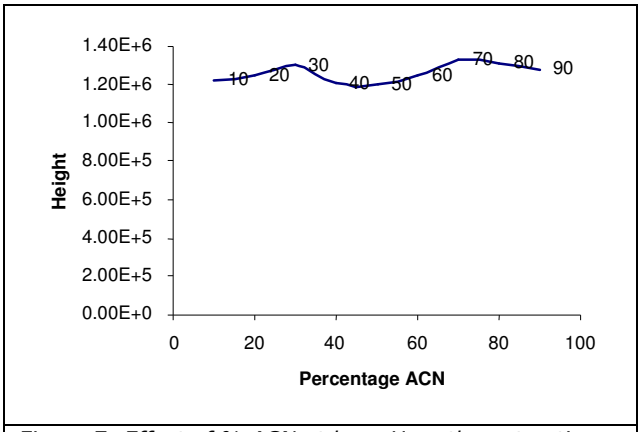


Figure 7: Effect of % ACN at low pH on the extraction recovery. Up to 90% ACN is possible without significantly decreasing the recovery.

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 *(Fig. 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.

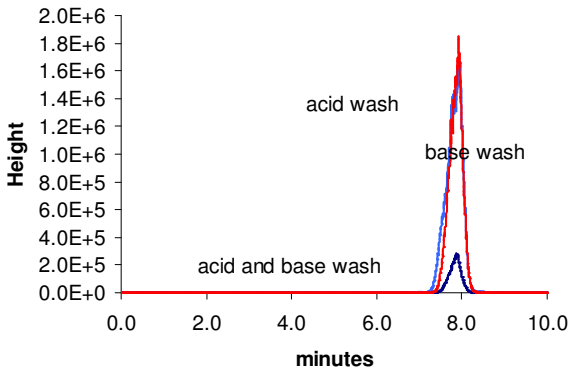


Figure 8: Overlay of the three wash routines

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 in new born calf serum. Samples contain 90 % serum (and what is the rest?). The standard stock solutions of Atenolol are made-up in water.
No protein precipitation has been performed (correct?).
Figure 9 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

Chromatograms

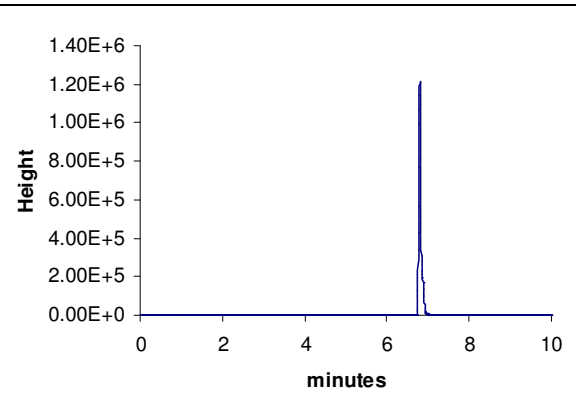


Figure 9: XLC-MS chromatogram representing 1000 ng/mL Atenolol in serum

Figure 10 shows a chromatogram of a blank serum - immediately after injection of the high standard solution. Carry-over is less than 20 % of LLOQ.

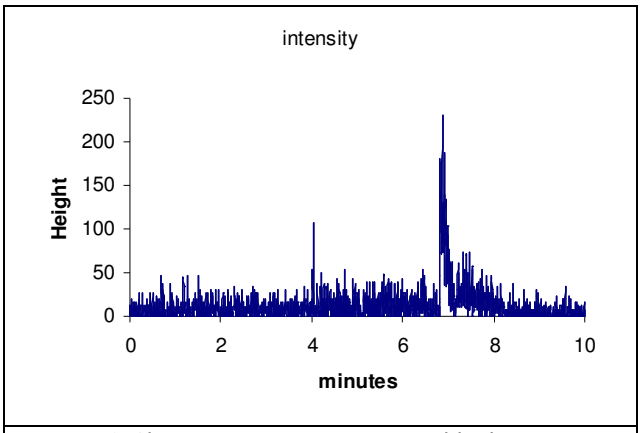


Figure 10:Chromatogram representing blank serum (less then 20% of LLOQ)

Linearity, Accuracy and Precision

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

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

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

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

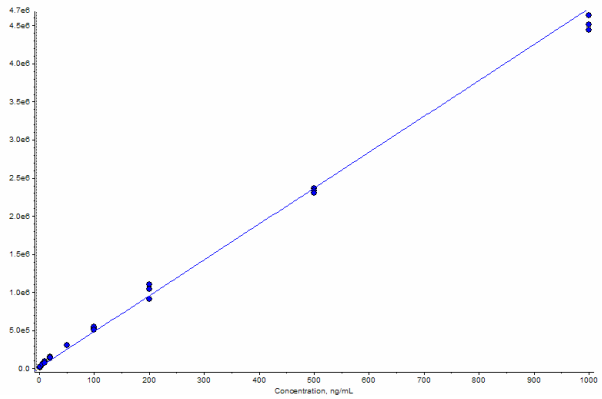


Figure 10: Calibration curve (peak area vs. concentration) of Atenolol with R^2=0.996

Conclusions

From this study it can be concluded that within a time frame of 2 days it is possible to develop a XLC-MS method for Atenolol with an absolute recovery >90%. Running a set of calibration standards yields a linear range from 1 to 1000 ng/mL (R^2 = 0.996), an accuracy between 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 consists of the sample preparation time + HPD focusing + LC-MS runtime. Since the sample prep + HPD focusing are executed in parallel with the LC, the total XLC-MS time is 9 minutes.

Mixed Mode Cation Exchanger

Spark's mixed-mode cation exchanger has a strong cation exchange group uniformly bonded on the polymeric surface unlike traditional silica-based mixed mode sorbents. 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.

About Spark

Since 1982 Spark has provided the HPLC and LC/MS markets with state-of-the-art autosamplers, column ovens and sample preparation solutions. Solid Phase Extraction with online elution into HPLC and LC/MS systems was pioneered by Spark and introduced in the early 90's. Spark, ISO 9001 certified, does basic research, product development, production, sales and marketing in-house, guaranteeing quality from start to finish. With 25% of the employees working in research and development, Spark continues to invest in the future, making sure delivering the solutions you need to improve your business results. Innovation and quality are the keywords when talking about our development efforts.

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