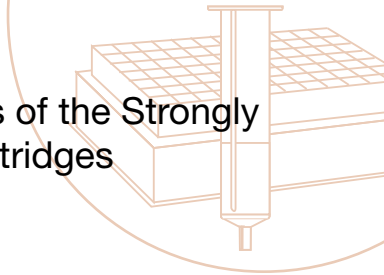


Three Minutes for the Extraction, Aggressive Cleanup and Analysis of the Strongly Polar Drug Acyclovir from Plasma using strata™ X On-line SPE Cartridges

Scott Waite, Art Dixon and Krishna Kallury
Phenomenex Inc., Torrance, CA, USA

Acyclovir (ACV, Zovirax) is an important antiviral drug, used extensively for treatment of herpes simplex and Varicella zoster in pregnant women. It is a synthetic purine nucleoside analogue derived from guanine, with a chemical structure of 9-(2-hydroxyethoxy)-methylguanine. It is a strongly polar molecule, with an octanol/water partition coefficient (log P) value of -1.56 and has a water solubility of 1.3 mg/mL at 25°C. It exhibits plasma protein binding levels of 9 to 33% and its bioavailability is around 15-30%. The physical properties of Acyclovir present a significant challenge for analytical method development from biological fluids, making it one of the "difficult-to-analyze" types of drugs.

With an aim to improve analytical sensitivity through more efficient removal of matrix components, off-line solid phase extraction (SPE) is commonly used for sample pretreatment of drugs in plasma samples. When silica-based C18 sorbents are used, an initial protein precipitation step involving treatment with perchloric acid has been frequently used.[1] After loading the sample recovered from the protein precipitation step on to the SPE tube, the wash step was performed with an aqueous solution and elution was effected with methanol. Due to the polarity of Acyclovir, only an aqueous wash condition was used. The resulting extracts showed considerable matrix constituents, some of which interfered with the HPLC peak of the drug. An alternative sample preparation method used Oasis® HLB from Waters. In this protocol, washing was done with water, followed by elution with 5% acetonitrile adjusted to pH 3.0 with phosphoric acid.[2] This low-percent organic in the elution solvent was designed to minimize the coelution of hydrophobic matrix components from the SPE sorbent, which would occur if stronger organic were used. These off-line SPE strategies are time-consuming and laborious while offering minimal removal of interfering impurities from the sample.

Here, we demonstrate a novel method for the rapid extraction and aggressive cleanup of Acyclovir plasma using an on-line strata-X cartridge. This method simplifies sample handling, completely eliminating the dry down step typically employed between SPE and LC analysis, offers aggressive cleanup of matrix components without impacting recoveries and reduces the time for sample preparation and analysis to three minutes.

Experimental

Materials

Acyclovir and Porcine Plasma were obtained from Sigma (St. Louis, MO). Lyophilized porcine plasma diluted 1:1 with water was spiked with the appropriate concentration of Acyclovir.

On-line SPE cartridge

An on-line SPE cartridge (20x2.0mm) packed with 25µm sorbent was used for the solid phase extraction of the polar drug probe. strata-X is a revolutionary, patent-pending, polar, functionalized, polymeric sorbent that is capable of exhibiting π-π, dipolar and hydrogen bonding retention mechanisms.

HILIC-MS Analysis

HILIC-MS analysis was carried out using an HP 1100 LC system (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump (1), binary pump (2), in-line degasser, DAD detector, autosampler coupled with a Bruker Esquire 2000 Ion-Trap MS analyzer, and a Phenomenex Synergi™ 10-port, 2-position electronic switching valve. HP Chemstation software (Version A.08.03) was used for data analysis. The detection was by Bruker 2000 ESI/MS.

In this application, we have made use of a Luna® NH_2 3µ 50 x 3.0mm column (Phenomenex, Torrance, CA) that can function in the hydrophilic interaction (HILIC) mode. It has been well established that the HILIC mode enables greater retention of very polar analytes and the conditions involve starting with a high organic and ending with a low organic mobile phase.[3]

The configuration for the valves for the on-line extraction-LC/MS system is shown in **Figure 1**. The heart of the system is the Synergi electronic switching valve, which regulates the flow of the mobile phase from the two pumps into the desired column. In the load position, the autosampler introduces the drug/plasma sample into the system and Pump 1 furnishes the 100% aqueous mobile phase at a high flow rate to push the sample into the extraction column (strata-X). Loading of the drug and elimination of plasma matrix components simultaneously occurs during this process, which takes about 1min. The valve is then switched to Pump 2, which supplies acetonitrile/water (80:20) at 1mL per minute in a direction opposite to the flow in the load position (see Figure 1, elution position). The drug is now eluted from the extraction cartridge into the analytical column (Luna Amino, HILIC mode) with this mobile phase. The analytical column further purifies the sample and introduces the sample into the electrospray chamber of the mass spectrometer and the relevant ions are recorded by the mass spectrometer. Total analysis time is just 3 minutes!!

HILIC conditions:	
Mobile Phase:	A = water B = acetonitrile
Flow Rate:	Pump 1 3mL/min, Pump 2 1mL/min
Load:	Pump 1 100% A
Elution:	Pump 2 80% B: 20% A
MS:	Bruker Esquire 2000 Ion-Trap MS analyzer
Source:	ESI-Negative mode
Scan Range:	m/z 100-400

Note: no buffers are used in this method.

RESULTS AND DISCUSSION

The extraction of Acyclovir from plasma was performed at two different concentrations (40ng/mL and 200ng/mL). This resulted in an on-column load of 2ng and 10ng, respectively. Previous results determined an on-column load of 200pg to be the limit of detection (data not shown). Recovery of Acyclovir, shown in **Table 1**, was greater than 92% with a RSD of <5% for the two different concentrations. From the structure of Acyclovir, it can be inferred that this drug can participate in polar interactions through hydrogen bonding between the amino and alcoholic moieties, dipole-

Three Minutes for the Extraction, Aggressive Cleanup and Analysis of the Strongly Polar Drug Acyclovir from Plasma using strata™ X On-line SPE Cartridges (cont'd.)

dipole interaction through the cyclic amide group and can also exhibit π-π interaction through its heterocyclic ring system. This results in good retention of Acyclovir on strata-X under on-line SPE conditions. **Figure 2** shows the mass spectrum of the purified Acyclovir in the negative ion mode, yielding an $[M-H]^-$ ion at m/z 223. The mass spectrum (top), as well as the extracted ion chromatogram (bottom), clearly demonstrate that no other peak of any significance is observed, indicating that the Acyclovir is totally free of any contaminants. Even at the high organic concentration in the mobile phase, the Acyclovir appears beyond 1 minute in the ion chromatogram, indicating the retention attained under the HILIC conditions on the Luna Amino column.

Table 1. Recovery of Acyclovir

Concentration	% Recovery	RSD % (n=6)
40 ng/mL	92.4	4.4
200 ng/mL	97.9	2.3

strata-X in the on-line SPE format offers several distinct advantages over the conventional, off-line SPE method. First, in contrast to silica-based C18, the polymeric strata-X sorbent is effective in retaining polar drugs such as Acyclovir. More aggressive washings result in cleaner chromatography since the coeluting matrix impurities can be washed away. In addition, recoveries using strata-X can be better for polar compounds as compared to those from turbulent flow on-line techniques where retention has been reported to be problematic even under on-line conditions.[4] Second, the on-line technique permits repeated injections of plasma samples, frequently in larger volumes compared to off-line SPE. This results in a lower cost per sample as compared to the single-use syringe barrel SPE tubes. Third, the sample handling is greatly simplified. With the direct injection of the sample as demonstrated by this technique, several time consuming sample preparation steps such as protein precipitation and the evaporation/reconstitution of the sample after SPE are completely eliminated. The result is a time-saving method where the analysis of the drug is completed in only three minutes.

In conclusion, we have demonstrated that strata-X on-line cartridges enable simultaneous extraction and concentration of very polar drugs like Acyclovir from plasma samples and yield clean extracts devoid of plasma matrix constituents. This rapid on-line extraction technology in combination with HILIC is a powerful tool for the analysis of polar drugs in plasma.

Acknowledgments

Special thanks to Tom Cleveland, Phil Koerner, Stanley Lok and James Teuscher for their critical review of this application note and helpful discussions.

References

1. *S.D. Brown, C.A. White, C.K. Chu and M.G. Bartlett,* ***J.Chromatogr. B,*** 772 (2002), 327-334.
2. *M. Fernandez, J. Sepulveda, T. Aranguiz and C. von Plessing,* ***J.Chromatogr. B,*** 791 (2003), 357-363.
3. *B.A. Olsen,* ***J.Chromatogr. A,*** 913 (2001), 113-122.
4. *J.L. Herman,* ***Rapid Commun.Mass Spectrom.*** 16 (2002), 421-426.

Figure 1. Schematic of the valve configuration for the on-line extraction-LC/MS system

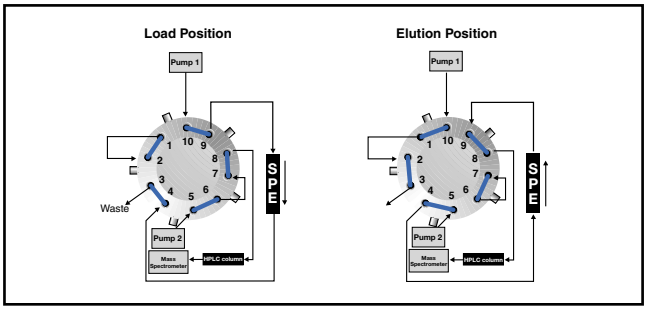
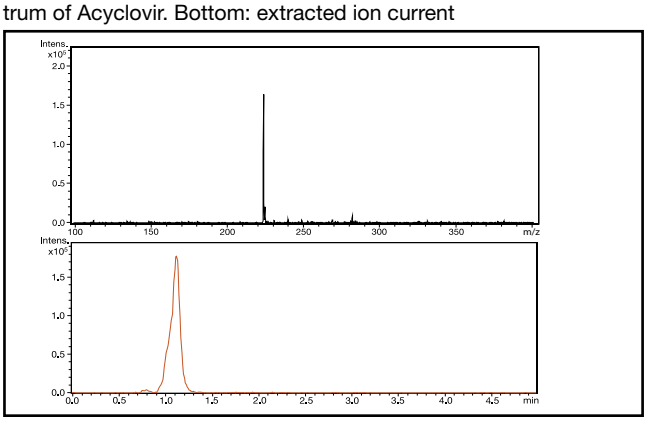


Figure 2. Analysis of Acyclovir extracted from Plasma using the Tandem On-line SPE/HILIC-MS System. Top: mass spectrum of Acyclovir. Bottom: extracted ion current



Ordering Information

strata-X is also available in syringe-barrel tubes and 96-well plates.

Order Number	Description	Units (Box)
8B-S100-TAK-TN	strata-X 30mg/1mL Tubes	100
8B-S100-UBJ-TN	strata-X 60mg/3mL Tubes	50
8B-S100-FBJ-TN	strata-X 200mg/3mL Tubes	50
8B-S100-HBJ-TN	strata-X 500mg/3mL Tubes	50
8B-S100-ECH-TN	strata-X 100mg/6mL Tubes	30
8B-S100-FCH-TN	strata-X 200mg/6mL Tubes	30
8B-S100-HCH-TN	strata-X 500mg/6mL Tubes	30
8B-S100-HDG-TN	strata-X 500mg/12mL Giga Tubes	20
8B-S100-JEG-TN	strata-X 1g/20mL Giga Tubes	20
8E-S100-NGB-TN	strata-X 96 Well Plates (5mg/well)	2
8E-S100-AGB-TN	strata-X 96 Well Plates (10mg/well)	2
8E-S100-TGB-TN	strata-X 96 Well Plates (30mg/well)	2
8E-S100-UGB-TN	strata-X 96-Well Plates (60mg/well)	2
00M-S033-B0-CB-TN	strata-X 25µm On-Line Extraction Cartridge	each
CH0-5845-TN	20mm Cartridge Holder	each

2600_L

Extraction of the Anti-Psychotic Drug Clozapine and its Two Major Metabolites from Plasma Using a strata™X On-Line Cartridge

Art Dixon, Krishna Kallury, Scott Waite
Phenomenex Inc., Torrance, CA USA

Introduction

Clozapine is a dibenzodiazepine derivative used for the treatment of severely ill schizophrenic patients. Its two major metabolites are des-methyl-clozapine and clozapine N-oxide. Accurate determination of plasma levels in patients is of great interest because of the potential for interactions with other drugs and also the high probability of patient non-compliance. Previous analytical methods have been reported for the quantification of clozapine and its metabolites in plasma, including GC analysis, liquid-liquid extraction or off-line SPE followed by HPLC assays with UV-Vis and MS detection (**Ref. 1**). These methods are time consuming, prone to error, result in poor recovery, and cannot be fully automated. A simple, reproducible and fully automated method for the accurate determination of clozapine, desmethyl-clozapine and clozapine N-oxide in plasma using a strata-X 25µm on-line SPE cartridge followed by LC/MS is presented.

Experimental

The HPLC analysis was carried out using an HP 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (1), binary pump (2), in-line degasser, DAD detector, and autosampler coupled with a Bruker Esquire 2000 Ion-Trap MS analyzer and a Synergi Fluid Processor 10-port, 2-position switching valve. HP Chemstation software (Version A.08.03) was used for data analysis. The HPLC columns used for this analysis were strata-X 25µm on-line SPE cartridge (20 x 2.0mm) and Luna 5µm C8(2) 50 x 3.0mm (Phenomenex, Torrance, CA, USA). The mobile phase composition and gradient is listed in Table 1. The detection was by MS. Lyophilized porcine plasma diluted 1:1 with water was spiked with the appropriate concentration of each drug. Standards were obtained from Sigma (St. Louis, MO, USA).

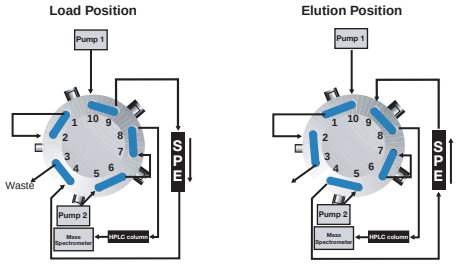
Table 1. On line Extraction conditions

Chromatographic conditions:

Mobile Phase:	A: 0.1% Formic Acid: Water
	B: 0.1% Formic Acid: Acetonitrile
Flow Rate:	**Pump 1** 3mL/min (Loading)
	Pump 2 1mL/min (Elution)
Isocratic Gradient:	**Pump 1** 85:15% A:B
	Pump 2 15-85% B in 3 min
Injection:	50µL diluted porcine plasma
MS:	Bruker Esquire 2000 Ion-Trap MS analyzer
Source:	ESI-Positive ion mode

Figure 1. Schematic of the valve configuration

for the on-line extraction-LC/MS system



Results and Discussion

The configuration used for the Synergi™ switching valve is shown in **Figure 1**. The heart of the system is the Synergi electronic switching valve, which regulates the flow of the mobile phase from the two pumps into the desired column. In the load position, the autosampler introduces the drug/plasma sample into the system and Pump 1 furnishes aqueous mobile phase at a high flow rate to push the sample into the extraction column (strata-X). Loading of the drug and elimination of plasma matrix components simultaneously occurs during this process, which takes about 1 min. The valve is then switched to Pump 2, which supplies mobile phase at 1mL per minute in a direction opposite to the flow in the load position (see **Figure 1**, elution position).

The extraction of clozapine, desmethyl-clozapine, clozapine N-oxide and loratadine (internal standard) from plasma is performed at five different concentrations (12-200ng/mL, corresponding to 0.6–10 ng injected on column). The linearity obtained with the protocol is shown in **Figure 2**. **Figure 3** shows an extracted ion chromatogram (EIC) of each drug. Recovery results of clozapine, desmethyl-clozapine and clozapine N-oxide are shown in **Table 2**. A recovery greater than 90% and an RSD% of less than 5% was obtained for each drug. When using online-SPE, the entire process of conditioning, loading, washing and elution of the analyte of interest into an MS detector can be achieved. strata-X 25µm on-line SPE cartridges allow for direct injections of plasma/serum samples into the LC system, eliminating time-consuming sample preparation steps such as liquid-liquid extraction or off-line SPE. Thus a faster procedure that allows for high sample throughput can be implemented. strata-X on-line SPE cartridges allow for a more cost effective process that is fully automated and superior to off-line SPE in sample capacity per day.

Figure 2. Linearity of compounds extracted from plasma using strata-X on-line cartridge

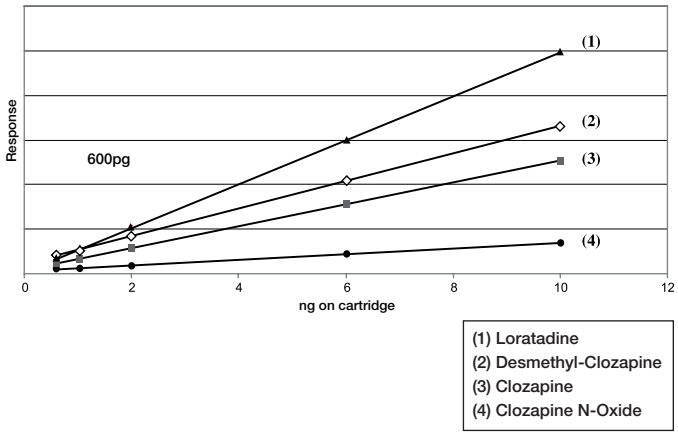


Figure 3. Analysis of Clozapine and Metabolites

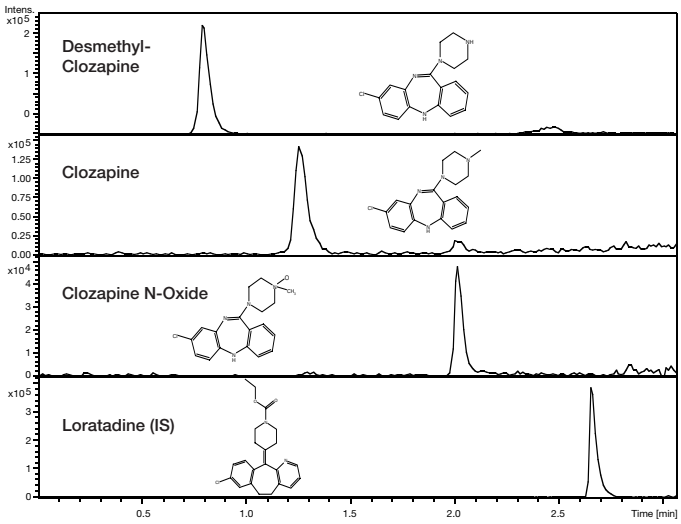


Table 2. Recovery Results

ID	Analyte	LogP	MW	r^2
1	Loratadine (I.S.)	5.20	382.9	0.9998
2	Desmethyl-Clozapine		312.0	0.9927
3	Clozapine	3.23	326.8	0.9961
4	Clozapine N-Oxide		342.1	0.9974

Analyte	Recovery % (RSD %)	
	12 ng/mL	40 ng/mL
Clozapine	100.5 (1.2)	99.5 (0.8)
Clozapine N-Oxide	97.3 (2.8)	99.1 (1.4)
Desmethyl-Clozapine	91.1 (3.0)	95.3 (2.6)

Acknowledgements

Special thanks to Phil Koerner, for his critical review of this application note and helpful discussions.

References

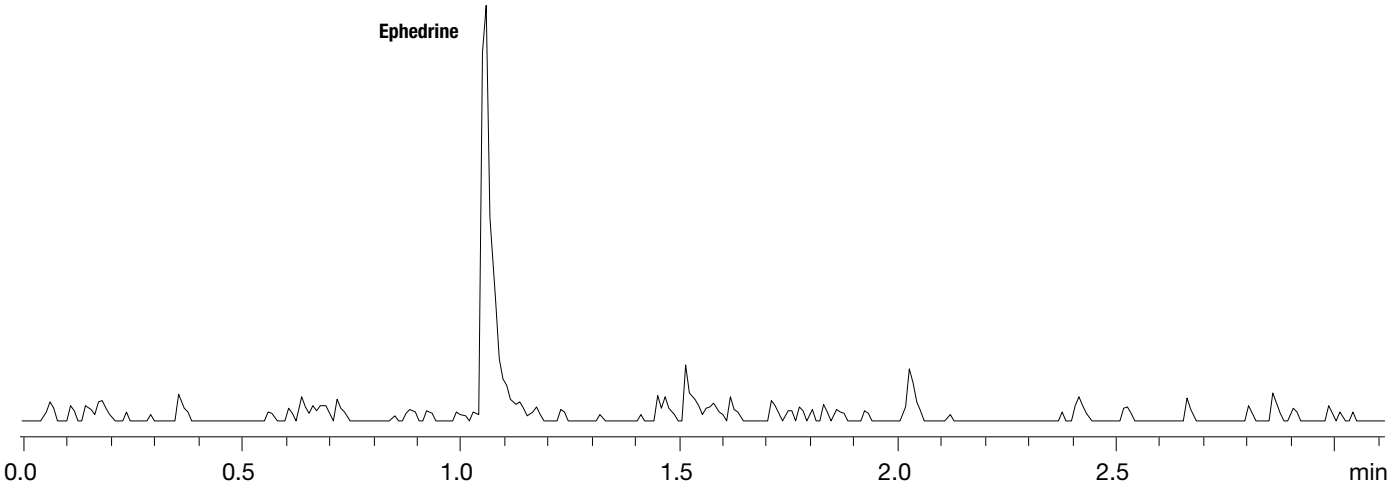
1. S.D. Brown, C.A. White, C.K. Chu and M.G. Bartlett, J.Chromatogr. B, **772** (2002), 327-334.

Ordering Information

strata-X is also available in syringe-barrel tubes and 96-well plates.

Order Number	Description	Units (Box)
8B-S100-TAK-TN	strata-X 30mg/1mL Tubes	100
8B-S100-UBJ-TN	strata-X 60mg/3mL Tubes	50
8B-S100-FBJ-TN	strata-X 200mg/3mL Tubes	50
8B-S100-HBJ-TN	strata-X 500mg/3mL Tubes	50
8B-S100-ECH-TN	strata-X 100mg/6mL Tubes	30
8B-S100-FCH-TN	strata-X 200mg/6mL Tubes	30
8B-S100-HCH-TN	strata-X 500mg/6mL Tubes	30
8B-S100-HDG-TN	strata-X 500mg/12mL Giga Tubes	20
8B-S100-JEG-TN	strata-X 1g/20mL Giga Tubes	20
8E-S100-NGB-TN	strata-X 96 Well Plates (5mg/well)	2
8E-S100-AGB-TN	strata-X 96 Well Plates (10mg/well)	2
8E-S100-TGB-TN	strata-X 96 Well Plates (30mg/well)	2
8E-S100-UGB-TN	strata-X 96-Well Plates (60mg/well)	2
00M-S033-B0-CB-TN	strata-X 25µm On-Line Extraction Cartridge	each
CH0-5845-TN	20mm Cartridge Holder	each
AV0-6086	Synergi 10-position Fluid Processor, Stainless Steel	each

On-line Extraction of Ephedrine from Plasma using strata™ X



SPE Cartridge:	strata-X 20x2.0mm on-line extraction cartridge plus holder
Order No:	00M-S033-B0-CB
Mobile Phase:	A: 0.1% Formic Acid in Water B: 0.1% Formic Acid in Acetonitrile
Flow rate:	Pump 1. 3mL/min, cartridge Pump 2. 1mL/min ,analytical column
Load:	Pump 1. 95:5, A:B
Elution:	Pump 1. 95:5, A:B

Analytical Column

HPLC Column:	Luna 5µ C18(2) 50mmx4.6mm
Order No.:	00B-4252-E0
Mobile Phase:	95:5, A:B
Flow rate:	1mL/min
Detection:	Bruker Esquire 2000 Ion-Trap MS analyzer ESI-Positive mode
Scan Range:	m/z 100-500
Sample:	1. Ephedrine

Ordering Information

Silica-Based

Order Number	Description
00M-S039-B0-CB-TN	Strata C18 on-line extraction cartridge 20µ 20x2.0mm

Polymer-Based

Order Number	Description
00M-S033-B0-CB-TN	strata-X on-line extraction cartridge 25µ 20x2.0mm

3772_L_5

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