XLC-MS for Therapeutic Drug Monitoring

E. Koster[1], M. Hilhorst[1], B. Ooms[1], H. Metting[2] and H. Niederländer[2]

1- Spark Holland Inc., 666 Plainsboro Road, Suite 1336, Plainsboro, NJ 08536, USA, Phone (1) 609 779 7250, Fax (1) 609 799 5250, E-mail: emile.koster@spark.nl
2- University Centre for Pharmacy, Department of Pharmaceutical Analysis, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands.

Objectives

- Develop a universal automated drug analysis system for therapeutic drug monitoring (TDM) that is sensitive, robust, compact, compatible with existing prep methods, fast enough to keep in pace with LC-MS/MS analysis, and that can easily switch between a large variety of assays, compounds and sample types.
- Investigate the potential of the full integration of solid phase extraction and LC-MS (XLC-MS).
- Develop an XLC-MS method for the determination of clozapine and its metabolites in serum to measure therapeutic levels (100 - 700 ng/mL).
- Validate the optimal method and use it for patient samples.

Methodology

The universal applicability of MS as detection technique has been a great help in streamlining the lab organization, especially in terms of equipment maintenance, method development and operator training. For sample preparation, just as desirable, such a universal approach has now been evolved by Spark Holland so that total automation and integration of front-end sample prep and LC-MS is achieved (see figure 1).

XLC (Symbiosis Pharma, Spark Holland)

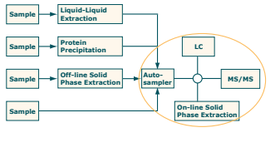

Figure 1: XLC, integration of eXtraction and LC separation

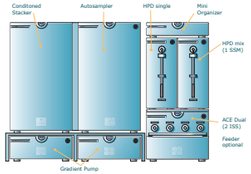

Figure 2: Symbiosis Pharma system

Extraction conditions:

Cartridge:	HySphere C18HD, 2x10 mm
Solvation:	1 mL acetonitrile, 5 mL/min
Equilibration:	1 mL water, 5 mL/min 1 mL 0.1M NH4Ac, pH 8.0, 5 mL/min
Sample load:	50 µL serum, partial loopfill 1 mL 0.1M NH4Ac, pH 8.0, 1.5 mL/min
Wash:	1 mL 15% methanol in water, 5 mL/min
Elution:	LC mobile phase

LC conditions:

Mobile phase:	25 mM NH4Ac/methanol (35/65), pH 4.5
Column:	Zorbax Eclips C18 XDB, 150 x 4.6 mm, 3 µm particles
Flow rate:	1 mL/min (split to 250 µL/min for ESI-MS)

MS (API2000, Applied Biosystems)

Monitoring mode:	SIM, positive
Ionspray voltage (IS):	5000 V
Dwell time:	200 ms
Corona discharge current:	3 µA (APCI)

	ESI	APCI
Curtain gas (psi):	20	40
Ion source gas 1 (psi):	70	90
Ion source gas 2 (psi):	70	30
Temperature (°C):	475	500

Table 1: MS potentials for clozapine and metabolites

Compound	[M+H]+ (m/z)	Declustering potential (V)	Focusing potential (V)
Clozapine (CLZ)	327.3	91	280
Desmethylclozapine (DMC)	313.3	81	350
Clozapine N-oxyde (NOX)	343.3	80	220
Mirtazepine* (MIR)	266.3	61	360

* Mirtazepine was used as the internal standard.

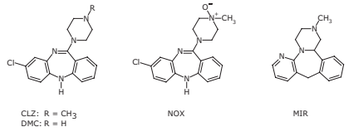

Figure 3: Structures of clozapine (CLZ), desmethylclozapine (DMC), clozapine N-oxide (NOX) and mirtazepine (MIR).

Results

Optimization of LC conditions

Initially the LC separation of CLZ and metabolites was coupled to UV and MS, so baseline separation is required.

- Separation efficiency is best between 60-70% methanol and pH 4-6.
- Elution order: tr DMC < NOX < CLZ (e.g. see figure 5)

Optimization of SPE conditions

The most suitable SPE sorbent was found by means of tray scanning. For this standard SPE conditions were used to extract CLZ and its metabolites from serum utilizing C8, C18, Resin GP and cyanopropyl SPE cartridges.

- HySphere C18HD gave the best extraction performance with respect to recovery (100% for CLZ and metabolites), peak shape and clean-up.

Figure 4: Example of a 10 x 2 mm cartridge.

SPE was further optimized by changing the extraction conditions. Various parameters could be tested in a short period due to the high degree of automation.

- CLZ, DMC and NOX are most efficiently trapped during sample loading at pH 8 due to compound basicity.
- Breakthrough of some compounds is observed when high flow rates are used for sample loading. 100% recovery is obtained at a sample loading flow of 1.5 mL/min.
- Washing with 15/85 methanol/water gives the best compromise between recovery (100%) and sample clean-up.
- Washing of the cartridge at higher flow rates gave better clean-up. Flow rates of 5 to 10 mL/min can be used during washing without analyte recovery loss.

Elution of the cartridge is performed with mobile phase and turned out to be ideal for these basic compounds as the mobile phase contains acid and a high percentage of methanol.

Optimization of MS conditions

- LC-MS was performed with both atmospheric pressure chemical ionization (APCI) and thermally assisted electro spray ionization (ESI).
- With both sources [M+H]+ ions are formed for all tested compounds.
- No other adducts like sodium and potassium were found.
- With both sources the detection limits of all tested compounds are below the low end of the therapeutic range (see table 2).

Table 2: Limit of detection (LOD, ng/mL) for CLZ and metabolites using APCI and ESI (s/n=3).

Compound	APCI	ESI
CLZ	0.13	0.40
DMC	0.14	0.23
NOX	1.00	0.43
MIR	0.19	0.26

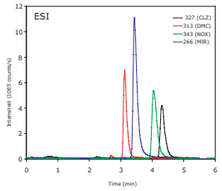

Figure 5. Extracted ion chromatogram of spiked serum containing 250 ng/mL of CLZ, DMC, MIR and NOX using XLC-MS with ESI interface

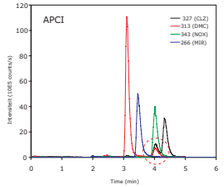

Figure 6. Extracted ion chromatogram of spiked serum containing 250 ng/mL of CLZ, DMC, MIR and NOX using XLC-MS with APCI interface

- For ESI, chromatographic resolution can be sacrificed for throughput. (see figure 5)
- For APCI, in-source reduction of NOX results in a fragment at the same m/z-value as CLZ, i.e., base line separation of CLZ and NOX is required. (see figure 6)

Method validation

Extraction recovery of the compounds is determined by comparing a direct injection of spiked serum in XLC-mode with a direct injection of a standard solution in LC-mode.

- No system hardware changes were required to switch between modes.
- Absolute recovery of CLZ and metabolites is 100%.

Linearity was evaluated over a concentration range extending the therapeutic range for clozapine (see figure 7).

- Good linearity is obtained for all compounds.
- Correlation coefficients were 0.9987 for NOX, 0.9998 for DMC and 1.000 for CLZ.
- Intercepts do not differ from zero at 95% confidence level.

The relative standard deviation (RSD) of the system is evaluated at therapeutic concentration levels.

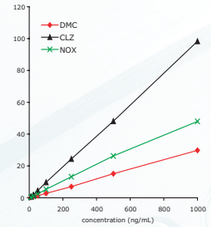

Figure 7. XLC-MS Calibration curves of CLZ, DMC and NOX in serum (50 µL injections, ESI interface)

Table 3: Within day precision of CLZ and metabolites.

Compound	RSD(%) ESI*
Clozapine (CLZ)	2.0
Desmethylclozapine (DMC)	5.1
Clozapine N-oxyde (NOX)	1.3
Mirtazepine (MIR)	1.6

* 50 µL injection of spiked serum (250 ng/mL), n = 9

The accuracy of the method was estimated by analysis of "QC samples" supplied by the Pharmaceutical Department of the University Hospital Groningen (The Netherlands).

Table 4: Method accuracy for CLZ using XLC-MS with ESI interface

	QC data	Experimental data (n = 2)		
Sample	CLZ (ng/mL)	CLZ (ng/mL)	Accuracy (%)	RSD (%)
1	103	101	97.6	0.3
2	255	253	99.1	5.8
3	372	334	89.8	2.4

Real patient samples were analyzed to check for possible interferences.

- In some cases additional peaks can be observed. An example is shown in figure 8.
- The additional peaks in figure 8 (tr 2.3 and 3.7 min) are attributed to CLZ metabolites that are well separated during LC but cleaved or fragmented in the MS-source.
- Single MS is not always selective enough to exclude interferences or to discriminate between co-administered drugs.

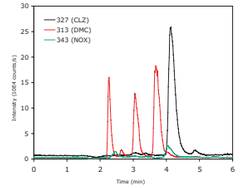

Figure 8. Extracted ion chromatogram of a patient sample using XLC-MS with ESI interface (CLZ 337 ng/mL, DMC 239 ng/mL and NOX 70 ng/mL.

XLC - High throughput

As shown in figure 9, injection, extraction and elution (LC-MS analysis) are run in parallel for highest throughput.

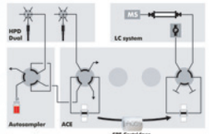

Figure 9: Schematical overview of XLC-high throughput

Because the SPE cycle time is only about 2 min, a shorter analytical column (Zorbax Eclips C18 XDB, 30 x 2.1 mm, 3 µm particles) was used to improve sample throughput.

The example in figure 10 demonstrates that:

- Sample prep can keep pace with LC-MS analysis.
- Separation efficiency (LC-MS analysis time) determines the throughput of the system.
- SPE cycle times of 2 min are feasible (throughput 30 samples/hr).

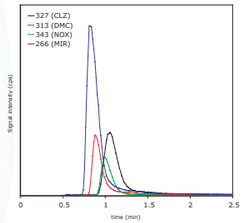

Figure 10. Extracted ion chromatogram of spiked serum containing CLZ (102 ng/mL), DMC (103 ng/mL), NOX (110 ng/mL) and MIR (113 ng/mL). XLC-MS is performed with short analytical column and ESI interface.

Conclusions

- The universal sample introduction approach for MS, based on seamless integration of front-end sample prep and LC-MS, is a very valuable tool for TDM.
- For LC-MS interfacing both APCI and ESI can be considered. APCI is preferred for detectability, ESI for selectivity.
- ESI was chosen as the LC-MS interface because chromatographic resolution could be partially exchanged for MS resolution and no ionization suppression was observed.
- Method optimization could be performed fast and easily as the selection of multiple solvents and the exchange of SPE cartridges is fully automated.
- Raw biological samples were directly analyzed by XLC-MS, so no pretreatment steps like dilution, protein precipitation and centrifugation are required.
- Absolute recoveries of 100% were easily obtained despite the high (97%) protein binding of clozapine.
- Good linearity (R > 0.999) is obtained for all compounds within their therapeutic range.
- The linear range and limit of detection depend on type of compound and detection technique used.
- Good method precision (max ~5%) and accuracy are obtained.
- XLC-high throughput can keep pace with LC-MS cycle times of about 2 min (30 samples per hour).
- XLC-MS for TDM will be further explored in the near future.

0053.845-1, October 2003