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High throughput therapeutic drug monitoring of clozapine and metabolites in serum by on-line coupling of solid phase extraction with liquid chromatography–mass spectrometry

H.A.G. Niederländer [a,*], E.H.M. Koster [b], M.J. Hilhorst [b], H.J. Metting [a], M. Eilders [a], B. Ooms [b], G.J. de Jong [a,1]

[a] *Pharmaceutical Analysis Group, University Center of Pharmacy, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands*
[b] *Spark Holland BV, P.O. Box 388, 7800 AJ Emmen, The Netherlands*

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Abstract

The characteristics of automated on-line solid phase extraction with liquid chromatography–mass spectrometry (SPE-LC–MS) are very amenable for flexibility and throughput in therapeutic drug monitoring (TDM). We demonstrate this concept of automated, on-line SPE-LC–MS for the analysis of clozapine and metabolites (desmethylclozapine and clozapine-*N*-oxide) in serum. Method development, optimisation and validation are described and a comparison with previously published methods for the determination of clozapine and metabolites in serum and plasma is made. Optimisation of chromatographic and SPE conditions for increased throughput resulted in SPE-LC–MS cycle times of only about 2.2 min, demonstrating the great potential of automated on-line SPE-LC–MS for TDM. The new method is shown to be clearly favourable, in particular in terms of ease of sample handling, throughput and detection limits. Recovery is essentially quantitative. Detection limits are at about 0.15–0.3 ng ml^{-1}, depending on the ionisation source used. Calibration follows a quadratic model for clozapine and its *N*-oxide and a linear model for the desmethyl metabolite (all cases: $R > 0.99$). Accuracy, evaluated at three concentration levels spanning the whole therapeutic range, shows that bias is less than 10%. Precision (intra – and inter assay) ranges from about 5% R.S.D. at the high end of the therapeutic range (700–1000 ng ml^{-1}) to about 20% R.S.D. (OECD[2] defined limit) at the lower limit of quantitation (∼50 ng ml^{-1}). The lower limit of quantitation is well below the low end of the therapeutic range at 350 ng ml^{-1}.

Keywords: High throughput; Therapeutic drug monitoring; Solid phase extraction; Liquid chromatography–mass spectrometry; Clozapine; Clozapine-*N*-oxide; Desmethylclozapine; Serum

1. Introduction

Therapeutic drug monitoring (TDM) is an essential element in medical treatment with drugs that have a narrow therapeutic range. Typical examples are anti-epileptics and anti-depressives. The increasing variety of drugs, the large variety in sample numbers (1–100) and the large variety in urgency (minutes–days) has resulted in a large variety of analytical methods [1]. Because many analysers do not allow easy change of methods, a growing number of analysers—demanding specialised personnel and space—are required in the TDM lab. Consequently, there is an increasing need for a universal automated drug analysis system that is sensitive, robust and compact and that can easily change between a large variety of assays even inbetween single samples.

Normally, biological samples require extensive sample clean-up. Protein precipitation has become a very popular method for biological sample clean-up, due to its simplicity [2]. However, over recent years, protein precipitation has become highly suspect for the occurrence of ionisation suppression [3], which is especially problematic in high throughput analysis. Liquid–liquid extraction techniques have long been the preferred method to isolate the analytes of interest from the sample matrix [4,5]. However, solid-phase extraction (SPE) is increasingly becoming the method of choice [6,7]. SPE can be performed

* Corresponding author. Tel.: +31 50 3636122/33336; fax: +31 50 3637582.
E-mail address: h.a.g.niederlaender@rug.nl (H.A.G. Niederländer).
[1] Present address: Department of Biomedical Analysis, University Utrecht, P.O. Box 8008, 3508 TB Utrecht, The Netherlands.
[2] OECD: Organisation for Economic Co-operation and Development.

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as a manual or automated batch-like procedure ("off-line"), or as a procedure that is fully integrated with the instrumental system for (liquid) chromatographic separation ("on-line") [6–9]. On-line SPE has the advantage that sample handling by an analyst is reduced, thus saving time for supervised operation, and avoiding mistakes and contamination. Moreover, as the whole sample is transferred to the chromatographic system after clean up by SPE, a significant pre-concentration factor—and thus a gain in sensitivity—can be achieved. Furthermore, instrumentation is now available that will perform conditioning, sample-application and clean-up on a SPE cartridge, while chromatographic analysis of a previous sample is running from another SPE cartridge at the same time ("concurrent SPE"), thus saving time. To some extent, these advantages can also be obtained for liquid–liquid extraction (LLE) and off-line SPE, if the procedure is automated ("robotised"). Furthermore, off-line sample clean-up can be performed parallel for a large number of samples, thus saving time. Ninety-six-well plate-based off-line SPE kits are now available for that purpose [8,10].

On-line SPE provides an automated sequential SPE process including on-line elution of the extract from the SPE cartridge into the LC column and the detector. Selectivity within such a system can be attained in each of the three stages. However, increasing selectivity for LC separation will normally influence throughput negatively, while increasing selectivity for MS detection cannot avoid potential matrix induced ionisation suppression [11]. Selectivity can therefore best be attained up-front during SPE. With dedicated optimisation of the SPE parameters, single quadrupole LC–MS should be selective enough to analyse a large range of drugs. This should make on-line SPE-LC–MS more accessible and affordable for the routine TDM laboratory. Considering the arguments provided, the method presented was developed applying concurrent SPE, coupled on-line with a fast LC separation and single quadrupole MS detection.

A typical example of a drug requiring TDM, is clozapine (CLZ). CLZ is an important neuroleptic drug against schizophrenia. Due to a severe side effect (agranulocytosis), occurring in 1 or 2% of the patients treated with CLZ [12], CLZ and its main metabolite desmethylclozapine (DMC) have to be routinely monitored in patient sera during therapeutic treatment. Another CLZ metabolite, which can be found in patient sera at significant concentrations, is clozapine-*N*-oxide (NOX). Though this metabolite itself is pharmacologically inactive, screening its concentration in serum or plasma can be interesting, as the conversion of CLZ to NOX is reversible [13]. Over the last decade, several liquid chromatographic methods for monitoring CLZ (and metabolites) in serum and/or plasma have been published. Sample clean-up is performed either by LLE [18,20–22,25,28,30], or by SPE, off-line [14,16,17,23,26,27], or on-line [15,19,24,29] with LC separation. In the case of on-line SPE, a cyanopropyl [15,24], C18 [19], or divinylbenzene co-polymer resin SPE stationary phase [29] is used.

In most cases, UV absorption detection is applied, thus requiring relatively long LC run times to attain sufficient chromatographic selectivity. Amperometric detection of CLZ and metabolites has been suggested by Raggi et al. [23] and, more recently, mass spectrometric detection bas been applied [25,29]. Though amperometric detection is more sensitive and selective than UV detection, the performance of MS detection, which is intrinsically highly selective, can be even better in this respect. Though, detection limits published by Aravagiri and Marder for a method using tandem MS [25] and Kollroser and Schober for a method using ion trap MS^2 [29] are not as good as those presented by Raggi et al. for a method using amperometric detection [23].

In this paper, we demonstrate the concept of flexible, automated, on-line SPE-LC–MS for the analysis of CLZ and its metabolites in serum. A high throughput method based on an automated cartridge exchange SPE system coupled to LC–MS is developed and optimised. As indicated above, detection was performed by single quadrupole MS in positive multiple selected ion monitoring (SIM) mode. Organic modifier percentage, pH and LC-column length are tailored to increase throughput extensively. Parameters for SPE (percentage organic modifier and pH for loading and washing) and MS (ionisation method and source parameters and ion optics) are optimised for selectivity and sensitivity. The resulting method is validated for linearity, accuracy, precision, limit of detection (LOD), lower limit of quantitation (LLOQ) and recovery. The performance of the method is compared with that of methods previously presented for the analysis of CLZ and metabolites in serum or plasma. Finally, the method is compared with a certified method for a number of patient serum samples.

2. Experimental

2.1. Solvents and chemicals

Methanol (HPLC quality) was purchased from Lab-scan Analytical Sciences (Dublin, Ireland), absolute ethanol (analysis grade) from Merck (Darmstadt, Germany) and ammonium-acetate (99.99%) from Sigma-Aldrich (St. Louis, MO, USA).

CLZ, DMC, NOX and mirtazepine (MIR), all >99% pure, were obtained as a gift from the Pharmacy Department of the Academic Hospital Groningen. Structures of these compounds are represented in Fig. 1. Foetal calf serum was also obtained as a gift from the Pharmacy Department of the Academic Hospital Groningen. Stock solutions (CLZ, DMC, NOX and MIR, at the 100 $\mu g\,ml^{-1}$ level) were prepared in absolute ethanol.

Prior to use, all buffer and wash solutions were filtered over a 0.45 μm filter and ultrasonicated for 15 min. Pure solvents were ultrasonicated for 15 min.

SPE cartridges, HySphere-C18-HD (10 mm × 2 mm, 7 μm particles) and HySphere-CN (10 mm × 2 mm, 7 μm particles), were purchased from Spark-Holland (Emmen, The Netherlands).

2.2. Instrumental

All analyses were performed on a solid phase extraction with liquid chromatography–mass spectrometry (SPE-LC–MS)

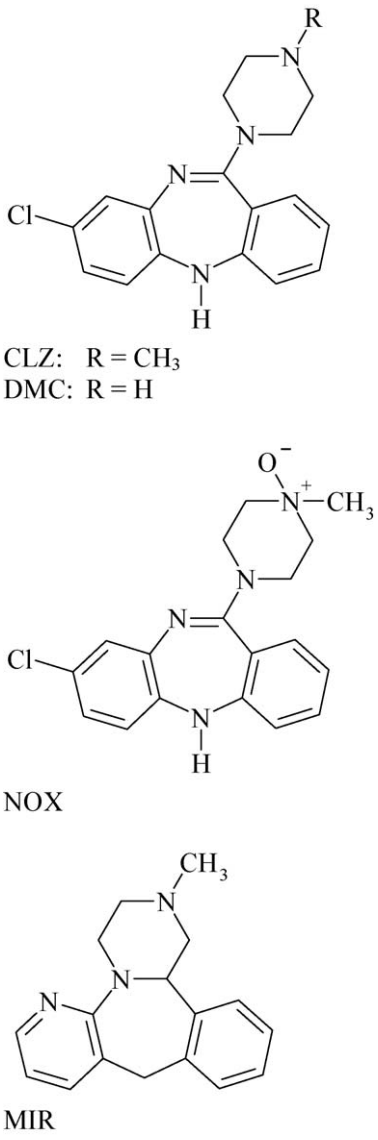

Fig. 1. Structures of clozapine (CLZ, MW: 326.13 nominal), desmethylclozapine (DMC, MW: 312.11 nominal), clozapine-*N*-oxide (NOX, MW: 342.12 nominal) and mirtazepine (MIR, MW: 265.16 nominal).

system consisting of a Prospekt-2 automated SPE system (Spark Holland) coupled on-line to an 1100 LC system (Agilent Technologies, Amstelveen, The Netherlands) and an API 2000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ont., Canada). The Prospekt-2 system consists of an endurance autosampler, an automated SPE cartridge exchanger (ACE), with two clamps for holding SPE cartridges, and a high-pressure dispenser (HPD) [31].

LC separations were run over a Zorbax Eclipse XDB-C18 (3.5 μm particle size) analytical column (Agilent Technologies, Amstelveen, The Netherlands), mounted with a Zorbax Eclipse XDB-C18 or XDB-C8 (5 μm particle size) guard column (Agilent Technologies). A 4.6 mm × 12.5 mm C-18 guard column was used in combination with a 4.6 mm × 150 mm analytical column. For increasing throughput, both a 2.1 mm × 50 mm and a 2.1 mm × 30 mm analytical column were used in combination with a 2.1 mm × 12.5 mm C-8 guard column.

2.3. Instrument settings

Fifty-microliter sample volumes were injected onto the SPE cartridges. Conditions for SPE extraction and washing are presented in the text. Elution to the analytical column and chromatographic separation was performed using an isocratic mobile phase; methanol–aqueous ammonium acetate buffer (25 mM). The pH (4.5 or 6.0) and modifier percentage (60–70% methanol, v/v) were adjusted to increase sample throughput (see text). Eluent flow rates are 1 ml min^{-1} with the 4.6 mm ID column and 200–300 μl min^{-1} (see text) for the 2.1 mm ID columns. Operating at 1 ml min^{-1}, the column effluent was split of 1:3 before coupling to the ESI MS interface.

The temperature of the TurboIonSpray® ESI interface was set at 475 °C. Curtain gas (nitrogen) pressure was maintained at 20 psi and both ion source gasses (dry and carbon filtered air) were fixed at 70 psi. The ion spray voltage was set at 5 kV.

When using the APCI interface, the vaporiser temperature was set at 500 °C. Curtain gas pressure was maintained at 40 psi and the ion source gasses were fixed at 90 and 30 psi, for "Gas 1" and "Gas 2" respectively. Finally, the corona discharge current was set at 3 μA.

The mass spectrometer was operated in selected ion monitoring (SIM) mode, monitoring the $[M+H]^+$ ions for all analytes. Settings for the individual ions monitored are: CLZ $[M+H]^+$ at 327.3 *m/z*, declustering potential 91 V, focussing potential 280 V; DMC $[M+H]^+$ at 313.3 *m/z*, declustering potential 81 V, focussing potential 350 V; NOX $[M+H]^+$ at 343.3 *m/z*, declustering potential 80 V, focussing potential 220 V; MIR $[M+H]^+$ at 266.3 *m/z*, declustering potential 61 V, focussing potential 360 V.

2.4. Validation procedure

In line with the matrix used for the Dutch program for round robin testing for therapeutic CLZ monitoring ("Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie", www.kkgt.nl), method development and validation were done using foetal calf serum. The validity of the method for human serum samples was verified by comparison of results for five patient serum samples with values independently obtained with the certified and round-robin tested method used at the hospital pharmacy of Groningen academic hospital.

Linearity, recovery, accuracy and precision (intra- and inter-day) were assessed for the method after optimization for throughput. The lower limit of quantitation for each component was estimated based on the validation results for accuracy and precision (bias and precision within 20% R.S.D.), whereas detection limits were calculated based on signal intensity and noise for the *m/z* trace of the compound of interest in the MS in SIM mode (S/N = 3). A single batch consisted of 36 samples (i.e. calibration standards, blancs and QC standards), including triplicates. When real samples (five samples, out of which three in duplicate) and recovery standards were included, a single batch consisted of 54 samples. Based on literature data [24] and the fact that complete analysis of a single batch of samples

takes less than 2.5 h, samples were considered to be sufficiently stable.

Linearity was assessed for a range of seven concentrations, in triplicate, on 5 consecutive days. The concentration range for CLZ and its metabolites was made from two stock solutions for each compound in absolute ethanol. For the internal standard (IS: MIR) only a single stock solution in absolute ethanol was prepared. Stock solutions were prepared by weighing an amount of 1–3 mg on a Mettler Toledo MT-5 mg balance (Mettler-Toledo, Tiel, The Netherlands) and dissolving in 5, 10 or 25 ml absolute ethanol in a volumetric flask to end up with a concentration of about 100 $\mu g\,ml^{-1}$. The exact concentrations obtained are: CLZ: 108.8 and 104.5 $\mu g\,ml^{-1}$, NOX: 99.0 and 94.2 $\mu g\,ml^{-1}$, DMC: 101.2 and 101.0 $\mu g\,ml^{-1}$, MIR: 105.3 $\mu g\,ml^{-1}$. Standards, containing CLZ, DMC and NOX, at the 10, 20, 250 and 1000 ng ml^{-1} concentration levels in foetal calf serum were prepared from a single stock solution, whereas the standards at the 50, 100 and 500 ng ml^{-1} concentration level were all prepared from the second stock solution. For MIR, a working solution was prepared by diluting the stock solution 50 times with absolute ethanol ([MIR] = 2.11 $\mu g\,ml^{-1}$). The IS was added to the samples on the day of the measurement by adding 100 μl of the MIR working solution (211 ng MIR) to 900 μl of the standard of the right concentration level in foetal calf serum, to end up with a MIR concentration of 211 ng ml^{-1}. Unless stated otherwise, CLZ, DMC and NOX concentrations presented in the text, tables and figures refer to the concentration before addition of the IS. Standards were measured in random order. Subsequently, linearity was assessed for peak area response ratios for the individual components and the IS, using one-way ANOVA (Microsoft® Excel 2002 SP-2).

Recovery, accuracy and precision were assessed at three concentration levels (low: 50 ng/ml level, middle: 500 ng/ml level, and high: 1000 ng/ml level). Samples were prepared in foetal calf serum, using an independent stock solution for each compound (CLZ: 105.3 $\mu g\,ml^{-1}$, NOX: 105.2 $\mu g\,ml^{-1}$, DMC: 101.0 $\mu g\,ml^{-1}$), as described for linearity. Samples for recovery were also prepared in water–methanol (85:15, v/v). Accuracy and precision were assessed on 5 consecutive days, in quintuplicate for each concentration. Standards were measured in random order. Precision was assessed for peak area response ratios, using one-way ANOVA. Recovery was assessed in triplicate at each concentration level on day 3 of the validation. Recovery was calculated based on comparison of absolute peak area for standards in foetal calf serum analysed with SPE-LC–MS with absolute peak area for standards in water–methanol (85:15, v/v) analysed with LC–MS.

Patient serum samples (five samples) were obtained from the pharmacy department of the university hospital Groningen (The Netherlands). Where sample size allowed, these samples were analysed in duplicate. Results were compared with values independently obtained with the certified and round-robin tested method used at the hospital pharmacy. This reference method is based on dual liquid–liquid extraction (dual-LLE), followed by reversed phase HPLC separation and UV-diode array detection (UV-DAD) of CLZ and DMC (no data for NOX are obtained with this independent method).

3. Results and discussion

3.1. Basic method optimisation

3.1.1. Liquid chromatography

In accordance with the majority of literature dealing with monitoring of CLZ and metabolites, a reversed phase (RP) chromatographic system, utilising a C18 stationary phase [14,16–18,20,23,24–29], has been chosen to separate CLZ, DMC and NOX. Other RP stationary phases reported in literature include C6 [21], C8 [17,20] and cyanopropyl (operated under RP conditions) [19,30]. A typical normal phase LC stationary phase (bare silica), but also run under reversed phase conditions, has been used twice [15,22].

Applying an isocratic mobile phase, consisting of methanol–25 mM ammonium acetate buffer pH 6.0 (65:45, v/v), baseline separation for CLZ and metabolites is accomplished. Under these chromatographic conditions, MIR (an antidepressant) can be selected as an IS, as it elutes in-between NOX and CLZ. Furthermore, MIR has structural features similar to those of CLZ and metabolites. To our knowledge, MIR is not likely to be prescribed in combination with CLZ. However, whenever MIR is prescribed in combination with CLZ [32,33], another IS should be selected.

3.1.2. Solid phase extraction

A Hysphere C18 HD SPE stationary phase was chosen for sample preparation purposes in this study. A cyanopropyl SPE stationary phase [15,24] was also considered, but did not show the desired extraction behaviour. Independent of pH value for sample loading and washing, recovery was low with the latter stationary phase (especially for NOX). Furthermore, instrument failures resulted from pressure build-up, probably caused by the high viscosity of the methanol–water mixtures used. The Hysphere C18 HD SPE stationary phase performed much better and provided sufficient selectivity to separate CLZ and metabolites from more polar matrix components in serum.

CLZ, DMC, NOX and the IS are all neutral and therefore efficiently trapped at pH 8.0. The analytes are strongly retained on the cartridge during washing with up to 10 ml of 15% (v/v) methanol in water, whereas most matrix components are washed from the cartridge based on cartridge effluent monitoring with UV detection at 210 and 261 nm (data not shown). Breakthrough was observed when washing at methanol percentages as low as ca. 30%. Therefore, washing with 15% of methanol was used for all further experiments. Finally, the compounds are eluted from the SPE cartridge with the LC mobile phase. An elution time of about 150 s, at the flow rate applied for LC separation, is required for complete elution of all compounds from the SPE cartridge to occur. Only at elution times below 120 s, incomplete recovery was observed.

3.1.3. Mass spectrometry

Previous publications only consider electrospray ionisation interfacing for LC–MS analysis of CLZ and metabolites [25,29].

We have investigated both thermally assisted electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) for interfacing LC separation with mass spectrometric detection. With both sources the $[M+H]^+$ ions of CLZ, CLZ metabolites and the internal standard are efficiently formed with all mobile phase compositions used. No other ions, including the ammonium and sodium adducts and possible fragment ions, are detected, with an exception for NOX. With the APCI interface, NOX also produces two "fragment" ions at the same *m/z* value as $(CLZ+H)^+$ and $(DMC+H)^+$, whereas with the ESI interface these ions were not found. These ions are probably a result of in-source reduction of NOX to CLZ and (thermal) elimination of OCH_2 from NOX to give DMC. Therefore, when analysing CLZ and metabolites with APCI ionisation, the chromatographic peaks of CLZ, DMC and NOX should be completely resolved to avoid interference by NOX. With both interfaces limits of detection for CLZ (see below) are well below the low end of the therapeutic range (350 ng ml^{-1} in serum [12,34,35]) for this drug. However, detection limits are about a factor of 2–3 better with the APCI interface. Nevertheless, the in-source conversion of NOX to CLZ and DMC requires complete separation of these compounds and thus limits the use of the APCI for high throughput. Therefore, the ESI source was selected for all further experiments.

Applying the settings selected above for SPE, LC and MS, chromatograms as shown in Fig. 2 are obtained. As baseline separation is achieved for all analytes, the selectivity provided by MS detection is not yet imperative and UV detection is still a possible alternative. However, once chromatographic resolution is sacrificed for throughput, UV detection is no longer an option. The resulting method requires a total analysis time of about 16 min (including sample preparation, chromatographic separation and MS detection), as dictated by the software controlling the system. As sample preparation can be carried out while chromatographic analysis of a previous sample is running, a cycle time of about 12 min can be achieved.

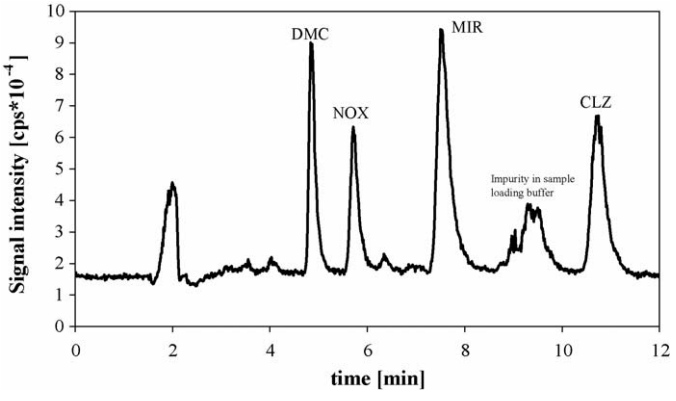

Fig. 2. Standard containing 10.5 ng/ml CLZ, 10.3 ng/ml DMC, 11.5 ng/ml NOX and 11.2 ng/ml MIR in serum. Total ion current (TIC) for the 4 SIM traces of the $[M+H]^+$ ions (see experimental for *m/z* values). Mobile phase (1 ml/min): methanol–25 mM NH_4Ac (pH 6.0) (65:35); Column: Zorbax Eclipse C18 XDB (150 mm × 4.6 mm, 3.5 μm particle size); Injection: 200 μl; MS interface: ESI (split 1:3).

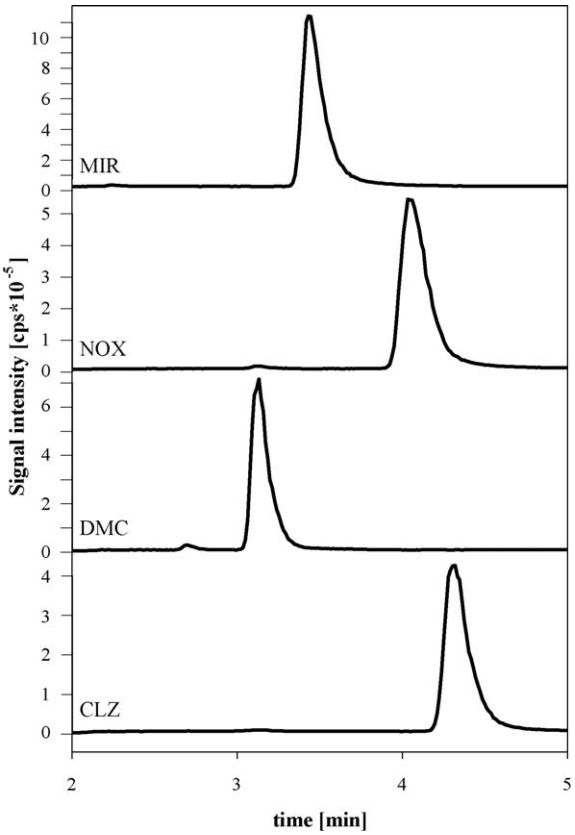

Fig. 3. Extracted ion chromatogram (XIC) ($[M+H]^+$ ions) of a standard, containing 102 ng/ml CLZ, 103 ng/ml DMC, 110 ng/ml NOX and 113 ng/ml MIR in serum (see experimental for *m/z* values). Mobile phase (1 ml/min): methanol–25 mM NH_4Ac (pH 4.5) (70:30); Column: Zorbax Eclipse C18 XDB (150 mm × 4.6 mm, 3.5 μm particle size); Injection 200 μl; MS interface: ESI (split 1:3). Traces are scaled individually in order to improve clearness.

3.2. Increasing throughput

The instrumentation used here for the analysis of CLZ and metabolites in serum performs sequential on-line SPE, while chromatographic analysis of a previous sample is running ("concurrent SPE"). Such a procedure is inherently efficient for sample throughput. In further optimising throughput with such a system, it should be realised that either sample preparation or chromatographic analysis will be the time limiting step.

When applying mass spectral detection, chromatographic resolution can be (partially) exchanged for mass spectral resolution, provided that matrix ionisation suppression does not adversely affect analyte quantification. Reduction of the retention on the chromatographic column results in a significant first increase in throughput. At a low pH (pH 4.5) and a high modifier percentage (70% methanol, v/v) CLZ, DMC, NOX and the IS elute from the 150-mm chromatographic column within 5 min (Fig. 3). A further increase in throughput can be achieved by reducing the column length. The analytes elute within 3 min when installing a 30-mm column (ID 2.1 mm, proportional flow rate 200 μl min^{-1}) and increasing the flow rate to 300 μl min^{-1} even reduces the chromatographic analysis time to about 2 min (Fig. 4). Though chromatographic resolution is now almost completely lost, the potential for quantitative analysis is unaffected,

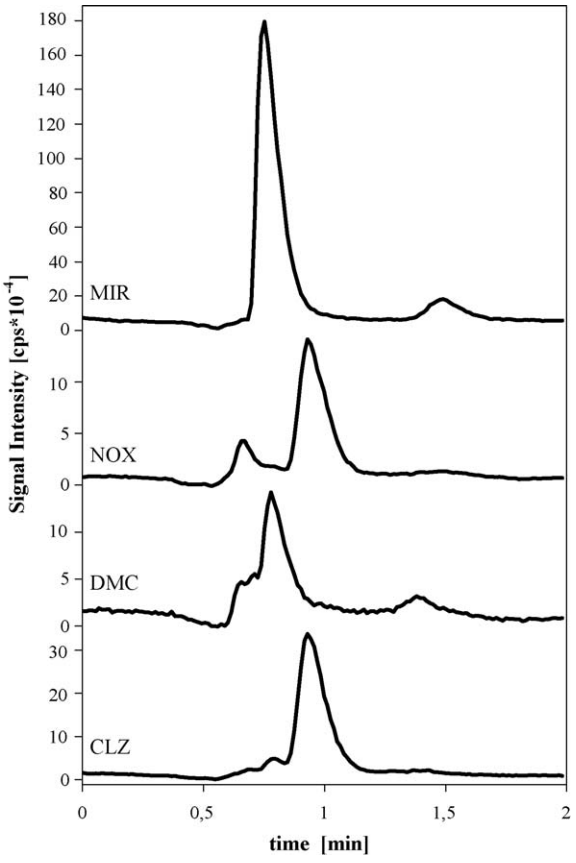

Fig. 4. Effect of reducing column size and increasing flow rate. Standard at the LLOQ level, containing 53 ng/ml CLZ, 53 ng/ml DMC, 51 ng/ml NOX and 211 ng/ml MIR in serum (see experimental for *m/z* values). Mobile phase (300 μl/min): methanol–25 mM NH_4Ac (pH 4.5) (70:30); Column: Zorbax Eclipse C18 XDB (30 mm × 2.1 mm, 3.5 μm particle size); Injection: 50 μl; MS interface: ESI (no split). Traces (extracted ion chromatograms (XIC) of the $[M+H]^+$ ions) are scaled individually in order to improve clearness.

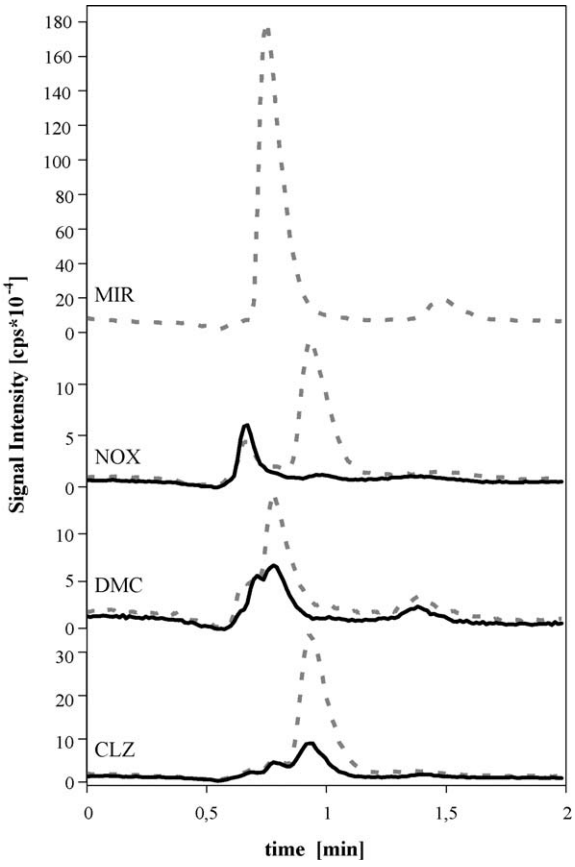

Fig. 5. Interferences from a blank serum sample at the *m/z* values of the analytes. The curves for the standard at the LLOQ level presented in Fig. 4 are included for reference (dotted grey lines). Mobile phase (300 μl/min): methanol–25 mM NH_4Ac (pH 4.5) (70:30); Column: Zorbax Eclipse C18 XDB (30 mm × 2.1 mm, 3.5 μm particle size); Injection 50 μl; MS interface: ESI (no split). Traces (extracted ion chromatograms (XIC) at the *m/z* values of the $[M+H]^+$ ions) are scaled individually in order to improve clearness.

as mass spectral resolution is unaltered and no matrix ionisation suppression occurs. Recovery at the low analyte concentrations shown in Fig. 4 remains essentially unaltered at ≥90% (see below). Furthermore, interferences from the serum at the *m/z* values of the analytes only become apparent at the LLOQ level. This is illustrated in the chromatogram for a blank serum sample, presented in Fig. 5.

With a chromatographic analysis time of only about 2 min, sample preparation may become the throughput-limiting step. Minimising the time required for sample preparation requires maximising the flow rates and minimising the volumes applied for the individual steps in the SPE procedure (i.e. activation of the sorbent, equilibration of the sorbent, sample loading, washing and elution to the chromatographic part of the system). The flow rate for these steps, with an exception for elution (determined by LC conditions), could be set to the maximum allowed by the instrumentation used (10 ml min^{-1}). Volumes were minimised to end up with a total sample preparation time with about the same duration as the chromatographic analysis time (i.e. about 2 min). The amount of methanol for activation of the SPE cartridges was reduced to 750 μl. A first equilibration step with pure water was omitted, as equilibration with buffer directly following the activation with methanol did not lead to precipitation of buffer salts. The amount of buffer required for injection loop flushing and equilibration of the cartridge was minimized at 1.75 ml. The amount of solvent (15% methanol) used to load the sample onto the SPE cartridge and wash out matrix components was reduced to 2 ml, without introducing matrix interferences during the elution step.

Further reductions in the volumes applied for sample preparation might even be possible. However, doing so will not lead to any further gain in throughput, as the chromatographic analysis time will then be limiting the throughput. An overview of sample preparation steps is presented in Table 1. Implementing the amendments made into a concurrent SPE-LC–MS method (i.e. performing SPE sample clean-up of a sample while chromatographic analysis of the previous sample is running), results in an analytical procedure for the analysis of CLZ and metabolites in serum with a throughput of 25 samples per hour.

3.3. Validation and application

Linearity was evaluated over a concentration range extending from 10 to 1000 ng ml^{-1}. This range extends from well

Table 1
Sample preparation steps

SPE step	Volume and solvent	Flow rate (ml min^{-1})
Place cartridge in left clamp		
Activation	0.75 ml methanol	10
Equilibration	1.75 ml buffer[a]	10
Inject (aspirate sample)	50 μl sample	–
Sample load and wash	2.00 ml 15% methanol[b]	10
Cartridge transfer form left to right clamp for elution		
Total time required is 132 s		

[a] Buffer is 0.1 mol l^{-1} NH_4Ac pH 8.0.
[b] About 500 μl of this volume never reaches the cartridge, because of the dead volume introduced by the sample loop which is now switched into the system.

below the minimum of the therapeutic range (ca. 350 ng ml^{-1}) to just beyond the maximum of the therapeutic range for CLZ (700–1000 ng ml^{-1}) [12,35]. As can be seen from Table 2, calibration curves for both CLZ and NOX are not linear over the concentration range studied. A non-linear behaviour for response with concentration is observed more often when ESI-MS detection is used for concentration series extending over a long range [36,37] and remains upon ratioing against an IS at a fixed concentration. This is a direct consequence of saturation of ESI response at concentrations above ±10 μM (=ca. 3000 ng ml^{-1} for CLZ), and is a fundamental characteristic of the ionisation mechanism for ESI. Though actual saturation only occurs as of about 10 μM, deviation from a linear calibration curve is generally already observed at significantly lower concentrations [36].

As deviation from linearity for the high throughput method already exceeds 3% at concentrations as low as 100 ng ml^{-1} for CLZ and 300 ng ml^{-1} for NOX, non-linear regression was used for calibration purposes. The results, applying a quadratic model for both CLZ and NOX, are summarised in Table 2. For DMC no deviation from linearity is observed and linear regression analysis was used. This is probably due to differences in basicity between DMC, NOX and CLZ, while the fact that CLZ and NOX practically co-elute will also add to the observed differences. The remaining lack of fit for CLZ is marginal, especially given the very high goodness of fit for the quadratic model (corrected $R^2 = \pm 0.997$ and F for regression = ±4000; $P(4000, 2, 18) = 1.45 \times 10^{-24}$). Moreover, accuracy and precision (next section) do not significantly improve when using a cubic model without lack of fit.

Table 2
Linearity

Parameter	CLZ[a]	DMC[a]	NOX[a]
Days	5	5	5
Concentrations	7	7	4
Replicates	3	3	3
Concentration range (ng ml^{-1})	10–1000	10–1000	10–1000
Response ratio	0.05–2	0.01–0.75	0.01–1
A[b]: intercept (error)	0.04 (0.02) −0.05 (0.03)	0.004 (0.007) −0.017 (0.011)	0.002 (0.01) −0.0034 (0.01)
B[b]: slope	± 0.003	±0.0007	±0.0014
C[b]	$\pm -1.10^{-6}$	*Zero by definition*	$\pm -5.10^{-7}$
Corrected R^2	0.994–0.998	0.993–0.999	0.999–1.000
F for lack of fit	6.3–34.0[c]	0.3–2.7[d]	0–0.4[e]

[a] Regression analysis was performed for individual days, ranges show the spread over 5 days.
[b] $y_{calc} = A + Bx + Cx^2$, intercept standard error (in parentheses) was evaluated at $P = 0.05$.
[c] $F_{crit.}$ $(P = 0.05, 4, 14) = 3.112$.
[d] $F_{crit.}$ $(P = 0.05, 5, 14) = 2.9582$.
[e] $F_{crit.}$ $(P = 0.05, 1, 8) = 5.3176$.

For NOX, an inaccurate stock solution was rejected, leaving a calibration curve at only four concentration levels. As NOX is not itself a pharmacologically active metabolite and no therapeutic limits have been set for this metabolite, monitoring NOX concentrations is primarily of interest because the transition of CLZ to NOX is reversible [13]. Consequently, a calibration curve at four concentrations was considered sufficient for NOX.

Accuracy and precision were evaluated at three concentration levels (low: 50 ng ml^{-1} level, middle: 500 ng ml^{-1} level, and high: 1000 ng ml^{-1} level), on 5 consecutive days, in quintuplicate for each concentration. The concentrations calculated from the response ratios were grouped in a 5-days-by-5-replicates matrix for each compound at each concentration level, and accuracy, inter- and intra-assay variation were calculated using one-way ANOVA. Results are shown in Table 3.

With an exception for DMC at the low concentration level, accuracy and precision are within the criteria required for method validation (i.e. bias and CV ≤15%, with an exception at the lower limit of quantitation (≤20%)) [38]. For DMC, the CV for precision (both intra- and inter-assay) at the low concentration level is just above 20%. However, given the fast decline, to a CV of about 5% at the medium concentration level, the 20% criterion for lower limit of quantitation is expected to be only slightly higher than 50 ng ml^{-1}. Precision is comparable with results obtained for other methods applying MS detection [25,29].

Limit of detection (LOD) and lower limit of quantitation (LLOQ) were calculated based on signal to noise ratio for the LC–MS response and criteria for accuracy and precision respectively. For LOD a signal to noise ratio (S/N) of 3 was adopted. For LLOQ the criterion presented in the previous paragraph (CV and bias ≤20%) was applied. For all three compounds, the low concentration level (50 ng ml^{-1}) is adopted as the LLOQ. For both CLZ and DMC percentage CV at this level is close to the criterion. Though for DMC precision at the 50 ng ml^{-1} level is slightly higher than 20%, the precision at the 500 ng ml^{-1} level is significantly lower. Though slightly higher, the actual LLOQ is therefore expected to be in the range of 50 ng ml^{-1}. For NOX both percentage CV and bias are well below this criterion at the 50 ng ml^{-1} level, but no further efforts were made to establish LLOQ at a lower concentration level. LLOQ values are well below the low end of the therapeutic range for CLZ (ca. 350 ng ml^{-1} [12,35]) and the toxicity limit/high end of the therapeutic range (DMC: 500–600 ng ml^{-1} [39], CLZ: 700–1000 ng ml^{-1} [12,35]) for these compounds. For NOX no limits have been defined.

Detection limits with the ESI interface are in the order of 0.3 ng ml^{-1}. However, it should be noted that, with an exception for NOX, a factor of about 2–3 better detection limits can be

Table 3
Accuracy and precision for CLZ, DMC and NOX at three concentration levels

Concentration level	Accuracy			Precision	
	Spiked (ng ml^{-1})	Mean	Bias (%)	CV intra-assay (%)	CV inter-assay (%)
Clozapine (CLZ)					
Low	52.65	57.51 (n = 25)	9.2	8.7 (df = 20)	17.0 (df = 4)
Medium	473.9	489.9 (n = 25)	3.4	3.6 (df = 20)	7.6 (df = 4)
High	947.7	998.6 (n = 25)	5.4	6.2 (df = 19)	14.6 (df = 4)
N-desmethyl-clozapine (DMC)					
Low	50.5	47.35 (n = 25)	−6.23	28.4 (df = 20)	22.2 (df = 4)
Medium	454.5	462.1 (n = 25)	1.7	6.9 (df = 20)	4.4 (df = 4)
High	909.0	910.6 (n = 25)	0.2	4.7 (df = 20)	6.0 (df = 4)
Clozapine-*N*-oxide (NOX)					
Low	52.6	60.5 (n = 25)	15.0	4.1 (df = 20)	10.5 (df = 4)
Medium	473.4	491.1(n = 25)	3.7	3.6 (df = 20)	7.1 (df = 4)
High	946.8	1036 (n = 24)[a]	9.4	5.6 (df = 19)	5.9 (df = 4)

Number of measurements (n) and degrees of freedom (df) in parentheses.
[a] A single concentration was rejected as an outlier based on a Grubb's test.

obtained with the APCI interface (i.e. whenever CLZ and NOX are separated by the chromatographic system). Detection limits with the ESI interface are slightly better than previously reported for CLZ and metabolites with ESI-tandem-MS and ESI-MS^2 [25,29]. Detection limits reported for UV detection at wavelengths ranging from 210 to 280 nm are generally much higher, but in a few cases detection limits in the low ng ml^{-1} range have been reported for CLZ and metabolites [14,21,28].

Recovery is calculated at the three concentration levels (low, medium and high) presented for assessing accuracy and precision. For both CLZ and NOX, recovery percentages calculated seem to increase significantly with increasing concentration, up to unrealistically high percentages at the concentration levels medium (∼500 ng ml^{-1}) and high (∼1000 ng ml^{-1}). These unrealistic recovery percentages are a direct result of the non-linear behaviour of the MS response for these two compounds (see linearity) in combination with significant band broadening on the SPE cartridge as compared to the band broadening for direct injections. For CLZ and NOX, the recovery percentages calculated at low concentration level (i.e. where the calibration curve is still linear) should therefore be taken as most reliable. At this low concentration level recovery percentages of 90% (R.S.D. = 4%, n = 3) and 84% (R.S.D. = 2%, n = 3) are obtained for CLZ and NOX respectively. It should be noted that problems due to differences in the extent of band broadening do not occur under normal operating conditions, as both standards and samples are analysed using SPE prior to LC–MS. Differences in peak broadening, as observed when comparing direct injections with injections undergoing SPE, as is the case for recovery assessment, will therefore not occur under normal operation conditions.

For DMC, no additional retention and only limited band broadening and signal intensity reduction is observed. Moreover, the calibration curve for DMC is linear, so that the peak area of a broadened peak will be identical to the area of a narrow peak. At the medium and high concentration levels the respective recovery percentages are 96% (R.S.D. = 2%, n = 3) and 107% (R.S.D. = 1%, n = 3). At the low concentration level, recovery for DMC is significantly less (62%, R.S.D. = 34%, n = 3)). This result is in agreement with the limited accuracy and precision obtained for this compound at this concentration level. The high recovery percentages obtained are in accordance with literature data for most other methods where sample preparation is performed over a reversed phase extraction material [14,16,17,23,26,29].

Patient serum samples, obtained from the hospital pharmacy at Groningen Academic Hospital (The Netherlands) were analysed for their CLZ, DMC and NOX content. Results were compared with values independently obtained with the certified and round-robin tested method used at the hospital pharmacy. The chromatogram for patient sample number 3 (see Table 4) is presented in Fig. 6. Both the SIM traces for DMC and NOX show an additional chromatographic peak at the solvent front (0.66 min). Additional peaks in patient samples were also detected by other researchers in plasma [20] and urine [40]. These peaks were attributed to other CLZ metabolites, such as hydroxy metabolites, or glucuronidated metabolites. Indeed, a hydroxy metabolite, for example, has the same mass (and thus m/z for the singly charged state) as NOX and would show up in the SIM trace for NOX. Alternatively, the additional peaks may also result from endogenous or exogenous compounds providing ions with the same m/z by chance.

Table 4
Patient serum samples

Sample	CLZ[a] (ng ml^{-1})	DMC[a] (ng ml^{-1})	NOX[a] (ng ml^{-1})
#1	332 ± 3.0% (341)	305 ± 1.0% (294)	48 ± 1.5%
#2	212 ± 4.2% (245)	211 ± 1.1% (192)	44 ± 0.8%
#3	294 ± 1.0% (325)	211 ± 3.1% (178)	59 ± 1.6%
#4	306 (339)	263 (203)	54
#5	741 (873)	453 (427)	62

Concentrations measured for CLZ, DMC and NOX. For CLZ and DMC, a comparison with independent values obtained with a certified and round-robin tested method is provided (values in parentheses).
[a] Where sample size allowed, samples were analyzed in duplicate. Deviation of the individual samples from the average is given in percentage.

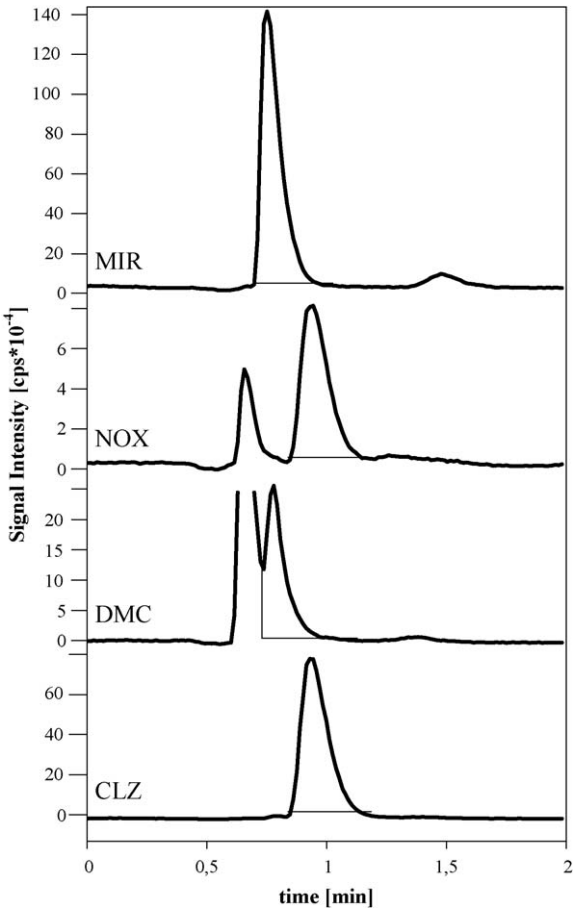

Fig. 6. Extracted ion chromatogram (XIC) ($[M+H]^+$ ions) of patient serum sample number 3, containing 294 ng/ml CLZ, 211 ng/ml DMC, and 59 ng/ml NOX, spiked with 211 ng/ml MIR (see experimental for *m/z* values). Mobile phase (300 μl/min): methanol–25 mM NH_4Ac (pH 4.5) (70:30); Column: Zorbax Eclipse C18 XDB (30 mm × 2.1 mm, 3.5 μm particle size); Injection: 50 μl; MS interface: ESI (no split). Traces are scaled individually in order to improve clearness. Base line definition as used for quantification purposes is included.

The quantification results for single MS in SIM mode are summarised in Table 4. There is a high degree of correlation between concentrations obtained with the method presented and the reference method. Absolute concentrations obtained for both CLZ and DMC only deviate from those obtained with the reference method by about 10% on average (underestimation for CLZ and overestimation for DMC), with an exception for DMC in sample number 4. For both DMC and CLZ deviations between the methods are sufficiently small to emphasize applicability of the high throughput method for TDM of CLZ and metabolites.

4. Conclusions

Our results show that automated concurrent on-line SPE-LC–MS has great potential for TDM. For the analysis of CLZ in serum, as a model system, a sensitive, selective, robust and fast method for the purpose of monitoring "blood levels" of CLZ (and metabolites; DMC and NOX) in schizophrenic patients receiving CLZ treatment is obtained. The method is based on SPE with a C18 stationary phase in a cartridge exchanger, followed by chromatographic separation with a C18 stationary phase and mass spectral detection. For LC–MS interfacing, both thermally assisted electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) have been evaluated. Detectability is shown to be better with the APCI interface, whereas selectivity is better with the ESI interface.

The throughput of the system has been increased by changing column length and mobile phase flow rate, modifier percentage and pH, allowing for cycle times of only about 2.2 min. This is a significant improvement as compared with any method previously published for CLZ and metabolites. Validation results are at least as good as for methods previously presented for the analysis of CLZ and metabolites in serum or plasma.

The possibilities for increasing throughput and the inherent prospect for automation, including the flexibility to analyse other drugs with the same system, make the developed system very appealing for TDM. The amenable characteristics of SPE sample preparation and MS detection are very important in this respect. It is only because of the effectiveness of sample clean-up and pre-concentration offered by SPE and the selectivity and sensitivity offered by MS detection that chromatographic separation can almost completely be omitted. Furthermore, as SPE of a sample runs in parallel with separation and detection of the previous sample (concurrent SPE), the time required for sample preparation is even less than with off-line parallel sample pre-treatment (e.g. using SPE in a 96-well format).

With respect to costs, the effectiveness of on-line SPE sample clean-up allows for the application of a simple and relatively cheap MS detector (i.e. without the ability to perform MS/MS), which is also very amenable from the perspective of a routine TDM laboratory.

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