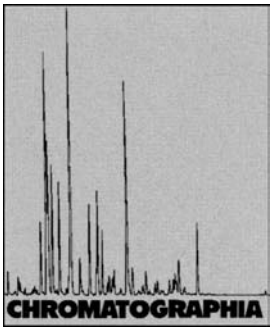
Multidimensional On-Line SPE for Undisturbed LC-MS-MS Analysis of Basic Drugs in Biofluids



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Abstract

In bioanalytical LC-MS-MS matrix effects influencing the ionization process are a major concern with respect to the quality of the results obtained. In general such matrix effects are directly related to an insufficient sample clean-up of the biofluids. In order to establish a MS-adequate clean-up procedure for basic analytes present in biofluids (e.g. urine, plasma) which is based on solid-phase extraction (SPE) principles a combination of tailor-made SPE column packings and automated column-switching was developed. This novel, multidimensional (MD) SPE platform relies on the combination of a SPE column packed with a restricted access material (RAM) allowing size-exclusion and reversed phase chromatography (SEC-RPC) and a second SPE column packed with a mixed-mode phase (MMP) allowing ion exchange and reversed phase chromatography (IEX-RPC). For the evaluation of this MD-SPE platform 8 tricyclic antidepressants and two metabolites were chosen as model analytes. In order to monitor matrix effects, i.e. ion suppression, postcolumn infusion experiments were performed and compared with a two-dimensional SPE column mode (SEC-RPC). The MD-SPE platform is highly efficient for removal of low and high molecular weight sample components which suppress ionization to varying extend. In addition electrospray ionization of the model analytes is not affected by inter- or intra-individual variations in the composition of the matrix investigated. It is also independent of the species the biofluids originate from. It was demonstrated that the MD-SPE platform has a generic potential with respect to on-line SPE of basic drugs having a p*Ka* > 6.5 and a moderate to low polarity and being present in different biofluids.

Keywords

Column liquid chromatography
Solid phase extraction
Column switching
Restricted access materials
Basic drugs in biofluids

Introduction

In pharmaceutical industry, liquid chromatography–tandem mass spectrometry (LC-MS-MS) is already an established method for quality control and quantification of drugs in different matrices. In the meantime, this hyphenated analytical tool is becoming more and more attractive for clinical chemical analysis, i.e. therapeutic drug monitoring (TDM).

However, despite of the high selectivity and specificity of modern mass spectrometers the analysis of highly complex samples such as blood, plasma and urine is often error prone. The main reasons for incorrect analyses are so called matrix effects. They are the result of a change in the ionization efficiency of the target analyte(s) which is caused by coeluting matrix components. Their presence can strongly influence the accuracy and precision of a MS method even if their signals are not recorded. Because of that, analyte signals can even be fully suppressed when analyzing samples with low analyte concentrations [1–4].

Especially electrospray ionization which is the predominant ionization technique in bioanalysis is very susceptible to matrix effects that are caused by low- and high molecular sample components. Due to the natural variation of endogenous compound concentrations within an individual, between individuals and between species these effects and thus false negative or false positive results are hardly predictable in analyzing biological fluids.

Because of these reasons, the favored dilute and shoot mode [5–8] for sample preparation, often is not suited for a reliable LC-MS-MS analysis of biofluids. On the contrary, a LC-MS-MS adequate pretreatment of biological fluids involves multiple steps to separate the target analyte(s) from interfering matrix constituents.

For clean-up of complex biofluids prior to LC-MS analysis solid-phase

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extraction (SPE) is the predominant technique. However, most of these off-, at- or even on-line SPE procedures rely on conventional sorbents which posses the drawback of being rather unselective. This is because not only the target analytes are extracted or even enriched but also other low as well as high molecular weight matrix components which potentially can interfere with the ionization process. This is particularly true for high molecular weight components, i.e. proteins, because conventional SPE sorbents are not biocompatible and cause non-specific adsorption and desorption respectively of proteins with on-line SPE. This, in turn, causes a clogging of the SPE column and shortens its lifetime significantly. Moreover, unspecifically bound proteins change the selectivity of the extraction step in an irreproducible manner. As a result, most sample clean-up protocols which use SPE columns (e.g. Oasis HLB or Cohesive HTC) in an on-line mode include a protein precipitation step in order to minimize these effects [9–12]. However, Polson et al. [13] showed that protein precipitation is not efficient enough to remove all matrix components that cause ion suppression.

From this it follows that a LC-MS adequate clean-up of biofluids which is based on SPE should include 1) complete removal of high molecular weight matrix components such as proteins, polysaccharides and nucleic acids; 2) complete removal of low molecular weight sample components which influence the electrospray ionization process; 3) extraction of a broad spectrum of analytes; 4) robustness, e.g. long SPE column lifetime; 5) total automation; 6) high speed and throughput respectively.

We have accomplished this, as described in this paper, with the development of a multidimensional SPE platform for extractive on-line clean-up and subsequent undisturbed LC-MS-MS analysis of drugs with basic properties such as antidepressants in crude biofluids such as plasma and urine.

The MD-SPE platform is composed of two commercially available SPE columns packed with special sorbents. The chromatographic separation power and selectivity of the platform is due to the use of at least three different chromatographic dimensions. The combined SPE columns fulfill the criteria for a multidimensional separation system [14,15].

Experimental

Reagents and Solutions

The tricyclic antidepressants amitriptyline (ATL), clomipramine (CMP), desipramine (DSP), doxepin (DXP), imipramine (IMP), maptrotiline (MTL), nortriptyline (NTL) and trimipramine (TMP) and the metabolites norclomipramine (NCP) and nordoxepin (NDX) were all obtained from Sigma-Aldrich (Steinheim, Germany). The internal standard (IS) d6-amitripyline (d6-ATL) was obtained from Euriso-top (Gif-sur-Yvette, France). Formic acid (min. 99.8%), ammonium acetate and acetonitrile (gradient grade) were from Merck KGaA (Darmstadt, Germany). Water and methanol (both HPLC grade) were obtained from J.T. Baker (Deventer, the Netherlands). Serum and urine samples from poultry, cattle and swine were provided by the Landesuntersuchungsanstalt of Saxony (Chemnitz, Germany).

Stock solutions containing the tricyclic antidepressants (100 μg mL^{-1}) and stock solution of IS (100 μg mL^{-1}) were both prepared using volumetric flasks by dissolving accurately weighted amounts of powder in methanol. All stock solutions were kept at +4 °C.

Calibration standards were prepared in drug free plasma to yield concentrations equal to 1, 5, 15, 25, 50, 100, 250, 500 and 1000 ng mL^{-1} for each antidepressant.

Three levels of plasma quality controls (5, 50 and 500 ng mL^{-1}) were prepared using the same procedure in order to evaluate precision, trueness, recovery and limit of quantitation of the method.

MD-SPE Set-up

The MD-SPE platform (Fig. 1) consists of a LiChrospher® ADS RP4 RAM SPE column (25 mm × 2 mm ID, 25 μm particle size; Merck KGaA, Darmstadt, Germany), an Oasis WCX MMP SPE column (20 mm × 2.1 mm ID, 30 μm particle size; Waters, Milford, USA) and an analytical LC column (Zorbax RX-C18, 75 mm × 4.6 mm ID, 3.5 μm particle size; Agilent, Waldbronn, Germany) which are connected by two 6-port switching valves. The mobile phases for the system are delivered by two model 1100 binary pumps (Agilent) and a model L-6200 HPLC pump (Merck KGaA). The autosampler HTC PAL (CTC Analytics, Zwingen, Switzerland) was programmed to control the two switching valves.

A triple quadrupole MS (Quattro micro, Waters, Milford, USA) equipped with an electrospray source operating in the positive mode was used for mass spectrometric detection. The spectra were recorded in multiple reaction monitoring (MRM) mode. The mass transitions of the tricyclic antidepressants used as model analytes were as follows: ATL (278.4 > 233.4), CMP (315.4 > 86), DSP (267.4 > 71.9), DXP (280.4 > 107.3), IMP (281.4 > 86), MTL (278.4 > 250.3), NCP (301.4 > 71.9), NDX (266.4 > 107.3) NTL (264.4 > 91.2), TMP (295.5 > 100.4) and d6-ATL (284.4 > 90.1). The dwell time for each of these transitions was 0.1 s. Source temperature was set to 130 °C, desolvation temperature to 330 °C, desolvation gas flow to 700 L h^{-1}, cone gas flow to 25 L h^{-1}, capillary voltage to 3.2 kV and cone voltage to 20 V.

MD-SPE Procedure

The operational procedure of the MD-SPE platform is shown in Table 1 and explained in the following. The untreated biofluid (injection volume 20 μL) is directly injected by the HTC PAL onto the RAM SPE column, where a size-selective fractionation of the sample takes place. High molecular weight sample components (MW > 15 kDa) are excluded from the inner pore surface and washed to waste within 1 min at a flow rate of 4 mL min^{-1} while low molecular weight sample components including the target analytes are extracted by RP partitioning. This step is performed using eluent E1 which is composed of 95% water and 5% acetonitrile (*v*/*v*). Thereafter, the first valve (V1) is switched and the retained low molecular weight components are transferred to the MMP SPE column at a flow rate of 3 mL min^{-1}. The low molecular weight components are then chemoselectively fractionated on the MMP SPE column. The target analytes are retained on the MMP material and the remaining matrix components are washed to waste within 1.5 min. For this step eluent E2 is used which is composed of 80% methanol and 20% 2 mM ammonium acetate (*v*/*v*). After this chemo-selective fractionation the second valve (V2) is switched

Table 1. Instrumental settings for the MD-SPE-LC-MS-MS procedure

Time (min)	Valve position		Pump E1		Pump E2		Pump E3	
	V1	V2	% B (E1)	Flow (mL min^{-1})	% B (E2)	Flow (mL min^{-1})	% B (E3)	Flow (mL min^{-1})
0.00	0	0	100	4.0	100	0.5	100	1.0
0.90	0	0	100	4.0	100	3.0	100	1.0
1.00	1	0	0	0.1	100	3.0	100	1.0
2.50	0	1	0	2.0	0	0.1	100	1.0
4.00	0	0	100	2.0	0	2.0	100	1.0
4.50	0	0	100	0.5	100	2.0	100	1.0
5.50	0	0	100	0.5	100	0.5	100	1.0
8.00	0	0	100	0.5	100	0.5	100	1.0

and the extracted target analytes are transferred to the analytical column at a flow rate of 1 mL min^{-1} with eluent E3, which is composed of 40% acetonitrile and 60% 25 mM ammonium acetate pH 3 (*v*/*v*). This eluent is splitted to a fifth before entering the MS. Finally, both valves are switched to their initial positions and both SPE columns are washed and reequilibrated. For the RAM SPE column the washing solution is acetonitrile and for the MMP SPE column an eluent composed of acetonitrile and 0.4% formic acid (99.6/0.4; *v*/*v*) was used.

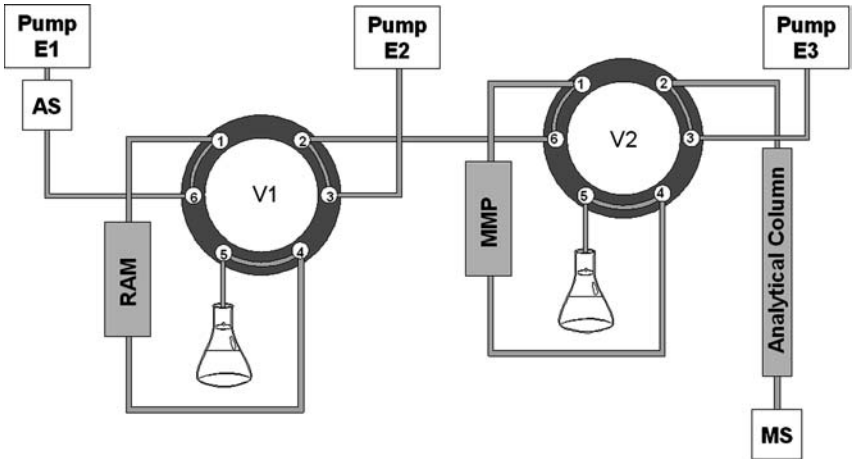

Fig. 1. Instrumental set-up of the MD-SPE platform coupled to a LC-MS-MS system. Positions of 6-port switching valves (V1 and V2) depicted correspond to the start of the MD-SPE-LC-MS-MS analysis cycle. The RAM SPE column is loaded with the sample by the autosampler (AS). The MMP SPE column and the analytical column are equilibrated by eluent E2 and E3 respectively

RAM SPE-LC Set-up and Procedure

For the evaluation of matrix effects the MD-SPE platform was compared with a two-dimensional on-line SPE method which applies only the RAM SPE column [16]. The instrumental set-up corresponds in part to that illustrated in Fig. 1. Position 2 of valve 1 is disconnected from valve 2 and directly connected to the analytical column and mass spectrometer respectively. Size-selective fractionation of the biofluids and simultaneous extraction of the analytes is identical to that performed on the MD-SPE platform. For the elution of the target analytes from the RAM SPE column onto the analytical column an eluent composed of 80% methanol and 20% 2 mM ammonium acetate pH 3 is used at a flow rate of 1 mL min^{-1}. After 3 min the valve is switched back to its initial position and the RAM SPE column is washed and reequilibrated as described above.

Monitoring of Matrix Effects

For the monitoring of matrix effects postcolumn infusion experiments were performed using an experimental set-up as described by King et al. [17].

A t-piece is placed between the outlet of the analytical column and the splitting device in front of the MS. A standard solution (1 μg mL^{-1}) of the target analytes, i.e. the tricyclic antidepressants is infused into the eluent stream at a flow rate of 10 μL min^{-1} and the corresponding MRM transitions of the analytes are recorded. After obtaining a steady baseline a blank sample (e.g. plasma or urine) is injected and processed either by the MD-SPE-LC system or the RAM SPE-LC system. Any eluting compound that interferes with the ionization of the target analytes leads to an elevation or a depression of the baseline which mirrors the matrix effects.

Validation

To verify the analytical quality of the described MD-SPE platform for the determination of the tricyclic antidepressants as model analytes a short validation of the overall analysis procedure was performed.

Nine plasma calibration standards were prepared and analyzed in three replicates. Calibration curves were calculated based on the measurement of the peak ratio of the antidepressants to that of the internal standard d6-amitriptyline. To fit the measured signals versus the concentrations of the calibration standards least square linear regression with a weighting factor of 1/x was used.

Quality control samples at three concentration levels (5, 50 and 500 ng mL^{-1}) were analyzed to assess trueness and precision of the MD-SPE platform. Five replicates were analyzed in each. For inter-day precision 3 quality control samples were analyzed the following day. The trueness was expressed by the relative percentage error (Er %), and the precision by relative standard deviation (RSD %).

Recovery was determined by comparing peak heights obtained from analysis of quality control samples using the

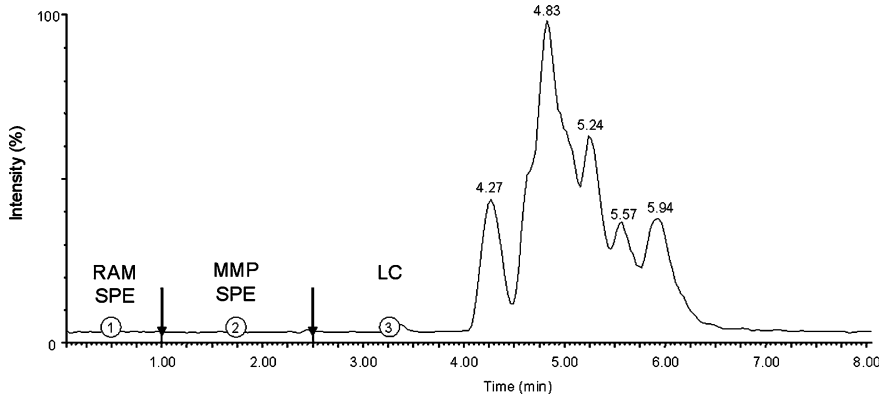

Fig. 2. Representative chromatogram of the investigated 8 antidepressants and two of their major metabolites obtained after MD-SPE-LC-MS-MS analysis of 20 μL of human plasma. **1** = Size-selective fractionation of high- and low molecular weight sample components. **2** = Chemo-selective fractionation of remaining low molecular weight sample components. **3** = Analytical LC-separation of target compounds. TIC of 10 MRM traces

MD-SPE platform with those obtained from analysis of standard solutions of the same concentrations (10 ng mL^{-1} for each antidepressant) after direct injection onto the analytical column.

Results and Discussion

MD-SPE Platform

Fig. 2 shows a representative chromatogram of the model analytes, i.e. 8 tricyclic antidepressants, 2 major metabolites and the internal standard d6-ATL, recorded after the direct injection and on-line processing of 20 μL of a spiked human plasma sample (25 ng mL^{-1}).

Size-selective fractionation of the raw, complex biofluids on the RAM SPE column takes only 60 s and thus is adequate for MS analysis. This very fast on-line sample clean-up could be achieved by choosing appropriate dimensions of the RAM SPE column, i.e. 25 × 2 mm, and a relatively high flow rate of the mobile phase used for the fractionation step. The addition of 5 vol % of acetonitrile to this mobile phase guarantees a quantitative displacement of drugs from their protein binding sites. The MD-SPE platform tolerates the injection of human plasma and urine up to a volume of 100 μL without changing any chromatographic parameters.

LiChrospher® ADS [18, 19], was chosen as RAM SPE column packing for several reasons. Due to its relatively low molecular weight cut-off of approximately 15 kDa LiChrospher® ADS excludes most of the high molecular weight compounds, e.g. proteins, present in biofluids from access and potential unspecific binding to the extractive stationary phase. LiChrospher® ADS SPE columns are available in a SPE-LC-MS adequate size and hydrophobicity including a C-4, C-8 and C-18 RP modification. The relative large particle size of 25 μm allows high flow-rates of up to 8 mL min^{-1} without a decrease of the chromatographic performance and a drastic increase of back pressure [20–22]. Finally, the lifetime of a SPE-column packed with LiChrospher® ADS material exceeds more than 2000 injections, each of 20 μL of human plasma [22].

Using this type of RAM SPE-column two chromatographic processes are performed simultaneously, namely reversed phase and size exclusion chromatography (RPC-SEC). Both chromatographic modes are orthogonal. Therefore the first SPE column represents a two-dimensional chromatographic system.

With respect to the packing material of the second SPE-column (MMP) we screened several weak cation exchange materials. Oasis WCX (Waters) turned out to be the most appropriate one with regard to ion-exchange capacity and hydrophobicity. By applying this mixed-mode polymer (MMP) as stationary phase an additional and orthogonal chromatographic mode is performed on the second SPE-column, namely ion-exchange chromatography (IEX). The described MD-SPE procedure thus relies at least on the 3 different chromatographic principles. In this context, it should be mentioned that for apolar analytes the retention mechanism of the alkyl-silica bonded phase of the RAM SPE column is different from the one of the mixed-mode polymer. The platform also fulfills another criterion for multidimensionality, namely comprehensiveness. However, this holds only for the low-molecular weight sample components being extracted on the first SPE-column.

The MD-SPE platform is rather flexible with regard to adjusting its chromatographic properties. In principle, all four chromatographic modes applied can be tuned for a given analyte which possesses basic properties and is present in different biofluids. With regard to the RAM SPE column the molecular weight cut-off can be varied or one can chose different RP modifications [23, 24]. With regard to the MMP SPE-column the pH of the mobile phase can be varied and the amount as well as the type of organic modifier can be adjusted so that only the target analyte is retained or desorbed. These measurements allow to fine-tune the MD-SPE platform for basic target analytes having a p*K*a value > 6.5. In order to demonstrate this, we investigated three additional model analytes which differ in their basicity and polarity, i.e. p*K*a and Log*P* value.

The antibiotic trimethoprim exhibits a p*K*a of 6.6 and a Log*P* of 1.43. Compared to the antidepressants described this analyte has a higher polarity and therefore a more apolar LiChrospher® ADS SPE column, i.e. a RP 18 modification was applied for the extraction of trimethoprim from human plasma. All other chromatographic conditions including valve switching times were identical to those for MD-SPE analysis of the antidepressants. For detection the transitions 291.4 > 123.3 m/z (quantifier) and 291.4 > 230.3 m/z (qualifier) were used. Overall analysis time was 4.5 min (data not shown).

The anesthetic ketamine has a p*K*a of 7.5 and a Log*P* of 2.58. It was extracted from spiked human plasma using a RP 8 modified LiChrospher® ADS SPE column. All other chromatographic conditions corresponded to those used for the analysis of antidepressants and trimethoprim respectively. Ketamine was detected using the MRM transitions 238.3 > 125.3 m/z and 238.3 > 162.9 m/z. Overall analysis time was 4.5 min (data not shown).

The third model analyte investigated to proof a generic applicability of the MD-SPE platform was the β-agonist

clenbuterol. For monitoring its abuse this drug preferentially is analyzed in urine of cattle and human beings [25–26]. Fig. 3 depicts the corresponding chromatogram obtained after MD-SPE-LC-MS-MS of clenbuterol in spiked human urine. The drug was detected using the MRM transition 277.3 > 203.0 *m/z*. Clenbuterol has a p*K*a of 8.86 and a Log*P* of 2.61 allowing the use of a RP 4 modified LiChrospher® ADS SPE column for the fractionation step. In order to obtain optimal separation, some chromatographic conditions had to be slightly changed. The chemoselective fractionation step was already terminated after 1 min. The composition of the mobile phase E2 (cf. Fig. 1) was acetonitrile / 2.5 mM ammonium acetate pH 6.0 (50/50, *v/v*). The analytical separation was achieved on a Reprosil Pur C18 Aq column (125 × 2 mm, 5 μm, Maisch, Ammerbuch, Germany) with a mobile phase E3 (cf. Fig. 1) of acetonitrile/50 mM ammonium acetate pH 3.0 (45/55, *v/v*).

Besides its selectivity, the MD-SPE procedure also stands out by its speed and throughput. The fully automated and highly selective clean-up of basic drugs in complex matrices is achieved in less than 150 s. This means that sample pretreatment prior to LC-MS analysis of these drugs is not the rate limiting step anymore.

Evaluation of Matrix Effects

For the monitoring of matrix effects, i.e. ion suppression, post-column infusion experiments were performed.

In order to evaluate the MD-SPE-(LC) procedure with regard to its impact on matrix effects we compared it with a two dimensional SPE method, i.e. RAM SPE-(LC) (see Experimental). For that purpose a standard solution of the model analytes (8 antidepressants and 2 metabolites) was infused into the eluent stream of the analytical column. After the corresponding MRM transitions had reached a constant baseline, a sample of distilled water, raw human plasma or raw human urine was injected onto the MD-SPE-LC or RAM SPE-LC system (Fig. 4).

As expected, injection of water revealed no ion suppression effects in both systems. The observed distortion of the baseline is due to valve switching. The

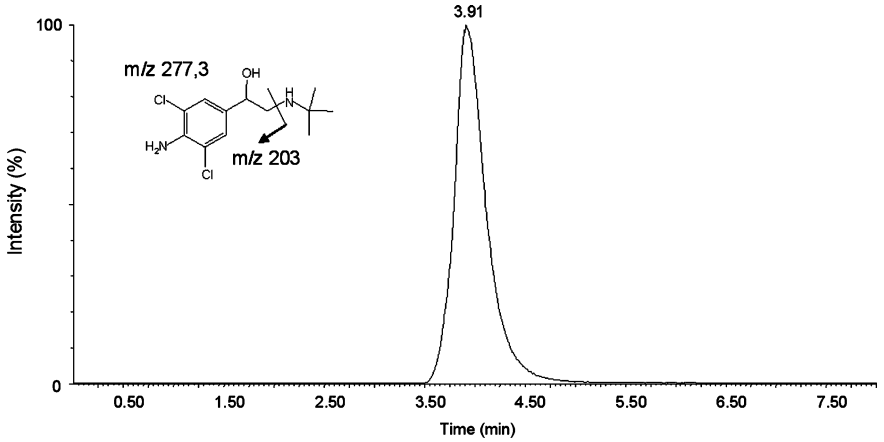

Fig. 3. MD-SPE-LC-MS-MS analysis of clenbuterol in spiked (500 ng mL^{-1}) human urine (20 μL injection). MRM transition 277.3 > 203 *m/z*

noise of the baseline is due to fluctuations of the syringe pump. However, the injection of a plasma or urine sample onto the RAM SPE-LC system caused a significant drop in the ionization yield (Fig. 4A). This suppression persisted for almost 4 min under the applied chromatographic conditions.

The matrix effects observed are more pronounced in urine (Fig. 4A, 3) presumably due to the higher complexity and concentration of low-molecular weight compounds in urine compared to plasma. The time window found for ion suppression covers the range where the model analytes elute (Fig. 4*). Detection and therefore quantification of these analytes are thus significantly disturbed, if not impossible.

Injection of the same plasma and urine sample onto the MD-SPE-LC system revealed no matrix effects. This means that the MD-SPE platform obviously removed all matrix components which potentially could suppress the ionization of the model analytes. This is further demonstrated by scanning the *m/z* range from 100 to 1000 (Fig. 5A and B). The MS-scan of blank human plasma and urine revealed no masses, i.e. no matrix components. The peaks recorded at 3.4 and 3.5 min are due to valve switching. These findings are also in accordance with those using a diode array detector (DAD). For detection of remaining matrix components the DAD was set to scan the wavelength from 220 to 400 nm [27].

Biofluids such as plasma and especially urine represent highly complex matrices, whose compositions can vary not only between individuals and species but also within an individual. This heterogeneity and variation, subsequently gives rise to varying matrix effects. In order to evaluate such matrix effects related to intra- and inter-individual as well as inter-species variations we first investigated 11 plasma and 6 urine samples from different healthy donors. For comparison we recorded infusion chromatograms, obtained using a RAM SPE-LC system (Fig. 6).

The distortion of the baseline between 1.5 and 2.0 min in the infusion chromatograms recorded in Fig. 6 is caused by valve switching. The noise of the baseline results from the fluctuations of the syringe pump. From the chromatographic profiles obtained from plasma samples from 11 individuals and urine samples from 6 individuals it is obvious that the observed matrix effects differ significantly between individuals.

Injection of the same plasma and urine samples, on the other hand, onto the MD-SPE-LC system revealed no matrix effects (Fig. 7). This means that the MD-SPE platform eliminates also those matrix components which are responsible for the inter individual variations with regard to the ionization of the model analytes.

Fig. 8A shows infusion chromatograms obtained with the RAM SPE-LC system after injection of different plasma samples taken from a volunteer. Plasma sample 1 represents the matrix under fasting conditions and shows the weakest ion suppression. Plasma sample 2 was taken 1.0 h after a large breakfast and revealed, as expected, the most pronounced ion suppression. Plasma samples 3 and 4 were taken 3.0 and 6.0 h after food intake and showed an almost identical ion suppression profile.

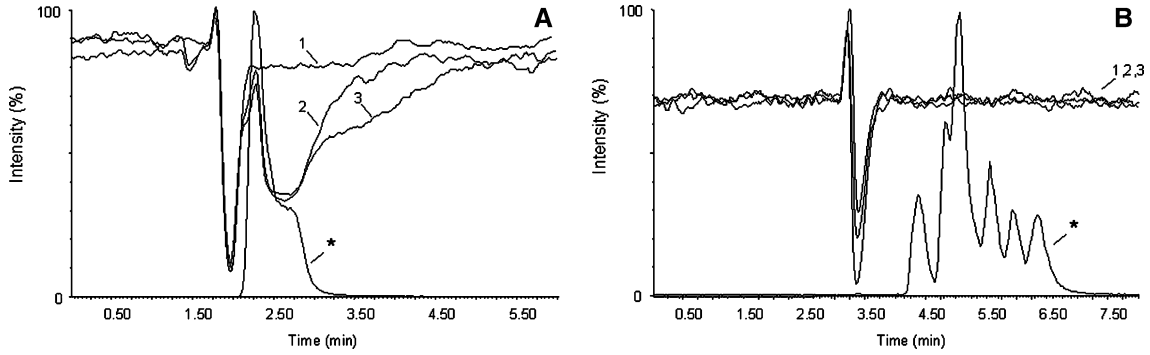

Fig. 4. Comparison of infusion chromatograms monitored via TIC of MRM transitions of the model analytes investigated. **A**) RAM SPE-LC-MS-MS system **B**) MD-SPE-LC-MS-MS system. Samples (20 μL) investigated: **1** = distilled water; **2** = human plasma; **3** = human urine.*) Overlays of representative LC-chromatograms of the analytes investigated after direct injection and on-line processing of spiked human plasma samples using the corresponding analysis system

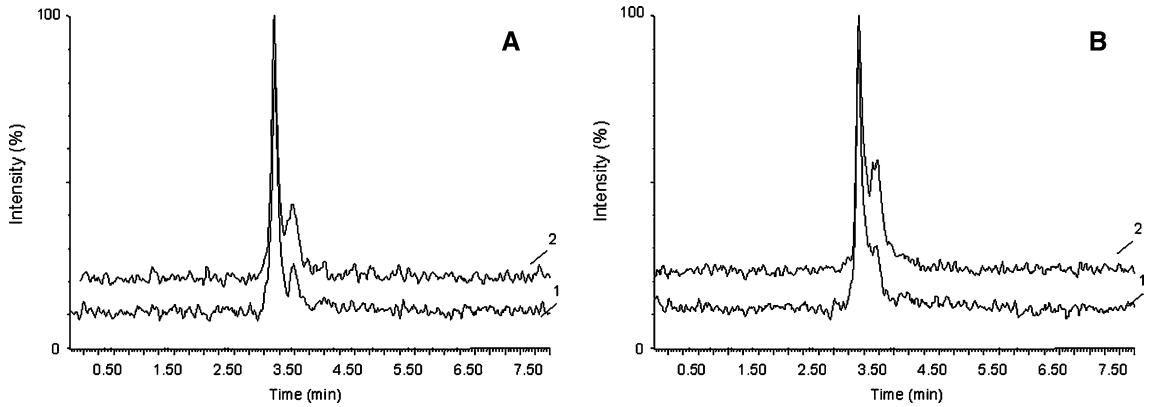

Fig. 5. Representative chromatograms of real samples obtained with the MD-SPE-LC-MS system and monitored via TIC of MS scans ranging from 100 – 1000 *m/z*. Injection volumes: **1** = 20 μL; **2** = 100 μL. **A**) Blank human plasma **B**) Blank human urine

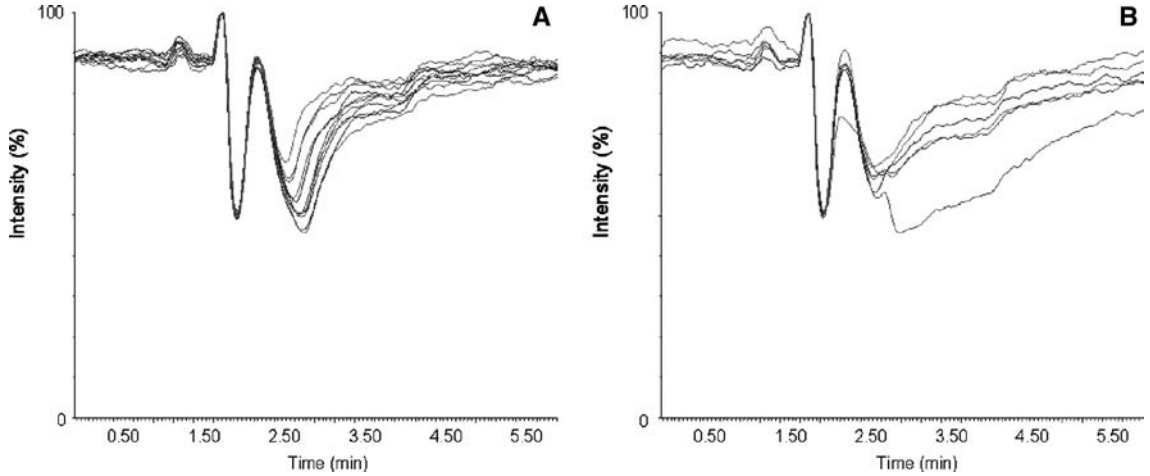

Fig. 6. Infusion chromatograms obtained with the RAM SPE-LC system and monitored via TIC of MRM transitions of the model analytes investigated. **A**) Spiked human plasma samples (20 μL) from 11 individuals **B**) Spiked human urine samples (20 μL) from 6 individuals

Again, injection of the same plasma samples onto the MD-SPE-LC system revealed no matrix effects (Fig. 8B). This demonstrates that the MD-SPE platform also effectively eliminates matrix components, supposedly triglycerides, which cause intra-individual variations of the ionization of the analytes investigated.

Finally, we investigated if the MD-SPE platform is also suitable for a MS adequate clean-up of biofluids originating from other species than man. The different biofluids (plasma and urine from man, serum and urine from cattle as well as swine and serum from poultry) were first analyzed with the RAM SPE-LC system (Fig. 9A) and then for comparison with the MD-SPE-LC system (Fig. 9B).

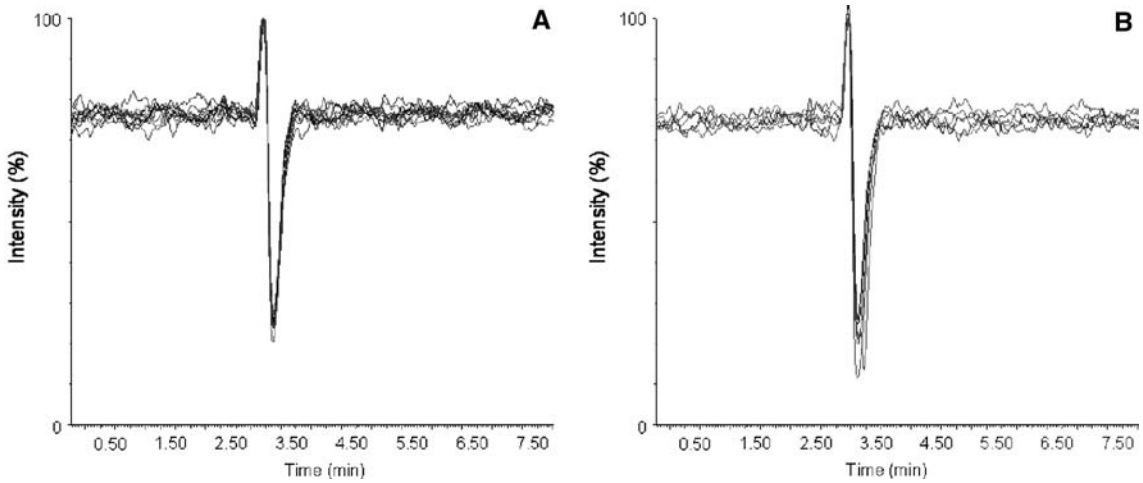

Fig. 7. Infusion chromatograms obtained with the MD-SPE-LC system and monitored via TIC of MRM transitions of the model analytes investigated. **A**) Spiked human plasma samples (20 μL) from 11 individuals **B**) Spiked human urine samples (20 μL) from 6 individuals

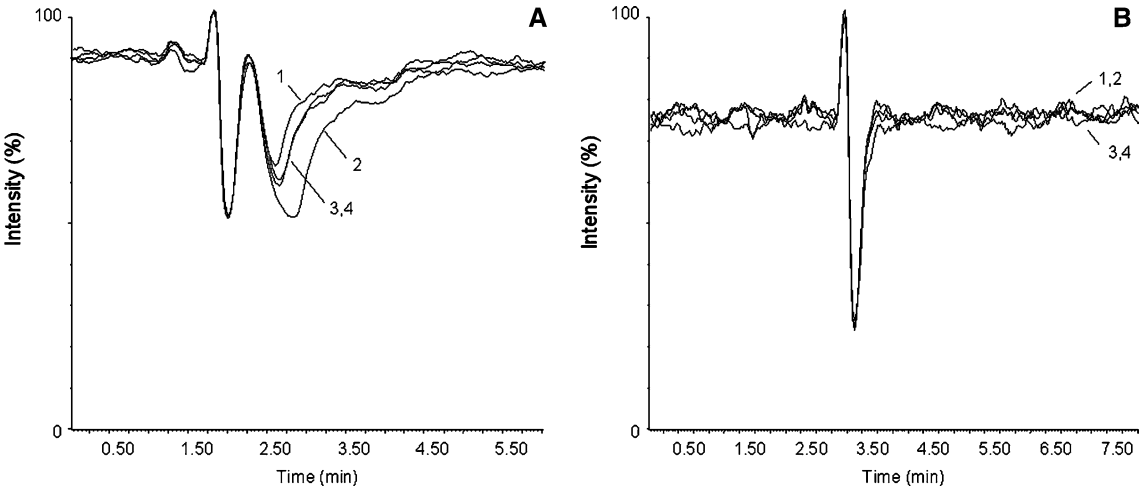

Fig. 8. Comparison of infusion chromatograms monitored via TIC of MRM transitions of the model analytes investigated. **A**) RAM SPE-LC-MS-MS system. **B**) MD-SPE-LC-MS-MS system. Plasma samples (20 μL) investigated: **1** = fasting conditions, **2** = 1.0 h, **3** = 3.0 h and **4** = 6.0 h after food intake

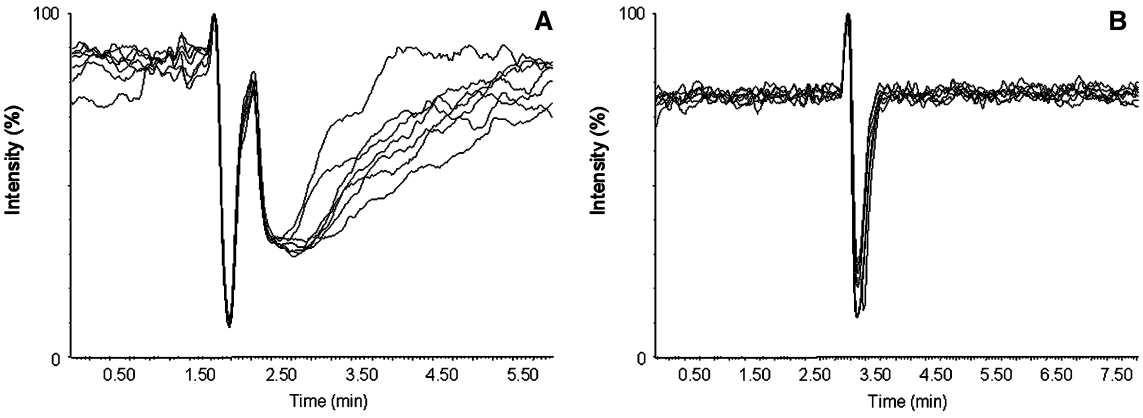

Fig. 9. Comparison of infusion chromatograms monitored via TIC of MRM transitions of the model analytes investigated. **A**) RAM SPE-LC-MS-MS system. **B**) MD-SPE-LC-MS-MS system. Samples (20 μL) investigated: plasma, serum and urine from man, swine, cattle and poultry

The biofluids from different species revealed different ion suppression profiles when processed with the RAM SPE-LC system. Matrix effects were weakest in serum from cattle and strongest in urine from cattle. The corresponding profiles of the other biofluids varied, but were within these boundaries.

Again, after injection of the same set of samples onto the MD-SPE-LC system no matrix effects could be detected. From these results it follows that the described MD-SPE platform can process different biofluids such as plasma, serum and urine which even

Table 2. Trueness and intra- and inter-day precision

Analyte	Spiked concentration (ng mL^{-1})	Calculated concentration (ng mL^{-1})	Trueness $n = 5$ (Er %)	Precision Intra-day $n = 5$ (%)	Precision Inter-day $n = 8$ (%)
ATL	5.0	5.0	0.4	3.8	4.5
	50.0	51.6	3.1	3.2	3.4
	500.0	511.5	2.3	1.8	2.2
CMP	5.0	5.0	0.2	2.7	2.7
	50.0	52.0	3.9	2.3	2.6
	500.0	512.2	2.4	2.1	2.3
DSP	5.0	5.2	4.5	2.2	2.6
	50.0	51.9	3.8	2.0	3.0
	500.0	504.5	0.9	1.8	1.9
DXP	5.0	5.0	0.8	3.1	4.2
	50.0	51.7	3.3	3.3	3.3
	500.0	491.7	−1.6	2.1	2.2
IMP	5.0	5.2	3.8	2.0	2.8
	50.0	51.9	3.7	2.3	2.5
	500.0	494.7	−1.1	2.0	2.1
MTL	5.0	5.2	4.6	3.0	3.3
	50.0	51.6	3.1	2.4	2.5
	500.0	516.2	3.2	2.0	2.2
NCP	5.0	5.2	4.2	3.0	3.3
	50.0	52.5	5.1	2.2	2.4
	500.0	498.6	−0.3	2.1	2.1
NDX	5.0	5.2	3.7	3.1	3.4
	50.0	51.1	2.3	2.9	3.1
	500.0	505.8	1.1	1.8	2.0
NTL	5.0	5.3	5.1	3.0	3.3
	50.0	51.5	2.9	2.1	2.2
	500.0	515.8	3.2	1.9	2.2
TMP	5.0	5.2	3.5	2.7	3.0
	50.0	51.2	2.4	2.1	2.1
	500.0	497.1	−0.6	2.0	2.0

Table 3. Concentration ranges, calibration curves, correlation coefficients, recovery, LOD and LLOQ of the MD-SPE-LC-MS-MS procedure

Analyte	Concentration range (ng mL^{-1})	Calibration curve $y = a\,x + b$	Correlation coefficient	Recovery (%)	LOD (ng mL^{-1})	LLOQ (ng mL^{-1})
ATL	5.0 – 1000	$y = 0.018\,x + 0.0051$	0.9990	101.9 ± 3.4	1.2	5.0
CMP	5.0 – 1000	$y = 0.083\,x + 0.0039$	0.9999	99.3 ± 1.3	1.0	5.0
DSP	1.0 – 1000	$y = 0.093\,x + 0.0090$	0.9998	99.6 ± 2.9	0.2	1.0
DXP	5.0 – 1000	$y = 0.040\,x + 0.0027$	0.9994	98.4 ± 2.5	0.9	5.0
IMP	5.0 – 1000	$y = 0.111\,x + 0.0081$	0.9996	100.7 ± 1.7	0.9	5.0
MTL	1.0 – 1000	$y = 0.030\,x + 0.0057$	0.9996	100.5 ± 2.3	0.2	1.0
NCP	1.0 – 1000	$y = 0.066\,x + 0.0069$	0.9999	100.5 ± 1.8	0.2	1.0
NDX	1.0 – 1000	$y = 0.039\,x + 0.0080$	0.9998	98.8 ± 2.1	0.4	1.0
NTL	1.0 – 1000	$y = 0.032\,x + 0.0034$	0.9992	98.1 ± 2.6	0.5	1.0
TMP	5.0 – 1000	$y = 0.103\,x + 0.0032$	0.9998	99.6 ± 2.1	1.0	5.0

can originate from different species. In addition, this is achieved without a change of chromatographic conditions and without a loss in performance with respect to the elimination of matrix effects.

Precision and Trueness

For therapeutic drug monitoring (TDM) intra- and inter-day precision is required to be within ± 20% at the lower limit of quatitation (LLOQ) and within ± 15% for higher concentrations. Trueness is required to be less than 20% at the LLOQ and less than 15% at higher concentrations [28]

The short validation of the intra-day and inter-day precision as well as trueness values for the MD-SPE-LC-MS-MS procedure remain within the required limits for all tricyclic antidepressants at the investigated concentrations (Table 2).

Linearity

The fully automated method for the determination of the model analytes, i.e. tricyclic antidepressants, in biofluids was linear from sub-therapeutic to overdose concentrations for all compounds investigated. Calibration curves, linear ranges and correlation coefficients are shown in Table 3.

Limit of Detection and Lower Limit of Quantitation

The limit of detection (LOD) was defined as the concentration of each analyte investigated giving a signal-to-noise ratio of 3. The lower limit of quantitation (LLOQ) was defined as the lowest concentration in the calibration curve. Data obtained for LLOQ, LOD and recovery of the analytes from human plasma are summarized in Table 3.

Conclusions

We were able to demonstrate, for the first time, that a distinct combination of chromatographic separation modes

applied for on-line SPE of biofluids, completely eliminates matrix effects otherwise encountered in ESI-MS detection of basic drugs.

The multidimensional SPE platform uses a combination of a RAM SPE column and a MMP SPE column and allows the direct injection and fully automated clean-up of different biological fluids as well as the undisturbed LC-MS-MS analysis of basic drugs.

The MD-SPE platform eliminates and compensates, respectively all varying degrees of matrix effects when analyzing different specimens such as plasma, serum and urine originating from an individual, from different subjects and from different species.

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