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Generic solid phase extraction–liquid chromatography–tandem mass spectrometry method for fast determination of drugs in biological fluids

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Abstract

A generic method was developed for the fast determination of a wide range of drugs in serum or plasma. The methodology comprises generic solid-phase extraction, on-line coupled to gradient HPLC with tandem mass spectrometric detection (SPE–LC–MS/MS). The individual components of the SPE–LC–MS/MS system were optimized in an integrated approach to maximize the application range and minimize the method development time. The optimized generic SPE–LC–MS/MS protocol was evaluated for 11 drugs with different physicochemical properties. Good quantification for 10 out of 11 of the pharmaceuticals in serum or plasma could be readily achieved. The quantitative assays gave recoveries better than 95%, lower quantification limits of 0.2–2.0 ng/ml, acceptable precision and accuracy and good linearity over 2–4 orders of magnitude. Carry-over was determined to be in the range of 0.02–0.10%, without optimization.

Keywords: Generic method; Drug analysis

1. Introduction

The combination of a rapidly increasing output from new, high-throughput drug discovery processes and the demand to bring products faster to the market results in a strong drive to accelerate the drug development process in the pharmaceutical industry. An essential, but time consuming element in the drug development process is bioanalysis, i.e. quantitative determination of drugs in biological fluids to support drug development. The successful development of atmospheric pressure interfaces has made LC–MS/MS the major tool for bioanalysis [1]. LC–MS/MS offers high specificity, good precision and accuracy, a wide dynamic range and high sensitivity. Due to its mass selectivity, it was expected that method de-

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velopment time and sample turnover time would be reduced significantly. However, matrix ion-suppression, a specific LC–MS interface related phenomenon, requires that the majority of biological matrix constituents are removed prior to LC–MS/MS analysis [2–5] making sample preparation a time consuming element in the development of LC–MS/MS bioassays. During the process of drug development the demands on sensitivity, precision and accuracy become more stringent, resulting in increasing assay development time (ranging from 1 day for ''quick and dirty'' discovery work up to several weeks for a fully validated assay applicable for (pre-)clinical study samples). Especially, for the analysis of samples from pre-clinical and clinical studies, high demands on specificity, accuracy and precision must be complied with in case the results are to be used for drug registration [6]. To speed up the method development process down the line of drug discovery, development and clinical study, we are investigating the possibilities of ''generic'' solid phase extraction (SPE) coupled on-line to fast ''generic'' gradient-LC with MS/MS detection [7] for fully automated bioassays with little or no method development. Apart from full automation, state-of-the-art on-line SPE also provides high precision and sensitivity [8] and a higher sample throughput as compared to liquid–liquid extraction or off-line SPE. The aim of this study is to formulate a generic set of conditions for SPE and gradient-LC, which can be used to develop quantitative bio-analytical SPE–LC–MS assays to support discovery work within a few hours. Further method optimization for application in drug development should require only minor modifications such as pH adjustment of the sample to increase recovery or SPE clean-up optimization to reduce ionization interference. Naturally, MS/MS conditions need to be optimized for every single analyte.

Several SPE sorbents and different extraction and elution conditions were tested for a set of model compounds spiked into serum or plasma. The generic on-line SPE–LC–MS/MS methodology was optimized and evaluated with respect to analyte diversity, method development time, sample throughput, quantification limit, precision, accuracy and carry-over.

2. Experimental

2.1. Materials and reagents

2.1.1. Reagents and chemicals

HPLC-grade acetonitrile was purchased from Labscan (Dublin, Ireland) and HPLC-grade water from J.T. Baker (Deventer, the Netherlands). Ammonium acetate was purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands). Phenol, salicylic acid, theobromine, caffeine, sulfadiazine, sulfamerazine, propranolol, carbamazepine, procainamide, ranitidine, acetaminophen, pentachlorophenol, theophylline and taxol were purchased from Sigma–Aldrich. Porcine serum was obtained from ICN Biomedicals (Aurora, Ohio, USA). Human plasma was obtained from Charterhouse (UK). A stock solution of taxol of 2000 μg/ml was prepared in acetonitrile. The stock solutions of sulfadiazine, sulfamerazine, propranolol, carbamazepine, procainamide, caffeine, ranitidine, theophylline, theobromine and acetaminophen were prepared in methanol at a concentration of 1000 μg/ml. All stock solutions were stored protected from light at 4–10 °C. Working solutions for spiking the serum and plasma samples were prepared from these stock solutions by dilution with acetonitrile/water (50/50, by volume) containing 0.1% formic acid.

2.1.2. Analyte solutions for breakthrough measurement

A solution of 20 μg/l pentachlorophenol was prepared by dissolving this compound in 300 ml acetonitrile followed by addition of 700 ml water. The pH was adjusted to 2 by adding hydrochloric acid. Then 20 μg/l solutions of phenol (pH 7), caffeine (pH 7), ranitidine (pH 9 and pH 7) and salicylic acid (pH 2 and pH 7) were prepared in water. The pH of each solvent was adjusted by adding hydrochloric acid or sodium hydroxide.

2.1.3. On-line SPE materials

The different cartridges evaluated for the generic on-line extraction were HySphere C_{18} (EC), 10×2 mm ID, with a particle size of 8 μm, HySphere Resin GP (General Purpose), 10×2 mm ID and 10×3 mm ID, with a particle size of 5–15 μm,

HySphere Resin SH (Strong Hydrophobic), 10×2 mm ID, with a particle size of 12–20 μm (all Spark Holland, Emmen, The Netherlands), PLRP-s, 10×2 mm ID, with a particle size of 15–25 μm (Polymer Laboratories, Shropshire, United Kingdom) and Oasis HLB, 10×2 mm ID, with a particle size of 15–25 μm (Waters, Milford, MA, USA. Trademark).

2.2. Instrumentation

2.2.1. On-line SPE and HPLC

A schematic diagram of the SPE–LC–MS system is presented in Fig. 1. A Gynkotek P 580 (Germering, Germany) binary high-pressure gradient pump delivered the mobile phase. On-line SPE is performed with a Prospekt system including a Triathlon autosampler for sample injection (all Spark Holland, Emmen, The Netherlands). Breakthrough measurements and validation experiments with serum have been performed with a Prospekt-1 system, validation experiments with plasma samples have been performed with a Prospekt-2 system. For recovery measurements with SPE–LC–UV, the analytical column was Hypersil ODS (C_{18}), 150×4.6 mm ID, 3 μm particle size (Thermo Hypersil, Bellefonte, PA, USA). For generic SPE–LC–MS/MS the analytical column was Nucleosil 120-3 C_{18}, 30×4 mm ID, 3 μm particle size (Machery Nagel, Dueren, Germany).

2.2.2. Tandem mass spectrometry

The applied tandem mass spectrometer was an API 3000 (PE Sciex, Concord, ON, Canada). Quantification of the analytes was performed in the positive ion mode. The turbo-ionspray interface was operated at a temperature of 400–450 °C and the applied orifice potential was 50–55 V. The applied scan mode was selective reaction monitoring, with a collision gas (N_2) setting of ''5''. Precursor, product

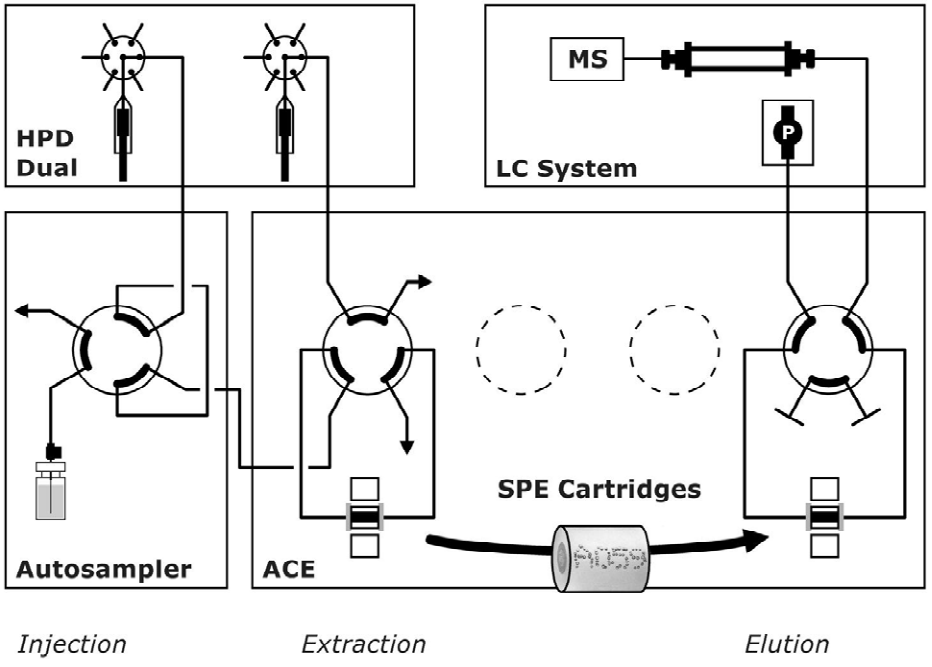


Fig. 1. System diagram for parallel or ''staggered serial'' on-line SPE–LC–MS with Prospekt-2. SPE solvents for solvation, equilibration, sample application and clean-up are provided by the high pressure dispenser (HPD) at programmable flow rates, independent of SPE cartridge backpressure. Sample is introduced by the autosampler. When SPE is completed, the cartridge is transferred to the elution clamp in the automatic cartridge exchange (ACE) module and the analytes are eluted from the SPE cartridge directly onto the LC column by the HPLC pump. After analysis, the SPE cartridge can be replaced automatically by a fresh one.

ion and collision energy (E_{col}) settings of each compound are listed in Table 1.

Data acquisition and analysis were performed using Sample Control and MacQuan software (MassChrom 1.0, PE Sciex, Concord, ON, Canada), respectively.

2.3. Experiments

2.3.1. Breakthrough measurements

Extraction capacity and desorption efficiency for different SPE sorbents were determined by recording breakthrough curves using the following procedure: a standard solution of the analyte is pumped directly to a UV detector (model 168 Diode Array Detector, Beckman, Fullerton, CA, USA, with System Gold Nouveau software) by the HPLC pump (see Fig. 1; MS replaced by UV, cartridge in the bypass position) to provide a constant, elevated absorbance signal. Next, the cartridge is placed in-line by switching the valve, causing the UV signal to drop to baseline level because the analyte is retained. When the capacity of the sorbent is exceeded (breakthrough), the signal starts to rise back to the original level. The volume of analyte solution passed through the cartridge before 10% breakthrough occurs defines the ''breakthrough volume'' and is our measure for the extraction capacity. The steepness of the up-slope after breakthrough is taken as a measure of desorption efficiency: the steeper the curve, the better desorption efficiency will be. A typical example of breakthrough curve measurement and comparison is shown in Fig. 2. All cartridges were solvated with 2.0 ml methanol and conditioned with 2.0 ml of the same solvent used for the analyte solution prior to the breakthrough test. The system was equilibrated with 2.0 ml analyte solution (cartridge in bypass position) before the cartridge was switched in-line. Analyte solution flow rate was 1.0 ml/min.

2.3.2. Generic on-line SPE–LC method

The following SPE program was used as the generic SPE protocol: The cartridges were solvated with 1.5 ml methanol (at 5.0 ml/min) in order to wet the packing material, and subsequently equilibrated with 1.5 ml water (at 5.0 ml/min) in order to obtain suitable conditions for analyte adsorption. Next, 100 μl of spiked serum or plasma was loaded onto the cartridge using 3.0 ml water (at 2.0 ml/min). Since less than 1 ml of water is required for quantitative transfer of the sample onto the cartridge, at least 2 ml water is used as wash solvent to remove matrix components. After completion of the SPE, the cartridge was switched in-line with the LC–MS/MS mobile phase to elute the analytes onto the analytical column. The elution was performed by a fast gradient at a constant flow rate of 2.0 ml/min. Mobile phase A consisted of acetonitrile/water (5/95, by volume) containing 0.1% formic acid and 10 m*M* ammonium acetate, mobile phase B consisted of acetonitrile/water (95/5, by volume) containing 0.1% formic acid and 10 m*M* ammonium acetate. The applied gradient was as follows: 0.1 min 100% A, from 0.1–1.3 min linear to 100% B, from 1.3 to 1.4 min 100% B, from 1.4 to 1.9 min linear back to 100% A. The column effluent was split to 400 μl/min before it entered the ESI source of the MS

3. Results and discussion

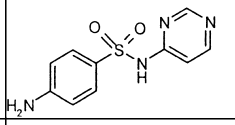
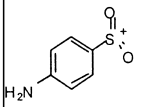
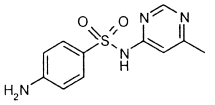
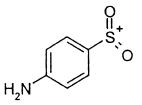
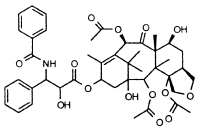
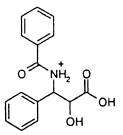
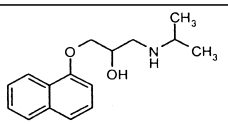
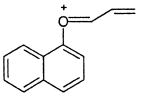
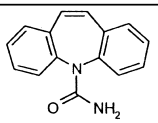
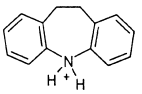
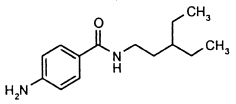
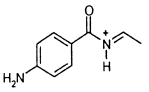
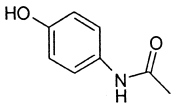
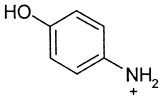
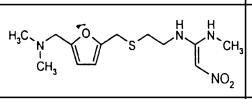
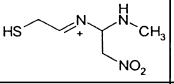
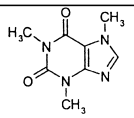
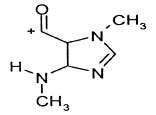
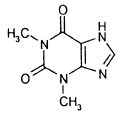
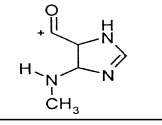
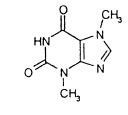
3.1. Generic on-line SPE–HPLC–MS/MS

3.1.1. SPE sorbent selection

A ''generic'' sorbent must be able to trap a broad range of compounds. In addition, efficient and complete desorption using HPLC–MS/MS compatible solvents is required, especially for on-line SPE, to maintain chromatographic separation efficiency and MS/MS sensitivity.

From the literature [9–12], it is evident that hydrophobic interaction is the most widely used trapping mechanism for SPE. Therefore a range of 5 different, relatively strong, hydrophobic sorbents has been selected for a comparative study on extraction capacity and desorption efficiency: HySphere C_{18}, PLRP-s, Oasis, HySphere Resin GP and HySphere Resin SH (all with dimensions 10×2 mm ID). C_{18} is silica based and is the least hydrophobic sorbent. PLRP-s, Hy-GP and Hy-SH are resin based and listed in increasing order of hydrophobicity. Oasis is a resin with polar and non-polar properties. For the five sorbents the breakthrough volume (see Section 2.3) was determined for five different analytes, dissolved in solutions with different pH; pentachlorophenol pH 2, phenol pH 7, caffeine pH 7, ranitidine

Table 1
Mass spectral information regarding the analytes used for this study and their selected reaction monitoring (SRM) conditions; the applied SRM conditions were: m/z $[M+H]^+ \rightarrow F^+$

name	Structural formula	*m/z* [M+H]$^+$	Fragment ion (F$^+$)[a]	*m/z* F$^+$	E_{Col} (eV)
Sulfadiazine		*251.4*		*156.2*	23.0
Sulfamerazine		*264.8*		*156.2*	30.0
Taxol		*854.5*		*285.9*	30.0
Propranolol		*259.8*		*183.0*	29.5
Carbamezepine		*237.6*		*193.8*	28.0
Procainamide		*236.4*		*163.4*	25.0
Acetaminophen		*152.2*		*110.2*	25.0
Ranitidine		*315.2*		*176.0*	25.0
Caffeine		*195.2*		*138.2*	30.0
Theophylline		181.2		*124.0*	28.0
Theobromine		181.2	—[b]	—[b]	—[b]

[a] Tentative fragment ion structure.
[b] No specific fragment ion could be identified.

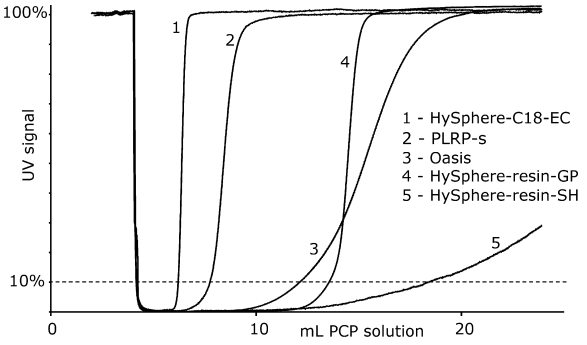


Fig. 2. Breakthrough curves for pentachlorophenol (PCP) on various sorbents. PCP solution: 20 μg/l in acetonitrile/water (30/70), pH 2, 1 ml/min.

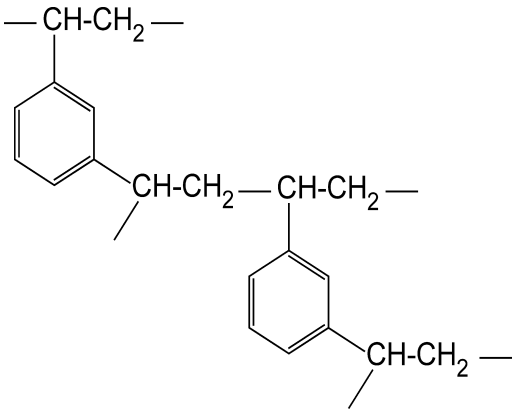


Fig. 3. Structure of DVB.

pH 9, ranitidine pH 7, salicylic acid pH 2 and salicylic acid pH 7. The breakthrough volumes for the various sorbents and analyte solutions are given in Table 2. From this table it is evident that Oasis, HySphere Resin GP and HySphere Resin SH show significantly higher extraction capacities than HySphere C_{18} (EC) and PLRP-s. From the curves in Fig. 2 it is clear that HySphere C_{18} (EC), PLRP-s and HySphere Resin GP provide better desorption efficiencies than Oasis and much better than HySphere Resin SH. Only HySphere Resin GP (a DVB resin, see Fig. 3) combines high extraction capacity with high desorption efficiency and was therefore selected as the generic sorbent for further experiments.

3.1.2. Evaluation of the generic SPE method by SPE–LC–UV

The proposed generic SPE method (as described in Section 2.3) with HySphere Resin GP as the sorbent, was derived from an existing application for the determination of theobromine, a very polar xanthine, using HySphere-GP for the on-line extraction from serum [13]. The clean-up after sample application is performed with water and is the minimum volume required to remove traces of sample matrix from the cartridge, without eluting the most polar compounds.

A model array of 11 pharmaceutical compounds (Table 1) was used to test the generic extraction protocol. The selected pharmaceutical compounds are highly diverse and vary from polar (e.g. theobromine) to non-polar (e.g. taxol), acidic (e.g. sulfadiazine) to basic (e.g. procainamide) and linear (e.g. ranitidine) to aromatic (e.g. carbamazepine). Extraction recovery for all analytes, spiked at relatively high concentrations in porcine serum, was determined by comparison with direct injection of aqueous standard solutions of the analytes (absolute recovery). To avoid possible interference caused by the sample matrix (like ionization suppression), the recovery for all analytes was determined with UV, rather than MS detection, using a relatively long

Table 2
Breakthrough volumes (ml) for various analytes on 5 different SPE sorbents

SPE cartridge	PCP pH 2	Phenol pH 7	Caffeine pH 7	Ranitidine		Salicylic acid	
				pH 9	pH 7	pH 2	pH 7
HySphere C_{18} (EC)	1.9	0.6	3.4	21	0.6	2.5	<0.2
PLRP-s	3.4	2.7	5.0	29	0.4	7.8	<0.2
Oasis	8.5	10	7.0	33	0.6	28	<0.2
HySphere Resin GP	10	9.0	17	>50	4.0	29	<0.2
HySphere Resin SH	14	16	>25	>50	18	>30	0.4

column (15 cm) with gradient elution. Solvent A was water with 0.1% formic acid, solvent B was acetonitrile, 0.1% formic acid. The gradient program was 5–95% B in 12 min with initial equilibration at 5% B for 2 min. More than 95% recovery was obtained for taxol, propranolol, carbamazepine, procainamide, caffeine, ranitidine, theobromine and theophylline, without any adaptations of the method. Sulfadiazine and sulfamerazine showed only partial recovery for serum samples. This was probably due to protein binding, since recovery for aqueous standards was >95%. Addition of 20 μl phosphoric acid per ml sample restored recovery (>95%) for both compounds. The very polar acetaminophen required more sorbent material to prevent breakthrough; 10× 3 mm instead of 10×2 mm cartridges provided >95% recovery.

3.1.3. Testing the generic SPE–LC–MS/MS method

To test the performance of the generic method for bioanalytical applications, all model compounds were spiked to serum and plasma, and the assays of the compounds were validated. It was possible to incorporate up to seven compounds in one assay to accelerate validation experiments. A typical example of such a seven-in-one result is shown in Fig. 4.

3.1.3.1. Specificity. To determine the specificity of the method, blank human plasma samples were analyzed to determine whether ''visible'' interfering peaks were present in the selected-reaction-monitoring (SRM) windows of the analytes. The chromatograms (not shown) obtained from the analysis of

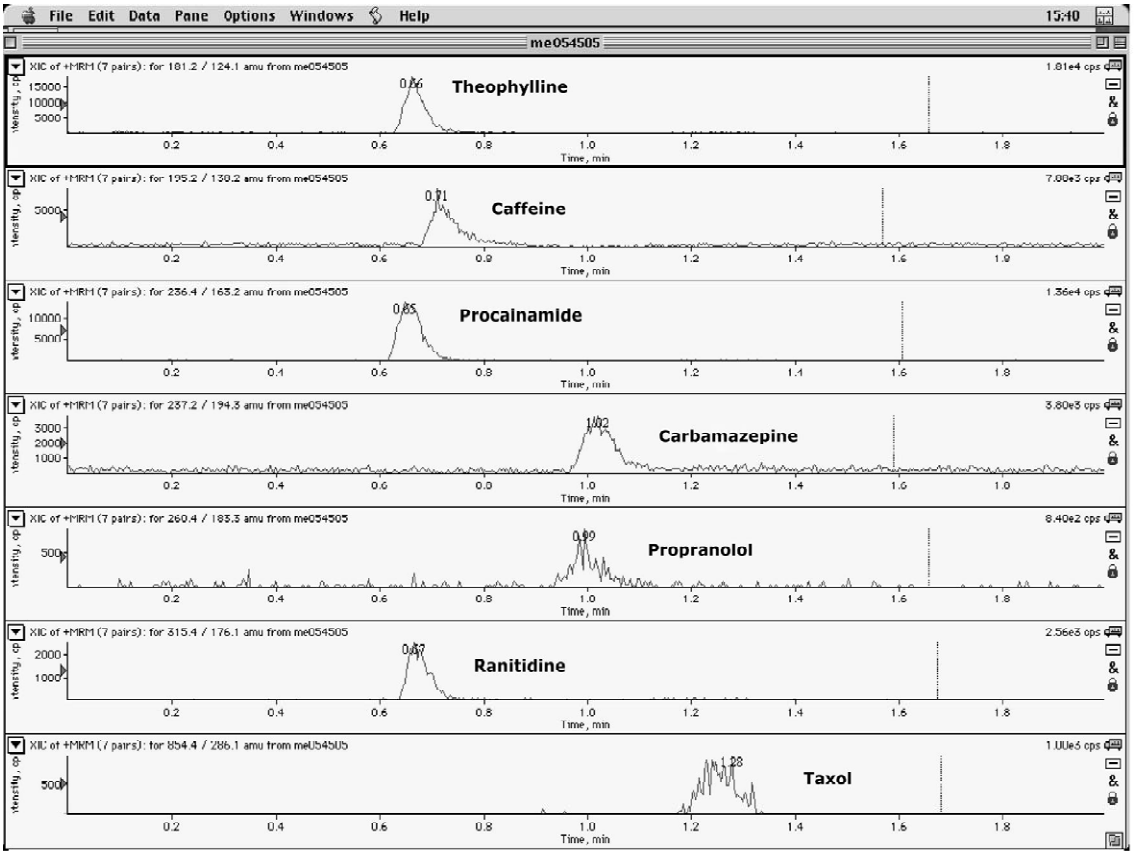


Fig. 4. On-line SPE–LC–MS/MS traces of seven drugs spiked to plasma at 1.0 ng/ml. MS in SRM mode. Conditions are according to Table 2.

blank human plasma did not show any peaks close to or at the retention time of the analytes.

3.1.3.2. Carry-over. The assays were not optimized with respect to carry-over; standard needle wash was used for the autosampler i.e. a single wash volume of 300 μl. Water/acetonitrile (80/20, by volume) was used as the wash solvent. The carry-over was determined using spiked and blank plasma samples and varied per compound (see Table 3). Carry-over was acceptable [6] for all model compounds over a range of two to three orders of magnitude. If required, carry-over can be further reduced by optimizing the wash procedures for autosampler and Prospekt.

3.1.3.3. Quantification limit. With recoveries >95% for all analytes, ionization and fragmentation efficiency are the two main parameters limiting the detection or quantitation by LC–MS/MS for the model compounds. The ionization efficiency is a compound-dependent parameter that is significantly influenced by mobile phase composition [14,15]. The ionspray process requires that the analytes already possess a charge when dissolved in the liquid phase [16–18]. Most model compounds are relatively basic, and thus it is plausible that positive ionspray conditions in combination with the chosen acidic gradient mobile phase conditions are optimal for the model compounds.

Optimization of the collision energy (E_{col}) was performed in order to maximize the abundance of the selected product ions of the model compounds that were monitored with the second mass filter. The optimal E_{col} settings for all model compounds are given in Table 1. However, for theobromine no optimal E_{col} could be defined; either no fragmentation occurred or too many fragmentation channels have opened, and as a result of that no SPE–LC–MS/MS assay could be developed for this compound.

The quantification limit of all the model compounds was determined in plasma using a Prospekt-2 system. Table 3 shows quantification limits in the range of 0.2–2.0 ng/ml.

3.1.3.4. Precision, accuracy and linearity. A brief assessment of precision, accuracy and linearity was performed in order to obtain a good indication of the analytical quality of assays based on the generic method. All model compounds were spiked to porcine serum in one batch and validation runs consisted of one set of calibration samples and three sets of QCs at four levels. Accuracy and repeatability (%RSD) was determined to be below 15% for all tested model compounds. No internal standards were used.

With >95% analyte recovery, linearity is limited mainly by the ionspray process [18]. Linearity was found to vary per compound from two orders of magnitude (taxol) up to four orders of magnitude (caffeine, theophylline). Correlation coefficients varied from 0.9953 to 0.9999 (Table 3).

Table 3
Performance overview of the generic on-line SPE–LC–MS/MS protocol, using Resin GP extraction cartridges; see for conditions Table 2 and Experimental section

Compound	LLOQ[a] (ng/ml)	Linear range (10^n)	R	Carry-over (%)
Sulfadiazine	1.0	3	0.9982	<0.05
Sulfamerazine	1.0	3	0.9998	<0.05
Taxol	1.0	2	0.9953	<0.05
Propanolol	1.0	3	0.9995	0.08
Carbamazepine	0.2	3	0.9997	<0.1
Procainamide	0.2	3	0.9995	0.07
Caffeine	0.2	4	0.9999	0.05
Ranitidine	0.25	2.5	0.9993	0.07
Theophylline	0.25	4	0.9995	0.02
Acetominophen	2.0	2.5	0.9992	<0.1
Theobromine[b]	–	–	–	–

[a] Determined with Prospekt-2.
[b] For theobromine no specific fragment ion could be identified, hence no MS/MS method could be developed.

3.2. General discussion

The generic SPE–LC–MS/MS protocol provides a new assay in almost no time. For ten out of eleven compounds tested in our laboratory, the generic protocol worked well, with little or no adaptations at all. Though the authors are well aware that it will not be possible to analyze every compound with this protocol, it is very likely to be at least a good starting point for further method development. In addition, assays based on the generic on-line SPE approach are able to analyze samples in a high-throughput mode: solid-phase extraction time requires only two min, a gradient LC–MS/MS run also takes two min, giving a total assay cycle time of four min when using the Prospekt-1 system and only two min when using the Prospekt-2 system in parallel mode (see Fig. 1).

Relatively simple adaptations have provided adequate results in cases when the first attempt did not provide acceptable results, as was shown for sulfadiazine and sulfamerazine (acid addition) and acetaminophen (3 mm ID cartridge instead of 2 mm ID). The pH is a parameter that can be used simply and effectively to improve either recovery or clean-up. Bringing the pH at least one unit below the pK_a for acidic, and above the pK_a of the conjugate acid for basic analytes, will usually increase analyte hydrophobicity dramatically and can thus be used to increase recovery or clean-up.

4. Conclusions

Generic solid-phase extraction coupled to fast generic gradient HPLC–MS/MS was successfully applied for the assay of 10 out of 11 highly diverse model compounds. For the 11th compound (theobromine) the generic SPE–LC method worked well (demonstrated with UV detection), but MS detection failed. The use of the generic SPE–LC–MS/MS protocol has eliminated method development completely for 7 model compounds and significantly reduced method development time for the other 3 compounds. The analytical results demonstrate the feasibility of on-line SPE–LC–MS/MS as a platform for the rapid assay development and sample processing all along the line of drug discovery, development and clinical study. Using the generic protocol as described here, a significant number of these assays is likely to be developed with very little or no method development time at all.

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