International Journal of Mass Spectrometry 296 (2010) 1–9

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

An integrated platform for fully automated high-throughput LC–MS/MS analysis of *in vitro* metabolic stability assay samples

Andreas H. Luippold [a], Thomas Arnhold [a], Wolfgang Jörg [a], Roderich D. Süssmuth [b,*]

[a] *Department of Drug Discovery Support, Boehringer Ingelheim Pharma GmbH & Co KG, Birkendorfer Str. 65, 88397 Biberach an der Riss, Germany*
[b] *Institut für Chemie, Technische Universität Berlin, Strasse des 17. Juni 124, 10623 Berlin, Germany*

ARTICLE INFO

Article history:
Received 15 May 2010
Received in revised form 10 June 2010
Accepted 5 July 2010
Available online 17 July 2010

Keywords:
In vitro ADME
Metabolic stability
High-throughput mass spectrometry
Automation
High-speed robotics

ABSTRACT

The evaluation of ADME properties contributes importantly to drug candidate selection and therefore is crucial for the drug discovery process. Providing a sufficiently high-throughput capability in laboratories dedicated to early pharmacokinetic studies will thereby shorten the entire drug discovery process. In this paper an integrated and fully automated LC–MS/MS-based platform is described, which enables the assessment of *in vitro* metabolic stability, a key ADME parameter. An ultra-rapid injection system was coupled to a triple quadrupole mass spectrometer and hard- and software were customized to perform sample identification, cleanup, MS compound optimization and sample measurement without manual interaction. Conventional chromatography was initially evaluated but was later replaced by solid phase extraction as the only purification step. Ultra-fast robotics, combined with a generic step gradient enables analysis times of 8 s/sample. Reproducibility and quality of data has been found fully comparable to data generated by validated LC–MS/MS methods. The system handles data for mass spectrometric compound optimization and MRM (Multiple Reaction Monitoring) analytics in a fully automated way and exhibits great potential for a generalized use in ADME replacing currently applied conventional LC–MS/MS approaches.

1. Introduction

The optimization of the drug discovery process made over the past decades has been facilitated by the increasing chemical synthesis capabilities using technologies such as combinatorial chemistry. It has also benefited by the implementation of high-throughput technologies to rapidly identify potent drug candidates from large compound libraries. One key remaining challenge related to ADME (absorption, distribution, metabolism, excretion), an integral part of early drug discovery processes, is the characterization of these compounds. Fast and robust technologies are needed to provide adequate high quality ADME data. In contrast to potency investigations in high-throughput screening (HTS) campaigns, ADME properties of potential drug candidates can only be addressed by simultaneously quantifying individual compounds. In this regard, mass spectrometric (MS) technologies are indispensable, since they deliver adequate sensitivity, selectivity, and speed for the analytical process. However, early investigation of ADME properties has not been able to keep pace with the increasing demands generated by the implementation of synthetic and screening technologies.

The timely delivery of valid ADME data is crucial for the fast progression of hit and lead candidates and constitutes an integral part of a drug discovery strategy. Over the past 15 years pharmaceutical companies increased specialization of laboratories dedicated to early drug discovery investigations. More recently, automation technologies such as pipetting robots have been established for ADME assays. In order to keep pace with the increased sample load, efforts were recently directed towards integrating new analytical methodologies with the aim to further shorten analysis times while maintaining data quality.

In early pharmaceutical research LC–tandem MS methodologies have provided the basis for pharmacokinetic investigation of lead compounds. In the past decade manifold approaches increasing throughput in the ADME field specifically addressed the analytical part of these processes. Most importantly, these approaches include rapid LC gradients using monolithic columns [1–3] or ultra-fast HPLC technologies based on short columns [4–8] and column packing material with smaller particle sizes [9–11]. All of these approaches significantly decreased analysis times compared to conventional LC–MS/MS systems. Hence, analysis times range between 0.1 and 2 min and are strongly dependant on the autosampler system ultimately limiting the pacing of the LC–MS system [7,12]. In order to further reduce analysis times multiplexing systems [1,13–18] or pooling approaches [3,19,20] have been reported as further advancements for shortening the analytical

* Corresponding author.
E-mail address: suessmuth@chem.tu-berlin.de (R.D. Süssmuth).

1387-3806/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2010.07.004

process. An aspect common to all these analyses involves sample preparation methods like solid phase extraction (SPE), liquid–liquid extraction (LLE) or protein precipitation (PPT) protocols, followed by linear chromatographic gradients in HPLC–MS measurements both processes are considered to be essential for the elimination of matrix effects. Furthermore, it has been shown that approaches combining fast HPLC and high resolution mass spectrometry allow omission of the compound optimization step necessary for tandem MS technology and generate both quantitative and qualitative data [9]. Recently the DART (direct analysis in real time) technology, a customized ionization-source replacing conventional chromatographic separation, has been coupled to an autosampler and used for assaying metabolic stability in the range of 50 s/sample [21].

We document herein for the first time a customized rapid and integrated analysis system (RIAS) which has been applied to a mass spectrometric evaluation of metabolic stability as a key parameter of ADME studies [22]. The new RIAS implements a RapidFire™ system, which in combination with a triple quadrupole mass spectrometer has previously been applied exclusively for the determination of enzyme inhibitors [23–30] via standard analysis of well-known marker substrate biotransformations. The above mentioned hardware components of the analytical setup have been harmonized to communicate with a specific master software. This master software controls the software of the rapid injection system and the mass spectrometer by means of a software solution based on LabVIEW developed in-house. The RIAS is able to operate automatically at 8 s/sample. Together with workflow tools covering other aspects of the entire process (such as experiment request, sample generation, data analysis and reporting), the approach results in a seamless automated data handling and data processing. The data generated by the RIAS have been compared to results generated with validated LC–MS/MS systems and the system has the potential for further uses in various ADME applications.

2. Experimental

2.1. Materials and chemicals

Acetonitrile (gradient grade) was purchased from Merck (Darmstadt, Germany), methanol (gradient grade) from J.T. Baker (Deventer, Netherlands), formic acid (p.a.) and trifluoroacetic acid (TFA, reagent grade) from Sigma–Aldrich (St. Louis, MO, USA) and water was obtained from an in-house water purification system (Elgastat Maxima HPLC, Elga Ltd., High Wycombe Bucks, UK). Ammonium acetate (p.a.) and ammonia solution (25%, p.a.) were purchased from Merck (Darmstadt, Germany). The test compounds alprenolol, buspirone, dextromethorphan, diclofenac, diltiazem and verapamil were purchased from Sigma–Aldrich (St. Louis, MO, USA), diazepam and midazolam from Roche Pharma AG (Grenzach-Wyhlen, Germany); the residual 159 proprietary compounds were synthesized in-house at Boehringer Ingelheim Pharma GmbH & Co KG (Biberach, Germany). All compounds were within a molecular mass range of 250 and 700 Da; calculations of logP and TPSA (total polar surface area) were performed with ACD/Labs (version 11, ACD Inc, Toronto, Ontario, Canada) and were found to have a log P of −0.7–9 and a total polar surface area (TPSA) of 12–139 Å^2. Cartridges for the RapidFire™ system were obtained from BioCius (Woburn, MA, USA), the 0.8 mL 96-well plates and the "Easy Peel" sealing mats were purchased from Thermo Scientific (Hamburg, Germany).

2.2. Sample preparation

Samples taken from an *in vitro* metabolic stability assay (compound concentration 1 μM) were taken at 0, 5, 15, 30 and 45 min and were subsequently quenched with a 2-fold volume of acetonitrile in order to precipitate protein and to stop enzymatic reactions. An aliquot of a proprietary generic internal standard at a concentration of 2 μM was added to each sample and samples were centrifuged at 4000 rpm and 4 °C (centrifuge 5810R, Eppendorf, Hamburg, Germany) for 20 min. The supernatants were transferred to 0.8 mL 96-well plates. The sample plates were heat-sealed and stored at −20 °C until analysis. Directly before analysis, plates were thawed and centrifuged at 4000 rpm for 5 min. Tuning solutions of the analyzed drugs were provided on separate plates for mass spectrometric compound optimization at a concentration of 1 μM in 50% methanol. All sample and tuning plates were barcode labeled and associated with a file containing all relevant analytical information e.g., compound name, molecular formula, experiment ID, position on the plate and internal standard code. The information in the corresponding file was readily accessed by the master software of the injection system via barcode reading.

2.3. Instrumentation

Analytical sample handling was performed by a rapid-injecting RapidFire™ autosampler system (BioCius, Woburn, MA, USA). The system consists of a plate handler equipped with a barcode scanner, a rapid sample injection device and a solid phase extraction cartridge. High-speed robotics (injection needle static in x/y-axis but movable in z-axis, plate nest movable in x/y-axis but static in z-axis) and fast switching rotary valves enable high flow rates. Since the original RapidFire™ system was capable of injecting only line-by-line from 96- or 384-well plates the system software was modified to enable use as a flexible autosampler. The system contains two wash stations located next to the plate nest to facilitate interim needle washes between sample washes. The wash stations are under continuous flow, filled with water and with methanol.

To facilitate the use of 0.8 mL 96-well plates, the plate handling device and the injection needle ("sipper") were adapted in-house. The sipper guide had to be beveled with a 30° angle in order to pierce reliably and without any punch-outs the sealing mats of the sample plates. As the new system was designed to run unattended, there is no method development and interactive pre-check in terms of method selectivity during the mass spectrometric analysis. Therefore, four troughs were built into the system next to the plate nest to enable blank matrix injections for the confirmation of selectivity of the analytical method.

The RapidFire™ technology is based on classic column switching [31] and the technical solutions developed for solvent management and column switching are depicted in Fig. 1A–C. Liquid sample is aspirated by a vacuum pump into a 10 μL sample loop for 250 ms (A) and subsequently flushed for 3000 ms onto a C4 cartridge (B) (3.8 μL bed volume; BioCius, Woburn, MA, USA) with the aqueous mobile phase. The solid phase extraction step retains the analyte while removing interfering matrix (e.g., buffer components). The analyte is desorbed and back-eluted from the cartridge for 3000 ms with an organic mobile phase and flushed into the mass spectrometer (C). The mass spectrometric detection was performed on a TSQ Vantage from ThermoFisher (San Jose, CA, USA). While performing the back flush into the mass spectrometer, the sample loop and relevant tubing were flushed with the organic mobile phase to prevent carry-over of analyte or matrix components into the next sample. Equilibration for the subsequent sample is performed in state A (Fig. 1) and requires another 500 ms. In order to further minimize carry-over effects, the wash station of the system was used to perform needle washes with pure water (100%) and pure methanol (100%) between samples. The solvent delivery setup consists of three continuously running and isocratically operating HPLC pumps (G1310A, Agilent, Waldbronn, Germany): Pump 1 (99.9% water, 0.09% formic acid and 0.01% TFA, flow rate

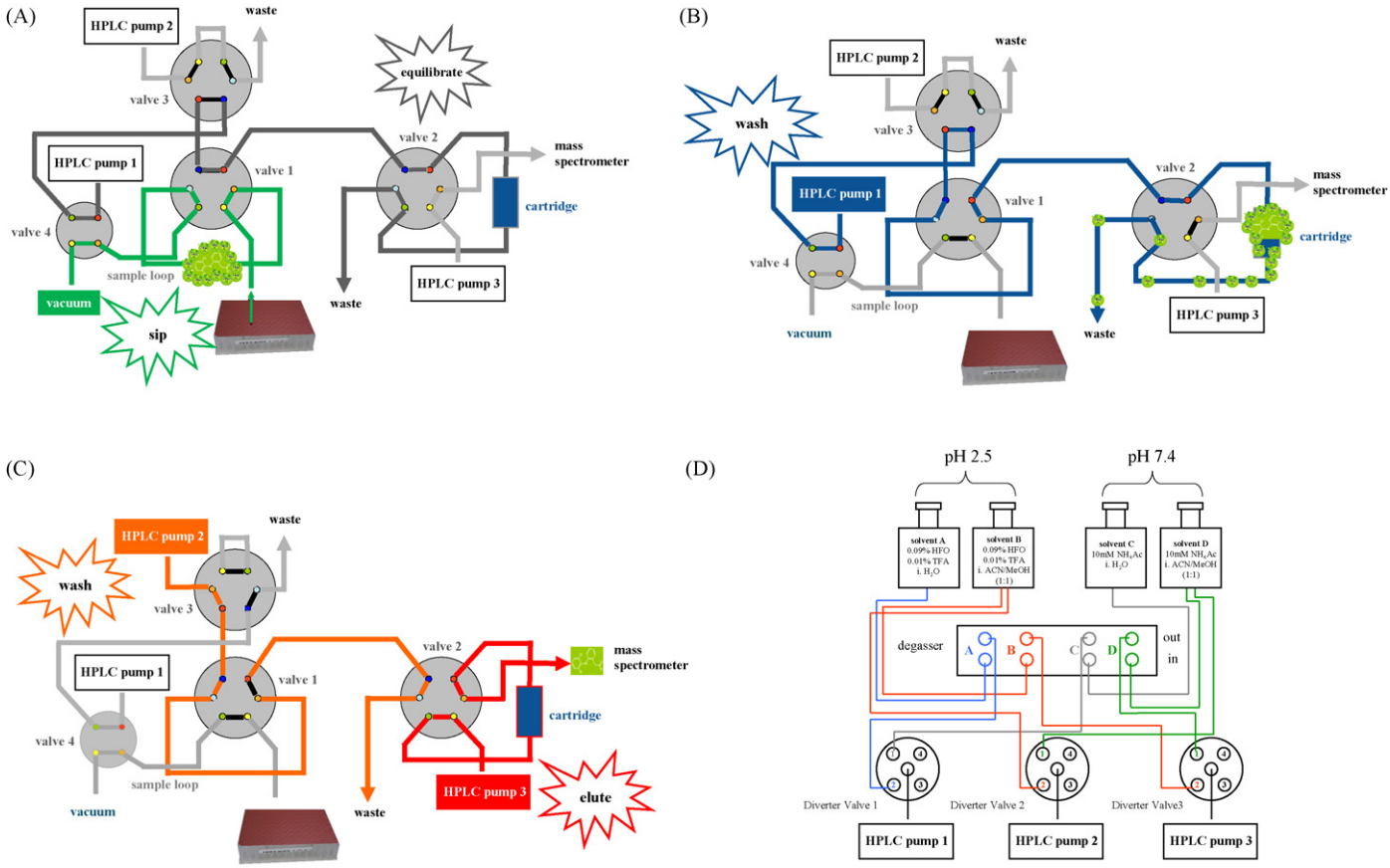

Fig. 1. Schematics solvent and sample delivery of the RIAS (rapid and integrated analysis system) based on RapidFire™ technology: High flow rates and fast switching valves allow a cycle time of ~8 s per sample depending on the sample matrix the combination. (A) The sample is aspirated into the sample loop, and adsorbed on a solid-phase extraction cartridge. (B) A subsequent aqueous solvent wash eliminates matrix components. (C) The backflush into the mass spectrometer is performed with organic solvent, while the sample loop is flushed with organic solvent to prepare aspiration of the following sample. (D) Schematics of automated solvent pH-switch: Solvent at pH 7.4 is used for compound optimization and sample measurement in negative ionization mode. Depending on the compound-specific ionization mode the diverter valves 1–3 are switched to pH 2.5 for sample measurement in positive ionization mode. Time needed for equilibration after switching pH solvent is less than 1 min.

1.25 mL/min) performs sample loading onto the cartridge and the SPE-wash. Pump 2 (99.9% acetonitrile/methanol (1:1, v:v), 0.09% formic acid and 0.01% TFA) runs at a flow rate of 1.25 mL/min and performs an organic flush of the sample loop and the tubing (rotary valves 1, 2 and 3; state C). Pump 3 is eluting the analytes from the cartridge into the mass spectrometer with the organic mobile phase from pump 2 (flow rate 1.25 mL/min).

The validated LC–MS/MS system consisted of two HPLC pumps (PU-980, Jasco, Groß-Umstadt, Germany), one binary pump (G1312A, Agilent, Walbronn, Germany), coupled to a 2000QTrap mass spectrometer (AB Sciex, Foster City, CA, USA) and was run with column switching technology and a throughput of 1 min/sample. This system was validated against linear gradient LC–MS/MS systems earlier.

2.4. MS compound optimization

A prerequisite for sensitive and selective "Multiple Reaction Monitoring" (MRM) analytics with triple quadrupole mass spectrometers is the determination of the compound-specific MRM transitions by tuning of MS parameters. State-of-the-art mass spectrometers automatically perform both compound optimization and sample measurement in a fully automated and software-aided way for compounds in positive and negative ionization modes. For this purpose the mass spectrometer was set to "optimization mode" by the QuickQuan™ (ThermoFisher, San Jose, CA, USA) software triggering the automated compound optimization and to "MRM mode" for quantitative analysis. Since for compound optimization pure compound solutions (1 μM) are used, the wash-step from the RapidFire™ system was reduced from 3000 to 1000 ms. A persistent mass spectrometric signal of the targeted compound during the optimization process was achieved by reducing the eluting flow rate to 0.3 mL/min. Timing of injections was set to deliver an analyte signal to the mass spectrometer upon request from the QuickQuan™ software, for both MS and MS/MS optimization. The mass spectrometric compound optimization takes less than 1.5 min yielding the basic mass spectrometric parameters ionization polarity (positive or negative), S-lense, CE voltage and, most importantly, the determination of the optimized MRM transition. Optimized parameters are stored in the QuickQuan™ database and can be automatically retrieved for sample measurement in MRM mode. In order to facilitate compound optimization for positive and negative ionization modes compound tuning is performed at pH 7.4. In the first step the analyte solution is loaded with aqueous 10 mM ammonium acetate onto the cartridge and eluted back into the mass spectrometer with organic solvent (95% acetonitrile/methanol (1:1, v:v) and 5% 10 mM ammonium acetate; pH 7.4).

In case of a higher ionization yield in negative ionization mode, pH 7.4 (solvent A: 10 mM ammonium acetate; solvent B: 95% acetonitrile/methanol (1:1, v:v) and 5% 10 mM ammonium acetate) is maintained for sample measurement. For samples displaying a higher signal response in positive ionization mode the system automatically switches from pH 7.4 to 2.5 (solvent C: 99.9% water, 0.09% formic acid and 0.01% TFA; solvent D: 99.9% acetonitrile/methanol

(1:1, v:v), 0.09% formic acid and 0.01% TFA), in less than 1 min. This was achieved by implementation of three diverter valves which were positioned behind the degasser of the isocratically working pumps of the RIAS. A scheme outlining the automated solvent pH-change is depicted in Fig. 1D. Individual changes of the high-throughput sampling system in wiring for signal handling were adapted in-house.

2.5. Software

The RapidFire™ control and interface software were customized and obtained from BioCius (Woburn, MA, USA). The mass spectrometry software QuickQuan™ 2.3, Xcalibur™ 2.0.7, XDK 1.0.2.15 and mass spectral data processing software QuickCalc™ 6.0.2 were purchased from ThermoFisher (San Jose, CA, USA). The database used to store processed MS data is Microsoft SQL Server 2005 (Microsoft Corporation, Redmont, WA, USA). The LabVIEW software (version 8.6.1) from National Instruments (Austin, TX, USA) was programmed in-house operating as master software for the entire system. The software is functional according to so-called "handshake" principles with the RapidFire™ system and the TSQ Vantage mass spectrometer. Final data analysis and calculation of compound half-lifes was performed by AssayExplorer 3.2 (Symyx, Sunnywale, CA, USA). Half-lifes were calculated in the Assay Explorer software by plotting an automated linear regression of ln % parent compound vs. time and calculating $\ln(2)/k$ where k represents the slope of the regression curve. Correlation (r^2) was calculated and visualized in TIBCO® Spotfire® version 2.2.0 (TIBCO®, Palo Alto, CA, USA).

3. Results and discussion

An efficient analytical system applicable to early drug discovery integrates hardware speed, robustness with reproducibility and sensitivity. According to these requirements, the goal was to establish a system for the automated and high-throughput mass spectrometric analytics applicable to metabolic stability investigations and additional *in vitro* ADME assays. The integrated and customized RapidFire™ system consists of a flexible autosampler with a fast sample injection module and an online solid-phase extraction unit capable of running multiwell-plates in 96- or 384-well format. A time-saving feature of this system is the movable *x*-/*y*-tray, which rapidly places the sample plate into programmable sample positions while the aspiration needle remains fixed. The customized implementation of assay-type barcoding principles into the autosampler hardware was a first improvement with regard to assay-type recognition and automated initiation of corresponding analytical processes. Thus sample plates from ADME assays can be introduced into the system without manual interaction. As a consequence, the system was capable of differentiating sample plates and plates for mass spectrometric compound optimization. Since for triple quadrupole mass spectrometers the determination of the compound-specific MRM-transitions and elementary voltages like the S-lense and collision energy are prerequisite for obtaining quantitative data, the system automatically performs both, compound optimization and sample measurement in a fully automated way. The challenge of optimizing mass spectrometric conditions was previously solved by flow-injection analysis (FIA) [32] constant infusion of the analyte solution [33,34]. Hence, most often switching between optimization and measurement involved manual hardware changes, e.g., columns had been bypassed to enable flow-injection or separate autosamplers were used for constant infusion via an additional valve [34]. In the present approach this issue was solved with a timed multiple injection series ("pseudo infusion") by applying a low flow rate (0.3 mL/min). Thus, the analytes elute slowly but continuously over time in a broadened peak shape suited for optimization procedures. Technical requirements enabling the specific timing and application of different flow rates are managed by the LabVIEW interface software which performs the bidirectional communication ("handshake") between the HT-autosampler and the mass spectrometer. With respect to the hierarchic order of system components, the LabVIEW software is the master software for the entire system and is the key for the communication and timing between the analytical devices and ultimately the decisive factor for the achieved level of automation and speed.

3.1. Workflow

Increasing throughput in sample analysis requires not only high speed analytical instrumentation but also a concomitant workflow optimization to achieve a compatible information flow. It is therefore indispensable to integrate the described high-throughput analytical system into the entire process. Within many pharmaceutical companies, ADME investigations and ADME data profiling are provided by central service units to different research projects. Central sample preparation laboratories generate different kinds of ADME samples which are then transferred to a central MS analysis unit. The central sample preparation units perform the desired type of ADME assay and transfers barcode labeled sample plates as well as file information per plate to the analytical unit. The details of the optimized entire workflow process will be described elsewhere.

Only a short peak review in the program QuickCalc™ is required obtaining an overview of the peak areas of an entire metabolic stability experiment for one compound. Subsequently, processed data are transferred into an SQL database. The SQL database serves as the retrieval layer for the data analysis software and result calculation engine (AssayExplorer), finally providing ADME parameters like half-lifes and upscaled *in vitro–in vivo* parameters, such as clearance and percent hepatic blood flow. The calculated data is finally reported to a global database allowing scientists to execute queries and assemble structure activity relationships (SAR). Thus, integration of the RIAS into the entire information flow of drug discovery avoids time-consuming and error-prone manual transactions of information. Beside data evaluation, manual intervention within the whole process is limited to sample feeding, solvent refilling and exchange of the cartridge after ca. 2500 samples injections.

3.2. Automation and speed

Since the RIAS (rapid and integrated analysis system) was set up to constitute an integral part of the drug discovery workflow it is capable of automatically exploiting information by barcode scanning. A prerequisite for information access is that all required information is provided on the computational network. This information comprises e.g., the assay type, compound name and other specific content necessary with regard to experiments and procedures, shared by all the assays robotics of the metabolic stability experiments as well as the RIAS and is applied as a trigger for activities of the system.

Time needed for automated compound optimization of a validated mass spectrometric system typically ranges around 1.5 min/compound for MS and MS/MS optimization and is principally independent of the type of *in vitro* assay. With the experimental setup of RIAS presented above, the speed of sample analysis was minimized to 8 s/sample which equals a throughput of 450 samples per hour. Technical factors reducing the sample to sample cycle time are the optimized microfluidic system including the fast switching rotary valves of the RapidFire™ as well as the quickly movable *x*-/*y*-tray. Of crucial importance is the abandonment of conventional chromatography, which has been reduced to

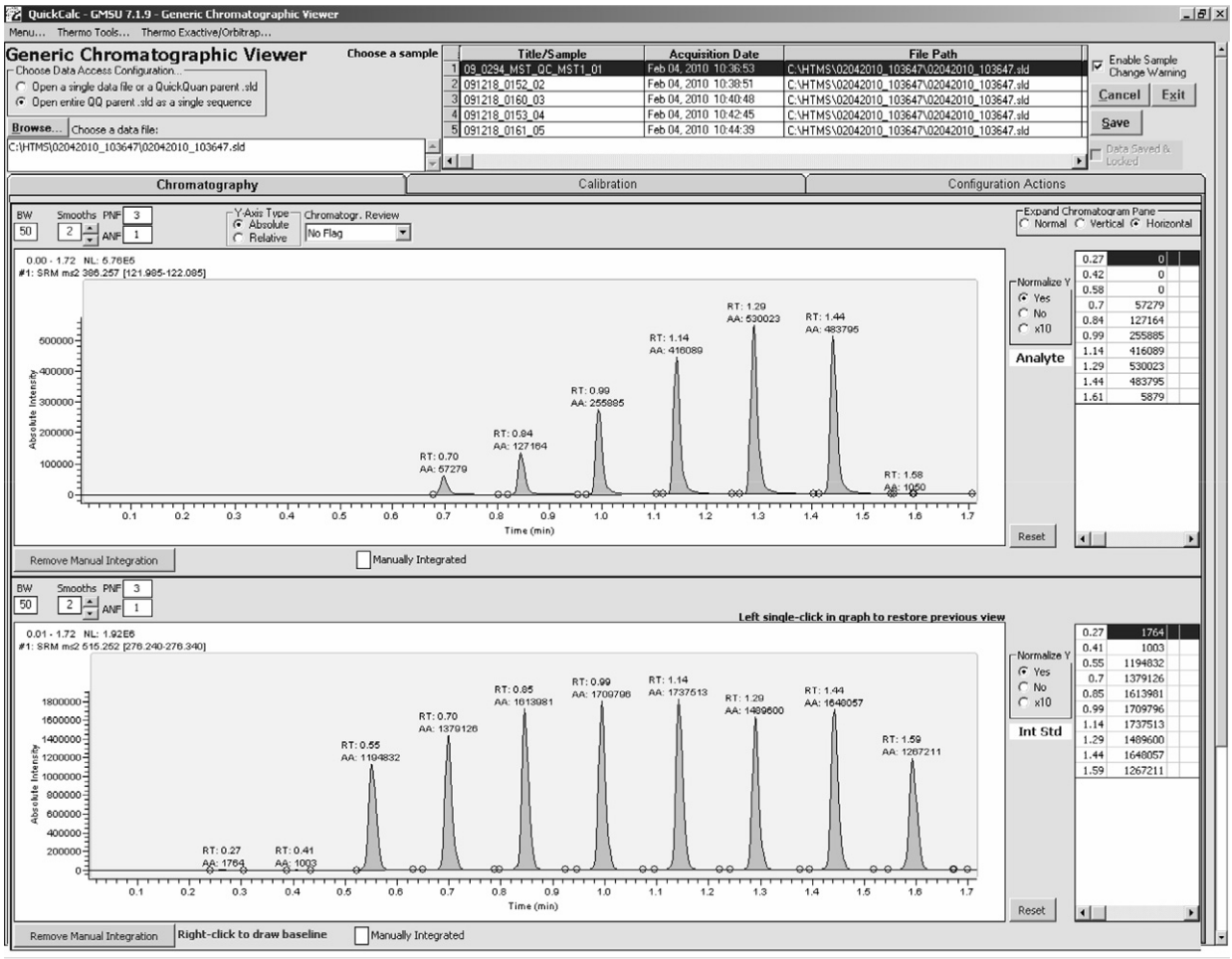

Fig. 2. HT-mode data processing. Typical data processing view of the HT–MS/MS system in high-throughput mode. After a quick peak review data are stored in a database for subsequent retrieval into data analysis software for further calculations. In this example buspirone is metabolized from human liver microsomes (upper MRM transition). In the lower MRM transition the peaks from first and final internal standard demonstrate blank matrix injections facilitating the selectivity and carryover check.

the application of a simple step gradient on a solid phase extraction cartridge as the only purification step.

In early drug discovery quantitation of NCEs (new chemical entities) has to be performed mostly without stable labeled internal standards (IS) due to their time consuming and therefore cost-intensive synthesis. Nevertheless the principal use of internal standards is advantageous and advisable in order to balance variability in the analytical process. Therefore generic internal standards constitute a compromise allowing the correction of interferences from matrix even if the IS are structurally different. This application of IS is used in both methods, conventional LC–MS/MS and RIAS. Application of conventional HPLC gradient methods commonly results in different retention times of the analyte and the IS. Thus, matrix components which co-elute with the analyte or IS may differ and may interfere differently with the ionization processes of the analyte or IS. Applying the RIAS, a step gradient simultaneously elutes both the analyte and the IS from the column with the same matrix components. As a consequence, potential quenching effects in the MS ionization process affect both, the analyte and the internal standard in a similar way.

3.3. Data processing

Data processing was performed in high-throughput mode which facilitates the peak review and reduces number of data files since all samples from an assay series are included in a single file [12,14,18]. To achieve accurate peak allocation as well as precise peak integration the switching time of valve 2 (Fig. 1C) of the HT-autosampler was monitored and automatically added to the MS raw data file during peak review in QuickCalc™ (Fig. 2). Processed MS raw data are stored in a database and can be retrieved from the data analysis software. Fig. 2 depicts an example of typical peak review in high-throughput mode of samples from a metabolic stability assay. The MRM transition on the upper panel depicts the analyte trace, the MRM transition on the lower panel the trace of the internal standard. Samplings of compounds from late time-points of the metabolic stability assay were injected first i.e., the analysis sequence was arranged from potentially low to high analyte concentrations, which minimizes sample to sample carry-over effects and thus errors in quantitation and half-lifes.

3.4. Carry-over and sensitivity

Once the integral workflow of the RIAS has been established the major requirements to the analytical system were minimal carry-over effects and a maximized sensitivity. In order to minimize sample carry-over the tubing of the rapid injection system is flushed with organic solvent after each sample injection shown in Fig. 1C). Furthermore one aqueous and one methanolic injection from the integrated wash station is executed between samples, in order to clean the sipping needle from the inside and from the outside. The example in Fig. 3 shows a representative carry-over

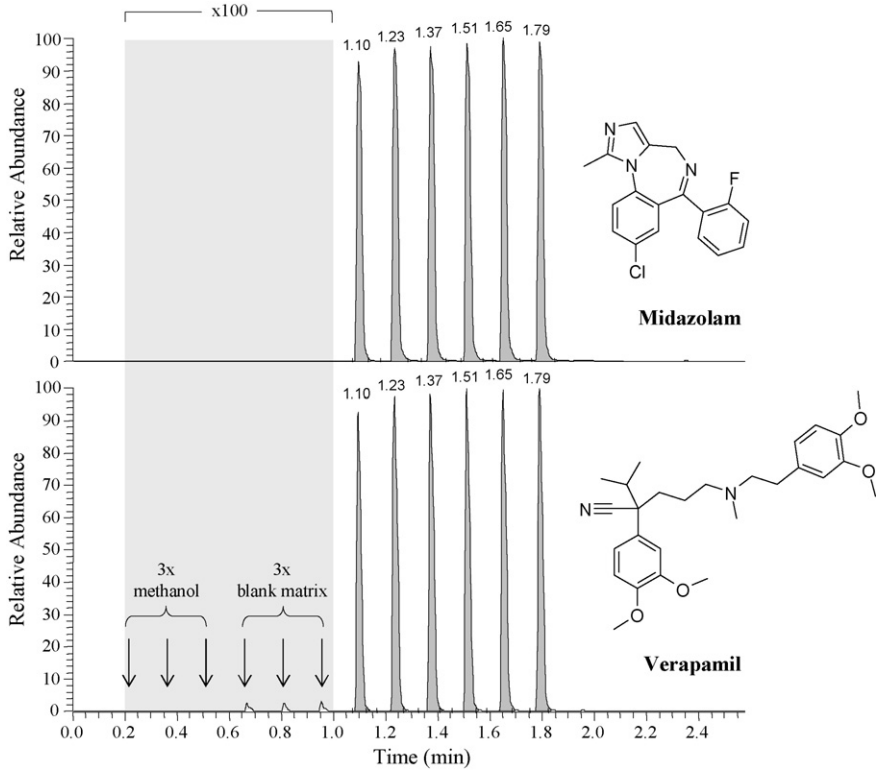

Fig. 3. Investigation of carry-over and signal-to-noise levels. Injection series of 3× methanol and 3× blank matrix from a metabolic stability experiment (human liver microsomes), followed by 6× a cocktail solution of midazolam and verapamil spiked into the abovementioned blank matrix (300 nM conc. each) and again 3× blank matrix and 3× methanol to demonstrate low carry-over and high sensitivity of the system. MRM signals in the time period from 0.2 to 1 min were amplified by a factor of 100 to show the high signal-to-noise level (3×10^5:1 for midazolam and 2×10^4:1 for verapamil). Carry-over for both analytes was 0.2% and the CV of peak area for the 6 injections was 2% for midazolam and 3% for verapamil respectively.

and signal-to-noise characteristics of the system. Hence, a cocktail of two classical phase I metabolism substrates, midazolam and verapamil (300 nM each) in a matrix of a metabolic stability assay with human liver microsomes was prepared. A sequence of three methanolic and three blank matrix injections was followed by six injections of the analytes, midazolam and verapamil, again followed by three injections of blank matrix. Almost no carry-over signal (0.2% for both, midazolam and verapamil) was observed and a high signal-to-noise level (3×10^5:1 for midazolam and 2×10^4:1 for verapamil) could be obtained as illustrated in Fig. 3 by amplifying the baseline in the region of blank matrix injections by a factor of 100.

3.5. Reproducibility

Reproducibility is a further important prerequisite for quantitative work and has been addressed at an early stage of validation of the system. The RapidFire™ system in general is sensitive to samples containing particles which might cause clogging of the microfluidic flow-path [25]. To prevent clogging all sample plates are centrifuged before analysis. A system suitability test is commonly performed before initiating the analysis of samples and checks the system regarding reproducibility, peak shape and sensitivity. As a result, reproducibility of the system was found to be comparable to conventional LC–MS/MS systems. This is exemplified by in Fig. 3 where a matrix of protein-precipitated incubations from a metabolic stability assay with human liver microsomes has been spiked with a solution of midazolam and verapamil (300 nM each) and injected six times. The coefficient of variation (CV) was 2% for midazolam and 3% for verapamil, respectively confirming excellent reproducibility for LC–MS/MS analytics in general.

3.6. Application to metabolic stability analysis

The RIAS, customized and validated according to the above mentioned criteria, was tested in a fully automated way, covering compound optimization (tuning plates) and sample measurement (sample plates) for metabolic stability experiments. The setup of the system enabled unattended processing of the provided 96-well plates. The results from 48 metabolic stability assays using human liver microsomes assaying a set of eight commercially available reference compounds are depicted in Fig. 4. The correlation of half-lifes ($t_{1/2}$) from a validated LC–MS/MS approach (x-axis) and half-lifes from the newly customized RIAS (y-axis) with the abovementioned reference compounds displayed an excellent correlation with a correlation coefficient (r^2) of 0.982.

The selected reference compounds represent high-, medium- and low-clearance drugs and are prevalently used to characterize enzymatic activities of phase I drug metabolizing enzymes, such as cytochrome P450 isoenzymes (CYP450). Buspirone and verapamil are highly cleared by N-dealkylation of CYP450 3A4 while midazolam is rapidly hydroxylated. Alprenolol and diclofenac represent high clearance drugs also underlying hydroxylation by CYP450 2D6 (alprenolol) and CYP450 2C9 (diclofenac). Dextromethorphan and diltiazem undergo moderate dealkylation by CYP450 2D6 and 3A4 while diazepam, a low clearance drug, is slowly hydroxylated and dealkylated by CYP450 2C19 and 3A4. The microsomal stability assay as well as mass spectrometric analytics were performed on the same day ($n = 2$) and repeated on 3 consecutive days (total $n = 6$) for each of the reference compounds.

The real-life applicability was demonstrated with an extended set of 470 metabolic stability experiments in various animal species. 167 different compounds, reference compounds as well as proprietary research compounds with a molecular mass range from

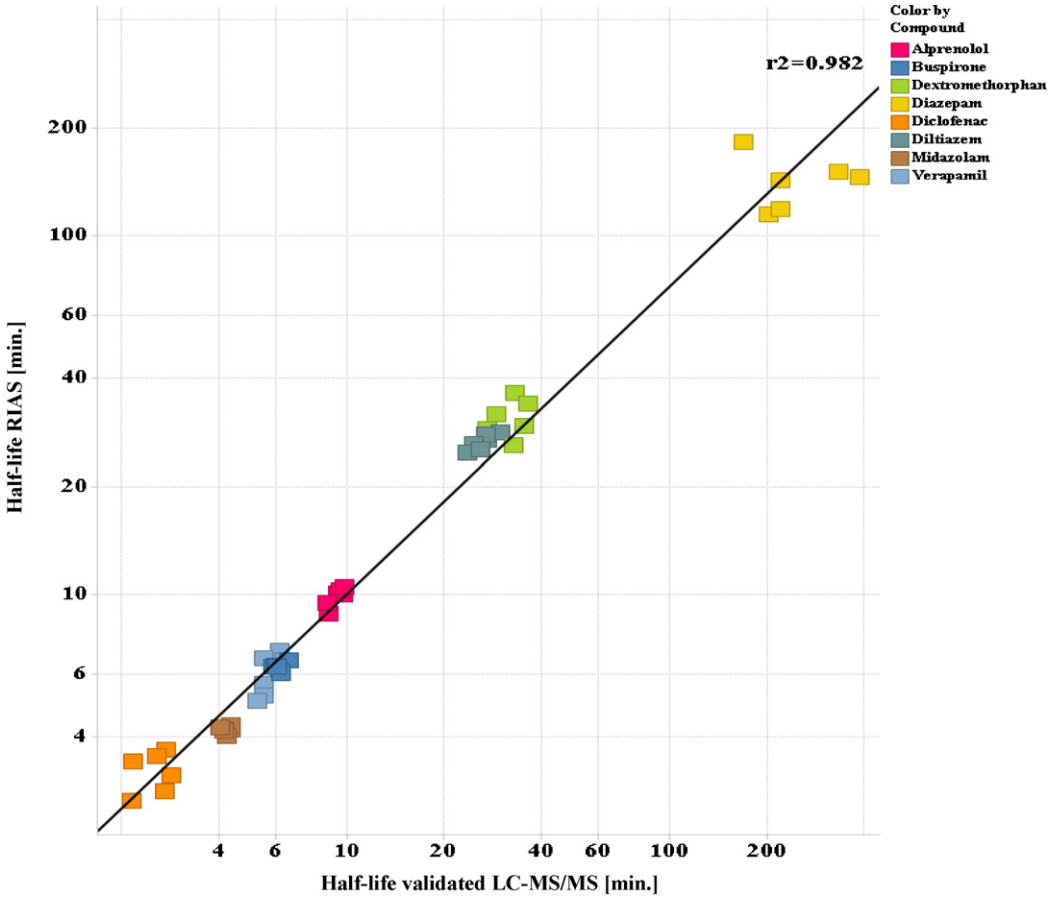

Fig. 4. Correlation of half-lifes of 8 model compounds in human liver microsomes. Correlation of half-lifes ($t_{1/2}$) derived from a validated LC–MS/MS system (x-axis) and the HT–MS/MS (y-axis) system "RIAS". Data were acquired from metabolic stability assays which were executed on 3 consecutive days ($n = 2$) for each analyte.

250 to 700 Da displayed a lipophilicity range (*c*log *P*) between −0.7 and 9, which is representative for early drug discovery programs. Since MS compound optimization in the RIAS is performed in a fully automated way the operator did not require a priori knowledge of the compound characteristics like molformula or structural information. The assays and mass spectrometric experiments have been performed under real-life research conditions, i.e., samples underwent multiple freeze and thaw cycles. In addition, sample measurement in the validated LC–MS/MS assay and the RIAS have not always been performed on the same day. Fig. 5 shows a high correlation ($r^2 = 0.828$) of half-lifes ($t_{1/2}$) between the validated LC–MS/MS approach (x-axis) and the RIAS (y-axis). Due to the limits of the biological experiments the last time point of sampling (sampling of the metabolic stability assay of a research compound) was taken at 45 min and an extrapolation by a factor of two (=90 min) was considered to be acceptable apart from influencing a certain variance of the half-lifes ($t_{1/2}$). In Fig. 5 the blue doted lines indicate the border of 90 min and compounds beyond these lines are considered to be stable. Sample degradation from multiple freeze and thaw cycles in connection with unknown stability characteristics of research compounds are considered as the probable reasons for outliers. During the data analysis process even small deviations in the regression plot of the assays time-points lead to big differences in half-lifes ($t_{1/2}$) and therefore cause a typical spread in the correlation plot for half-lifes beyond 90 min. Since outliers are found for compounds measured by validated LC–MS/MS and by the RIAS an equality of both methods with regard to reliability of half-lifes is assumed.

The entire analytical process (MS compound optimization and sample measurement) for 470 experiments lasted 120 h on the vali-

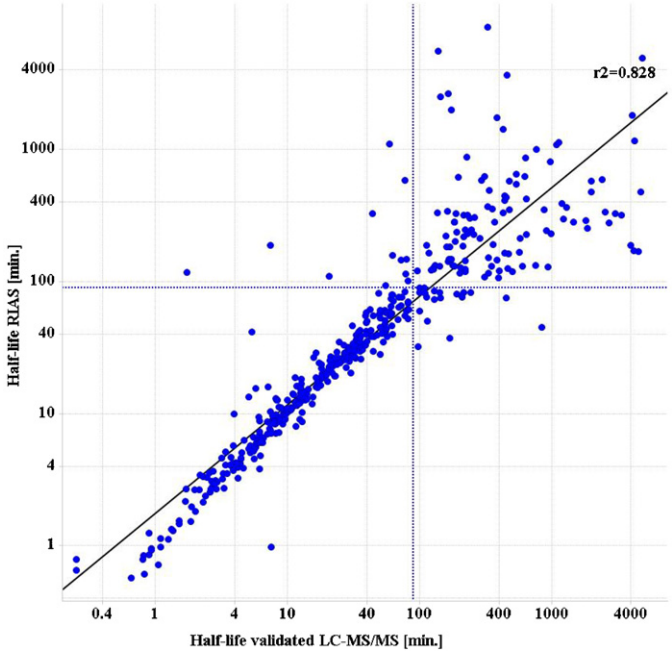

Fig. 5. Correlation of half-lifes of 167 compounds from 470 metabolic stability experiments. Correlation of half-lifes ($t_{1/2}$) derived from a validated LC–MS/MS (x-axis) system and the RIAS (y-axis). All data were obtained after fully automated compound optimization with the new system. Samples from different species were derived from 470 metabolic stability experiments with 167 compounds in different species. Cycle time from sample to sample is 8 s based on RapidFire™ technology compared to 1 min with the validated approach.

dated system but only 12 h on the RIAS – corresponding to 2338 fast injections (3 s each) for MS tuning and 4700 injections (8 s each) for sample measurement. By speeding up a factor of 10 while keeping data quality for reference compounds as well as for a representative set of research compounds, we consider the RIAS as a superior system in generating data from metabolic stability assays compared to the previously applied validated LC–MS/MS.

3.7. Limitations and future directions

The setup of the HT-autosampler and mass spectrometer, controlled by a tailored software solution, provides optimized speed of sample measurement and thus data generation of metabolic stability assays. The current limitation for further increasing sampling rate is the scan speed of the mass spectrometer. Analyte signals originating from the RIAS usually show a peak width at baseline of 2 s. Assuming that 10 data points are required to describe a peak with adequate precision, a narrow peak width implies a trade-off between scan speed of the mass spectrometer and the number of data points from MRM transitions. Furthermore, the new high speed makes demands on the capacity of the system components. A throughput of 450 samples/h extrapolated to 24 h equals a theoretical throughput of 10,800 samples per day. However, in our applications the solid-phase extraction cartridge of the system is exhausted already after 2500 injections. This necessitates a manual cartridge exchange approximately every 4 h. Most recent technical modifications of the RapidFire™ system have addressed this issue by implementing an automated cartridge exchange device. Furthermore, further potential to decrease the sample to sample time may be achieved if the mass spectrometer scan speed could be reduced.

In the present contribution, the speed and robustness of the RIAS (rapid and integrated analysis system) has been successfully demonstrated using metabolic stability experiments as an example. It remains to be seen whether other assays, e.g., *in vitro* permeability like CaCo-2 and PAMPA can be accelerated similarly, thereby integrating the RIAS into other ADME processes. Currently, the use of this HT–MS/MS in bioanalytics of pharmacokinetic and toxicokinetic samples should be considered critically. Since the system is based on a step gradient with a concomitant elution of all adsorbed compounds, *in-source* fragmentation of metabolites (e.g., glucuronides) to the parent compound could lead to higher, falsely higher parent compound concentrations. Although the system has not yet been evaluated for phase II metabolites or *in vivo* samples, advanced MS pre-filter technology [35–39] may extend the application area of the RIAS to phase I and phase II metabolite samples.

4. Conclusions

The customized RIAS (rapid and integrated analysis system) described provides a robust tool for high-throughput analysis of *in vitro* metabolic stability experiments including barcode-mediated and automated compound optimization and sample measurement. Integration into the ADME workflow including experiment request, sample preparation up to the final result calculation and reporting has been achieved. Matrix and carry-over effects have been reduced by a sophisticated column switching. A sample to sample time of 8 s was achieved which is significantly faster than current conventional LC–MS/MS systems. The excellent correlation of eight known drugs and 159 proprietary Boehringer Ingelheim compounds between validated LC–MS/MS and RIAS data proves robustness, reliability and competitiveness of the system. The RIAS appears to have the potential being employed also for other ADME applications, e.g., CaCo-2 and PAMPA thus becoming a universal and fully automated system for a considerable range of *in vitro* ADME applications.

Acknowledgements

The authors would like to thank the following colleagues from Boehringer Ingelheim (Biberach, Germany): Beate Krüger and Christian Kneer for their valuable contribution to integrate the described system into the entire workflow, Dr. Daniel Bischoff for fruitful discussions, Dr. Brian Guth for his revision of the manuscript and Dr. Klaus Klinder and Dr. Kurt Schumacher for their support of the project.

Finally, we greatly acknowledge the collaborative spirit of Dr. Can Oezbal from BioCius, Nicholas Duczak from ThermoFisher Scientific and Dr. Larry Elvebak from Gubbs Inc.

References

[1] Y. Deng, J.T. Wu, T.L. Lloyd, C.L. Chi, T.V. Olah, S.E. Unger, Rapid Commun. Mass Spectrom. 16 (2002) 1116–1123.
[2] N. Ishizuka, H. Kobayashi, H. Minakuchi, K. Nakanishi, K. Hirao, K. Hosoya, T. Ikegami, N. Tanaka, J. Chromatogr. A 960 (2002) 85–96.
[3] S.X. Peng, A.G. Barbone, D.M. Ritchie, Rapid Commun. Mass Spectrom. 17 (2003) 509–518.
[4] L. Di, E.H. Kerns, Y. Hong, T.A. Kleintop, O.J. McConnell, D.M. Huryn, J. Biomol. Screen. 8 (2003) 453–462.
[5] K.W. Dunn-Meynell, S. Wainhaus, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 19 (2005) 2905–2910.
[6] L. Gao, X. Cheng, J. Zhang, D.J. Burns, Rapid Commun. Mass Spectrom. 21 (2007) 3497–3504.
[7] K. Heinig, J. Henion, J. Chromatogr. B: Biomed. Sci. Appl. 732 (1999) 445–458.
[8] L.A. Romanyshyn, P.R. Tiller, J. Chromatogr. A 928 (2001) 41–51.
[9] K.P. Bateman, M. Kellmann, H. Muenster, R. Papp, L. Taylor, J. Am. Soc. Mass Spectrom. 20 (2009) 1441–1450.
[10] R.S. Plumb, W.B. Potts III, P.D. Rainville, P.G. Alden, D.H. Shave, G. Baynham, J.R. Mazzeo, Rapid Commun. Mass Spectrom. 22 (2008) 2139–2152.
[11] T. Yamashita, Y. Dohta, T. Nakamura, T. Fukami, J. Chromatogr. A 1182 (2008) 72–76.
[12] J.S. Janiszewski, K.J. Rogers, K.M. Whalen, M.J. Cole, T.E. Liston, E. Duchoslav, H.G. Fouda, Anal. Chem. 73 (2001) 1495–1501.
[13] K. Heinig, F. Bucheli, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 795 (2003) 337–346.
[14] J.S. Janiszewski, T.E. Liston, M.J. Cole, Curr. Drug Metab. 9 (2008) 986–994.
[15] D.B. Kassel, Curr. Opin. Chem. Biol. 8 (2004) 339–345.
[16] R.C. King, C. Miller-Stein, D.J. Magiera, J. Brann, Rapid Commun. Mass Spectrom. 16 (2002) 43–52.
[17] K.M. Whalen, K.J. Rogers, M.J. Cole, J.S. Janiszewski, Rapid Commun. Mass Spectrom. 14 (2000) 2074–2079.
[18] Z. Yan, C. Lu, J.T. Wu, L. Elvebak, A. Brockman, Rapid Commun. Mass Spectrom. 19 (2005) 1191–1199.
[19] M.J. Kim, H. Kim, I.J. Cha, J.S. Park, J.H. Shon, K.H. Liu, J.G. Shin, Rapid Commun. Mass Spectrom. 19 (2005) 2651–2658.
[20] A. Tolonen, A. Petsalo, M. Turpeinen, J. Uusitalo, O. Pelkonen, J. Mass Spectrom. 42 (2007) 960–966.
[21] S. Yu, E. Crawford, J. Tice, B. Musselman, J.T. Wu, Anal. Chem. 81 (2009) 193–202.
[22] A.H. Luippold, T. Arnhold, W. Joerg, K. Klinder, K Schumacher, A novel and integrated platform for fully automated high-throughput LC–MS/MS analysis of in vitro ADME samples, in: 57th ASMS Conference, Philadelphia. Ref Type: Conference Proceeding, 2010.
[23] C.D. Forbes, J.G. Toth, C.C. Ozbal, W.A. Lamarr, J.A. Pendleton, S. Rocks, R.W. Gedrich, D.G. Osterman, J.A. Landro, K.J. Lumb, J. Biomol. Screen. 12 (2007) 628–634.
[24] T.G. Holt, B.K. Choi, N.S. Geoghagen, K.K. Jensen, Q. Luo, W.A. Lamarr, G.M. Makara, L. Malkowitz, C.C. Ozbal, Y. Xiong, C. Dufresne, M.J. Luo, Assay Drug Dev. Technol. 7 (2009) 495–506.
[25] M. Jonas, W.A. Lamarr, C. Ozbal, Comb. Chem. High Throughput Screen. 12 (2009) 752–759.
[26] C.C. Ozbal, W.A. Lamarr, J.R. Linton, D.F. Green, A. Katz, T.B. Morrison, C.J. Brenan, Assay Drug Dev. Technol. 2 (2004) 373–381.
[27] A.K. Quercia, W.A. Lamarr, J. Myung, C.C. Ozbal, J.A. Landro, K.J. Lumb, J. Biomol. Screen. 12 (2007) 473–480.
[28] A.K. Shiau, M.E. Massari, C.C. Ozbal, Comb. Chem. High Throughput Screen. 11 (2008) 231–237.
[29] P. Soulard, M. McLaughlin, J. Stevens, B. Connolly, R. Coli, L. Wang, J. Moore, M.S. Kuo, W.A. Lamarr, C.C. Ozbal, B.G. Bhat, Anal. Chim. Acta 627 (2008) 105–111.
[30] A. Brown, S. Bickford, P. Hatsis, J. Amin, L. Bell, S. Harriman, Rapid Commun. Mass Spectrom. 24 (2010) 1207–1210.
[31] W. Roth, K. Beschke, R. Jauch, A. Zimmer, F.W. Koss, J. Chromatogr. 222 (1981) 13–22.
[32] L.E. Chovan, C. Black-Schaefer, P.J. Dandliker, Y.Y. Lau, Rapid Commun. Mass Spectrom. 18 (2004) 3105–3112.

[33] K. Geddes, G. Adamson, N. Dube, S. Crathern, R.C. King, Rapid Commun. Mass Spectrom. 23 (2009) 1303–1312.
[34] K. Kieltyka, J. Zhang, S. Li, M. Vath, C. Baglieri, C. Ferraro, T.A. Zvyaga, D.M. Drexler, H.N. Weller, W.Z. Shou, Rapid Commun. Mass Spectrom. 23 (2009) 1579–1591.
[35] P. Hatsis, A.H. Brockman, J.T. Wu, Rapid Commun. Mass Spectrom. 21 (2007) 2295–2300.
[36] P. Hatsis, J.T. Kapron, Rapid Commun. Mass Spectrom. 22 (2008) 735–738.
[37] P. Hatsis, G. Valaskovic, J.T. Wu, Rapid Commun. Mass Spectrom. 23 (2009) 3736–3742.
[38] J.T. Kapron, M. Jemal, G. Duncan, B. Kolakowski, R. Purves, Rapid Commun. Mass Spectrom. 19 (2005) 1979–1983.
[39] Y.Q. Xia, M. Jemal, Anal. Chem. 81 (2009) 7839–7843.