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

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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

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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