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Systematic evaluation of commercially available ultra-high performance liquid chromatography columns for drug metabolite profiling: Optimization of chromatographic peak capacity

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ABSTRACT

The present study investigated the practical use of modern ultra-high performance liquid chromatography (UHPLC) separation techniques for drug metabolite profiling, aiming to develop a widely applicable, high-throughput, easy-to-use chromatographic method, with a high chromatographic resolution to accommodate simultaneous qualitative and quantitative analysis of small-molecule drugs and metabolites in biological matrices. To this end, first the UHPLC system volume and variance were evaluated. Then, a mixture of 17 drugs and various metabolites (molecular mass of 151–749 Da, log *P* of −1.04 to 6.7), was injected on six sub-2 μm particle columns. Five newest generation core shell technology columns were compared and tested against one column packed with porous particles. Two aqueous (pH 2.7 and 6.8) and two organic mobile phases were evaluated, first with the same flow and temperature and subsequently at each column's individual limit of temperature and pressure. The results demonstrated that pre-column dead volume had negligible influence on the peak capacity and shape. In contrast, a decrease in post-column volume of 57% resulted in a substantial (47%) increase in median peak capacity and significantly improved peak shape. When the various combinations of stationary and mobile phases were used at the same flow rate (0.5 mL/min) and temperature (45 °C), limited differences were observed between the median peak capacities, with a maximum of 26%. At higher flow though (up to 0.9 mL/min), a maximum difference of almost 40% in median peak capacity was found between columns. The finally selected combination of solid-core particle column and mobile phase composition was chosen for its selectivity, peak capacity, wide applicability and peak shape. The developed method was applied to rat hepatocyte samples incubated with the drug buspirone and demonstrated to provide a similar chromatographic resolution, but a 6 times higher signal-to-noise ratio than a more traditional UHPLC metabolite profiling method using a fully porous particle packed column, within one third of the analysis time. In conclusion, a widely applicable, selective and fast chromatographic method was developed that can be applied to perform drug metabolite profiling in the timeframe of a quantitative analysis. It is envisioned that this method will in future be used for simultaneous qualitative and quantitative analysis and can therefore be considered a first important step in the Quan/Qual workflow.

1. Introduction

Over the last few years, the field of liquid chromatography has been rapidly evolving. Pressured mainly by the pharmaceutical industry demanding faster separations to increase productivity and speed and to reduce costs, column manufacturers introduced columns with sub-2 μm fully porous particles and, more recently, with solid core particles to improve the efficiency of separations. In order to fully benefit from these stationary phases, a liquid chromatography (LC) instrument should have a low dwell volume, system volume, system variance and injection cycle time, and should be able to operate under high pressures [1]. When used with the conventional high performance liquid chromatography instruments (HPLC), the efficiency gained with the small particle sizes is lost due to the large system volume and variance as compared to

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that of the column [2]. Therefore, to keep up with the progression in the stationary phases, a new generation of instruments, the ultra-high performance liquid chromatography (UHPLC) instruments, was developed and first launched in 2004 [3]. Since then, continuous efforts have been made to further reduce the system volumes and variances. The current state-of-the-art instruments have a system volume in the range of 10 μL, a system variance (dispersion) as low as 1–3 μL^2 and can stand pressures up to 1300 bar [1].

Also the area of mass spectrometry is evolving. While the triple quadrupole mass spectrometer (TQMS) employing single or multiple reaction monitoring has been the workhorse of the bioanalytical laboratories for decades, the use of high resolution mass spectrometers (HRMS) is now increasingly being adopted. The obvious advantage that full scan HRMS data provides more information than SRM or MRM data was for example demonstrated in a publication on HRMS in regulated quantitative bioanalysis, wherein Fung and coworkers described using the full-scan data not only to analyze their compounds of interest, prednisone and prednisolone, but also to obtain qualitative information on phospholipids and other endogenous compounds as well [4]. The widespread use of HRMS for quantitative analysis is still limited by various factors, like the large data files and the resulting problems with data storage and handling, the lower sensitivity as compared to TQMS, the current lack of expertise in quantification with HRMS and the price tag of the instrumentation. Still, with the increasing ease-of-use and the decreasing costs, it is expected that the use of HRMS in quantitative bioanalysis will continue to expand.

Currently we are at a stage that the combination of the above described developments toward more efficient chromatography and toward the use of HRMS for quantitative purposes can lead to a paradigm shift in the classical workflow of the drug metabolism and pharmacokinetics (DMPK) departments in the pharmaceutical industry. At the moment, quantitative bioanalysis is mainly focused on high-throughput (U)HPLC separations followed by SRM analysis, while the identification and elucidation of drug metabolites is characterized by relatively long (U)HPLC separations, *viz.* 10 min up to hours, followed by HRMS analysis. The new approaches could favor the integration of high-throughput quantitative bioanalysis and drug metabolite profiling in one department to leverage the use of expensive instruments and limit the analysis time.

The idea of an integrated quantitative and qualitative workflow (Quan/Qual) has been proposed in several reviews [5,6], and various publications describe Quan/Qual assays for specific compounds [7,8]. Also the performance of recently commercialized sub-2 μm solid core particles has been described in theory [9,10], but the practical possibilities of these columns for Quan/Qual purposes have yet to be demonstrated. In the present study we explore these possibilities, focusing on their practical potential for high-throughput metabolite profiling. The aim was to develop an LC method that (i) is fast (*i.e.* total runtime <5 min), (ii) provides a high resolution separation to accommodate qualitative analysis, (iii) is generically applicable for a wide range of small molecule drugs and metabolites and (iv) is easy-to-use in daily practice. To achieve this, the instrumental setup of the available LC-system was evaluated and optimized and various recently commercialized columns were systematically evaluated for peak capacity, peak asymmetry and generic applicability. The final method was tested on *in vitro* rat hepatocyte incubation samples, to demonstrate its capability.

2. Experimental

2.1. Chemicals, columns

Water was obtained from a Milli-Q Purification System from Millipore (MA, USA). Acetonitrile, methanol (both Ultra LC–MS grade) and isopropanol (HPLC–MS grade) were supplied by Actu-All Chemicals (Oss, the Netherlands) and dimethylsulfoxide (DMSO, ≥99.7%) by Biosolve (Valkenswaard, the Netherlands). Formic acid (99%) was purchased from Acros Organics (Geel, Belgium) and ammonium acetate (>98%) from Sigma–Aldrich (Gillingham, UK). Also the drug product standards of acetaminophen, tolbutamide, 19-norethindrone, omeprazole, prednisone, buspirone hydrochloride, (±)-verapamil hydrochloride, nefazodone hydrochloride and loperamide hydrochloride were obtained from Sigma–Aldrich. Abiraterone was supplied by Cambridge Major Laboratories, Inc. (WI, USA). Cilag AG (Schaffhausen, Switzerland) provided darunavir ethanolate and midazolam was obtained from Actavis (Dublin, Ireland). 1′-hydroxy midazolam and 4-hydroxy midazolam were obtained from Toronto Research Chemicals (Toronto, Canada).

Janssen Research and Development (Beerse, Belgium) provided the drug product standards of galantamine hydrobromide, rilpivirine, risperidone, bedaquiline and simeprevir. The drug metabolite standards 4-acetaminophen sulphate potassium salt and p-acetamidophenyl β-D-glucuronide sodium salt were purchased from Santa Cruz Biotechnology, Inc. (CA, USA).

Williams E medium and dexamethasone, used for the *in vitro* metabolism experiments, were obtained from Sigma; fetal bovine serum and L-glutamine from Invitrogen (Bleiswijk, the Netherlands) and 1000× insulin, transferrin, selenium mixture from BD (Le Pont de Claix Cedex, France).

The Acquity UPLC BEH C18 1.7 μm column (50 mm × 2.1 mm, 130 Å), Cortecs UPLC C18 1.6 μm and Cortecs UPLC $C18^+$ 1.6 μm column (both 50 mm × 2.1 mm, 90 Å) were kindly provided by Waters (Milford, MA, USA). Phenomenex Inc. (Torrance, CA, USA) kindly provided the Kinetex 1.3 μm C18, Kinetex 1.7 μm XB-C18 and Kinetex 1.7 μm Phenyl-Hexyl columns (all 50 mm × 2.1 mm, 100 Å).

2.2. Methods

2.2.1. The standard mixture

Stock solutions of acetaminophen, tolbutamide, galantamine, norethindrone, the two hydroxylated midazolam metabolites, omeprazole, abiraterone, prednisone, rilpivirine, buspirone, risperidone, verapamil, nefazodone, loperamide, darunavir, bedaquiline, simeprevir, p-acetaminophenyl beta-D-glucuronide and 4-acetaminophen sulphate were prepared in DMSO at 2 mg/mL. Midazolam was purchased in solution at 5 mg/mL. A working solution containing a 4 μg/mL mixture of all compounds in DMSO was also prepared. Upon analysis, a volume hereof was diluted to the desired concentration with a final composition of DMSO: acetonitrile: water 1:6:7 (v/v/v).

2.2.2. Mass spectrometry

The mass spectrometer (MS) used was a Synapt G2S (Waters, Manchester, UK), which was equipped with an electrospray ionization probe and operated in positive ionization mode. Lock-mass calibration was performed with 0.1 mg/mL leucine-enkephalin (*m/z* 556.2771). The MS parameters were optimized by both direct infusion and flow injection analysis of a dilution of the standard mixture.

The capillary voltage was set at 0.7 kV, the sampling cone at 40 V, the source offset at 50 V, the nitrogen cone gas and desolvation gas flow rates at 50 L/h and 800 L/h, respectively, the source and desolvation temperature at 125 °C and 500 °C, respectively, the trap and transfer collision energy at 4.0 eV and 2.0 eV, respectively, the scan time at 100 ms and the lock-mass infusion rate at 10 μL/min. The time-of-flight analyzer was operated in V-mode (resolution mode) optics and mass spectral data were acquired over the range *m/z* 50–1200. For the experiments with an LC flow rate above 0.5 mL/min, the desolvation gas flow rate was changed to 1200 L/h, the source and desolvation temperatures to 150 °C and