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Journal of Chromatography B, 788 (2003) 331–337
JOURNAL OF CHROMATOGRAPHY B
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Fast analysis using monolithic columns coupled with high-flow on-line extraction and electrospray mass spectrometric detection for the direct and simultaneous quantitation of multiple components in plasma

Hang Zeng*, Yuzhong Deng, Jing-Tao Wu

Discovery MAP Group, Bristol-Myers Squibb Company, Route 141 and Henry Clay Road, Wilmington, DE 19880, USA

Received 26 February 2002; received in revised form 30 December 2002; accepted 30 December 2002

Abstract

In this work, monolithic columns were used as a fast separation tool for multiple-component quantitative liquid chromatography–tandem mass spectrometry (LC–MS–MS) assays of drug candidates in biological fluids. A considerably reduced runtime was achieved while maintaining good chromatographic separations. This significantly improved separation speed demanded higher throughput on sample extraction. To this end, monolithic separations were coupled on-line with high-flow extraction, which allowed for the fast extraction and separation of samples containing multiple analytes. An evaluation of this system was performed using a mixture of fenfluramine, temazepam, oxazepam, and tamoxifen in plasma. A total cycle time of 1.2 min was achieved which included both sample extraction and subsequent monolithic column separation via column switching. A total of over 400 plasma samples were analyzed in less than 10 h. The sensitivity and responses were reproducible throughout the run. The system has been routinely used in the authors' laboratory for high-throughput quantitation of compounds in biological fluids in support of drug discovery programs. The assay for samples from a 9-in-1 dog pharmacokinetic study is shown as an example to demonstrate the capability of this system.

Keywords: Monolithic columns; Multiple-component analysis

1. Introduction

The quantitation of compounds in plasma is an essential step in the determination of the pharmacokinetic (PK) properties of new chemical entities. In an accelerated drug discovery environment, an ever-increasing number of compounds are put into PK screening for lead optimization. As a result, new analytical methodologies are required to improve the throughput of quantitation of compounds in plasma.

As one of the approaches to reduce animal costs and workload, the simultaneous dosing of multiple compounds to a single animal followed by multiple-component LC–MS–MS assay (the *N*-in-1 approach) has been used. In many cases, the *N*-in-1 approach has proved to be an effective way to improve the throughput of PK screening [1–5]. Because this

*Corresponding author. Current address: Bioanalytical/DMPK, Millennium Pharmaceuticals, Inc., 270 Albany Street, Cambridge, MA 02139, USA. Tel.: +1-617-374-7715; fax: +1-617-551-7973.
E-mail address: hzeng@mpi.com (H. Zeng).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00040-0

strategy is mainly used in the lead optimization stage, most of the compounds in an *N*-in-1 pool are structural analogs. Also, each of these compounds can form numerous metabolites. The specificity offered by MS–MS alone is often inadequate for these applications. Thus, chromatography becomes essential for achieving sufficient specificity of the assay. Therefore, an effective high-throughput LC–MS–MS method for multiple-component assay from plasma should include high-quality chromatographic separations.

Plumb et al. [6] and the present authors [7] recently reported the use of monolithic columns for high-throughput quantitation in biofluids. In this approach, a column made of a single piece of silica rod was used instead of conventional particulate columns. The monolithic column possesses a unique biporous structure [8–10]. The mesopores (2 to 20 nm) located on the silica skeleton provide the large surface area needed to achieve sufficient capacity while the larger through pores (0.5 to 8 μm) reduce flow resistance, allowing the use of high flow-rates at considerably reduced backpressure. Thus, columns of conventional lengths (5 or 10 cm or more) can be used with a high flow-rate to achieve sufficient resolving power. In addition, the dependency of separation efficiency on flow-rate is relatively small with monolithic columns. Therefore, high separation efficiency can be maintained at increased flow-rate, resulting in short runtimes. A total separation cycle of 1 to 2 min can be achieved for either a single- or multiple-component analysis.

Samples in plasma are usually not directly compatible with LC–MS–MS analyses [8]. Sample extraction or cleanup is thus generally required before analyses. This has been done using protein precipitation, liquid–liquid or solid-phase extraction. This process is labor intensive and time consuming. Although the use of various automation tools helps to improve the throughput in this process [9–12], the high-speed separation achieved using monolithic columns often shifts the bottleneck back to the sample extraction step. An alternative sample extraction method that generated a lot of interest more recently is the direct injection of plasma using an on-line high-flow extraction method [5,13,14]. Besides using only conventional HPLC equipment, a major advantage of this method over off-line solid-phase extraction using liquid handlers is that it embeds the sample preparation step into the chromatographic separation step and thus eliminates most of the sample preparation time.

In this work, we have coupled high-speed monolithic column separations with a high-flow on-line extraction column switching method previously reported [5,14]. Both the separation and extraction columns were operated at a cycle time of 2 min or less at a flow-rate of 4 mL/min. The coupling with a high-flow on-line extraction column allowed for the direct and reliable injection of large volumes of plasma.

2. Experimental

2.1. Chemicals and materials

All the commercially available compounds and mobile phase additives were purchased from Sigma (St. Louis, MO, USA). All HPLC grade solvents were from EM Science (Gibbstown, NJ, USA). In-house deionized water, purified with a Milli-Q water purification system purchased from Millipore (Bedford, MA, USA), was used for HPLC and sample preparation. Compounds used in *N*-in-1 PK analysis were synthesized at the Bristol-Myers Squibb Company (Wilmington, DE, USA).

2.2. Sample preparation

Aliquots of 100 μL of plasma samples were transferred from the sample tubes to a 96-well plate using a Packard liquid handler. An equal volume of the internal standard in water was added to the samples. Then 10 μL standard working solution in mixed solvent [water–acetonitrile–formic acid (95:5:0.1, v/v)] was spiked into the plasma mixture to form standards and quality control (QC) samples with a concentration range of 2.5–5000 ng/mL. The samples were ready for injection after vortex mixing for 1 min.

2.3. Chromatography and on-line extraction system

Two binary high-pressure mixing HPLC pumps were used for this work as depicted in Fig. 1. A

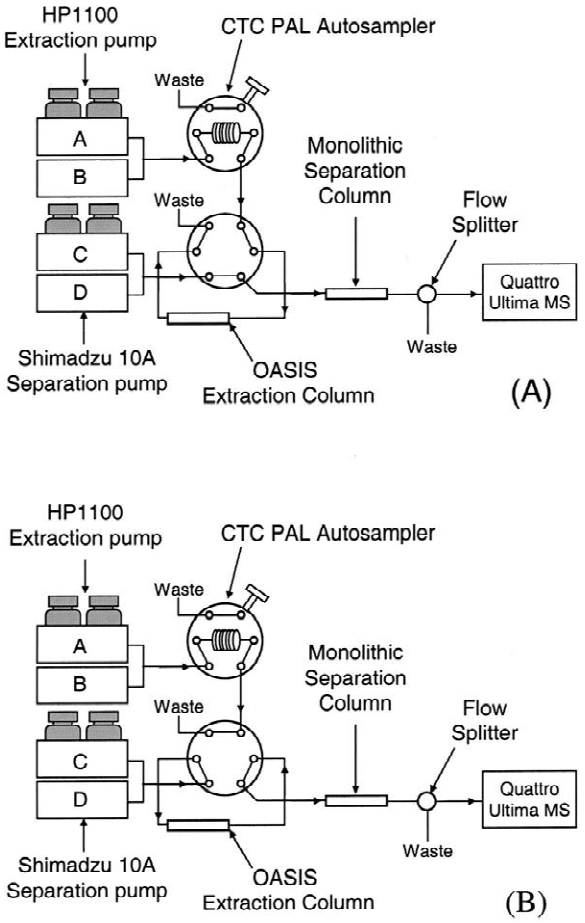

Fig. 1. Schematic diagram of the instrumental setup and configuration showing two valve positions: (A) extraction mode and (B) elution and separation mode.

HP1100 pump (Hewlett-Packard, Waldbronn, Germany) was used to deliver a high flow through the extraction column to load and wash the sample and subsequently to flush and equilibrate the extraction column. A Shimadzu LC-10AD-vp pump (Shimadzu, Columbia, MD, USA) was used to deliver a fast gradient flow to elute the analytes from the extraction column and to perform the separation on a monolithic silica column. A CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC, USA) was used to inject plasma samples. The 54-vial sample trays or 96-well plates used can be refrigerated in a sample cooling stack at temperatures of 4–10 °C. This autosampler also allowed for the use of two separate wash solvents for both the syringe and the injector. An aqueous solvent was used before a high organic content solvent to avoid plugging resulting from protein precipitation.

A C_{18} monolithic column (Chromolith Performance, RP-18e, 4.6×100 mm, Merck, Darmstadt, Germany) was used as the separation column. A reversed-phase extraction column (Oasis HLB, 1×50 mm, Waters, Milford, MA, USA) was used for high-flow extraction.

2.4. Electrospray ionization mass spectrometry

A triple quadrupole mass spectrometer (Quattro Ultima, Micromass, Manchester, UK) with a Z-spray ionization source was used as the mass detector. The positive ion electrospray ionization mode was used for all studies in this work. The mass spectrometer was operated in multiple reactions monitoring (MRM) mode with a unit mass resolution on both mass analyzers. Dwell times of 80 ms per channel with an inter-channel delay of 20 ms were used for studies in this work. The flow from the separation column at 4 mL/min was split to 0.4 mL/min using a purge valve (Valco Instrument, Houston, TX, USA) before introduction into the mass spectrometer.

2.5. System operation and control

After the plasma sample (50 μL) was injected by the CTC autosampler, the sample was loaded onto the extraction column by solvent A, HPLC grade water, at a flow-rate of 4 mL/min. At 0.6 min after injection, the valve switched to the other position, which put the extraction and separation columns in tandem in the flow path of the separation pump (Shimadzu). The separation pump then started a gradient using solvents C (water with 0.1% formic acid) and D (acetonitrile with 0.1% formic acid) to elute the retained analytes from the extraction column to the separation column using a flow-rate of 4 mL/min. A linear gradient of D from 10 to 90% was used. The effluent from the analytical column was directed to the mass spectrometer electrospray source with a 1 to 9 split. An elution step of 0.5 min was given before the switching valve was switched back to the original position (configuration 1 in Fig. 1). The extraction pump then started to deliver solvent

B, methanol with 0.1% formic acid, at a flow-rate of 4 mL/min for 0.5 min to flush the extraction column. For the last 0.4 min in the run cycle, the extraction pump delivered solvent A at 4 mL/min to recondition the Oasis extraction column for the next sample. The run time for the assay of an analyte mixture of four test compounds was 1.2 min; and the run time for the dog 9-in-1 PK study was 2 min.

For system control and synchronization, a Pentium III 733 MHz Compaq computer with Masslynx 3.4 was used to control the mass spectrometer through a TDAT board and the HP1100 pump via an HPIB board, respectively. The CTC autosampler was controlled by its own keypad. The Shimadzu pump system with a built-in switching valve was controlled by the Shimadzu controller. The Quattro Ultima MS, HP1100 pump, Shimadzu pump and CTC autosampler were also connected by contact closures to synchronize time events. The CTC autosampler injected the sample and sent a contact closure signal to the HP1100 pump to start its time program. The HP1100 pump then sent out two closure signals to the mass spectrometer and the Shimadzu pump controller to start the data acquisition and the time program on the Shimadzu pump, respectively. Before the end of each run cycle, the HP1100 pump sent out another contact closure signal to CTC to inject the next sample.

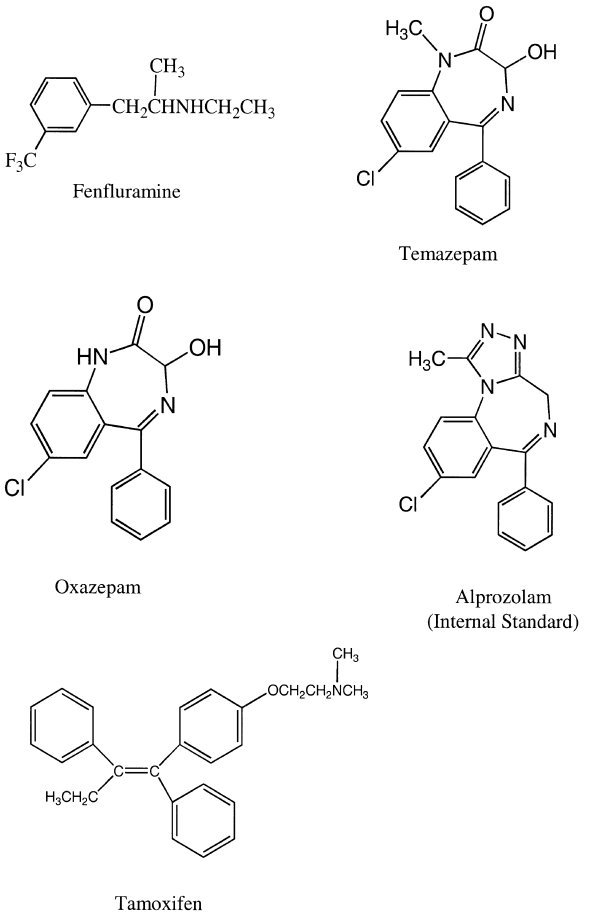

Fig. 2. Chemical structures of compounds used in the evaluation.

3. Results and discussion

In order to evaluate this high-throughput system, a test was performed using a mixture containing five commercially available compounds. These five compounds were alprozolam, fenfluramine, oxazepam, temazepam, and tamoxifen. Alprozolam was used as the internal standard in the test. The structures of these compounds are shown in Fig. 2. In this test, this mixture was spiked into rat plasma at different concentrations to form a seven-point standard curve from 2.5 to 5000 ng/mL. This plasma standard curve was repeatedly injected onto the system with four blank plasma injections between two sets of curves. The run cycle time was 1.2 min. During the first half of the runtime (0 to 0.6 min), the samples were extracted on the extraction column while the analytical column (the monolithic column) was re-equilibrated from the gradient of the previous sample. During the second half of the runtime (0.6 to1.2 min), the separation of the analytes was performed on the analytical column while the extraction column went through a recycle process to prepare itself for the next sample. Therefore, the extraction and separation processes were carried out in parallel to enhance throughput. A total of 407 plasma injections were made continuously within 10 h.

The system was found to be very rugged for the direct analysis of plasma samples. The back pressures on both the extraction column and the monolithic column showed less than 30% increase after over 400 plasma injections. Representative MRM chromatograms for a 250 ng/mL plasma

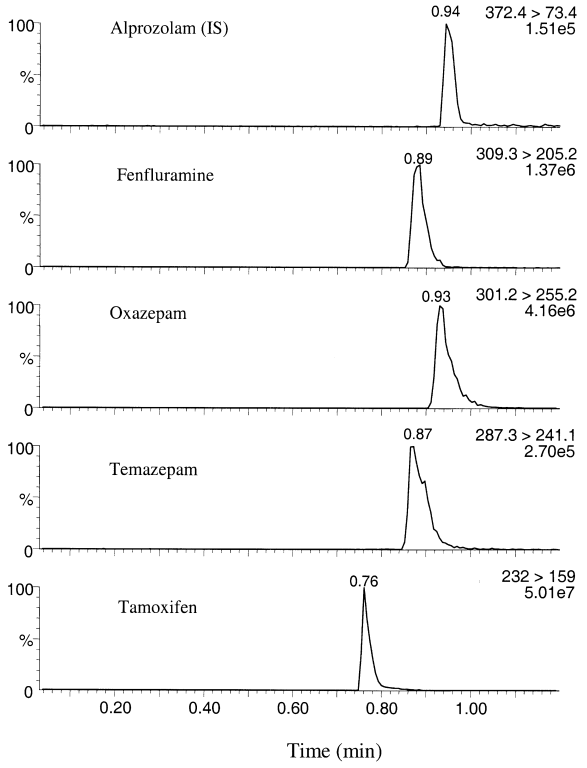

Fig. 3. MRM chromatograms for a direct injection of a rat plasma sample containing the four compounds used in the evaluation.

standard of the four compounds and the internal standard are shown in Fig. 3. In our experience, the extraction column can usually last for at least three batches of this size before its back pressure doubles its original value and it should then be discarded. The monolithic columns last much longer, for about 5000 injections. Even after the back pressure rises, the monolithic column can still be used by connecting it in a reversed flow direction. Similar separation quality can be achieved with a much-reduced back pressure.

A quantitative assessment of the sensitivity (slope of curve) and peak response for the four compounds is summarized in Table 1. In order to compare the system performance throughout the run, the average response and sensitivity at the beginning, in the middle, and at the end of the batch of 400 injections are listed. The relative standard deviation (RSD%) among the samples run at different positions in the batch was no more than 15% for both sensitivity and response. The RSD of the response at the LOQ was less than 20%.

The evaluation using the above four compounds demonstrated that this system was able to provide reliable and reproducible quantitative data for plasma samples containing multiple analytes. The system was therefore routinely used in the authors' laboratory for pharmaceutical bioanalysis in support of drug discovery programs. One of these examples is a 9-in-1 pharmacokinetic screening in the dog. In this study, a total of nine discovery compounds were dosed as a cassette to two dogs via intravenous (i.v.) infusion at a dose of 0.2 mg/kg for each compound. The plasma samples were collected and were directly injected on the system after mixing with an analog internal standard. In order to achieve cleaner samples and better chromatographic resolution, 1 min was used for the extraction and separation. Recycling of the gradient on the extraction and separation columns was performed in parallel to the separation and extraction processes, resulting in a total run time of 2

Table 1
Summary of average response and sensitivity for the analytes injected at the beginning, in the middle, and at the end of the batch[a]

	Average response for 1000 ng/mL (n=3)			RSD (%)	Average slope of curve ($\times 10^{-4}$) (n=3)			RSD (%)
	Front	Middle	End		Front	Middle	End	
Fenfluramine	6.4539	7.5178	7.9659	10.2	61.0	76.1	81.7	14.1
Oxazepam	0.0504	0.0497	0.0482	2.3	0.485	0.444	0.461	4.4
Temazepam	1.0171	0.8721	0.9946	8.1	9.77	9.29	9.69	2.7
Tamoxifen	0.0127	0.0164	0.0172	15.1	0.141	0.191	0.182	15.0

[a] Three sets of curves (seven standard/set) were used for linear regression calculations with a weighting of $1/x^2$. The average slopes were calculated with seven-point fits.

min. A set of MRM chromatograms for a sample collected 1 h after i.v. infusion is shown in Fig. 4. Although these nine compounds were all close structural analogs, adequate chromatographic resolution was achieved on the monolithic column with MS detection. A lower limit of quantitation (LLOQ) of 1 n*M* was achieved for seven of the nine compounds and a LLOQ of 2.5 n*M* was achieved for the other two compounds. These LLOQ values are fully comparable to conventional LC–MS–MS assays for these classes of compounds. The average concentration–time profile for all these nine compounds as a result of this high-throughput assay is shown in Fig. 5. Compared with conventional LC–MS–MS systems, the approach described in the report has a clear advantage in throughput for both sample preparation and chromatographic separation, which are often the bottlenecks in bioanalysis. More importantly, this throughput enhancement was achieved without sacrificing the quality of the separation efficiency and sensitivity. The disadvantage of this method is that the system carryover for some compounds may be higher due to insufficient flush for the on-line extraction system.

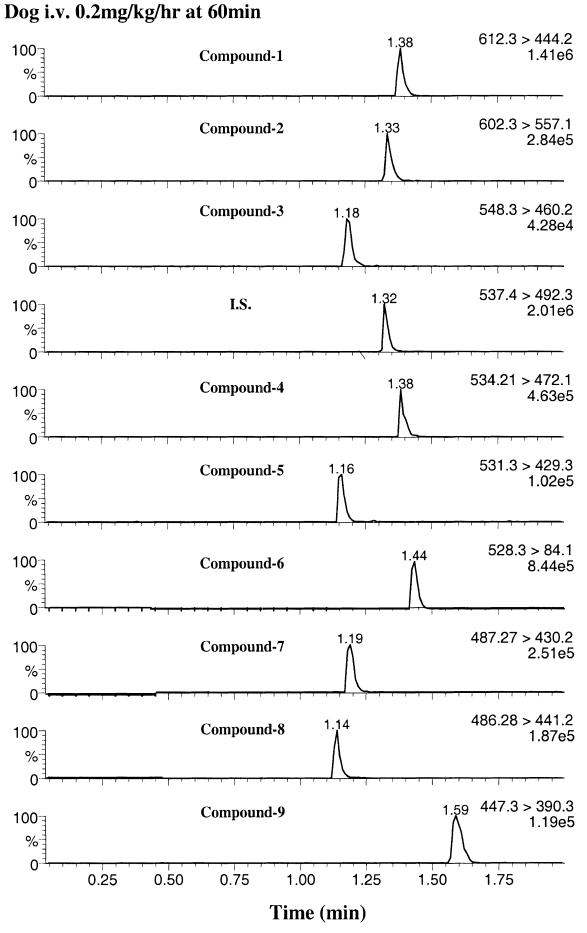

Fig. 4. MRM chromatograms for a plasma sample collected 60 min after an i.v. infusion of a 9-in-1 pharmacokinetic study in the dog.

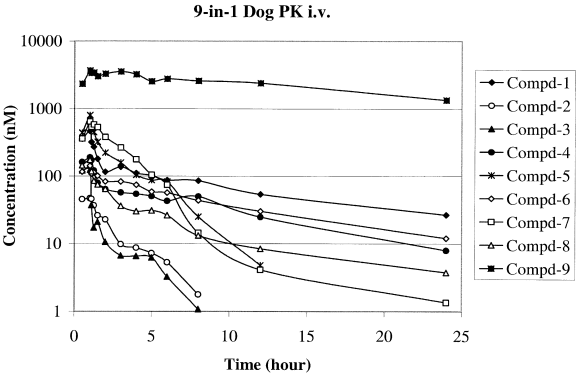

Fig. 5. Plasma concentration–time profile for the 9-in-1 pharmacokinetic study using the data generated on the system described in this work.

4. Conclusions

A new LC–MS–MS system has been developed to perform high-throughput quantitation of compounds in dog plasma. This system combines high-speed separation on monolithic columns with a fast and rugged on-line high-flow extraction method. A total extraction and separation cycle time of 2 min or less can be achieved for samples containing multiple analytes in plasma. In an evaluation of over 400 plasma injections, reproducible quantitative data were obtained for all the four analytes in the evaluation. This system has been routinely used in the authors' laboratory for high-throughput quantitation of drug candidates in plasma, urine and other biological fluids.

Acknowledgements

The authors would like to thank Dr. Steve Unger for the insightful discussions on monolithic columns and their potential applications in bioanalysis. The discovery support staff are also acknowledged for the design and implementation of the *N*-in-1 dog PK study.

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