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Generic three-column parallel LC–MS/MS system for high-throughput in vitro screens

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Abstract

A parallel three-column LC–MS/MS system for quantitative high-throughput in vitro screens is described. The robust novel system is composed of three LC pumps, an autosampler and a triple quadrupole mass spectrometer used in combination with three valves and three analytical LC columns in parallel configuration. Two of the three valves work in unison to select which column receives the injection, and the third valve selects which column is to be in line with the mass spectrometer. Improved sample throughput is achieved without sacrificing chromatographic separation quality or sensitivity. To demonstrate the applicability of the system, pools of five compounds (phenacetin, *S*-mephenytoin, bufuralol, midazolam and clomethiazole) were analyzed, together with an internal standard. The results show that the sample throughput can be increased significantly by reducing analysis time to 3 min per sample as compared to 8 min with a general gradient single-column system. Analysis of the five compounds shows an accuracy of 81–108% and a precision (given as relative standard deviation) of 1.5–14%. The system was further applied to samples from a metabolic stability assay in liver microsomes.

Keywords: Drug discovery; In vitro screens

1. Introduction

High-throughput absorption, distribution, metabolism and excretion (ADME) programs are becoming common throughout the pharmaceutical industry to aid in the rapid discovery of new candidate drugs [1]. Due to advancements in robotics, fully automatic procedures have been developed for metabolic stability and permeability assays. The increasing number of samples generates a large analysis workload, which has led to a constant need for more rapid quantification methods [2–4]. One of the most powerful techniques currently available for pharmaceutical analysis is liquid chromatography–tandem mass spectrometry (LC–MS/MS). In recent years, a number of new approaches have been applied in order to increase the LC–MS/MS throughput. The use of parallel HPLC systems combined with tandem mass spectrometry has proved useful to accelerate the analytical process [5–12]. A major disadvantage of some of these systems is that they require a significant capital investment in equipment, such as extra binary pumps, extra autosamplers, new computer software and/or a MUX multiple sprayer interface [5,9,10].

In the present paper, we describe a parallel three-column LC–MS/MS system that requires relatively low financial input. In this system, the time needed to equilibrate a column after gradient elution, is used to separate and detect the following sample by switching to the next column. With a three-column system, as compared to a two-column system [6], it is possible to utilize the time when injection takes place and while separation has started but no peak has yet been eluted. This is practically possible without extra investments. While analysis takes 3 min per sample with our system, a comparable one-column system with the same autosampler including the same binary pump and a corresponding 50 mm column would need 8 min. With a three-column system 1 min is gained utilizing the injection time. Two min can be saved making use of the time while the gradient is recycled. By applying a step gradient instead of a gradient created in a mixing chamber with a large

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dead volume, the elution process can be shortened down additionally 2 min. A comparable two-column system using corresponding 50 mm columns would be able to perform a sample analysis in 5 min, utilizing the injection time and the time while the gradient is recycled for parallel acquisition. Using a three-column system in combination with sample pooling significantly enhances sample throughput.

Synchronization of injection, choice of column and detection for each sample is accomplished by three co-operating valves. This system, developed for automated parallel LC–MS/MS analysis, is designed to meet requirements in terms of speed, capacity and precision. A generic step gradient LC system was created that copes with the extensive demands of structurally different compounds passing through the drug discovery process. Compounds with widely varying retention properties have to be covered by a single LC method. At the same time, separation has to be adequate to remove matrix traces and solvents from the in vitro assay. The system allows a great variety of settings, such as columns, mobile phases, detection mode and acquisition window. It can easily be adapted to other applications such as isocratic runs or detailed LC method development.

In order to evaluate the capability and the performance of this parallel three-column LC–MS/MS separation system, quantifications of a pool of the five compounds phenacetin, *S*-mephenytoin, bufuralol, midazolam and clomethiazole were performed. The system was further applied to samples from a metabolic stability assay in liver microsomes.

2. Experimental

2.1. High-performance liquid chromatography

The HPLC system consisted of a Waters Alliance 2790 autosampler (Waters, Milford, MA, USA), containing a binary pump used in isocratic mode (pump 1), two Shimadzu LC-10AD VP isocratic pumps (Shimadzu Corporation, Kyoto, Japan) (pumps 2 and 3), one Vici six-ports multiposition valve (VICI AG, Schenkon, Switzerland) and two Rheodyne six-ports switching valves (Rheodyne, L.P., Rhonert Park, CA, USA).

The Masslynx 3.5 software was used to control the autosampler, the valves and the mass spectrometer. Pump control for the two Shimadzu pumps was carried out separately. Chromatographic separations were performed using three Xterra MS C8 3.5 μm, 100 mm × 2.1 mm i.d. columns from Waters (Milford, MA, USA). All columns came from the same batch.

A binary gradient system was used at a flow rate of 0.2 mL/min. The solvents consisted of a mixture of acetonitrile:water:glacial acetic acid, solvent A (2:98:0.1, v/v/v) and solvent B (65:35:0.1, v/v/v). The injection volume was 25 μL.

2.2. Mass spectrometer

LC–MS/MS analysis was performed using electrospray ionization with multiple reaction monitoring on a Quattro Micro (Micromass UK Limited, Manchester, UK).

For all compounds, the MS instrument was operated in the positive ion electrospray ionization mode. The dwell time for each transition was 0.2 s. The desolvation and source temperature were 250 °C and 120 °C, respectively. The cone and desolvation gas flows were 130 L/h and 920 L/h, respectively. Nitrogen and argon were used as cone and collision gases, respectively. Transitions, cone voltage and collision energy used for each analyte and the internal standard were as described in Table 1.

2.3. Signaled events

The parallel three-column switching system is presented in Fig. 1. The three-valve system is composed of two six-port valves working in unison to select which of the three columns receives the injection and a six-port multiposition valve, positioned after the columns, selecting which column is to be in line with the mass spectrometer.

Three alternating LC methods, applied to pump 1 (located in the autosampler), control the valve switching as well as the injection procedure and solvent flow. Each of the three possible combinations of valve positions in the flow path shown in Fig. 1 is represented in one of the three LC methods.

By controlling the positions of the valves, the currently running method determines which of the three columns receives the injection, which is equilibrated and which is in line with the mass spectrometer. Method A is used for detection on column 1, method B on column 2 and method C on column 3.

Due to the structural diversity of the analytes, it was important to create a chromatographic system that is characterized by retention times within a narrow window and sufficient separation. To meet these demands, the analytes were exposed to a step gradient (Fig. 2). After running the sample 2 min on pump 1 with low eluent strength (solvent A), pump 2 with strongly eluting solvent B is switched immediately onto the analyte's column creating a passive solvent gradient. One minute later, the analyte's column is positioned in line with the MS and the acquisition starts. At the time when all data is recorded, the column is switched to pump 3 providing solvent A to equilibrate the column. The whole process runs parallel sequential on all three columns, as illustrated in Fig. 2.

As a consequence of the parallel sequential injection, the peak that is detected in the current acquisition time window has been injected at the start of the previous window. The injections in the analysis list are therefore displaced one step.

During the injection process, lasting exactly 1 min, no acquisition is possible. The autosampler is washing the needle, replacing volume, preparing syringe, selecting well, drawing

Table 1
Mass spectrometer settings

Compound	*m/z*	Transition	Cone voltage (V)	Collision energy (eV)
Clomethiazole	161.85	112.72	37	22
Phenacetin	180.04	109.81	37	22
S-Mephenytoin	219.10	134.00	28	16
Bufuralol	262.10	133.00	28	28
Bupivacaine (internal standard)	289.00	140.00	30	20
Midazolam	326.01	291.17	46	22

sample and loading sample. All these processes have to be completed before the autosampler gives the triggering signal to start the acquisition to the mass spectrometer. Integrating this "dead time" into the switching cycles requires longer retention times compared to the conventional one-column system. For this reason, 100 mm columns were selected.

2.4. Reagents

Acetonitrile, glacial acetic acid and potassium dihydrogen phosphate were purchased from Merck KGaA (Darmstadt, Germany). Deionized water, 18.2 mΩ, was taken from a Milli-Q system (Millipore, Bedford, MA, USA).

The test compounds phenacetin and midazolam maleate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). (*S*)(+)-Mephenytoin was received from Toronto Research Chemicals (North York, Canada), +/− bufuralol hydrochloride salt from Ultrafine Ltd. (Manchester, UK). Clomethiazole edisilate and bupivacaine hydrochloride monohydrate were synthesized at Process R&D AstraZeneca (Södertälje, Sweden). β-Nicotine amide adenine di-nucleotide phosphate, reduced form β-NADPH tetra sodium salt was obtained from Sigma Chemical Co. Microsomes were prepared at Research DMPK & Biomarkers, AstraZeneca (Södertälje, Sweden) in July 2002.

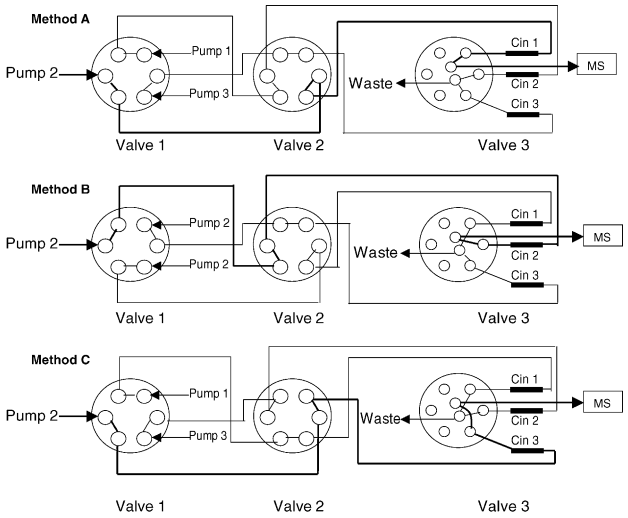

Fig. 1. Schematic representation of the column switching system.

2.5. Sample preparation

Appropriate amounts of the compounds were dissolved in 50 mM phosphate buffer, pH 7.4, to obtain stock solutions of 200 μM. To receive the five-in-one multiple component samples, a pooled stock solution was prepared containing equal volumes of each compound's stock solution. Standard solutions were then prepared by dilution of the pooled stock solutions with 50 mM phosphate buffer (pH 7.4):acetonitrile (50:50, v/v) to give 10, 20, 100, 200 and 400 nM.

Quality control solutions of the test compounds were prepared and pooled according to above. Quality control samples were prepared by dilution of the pooled quality control solution with 50mM phosphate buffer (pH 7.4):acetonitrile (50:50, v/v) to give concentrations of 15, 150 and 300 nM. Two solutions at each concentration level were prepared.

The internal standard was prepared by diluting a 1.39 mM stock solution of bupivacaine with deionized water to give 3000 nM.

Aliquots of 50 μL of internal standard (3000 nM in water) were added to each of the glass tubes of a 96-deep well plate, using a Quadra 96 workstation (Tomtec Inc., Hawden, CT, USA). Aliquots of 450 μL of the appropriate standard or quality control solutions were then pipetted into the glass tubes. After vortexing, the samples were ready for injection.

One set of a calibration curve at five concentration levels and duplicate quality control samples at three concentration levels were analyzed as described above.

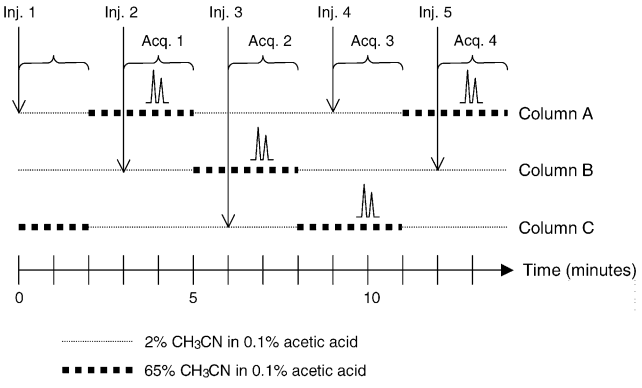

Fig. 2. Schematic diagram of time events in the three-column LC–MS/MS system. Sequentially, the samples are exposed to a step gradient on respective column. The peak that is detected in the current acquisition time window has been injected at the start of the previous window.

2.6. Metabolic stability samples

Samples from a metabolic stability assay in liver microsomes were analyzed. The aim of the study was to investigate if a cocktail approach could be used when comparing different species and strains in terms of their metabolic stability of human cytochrom P450 probe substrates. The compounds phenacetin, *S*-mephenytoin, bufuralol, midazolam, clomethiazole were analyzed. Standard solutions were pooled and prepared by dilution with boiled microsomes. Acetonitrile was added in equal volume to give the concentrations 0, 25, 125, 250, 1000 and 1500 nM.

The compound of interest (pooled) in a concentration of 2 μmol/L in 50 mmol/L phosphate buffer, pH 7.4, was preincubated at 37 °C for 10 min together with a liver microsomal preparation of 0.5 mg/mL. The microsomal incubations were carried out in a shaking incubator. Reaction was initiated by the addition of NADPH giving a final concentration of 1 mmol/L and continued for 0, 5, 15, 30 and 60 min. Reactions were terminated by the addition of 100 μL ice-cold acetonitrile. The samples were kept on ice for 30–60 min on ice to let the proteins fall. Samples were centrifuged for 10 min at 1900 × *g* to sediment precipitated protein. The supernatant containing buffer:acetonitrile, 1:1, and internal standard (bupivacaine) was analysed using the three-column parallel LC–MS/MS system. All pipetting was done using a Genesis RSP 200 robotic system from Tecan (Maennedorf, Switzerland).

3. Results

The quantification of phenacetin, *S*-mephenytoin, bufuralol, midazolam and clomethiazole was performed using a new parallel three-column LC–MS/MS system. The compounds were analyzed in a *N*-in-one assay, where standard and quality control samples contain all five compounds together in one vial. Standard solutions were injected five times at each concentration level. Duplicates of quality control samples were injected five times at each concentration level, resulting in ten acquisitions at each level. Peaks were integrated using the Masslynx processing software. Calibration curves were constructed by plotting peak area ratios of analyte to internal standard against concentrations of each analyte using quadratic regression. Back-calculated sample concentrations were determined by interpolation from the appropriate calibration curve.

A more than doubled throughput was achieved compared to a single-column system. A summary of the results is shown in Table 2. Analysis of the five compounds shows an accuracy (calculated as a percentage of the theoretical value) of 81–108% and a precision (expressed as relative standard deviation, R.S.D.) of 1.5–14%. The absence of interfering peaks was ascertained by running blank samples through the assay procedure. The coefficient of determination was between 0.999757 and 0.999997 for the five compounds.

Chromatographic stability on each column as well as across all three columns was investigated from the internal standard used in the evaluation (bupivacaine). Comparing response and retention time of bupivacaine collected from 60 injections per column shows that although the columns come from the same batch the response differs up to 20% between columns. The retention time, however, was almost constant on the three columns with a difference of 2%. One way of solving this problem is to place the same compound at the same column through the whole analysis and use a macro to sort out all different compounds on the three columns. Using this set up, different sorts of columns can be used. Another

Table 2
Precision (expressed as relative standard deviation, R.S.D.) and accuracy (calculated as a percentage of the theoretical value) data for the compounds *S*-mephenytoin, midazolam, bufuralol, clomethiazole and phenacetin from pooled samples

	Calibration curve samples (n = 5) (nmol/L)					Quality control samples (n = 10) (nmol/L)		
	10	20	100	200	400	15	150	300
S-Mephenytoin								
R.S.D. (%)		14	5.9	6.0	5.0	8.0	4.1	7.4
Accuracy (%)		106	106	101	104	104	89	96
Midazolam								
R.S.D. (%)	3.8	4.4	1.7	1.5	1.6	7.1	2.3	5.2
Accuracy (%)	94	97	102	98	99	84	87	92
Bufuralol								
R.S.D. (%)	7.8	5.4	4.2	1.7	3.3	5.6	2.6	3.4
Accuracy (%)	96	94	103	99	99	96	96	98
Clomethiazole								
R.S.D. (%)	7.8	4.0	5.3	4.5	4.0	5.8	3.0	6.5
Accuracy (%)	108	100	105	103	102	81	81	82
Phenacetin								
R.S.D. (%)	3.3	6.9	5.2	3.5	3.8	4.3	2.3	4.1
Accuracy (%)	103	97	105	100	99	94	90	89

way is to put standards on each column and quantify each sample against the standard on the same column.

The parallel LC–MS/MS system described was used for the assay of samples obtained from liver microsomal incubations. These in vitro incubations were prepared to investigate microsomal metabolism of cytochrom P450 substrates (human probe substrates). A comparison was made between microsomes from different species and strains. Samples were prepared as described in the metabolic stability samples section, and analysed by direct injection into the LC–MS/MS system. It was chosen to place the same compound at the same column through the whole analysis. Sample peaks were separated from matrix components using a step gradient (Fig. 2).

Standard solutions were prepared by spiking boiled blank microsomes with an appropriate volume of a pooled standard sample, as described in the previous section. Calibration curves were constructed by quadratic regression from sets of standard solutions run at the beginning and end of each assay. Analysis of the five compounds shows an accuracy (calculated as a percentage of the theoretical value) of 93–106% and a precision (expressed as relative standard deviation) of 0.1–11%. The coefficient of determination was between 0.9866 and 0.9981 for the five compounds. Quantification of the assay samples was achieved by reference of the peak area ratios for each sample to internal standard against the relevant calibration curve. The deviation in retention times (calculated as R.S.D.) for all standards and samples of the same compound was 3%. The analysis, comprising 300 injections (∼1500 samples), was performed in 15 h.

4. Discussion

An excellent advantage of this system is to accelerate the high-throughput screening process with low financial input compared to commercially available solutions. The required instrumentation is often already available or relatively inexpensive to gain. No gradient pumps are necessary to rapidly obtain gradient like and satisfying chromatographic separation. Furthermore, the user is not bound to a particular vendor's application but is free to combine the most suitable instrumentation. Analysis run time is accelerated and sample throughput increased by optimizing sample inlet into the MS.

Eluting analytes parallel sequential on three columns allows the MS to continuously analyze the window of interest, while separation phases of minor interest in the beginning and at the end of the chromatographic cycle can be run simultaneously. Since the MS is coupled to the column loaded with the analyte only during the relevant period of elution, contamination of the MS with spurious peaks eluted outside the acquisition window can be avoided.

Although no acquisition is possible during the injection process, at the same time both separation and column equilibration can be performed all over the chromatographic system without slowing down the analysis.

Prediction of the time needed for the injection process is a requirement for synchronizing events throughout the system. In the system described, the injection process takes exactly 1 min. It is a future prospect to further decrease analysis time by using instrumentation that allows a faster injection process. This could facilitate the use of shorter columns, leading to faster retention times, better peak shapes and, ultimately, higher reproducibility and speeding up the entire separation cycle. A system, under development at our lab, is aiming to achieve a 2 min analysis time per sample and to allow acquisition during injection.

Flexibility is another major advantage. It is easy to adopt the system to a conventional one-column LC–MS/MS or even LC/MS configuration if desired. As all valves are controlled by the LC-methods, configuration can be changed quickly on-screen without physically rearranging the system. By using the equipment described it is possible to run the system in isocratic as well as in gradient mode. If desired even more gradient pumps can be applied. The system can also be used with three different kinds of columns if needed. However, the mobile phases have to be the same for all three columns.

The parallel LC–MS/MS system described has been successfully used to obtain information on a number of drug candidate molecules, with respect to their metabolic stability in microsomes from different species. This generic method has proved to be robust and reliable. All compounds use to be successfully quantified. The analysis is performed quickly (300 injections (1500 samples) in 15 h) and with stable retention times for all compounds. This demonstrates that the three-column system offers an adequate analysis technique for these types of in vitro screening assays.

5. Conclusions

The three-column parallel LC–MS/MS system provides more than a 2.5-fold increased throughput in terms of speed, as compared to a generic single-column system. The optimizations were achieved with low financial input by combining basic and often already available instrument modules to a parallel system. Improved sample throughput is achieved without sacrificing chromatographic separation quality or sensitivity. The quantification of five compounds and the analysis of samples from a metabolic stability assay proved the applicability and robustness of the system. It is found to be suitable for high-throughput ADME screening.

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