

Ultra-high capacity small molecule chips for the quantification of pharmaceuticals using triple-quadrupole mass spectrometry

Application Note

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Abstract

The pressure to save active pharmaceutical ingredients drives the industry to scale-down laboratory processes. This puts the burden on the chemist to choose the appropriate analytical platform that can handle smallest sample quantities – a challenge that drove Agilent to develop the Agilent 1200 Series Nanoflow HPLC-Chip/MS system for high-sensitivity analyses of small molecules. This Application Note presents a technical evaluation of the novel ultra-high capacity small molecule HPLC-Chip for the quantitative analysis of pharmaceuticals using the Agilent 6410 triple quadrupole mass spectrometer. The evaluation demonstrates:

- Excellent precision, chip-to-chip reproducibility, lifetime and system linearity
- A 100-fold increase in sensitivity compared to conventional LC/MS, following quantitative enrichment of compounds comprising a wide polarity range
- Excellent, low limits of detection in biological samples such as blood plasma, saliva and urine

Agilent Equipment:

- 1200 Series Nanoflow LC
- Small Molecule HPLC-Chip
- 6410 Triple Quadrupole MS

Application Area:

 Pharmacokinetics studies during preclinical drug development



Introduction

The creation of a new pharmaceutical drug consumes vast amounts of capital. For instance, drug discovery and development alone may require investments of more than double-digit million dollar sums per lead candidate. The competition is intense and cost savings may become essential, even for leading pharmaceutical companies. Substantial savings can be achieved by the miniaturization of laboratory processes provided the quality of the results is uncompromised¹. A prime example for this situation exists in the drug metabolism and pharmacokinetics (DMPK) laboratory where PK studies are carried out in the preclinical development phase.

During PK-studies the potential lead compound is administered to animals from which blood is taken over the course of time to monitor drug absorption and decay using quantitative LC/MS analysis. Here, the cost per study may be reduced markedly by using smaller animals. For example, mice require much less active pharmaceutical ingredient than dogs. However, the animal's size sets the limit to the blood volume that can be drawn per unit time and serial bleeding of few or even only one animal, which reduces the variability in the TK profile, results in even lower volumes per bleed. Consequently, studies with small animals set extreme demands on the analytical system with respect to both the capability to handle small sample volumes and detection sensitivity.

The Agilent HPLC-Chip/MS system, first launched in 2005, fulfils

these strong demands. The chip incorporates an enrichment column and a nano-analytical column from which a very short and narrow channel leads to the chip's nanospray tip (figure 1). Miniaturization of the analytical column dimensions and the resulting low flow rates of a few hundredths of nanoliters, result in less sample dilution and improved overall analyte ionization efficiency. This ultimately leads to the pronounced sensitivity advantage of the chip compared to conventional LC-ESI-MS^{2,3}. Concentration of the analytes is integrated in the chip and yields even further increases in sensitivity. At the same time, higher throughput can be achieved, since preparation for biological samples can be reduced to only protein precipitation and centrifugation prior to direct injection onto the $chip^4$.

The chip is made of biocompatible, inert polyimide layers. Channel and surface structures are formed by laser ablation. This is the reason why dead volumes, extra-column volume and leakprone connections do not exist, so that the chip provides uncompromised chromatographic performance. In this way, the Agilent HPLC-Chip/MS system has transformed nanoflow-LC/MS into a robust plug-and-play technology for day-to-day use^{5,6}. The chip system can now be coupled to the entire portfolio of Agilent's mass spectrometers, providing for automated sample enrichment in addition to highest sensitivities for the wide range of routine qualitative and quantitative small molecule pharmaceutical applications^{3,4,7}. The challenge with pharmaceuticals lies in the wide variety of polarities present. It has to be

ensured that none of the molecules are lost during the on-chip enrichment process. In this study, we illustrate the design of a new ultra-high capacity small molecule HPLC-Chip, which features a 500 nL enrichment column and a 150 mm x 75 µm analytical column. We present data of the technical evaluation for quantification of small molecules in combination with the Agilent 6410 triple quadrupole MS. The test mix contained four pharmaceutical compounds, covering a wide range of polarities (logP 0 to 4.2).

Experimental

Chemicals

Human plasma and all standard chemicals, atenolol, atropine, metoprolol, propranolol, imipramine, caffeine, quinine, formic acid (FA), were purchased from Sigma-Aldrich. HPLC grade water was either purchased from J.T. Baker or freshly taken from a MilliQ water purification system. HPLC grade acetonitrile (ACN) was purchased from Merck.

Sample preparation

A 2 mg/mL stock solution of each compound was prepared in ACN/water, 50:50 v/v (0.1 % FA). To obtain different concentrations, compounds were mixed and diluted in the same mobile phase as used for sample loading. Prior to analysis of plasma samples, proteins were precipitated by addition of acetonitrile (3:1 ratio of agent-to-sample). After centrifugation at 13,000 rpm for 10 min, the supernatant was transferred to a fresh vial and evaporated in a SpeedVac concentrator and the pellet was redissolved in the original volume of HPLC loading mobile phase.

HPLC-Chip/MS system

The Agilent MassHunter software suite included LC/MS data acquisition, qualitative analysis and quantitative data processing. The Agilent 1200 Series Nanoflow LC system comprised a micro wellplate autosampler with sample thermostat, micro-degasser, capillary pump and nanoflow pump. The Agilent HPLC-Chip/MS interface provided all connections between the HPLC-Chip, capillary pump, autosampler, nanoflow pump and Agilent 6410 triple quadrupole mass spectrometer. The configuration of the new ultra-high capacity small molecule HPLC-Chip (UHC-chip) is shown in figure 1B. The UHC-chip included a 500 nL enrichment column and a $150 \times 0.075 \text{ mm ID separa-}$ tion column that were both packed with ZORBAX 80A SB-C18 5 µm stationary phase. All components, including the nano-electrospray emitter (10 µm ID), were integrated onto the chip...

Unless otherwise stated, solvent A was 0.1% formic acid (FA) in water and solvent B was 0.1 % FA in acetonitrile. The capillary pump (for sample loading) delivered 2 % B isocratic mobile phase at 4 µL/min. The nanoflow pump gradient (for sample analysis) was 2–32 % B in 5 min and then to 80 % B in 3.5 min, which was held for 1 min to flush out thoroughly all potential column contaminants, especially those arising from plasma samples. Post run time was 9 min at 2 % B and flow rate was 0.3 µL/min. To ensure optimized nanoflow and fast gradient response, the nanoflow pump is equipped with active split EMPV technology⁸. Following 1 μL sample injections, the enrichment column was flushed with a certain volume of loading mobile

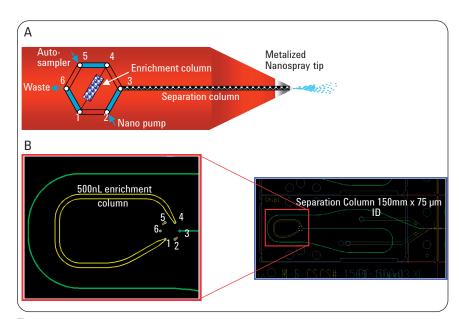


Figure 1
Chip schematic (A) and blueprints of ultra-high capacity (UHC) small molecule chip obtained using light microscopy (B).

phase (defined by the injection flush volume, IFV, of 4 µL). Here, the intelligent sample loading (ISL) feature of the Agilent MassHunter software was used to calculate automatically the time required for optimum sample loading^{6,9}. Following sample loading, the chip cube microvalve was switched automatically from enrichment to analysis mode with simultaneously start of the nanoflow pump gradient and MS data acquisition. For best peak shapes, the UHC-chip was operated in back-flush mode (figure 1A) with sample loading at port 5 and waste line at port 6.

Chemical background typically present in ambient laboratory air can significantly reduce overall sensitivity¹⁰. To eliminate this problem, the HPLC-Chip spray chamber was isolated completely from ambient laboratory air contamination. Drying gas flow was maintained at 4 L/min using a mixture of 3 L nitrogen and 1 L of filtered dry air at a constant temperature of 300 °C. Data acquisition was performed in the positive ionization mode. Capillary voltage was -1900 V with an endplate offset of -500 V. The infusion chip was used to determine the optimum conditions for all MRM experiments with 0.1 ppm single drug solutions. Table 1 gives

Compound	Precursor ion [Fragmentor voltage]	Quantifier [Collision voltage]	Qualifier [Collision voltage]
Atenolol	267.1 [140]	145.0 [25]	74.1 [20]
Atropine	290.1 [180]	124.1 [20]	93.0 [35]
Metoprolol	268.2 [160]	116.1 [15]	56.0 [30]
Imipramine	281.2 [140]	58.0 [45]	86.0 [15]

Table 1
Optimized MRM conditions for four-compound mix.

compounds and masses of precursors, quantifiers and qualifiers, and optimized fragmentor and collision voltages. Dwell times were chosen to obtain at least 12 data points across a peak. Quadrupoles were operated at unit resolution (0.7 amu at FWHM). Δ EMV = 700 V. The signal-to-noise (S/N) ratio was calculated from the height of the peak divided by 3 times RMS noise.

To reduce any potential system contamination and/or prevent enrichment column clogging from standards or samples containing biological matrices, an in-line filter (1 µm titanium, Upchurch, M-548) was attached between the autosampler and HPLC-Chip cube. To minimize carryover after drawing the sample and prior to injection, the outer surface of the injector needle was flushed with ACN/water, 50:50 v/v (0.25 % FA) for 5s in the autosampler flush port. After switching from enrichment to analysis mode, the autosampler injection valve was set to bypass in order to flush connecting tubing and in-line filter with 90 % B for 2 min.

Results and discussion

Pre-concentration of wide polarity and concentration ranges

Pharmaceutical compounds covering a wide polarity range (logP values ranging from 0 to 4.2) were chosen to evaluate the performance of the UHC-chip. Figure 2 shows extracted ion chromatograms of seven drugs with atenolol (logP 0) as the first and imipramine (logP 4.2) as the last eluting peak. Chromatogram A shows the results for the standard

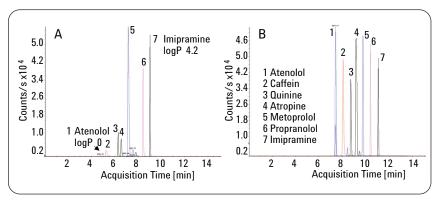


Figure 2
Analysis of a seven-compound mixture with a hydrophobicity range from logP 0 to 4.2. Loading mobile phase: ACN/water, 2:98 v/v (0.05%TFA). Gradients were performed as described in the experimental section. Panel A shows the EIC for the 40 nL standard enrichment column small molecule chip and panel B for the 500 nL enrichment column on the UHC small molecule chip.

small molecule chip with a 40 nL enrichment column. Analysis of this mixture on a small volume enrichment column revealed a marked correlation between analyte hydrophobicity and detected signal intensity. These results were in agreement with the preconcentration process on a reversed phase (RP) column after loading the sample onto the enrichment column, mobile phase was pumped through in order to remove contaminants potentially interfering with MS analysis (for example, salts). The volume of mobile phase was user-defined by the injection flush volume (IFV) in the Agilent MassHunter software. Thus, the enrichment process was simply an isocratic elution at very low % B. Since compound migration speed through the enrichment column depended on the hydrophilicity of the molecule, hydrophobic compounds were trapped⁷. whereas very polar compounds came through because the enrichment column volume was very small compared with the volumes of mobile phase flushed through it.

The 500 nL enrichment column in the new UHC-chip has more than 12 times higher capacity than the standard 40 nL enrichment column small molecule chip making it much more suitable to retain hydrophilic compounds. Figure 2B shows the analysis of the same sample mix as in figure 2A but using the UHC-chip. Responses of all peaks were similar, which indicated retention of all compounds over the entire polarity range (logP 0 to 4.2).

The ability of the UHC-chip to retain quantitatively compounds over a wide polarity and concentration range was demonstrated by increasing the injection volume while maintaining the concentration of the mixture constant. Figure 3 illustrates the results of this experiment where a mixture containing equal amounts (1 ng/mL) of atenolol, atropine, metoprolol and imipramine was analyzed in this fashion. Imipramine, metoprolol and atropine showed retention with a linear increase in response across the entire range of injection volumes with correlation factors close to unity. The result for

atropine is shown representatively in figure 3A. The UHC-chip demonstrated retention for at least 6 µL of the sample mix. This corresponds to at least 20 pg of drug on the enrichment column. In comparison, on the standard small molecule chip, a significant fraction of atropine was lost during the enrichment process when injecting only 1 µL (figure 2A). The results for atenolol (logP 0), are shown in figure 3B. While only a minute amount of this hydrophilic analyte was retained on the 40 nL enrichment column, a linear plot was obtained up to 3 µL injection volume with the UHC-chip.

Precision, chip-to-chip reproducibility and robustness

To demonstrate retention time precision, the four-compound mix (1 pg/μL of each compound) was consecutively injected ten times on the UHC-chip. Figure 4 shows the superimposed MRM-quantifier chromatograms and relative standard deviation (%RSD) values for retention time (RT) and peak area (PA) for all compounds. A maximum RSD value of 0.2 % was obtained for hydrophilic compounds such as atenolol. The RSDs for atropine, metoprolol and imipramine were below 0.1 %. While peak area precision for imipramine was 8.1 %, all other compounds exhibited peak area precision typically below 5.0 %.

Chip-to-chip reproducibility was evaluated with eleven individual UHC-chips, originating from different production batches. Eight runs per chip were performed and average retention times for each compound were calculated. The RSD for chip-to-chip reproducibility was calculated individually for each analyte whereby the %RSD

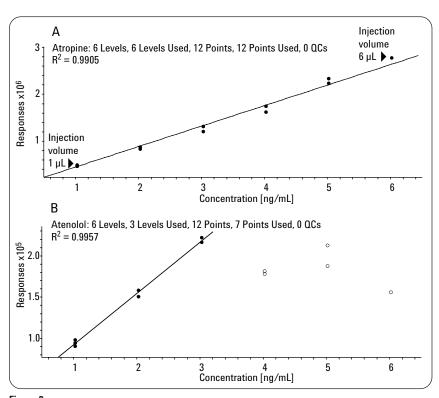


Figure 3 Representative results obtained for atropine (A) and atenolol (B) from repeated injections of the four-compound mix (each compound 1 pg/ μ L) with increasing injection volume from 1 to 6 μ L.

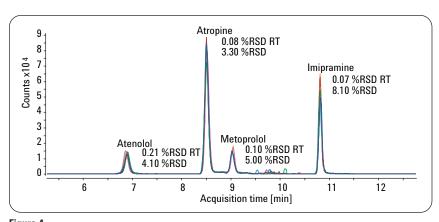


Figure 4 Superposition of quantifier chromatograms obtained from ten consecutive injections of the four-compound mix (each compound 1 pg/ μ L). To the right of the peaks, % RSD for retention time (RT) and for peak area (PA).

was below 1 %. The average %RSD of 0.71 demonstrated excellent chip-to-chip reproducibility (table 2). The lifetime of the chip nano-electrospray emitter was evaluated by continuous spraying a solution of

either 2:98 or 90:10 v/v ACN/water (0.1 % FA). After more than 2 weeks of continuous operation, the spray remained stable without any failures.

The chromatographic life-

time of UHC-chips was assessed by injecting repeatedly the four-compound mix (1 pg/ μ L each compound). For each of the chips studied the number of injections always exceeded 500. Retention, chromatographic efficiency and peak shape were stable over the whole period of time investigated (table 2).

Working with biological matrices required additional system care in order to avoid clogging of the enrichment column due to debris from the sample or biomolecules (for example, protein precipitation). Standard workflow protein precipitation proved to be sufficient in combination with the use of an inline filter in the loading path. Additional flushing is recommended to minimize carry-over from injection to injection (see experimental section). Following these guidelines allowed the analysis of between 200 to 500 samples of blood plasma samples spiked with the four-compound mix per UHC-chip (2 chips studied).

The sample peak capacity of the UHC-chip was determined by a 1 µL injection of a mixture of 10 tryptically digested proteins (100 fmol/µL). Sample peak capacity was calculated from the difference in retention times of the first and the last real gradient peak divided by the average peak width determined over the 60 min gradient (2 to 75 % B in 60 min, 0.3 µL/min, MS in scan mode). A sample peak capacity of 200 was achieved (table 2).

Increase in system linearity and sensitivity

The linearity of the UHC-chip/MS system was investigated using

Test	No. of chips	Result
Chip-to-chip reproducibility	11	0.71 % RSD
Spray tip lifetime	2	> 2 weeks continuous spray
Lifetime with four-compound mix	4	> 500 injections
Lifetime with four-compound mix in plasma	2	200–500 injections
Sample peak capacity	1	200

Table 2 Result summary.

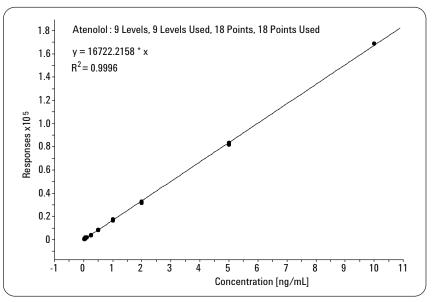


Figure 5
Plot of peak area against concentration (0.02 to 10 ng/mL) for atenolol.

dilutions of the four-compound mix over a range 20 pg/mL to 10 ng/mL for each compound. Figure 5 shows the excellent linearity of peak area against concentration obtained for atenolol. Similar results were obtained for atropine, metoprolol and imipramine with correlation factors always better than 0.99 (data not shown).

To estimate the sensitivity increase achieved through the routine use of nano-electrospray with the UHC-chip, it was compared with standard LC/MS using the Agilent API electrospray source. Switching between HPLC-Chip/MS and ES source takes only a few minutes, allowing the use of the same triple quadrupole MS instru-

ment. The 1 pg/µL four-compound mix was injected onto a ZORBAX SB C18 narrow bore column with comparable length to the chip's separation column. Linear flow velocities were adapted according to column IDs. More details on the analysis are given in figure 6, which shows quantifier chromatograms obtained with a narrow-bore column (A) and UHCchip (B). The use of the UHC-Chip led to an increase in response by a factor of about 100. This agreed well with earlier findings during metabolite ID experiments with buspirone³.

Analysis of blood plasma samples Enabling the routine use of high sensitivity nano-electrospray

LC/MS in combination with the ability to automate sample enrichment from very small blood plasma samples has the potential to immensely reduce both cost and data variability in the in vivo DMPK laboratory. To assess potential UHC-chip/MS performance with real samples, low amounts of atropine and imipramine were spiked in blood plasma. Samples were processed similar to DMPK workflows that are followed by the pharmaceutical industry (see experimental section) and subsequently analyzed by UHCchip/MS. Chromatograms for quantifier and qualifier obtained from the Agilent MassHunter quantitative analysis software following the injection of the sample mix containing 10 fg/µL of each compound are shown in figure 7. For 10 fg of imipramine, m/z 86 gave the highest signal intensity. Despite this, m/z 58 was chosen as quantifier because it showed better linearity than m/z 86 in the system linearity tests (above). For both imipramine (S/N 10) and atropine (S/N 42) the limit of detection was below the 10 fg level.

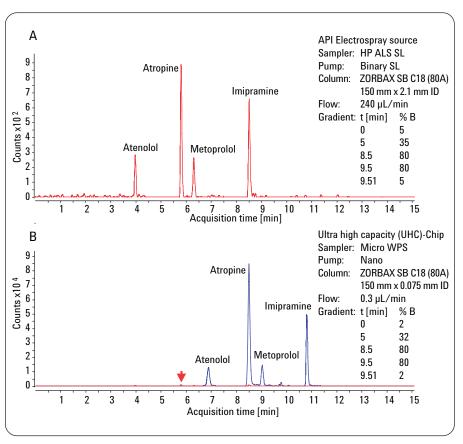


Figure 6 Quantifier chromatograms obtained for the four-compound mix (each compound 1 pg/ μ L) using narrow bore LC/MS (A) and UHC small molecule chip/MS (B). The bottom chromatogram represents an overlay of (A) and (B); the red arrow marks the position of the atropine peak.

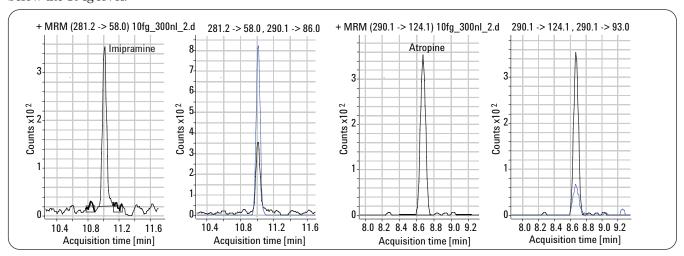


Figure 7
Quantifier chromatograms (black peak) and quantifier/qualifier (blue peak) overlay for atropine and imipramine obtained from an injection of 10 fg in 1μL plasma (injection volume: 1 μL).

Conclusion

The design of the new Agilent ultra-high capacity small molecule chip is illustrated (figure 1) and features a 500 nL enrichment column and 150 mm x 75 µm separation column with integrated nanoelectrospray emitter. In this study, UHC-chips were evaluated for quantitative nano-electrospray analysis of small molecules in combination with the Agilent 6410 triple quadrupole MS. The test mix contained four pharmaceutical compounds, covering a wide range of hydrophilicities (logP 0 to 4.2) and concentrations. At sequence start, automated analyte loading and pre-concentration was performed on the chip's enrichment column. Quantitative and reproducible pre-concentration was established for the entire range of hydrophilicites investigated - sample loads up to at least 20 pg of drug were demonstrated (figures 2 and 3). Excellent data were obtained for retention time and peak area precision, chip-to-chip reproducibility and chip lifetime with aqueous and blood plasma samples. Sample peak capacity was determined to be 200 (figure 4 and table 2). The UHC-chip/MS system's linearity was shown with correlation factors approaching unity (figure 5). When comparing narrow bore LC/MS using an API electrospray source with nanoelectrospray using the UHC-chip on the same triple quadrupole MS system, the chip configuration exhibited a 100 fold increase in sensitivity (figure 6). Plasma samples containing the drugs atropine and imipramine were analyzed at an absolute on-column level of 10 fg. Even this very low concentration resulted in prominent peaks, which were well above the lowest limit of detection (figure 7). With this new

concept of nanoflow HPLC-UHC-chip/MS, which provides for unequivocal sensitivity and thus the capability to handle smallest sample volumes, a new era has started for DMPK laboratories. Reduced sample volumes in animal or pediatric studies commensurate with ethical arguments and in addition could yield substantial cost-savings and higher quality results.

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