HPLC-Chip/Triple-Quadrupole MS for quantification of pharmaceuticals in diminishing small volumes of blood

Application Note

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Abstract

The reduction of rodent size and number is accompanied by the cutback of the amount of the active pharmaceutical entity per PK-study. This ultimately leads to substantial cost-savings in the DMPK-laboratory. Yet, small animals can only donate a limited sample volume in particular when serial sampling studies are performed to increase PK data quality. This Application Note

- demonstrates the quantification of drugs extracted from only 10 µL volumes of whole blood using the HPLC-UHC chip and an Agilent 6410 Triple Quadrupole system (QQQ),
- provides a simple sample extraction procedure utilizing dried blood spots (DBS), that exhibited excellent recovery and precision for our test compound, and
- corroborates the outstanding sensitivity of Agilent HPLC-chip/MS systems and their robustness for the analysis of extremely complex biological matrices.[1]

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Introduction

Using HPLC-chips coupled to the Agilent 6410 QQQ allows for substantial cost savings in the DMPK laboratory.[1] Based on the system's outstanding detection sensitivity, the volumes of blood drawn in animal PK-studies can be reduced considerably, which would commensurate with ethical arguments and could also be envisaged for pediatric studies. This permits the reduction of animal size and a concomitant cutback of the amount of the active pharmaceutical entity per PK-study. Furthermore, it can increase PK-data quality due to the ability to serially sample blood from the same small-size rodent without jeopardizing its physiological welfare and without increasing recovery periods.[2]

Our recent article introduced the ultra high capacity small molecule chip (UHC-chip), which was especially designed for these small molecule applications.[1] Quantitative pre-concentration on the chip-integrated enrichment column was demonstrated using pharmaceuticals covering a broad range of hydrophilicities (logP 0 to 4.2). The 100 fold increase in sensitivity of the UHC-chip/MS system, compared to the same Agilent 6410 QQQ system equipped with the API electrospray source, allowed for detection of 10 fg absolute on-column levels of drugs in blood plasma. We further showed excellent retention time and peak area precision, chip-to-chip reproducibility and chip lifetime with aqueous and blood plasma samples.

Barfield et al. recently developed and fully validated a method for the quantification of acetaminophen in dog blood.[3] They spotted 15 µL blood onto FTA®Elute Micro Cards, punched an area of 3 mm diameter from the dried blood spot, extracted using methanol, and after centrifugation quantified their compound of interest down to a level of 0.10 µg/mL full blood using HPLC/MS.

Inspired by this work, the first objective of our current study was to develop a method for the quantification of a pharmaceutical test-compound from 10 µL of full blood using the UHC-Chip/6410B QQQ system. We aimed for a feasibility study with an extraction procedure similar to that employed in reference 3.

We also targeted to corroborate the major sensitivity advantage of Agilent 1200 Series HPLC-Chip/MS systems, which should yield low detection limits of the drug in blood-extracts.[1,4,5]
The third objective was to substantiate the robustness of the HPLC-chip system for the analysis of extremely complex biological matrices such as those contained in plasma or blood.[1]

Experimental

Chemicals

Rat blood was from Novartis Pharma AG (Basel, Switzerland). All standard chemicals (nortriptyline, formic acid (FA), Trifluoro acetic acid (TFA)) were from Sigma-Aldrich (Germany). HPLC grade water was from Burdick & Jackson (USA). HPLC grade acetonitrile (ACN) was from Merck (Germany).

Sample preparation

A 2 mg/mL stock solution of nortriptyline was prepared in ACN/water [50:50 v/v (0.1 %FA)]. For pre-concentration experiments, dilutions of this stock were made in ACN/water [0:100, 10:90, 15:95 & 20:80 v/v (0.1 %FA)]. Blood-standards: 10 µL of standard dilutions of nortriptyline in ACN/water [10:90 v/v (0.1 %FA)] were spiked into 90 µL of rat blood to obtain a blank and five concentrations in the range 0.05 to 15.0 ng/mL.

Optimized sample extraction procedure

FTA®Elute MicroCards (FTA-cards) were from Whatmann (USA). These contain chemicals embedded in a fiber matrix, which lyse cell membranes and denature proteins on contact; pathogens are also inactivated.[6]
Volumes of 10 µL of spiked blood were spotted onto FTA-cards. 1.20 mm diameter disks were punched from the dried blood spot in the manner shown in figure 1 using a Harris Uni-CoreTM puncher (Sigma-Aldrich, Germany). Figure 1 illustrates the optimized extraction procedure: Disks were placed in a 0.5 mL Eppendorf tube and 20 µL of ACN/water 75:25 v/v were added. Extraction was achieved by vortex-mixing for a few seconds and gentle shaking for 30 minutes using an orbital shaker. After centrifugation at

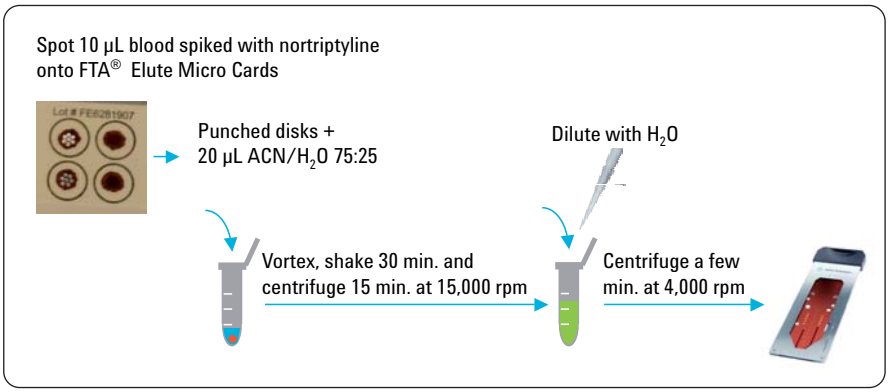

Figure 1
Procedure used to extract nortriptyline from whole blood.

15,000 rpm for 15 minutes, the blood-extract was diluted with water by a factor 7.5 to give samples containing 10 %ACN in order to ensure quantitative pre-concentration of nortriptyline on the chip. Samples were shortly centrifuged prior to injection at 4,000 rpm.

Agilent 1200 Series HPLC-chip/Agilent 6410B QQQ MS system

The Mass Hunter software suite includes LC/MS data acquisition, qualitative analysis and quantitative data processing. The Agilent 1200 Series Nano LC system consisted of a micro well-plate autosampler with sample thermostat (G1377A, G1330B), micro-degasser (G1379B), capillary pump (G1382A) and nanoflow pump (G2225A). All connections between the HPLC-chip, capillary pump, autosampler, nanoflow pump and Agilent QQQ mass spectrometer (G6410B) were accomplished with the Agilent HPLC-chip/MS interface (G4240A) – the chip cube. *Note: The blue capillary (1000 x 0.075 mm ID; G4240-87303) that is usually used for infusion experiments replaced the loading capillary used in the standard set-up (yellow capillary: 1050 x 0.025 mm ID; G4240-87301). 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.*

The UHC small molecule chip (G4240-63001 SPQ115) configuration was illustrated elsewhere.[1] It features a 500 nL enrichment column and a 150 x 0.075 mm ID separation column both packed with ZORBAX 80A SB-C18 5 µm stationary phase. All components including the nano electrospray emitter (10 µm ID) are integrated onto the chip. It is recommended to operate UHC-Chips in the back-flush mode. Solvent A was 0.1 % formic acid (FA) in water and B 0.1 % FA in ACN. The capillary pump loaded the samples in a 2%-B mobile phase at flow 8µL/min. Table 1 gives the optimized conditions for nano pump gradient (sample analysis) and a flush gradient delivered by the capillary pump in parallel to sample analysis, that thoroughly flushes loading path and enrichment column. This flushing gradient helps to reduce carry-over and to maintain system robustness when analyzing blood samples. *Important: During delivery of the flush gradient, the autosampler must be switched to bypass!*

Injections: 5 µL volumes using the overlapped injection function and injection flush volume 8 µL[7,8]. Needle wash was performed for 20 s using a wash solution of ACN/water 50:50 v/v (0.1 % TFA). Gradient Delay Reduction (GDR) is a new feature which is now incorporated into the MassHunter acquisition software. GDR compensates for the delay volume in the analysis-path and starts the nano-gradient accordingly prior to the switch of the chip cube microvalve into analysis mode. The result is that the nano gradient arrives significantly earlier at the column head thus reducing analysis time. GDR and overlapped injection led to cycle times for blood-extract analyses below 13 minutes when using the gradient given in Table 1.

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 -1750 V with an endplate offset of -500 V. The infusion chip (G4240-61002) was used to determine optimum MRM conditions with a 2 ppm single drug solution. Table 2 gives the precursor mass and fragments of nortriptyline, and optimized fragmentor and collision voltages. Dwell time was chosen to obtain at least 20 data points across the peak. Quadrupoles were operated at unit resolution (0.7 amu at FWHM). ΔEMV = 500V. The signal-to-noise (S/N) ratio was calculated by height of the peak over 3 x RMS of the continuous section of the mass chromatogram to the left and the right of the peak.

Nano pump			Capillary pump			Chip cube
Time [min]	%B	Flow (µL/min)	Time [min]	%B	Flow [µL/min)]	Time [min]
0	10	0.3	0	2	8	6 = enrichment
5.7	60	0.3	3.5	60	20	
6.5	60	0.3	5.7	60	20	
7	10	0.3	5.71	60	10	
			6	60	8	
			6.01	2	8	

Table 1
Optimized conditions for nanoflow pump, capillary pump and chip cube during 10.5 min run. Note: The chip cube microvalve switches back to enrichment position at 6 min; thus the enrichment column also experiences a flush with ACN/water [60:40 v/v (0.1 % FA)].

Compound	Precursor ion [Fragmentor voltage]	Fragments [Collision voltage]		
Nortriptyline	264.1 [140]	233.1 [10]	117.1 [20]	105.1 [20]

Table 2
Optimized MRM conditions.

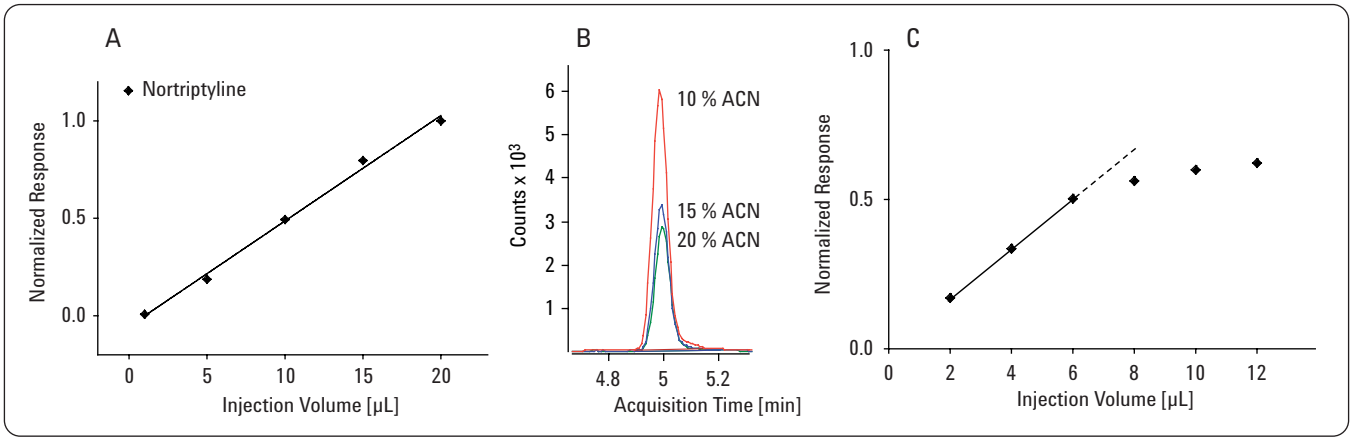

Figure 2
(A) Plot: Response vs injection volume obtained from repeated injections of nortriptyline (100 pg/mL) in mainly aqueous solution with increasing injection volume from 1 to 20 µL. Normalized response = response measured divided by maximum response (here obtained from 20 µL injection). (B) MRM-chromatograms (*m/z* 233.1 → 105.1) obtained from 4 µL-injections of nortriptyline dissolved in 10, 15 and 20 % ACN solutions. (C) Same plot as in (A) but with nortriptyline dissolved in a 10 % ACN solution.

Results and Discussion

Quantitative pre-concentration of nortriptyline

To investigate the pre-concentration performance of the UHC-chip with nortriptyline (logP 4.74), a 100 pg/mL-standard was prepared in mainly aqueous solution. Although chips are design for low injection volumes, in this test up to 20 µL standard was injected. Figure 2 panel (A) shows the proportional relationship between response against injection volume, which demonstrates quantitative enrichment over the entire range of volumes injected.

With an outlook that blood-extracts would contain higher %-organic, the enrichment behaviour of nortriptyline was also assessed in sample solutions containing ACN in the range 10-20 %. Figure 2 panel (B) shows the MRM-chromatograms obtained from a 4 µL injection at constant nortriptyline concentration. The 10 % ACN solution gave the best response, which may indicate a compound loss during the enrichment process with 15 and 20 %ACN solutions. Figure 2 panel (C), demonstrates quantitative pre-concentration of nortriptyline in 10 %ACN solutions up to 6 µL injection volume. At 8 µL and above the plot develops a plateau indicating partial sample break through. Conclusively, a 5 µL injection volume was chosen for all following experiments.

Extraction dependence on organic proportion

To investigate the optimum percentage of ACN for best recovery in the extraction procedure, a 3 mm diameter disk was punched from a 10 µL dried blood spot and extracted using 100 µL of solutions with varying content of ACN in the range 10 to 80 %. A 30 µL aliquot of each extract was evaporated to dryness using a speed-vac concentrator. Subsequently all residues were reconstituted in the same 30 µL-volume of a 10 % ACN solution. 70 % and 80 %ACN extractions gave the best results. This is illustrated in figure 3 showing MRM-chromatograms for 10 % and 70 % extractions, respectively. The peak areas suggested about 5-fold better recovery using 70 %ACN compared to 10 %ACN in the extraction solvent.

Result quality dependence on the nanoflow pump gradient

Table 3 shows nine nanoflow pump gradients tested to separate a nortriptyline extract. Figure 4 shows the superimposed MRM-chromatograms obtained with the nine gradients employed.

Nanoflow pump gradient %B	Time #1	#2	#3	#4	#5	#6	#7	#8	#9
10	0	0	0	0	0	0	0	0	0
60	3.2	3.7	4.2	4.7	5.2	5.7	6.2	6.7	7.2
60	4.0	4.5	5	5.5	6	6.5	7	7.5	8
10	4.5	5	5.5	6	6.5	7	7.5	8	8.5

Table 3
Nine nanoflow pump gradients with varying slopes; flow rate = 0.3 µL/min.

The general trend observed is that increased gradient time is accompanied by increased response (peak area) for nortriptyline. Response maximum was found for gradient #6.

Such behavior could arise for instance from insolubility effects that occur when the gradient-slope is too steep for the compound to remain soluble in the mobile phase. This however would predominantly apply for hydrophilic compounds in such a reversed phase separation. It is therefore conceivable that nortriptyline (logP 4.74) is most likely not markedly affected with the gradient ranging from 10 to 60 % B.

Another explanation for the findings in figure 4 could be an ionization suppression effect caused by the biological matrix potentially present in the sample. In this case one would expect a longer gradient to improve the separation of the compound of interest from the matrix and thus less ionic suppression effects. In turn, this should also yield better peak area precision, which is especially beneficial in analyses that do not use an internal standard. Indeed, peak area precision improved with increasing gradient time. For instance: triplicate analyses with gradient #1 and #6 gave peak area % RSD values of 12 and 2 %, respectively.

Thus, gradient #6 was chosen for all following experiments.

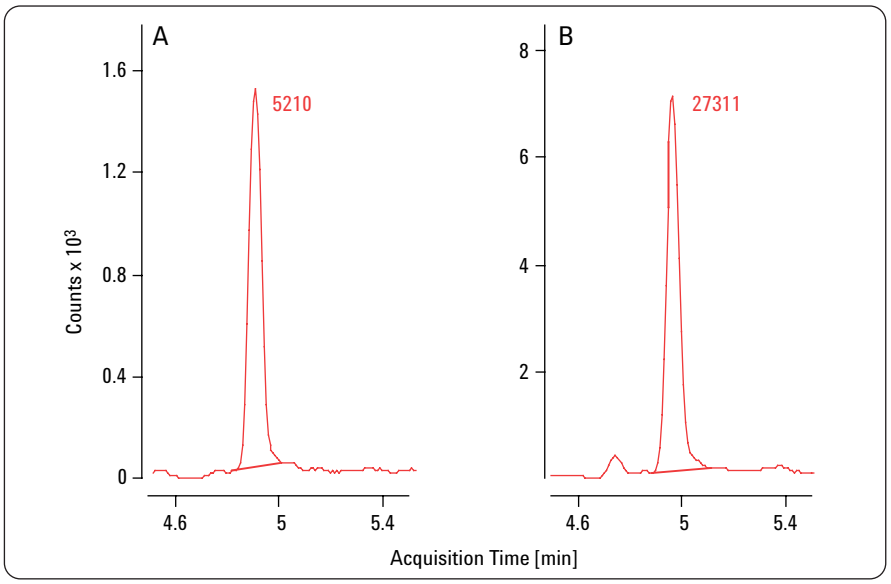

Figure 3
Representative MRM-chromatograms (m/z 233.1 → 105.1) obtained for nortriptyline after extraction from a 3 mm disk of a dried blood spot using 100 µL of extraction solution containing 10 % ACN (A) and 70 % ACN (B). 30 µL of each extract was evaporated to dryness and reconstituted in the same 30 µL-volume of a 10 % ACN solution. The numbers shown are peak areas.

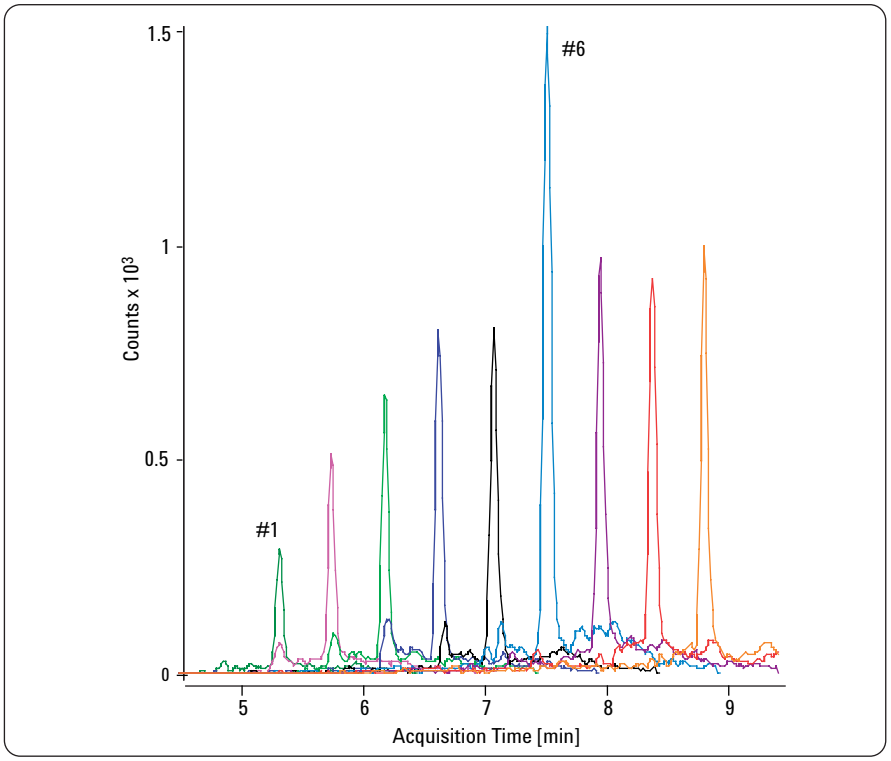

Figure 4
Superimposed MRM-chromatograms (*m/z* 233.1 → 105.1) obtained from notriptyline blood-extracts using linear gradients from 10 to 60 % ACN with varying slopes shown in table 3.

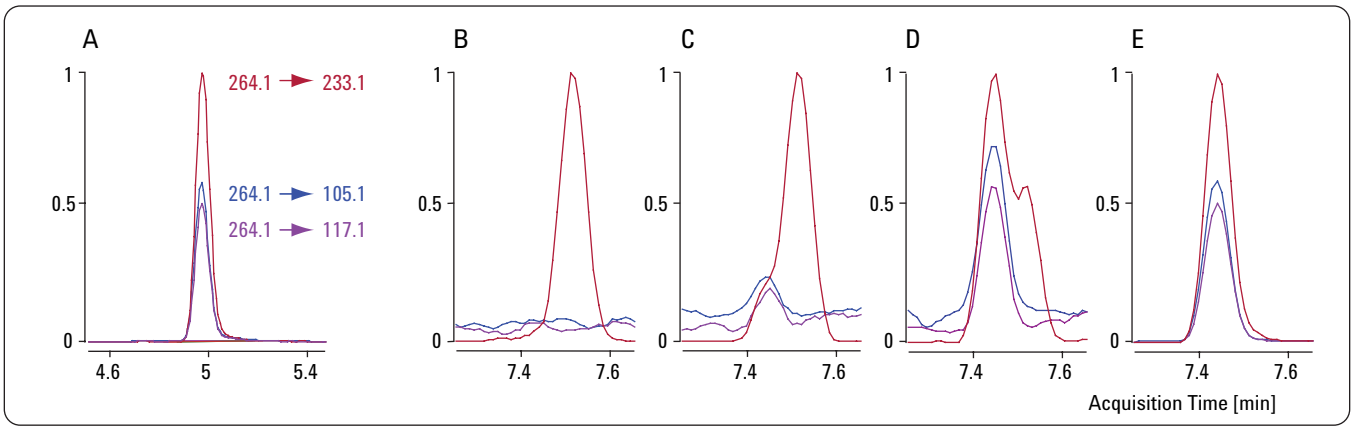

Figure 5
MRM-chromatograms obtained for three transitions of nortriptyline (*m/z* 264.1 → 233.1, 105.1 and 117.1). (A) Notriptyline standard in 10 % ACN solution (using a fast gradient here). (B-E) Results obtained from different concentrations of nortripyline extracted from dried blood spots; (B) blood-blank (C) 0.05 ng/mL (D) 0.5 ng/mL (E) 15 ng/mL.

Detection-specificity

Figure 5 (A) shows MRM-chromatograms obtained from a nortripytline standard in solution (here using gradient #1; table 3). The most abundant signal was found for the transition *m/z* 264.1 → 233.1, followed by transitions to 105.1 and 117.1. Originally chosen as the quantifier, *m/z* 233.1 could however not be used as such with the blood samples, since blank blood-extracts gave a signal for this transition at a similar retention time to that of nortriptyline. There was no signal observed in the blank-extract for the other two transitions as shown in figure 5 (B). Thus for blood-extracts, *m/z* 105.1 was chosen as the specific quantifier for nortriptyline.

It is still interesting to follow how this nortriptyline/matrix interference relationship develops along the concentration range. Figure 5 (C) shows MRM-chromatograms for the lowest standard. There is a shoulder in the 233.1-trace that originates from nortriptyline; this origin is clearly confirmed by signals obtained for *m/z* 105.1 and 117.1. At higher concentration nortriptyline becomes more prominent and the abundance of the transition 233.1 exceeds that of the matrix interference (Figure 5 (D)). At last the matrix peak becomes insignificantly small and disappears under the large trace corresponding to *m/z* 233.1 and stemming from notriptyline (figure 5 (E)).

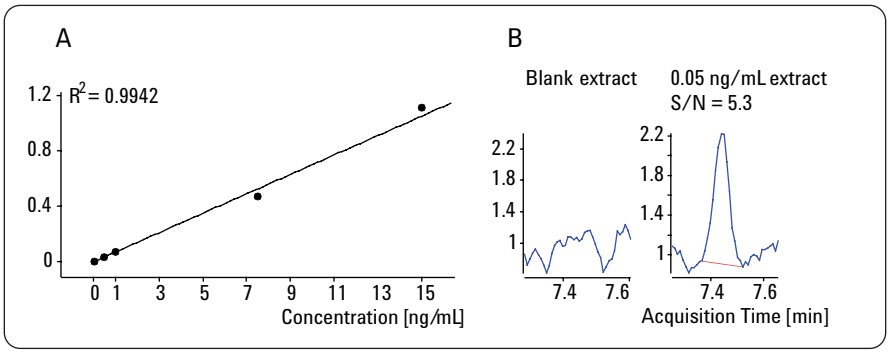

Figure 6
(A) Representative plot of peak area vs concentration obtained for nortrityline-extracts over the range 0.05 to 15 ng/mL full blood. (B) MRM-traces for quantifier transition *m/z* 264.1 → 105.1 for the blank-extract and the LLOQ.

Quantitation of nortriptyline, precision of the extraction procedure and recovery

Figure 6 (A) displays a representative plot of peak area vs concentration obtained for nortrityline-extracts over the range 0.05 to 15 ng/mL full blood. Good linearity was obtained with a correlation factor 0.9942 of the curve, which was obtained from a weighted 1/x linear regression.
Figure 6(B) shows MRM-traces for transition *m/z* 264.1 → 105.1 for the lowest concentration analyzed (0.05 ng/mL blood; S/N 5.3) and for the blank-extract. Specificity of the method is assured by the lack of signal in the blank.

To assess intra-day precision of the extraction procedure three concentrations of notriptyline in blood were chosen: 0.05, 0.5 and 7.5 ng/mL Each concentration was spotted, punched and extracted three times and subjected to chip/QQQ quantification in triplicate. The average was taken over all measurements (within one concentration) and RSD-values were calculated.
For the standard 0.05 ng/mL blood, S/N ratio always exceeded 5 and the % RSD-value of 16 % was well in agreement with the recommendation by the FDA for LLOQ (accuracy of this standard in correspondence to the calibra-

tion curve was within 80-120 %).[9] Standard 0.5 ng/mL gave an acceptable RSD-value of 14 % and standard 7.5 ng/mL an excellent RSD-value of 6 %.

For an indication of recovery, a blood-blank and the 7.5 ng/mL standard were extracted. Here, the entire blood spot was used for extraction in order to exclude errors which might originate from heterogeneous distribution of nortripyline across the spot. Instead of diluting the blank-extract with pure water, a freshly prepared standard of nortriptyline (in water) was used for dilution, so that an equivalent concentration was obtained to that expected in the blood spot. The average response was calculated from three injections of each, the extract and post-extracted spike (spiked blank-extract). These values were used to determine recovery as recommended by Zweigenbaum et al.[10] (Response of extracted-spike x 100 / Recovery of post-extracted spike). A value of 101 % was calculated.

Robustness of the HPLC-chip system
The HPLC-chip system employed during this study experienced over 500 injections of blood-extracts. During the whole analysis-period only the capillary connecting the 6-port valve in the autosampler and the inline filter in the loading path had to be replaced once. Yet, pressure drops due to UHC-chip enrichment and analytical column have not changed markedly. Figure 7 panel (A) shows overlaid quantifier-chromatograms obtained from three consecutive injections. These were extract-analyses numbers 350 to 352 and were produced just after the decision for the final gradient was made (gradient #6 in table 3). Panel (B) shows the chip performance about 150 injections later. Retention time has not shifted significantly (1.2 s) and chromatographic performance was reasonably stable with an increase in peak width measured at half height from about 4 to 5 s over the period of these approximately 150 injections.

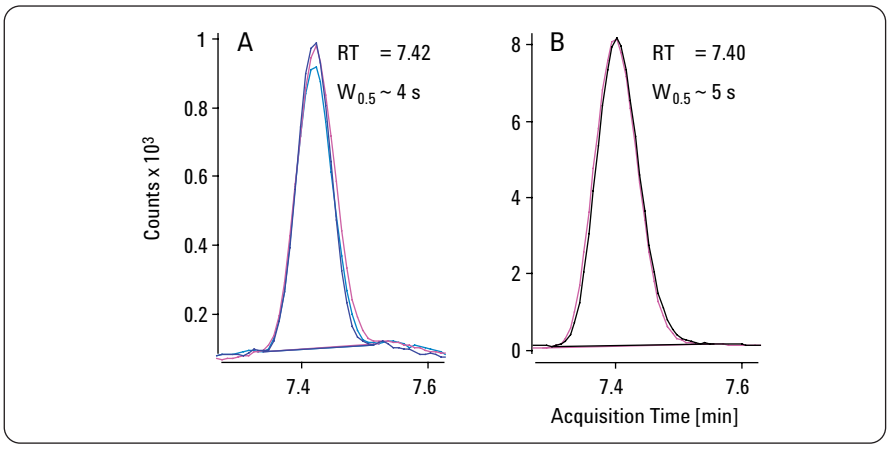

Figure 7
Superimposed quantifier-chromatograms obtained from blood extract analyses number 350-352 (A) and 511-512 (B).

Conclusion

We developed a method to specifically quantify our test drug nortriptyline out of 10 µL of whole blood utilizing the Agilent 1200 Series HPLC-chip/Agilent 6410B QQQ system.

Major steps in the optimized extraction procedure were spotting the blood onto FTA®Elute MicroCards, punching disks from the dried spot, extraction of nortriptyline using 75 %ACN solutions and the dilution step to obtain sample solutions with 10 %ACN content (figure 1). 5µL of these extracts were quantified employing the UHC-chip, which was specially designed for such small molecule applications.[1]

Recovery obtained from our optimized extraction process was 101 %. Intra-day precision ranged from 6 % to 16 %, respectively, at higher and the lowest concentration quantified, which was well in accordance with recommendations made by the US-FDA[9].

Linearity of the plot response vs concentration was shown in the range 0.05-15 ng/mL with the correlation factor approaching unity (figure 6). Excellent peak area precision with %RSD-values in the range of 2 % indicated interferences from the biological matrix to be most likely unimportant.

For this method the suggested LLOQ was 0.05 ng/mL for nortriptyline when extracted from blood. This is a remarkable result considering the LLOQ reported for acetaminophen (0.1 µg/mL) when analyzed from small volumes of blood (15 µL) using a similar extraction procedure and (conventional) HPLC/MS detection[3] (although care has to be taken when comparing results obtained by MS-detection with different compounds).

In addition we showed that HPLC-chip systems show great robustness even when used for the analysis of extremely complex biological matrices such as those contained in blood. More than 500 blood-extracts were analysed without significant change in pressure drop over the entire period of analysis while retention and chromatographic performance was stable (figure 7).

References

1.
Buckenmaier S., Vollmer M., Trojer L., Emotte C., "A small molecule chip for the high-sensitivity quantification of pharmaceuticals using triple quadrupole MS" *The Column, May 2008, 4, 20.* **2008.**

2.
Diehl K.H., Hull R., Morton D., Pfister R., Rabemampianina Y., Smith D., Vidal J-M, Van de Vorstenbosch C., "A good practice guide to the administration of sunstances and removal of blood, including routes and volumes." *J. Appl. Toxicol., 21, 15-23,* **2001.**

3.
Barfield M., Spooner N., Lad R., Parry S., Fowles S., "Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies." *J. Chromatography B, 870, 32-37*, **2008.**

4.
Vollmer M., Fandino A., Gauthier G. „Simultaneous assessment of drug metabolic stability and identification of metabolites using HPLC-chip/iontrap mass spectrometry". *Agilent Application Note 5989-5129EN.*

5.
Vollmer M., Buckenmaier S. "Using nanospray HPLC-chip/TOF for routine high sensitivity metabolite identification" *Agilent Application Note 5989-5938EN.*

6.
Information provided by Whatmann.

7.
Yin, H. and Killeen, K. "The fundamental aspects and applications of Agilent HPLC-Chip." *J. Sep. Sci. 30, 1427-1434,* **2007.**

8.
Ghitun M., Bonneil E., Cote E., Gauthier G.L., Thibault P.,"Advantages of using Intelligent Sample Loading with the HPLC-Chip for automated sample enrichment." *Agilent Application Note, 5989-5222EN,* **2006.**

9.
Guidance for the industry – Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, *Centre for drug Evaluation and Research, May* **2001.**

10.
Zweigenbaum, J., Henion, J., "Bioanalytical High-Throughput Selected Reaction Monitoring LC/MS determineation of Selected Estrogen Receptot Modulators in Human Plasma: 200 Samples/DAY." *Analytical Chemistry, 72, 2446-2454,* **2000.**

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