High Speed, Ultra-High Sensitivity, and Robustness Needed for the Quantitation of Pharmaceuticals in Blood Plasma

Application Note

Drug discovery and development: Drug metabolism and pharmacokinetics (DMPK)

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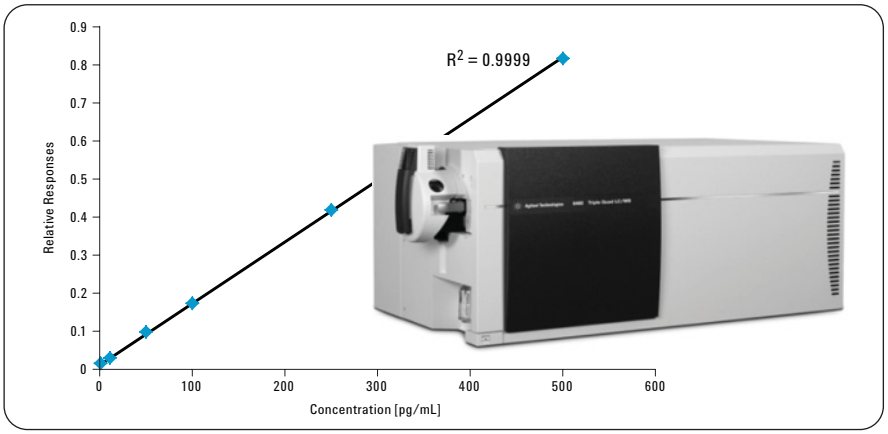

Abstract

This Application Note demonstrates the performance of an Agilent 1200 Series Rapid Resolution LC (RRLC) system coupled to an Agilent 6460A Triple Quadrupole (QQQ) mass spectrometer for the bioanalysis of human plasma.

High throughput analysis is shown for a test pharmaceutical and an internal standard with both drugs eluting well within a 1 minute chromatographic window.

Ultra-high sensitivity is demonstrated by linearity across a concentration range typically covered in human microdosing pharmacokinetic studies (1–500 pg/mL plasma).

Robustness and reproducibility are shown for this concentration range over a 5-day period during which the LC/MS system was exposed to more than 1000 plasma samples.

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Introduction

The use of LC/MS for the quantitative analysis of pharmaceutical drugs in physiological samples such as blood plasma or serum may be considered the hallmark of pharmacokinetic (PK) studies in drug discovery. PK profiles obtained from LC/MS analyses provide bioavailability and half-life information for drug candidates that is utilized to compare and select lead compounds for further investigation. The pharmaceutical industry demands sensitive, selective, and robust analytical methods but at the same time maintaining high throughput that allows generating this information quickly to make fast decisions. The concept of first-in-human trials (human microdosing), which accounts for often misleading extrapolation of PK data obtained from in-vitro or animal trials to human dosing, set even more stringent demands on bioanalytical sensitivity–the quantity of drug used for microdosing humans has to be less than a 1/100th of the therapeutic dose that is predicted from animal and in-vitro models [1–8].

Aim

An experiment was conducted to substantiate the high-throughput, ultra-high sensitivity, and robustness capabilities of the Agilent 1200 Series Rapid Resolution LC (RRLC) system coupled to the Agilent 6460A QQQ mass spectrometer. Human plasma was spiked with test drugs in a concentration range typically quantified in human microdosing studies (1–500 pg/mL plasma). Following plasma work-up a sequence of more than 1000 samples was analyzed in a trial over five working days (for example, 200 injections were made per day). Each sample sequence was followed by a standard series of drug in plasma that served to evaluate system performance.

Experimental

Equipment

- Agilent 1200 Series RRLC system comprising binary pump SL, micro vacuum degasser, thermostatted column compartment SL and Autosampler SL.
- Agilent 6460A QQQ mass spectrometer
- Agilent 20-port vacuum manifold processing station
- Agilent SampliQ Optimized Polymer Technology (OPT) cartridges (30 mg)
- Agilent ZORBAX C18 Eclipse Plus column 2.1 mm × 50 mm, 1.8 µm
- Agilent Mass Hunter Workstation software for instrument control, data acquisition, and data processing

LC Method

Solvent A	Water [0.1% formic acid (FA)]	
Solvent B	Acetonitrile (ACN) (0.1% FA)	
Gradient	t (min)	%B
	0	30
	0.1	30
	0.95	60
	1	60
	1.01	90
	1.20	90
	1.21	30
Flow	0.8 mL/min	
T (column)	40 °C	
Stop time	1.5 min	
Post time	2.5 min	
Injection volume	8 µL	
Needle wash	10 s with ACN/water 60:40 v/v (0.1% TFA)	

MS method

Scan type	MRM. MassHunter Optimizer software used to obtain settings given in Table 1.
Ionization mode	ESI positive with Agilent Jet Stream Technology
Gas temp	300 °C
Gas flow	11 L/min
Nebulizer	20 psi
Capillary	2000 V
Sheath gas temp	400 °C
Sheath gas flow	12 L/min
Nozzle voltage	0 V

Chemicals

Human blood plasma and all standard chemicals [nortriptyline, trimipramine, formic acid (FA), trifluoro acetic acid (TFA)] were from Sigma-Aldrich (Germany). HPLC grade water was from Burdick & Jackson (USA). HPLC grade ACN and MeOH were from Merck (Germany).

Plasma work-up

Calibration standards: 190-µL human plasma was spiked with 10 µL reference standard of nortriptyline (in ACN/water 70:30 v/v) to obtain blanks and six concentrations in the range of 1 to 500 pg/mL. 400 µL of 2% FA and 20 µL of the ISTD solution (trimipramine) were added. The mixture was vortexed.

Agilent SampliQ OPT cartridges (30 mg) were conditioned with 1 mL MeOH and 1 mL water (0.1% FA). The sample mixtures were loaded and washed with 1 mL 0.1% phosphoric acid followed by 1 mL 5% ACN solution. Elution was with 200 µL of solutions of ACN/water 70:30 v/v. The extracts were diluted by

Compound	ISTD	Prec ion	MS1 res	Prod ion	MS2 res	Dwell	Frag (V)	CE (V)	Polarity
Trimipramine	X	295.2	Unit	100.1	Unit	30	126	12	Positive
Nortriptyline		264.2	Unit	233.1	Unit	30	115	10	Positive
Nortriptyline		264.2	Unit	117.1	Unit	20	115	18	Positive
Nortriptyline		264.2	Unit	105.1	Unit	20	115	18	Positive

Table 1
MRM settings determined automatically using MassHunter Optimizer

a factor of 2 with water to obtain solvent compositions similar to that in the starting mobile phase. Plasma samples were worked-up the same way. Prior to injection samples were centrifuged for 5 minutes at 4,000 rpm.

Results and discussion

Matrix effect and recovery

Matrix factor and recovery obtained with the new SampliQ OPT material were determined as suggested by Zweigenbaum et al. using equation (1) and (2), respectively[9,10]. Two standard concentrations were used: 500 and 100 pg/mL nortriptyline. Spiked plasma and blanks were extracted (as described in the plasma work-up section). The blank-extracts were evaporated under a gentle stream of nitrogen and reconstituted with the reference standard. In this way, equivalent concentrations of nortriptyline were obtained in the extracted spike, post-extraction spiked blank, and reference standard.
1 µL injections were made in duplicate and averages taken for calculation. Recovery of nortriptyline was 97 and 95% respectively for 500 and 100 pg/mL standards. Matrix factor measurement gave a value of 0.8. Similar results were obtained for the ISTD trimipramine.

Equ. (1):
$$\text{Matrix factor} = \frac{\text{Response (post – extraction spiked blank)}}{\text{Response (reference standard)}}$$

Equ. (2):
$$\text{Recovery} = \frac{100 \times \text{Response (extracted spiked)}}{\text{Response (post – extraction spiked blank)}}$$

High throughput LC/MS method

Figure 1 shows a typical MRM-chromatogram obtained from a SPE-extract. The concentration of nortriptyline in plasma prior to extraction was 500 pg/mL. Nortriptyline and the ISTD both elute well within a 1 minute window demonstrating high throughput LC/MS analysis. Use of alternating column regeneration would increase throughput even further; cycle times (including injection, sample analysis, and column regeneration) was shown using the RRLC/QQQ system of less than 1.5 minutes for such PK-analyses[11].

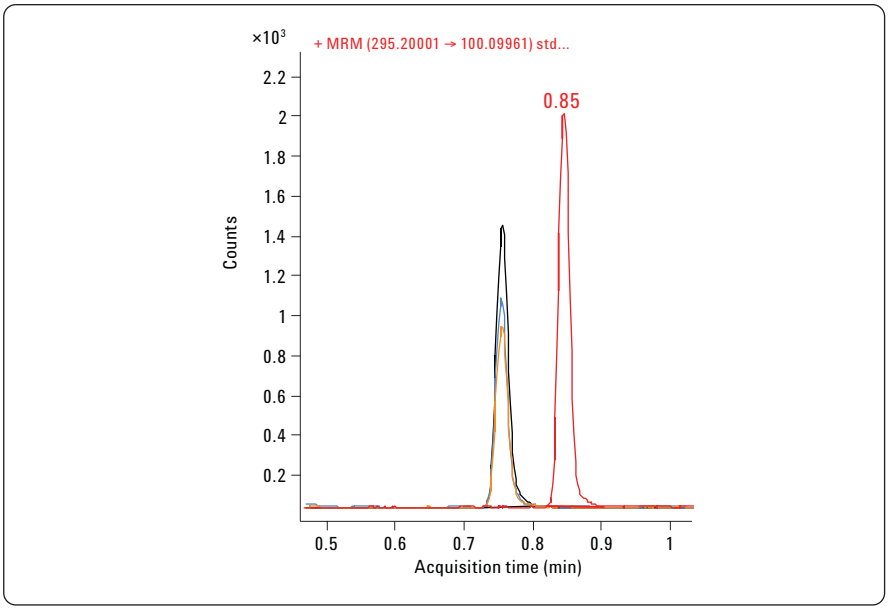

Figure 1
Demonstration of high-throughput analysis. MRM chromatograms obtained for 500 pg/mL nortriptyline and ISTD (red peak) in plasma. For nortriptyline quantifier (black) and qualifiers (blue and orange) are shown.

Sensitivity

Figure 2 shows the calibration curve of nortriptyline over a typical human microdosing range (1 to 500 pg/mL blood plasma) acquired at day 1 of this trial. Linearity was excellent with the correlation factor approaching unity (R^2 = 0.9999). Figure 3 shows corresponding MRM-chromatograms. Top chromatograms (bl) give results for the blank-extracts. There were no interfering peaks in elution positions of nortriptyline and the ISTD–illustrating selectivity. The 1 pg/mL nortriptyline-extract (~ 4 fg absolutely on-column) gave a prominent peak with good S/N ratios (for example, S/N = 14 with signal height over noise = 3 x RMS in the range 0.1–0.7 and 0.8–1.3).

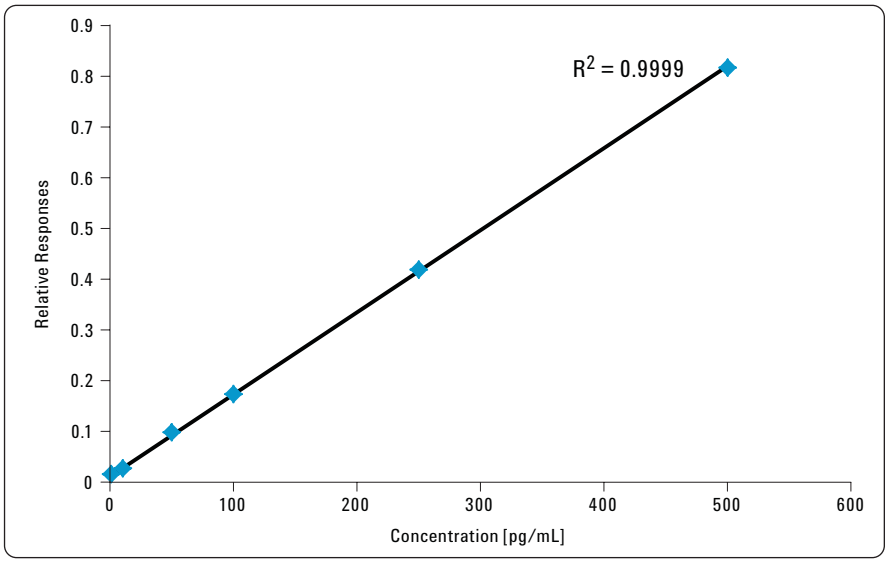

Figure 2

Demonstration of sensitivity and linearity in a typical human microdosing range. Plot of relative responses (nortriptyline/ISTD) vs. concentration of nortriptyline in blood plasma obtained on the first day of the five day experiment during which more than 1000 plasma samples were run.

System robustness/reproducibility

Continual exposure of the analytical system to blood matrix could impact the reproducibility of the bio-analytical method, for instance due to accumulation of non-volatile matrix constituents in the MS – inlet region. System robustness is particularly critical in ultra-high sensitivity analyses such as those performed in human microdosing studies.

Agilent's patented orthogonal spray sources (for example, ESI, APCI, MMS, or APPI) were designed to provide best signal-to-noise characteristics and robust operation ensuring reproducibility in high sensitivity analyses. The Agilent Jet Stream Technology that equips the Agilent 6460 QQQ's lets us achieve even higher detection sensitivity at even less maintenance.

To corroborate this robustness a sequence of more than 1000 plasma samples was analyzed over the course of five working days (for example, 200 injections per day). These 200 samples were followed by the standard series.

Note: Prior to analysis each day, the MS – inlet got a squirt of MeOH.

Figure 3, panel A shows MRM-chromatograms obtained for nortriptyline on day 1, day 2, and day 5. There was no marked variation in system performance (retention, peak shape, area, height, and S/N ratio) for this lowest level quantified during this 5-day trial. Figure 3 panel A, bl-day 1/5 is an overlay of blanks injected prior to the calibration series analyzed at day 1 and day 5; there was no change in noise observed.

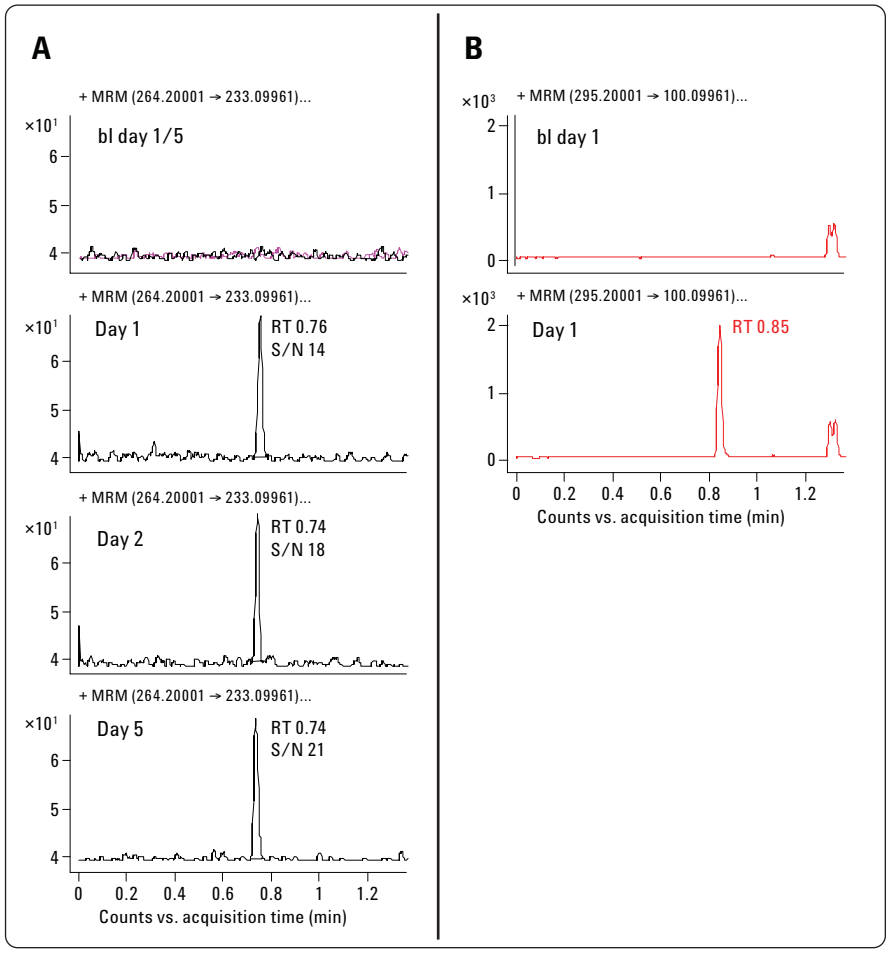

Figure 3
Demonstration of sensitivity and system reproducibility. MRM-chromatograms at the lowest concentration level (1 pg/mL) of nortriptyline in plasma. **A**: Nortriptyline results obtained at day 1, 2 and 5 of the 5-day trial during which more than 1000 plasma samples were run. bl day 1/5: Overlay of blanks injected prior to cal. series at day 1 (black) and day 5 (purple). **B**: Results for ISTD. RT = retention time and S/N = signal-to-noise ratio. Noise = 3 × RMS in the range 0.1–0.7 and 0.8–1.3.

Figure 4 shows the superposition of five calibration series run each day during the 5-day trial. The correlation factor (R^2 = 0.9996) demonstrates inter-day reproducibility. Variation of relative response was insignificant (for example, %RSD [1 pg/mL] = 2.4).

Figure 5A shows the plot of calculated concentration versus expected concentration with a correlation factor R^2 = 0.9997, which illustrates the detection accuracy to be maintained despite exposure to over 1000 plasma samples during the 5-day trial.

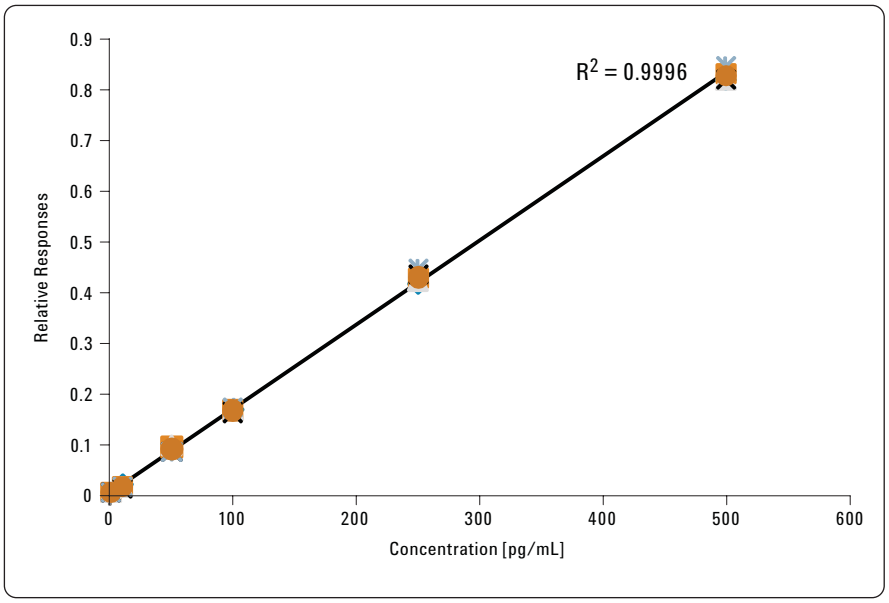

Figure 4
Demonstration of system robustness. Five calibration plots (as in Figure 1) obtained during the analysis of more than 1000 plasma injections overlaid. R^2 was calculated from the average response values.

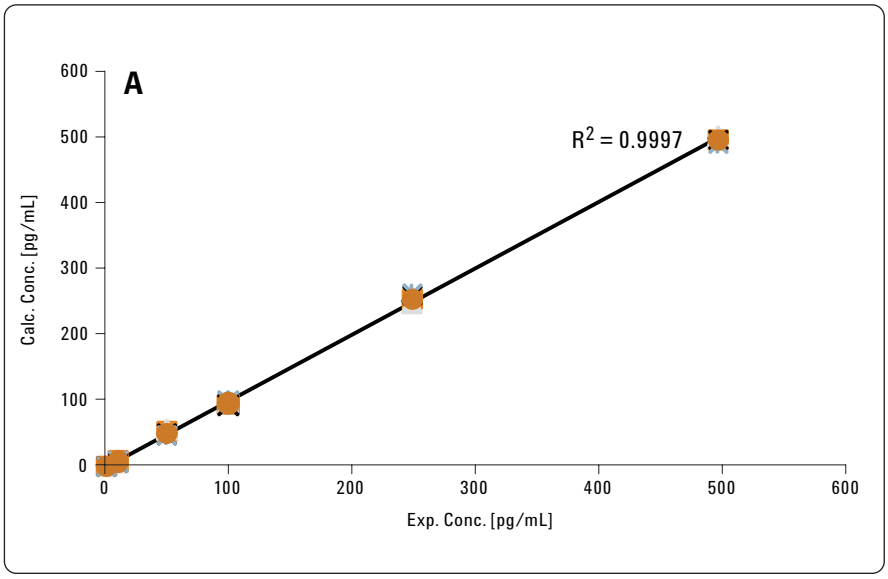

Figure 5A
Demonstration of accuracy-stability. A: Shows overlay of five plots of calculated concentration (from cal. curves) vs. expected concentration corresponding to the curves displayed in (Figure 4). R^2 was calculated from the average values.

Figure 5B shows that the percent accuracy for even the lowest level of quantification (1 pg/mL) to typically give little variation. The only time a 10% interval was exceeded was on day two. One FDA acceptance criteria for the lowest limit of quantitation (LLOQ) is an accuracy of 80% to 120%[12]. At higher concentration levels (here shown as 500 pg/mL) variation was insignificant. %RSD-values calculated for the 1 and 500 pg/mL levels over the course of the five days were 7.5 and 0.8%, respectively.

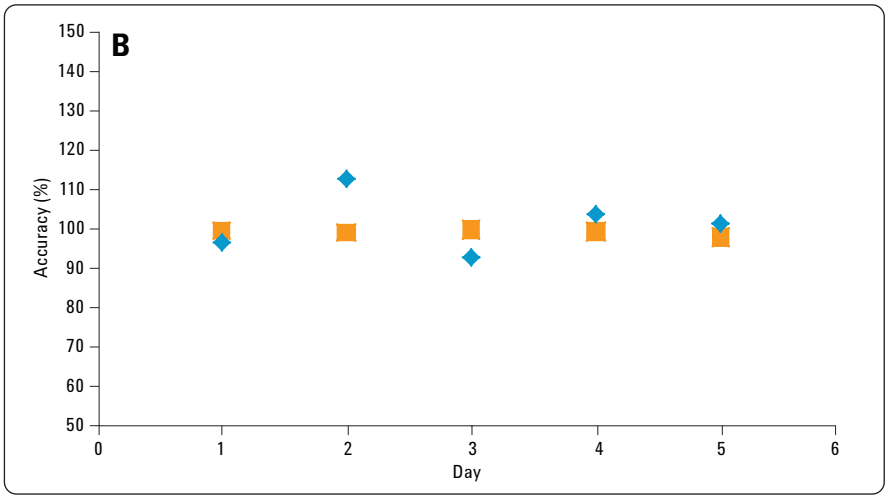

Figure 5B
Demonstration of accuracy-stability. B: Shows 5-day % accuracy development at lowest (1 pg/mL = blue diamond) and highest (500 pg/mL = orange square) level quantified.

Conclusions

The pharmaceutical industry requests high throughput, ultra-high sensitivity, and robustness for PK trials in blood plasma to being able to make decisions fast and to fail non-drug like candidates at the lowest development cost possible.

This Application Note demonstrates the Agilent 1200 Series RRLC system coupled to the Agilent 6460A QQQ to fully encounter these requests.

High throughput: Both drugs extracted from plasma eluted well below 1 minute (see Figure 1).

Ultra-high sensitivity: Quantification was performed in a concentration range typically used in human microdosing studies (1–500 pg/mL plasma). The amount of lowest quantitation (4 fg absolutely on column) gave prominent signals with good S/N ratios (Figure 3). Linearity was excellent: $R^2 = 0.9999$ (Figure 2).

Robustness/Reproducibility: A 5-day trial during which the LC/MS system saw more than 1000 plasma samples was conducted. The calibration series ran every 200 injections served to evaluate system performance (linearity in Figure 4, accuracy in Figure 5, and signals obtained at the lowest quantitation level over the course of the 5-days in Figure 3). The performance remained remarkably stable.

References

1.
Mike S. Lee, "LC/MS Applications in drug development,"Wiley-Interscience, 2002.

2.
Rang H.P., "Drug Discovery and Development - Technology in Transition," Elsevier Limited, 2006.

3.
I. R. Wilding, J. A. Bell, "Improved Early Clinical Development through human microdosing studies," DDT, 2005, 10, 890-894.

4.
N. Yamane, et al., "Microdose Clinical Trial...," *J.Chromatogr.B*, 2007, 858, 118-128.

5.
"European Agency for the Evaluation of Medicines for Human Use (EMEA), Position Paper on the Nonclinical Safety Studies to Support Clinical Trials with a Single Microdose," CPMP/SWP2599/0, 28 January 2003.

6.
"Guidance for Industry, Investigators, and Reviewers Exploratory IND Studies," Food and Drug Administration, January 2006.

5.
G. Lappin, et al., "Use of Microdosing to predict Pharmacokinetics at the therapeutic Dose: Experience with 5 drugs," Clinical Pharmacology Therapeutics, 2006, 80, 203-215.

8.
S. Pedraglio, et al., "New Perspectives in Bio-Analytical Techniques for Preclinical Characterization of Drug Candidate: UPLC-MS/MS in *In-Vitro* Metabolism and Pharmacokinetic Studies," *J.Pharmacaeutical and Biomedical Analysis*, 2007, 44, 665-673.

9.
J. Zweigenbaum, J. Henion, "Bioanalytical High-Throughput Selected Reaction Monitoring LC/MS Determination of Selected Estrogen Receptor Modulators in Human Plasma: 200 Samples/DAY." *Analytical Chemistry*, 72, 2446–2454, 2000.

10.
D. A. Wells, "High Throughput Bioanalytical Sample Preparation - Methods and Automation Strategies" *Elsevier Limited*, 2003.

11.
M. Frank, "Improving the Productivity of Pharmacokinetic Parameter Determination," Agilent Technologies publication 5989-7648EN.

12.
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

www.agilent.com/chem/qqq

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