Simultaneous assessment of drug metabolic stability and identification of metabolites using HPLC-Chip/ion trap mass spectrometry

Application Note

Martin D. Vollmer
Anabel S. Fandino
Georges L. Gauthier

Abstract

In vitro drug metabolism assays like metabolic stability and identification of metabolites play an important role in the early understanding of *in vivo* pharmacokinetic characteristics and help to discard non-drug like compounds that would fail during later stages of development. Identifying compounds with poor pharmacokinetic properties early enough leads to enormous savings of resources. Thus, fast screening methods are required to screen the large collection of new chemical entities that need characterization in the early stage of drug discovery. Typically metabolic stability assessment is performed at 1-4 µM substrate concentration to mimic physiological concentrations while identification of metabolites requires an additional incubation at the 30-50 µM substrate concentration to generate detectable levels of major and minor metabolites.

In this Application Note we investigate the ability of automated nanospray LC/MS using HPLC-Chip/MS technology with an ion trap mass spectrometer to simultaneously measure the metabolic stability and identify metabolites of the drug buspirone at 1 µM concentration following incubation with rat liver S9 fractions. The high sensitivity of the HPLC-Chip/MS approach allowed performing both assays simultaneously. The percentage of drug remaining after 28 minutes and the identified metabolites for the 1-µM incubation are in good agreement with those reported in the literature.

Agilent Technologies

Introduction

"Fail fast, early and cheap" is the current paradigm adopted by the pharmaceutical industry. Different types of *in vitro* ADME assays (Absorption, Distribution, Metabolism, Excretion) are performed in order to characterize new chemical entities (NCEs) and discard non-drug-like compounds early in the drug discovery lifecycle. Drug metabolic stability and identification of metabolites are the two primary filters that are applied to ensure that compounds are metabolically stable. In addition, the identification of metabolites allows the chemist to locate metabolically susceptible sites in the molecules ("soft spots"). The medicinal chemistry groups then use this information to synthesize molecules with superior pharmacokinetic properties like longer half-life or improved bioavailability[1]. Stand-alone drug metabolic stability measurements are typically carried out at substrate concentrations of 1-4 µM in order to mimic physiological concentrations of the drug[2-4]. Fast gradient LC methods coupled with triple quadrupoles operated in the selected reaction monitoring (SRM) mode is the preferred analytical technique. Since metabolic stability incubations are performed at very low levels of substrate, discovery and identification of metabolites typically requires a second incubation at the 30-50 µM substrate concentration in order to generate detectable metabolite levels and good quality product ion spectra of all major and minor metabolites. This second assay requires not only one or more additional incubations but also requires additional LC/MS analysis and data-processing time.

In this Application Note, we show the ability of automated nanospray LC/MS using HPLC-Chip/MS technology with an ion trap mass spectrometer to perform measurement of metabolic stability and identification of metabolites in a single run at the 1 µM level using buspirone as test substance.

Experimental

Reagents and supplies

The substance buspirone hydrochloride and the biochemicals beta-nicotinamide adenine dinucleotide phosphate sodium salt (NADP) and isocitric dehydrogenase (IDH) were purchased from Sigma-Aldrich (Taufkirchen, Germany). DL-isocitric acid trisodium salt was purchased from MP Biomedicals Inc. (Eschwege, Germany). Untreated male Sprague Dawley rat liver S9 homogenate (pool of 203) was purchased from XenoTech, LLC (Kansas, USA). All other reagents and organic solvents were of analytical grade and from VWR (Darmstadt, Germany).

Incubation of buspirone with rat liver S9 fractions

The incubation mixtures for phase I metabolism consisted of an amount of S9 preparation equivalent to 1 mg protein, substrate at 1 µM concentration (buspirone from a 0.1 mg/mL stock solution in water), 1.2 mM NADP, 2.5 U isocitrate dehydrogenase, 5 mM isocitrate and 5 mM magnesium chloride in 0.1 M phosphate buffer (pH 7.4) up to a total volume of 1000 µL. Incubation was carried out with constant agitation at 37 °C for 0 minutes (n=3) and 28 minutes (n=3), whereby the reaction was stopped by adding 100 µL perchloric acid and 60 µL acetonitrile followed by centrifugation for 15 minutes at 10,000 RPM. The supernatant was evaporated using a SpeedVac concentrator and reconstituted in 1 mL water, 0.1 % formic acid. 1 µL of the solution was injected for analysis.

HPLC-Chip/MS system

Components and flow path of the HPLC-Chip/MS system were described in previous application notes[5-7]. The HPLC-Chip is fabricated from inert polyimide using UV laser ablation in combination with vacuum lamination of the polyimide film to create a multilayer microfluidic device. Open micro-channels are packed with reversed-phase column materials to create HPLC columns. Metals are applied by thin film deposition onto the polymer film surfaces to produce the electrical contacts for electrospray ionization. Figure 1 shows a schematic of the HPLC-Chip. For the experiments described here, the following components were integrated onto the HPLC-Chip (G4240-65001):

- A 40-nL enrichment column packed with ZORBAX 80 SB-C18, 5-µm particle size
- A 0.075 x 43 mm analytical column packed with ZORBAX 80 SB-C18, 5-µm particle size.
- All connections between the two columns and between the analytical column and the nanospray emitter
- The nanospray emitter (10-µm ID).

The HPLC-Chip is inserted into the HPLC-Chip/MS interface (HPLC-Chip cube). This interface

provides all fluid connections to the Agilent 1200 Series nanoflow LC system and ensures efficient coupling of the nanospray emitter to the Agilent 6330 Ion trap LC/MS. The HPLC-Chip cube includes the HPLC-Chip loading and ejection mechanism, a microvalve for flow switching and fluid connection ports for the nano-LC and microwell-plate autosampler. The HPLC-Chip/MS interface mounts directly on the MS source and includes a miniature CCD camera for spray visualization. The HPLC-Chip/MS interface is a standard module within the Agilent 1200 Series LC system and is fully controlled by the Agilent ChemStation software. The HPLC-Chip/MS interface can also be used with the Agilent 6210 Time-of-Flight LC/MS and has been applied to biomarker studies [7]. HPLC-Chips can be replaced in seconds. When a HPLC-Chip is loaded, leak-tight fluid connections are automatically established as the chip is sandwiched between the rotor and stator of the built-in multi-port microvalve. The rotor and stator dock onto the chip and establish a flow path from the nano-LC to the ports on the chip surface (figure 2). Fast movement of the rotor ensures reliable switching between sample loading and sample analysis positions on the HPLC-Chip. The loading mechanism in the HPLC-Chip interface precisely and automatically positions the nanospray emitter orthogonal to the MS inlet for maximum sensitivity and robustness.

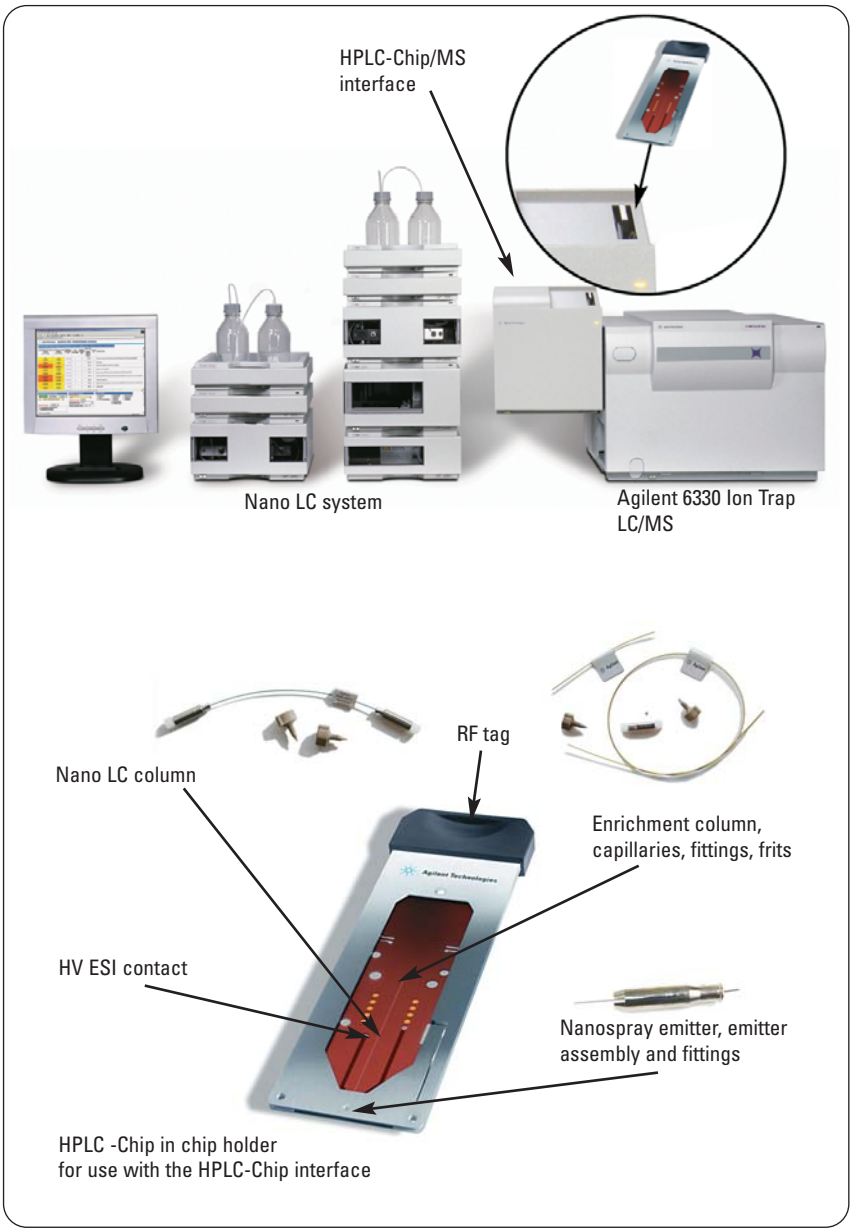

Figure 1
Agilent HPLC-Chip/MS system.

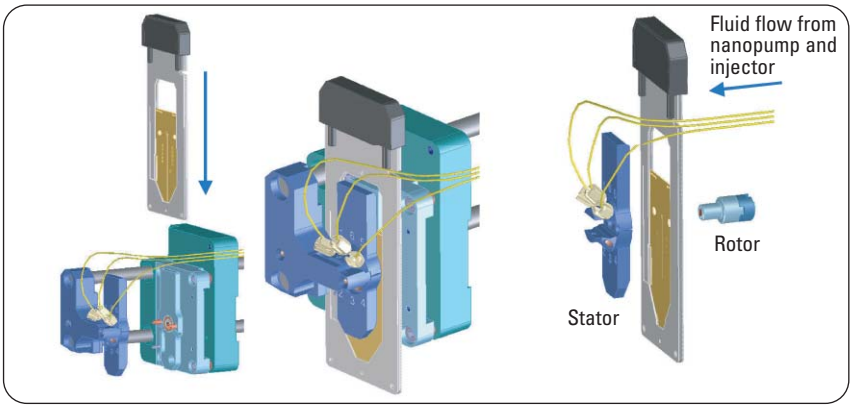

Figure 2
Fluid connections to the HPLC-Chip.

Chromatographic conditions

The HPLC part of the system was composed of following Agilent 1200 modules:

- 1200 Series nanopump with degasser and solvent cabinet
- 1200 Series capillary pump
- 1200 Series microwell-plate autosampler (thermostatted)
- HPLC-Chip/MS interface

Solvent A contained water with 0.1 % formic acid and solvent B consisted of acetonitrile with 0.1 % formic acid. The gradient of the analytical pump started with 2 % B, reached 70 % B after 10 minutes and was set back to 2 % B after 10.01 minutes with a post run time of 8 minutes at 2 % B. Flow rate was 0.3 µL/min. Enrichment of the analytes prior to gradient start was performed at 4 µL/min 2 % B using the loading pump. The injection volume was 1 µL. For sample loading the ISL (intelligent sample loading) feature of the HPLC-Chip ChemStation (version B01.03) menu was used. This feature allows sample loading onto the enrichment column during the prerun. A user defined injection flush volume plus the injection volume of the sample is precisely delivered by the loading pump to the enrichment column while the microvalve is in the enrichment mode. Once the sample is loaded, the microvalve is automatically switched to the analysis position. Sample loading time and data acquisition start time are always optimised by the ILS software. A 4-uL injection flush volume was selected for buspirone metabolite analysis.

Mass spectrometric conditions

An Agilent 6330 Ion Trap LC/MS was used for MS and MS^n data acquisition. The ion trap analysis was performed in positive mode. The drying gas flow was 3 L/min of nitrogen. The HPLC-Chip interface uses an enclosed source design to eliminate polydemethylsiloxane background contamination from the laboratory air[8]. To achieve low background conditions, and additional 1 L/min of filtered air was added to the drying gas. The drying gas temperature was 300 °C. Capillary voltage was set at -1800 V with an endplate offset of -500 V. Skim 1 was at 40 V, capillary exit was 117 V and trap drive was 34.5 V. Scan mode was ultrascan. Scan range was 100-500 m/z (MS) and 100-500 m/z (MS/MS). The Ion Charge Control (ICC) parameters were as follows: target: 500,000, maximum accumulation time: 150 ms and averages: 1. The automatic MS^2 conditions were as follows: number of precursors: 3, active exclusion: on, exclude after 5 spectra, release after 0.1 min, isolation width: 4 m/z, fragmentation amplitude: 1.3 V and CID voltage ramping (Smart-Frag on): 30-200 %.

Data analysis

Data analysis was performed using the Agilent 6330 Ion Trap LC/MS mass spectrometer version 6.0 software. The buspirone metabolites were identified by search of the expected MH^+ values in the MS traces as well as searching for diagnostic fragments in the MS/MS spectra. These fragments are m/z 168 (A), m/z 219 (B), m/z 180 (C), m/z 222 (D), m/z 150 (E), m/z 265 (F), m/z 122 (G) and m/z 291 (H) for buspirone[9,10] (figure 3).

Measurement of metabolic stability

The metabolic stability was determined by comparing the peak areas of buspirone measured at 28 min (t_{28} sample, metabolised compound, n=3) with that at 0 min (t_0 sample, unchanged test compound, n=3), and was calculated as follows:
% remaining = [Average Area t_{28}/ Average (Areas t_0)] x 100 %
metabolized = [100 - % remaining]

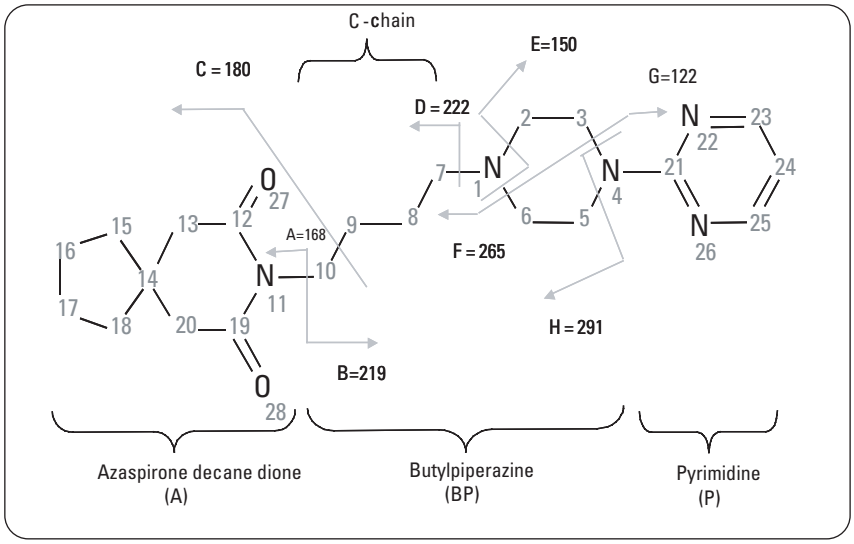

Figure 3
Buspirone diagnostic fragments according to Zhu et al.[10]

Following incubation, the expected percentage of remaining buspirone was approximately 5 % according to previously reported buspirone metabolic stability data[11]. Therefore, linearity test were performed over the range of 5 to 100 % remaining buspirone.The t_0 minute sample was used to prepare 1:20, 1:10 and 1:2 dilutions in 0.1 % formic acid (n=3 injections each), which were measured in the same sequence together with t_0 and t_{28}.

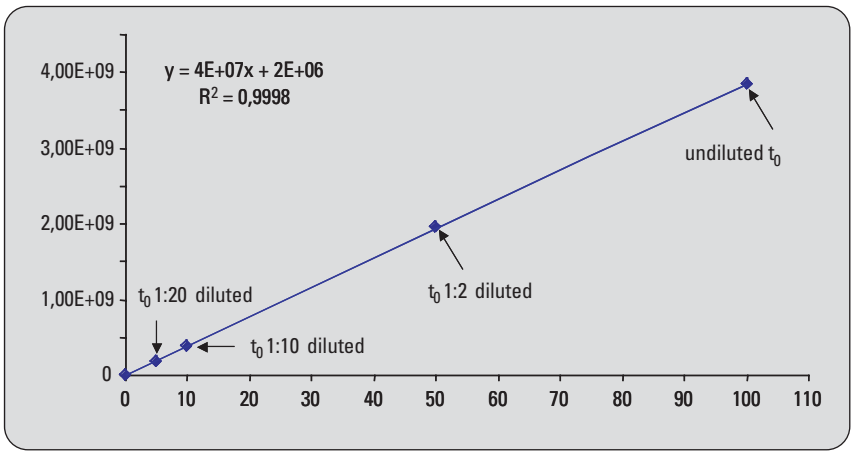

Figure 4
Linearity test. Simulated percentage range of remaining buspirone (5% to 100%) using undiluted t_0 and t_0 diluted 1:2, 1:10 and 1:20.

Results and discussion

Simultaneous measurement of drug metabolic stability and identification of phase I metabolites

The superior sensitivity of the nanospray HPLC-Chip/MS approach allows the quantification of buspirone and the simultaneous identification of buspirone metabolites using an injection volume of 1 µL.

Drug metabolic stability

Buspirone was incubated at 1 µM with rat liver S9 fraction during 0 min (t_0) and 28 min (t_{28}) and its disappearance was measured (n=3 injections for each t_0 and t_{28}). An extracted ion chromatogram of the MH^+ ion from buspirone (MH^+ 386) was obtained for all samples and the peak areas were determined using the Agilent 6330 Ion Trap LC/MS mass spectrometer version 6.0 software. The results obtained from the linearity test demonstrate that the HPLC-Chip/MS Ion Trap system can provide linear results over the expected percentage range of remaining buspirone (figure 4). The remaining percentage of buspirone observed at 28 min was 4 % ± 0.1 % (coefficient of variation = 3 %) and in good agreement with previously reported data[11]. Figure 5 shows the extracted ion chromatograms for buspirone at t=0 min and t=28 min.

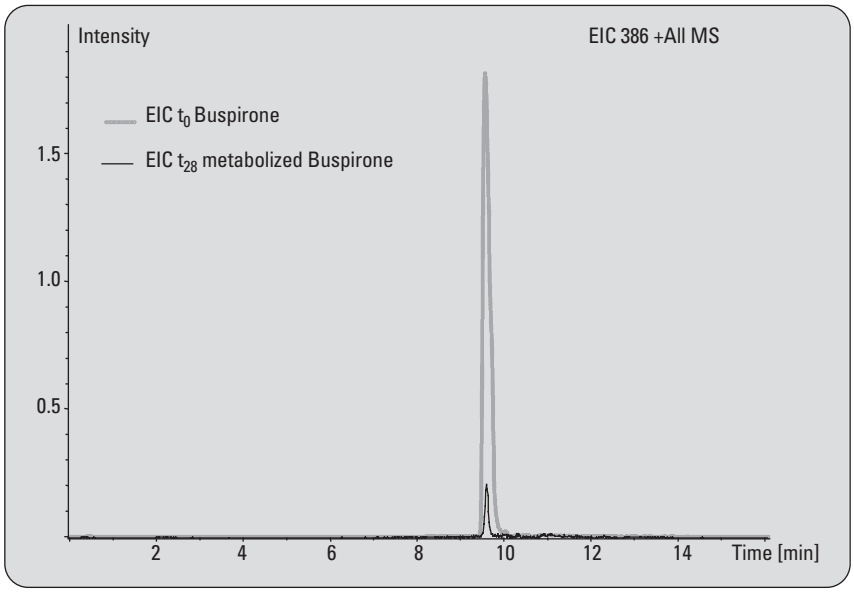

Figure 5
Phase I metabolism of 1 uM Buspirone.

Identification of metabolites

Due to the excellent chromatographic performance and high sensitivity of the HPLC-Chip/6330 Trap LC/MS system, good quality metabolite product ion spectra are generated even at 1 µM buspirone concentration. The advanced data-dependent acquisition capabilities of the 6330 Trap software in com-

bination with the fast data acquisition rate of the 6330 Trap were used to increase the amount of unique metabolite data. Overlaid base peak chromatograms of buspirone incubations at 0 min and 28 min (figure. 6) clearly demonstrate the conversion of buspirone to its phase I metabolites and highlights the good separation capabilities of the HPLC chip. Major and minor buspirone phase I metabolites like hydroxybuspirone (MH^+ 402), dihydroxybuspirone (MH^+ 418), N,N-desethyl buspirone (MH^+ 360) and N,N-desethyl hydroxybuspirone (MH^+ 376), which were previously only identified in 30 µM incubations [9], could also be identified in 1 µM incubations with very good quality product ion spectra. Even low level metabolites like N,N-desethyl buspirone and its hydroxylated form could be unequivocally detected at this low level in vitro incubation using an injection volume of 1 µL ([2] 330 pg total drug injected). Identified buspirone metabolites and representative fragment ions are shown in table 1. Several isomers were found for hydroxylated (m/z 402) and dihydroxylated buspirone derivates (m/z 418). Major sites of hydroxylation are the azaspirone decane dione (A) or the pyrimidine (P) substructures of the molecule (figure 3). Presence or absence of specific product ions are diagnostic for hydroxylation of the different substructures. Single oxidation was detected in 6 different isobars (figures 7 and 8) among them five were hydroxylated buspirones and the one with the latest retention time was buspirone N-oxide. Fragment ions of m/z 138 and m/z 166 indicate single hydroxylation at the pyrimidine (P) and pyrimidine/piperazine structures (P and partially BP) respectively and presence of m/z 196 and/or m/z 238 are diagnostic for single hydroxylations at the azaspirone decane dione (A) not or including butyl residue (figures 3 and 8). For the dihydroxylated buspirone eight different isomers were identified.

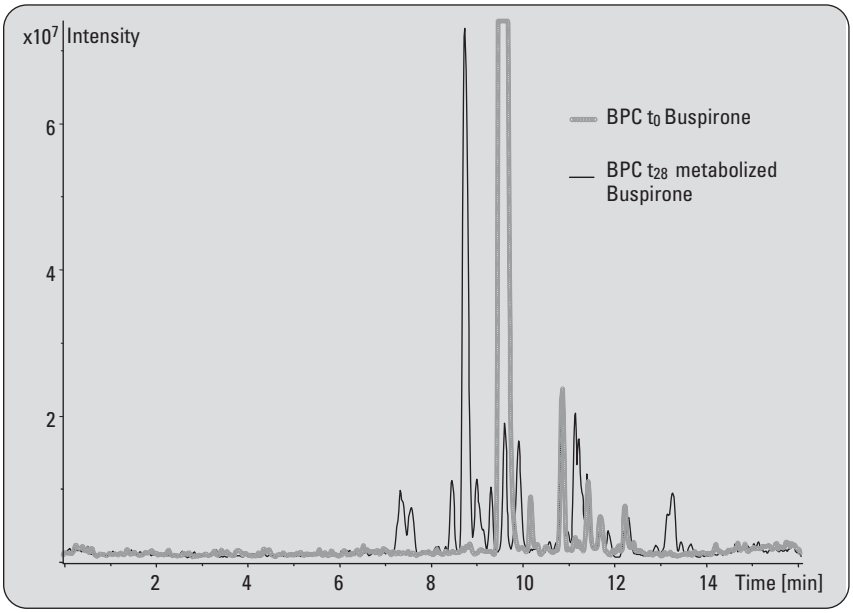

Figure 6
Conversion of buspirone to the phase I metabolites after 0 and 28 min of incubation time.

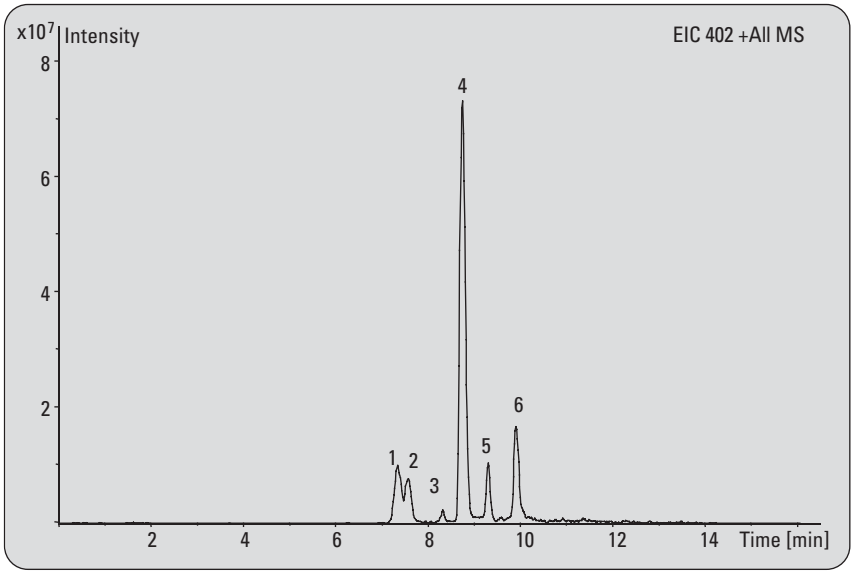

Figure 7
Extracted ion chromatogram of m/z 402. 6 isobars were detected with good quality MS/MS spectra.

Figure 9 shows as an example the MS^2 and MS^3 spectra of buspirone N-oxide. Fragments of m/z 277 and m/z 177 are diagnostic for the buspirone N-oxide identity [9,10]. The good quality MS^2 and MS^3 spectra obtained at the 1 µM substrate concentration allowed for simultaneous structure elucidation of the detected metabolites and assessment of metabolic stability. The ability to perform both metabolic stability and metabolite identification in the same analysis at the 1 µM substrate concentration significantly increases the overall throughput of this type of study.

Conclusion

The microfluidic HPLC-Chip coupled to the Agilent 6330 Ion Trap LC/MS allowed for simultaneous assessment of metabolic stability and identification of metabolites using low level in vitro incubations at substrate concentration of 1 µM. The key factors that make the simultaneous measurement possible are:

- High sensitivity and excellent chromatographic separation achieved with nanospray LC/MS when using the integrated HPLC-Chip device.
- Excellent MS and MSn data achieved at the 1-µM substrate concentration level with the high sensitivity and fast acquisition Ion Trap mass spectrometer operated in data-dependent acquisition mode.
- The robustness, reliability and ease of use of the HPLC-Chip/MS system allowing routine use of nanospray LC/MS for this application.

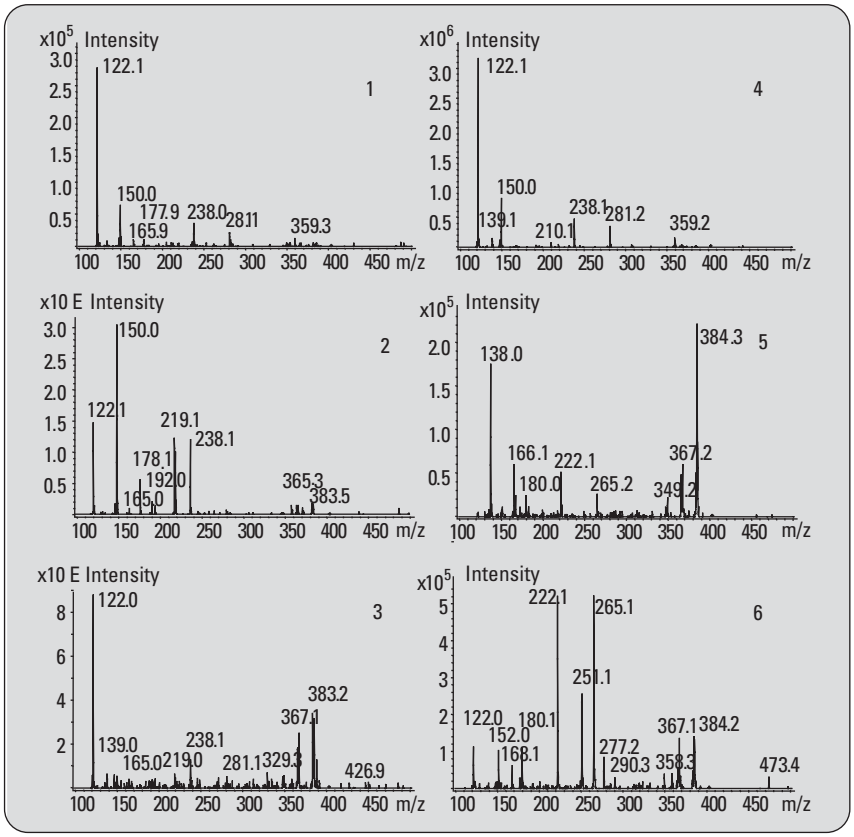

Figure 8
MS^2 spectra from all detected 402 isobars containing a single oxidation.

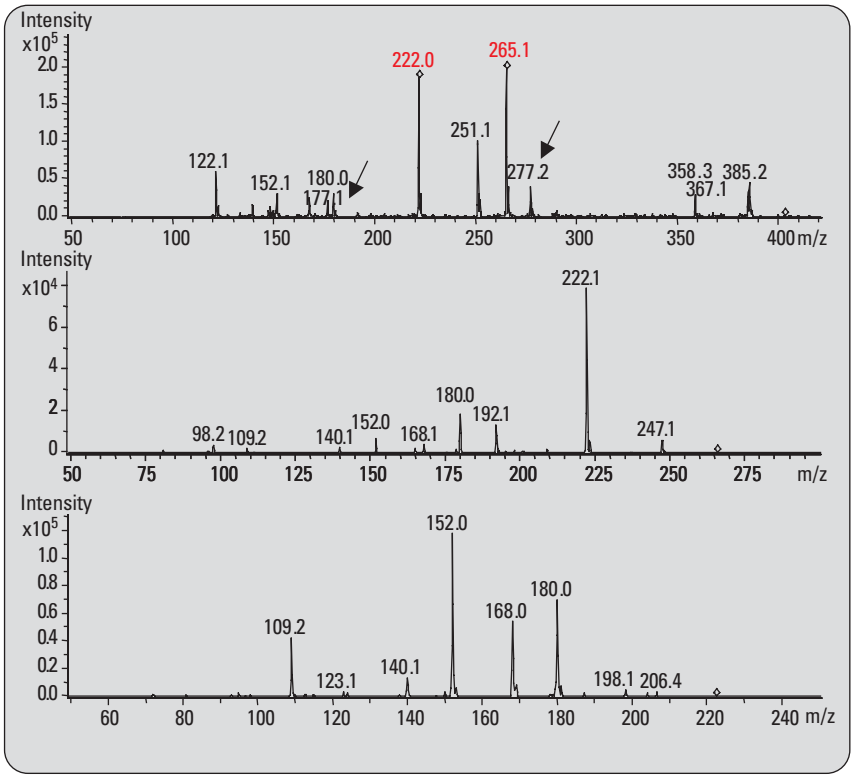

Figure 9
MS/MS and MS^3 from buspirone N-oxide.

Mass M+H	Retention time (min)	Metabolite	122	138	150	166	222	238	other
418	6.55	Dihydroxybuspirone	X						196
									254
418	6.74	Dihydroxybuspirone	X			X		X	
418	7.04	Dihydroxybuspirone		X		X			
402	7.32	Hydroxybuspirone	X		X			X	196
402	7.57	Hydroxybuspirone	X		X			X	219
									196
418	7.90	Dihydroxybuspirone	X					X	168
418	8.09	Dihydroxybuspirone	X						194
376	8.16	N,N-desethyl hydroxybuspirone	X						168
									281
402	8.30	Hydroxybuspirone	X		X			X	
418	8.44	Dihydroxybuspirone		X		X		X	281
418	8.69	Dihydroxybuspirone	X						
402	8.73	Hydroxybuspirone	X		X				281
360	9.04	N,N-desethylbuspirone	X				X		265
418	9.11	Dihydroxybuspirone	X					X	
402	9.28	Hydroxybuspirone		X		X	X		
386	9.59	Buspirone	X		X		X		265
402	9.89	Buspirone N-oxide	X				X		277
									265
									251
									177

Table 1
Identified metabolites and representative MS/MS fragment ions from buspirone phase 1 metabolism (figure 3).

References

1.
Nassar AF, Talaat RE. "Strategies for dealing with metabolite elucidation in drug discovery and development." *Drug Discovery Today. 9: 317-327*, **2004.**

2.
Korfmacher WA, Palmer CA, Nardo C, Dunn-Meynell K, Grotz D, Cox K, Lin C, Elicone C, Liu C, Duchoslav E. "Development of an automated mass spectrometry system for the quantitative analysis of liver microsomal incubation samples: A tool for rapid screening of new compounds for metabolic stability." *Rapid Communications in Mass Spectrometry.13: 901-907*, **1999.**

3.
Ansede JH, Thakker DR. "High-throughput screening for stability and inhibitory activity of compounds toward cytochrome P450-mediated metabolism." *J. Pharm. Sci. 93 (2): 239*, **2004.**

4.
Wring SA, Silver IS, Serabjit-Singh CJ. "Automated quantitative and qualitative analysis of metabolic stability: a process for compound selection during drug discovery." *Methods Enzymol. 357: 285*, **2002.**

5.
"Comparison of HPLC-Chip/MS with conventional nanoflow LC/MS for proteomic analyses." *Agilent Application Note, Agilent publication number 5989-3538EN*, **2005.**

6.
Yin H, Killeen K, Brennen R, Sobek D, Werlich M, van de Goor T. "Microfluidic chip for peptide analysis with an integrated HPLC column, sample enrichment column, and nanoelectrospray tip." *Analytical Chemistry. 77: 527-533*, **2005.**

7.
Fortier MH, Bonneil E, Goodley P, Thibault P. "Integrated Microfluidic Device for Mass Spectrometry-Based Proteomics and Its Application to Biomarker Discovery Programs."*Analytical Chemistry. 77: 1631-164*0, **2005.**

8.
Schlosser A, Volkmer-Engert R. "Volatile polydimethylcyclosiloxanes in the ambient laboratory air identified as source of extreme background signals in nanoelectrospray mass spectrometry." *J.Mass Spectrom. 38: 523-525*, **2003.**

9.
"Automated software-guided identification of new buspirone metabolites using capillary LC coupled to ion trap and TOF mass spectrometry." *Agilent Application Note, Agilent publication number 5989-4146EN*, **2005.**

10.
Zhu M, Zhao W, Jimenez H, Zhang D, Yeola S, Dai R, Vachharajani N, Mitroka J. "Cytochrome P450 3A-mediated metabolism of buspirone in human liver microsomes." *Drug Metabolism and Disposition, 33: 500-507*, **2005.**

11.
Whitney J, Sanders M, Josephs J, Hail M, Nugent K. "Automated High Throughput metabolic stability and detailed metabolite profiling." *20th Annual International Symposium on Laboratory Automation and Robotics (ISLAR), Boston, MA.*, **2002.**

Anabel Fandino is Application Chemist, Martin Vollmer is R&D Scientist and Georges Gauthier is Product Manager for the HPLC-Chip Technology program, all at Agilent Technologies, Waldbronn, Germany.

www.agilent.com/chem/ms

Published May 1, 2006
Publication Number 5989-5129EN

Agilent Technologies