Using nanospray HPLC-Chip/TOF for routine high sensitivity metabolite identification

Application Note

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Abstract

Nanospray LC-MS analysis of dextromethorphan (DEM) and its metabolites was performed with the Agilent 1200 Series HPLC-Chip/TOF system. Automated nanospray HPLC-Chips with the high sensitivity TOF allowed detection of all DEM metabolites, following incubation at concentration levels 100-fold lower than normally used. In addition, system response was linear over a wide concentration range for metabolites even when spiked into human plasma and urine. LOD's for the “real-life” samples were in the high attmol to low fmol-range. Sample preparation was simplified to acidic precipitation by perchloric acid and a short centrifugation step. Simplified sample preparation, accurate mass measurements, excellent chromatographic reproducibility and a robust easy-to-use nanospray chip system make this set-up optimal for use in a routine environment allowing higher throughput and reduced sample preparation cost.

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Introduction

To “fail fast, early and cheap”, analytical methodologies must be able to deliver routine high throughput and high sensitivity measurements when investigating drug metabolism during early drug discovery. Identifying metabolically labile points, the so called “soft spots”, on candidate molecules during drug development will improve metabolic stability and allow for early safety assessment[1]. In most cases, LC/MS is the method of choice because of the high throughput, automation and high sensitivity achieved with modern LC/MS systems.

Metabolizing enzymes will act on drug molecules at many different sites often leading to the creation of isobars. Chromatographic performance of the LC/MS system must therefore ensure complete separation of these isobaric metabolites while maintaining the required high throughput, automation and sensitivity objectives. Nanospray LC/MS combines both high resolution chromatography and high sensitivity MS detection and is used for many ultra-trace analysis applications[2]. The integration of all nanospray components directly on the HPLC-Chip delivers the robustness and reliability required for routine automated unattended nanospray LC/MS operation[3]. Superior sensitivity is achieved from the combination of extremely reproducible nanoflow, seamless zero dead volume connections and stable nanospray performance due to the nanopump and HPLC-Chip design[4]. Automation of sample loading and pre-concentration of the sample directly onto the chip enrichment column, combined with short flow paths and low delay volumes in the chip channels, enables high-throughput, fast-gradient operation. Finally, the superior mass accuracy of the Agilent 6210 TOF LC/MS allows unambiguous metabolite identification.

The metabolism of DEM has been extensively investigated and has been used as a model drug to distinguish between “poor” and “efficient” cytochrome P4502D6 metabolizers[5]. In addition, DEM metabolism is also extensively used to assess the inhibition of Cyp2D6 pathways by novel chemical entities in the early drug development process[6]. Therefore, the metabolism of DEM represents an ideal target system to investigate the analytical capability of the nanospray HPLC-Chip/MS system for the early drug discovery phase.

During microsomal phase I and phase II metabolism (figure 1), DEM (m/z 272) is either demethylated to 3 MM (m/z 258) or converted to the isobar dextrorphan (DOR). The latter compound may be further metabolized to 3 OM (m/z 244) or directly glucuronidated to dextrorphan glucuronide (DORGlu, m/z 434). 3 OM is further metabolized by phase 2 metabolism to 3-hydroxymorphinan glucuronide (3 OMGlu, m/z 420).

In this Application Note, we demonstrate the performance of the Agilent 1200 Series nanospray HPLC-Chip/MS system and Agilent 6210 TOF LC/MS for automated, high resolution and high sensitivity analysis for early drug discovery. Using DEM metabolism as our model system, metabolites were analyzed from in vitro assays as well as from complex biological matrices such as plasma and urine.

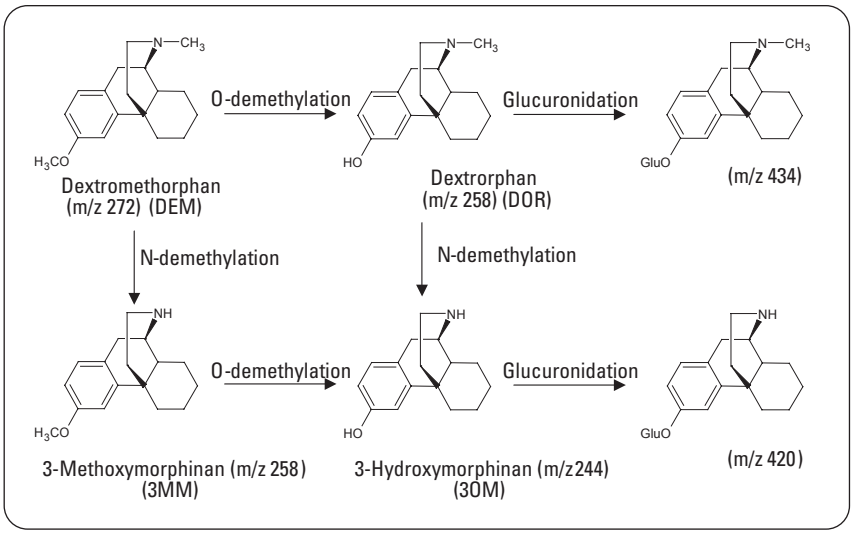

Figure 1
Metabolism of Dextromethorphan by Cytochrome P4502D6.

Experimental

In vitro incubation

The incubation mixtures for phase I and phase II in vitro metabolism consisted of an amount of S9 preparation equivalent to 1 mg protein, 0.1 µM substrate (dextromethorphan from a 50 µM stock solution in water), 1.2 mM NADP, 5 U isocitrate dehydrogenase, 5 mM isocitrate and 5 mM magnesium chloride, UDPGA (50 mM) in 0.05 M phosphate buffer (pH 7.4) made up to a total volume of 1000 µL. The sample was incubated with constant agitation for 60 minutes. With the exception of co-factors, controls contained the same components.

Sample preparation

Perchloric acid in a ratio of 1:18 of agent-to-sample was used for protein precipitation in plasma, urine and in vitro assay samples. After centrifugation at 13,000 rpm for 5 min, the supernatant was injected directly onto the chip enrichment column.

Agilent 1200 Series HPLC-Chip and Agilent 6210 TOF LC/MS system

The Agilent 1200 Series HPLC-Chip system includes the micro well-plate autosampler (thermostatted), nanopump, capillary sample loading pump, HPLC-Chip interface (chip cube), nanospray chip source and the Agilent 6210 TOF LC/MS. The entire system is controlled by the Agilent MassHunter software and allows the chip cube to automatically load and position the chip in the spray chamber. Leak-tight fluidic connections from the chip to nano pump, injector and waste are established by sandwiching the chip between the stator and rotor of a novel multi-port microvalve in the chip cube. The microvalve rotor switches between the sample loading and sample analysis position, directly on the chip's surface[7]. The chip configuration used included a 40 nL enrichment column and 43 x 0.75 mm ID analytical column, both packed with ZORBAX 80SB-C18 5 µm stationary phase. All components, including the nanospray emitter (10 µm ID), are integrated on the chip. Direct connection of the column outlet to the nanospray emitter ensures minimum post column peak dispersion, which results in superior peak shape and sensitivity.

Solvent A = 0.1 % formic acid in water and B = 0.1 % formic acid in acetonitrile. The capillary pump (sample loading) delivered an isocratic 2 % B mobile phase at 4 µL/min. Nano pump gradients (sample analysis) were 2 to 90 % B in 6 or 2.5 min with post run times at 2 % B of 6 min, respectively; flow rate was 0.6 µL/min. To ensure optimized nanoflow and fast gradient response, both capillary and nanopump flow were controlled utilizing nanoflow sensors and active split EMPV technology[8]. Following 1 µL sample injections, the enrichment column was flushed with 4 µL mobile phase (capillary pump). Upon switching to the analysis position, the gradient immediately started. The drying gas temperature was 300 °C, the flow was 3.5 L/min (2.5 L nitrogen and 1 L of filtered air). Data acquisition occurred in the positive ionization mode. Capillary voltage was –1900 V with an endplate offset of –500 V. Recorded mass range was 150-800 m/z, fragmentor voltage, skimmer voltage and octopole RF were set to 250 V, 60 V and 200 V, respectively. Reference mass correction was activated using reference masses of 445.12 and 519.14. The HPLC-Chip nanospray source is enclosed, eliminating chemical background contamination from the laboratory air, further enhancing TOF nanospray sensitivity. The Agilent MassHunter Workstation A02.01 software was used for system control and data analysis was performed with the Analyst QS 1.1.

Chemicals and reagents

Human plasma, male Sprague-Dawley rat S9 pooled liver preparations and commercially available standards of the dextromethorphan metabolism (3- methoxymorphinan [3 MM] and 3 hydroxymorphinan [3 OM]) were obtained from Sigma-Aldrich, as well as all standard chemicals used in this study. Blank urine for spiking experiments was obtained from a male human volunteer.

Results and discussion

Metabolite ID of in vitro assays at low analyte concentration on HPLC-Chip TOF

In standard assays, in vitro metabolism is usually conducted at concentrations of 10-30 uM in order to allow for the identification of major and minor metabolites. In our study we were able to conduct the experiment at a 100-fold lower concentration (100 nM). Sample handling was reduced to acidic precipitation by perchloric acid and a short centrifugation step. The resulting supernatant was injected directly without any fur-

ther treatment. The sample was loaded onto the enrichment column of the HPLC-Chip where pre-concentration and desalting takes place with a higher flow rate (4 µL/min). After switching the rotor of the nano valve, the sample is desorbed from the enrichment column and transferred to the analytical column by using nanoflow rate gradients.

An in vitro incubation of dextromethorphan with S9 liver microsomes was analyzed with two different gradients (2.5 min and 6 min gradient length). The typical elution profile for the fast gradient of dextromethorphan metabolism is shown in figure 2 (total run time 9.5 min). All known metabolites, including glucuronidated phase 2 metabolites, were identified even when using steep gradients and at low concentration (100 nM assay). The isobars DOR and 3MM were clearly baseline separated with both gradients allowing for the identification of metabolites with identical or similar m/z values. Outstanding chip chromatographic performance is demonstrated by narrow and highly symmetrical peaks (figure 3). For the fast gradient separation of the in vitro incubation mixture, the average peak width of phase 1 metabolites at half height was only 4 s.

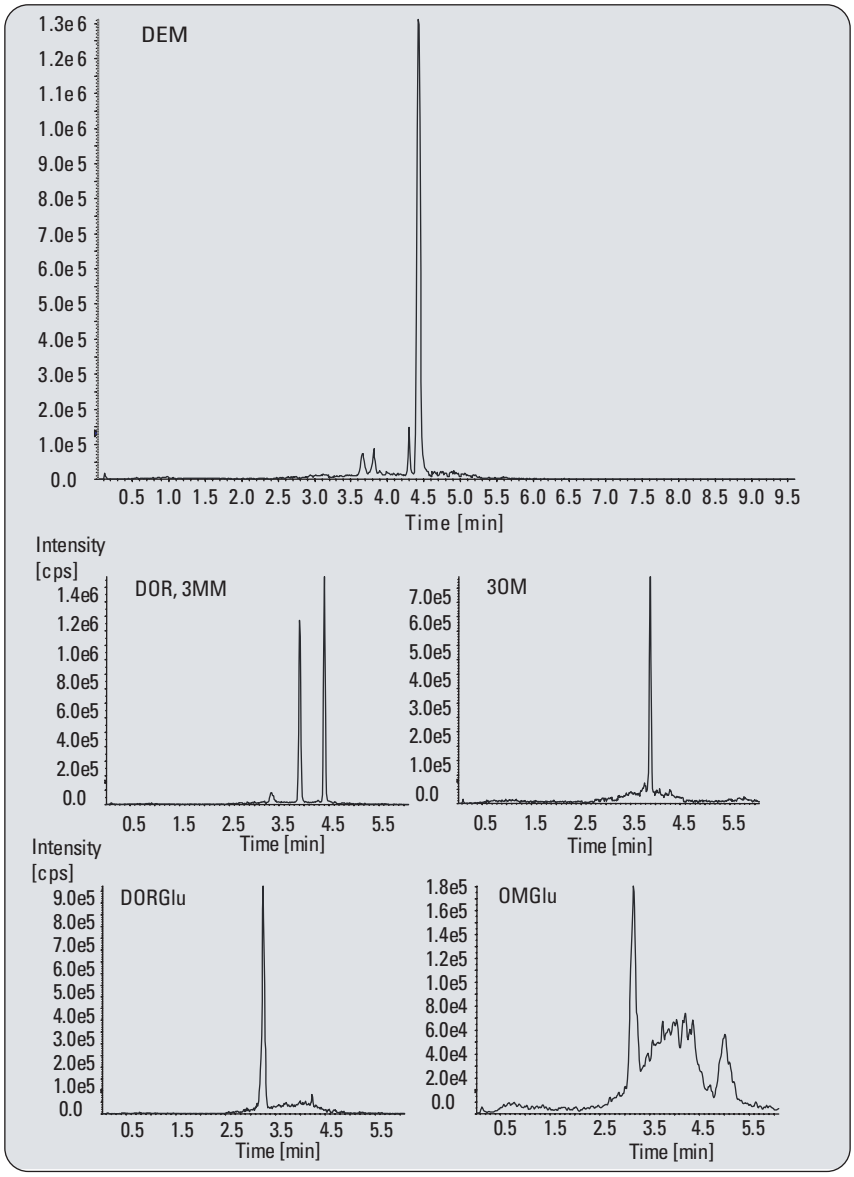

Figure 2
EICs for HPLC-Chip-MS (TOF) analysis of DEM metabolites obtained from an 100 nM *in-vitro* assay. Concentration on column 100 fmol. Gradient length 2.5 min, total run time 9.5 min.

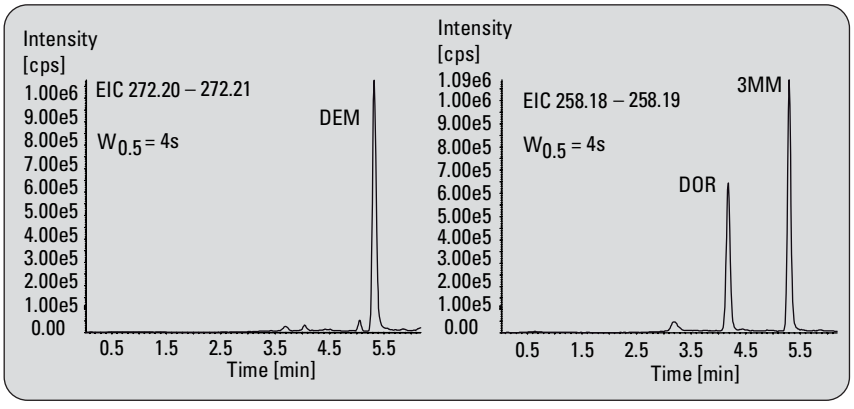

Figure 3
Peak width for DEM, DOR and 3MM in HPLC-Chip-MS (TOF) when using a 6 min gradient.

Retention time reproducibility, mass measurement and peak area

An essential parameter for identity confirmation of isobaric metabolites is retention time (RT) reproducibility. In our study , the RT reproducibility was better than 0.4 % RSD for phase 1 metabolites from dextromethorphan when using the 6 min gradient, and only the hydrophilic early eluting glucuronic acid metabolites exhibited RSDs above this value (table 1). The precision of retention times is demonstrated by an overlay of 10 consecutive EICs for the isobaric 258 m/z analytes (figure 4). Even for fast gradients (2.5 min), the reproducibility of retention time was still remarkable with relative standard deviations of less than 1.7 % for all metabolites (data not shown). This reflects the accuracy of the nanoflow pump active split EMPV for precise and robust fast gradient performance at nano flow rates (600 nL/min).

Mass precision for phase 1 metabolites was always better than 2 ppm, and for the glucuronidated phase 2 analytes it was always better than 3 ppm (table 1). Peak area reproducibility values were achieved between 1.7 % and 10.4 % RSD. Both results demonstrate the excellent performance of the Agilent 6210 TOF LC/MS system.

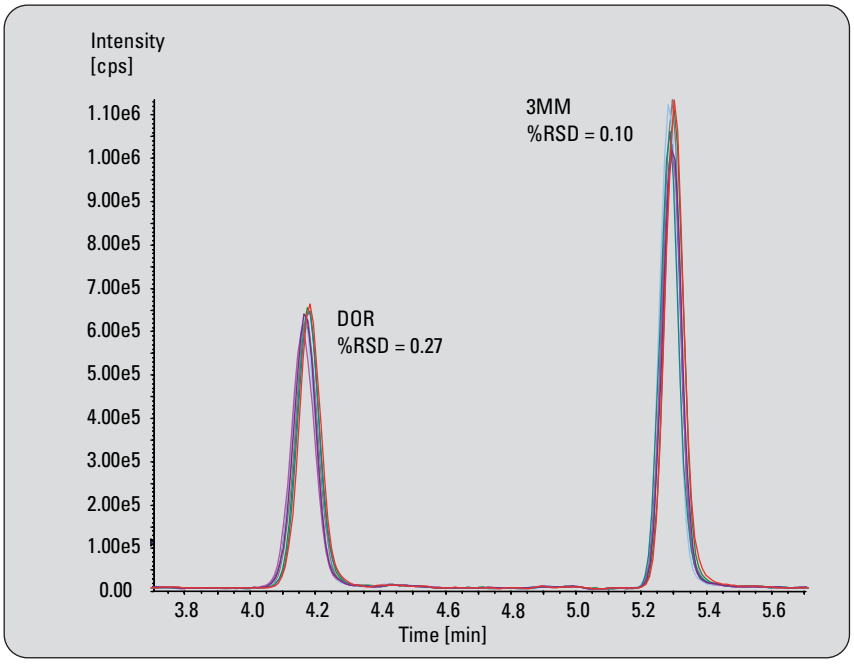

Figure 4
Overlay of EICs (m/z 258) for DOR and 3MM from 10 consecutive of the 100nM *in-vitro* metabolism assay.

Compound m/z [Da]	Average RT [min]	RSD RT [%] n= 10	Average precision of m/z in ppm (n=5)	Reproducibility of peak area %RSD (n=5)
Dextrorphan (DOR) 258.1857	4.17	0.27	1.70	2.10
3-Methoxymorphinan (3MM) 258.1857	5.30	0.10	2.00	3.40
3-Hydroxymorphinan (3OM) 244.1701	4.14	0.37	1.80	3.90
Dextromethorphan (DEM) 272.2017	5.31	0.16	1.70	1.70
Dextrorphan-glucuronide 434.2178	2.98	1.18	2.70	5.40
3-Hydroxymorphinan-glucuronide 420.2022	2.88	1.29	3.00	10.40

Table 1
Reproducibility of retention times, mass precision and peak area for a 6 min gradient applied to the separation of DEM metabolites on HPLC-chip/TOF.

Highly sensitive detection of DEM metabolites in biological matrices using HPLC-Chip/TOF

DEM and its commercially available metabolites 3MM and 3OM were dissolved in distilled water containing 0.1 % formic acid. A dilution series was made-up to produce concentrations ranging from 0.01 to 250 pmol/mL. A volume of 1 µL was injected three times for each solution and an analysis was performed using the six minute gradient. Peak areas were obtained from EIC's and the averages of replicate injections were calculated.

Figure 5A shows a plot of peak area versus sample concentration. System linearity ranges from 0.1 to 10 pmol/mL for DEM and 3MM, and up to 25 pmol/mL for 3OM. The given R2-factors exhibit good correlation to the linear regression lines. Figure 5B represents a zoom of the lower concentrations (0.1 to 2.5 nM). The limit of detection (LOD) was determined at 0.1 pmol/mL. In other words, using the HPLC-Chip/TOF technique we were able to detect as low as 100 attmol of DEM, 3MM and 3OM on column.

Since the challenge of metabolite detection and identification is, in general, to detect low concentrations of drug metabolites in a highly complex matrix background, serum and urine were spiked with different concentrations of DEM, 3MM and 3OM and subjected to analysis by the HPLC-Chip/TOF method. The serum is challenging due to the high protein concentration, which could easily cause blocking of microseparation devices such as the HPLC-Chip. To eliminate this problem, protein precipitation was performed as described in the experimental section. Within the concentration range investigated (0.5 to 10 pmol/mL), excellent linearity was achieved with R2-factors closely matching the regression line (figure 6). RSD's for peak area were calculated using six

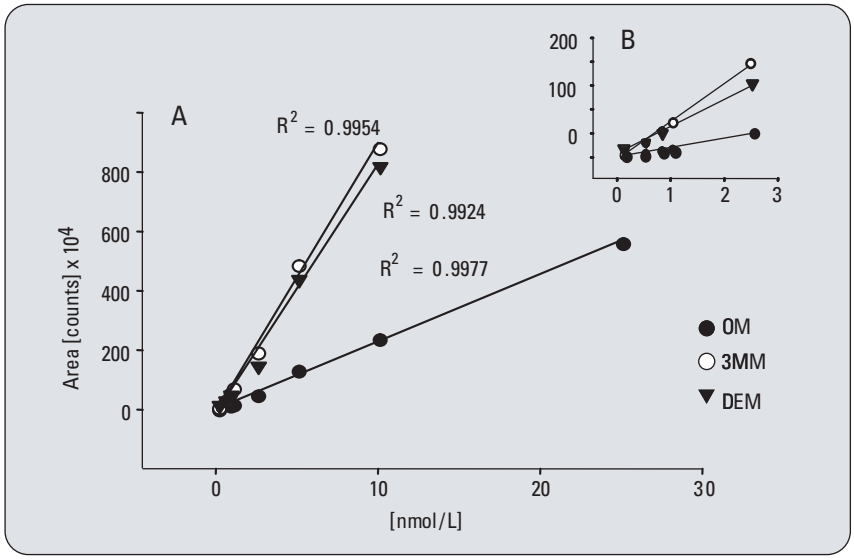

Figure 5
Linearity plot of peak area versus concentration of DEM, OM, 3MM in water. A: 0-25nM, B: 0-2.5 nM.

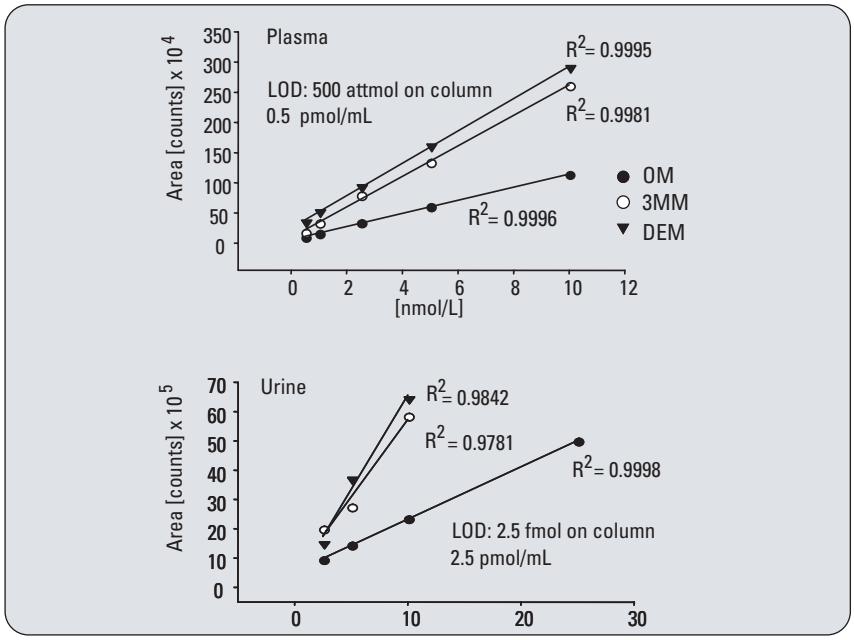

Figure 6
Linearity plot of peak area versus concentration of DEM, OM, 3MM spiked in human serum and urine.

injections per concentration. In all cases the RSD for peak area was below 10 %. The limit of detection in human plasma was 500 attmol on column (0.5 pmol/mL).

Drug excretion often happens via the renal pathway. Measurement of metabolites in urine requires minimal clinical intervention and sampling is easier than with blood derivatives. Urine, however, contains high concentrations of salts and small molecules, and the matrix background is therefore challenging for MS detection. In this study, human urine was spiked with DEM and associated metabolites. The limit of detection for the HPLC-Chip/TOF system in urinary samples was measured at 2.5 fmol on column. Again, good linearity could be obtained up to at least 10 pmol/mL for all metabolites (figure 6).

For both biofluids, the HPLC-Chip/TOF system proved to be a robust, reliable and sensitive technique. No failure due to column blockage was observed during the study and the ease of use, robustness and reliability of the nanospray HPLC-Chip makes this technology an ideal tool for routine, automated day-to-day use.

Conclusion

Ease of use, robustness and reliability of the automated nanospray HPLC-Chip system, as well as high sensitivity and mass accuracy of the Agilent 6210 TOF mass spectrometer make this system an ideal tool for unattended routine use for early drug discovery assays.

Our performance investigation using dextromethorphan metabolism validates the ability of this platform to perform metabolite ID for in-vitro assays at the 100 nM level. This is 100-fold lower than what is routinely used with standard LC/MS systems. Retention time reproducibility is excellent with %RSD of less than 0.4% for phase 1 metabolites. Average mass precision over repeated runs is better than 2 ppm. High sensitivity was achieved with standards (LOD 100 attmol on column) and also in complex biological matrices such as serum (LOD 500 attmols on column) and urine (LOD 2.5 fmol on column). Protein precipitation with perchloric acid is simple and very efficient. This resulted not only in a large number of injected “real-life” samples without any performance loss but also avoided time-consuming evaporation and reconstitution steps prior to injection. This increased throughput and decreased cost per sample.

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