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RESEARCH ARTICLE

Driving efficiency in a high-throughput metabolic stability assay through a generic high-resolution accurate mass method and automated data mining

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ABSTRACT

Improving analytical throughput is the focus of many quantitative workflows being developed for early drug discovery. For drug candidate screening, it is common practice to use ultra-high performance liquid chromatography (U-HPLC) coupled with triple quadrupole mass spectrometry. This approach certainly results in short analytical run time; however, in assessing the true throughput, all aspects of the workflow needs to be considered, including instrument optimization and the necessity to re-run samples when information is missed. Here we describe a high-throughput metabolic stability assay with a simplified instrument set-up which significantly improves the overall assay efficiency. In addition, as the data is acquired in a non-biased manner, high information content of both the parent compound and metabolites is gathered at the same time to facilitate the decision of which compounds to proceed through the drug discovery pipeline.

KEYWORDS metabolic stability, high-resolution mass spectrometry, accurate mass, ultra-high performance liquid chromatography

INTRODUCTION

A recent trend in the drug discovery and development process is to shift the starting point of drug metabolism and pharmacokinetic (DMPK) studies from the late developmental stage to the discovery stage so that a critical decision in the development chain can be made as early as possible to save tremendous amount of time and cost. DMPK study for early drug discovery involves various automated *in vitro* assays such as metabolic stability, cytochrome P450 (CYP) inhibition and induction, and cell permeability study (Di et al., 2009). Metabolic stability is regarded as the most important frontline screen as it plays an important role in determining the overall pharmacokinetic properties of a new drug substance (Tolonen et al., 2009). The metabolic properties of parent drugs as well as their relevant metabolites generated during microsomal incubation provide essential clues to pinpoint promising drug candidates and address their potential issues. As hundreds of discrete compounds need to be screened each week, improving the throughput of metabolic stability assays in a cost-effective manner is highly desired in early DMPK projects (Chu and Nomeir, 2006; Tolonen et al., 2009).

To acquire the most time-efficient results, researchers choose to apply fast generic liquid chromatography-mass spectrometry (LC-MS) approaches to assay large collections of drug candidates. Ultra-high performance liquid chromatography (U-HPLC) has gained high popularity in drug discovery arena because of its superior speed, sensitivity and separation efficiency compared with regular HPLC (Pedraglio et al., 2007). The run time can be shortened to 2–5 min/sample for quantitative bioanalysis or semi-quantitative metabolic stability assays (Pedraglio et al., 2007). U-HPLC is typically connected with a triple

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quadrupole (QqQ) mass spectrometer (MS) or hybrid triple-quadrupole linear ion trap (QqQ_{LIT}) MS so as to monitor the quantitative changes of parent drug concentrations by selected-reaction-monitoring (SRM) analysis. Despite of being a specific and sensitive approach for bioanalysis, SRM approach is laborious and time-consuming because SRM parameters have to be optimized for each compound in the queue and there are hundreds of them that need to be assayed on a weekly basis in a regular DMPK laboratory (Chu and Nomeir, 2006; Tolonen et al., 2009). An additional drawback of QqQ-based targeted analysis is that no data on the drug metabolites can be acquired unless a limited number of known metabolites were predicted prior to the experiment. As a result, the important information of these biotransformation products, particularly unknown metabolites, were lost in most QqQ-based workflows.

To achieve simultaneous analysis of both the parent drugs and their major metabolites, a variety of approaches using different mass analyzers have been explored, in support of either *in vitro* metabolic stability or *in vivo* pharmacokinetic (PK) studies. Several laboratories have established 'anticipated SRM channels' based on the fragmentation patterns of parent compounds in order to monitor putatively predicted metabolites alongside the parents using quadruple-based instruments (Poon et al., 1999; Tiller and Romanyshyn, 2002; Gao et al., 2007). The unit mass accuracy of quadruple analyzers, however, often leads to ambiguous metabolite identification in complex biological matrixes where many isobaric substances are present. To raise the confidence of identifying putative metabolites, Shou et al. combined anticipated metabolite SRMs with SRM-triggered full MS/MS scans in QqQ_{LIT} (Li et al., 2005; Shou et al., 2005). However, this innovative approach is limited by the relatively low sensitivity of the linear ion trap integrated in this instrument. One to three major metabolites were found from microsomal incubation samples of five test compounds, and full MS/MS spectra show weak signals of product ions. Notably, no metabolites were detected from midazolam using anticipated SRM method (Shou et al., 2005), because the fragmentation behavior of these metabolic products is radically different from the parent drug, making SRM prediction inaccurate (Shou et al., 2005). Furthermore, any unknown metabolites beyond prior knowledge are missed in this type of assay. Therefore, all the current SRM-based approaches fail to provide a universal solution to both drug and metabolite screening.

High resolution mass spectrometry (HRMS) has emerged as an attractive approach for early-phase DMPK applications (Tolonen et al., 2009). This approach relies on high resolution and high mass accuracy in full MS scans to derive or confirm the empirical formula of analytes within a wide mass range. One type of HRMS, time-of-flight (TOF) or the hybrid Q-TOF system, has been widely exploited for metabolite identification and quantitation. A few successful applications of TOF instruments coupled to regular HPLC or U-HPLC have shown unique strengths over the SRM-based methods for metabolite screening and stability assays (Castro-Perez et al., 2005; O'Connor et al., 2006; Nägele and Fandino, 2007; Tiller et al., 2008). However, most TOF instruments are cost-prohibitive and require frequent calibrations and continuous infusion of a reference mass to maintain sufficient mass accuracy.

The hybrid linear ion trap (LTQ)-Orbitrap MS is another type of HRMS which provides exceptional mass resolution (up to 100,000 FWHM) while maintaining good sensitivity (Hu et al., 2005). Excellent mass accuracy (<5 ppm) is sustained for days without re-calibration or infusing a reference mass. A rapidly growing number of qualitative studies using LTQ-Orbitrap have been reported for structural characterization of drug metabolites (Li et al., 2007; Lim et al., 2007; Ruan et al., 2008; Li et al., 2009). More importantly, the quantitative capability of Orbitrap was investigated on its full MS scans at 15 K mass resolution by comparison with SRM scans using QqQ (API 4000). The authors reported equivalent performance between the two platforms in regard to sensitivity and dynamic range for the analysis of 15 drug candidates (Zhang et al., 2009).

A lately commercialized HRMS, Exactive, is built on Orbitrap technology and delivers superior resolution and mass accuracy to guarantee confident discrimination of isobaric compounds in complex samples. It is anticipated to be more accessible than LTQ-Orbitrap for routine DMPK screening projects due to significantly reduced size and cost. However, it is still under debate whether the scan speed of Orbitrap instruments is sufficient to quantify sharp peaks eluted by U-HPLC. It has been a concern that the Orbitrap may suffer from slow data acquisition, thus lowering detection sensitivity, compared with TOF instruments (Rousu et al., 2010). In the presents study we conducted a comprehensive examination of the speed, sensitivity and selectivity of Exactive in conjunction with U-HPLC for metabolic stability assays. A rapid U-HPLC method with a 2-min gradient was applied to meet the needs of throughput in routine DMPK practice, and Exactive functioned at 25–50 K mass resolution to warrant high selectivity for compound identification in complex biological matrices. Qualitative and quantitative information of both the parent drugs and all major metabolites were obtained simultaneously, whereas relative quantitation of the parent drugs were compared side by side between HRMS and SRM approach.

RESULTS AND DISCUSSION

Performance of Exactive™ MS coupled to a fast generic U-HPLC method

The performance of the Exactive™ mass spectrometer operating at high resolution was examined to determine whether the instrument provides sufficient scan speed to be

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compatible with the typical width of U-HPLC peaks (2–4 s). The microsomal incubation samples were separated on U-HPLC using a 2-min gradient at a high flow rate (0.7 mL/min). The extracted ion chromatograms (XICs) of a test compound propanol measured at 10, 25 or 50 K mass resolution in the 30 min-incubation sample are shown in Fig. 1 (left panel). Although increased resolution leads to reduced scan speed, there are still 6 to 8 data points acquired on the parent ion even at 50 K resolution (Fig. 1C), which meets the general requirement for semi-quantitation. Excellent mass accuracy (mass error < 1.5 ppm), only by one-time external calibration, was easily maintained across the elution profile of this parent compound (Fig. 1; right panel). We noticed, however, in the cases of some other test compounds, interferences of very close m/z values may be present and affected confident assignment of the compound of interest. An example is shown in Fig. 2A, where a peak at 326.0859 m/z was present in the spectrum acquired at 10 K mass resolution on midazolam stability sample. This peak is very likely to be misidentified as the parent drug with a theoretical m/z of 326.0855, but it turned out to be a product merged from several interfering peaks which cannot be resolved at a

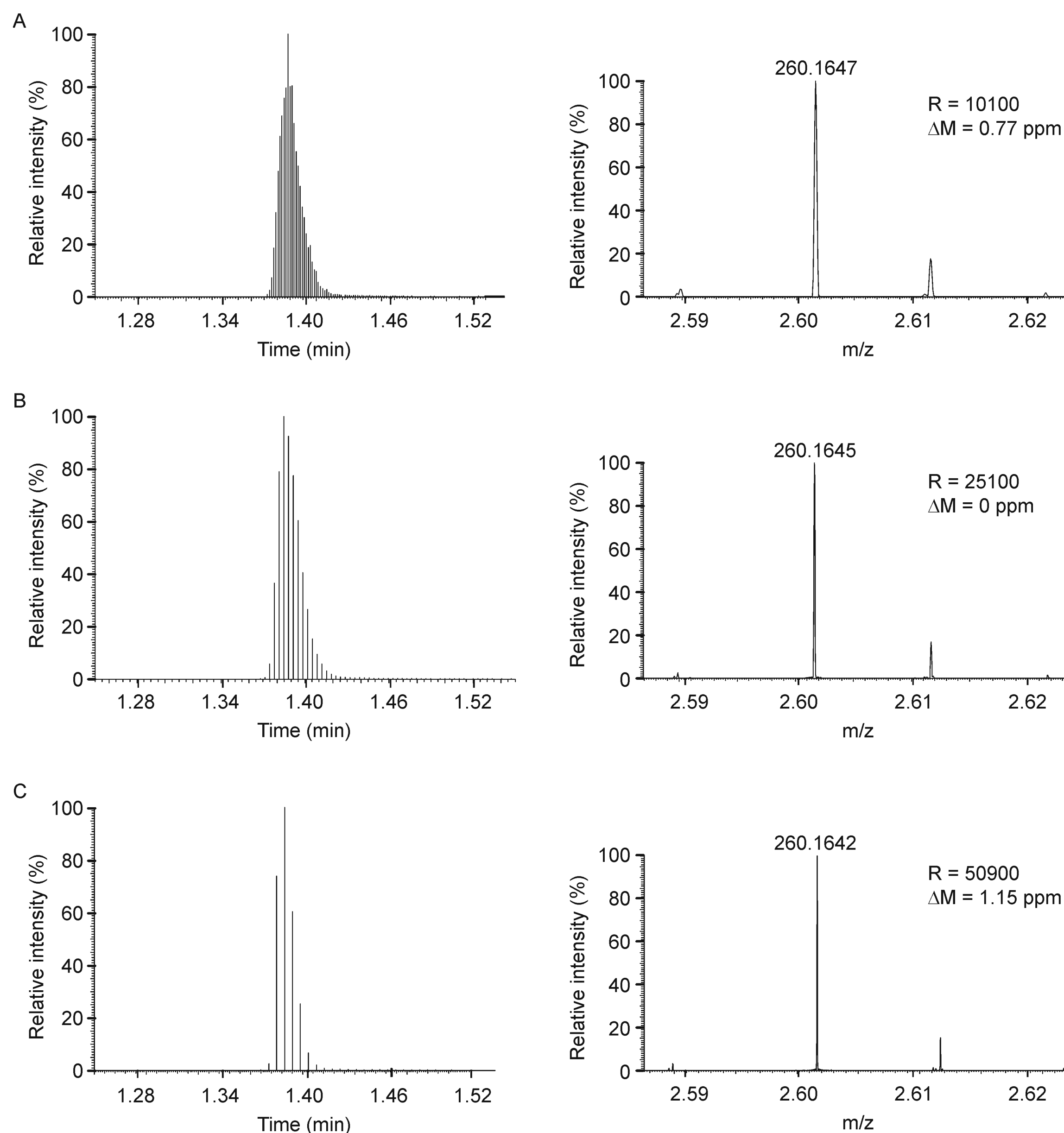

Figure 1. Extracted ion chromatograms of the parent compound propranolol based on its accurate mass with 5 ppm mass tolerance (left) and the mass spectra of its monoisotopic peak at different resolution (right). Individual scans represented by sticks are correlated with defined mass resolution: (A) 10 K FWHM, (B) 25 K FWHM, (C) 50 K FWHM. Actual resolution and mass accuracy are annotated in the spectrum.

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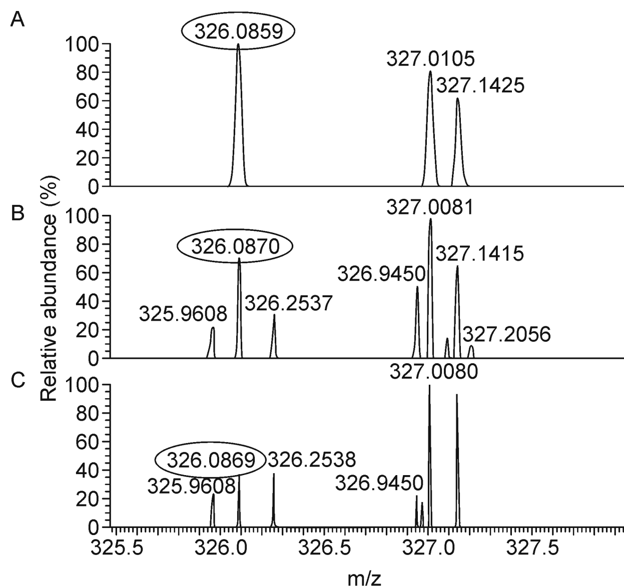

Figure 2. Zoomed-in mass spectrum of interferences present in the midazolam 30 min-incubation sample, measured by HRMS at 10 K (A), 25 K (B), or 50 K (C) resolution. The peak of the interference with a closest m/z to the parent drug (theoretical m/z = 326.0855) was highlighted in a circle in each spectrum, and it is only distinguishable and can be ruled out by 2 ppm mass window when the Exactive mass spectrometer scans at 25 K or higher resolution. HRMS, high resolution mass spectrometry.

relatively low resolution (Fig. 2A). The interferences are only distinguishable when scanning at higher mass resolution such as 25 K and 50 K (Fig. 2B and 2C). A mass window of 2 ppm for extracting parent ions measured at 25 K and 50 K resolution enables exclusion of interferences, thus preventing compound misidentification. Therefore, all the samples in our study were analyzed at 25 K or 50 K resolution in order to warrant higher reliability of detecting specific analytes in complex matrices.

Parent metabolic stability profiled by HRMS is consistent with SRM-based results

Seven commercial drugs of different pharmacokinetic properties were selected to assess the quantitative performance of the Exactive mass spectrometer in full-scan mode for metabolic stability assays. Quantitation of the parent drug at different time points was relative to time point zero (T0), which was defined to be a starting point when no metabolism occurred and thus 100% parent remained. This type of quantitative assay is typically conducted on a triple quadrupole instrument that monitors the disappearance of parent compounds via a specific SRM transition. To compare the quantitative results from this standard method with those from the HRMS approach, each microsomal stability sample was analyzed using optimized SRM on individual parents (Table 1). Unlike SRM method, identification of the parent compounds using HRMS does not require any compound-specific method development, and the assay was performed in a generic and efficient manner. One U-HPLC-MS method with generic settings was applied to all sample analyses, and the data was subsequently processed by extracting the m/z values of specific parent ions around their theoretical monoisotopic m/z with a 5 ppm mass window. The theoretical and experimentally measured m/z values for detecting different parent drugs are summarized in Table 1. The actual mass accuracy was found to be better than 1.5 ppm for all parent drugs.

Quantitative change of the parent drug over the time course of incubation is represented by the peak intensity of XICs, assuming a linear response over the concentration range from nmol/L to μmol/L for the incubation samples. An internal standard signal was used to normalize the XIC peak response. An example is illustrated in Fig. 3 for the relative quantitation of diclofenac detected at 25 K or 50 K mass resolution. Metabolic stability of this drug can be plotted based on normalized XIC responses at specific time points relative to T0. As expected, the stability plots of diclofenac obtained using different approaches are in close agreement (Fig. 4).

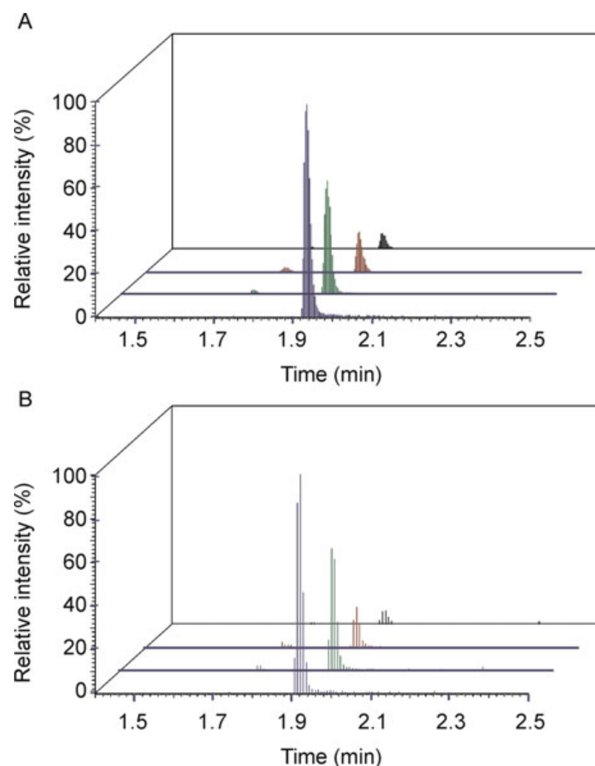

Figure 3. Disappearance of the parent compound diclofenac over the course of incubation. The remaining percentage of diclofenac relative to T0 is measured by HRMS at 25 K (A) or 50 K (B) mass resolution. HRMS, high resolution mass spectrometry.

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Table 1 Identification of specific parent drugs using SRM or HRMS approach

Parent drug	SRM[a]	Theoretical *m/z*	Measured *m/z*	Accuracy (ppm)[b]
Warfarin	309.1/251.1	309.1121	309.1118	1.0
Propranolol	260.1/116.1	260.1645	260.1642	1.2
Dextromethorphan	272.2/171.1	272.2009	272.2006	1.1
Bufuralol	262.2/188.1	262.1802	262.1798	1.5
Testosterone	289.2/109.0	289.2162	289.2159	1.0
Diclofenac	296.0/214.0	296.0240	296.0237	1.0
Midazolam	326.1/291.2	326.0855	326.0855	0.0
Glyburide (IS)	494.1/369.1	494.1511	494.1508	0.6

[a] Specific SRM transitions for detecting individual parent drugs are described.
[b] Mass accuracy refers to the mass deviation of the measured m/z value by HRMS approach from its theoretical m/z value. SRM, selected-reaction-monitoring; HRMS, high resolution mass spectrometry.

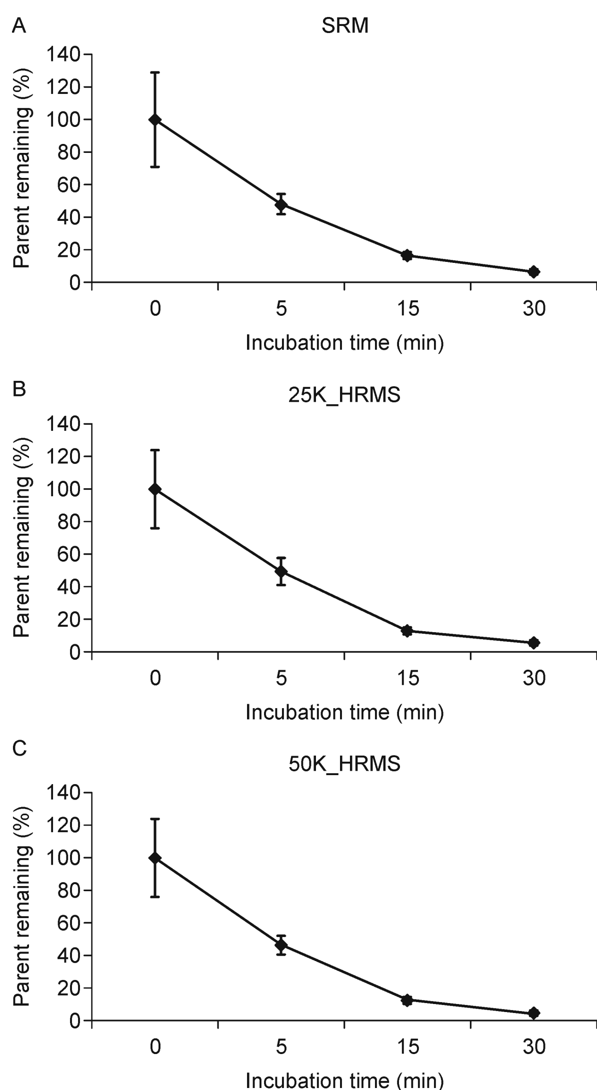

Figure 4. Metabolic stability plot of the parent drug diclofenac derived from SRM or HRMS data. Error bars represent standard deviation of the remaining percentage of the parent from three experimental replicates. SRM, selected-reaction-monitoring; HRMS, high resolution mass spectrometry.

The *in vitro* half-life of each drug ($t_{1/2}$) derived from the microsomal incubation data reflects the metabolic clearance *in vivo*, which is one of the major determinants of *in vivo* pharmacokinetic properties of the drug, and therefore provides valuable information for compound selection and optimization at the drug discovery phase. As shown in Table 2, the values of $t_{1/2}$ obtained for each drug using the HRMS-based method fall into the same range as those obtained from the traditional SRM-based method, no matter whether the drug possesses high, moderate, or low stability. Notably, scanning at 50 K resolution using HRMS method does not significantly compromise $t_{1/2}$ measurement whereas it is expected to offer a better capability in excluding interfering species.

Temporal changes of major metabolites of parent drugs are simultaneously determined by HRMS

Because of the unbiased nature of HRMS method, information can be gathered not only on the parent compounds but also on a variety of major metabolites with little need of prior knowledge about the metabolic pathways involved. A list of the 50 most commonly observed biotransformations occurring in phase-I and phase-II metabolism were searched using Thermo Scientific MetQuest automated data mining software to find putative metabolites with mass deviation of less than 5 ppm from the predicted values. Quantitative information is obtained from the XIC peak responses of specific metabolites so that temporal changes of metabolite abundances are profiled in the same assay of parent drug stability. As shown in Fig. 5, a few representative metabolites converted from specific drugs were identified and their relative changes of abundance compared to T0 were determined using HRMS data. Different trends of quantitative changes of metabolites were observed, indicating it is not always the case that metabolites are produced to the maximal amount at the last time point. Therefore, the quantitative profiles of metabolite abundances provide essential clues about the metabolic properties of the parent compounds.

With the assistance of data mining software, we were able

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Table 2 Calculated *in vitro* half-life ($t_{1/2}$) of individual parent drugs based on quantitative data acquired using SRM or HRMS approach

Parent drug	$t_{1/2}$ (min)		
	By SRM	By HRAM (25 K)	By HRAM (50 K)
Warfarin	225.00	165.00	208.00
Propranolol	52.40	51.00	55.00
Dextromethorphan	46.00	43.60	41.00
Bufuralol	46.30	45.40	51.50
Testosterone	7.33	7.02	6.54
Diclofenac	7.53	7.13	6.73
Midazolam	3.37	3.19	3.03

Abbreviations are the same as in Table 1.

to easily identify major metabolites converted from each parent drug and quantified their relative changes at individual time points of incubation. A complete list of metabolites with their retention time, mass accuracy and peak time (referring to the time point of maximal abundance) is provided in supporting information (Table S1). A representative summary of metabolite identification and quantitation for the parent drug dextromethorphan is illustrated in Table 3. Here several metabolites from the parent compound were identified based on accurate mass measurement and relative quantitation was performed simultaneously to find out the peak time for each metabolite. The mass deviation for detection of all the metabolites is within 2 ppm. Another parameter in Table 3, % parent peak which refers to the percentage of peak response for individual metabolites relative to that for the parent compound, is determined by relative XIC responses. It should be noted that MS response is dependent on analyte ionization efficiency and thus not a strictly quantitative measure of actual abundances, the exact relative amount of individual metabolites *vs* the parent drug can be calculated with the % parent peak values listed in Table 3 calibrated by response factors to normalize MS signals of different metabolites.

MATERIALS AND METHODS

Chemicals and reagents

All seven commercial drugs, NADPH, UDPGA and internal standard glyburide were purchased from Sigma Aldrich. Methanol, water and

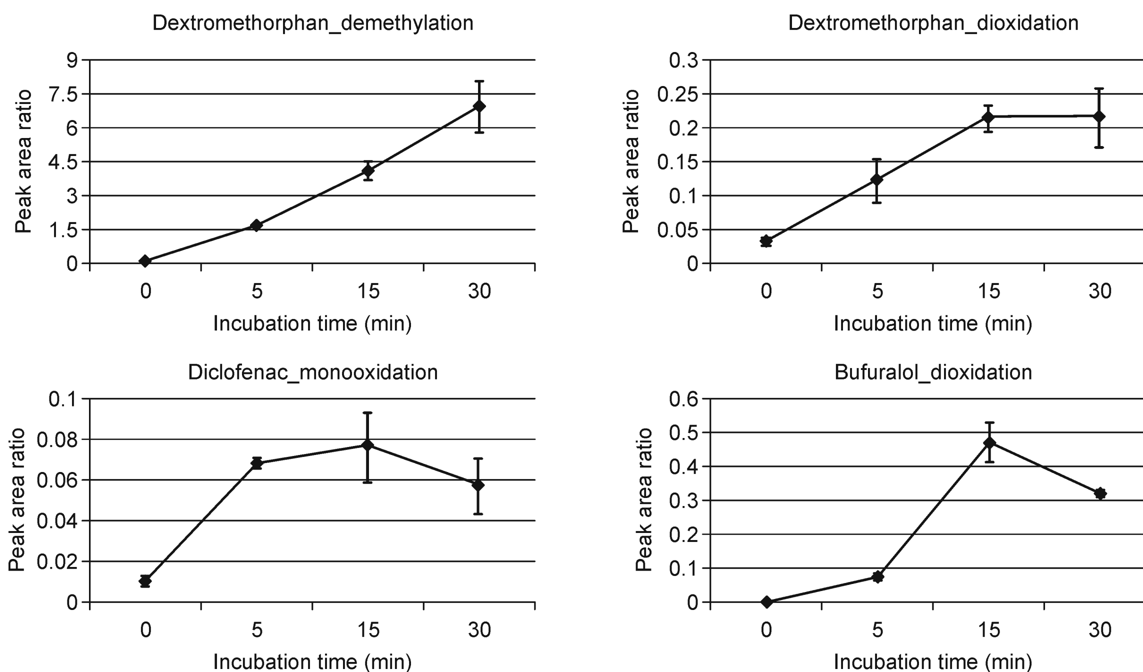

Figure 5. Quantitative changes of selected major metabolites transformed from specific parent drugs during incubation. Metabolites were identified and quantified using accurate mass measurement on an Exactive mass spectrometer at 25 K resolution. Error bars represent standard deviation of normalized peak responses for individual metabolites from three experimental replicates.

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Table 3 Major metabolites of dextromethorphan identified by HRMS approach and their quantitative information acquired simultaneously

Met ID	RT (min)	Putative modification	Formula	Mass accuracy (ppm)	Peak time	% Parent peak
M1	0.73	Glucuronidation (O, N, S) & demethylation	$C_{23}H_{31}NO_7$	1.2	T30	0.5
M2	1.00	Mono-oxidation & demethylation	$C_{17}H_{23}NO_2$	0.7	T30	0.3
M3	1.11	Hetero oxide reduction & hydrogenation	$C_{18}H_{27}N$	−1.9	T30	0.3
M4	1.11	Demethylation	$C_{17}H_{23}NO$	−0.9	T30	88.9
M5	1.15	2X-Demethylation	$C_{16}H_{21}NO$	0.2	T30	0.9
M6	1.27	Mono-oxidation	$C_{18}H_{25}NO_2$	0.8	T30	1.3
M7	1.37	Di-oxidation	$C_{18}H_{25}NO_3$	0.3	T15-T30	0.2
M8	1.46	Mono-oxidation	$C_{18}H_{25}NO_2$	0.2	T30	5.1
M9	1.46	Demethylation	$C_{17}H_{23}NO$	0.2	T30	4.5
M10	1.63	Glucuronidation (O, N, S)	$C_{24}H_{33}NO_7$	−0.2	T15	0.4

Retention time (RT) of the parent drug is 1.42 min. Peak time indicates the time point at which the maximal amount of that particular metabolite was observed. % Parent peak refers to the percentage of peak response for individual metabolites relative to that for the parent compound. HRMS, high resolution mass spectrometry.

formic acid, all of LC-MS grade, were from Thermo Fisher Scientific. Human liver microsomes were from BD Biosciences Discovery Labware (Woburn, MA, USA).

Microsomal incubations

Incubations of human liver microsomes were conducted at 37°C with 0.25 mg/mL of microsomal proteins in 0.1 mol/L phosphate buffer, at a compound concentration of 1 μmol/L, in the presence of 1 mmol/L nicotinamide adenine dinucleotide phosphate (NADPH) and 1 mmol/L uridine 5-diphosphoglucuronic acid (UDPGA) as cofactors and 2 mmol/L $MgCl_2$ in a total volume of 500 μL. Aliquots were taken at multiple time points (5, 10, 15, and 30 min) and quenched with 2 volumes of cold acetonitrile containing glyburide (2 nmol/L) as internal standard. Samples were centrifuged for 15 min at 3000 *g* and the supernatant was transferred to 96-well plates. Each sample was diluted with equal amount of ddH_2O before LC-MS analysis.

Ultra-high performance liquid chromatography

Sample injection from 96-well plates was performed with the CTC Analytics PAL autosampler (LEAP Technologies Inc., Carrboro, NC, USA). The injection volume was 3 μL for bufuralol, propranolol, dextromethorphan, midazolam and warfarin; 10 μL for testosterone and diclofenac. Separation of the analytes was performed on Accela U-HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a C_{18} column of 1.7 μm particle size, 50-mm length and 2.1-mm i.d (Acquity UPLC BEH C18, Waters). The chromatography was developed using 5% methanol in water, 0.1% formic acid (mobile phase A) and 95% methanol in water, 0.1% formic acid (mobile phase B) at a flow rate of 0.7 mL/min. A linear gradient progressed from 10% B to 95% B in 1.5 min, and then maintained at 95% B for 0.5 min. The equilibration time was 0.5 min.

High resolution mass spectrometry

U-HPLC was connected to the Exactive™ mass spectrometer (Thermo Fisher Scientific, USA) equipped with a heated-ESI source, operating in positive ion mode. The instrument was calibrated by infusion of a standard mixture of caffeine, MRFA tetrapeptide, and Ultramark. The initial mass calibration was applied throughout the sample analysis, and no internal reference was injected. Standard ESI source conditions compatible with the flow rate were applied: spray voltage 3500 V, heater temperature 450°C, capillary temperature 260°C, sheath gas 45, and auxiliary gas 10. Mass scan range is 100–1000 m/z. Mass resolution was set at 10 K, 25 K or 50 K with corresponding scan speed of 10 Hz, 4 Hz, or 2Hz.

Triple quadrupole mass spectrometry

The same U-HPLC was connected to a high-end QqQ instrument Vantage™ (Thermo Fisher Scientific, USA) equipped with a heated-ESI source, operating in positive ion mode. This platform was developed for the quantitative analysis of parent compounds alone. Precursor and product ions of each test compound were obtained by infusing 1 μg/mL solutions of the standard substance at 5–10 μL/min to a tee-connector between the outlet of the U-HPLC flow (0.7 mL/min) and the ESI source. S-lens and collision energy were optimized for each SRM transition. The dwell time for SRM channels monitoring either the parent or the internal standard was set at 40 ms.

Data analysis for compound identification and relative quantitation

Qual Browser in Xcalibur (Thermo Fisher Scientific, USA) was used for viewing and processing of raw data files. Parent compounds were identified based on their accurate mass-to-charge (m/z) values with deviation of less than 5 ppm from the theoretical ones. Major

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metabolites that are derived from a specific parent drug and present in 30 min-incubation samples were identified using MetQuest (Thermo Fisher Scientific, USA). A list of 50 most commonly observed biotransformations in phase-I and phase-II metabolism were searched in MetQuest in order to find putative metabolites with mass deviation of less than 5 ppm from the predicted values. Among the metabolites identified by Metworks, only those showing chromatograms with signal-to-noise ratios above 80 were retained to ensure good LC peaks for relative quantitation.

MetQuest was also used for relative quantitation of parent drugs and metabolites over the time course of incubation. All compounds were identified by their theoretical mass with a tolerance of 5 ppm, and the XIC responses were normalized using the internal standard. The normalized XIC responses of individual compounds were then used to generate metabolic stability plots. Half-life of each drug was automatically calculated using integrated parameters in MetQuest for human liver microsomes.

CONCLUSIONS

A highly efficient quantitative/qualitative metabolic stability assay was established using HRMS that shows significant advantages over triple quadrupole-based methods with regards to overall throughput, data quality, and information content. The workflow integrating fast-gradient U-HPLC with the Exactive MS scanning at high resolution followed by automated post-acquisition data processing provides specific advantages over traditional methods including: (1) analytical time is significantly reduced with no need for MS/MS optimization; (2) information content is expanded by monitoring both the parent and various possible metabolites; (3) post-run data re-interrogation allows users to identify and quantify additional components as they become of interest in later research stage.

As a perspective, the Exactive system is able to perform all-ion-fragmentation so that structural details of metabolites can be acquired at high confidence to pinpoint biotransformation sites of drug candidates and distinguishing isobaric components. By combing full-mass and all-ion-fragmentation scans, we envision HRMS approach based on the Exactive system would tremendously increase the throughput of various DMPK assays to facilitate the decision of which compounds to proceed through the pipeline.

ABBREVIATIONS

DMPK, drug metabolism and pharmacokinetic; HRMS, high resolution mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; PK, pharmacokinetic; SRM, selected-reaction-monitoring; TOF, time-of-flight; U-HPLC, ultra-high performance liquid chromatography

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