ORIGINAL PAPER

High-resolution mass spectrometry for integrated qualitative and quantitative analysis of pharmaceuticals in biological matrices

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Abstract Quantitative and qualitative high-resolution (HR) dependent and independent acquisition schemes on a QqTOF MS (with resolving power 20,000–40,000) were investigated for the analysis of pharmaceutical compounds in biological fluids. High-resolution selected reaction monitoring (HR-SRM) was found to be linear over three orders of magnitude for quantitative analysis of paracetamol in human plasma, offering a real alternative to triple quadrupole LC–SRM/MS. Metabolic stability of talinolol in microsomes was characterized by use of three different acquisition schemes: (i) information-dependent acquisition (IDA) with a TOF MS experiment as survey scan and product-ion scan as dependent scan; (ii) MS^{ALL} by collecting TOF mass spectra with and without fragmentation by alternating the collision energy of the collision cell between a low (i.e., 10 eV) and high setting (i.e., 40 eV); and (iii) a novel independent acquisition mode referred to as "sequential window acquisition of all theoretical fragment-ion spectra" (SWATH) or "global precursor ions scan mode" (GPS) in which sequential precursor ions windows (typically 20 u) are used to collect the same spectrum precursor and fragment ions using a collision energy range. SWATH or GPS was found to be superior to IDA or MS^{ALL} in combination with UHPLC for qualitative analysis but requires a rapidly acquiring mass spectrometer. Finally, the GPS concept was used for QUAL/QUAN analysis (i.e. integration of qualitative and quantitative analysis) of bosentan and its metabolites in urine over a concentration range from 5 to 2,500 ng mL^{-1}.

Introduction

In the drug-discovery process, the two major applications of liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) are quantitative analysis of the parent drug and identification of the metabolic biotransformation products. Triple quadrupoles (QqQ), the instruments most often used in the selected reaction monitoring mode for sensitive, accurate, and precise quantification [1–3], also enable qualitative analysis with information acquisition schemes using full scan, precursor ion scan, selected reaction monitoring, or neutral loss scan as survey experiments and product ion scan as dependent experiments. Despite the high selectivity of QqQ, the major drawback is the moderate sensitivity for qualitative analysis. Therefore, in most cases, the samples are analyzed twice: first on a QqQ for quantification and second on an ion trap (IT) or a quadrupole-time-of-flight (QqTOF) for qualitative analysis. Integration of qualitative analysis and quantitative analysis (QUAL/QUAN) is, then, of great interest for drug discovery in which sample throughput and/or sample volume is critical and more high-quality data are needed to improve the drug-selection process. These schemes have already been explored on various mass spectrometers, including triple quadrupoles, triple quadrupole linear ion traps, and quadrupole-time-of-flight, but have not gained wide acceptance mostly because of performance limitations of the MS instrumentation and workflow complexity [4–8]. With most

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atmospheric pressure ionization techniques, because of the variable analyte response factor and matrix effects [9], absolute quantification is mainly performed using reference compounds and relative quantification remains possible to follow the pharmacokinetic profile of metabolites.

Although for over two decades low-resolution instrumentation was mostly used to find metabolites and elucidate their structures, high-resolution (HR) mass spectrometry is becoming increasingly essential for metabolite identification [10, 11]. In practice, two categories of mass spectrometer can achieve high resolution (resolving power RP > 30,000) with good mass accuracy (<5 ppm)—TOF-MS and Fourier transform (FT)-MS, including the FT ion cyclotron resonance and the Orbitrap. The significant difference between the two types of instrument is the duty cycle [12]. Liquid chromatography is extremely important in analyte preconcentration and sample cleanup, and in recent years significant progress in separation efficiency in liquid chromatography has been realized with the development of sub-2 μm particles or core-shell technology, resulting in LC peak widths of only a few seconds, which challenges the MS duty cycle [13]. High-resolution mass spectrometry is an attractive alternative to quadrupole SRM quantification because almost no tuning is required and large numbers of analytes can be analyzed simultaneously. However, similar sensitivity, precision and accuracy must be achieved with these instruments. With resolving power RP higher than 20,000, selected ion monitoring quantification started to attract interest, and recent work has shown the potential of new developments in mass spectrometric equipment for alternative approaches to SRM for quantitative analysis [14].

This work explores alternative approaches including targeted and not targeted MS and MS–MS approaches based on high-resolution mass spectrometry with resolving power above 20,000 in combination with fast chromatography for QUAL/ QUAN analysis. The first part focuses on high-resolution selected reaction monitoring quantification of paracetamol in human plasma. The second part is dedicated to a novel acquisition strategy for drug metabolism based on multiple precursor ion windows, denoted sequential window acquisition of all theoretical fragments (SWATH) or global precursor scan (GPS), which is compared with commonly used information-dependent acquisition (IDA) and MS[ALL] acquisition schemes. The last section is devoted, using analysis of bosentan and its metabolites in urine as an example, to description of the benefits and limitations of SWATH for QUAL/QUAN approaches.

Experimental

Chemicals and reagents

Methanol (MeOH) and acetonitrile (MeCN) were obtained from VWR (Leuven, Belgium). Ammonium acetate, formic acid (FA), and acetic acid were obtained from Merck (Darmstadt, Germany). Acetaminophen, (paracetamol, APAP) was obtained from Sigma (Buchs, Switzerland). Acetaminophen-cysteine (APAP-CYS) trifluoroacetic acid salt, acetaminophen-glucuronide (APAP-GLU) monosodium salt, and the deuterated analogue of acetaminophen (APAP-d4) were purchased from Toronto Research Chemicals (Toronto, Canada). Bosentan, its metabolites (desmethylbosentan (M1), hydroxybosentan (M2), and hydroxydesmethylbosentan (M3), and their d4 analogs, were obtained from Roche (Basel, Switzerland) and talinolol was kindly provided by Arzneimittelwerk Dresden (Dresden, Germany). Clonazepam was provided by Lipomed (Arlesheim, Switzerland) and oxycodone by Cambridge Isotope Laboratories (Andover, MA, USA). Rat liver microsomes (RLM) and human liver microsomes (HLM) were obtained from BD Biosciences (Allschwill, Switzerland).

Analysis of acetaminophen in human plasma

Sample preparation

Protein in 50-μL plasma samples was precipitated by addition of the ice-cold solvent mixture MeOH–EtOH (1:1 v/v) in the ratio 4:1. Calibration standards and ISTD, APAP-d4, were added at this step (40 μL of solutions in water added in total). Samples were then mixed on a Thermomixer (Vaudaux-Eppendorf, Buchs, Switzerland) for 10 min at 15 °C and 1,400 rpm. Subsequent centrifugation was performed at 4 °C and 14,000g for 12 min. The supernatant was then removed and evaporated to dryness. Sample reconstitution was performed in 100 μL 5 mmol L^{-1} ammonium acetate buffer (pH 4.75), subsequently mixed for 10 min at 15 °C and 1,400 rpm. Human plasma (citrate) was provided by the University Hospital of Geneva.

LC–MS–MS analysis

HPLC was performed with an UltiMate 3000 micro-LC (Dionex, Germering, Germany), configured in binary high-pressure gradient mode and controlled by Chromeleon 6.70 software (Dionex). Mobile phase A was 5 mmol L^{-1} ammonium acetate buffer (pH 4.75) and mobile phase B was MeOH. The starting composition of the mobile phase was 5% B; this was held for 0.5 min, then changed to 25% B in 4.5 min, then to 80% B in 3 min (hold for 2 min), then back to 5% B in 0.1 min, and equilibrated for 9 min for the next injection. The column oven and the flow rate were set at 30 °C and 1 mL min^{-1}, respectively. Samples (10 μL) were injected on to an ODS-AQ 4.6 mm I.D. × 150 mm, S-5 μm particle, reversed-phase column (YMC Europe, Dinslaken, Germany) with a 2.0 mm I.D. × 4.0 mm C$_{18}$ guard column (Phenomenex, Torrance, CA, USA). Mass spectrometric

detection was performed with a quadrupole-TOF MS (TripleTOF 5600, AB Sciex, Concord, Canada) operated in positive mode with a DuoSpray ion source in high-sensitivity mode at a resolving power (full width at half maximum, FWHM, at m/z 400) of approximately 20,000 in MS–MS and 30,000 in MS mode. The automated calibration device system (CDS) was set to perform an external calibration every three samples. The source conditions were: temperature 700 °C, curtain gas (CUR) 25, GS1 and GS2 at 60 and 70 (laboratory frame), respectively, and ion-spray voltage floating (ISVF) at 5.5 kV. Collision energies (CE) were set to 20 eV for APAP and APAP-d4, 13 eV for APAP-GLU, and 35 eV for APAP-CYS.

The acquisition method was composed of three different periods:

- period 1 (0–5.55 min) a product ion scan (precursor m/z 328.1, mass range m/z 100–1000, accumulation time 250 ms) and a TOF-MS scan (mass range m/z 100–1000, accumulation time 200 ms);
- period 2 (5.55–6.61 min) a product ion scan (precursor m/z 271.1, mass range m/z 100–1000, accumulation time 250 ms) and a TOF-MS scan (mass range m/z 100–1000, accumulation time 200 ms); and
- period 3 (6.61–8.0 min) a product-ion scan (precursor m/z 152.1, mass range m/z 100–1000, accumulation time 250 ms) and product ion scan (precursor m/z 156.1, mass range m/z 100–1000, accumulation time 250 ms).

The duty cycle time for each period was 500 ms. Quantification was performed with MultiQuant 1.2 software (AB Sciex). The integration algorithm was MQL with Gaussian smooth width of two points using a linear regression model with $1/x^2$ weighting. The extraction window for HR-SRM mode was set at 0.025 u for the fragment ions: APAP-GLU m/z 152.1, APAP-CYS m/z 140.0, APAP m/z 110.1, and APAP-d4 m/z 114.1.

Analysis of talinolol incubation in microsomes

For microsomal incubations of talinolol, all concentrations given hereafter are final concentrations in the incubation mixtures. Pre-incubation at 37 °C in a Thermomixer was performed for 3 min after mixing of an appropriate volume of phosphate buffer (100 µL minus the volume of the other components), 1 mg mL^{-1} microsomes (RLM or HLM; 10 µL) and 1 µmol L^{-1} talinolol. NADPH (1.2 mmol L^{-1}) was then added and incubation was again performed at 37 °C before quenching after different times (0.25, 0.5, 0.75, 1, 2, 4, and 6 h) by adding 400 µL MeOH–EtOH (1:1 v/v) with ISTD (0.781 µg mL^{-1} oxycodone and clonazepam in the precipitation solvent). Subsequent centrifugation for 10 min at 4 °C and 12,000g was then performed and the supernatant was removed and evaporated to dryness

under vacuum in a Univapo 150 ECH concentrator (Uniequip, Planneg, Germany). These fractions were kept dry at −20 °C until LC–MS(–MS) analysis.

The human and rat microsomal incubations of talinolol were analyzed on an UltiMate 3000 RSLC system from Dionex (Germering, Germany) controlled by Chromeleon 6.70 software (Dionex) and hyphenated to a TripleTOF 5600 mass spectrometer (AB Sciex) operating at a resolving power of approximately 40,000 (FWHM at m/z 400). Automated external calibration was performed every three samples. Mobile phase A was 0.1% formic acid (FA) in water and mobile phase B was 0.1% FA in MeCN. The flow rate was set at 600 µL min^{-1} The binary gradient was linear from 10% to 60% in 5 min The LC column was a Kinetex C$_{18}$ (2.1 mm I.D. × 100 mm, 1.7 µm, Phenomenex) and was thermostatted at 50 °C. The injection volume was of 5 µL. Oxycodone and clonazepam were used as internal standards.

Three data acquisition schemes were investigated:

1. *Information-dependent acquisition mode (cycle time 350 ms)*: HR TOF as survey scan (accumulation time 100 ms) and HR MS–MS as dependent scan with a CE range 20–50 eV (four MS–MS dependent experiments) (accumulation time 50 ms);
2. *MS^{ALL} (cycle time 350 ms)*: HR TOF MS at low energy (CE=10 V) (accumulation time 150 ms) and HR TOF MS at high energy (CE=35 V) (accumulation time 150 ms);
3. *Sequential window acquisition of all theoretical fragment ions spectra (SWATH) (cycle time 375 ms)*: 15 PIS (Q1 20 u window) (accumulation time 25 ms each PIS, CE range 20–50 eV).

For all experiments, the TOF mass range was m/z 50–650. MS data acquisition was performed using Analyst TF 1.5.1 and data processing was performed using PeakView 1.1 software (AB Sciex).

LC-MS analysis of bosentan and its metabolites in urine

Urine was spiked with bosentan and its three metabolites (M1–M3) to obtain calibration and QC samples containing 5, 10, 25, 50, 100, 250, 500, 1000, and 2500 ng mL^{-1}). Urine was spiked with the d4 analogues used as internal standards to obtain a final concentration of 100 ng mL^{-1}. Before analysis the urine was diluted five times with water and 5 µL was injected on to the LC system. The analytes were separated on a Luna C18(2) (2.0 mm I.D. × 100 mm, 2.5 µm) LC column (Phenomenex, Brechbühler, Switzerland) in gradient mode using an UltiMate 3000 RSLC system from Dionex (Germering, Germany) controlled by Chromeleon 6.70 software. Mobile phase A was 0.1% FA in H$_2$O and mobile phase B was 0.1% FA in MeCN. The gradient started at 40% B and reached

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90% B in 4 min The flow rate was of 0.4 mL min^{-1} and the column was thermostatted at 40 °C. MS analysis was performed with a TripleTOF 5600 mass spectrometer in SWATH acquisition mode with eight Q1 windows of 20 u from m/z 494 to m/z 638. There was an overlap of 2 m/z between each Q1 window. The TOF mass range was m/z 50–1000. Seven product ion experiments were further performed with Q1 set at unit resolution for the precursor ions: 538.3 (M1), 542.3 (M1-d4), 552.3 (Bosentan), 556.3 (Bosentan-d4), 554.3 (M3), 568.3 (M2), and 572.3 (M2-d4). The TOF mass range was m/z 50–650. The accumulation time was set for all experiments at 25 ms for a total cycle time of 375 ms. Nitrogen was used as collision gas and the collision energy was ramped from 20 to 60 eV. Quantification was performed with MultiQuant 2.0 software (AB Sciex). The integration algorithm was MQL, applying a standard Gaussian smoothing algorithm with a half-width equal to 1 point and using Wagner regression fit. The extraction window was set at 25 mDa for the bosentan, its metabolites and the d4 internal standards.

Results and discussion

Quantification of acetaminophen in human plasma by HR-SRM

Triple-quadrupole mass spectrometers have become the instruments most widely used for quantitative LC–MS analysis in the selected reaction monitoring mode (SRM). The objective of this work was to evaluate the quantitative performance (i.e., precision, accuracy, and linearity) of the QqTOF instrument (TripleTOF 5600) in MS–MS mode for quantitative analysis of APAP in human plasma. The major advantage of high resolution MS–MS on a QqTOF, also referred as HR-SRM, compared with SRM is the limited MS method development. Indeed with HR-SRM acquisition, the full-scan product-ion spectrum is recorded, and for quantitative analysis there is no need to define the product ion in advance; moreover, the MS–MS spectrum enables compound structural characterization to be performed. The analytes were isolated from the matrix by protein precipitation and separated by an LC gradient before MS detection using positive polarity electrospray. The method was found to be linear over three orders of magnitudes (3.573 to 10,000 ng mL^{-1}). Precision and accuracy for APAP quality controls (QC) are listed in Table 1 and were better than 15%. The dynamic range and limit of quantification were found to be similar to those of a triple-quadrupole instrument (4000 QTRAP, AB Sciex; data not shown), demonstrating the quantitative ability of the HR-SRM mode for APAP. Representative chromatograms obtained from a blank plasma sample and from a quality-control sample at the limit of quantification are shown in Fig. 1a and b. The LC–HR-SRM traces of two other monitored metabolites: APAP-GLU, a phase II metabolite of APAP, and APAP-CYS, a further metabolite of the APAP-glutathione metabolite, are also presented in Fig. 1. The APAP-GLU trace of the blank plasma shows an interfering ion at t_r=4.16 min, which is

Table 1 Precision and accuracy for quantification of APAP in human plasma (calibration and QC samples)

Calibration standards	Conc. (ng mL^{-1})	Measured concentration (ng mL^{-1})			Mean (ng mL^{-1})	RSD (%)	Accuracy (%)
		Day 1		Day 2			
		Series 1	Series 2	Series 3			
Cal 1	3.573	3.695	3.497	3.386	3.526	4.4	98.7
Cal 2	6.253	5.966	6.350	6.768	6.361	6.3	102
Cal 3	15.63	15.25	16.62	15.92	15.93	4.3	102
Cal 4	40.02	38.41	38.15	39.83	38.80	2.3	96.9
Cal 5	100.1	103.3	103.8	103.6	103.6	0.2	103
Cal 6	250.1	244.8	258.2	254.8	252.6	2.8	101
Cal 7	640.3	680.9	677.3	656.6	671.6	2.0	105
Cal 8	1601	1651	1586	1565	1601	2.8	100
Cal 9	4002	4131	3846	3828	3935	4.3	98.3
Cal 10	10010	9404	9108	9435	9316	1.9	93.1
QC							
LLOQ	3.573	3.060	3.784	3.881	3.575	13	100
Low	6.253	5.918	6.711	6.005	6.211	7.0	99.3
Medium	250.1	244.2	253.9	265.9	254.7	4.3	102
High	10010	9174	9348	9075	9199	1.5	91.9
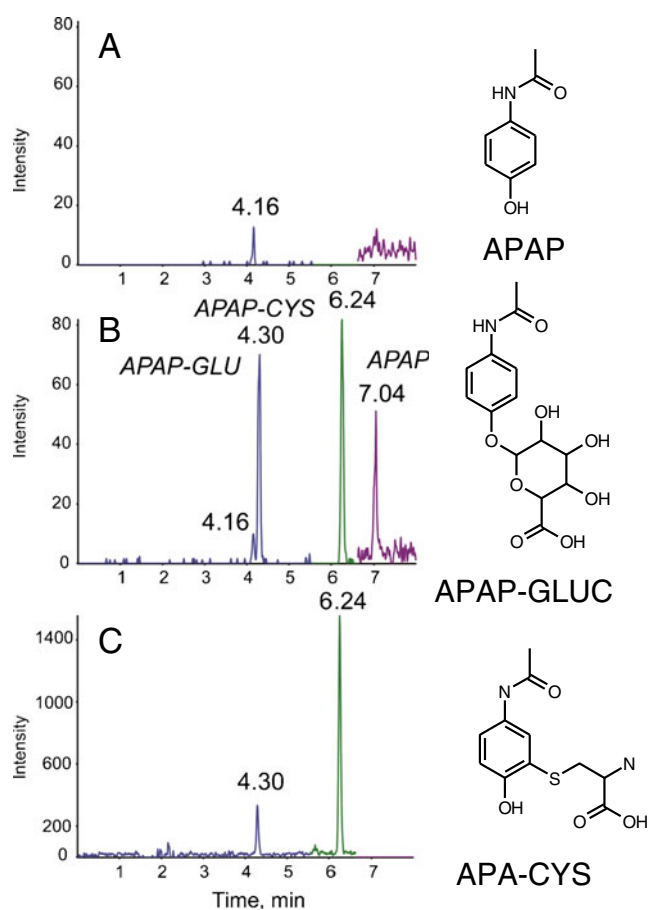
Fig. 1 LC–MS traces of APAP, APAP-CYS, and APAP-GLU in human plasma. (**A**) HR-SRM traces obtained from blank plasma. (**B**) HR-SRM traces obtained from an APAP quality-control sample at the LOQ (3.573 ng mL^{-1}). (**C**) HR-SIM traces obtained from an APAP quality-control sample at the LOQ (3.573 ng mL^{-1})

well separated from APAP-GLU eluting at t_r=4.3 min and is small enough to not affect quantitative performance. Interestingly, the extracted ion current profile in TOF mode at RP 30,000 (i.e., high resolution selected ion monitoring, or HR-SIM) does not show the interference (Fig. 1c). It is generally accepted that MS–MS provides additional selectivity and therefore better limits of quantification. However, one must remember that isolation of the precursor ion is performed at unit mass. In this case, several ions were observed in 1-u precursor windows at m/z 328 in TOF mode, and one of these generated isobaric fragment interference. To define which acquisition mode between HR-SIM and HR-SRM is optimum for quantification performance, and which RP is required, remains a difficult task. Certainly a resolving power of 10,000 is not sufficient and it is considered that an RP of 30,000 seems to be adequate for HR-SIM analysis [15]. However, HR-SRM gains from better signal-to-noise ratio and, in general, several product ions are available for quantitative analysis. Ultimately, which approach will be more efficient is

certainly analyte and limit of quantification-dependent and the use of both approaches is highly recommended.

## LC–MS analysis of talinolol incubations in liver microsomes

Investigation of metabolic stability in microsomes is an important step in early drug characterization. Degradation of the parent drug can be measured on a triple-quadrupole instrument by using the selected reaction monitoring mode, and the metabolites can be detected by using subsequent experiments, for example targeted product ion, neutral loss, or precursor ion scan modes. However, triple-quadrupole instruments suffer from limited sensitivity in these modes and, generally, product-ion spectra are recorded on ion-trap or triple-quadrupole linear ion-trap instruments. High-resolution mass spectrometry offers unique possibilities for quantification of the parent drug but also for identification of metabolites. Quantification can be performed in HR-selected-ion monitoring mode post-acquisition or in HR-selected reaction monitoring mode, and metabolites can be identified using either predictive metabolism [16] or mass defect filtering [17].

Talinolol is a highly efficient and selective β$_1$-adrenoceptor blocking agent. Oxidation at the cyclohexyl moiety has been described as the major metabolic pathway generating several metabolites hydroxylated in different stereochemical positions [18]. After incubation for 2 h of a 1 μmol L^{-1} solution talinolol in rat liver microsomes, six hydroxylated metabolites could be identified (Fig. 2, M1–M6) by use of mass-defect filtering. A mass defect filter is based on the shift of the mass defect from the parent drug (the non-integral value from the nominal value) to its metabolites. The filter (windows of 25 mDa for phase I metabolites) retains m/z ions, defects of which are close to that of the parent drug, and interfering ions are automatically removed. Elemental formulas are generated with accurate
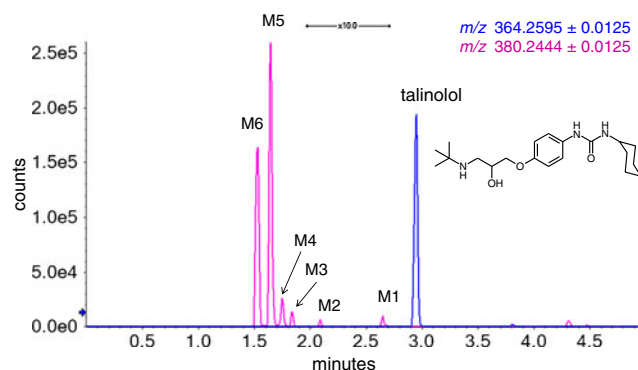
Fig. 2 Extracted ion current profile for talinolol and its hydroxylated metabolites from MS-TOF acquisition at a resolving power of 30,000 after incubation of talinolol with rat liver microsomes for 2 h

Fig. 3 Mass spectrum of
metabolite M4 at retention time
1.74 min. (A) Information-
independent acquisition mode
with a dependent product-ion
spectrum, Q1 at unit mass. (B)
MS^ALL, high-energy experi-
ment. (C) SWATH spectrum
with Q1 window m/z 374–394
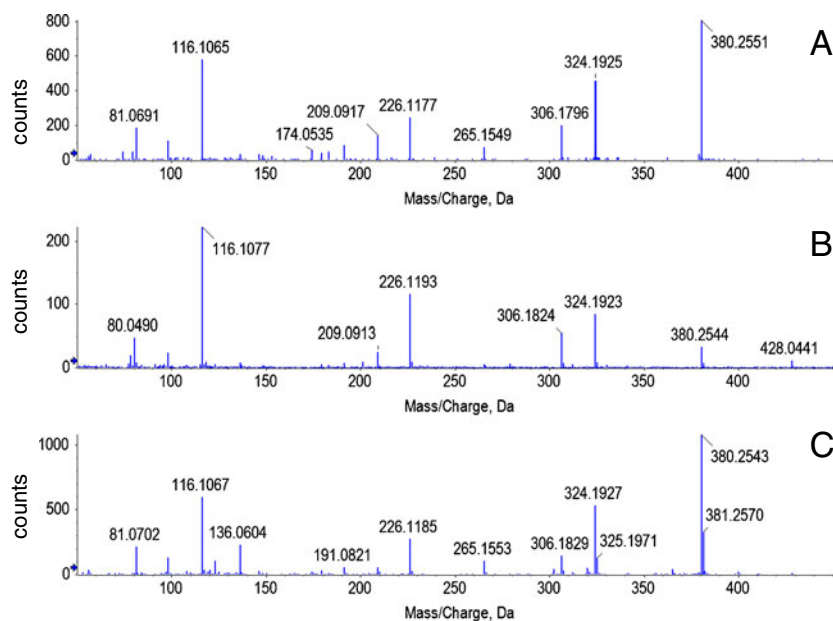
mass and isotopic distribution fit. The decrease of the parent
drug and the increase of the metabolites during incubation
for 6 h could be quantified post-acquisition by use of HR-
SIM. Chromatographic resolving power is essential in drug
metabolism studies, particularly when many isobaric metab-
olites are generated. With our chromatographic arrangement
using 1.7-μm particle columns, the chromatographic peak
width was approximately 1–2 s. For quantitative analysis,
approximately 10–12 data points are required, meaning that
the total duty cycle of all experiments should not exceed
350 ms to maintain chromatographic performance. Whereas
HR full scan can be used to find and quantify metabolites,
generation of product-ion spectra remains essential for char-
acterization of structure. Typical metabolite identification is
based on information-dependent acquisition (IDA) in which
HR full scan serves as survey scan and four product ion
spectra are recorded as dependent scans by applying on-the-
fly selection criteria to the survey scan, for example mass
defect filtering. The product-ion spectrum of metabolite M4
is illustrated in Fig. 3a. Hydroxylation is a major metabolic
process and hydroxylation of talinolol can occur at the tert-
butyl, benzyl, or cyclohexyl moiety. Spectra interpretation is
often challenging and a variety of approaches can be con-
sidered, for example:

1. comparison with synthetic reference; or
2. use of prediction software based on fragmentation rules.

Accurate mass is particularly useful for interpretation of
MS–MS spectra because it enables calculation of elemental
formulae for the fragments and definition of whether they are
even or odd-electron ions [10]. In this instance we used a
bottom-up approach to perform spectral interpretation on the
basis of the structural constraints of the talinolol and the

Table 2 Experimental arrangement of talinolol LC–MS analysis after
two incubations in rat liver microsomes Information-dependent
acquisition

	Q1 window m/z	TOF range m/z	CE (eV)	Dwell time (ms)
1	Open	50–650	10	100
2	Unit	50–650	20–60	50
3	Unit	50–650	20–60	50
4	Unit	50–650	20–60	50
5	Unit	50–650	20–60	50
MS^ALL				
	Q1 window m/z	TOF range m/z	CE (eV)	Dwell time (ms)
1	Open	50–650	10	150
2	Open	50–650	35	150
SWATH				
	Q1 window m/z	TOF range m/z	CE (eV)	Dwell time (ms)
1	300–320	50–650	10–40	25
2	318–338	50–650	10–40	25
3	336–356	50–650	10–40	25
4	354–364	50–650	10–40	25
5	372–392	50–650	10–40	25
6	390–410	50–650	10–40	25
7	408–428	50–650	10–40	25
8	426–446	50–650	10–40	25
9	444–464	50–650	10–40	25
10	462–482	50–650	10–40	25
11	480–600	50–650	10–40	25
12	498–518	50–650	10–40	25
13	516–536	50–650	10–40	25
14	534–554	50–650	10–40	25
15	552–572	50–650	10–40	25
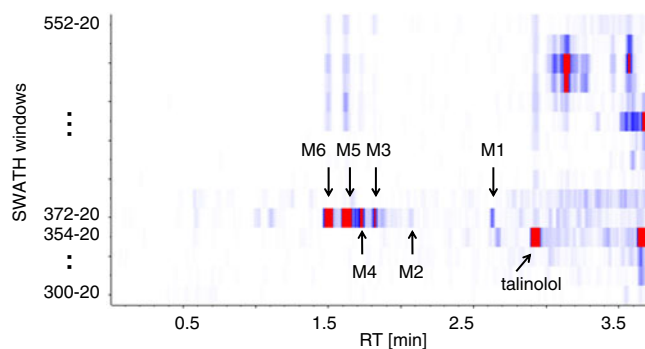
Fig. 4 Heat map of SWATH experiments (15 product-ion experiments) of the LC–MS analysis after incubation of talinolol with rat liver microsomes for 2 h. Blue (zero), red max signal corresponding to 10% of base peak

measured fragments. PeakView software has a fragment pane option that calculates all possible even or odd-electron fragments by cutting existing bonds. For the fragments of metabolite M4, *m/z* 209 and 226 ions were found to be specific for the unchanged benzyl moiety. At that stage, it could be postulated that hydroxylation occurred at the *tert*-butyl or cyclohexyl moiety. In a second iteration, both possible structures were submitted to the fragment pane routine and it was found that fragments at *m/z* 324 and *m/z* 116 were specific for the structure with the hydroxylation at the cyclohexyl moiety. M2 to M6 could be identified as talinolol hydroxylated at the cyclohexyl moiety, and M1 was oxidized at the *tert*-butyl moiety. The major advantage of the bottom-up approach compared with the use of fragmentation rules is that the software is issued to match the fragment ions with the structural and elemental composition constraints of the parent drug in a first iteration and is independent of fragmentation rules that are compound-dependent.
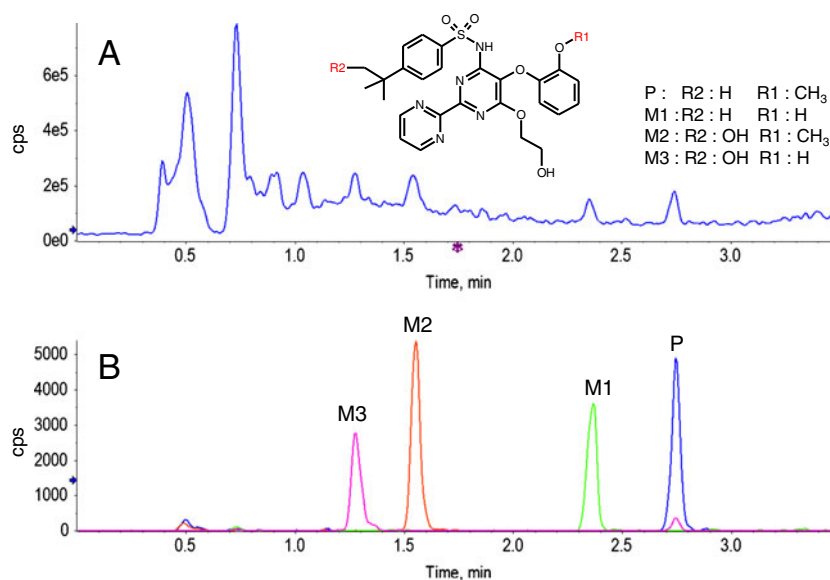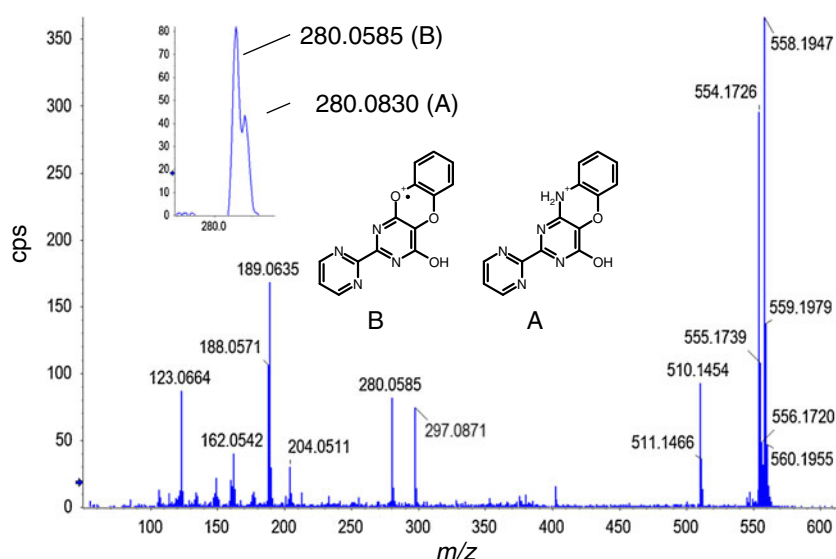
IDA is a powerful supervised MS(–MS) acquisition technique but has two main limitations:

1. some relevant precursor ions will not be selected for performing collision-induced dissociation whatever selection criteria are defined; and
2. quantification is challenging because no continuous data are collected during the complete LC peak elution.

In combination with liquid chromatography, unsupervised MS and MS–MS spectra generation have been described and are generally referred as MS^E or MS^{ALL}. In such an arrangement, data are collected on a high-resolution instrument typically of QqTOF geometry with the quadrupole operating in wideband mode and collecting, alternately, mass spectra with and without fragmentation by setting the collision energy of the collision cell in low (i.e. 10 eV) and high mode (i.e. 40 eV). Because only two experiments are necessary, the MS duty cycle is far less critical compared with IDA or multiple product ion scan experiments. The background-subtracted MS^{ALL} spectrum (high-energy) of metabolite M4 (talinolol incubation 2 h) is illustrated in Fig. 3b. Most of the ions present in Fig. 3a are present in this spectrum. Compared with the product-ion spectrum obtained by unit mass Q1 isolation, the MS^{ALL} experiment has no selection of the precursor ion and additional ions are observed at *m/z* 80 or *m/z* 428 which do not originate from the hydroxylated talinolol precursor ion but arise from co-eluting interferences. Data processing can be used to improve the quality of the spectra but does not enable generation of high-quality spectra [19] from low-intensity peaks, for example those of metabolites M2 and M1. MS^{ALL} theoretically enables generation of MS–MS data for any precursor ion but suffers from lack of selectivity, because of the absence of precursor ion selection, and therefore challenges data processing, in particular with dirty

Fig. 5 LC–MS analysis of bosentan and its metabolites spiked at 100 ng mL^{-1} in urine. (**A**) Total ion chromatogram obtained from 15 MS–MS experiments (eight SWATH and seven product-ion experiments). (**B**) Extracted ion current profile from SWATH windows using a 0.025-u window

Fig. 6 SWATH spectrum of metabolite M3 from Fig. 5, with Q1 window *m/z* 548–568

samples. On the other hand, on the LC time scale, using unit Q1 window, a given mass range (i.e. *m/z* 50-650) would result in 600 MS–MS experiments being performed.

An interesting approach to keep the benefits of the MS^{ALL} approach and to reduce sample complexity is to use multiple Q1 precursor ion windows at a given width (i.e. 25 u). This independent acquisition method is referred as sequential window acquisition of all theoretical fragment ions spectra (SWATH) and has been described for analysis of peptides [20, 21]. It has also been used for qualitative analysis of low-molecular-weight compounds, and was called global precursor ions scan mode (GPS) [22]. The talinolol sample incubated for 2 h with rat liver microsomes was analyzed using 15 production-ion scan experiments (*m/z* 50–650) with Q1 set at an increasing 20 u window (Table 2). To maintain a reasonable duty cycle of 375 ms for UHPLC, the duty cycle for each experiment was set to 25 ms on the QqTOF instrument. In contrast with MS^{ALL}, for which two experiments are necessary, in SWATH mode intact precursor and fragment ions are collected in the same spectrum. This is achieved by using a collision energy ramp in the collision cell (i.e. 10, to 45 eV) from low to high. Each acquired Q1 isolation window can be processed individually by applying a variety of filtering techniques, for example isotope filter or mass defect filter. An elegant way to visualize the data is to use heat-map representation as illustrated in Fig. 4. It can be clearly seen that metabolites M1–M6 are measured in the same window (*m/z* 372–392) whereas talinolol is measured in a different one (*m/z* 354–374). In this analysis, the mass range covered was 272 u, which is sufficient to investigate phase I metabolites. The Q1 isolation window was set to 20 u and centered on the *m/z* value of talinolol. To cover phase I and II metabolites variable windows sizes should be defined. In a SWATH experiment, all the relevant data are collected in a single LC–MS analysis and the data can be processed post-acquisition using any strategy. The product

ions spectrum of metabolite M4 is presented in Fig. 3c and, compared with the MS^{ALL} experiment, no interferences are observed around low mass fragments. In contrast with the MS^{ALL} experiments, metabolites M1 and M2 could also clearly be characterized from their product-ion spectra. As a matter of fact, SWATH has several advantages over MS^{ALL}:

1. precursor and fragment ions in the same spectrum;
2. fewer precursor ion interferences; and
3. quantitative analysis can be performed on the precursor ion or on any fragment ions.

QUAL/QUAN analysis of bosentan in urine by sequential window acquisition of all theoretical fragment ions spectra or global precursor ions scan

Bosentan (Tracleer) is an oral dual endothelin receptor antagonist which is currently approved for treatment of

Table 3 Precision and accuracy for analysis of bosentan and its desmethylhydroxy metabolite in urine, on the basis of quality-control samples

	ng mL^{-1}	n=1	n=2	n=3	Average	RSD (%)	Accuracy (%)
Bosentan							
QC01	5	4.42	4.40	4.93	4.58	6.6	91.6
QC02	10	10.6	10.8	10.2	10.6	2.9	105
QC06	250	252	306	296	285	10	114
QC08	1000	651	1182	1181	1005	31	101
Desmethylhydroxybosentan							
QC01	5	4.29	4.19	3.53	4.00	10	80
QC02	10	8.80	7.21	7.78	7.90	10	79
QC06	250	301	259	232	265	14	106
QC08	1000	1080	897	1101	1026	11	103

pulmonary arterial hypertension (PAH) [23]. Bosentan forms three metabolites (Fig. 5): M1, desmethylbosentan, M2, hydroxy bosentan, and M3, desmethylhydroxylbosentan. Several LC–MS–MS methods have been developed to quantify bosentan and its metabolites in plasma using the selected reaction monitoring mode [24] and the fragmentation mechanism has been extensively investigated [10, 25]. Bosentan was used as a model compound to further investigate the potential and limitations of GPS for QUAL/QUAN approaches. In this study the analytes were analyzed from 5 to 2,500 ng mL^{-1}, in urine, because urine resulted in a more complex endogenous background (Fig. 5a) than plasma. With GPS analysis, only one type of spectrum is generated, namely a product-ion spectrum. By use of collision energy spread, typically of 40 V, the precursor ions are still present in the spectrum and also fragment ions can be recorded over the complete mass range (Fig. 6) and can be used for quantification. For bosentan and its metabolites, deuterium labeling (d4) of the internal standards is located on the hydroxyethoxy side chain and the product-ion spectra of the d0 and d4 analogs give exactly the same fragments. Therefore, with a Q1 isolation window of 20 u it is not always possible to perform quantitative analysis in HR-SRM mode with isotopically labeled internal standards. However, it is still possible to perform HR-selected ion monitoring (HR-SIM) quantitative analysis on the precursor's ions, on the basis of their different m/z. Precision and accuracy for quality-control samples of bosentan and its desmethylhydroxyl metabolite over the concentration range 5–2,500 ng mL^{-1} are summarized in Table 3. Considering the accumulation time was only 25 ms, the results were found to be acceptable for drug-discovery work. If the objective is to follow the parent drug pharmacokinetics by use of HR-SRM mode, one can also combine GPS experiments with classical product-ion scans in which the Q1 isolation window is 1 u. As discussed previously, the major interest in SWATH or GPS experiments is to collect all information in a single LC–MS analysis, which is of great interest for identification of biotransformation of the parent drug and for absolute and relative quantification. Bosentan remains one of the first examples of fragmentation at low energy involving a radical fragmentation of an even-electron ion at the sulfonamide bond [25]. Also, two different fragmentation mechanisms illustrated for the different metabolites involve loss of radical hydroxyl ($^{\cdot}$OH, m/z 280.0585) or neutral ammonia (NH$_3$, m/z 280.030) both of 16 u. A resolving power of 10,000 was not sufficient to clearly differentiate both mechanisms and a RP of at least 20,000 was necessary to distinguish these fragments, as illustrated in Fig. 6. Because many pharmaceutical compounds are composed by C, H, N, O, or S,

an RP lower than 20,000 is considered to be critical for MS and MS–MS spectra interpretation.

Conclusions

High-resolution mass spectrometry on a rapid acquisition (<50 ms) QqTOF instrument with resolving power above 20,000 leads to new possibilities in QUAL/QUAN analysis. First, it enables the analyst to benefit fully from the increased separation performance of fast or ultra-high performance liquid chromatography without compromising chromatographic performance. Second, in quantitative analysis of acetaminophen in the HR-SRM mode, similar quantitative performance could be achieved as for quadrupole-type instruments. For QUAL/QUAN drug-discovery work, several dependent and independent MS strategies were considered including: targeted product ions scan, information-dependent acquisition, MS^{ALL}, and the sequential window acquisition of all theoretical fragment ions spectra concept. Compared with MS^{ALL}, global precursor ions scan (GPS), which is the low-molecular-weight version of SWATH, resulted in significant performance improvement and simplified data processing, because all the information is collected in a single LC–MS run and precursor and fragment ions are recorded within the same spectrum. Although the potential of GPS has been demonstrated for drug metabolism, it could also be applied in environmental, toxicology, doping, or food analysis, and in metabolomics investigations.

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