Journal of Chromatography B, 879 (2011) 2919–2927

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Full-scan high resolution accurate mass spectrometry (HRMS) in regulated bioanalysis: LC–HRMS for the quantitation of prednisone and prednisolone in human plasma

Eliza N. Fung*, Yuan-qing Xia[1], Anne-Francoise Aubry, Jianing Zeng, Timothy Olah, Mohammed Jemal**

Research & Development, Bristol-Myers Squibb Co., Route 206/Province Line Road, Lawrenceville, NJ 08543, United States

ARTICLE INFO

Article history:
Received 31 May 2011
Accepted 19 August 2011
Available online 26 August 2011

Keywords:
High resolution accurate mass spectrometry
Prednisone and prednisolone
Regulated bioanalysis
LC–MS/MS
LC–HRMS

ABSTRACT

A liquid chromatography–full scan high resolution accurate mass spectrometry (LC–HRMS) method for quantifying prednisone and prednisolone in human plasma using a quadrupole time-of-flight mass spectrometer (Q-TOF) was developed. Plasma samples were extracted using a liquid–liquid extraction procedure. Full scan data were acquired in the TOF only mode and extracted ion chromatograms were generated post-acquisition with the exact masses of the analytes. The calibration range was 5–2500 ng/mL, with a Lower Limit of Quantitation (LLOQ) of 5 ng/mL. The assay accuracy was between 98.4% and 106.3%. The between-run (inter-day) and within-run (intra-day) precision were within 1.7% and 2.9%, respectively. The matrix effect was between 0.98 and 1.10 for the six different lots of human plasma evaluated. Pooled incurred samples were analyzed by the method and the results matched those obtained from an LC–MS/MS method. In addition, qualitative information on phospholipids, and other endogenous components were also extracted from the full-scan data acquired.

1. Introduction

Reversed-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS) is widely used for the quantification of drugs, metabolites and biomarkers in biological matrices in support of drug discovery and development [1–9]. Whereas, qualitative analysis of drugs and their metabolites has been traditionally performed on different platforms of high resolution accurate mass spectrometry (HRMS) such as Q-TOF and Orbitrap mass spectrometers because of their high mass resolution [10–14]. Advantages may be gained through using a single platform that combines selectivity and sensitivity, so that both quantitative and qualitative information from the specimens could be collected simultaneously. With the advances in the latest generation of high resolution accurate mass spectrometers, it has become feasible to perform both quantitative and qualitative analysis of drugs, their metabolites and biomarkers on a single platform. This approach has been successfully applied to non-regulated bioanalysis, residue analysis of pesticides, and analysis of veterinary drugs in animal based food [15–22]. This approach can potentially be applied to the regulated area.

In LC–HRMS, total ion chromatograms (TIC's) are acquired over a pre-defined m/z range (e.g. 100–1600 m/z) with a pre-set mass resolution on the mass spectrometer. Extracted ion chromatograms (EIC's) are generated post-data acquisition from the TIC's with the exact masses of the target analytes and a pre-defined mass extraction window (MEW). Quantitative information is then obtained from the EIC's, similar to that of selected reaction monitoring (SRM)-based method. Unlike SRM-based methods, in which the triple quadrupole mass spectrometers are typically set at unit resolution, with full width at half maximum (FWHM) of 0.7 Da for data acquisition, different mass resolutions are typically available on full-scan mass spectrometers, depending on the type of mass spectrometers used. Higher mass resolution in general provides better selectivity especially in complex sample matrix [16–20]. However, higher mass resolution may come at the expense of dynamic range for Q-TOF systems, or increasing scan time for Orbitrap-based mass analyzers [18,20,21]. Another important factor to consider for HRMS analysis is the choice of MEW used to generate the EIC's. The accuracy of the area value obtained for the target analyte chromatographic peak in the EIC may depend on the MEW. A large MEW may allow interfering peaks to be extracted into the EIC, while a very

* Corresponding author. Tel.: +1 609 252 5685.
** Corresponding author. Tel: +1 609 252 3572.
E-mail addresses: ngakiteliza.fung@bms.com (E.N. Fung), mohammed.jemal@bms.com (M. Jemal).
[1] Current address: AB Sciex, 500 Old Connecticut Path, Framingham, MA 01701, USA.

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.08.025

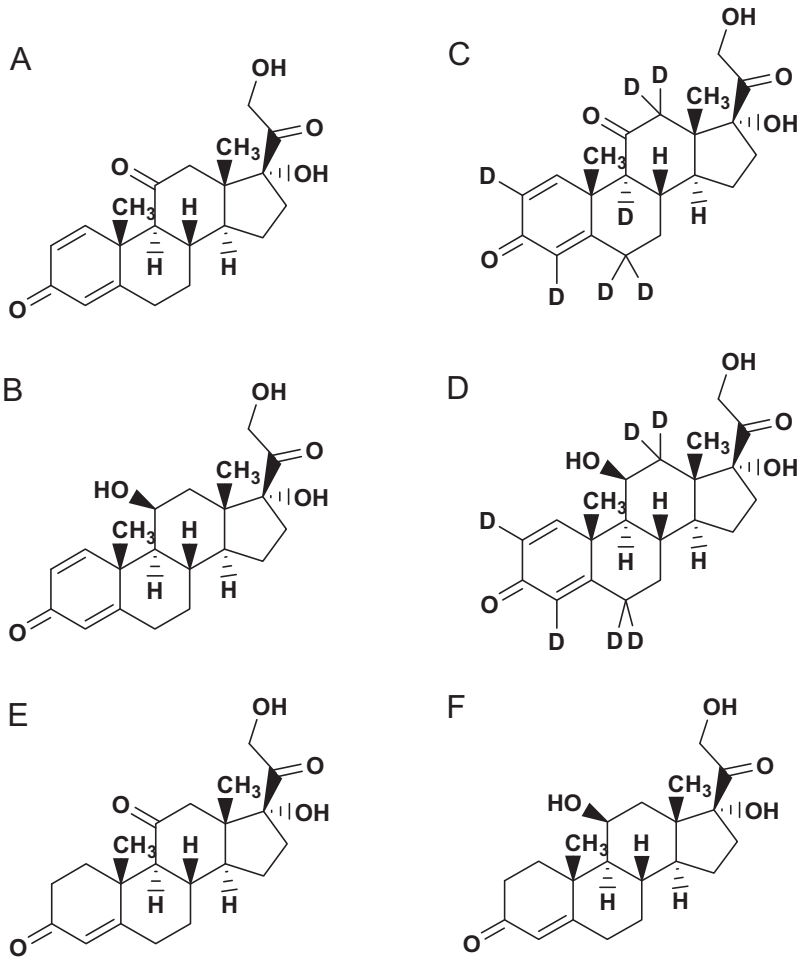

Fig. 1. Structures of (A) prednisone (exact mass of $[M+H]^+$ ion = 359.1853), (B) prednisolone (exact mass of $[M+H]^+$ ion = 361.2010), (C) prednisone-d7 (exact mass of $[M+H]^+$ ion = 366.2298), (D) prednisolone-d6 (exact mass of $[M+H]^+$ ion = 367.2386), (E) cortisone (exact mass of $[M+H]^+$ ion = 361.2010), (F) cortisol (exact mass of $[M+H]^+$ ion = 363.2166).

narrow MEW may give false negative results [18–20]. The choice of mass resolution and MEW has direct impact on the selectivity, sensitivity and linearity of the method, and therefore has to be carefully evaluated during method development.

In order to assess the feasibility of using LC–HRMS in regulated bioanalysis, we evaluated its performance on the quantitation of prednisone and prednisolone in human plasma against criteria commonly adopted for regulated bioanalysis [23]. Prednisone (Fig. 1A) is a commonly prescribed glucocorticoid to reduce symptoms such as swelling and allergic reactions. Prednisone is metabolized in the liver to its active form, prednisolone (Fig. 1B) [24]. A number of analytical methods with Lower Limit of Quantitation (LLOQ) in the range of 0.1–15 ng/mL have been reported previously [25–31]. Herein, we present the results of our evaluation of the LC–HRMS method with regard to sensitivity, selectivity, accuracy, precision, matrix effect and incurred sample analysis.

2. Experimental

2.1. Chemicals

Human plasma was obtained from Bioreclamation (Hicksville, NY, USA). Reference standards of prednisone and prednisolone were obtained from U.S. Pharmacopeia (Rockville, MD, USA). Prednisone-d7 and prednisolone-d6 (Fig. 1C and D) were obtained from SynFine (Richmond Hill, Ontario, Canada) and C/D/N Isotopes (Pointe-Claire, Quebec, Canada), respectively. Acetonitrile and methanol (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate was obtained from Fluka (St. Louis, MO, USA). Formic acid was obtained from EMD Chemicals (San Diego, CA, USA). High-purity water was obtained with a Barnstead Nanopure Diamond water purification system (Dubuque, IA, USA).

2.2. Preparation of stock solutions of prednisone and prednisolone and their internal standards

Stock solutions of prednisone and prednisolone were prepared in acetonitrile at 1.00 mg/mL with two separate weighings. Stock solutions of prednisone-d7 and prednisolone-d6 were prepared in acetonitrile at 1.00 mg/mL. A combined working solution of prednisone and prednisolone at 10 μg/mL was prepared by diluting the appropriate volumes of the stock solutions with acetonitrile. A combined internal standard working solution (ISWS) was prepared by diluting the internal standard stock solutions to 500 ng/mL with acetonitrile. All solutions were stored at 4 °C.

2.3. LC–MS/MS analysis by triple quadrupole mass spectrometer

A Shimadzu 10ADvp binary pump (Shimadzu, Columbia, MD, USA) was used for the LC–MS/MS analysis. The column was a

Zorbax Eclipse Plus C18, 2.1 × 50 mm, 3.5 μm (Agilent Technologies, Santa Clara, CA, USA.). A CTC HTC-PAL autosampler (Leap Technologies, Carboro, NC, USA) equipped with the self-wash kit was used. Both the HPLC pump and autosampler were controlled by Analyst™ 1.4.2 (AB Sciex, Foster City, CA, USA). The mobile phases were 10 mM ammonium formate – 0.1% formic acid in water (A) and 10 mM ammonium formate – 0.1% formic acid in 80% methanol: 20% acetonitrile (B). The flow rate was 0.3 mL/min and column temperature was operated at ambient temperature. The following gradient elution was used, starting at 40% B, then increasing to 63% B in 3.0 min, increasing again to 90% B in 0.1 min, holding at 90% B for 1.0 min and then lowering back to 40% B in 0.1 min. The total run time was 4.5 min. The autosampler temperature was set at 10 °C.

The samples were analyzed in positive ion mode using the TurboIonSpray® interface (TIS) of Sciex API 4000 triple quadrupole mass spectrometer. The following MS/MS conditions were used: ionspray voltage (IS), 4 kV; declustering potential (DP), 70 V; collision energy (CE), 40 eV; collision cell exit potential (CXP), 10 V; entrance potential (EP), 10 V; source temperature, 400 °C. Nebulizer gas (GS1 and GS2), curtain gas (CUR) and collision gas (CAD) were set to 40, 60, 18 and 6, respectively. The transitions monitored were: m/z 359 → m/z 147 for prednisone and m/z 361 → m/z 147 for prednisolone, m/z 366 → m/z 150 for prednisone-d7 and m/z 367 → m/z 150 for prednisolone-d6. The instrument was operated, data collected, and peak integration was processed using Analyst™ 1.4.2 (AB Sciex). The integrated peak areas were imported into Watson® (Thermo Fisher Scientific, Waltham, WA) and all concentration calculations were performed in Watson®.

2.4. LC–HRMS analysis by full-scan high resolution accurate mass spectrometer

An Agilent 1290 binary pump and autosampler (Agilent Technologies) were used for the LC–HRMS analysis. The column was a Zorbax Eclipse Plus C18, 2.1 mm × 50 mm, 1.8 μm (Agilent Technologies). Both the pump and autosampler were controlled by MassHunter (Agilent Technologies). The mobile phases were the same as those used in LC–MS/MS analysis. The flow rate was 0.5 mL/min and column temperature was set at 45 °C. The following gradient elution was used, starting at 35% B and increasing to 50% B in 2.7 min, increasing again to 90% B in 0.2 min, holding at 90% B for 0.7 min and then lowering back to 35% B in 0.1 min. The total run time was 4.0 min. The autosampler temperature was set at 10 °C.

The samples were analyzed in positive ion mode using the Dual ESI interface of Agilent 6530 quadrupole-time of flight (QTOF) mass spectrometer (Agilent Technologies). The source conditions were as follow: gas temperature, 350 °C, drying gas at 12.5 L/min, nebulizer gas at 50 psig, Vcap at 3500 V. For the TOF experiments, the fragmentor was set at 165 V, skimmer at 65 V and OCT 1 RF Vpp at 750 V. The mass range scanned was 100–1600 m/z and at a rate of 2 spectra/s. The resolution was set at 10,000. Data were acquired in both profile and centroid modes, while the quantitation was performed on the centroid data. The chromatograms of individual compounds (extracted ion chromatograms, EIC's) were extracted with the exact mass of each compound using a mass extraction window (MEW) of 40 ppm. The peak integration was processed using MassHunter (Agilent Technologies). The integrated peak areas were imported into Watson® (Thermo Fisher Scientific) and all concentration calculations were performed in Watson®.

In a separate experiment, an ion source equipped with Jet Stream Thermo Gradient Focusing Technology was used to further improve the sensitivity. The Vcap was lowered to 3000 V, and nozzle voltage was set to 200 V. All other parameters were the same as the Dual ESI source.

2.5. Human plasma standard and QC samples of prednisone and prednisolone

The calibration curves consisted of 8 concentrations in the range of 5.00–2500 ng/mL, prepared by serial dilution from the 10 μg/mL combined working solution of prednisone and prednisolone (Section 2.2). The concentrations of the plasma standards were 5.00, 10.0, 25.0, 100, 400, 1250, 2000 and 2500 ng/mL. The plasma standards were freshly prepared on the day of use.

A separate set of plasma standards in the range of 0.500–2500 ng/mL were also prepared by serial dilution from the 10 μg/mL combined working solution of prednisone and prednisolone for evaluating the Jet Stream Thermo Gradient Focusing Technology.

For QC samples, the stock solutions of prednisone and prednisolone were prepared from separate weighings than those used for the standards. QC samples at 10,000 ng/mL were prepared by diluting these stock solutions with blank human plasma. Other QC samples at 5.00, 15.0, 120, 1250 and 1875 ng/mL were prepared from serial dilution of the 10,000 ng/mL QC sample with blank human plasma. The QC samples were stored at −20 °C.

2.6. Liquid–liquid extraction procedure for human plasma

The plasma standards, QC samples and blank plasma were extracted by liquid–liquid extraction (LLE) as detailed here. A 150 μL volume of sample was mixed with 50 μL of ISWS, 50 μL of 1 M ammonium formate at pH 5, 650 μL of methyl *tert*-butyl ether, followed by shaking for 20 min. The organic layer was separated by centrifugation. Approximately 420 μL of the organic layer was transferred to a clean microtube and evaporated to dryness. The extraction recovery was 68–69% for both analytes and their internal standards. The dried extract was reconstituted in 150 μL of 70% water and 30% acetonitrile. An 8 μL portion of the reconstituted extract was injected into the LC–HRMS system or LC–MS/MS system.

2.7. Evaluation of performance of the LC–MS/MS method

Two sets of calibration curves were used to bracket each run. The accuracy, inter-day and intra-day precision were assessed by analyzing QC samples in six replicates in each of the three accuracy and precision (A&P) runs. Dilutional linearity was established by analyzing the dilution QC (10,000 ng/mL) in six replicates in each of the three A&P runs. The dilution QC was diluted 20 times with blank plasma and the diluted samples were processed with the rest of the plasma standard and QC samples. The selectivity of the assay was assessed by processing blank plasma from 6 different lots as single blanks (blanks with IS) and double blanks (blanks without IS). The Lower Limit of Quantitation (LLOQ) was assessed by using both LLOQ QC at 5.00 ng/mL and six LLOQ samples prepared in six different lots of blank human plasma at 5.00 ng/mL. The matrix effect was evaluated by comparing the peak areas of extracted blank samples spiked with analytes to those of neat solutions at 15 ng/mL.

Human plasma samples were collected in tubes containing EDTA from subjects who entered a clinical study. Prednisolone at 10 mg was given to the subjects as an oral solution. Human blood samples were collected up to 72 h after drug administration. Human plasma was harvested by centrifuging the blood for 5 min at 2000 × g at 4 °C. The human plasma was separated and stored at −20 °C. For these experiments, samples with the same timepoint from three subjects were pooled. These samples together with plasma standards and QC samples were extracted with the LLE method described in Section 2.5. The extracted samples were analyzed with both the LC–MS/MS and LC–HRMS methods. The data obtained from the LC–MS/MS method served as references for

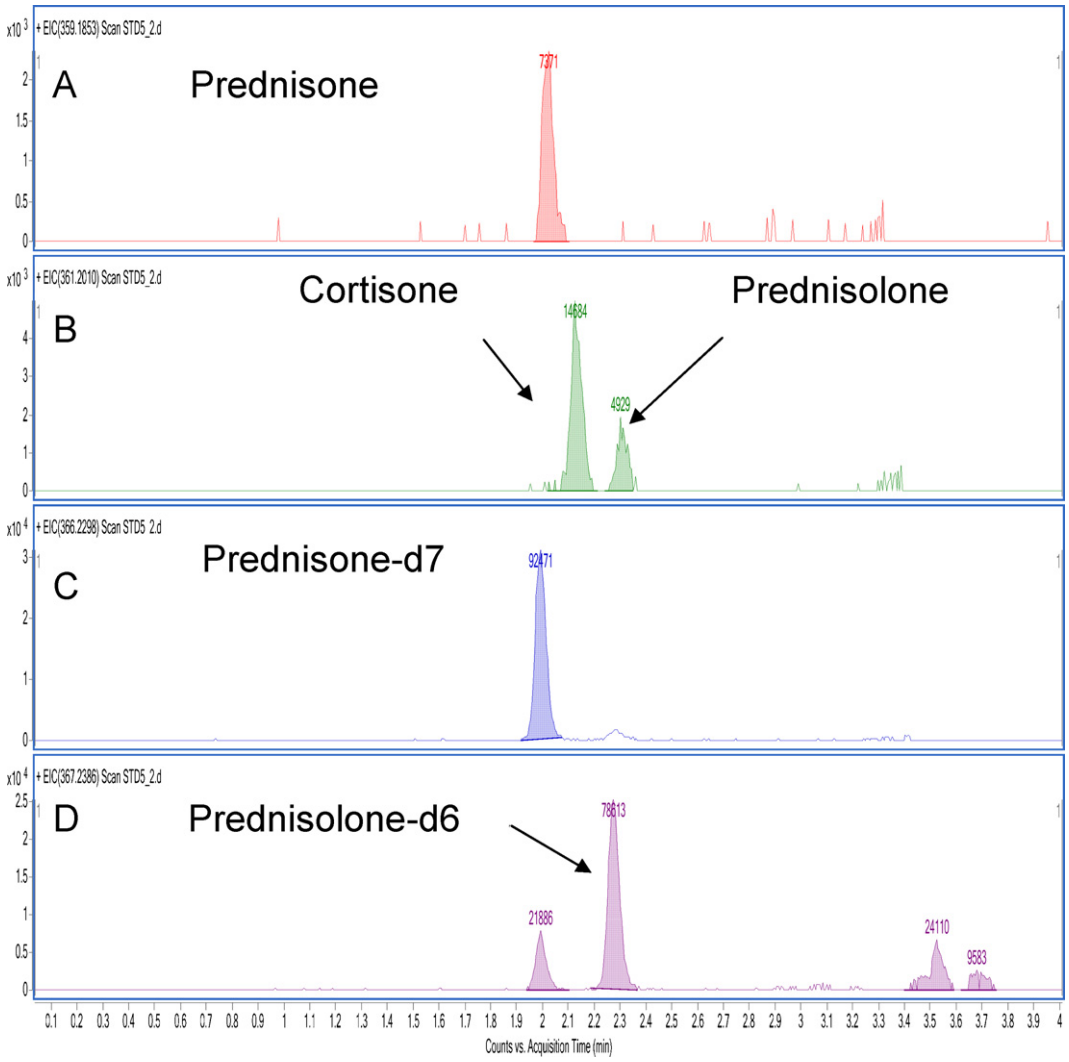

Fig. 2. Extracted ion chromatograms (EIC's) of (A) prednisone, (B) prednisolone, (C) prednisone-d7 and (D) prednisolone-d6 at LLOQ level (5 ng/mL) in human plasma by LC–HRMS.

evaluating the accurate determination of prednisone and prednisolone in incurred samples by the LC–HRMS method.

3. Results and discussion

3.1. Development of LC–HRMS method

Unlike LC–MS/MS methods, which require identifying the product ions and their optimal collision energies and the source parameters such as gas flow rate and temperature, LC–HRMS methods, in general, only require optimizing the source parameters. This allows the use of a single set of instrument parameters and thereby greatly simplifies the method development, especially if a large number of compounds are to be analyzed. Extracted ion chromatograms (EIC's) on individual compounds can then be obtained post data acquisition. Besides targeted analytes, information from other components that are present in the extracted samples can also be obtained similarly. These can be used to provide insights in potential assay interference, such as endogenous phospholipids co-eluting with targeted analytes.

3.1.1. Optimization of liquid chromatographic condition

With their high resolving power, full-scan high resolution accurate mass spectrometers offer another orthogonal dimension of separation in addition to the chromatographic separation. Even at a modest mass resolution of 10,000 and a relatively wide extraction window of 40 ppm, a LLOQ of 5 ng/mL can be achieved easily for small molecules such as prednisone (exact mass of 359.1853) and prednisolone (exact mass of 361.2010) with very low background (Fig. 2A and B). Special attention is needed, however, if isobaric compounds are present in the samples. With their identical exact masses, these compounds are not separated in the *m*/*z* dimension, and care is needed to ensure that they are separated chromatographically. This is illustrated by prednisolone, with an exact mass of 361.2010, which is isobaric with a naturally occurring glucocorticoid, cortisone (Fig. 1E, exact mass of 361.2010). Cortisone is present in plasma samples at various concentrations and it is, therefore, essential to separate cortisone from prednisolone for accurate measurement of prednisolone. Using methanol as organic modifier and a relatively shallow LC gradient, we were able to separate these two compounds (Fig. 2B).

3.1.2. Optimization of mass spectrometric parameters

During method development, we initially used a scan rate of 3 spectra/s for data acquisition in combination of a LC flow rate of 0.6 mL/min to insure that a sufficient numbers of data points were collected across the chromatographic peaks for robust quantitation. With data collected in the profile mode, the amount of

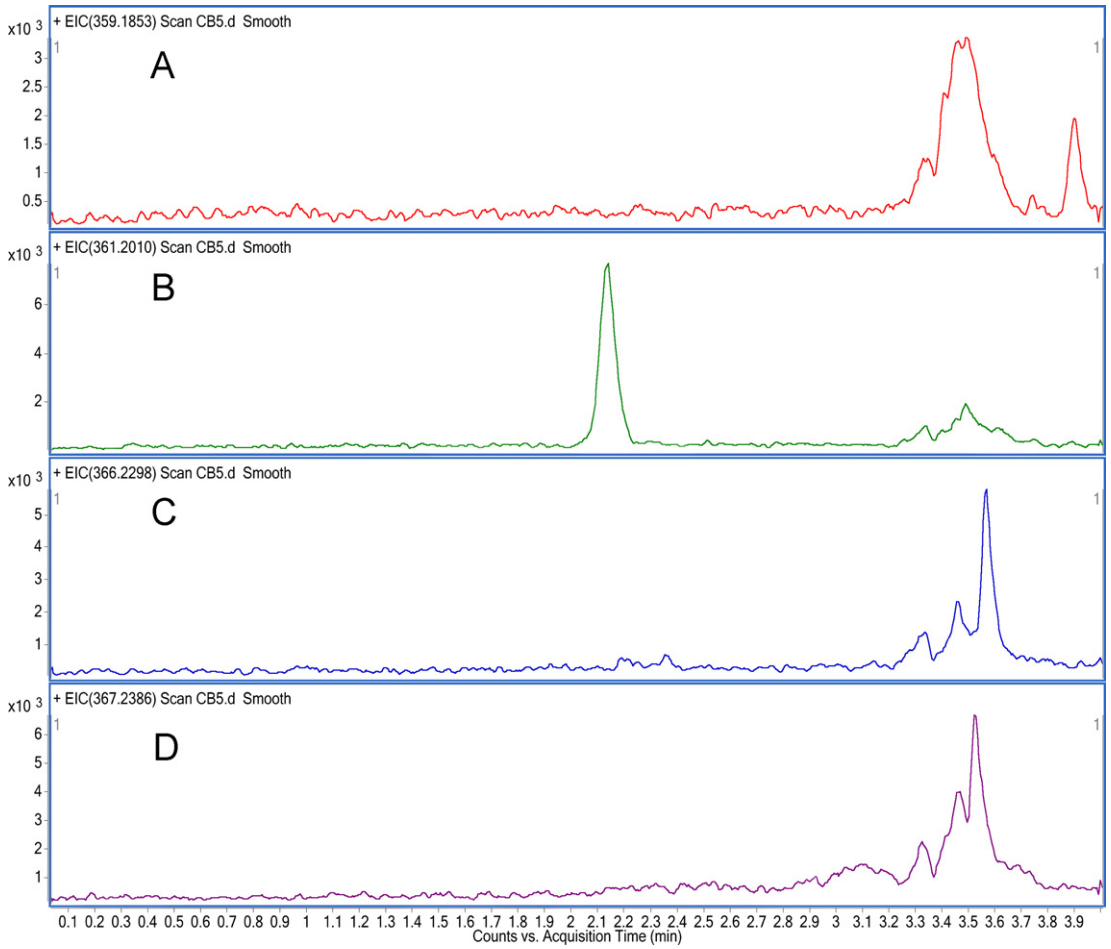

Fig. 3. Extracted ion chromatograms (EIC's) of (A) prednisone, (B) prednisolone, (C) prednisone-d7 and (D) prednisolone-d6 of double blank sample in human plasma by LC–HRMS. Cortisone is present in the prednisolone EIC (B).

data collected was enormous and it became difficult to process the data efficiently. We then evaluated the feasibility of using a scan speed of 2 spectra/s and lowering the LC flow rate to 0.5 mL/min. With these parameters, we were able to collect at least 15–18 data points across the chromatographic peaks which were more than sufficient for quantitation. It should be noted that when a higher LC flow rate is desired for fast chromatography, one should optimize the scan rate to make ensure a sufficient number of data points are collected.

In terms of mass resolution, Xia et al. [18] demonstrated that a mass resolution of 20,000 is adequate to achieve accurate and precise quantitation in plasma extract. During method development, our initial attempt was to use mass resolution of 20,000 with profile mode for data acquisition. However, our experience indicated that due to detector saturation at the high end, increased resolution came at the expense of a narrower dynamic range. We then explored the possibility of using lower resolution of 10,000 and centroid mode since detector saturation is less severe. Close examination of the chromatograms for the LLOQ and double blank samples (Figs. 2 and 3) obtained with the 10,000 resolution setting did not detect any additional peaks at the retention time of the analytes of interest, and good linearity was obtained from 5 to 2500 ng/mL. The lack of interference in this case was also partly attributed to the use of LLE as the extraction method which yielded cleaner samples. During method development, it is therefore important to carefully evaluate the effect of mass resolution, the mode of data acquisition (centroid vs. profile) on linearity, and potential presence of interfering endogenous peaks under the sample extraction procedure employed. With the TOF mass spectrometer we used for this work, the dynamic range obtained with mass resolution of 20,000 was 5–1250 ng/mL. It should be noted that with the newer generation of TOF mass spectrometers, it is feasible to extend the dynamic range at higher resolution settings.

After full-scan data were acquired, extracted ion chromatograms for each of the analytes and their internal standards were generated with a specified MEW for all samples. A wide MEW could introduce unwanted interference or higher background by extracting responses from compounds with similar m/z's to the analyte, along with the analyte of interest. On the other hand, a very narrow MEW increases the probability of reporting a false negative at low concentrations, especially in centroid mode [18]. For our LC–HRMS method, we found that negligible background noise was observed with a relatively wide MEW of 40 ppm. In addition, we did not observe any detrimental effect on the accuracy and precision of the method, even at low concentrations. This again was likely attributed to the use of LLE, which gave a relatively clean extract. A narrower MEW may be needed for cruder extracts obtained with protein precipitation procedures. It is therefore important to carefully evaluate the effect of MEW on accuracy and precision of the quantitation, the potential presence of interfering endogenous peaks, or false negative at low concentrations during method development, and potentially during sample analysis phase.

Table 1
Accuracy and precision for quantifying prednisone and prednisolone in human plasma by LC–HRMS and LC–MS/MS.

	Nominal conc. (ng/mL)	LLOQ (5.00)	Low (15.00)	GM (120.00)	Mid (1250.00)	High (1875.00)	Dilution (10,000.00)
LC–HRMS prednisone	Mean observed conc.	5.33	15.06	118.10	1310.09	1950.28	10,396.13
	Accuracy	106.6	100.4	98.4	104.8	104.0	104.0
	Inter-day precision (%CV)	4.3	0.7	0.3	2.2	0.0	0.0
	Intra-day precision (%CV)	3.7	2.7	1.6	1.7	2.4	2.2
	n	18	18	16	18	18	18
LC–MS/MS prednisone	Mean observed conc.	5.25	15.62	124.12	1274.67	1894.73	10,285.11
	Accuracy	105.0	104.1	103.4	102.0	101.1	102.9
	Inter-day precision (%CV)	2.5	0.0	0.6	0.0	0.7	0.0
	Intra-day precision (%CV)	4.5	2.7	2.3	2.7	1.6	2.3
	n	18	18	18	18	18	18
LC–HRMS prednisolone	Mean observed conc.	5.42	15.33	122.05	1328.38	1976.58	10,447.74
	Accuracy	108.4	102.2	101.7	106.3	105.4	104.5
	Inter-day precision (%CV)	3.1	1.3	1.7	1.3	0.9	1.1
	Intra-day precision (%CV)	7.4	2.9	2.6	2.0	1.5	2.3
	n	18	18	16	18	18	18
LC–MS/MS prednisolone	Mean observed conc.	5.34	16.03	129.30	1307.95	1924.55	10,626.57
	Accuracy	106.8	106.9	107.8	104.6	102.6	106.3
	Inter-day precision (%CV)	3.0	2.4	0.0*	1.1	0.0*	0.0*
	Intra-day precision (%CV)	4.2	2.5	1.7	1.7	1.9	1.7
	n	18	18	18	18	18	18

Data extracted from Watson ANOVA table.

3.2. Evaluation of performance of LC–HRMS method

In order to assess the feasibility of applying full scan HRMS to regulated bioanalysis, we evaluated its performance on the quantitation of prednisone and prednisolone in human plasma in terms of sensitivity, selectivity, accuracy, precision and matrix effect required for the regulated bioanalysis. We also compared the results from the LC–HRMS-based analysis of incurred samples with those results obtained from a validated LC–MS/MS method.

3.2.1. Accuracy and precision

The accuracy and precision of the method were evaluated by analyzing spiked human plasma QC samples against freshly prepared human plasma standards in three separate A&P runs. As shown in Table 1, the LC–HRMS method provides accurate and precise measurement of analytes with regard to the requirements of regulated bioanalysis [23]. The between-run (inter-day) and within-run (intra-day) precision of the LC–HRMS method for all levels of analytical QC's (low, GM, mid and high) were within 1.7% and 2.9%, respectively. The assay accuracy was between 98.4% and 106.3%. Furthermore, the accuracy and precision achieved by the LC–HRMS method was similar to those obtained by the LC–MS/MS method. Dilutional linearity was also demonstrated as shown by the data on dilution QC in Table 1.

In addition, we have evaluated the measured mass accuracy of prednisone and prednisolone at different plasma concentrations. It is important to examine the mass accuracy since it provides additional information on potential interferences from endogenous components. If there is a co-eluting endogenous component with a m/z ratio that is close to the analyte's (for example, a mass difference of only 20 ppm at a resolution of 10,000), it can potentially interfere with the measured mass accuracy because the measured mass is a summation of the exact masses of the analyte and the interference. This effect is more prominent at low analyte concentrations. An interfering component (especially one with much higher ion intensity) will shift the measured mass of the analyte from the theoretical mass. On the other hand, the measured mass of the analyte may shift at a very high concentration due to the flattening of the top (apex) of mass peak, making it difficult for the system to assign the correct mass. With the current LC–HRMS method, the mass accuracy at the apex of the chromatographic peak was excellent at all plasma concentrations (<6 ppm) (Table 2). Taken together with the fact that the peak areas were proportional with analyte concentration, it can be concluded that there was no significant interference from endogenous components, nor significant saturation of the detector.

3.2.2. Linearity and Limit of Quantitation (LLOQ)

The LC–HRMS method had acceptable linearity in the concentration range of 5.00–2500 ng/mL for both prednisone and prednisolone, with $r^2 > 0.99$ for all standard curves of prednisone and prednisolone in the three A&P runs. The regression model used was linear with $1/x^2$ weighting. The analytes had excellent peak signals at LLOQ level (Fig. 2). The LLOQ of the assay was established by analyzing LLOQ samples prepared by spiking prednisone and prednisolone to six different lots of human blank plasma at 5.00 ng/mL, and replicate analysis of QC samples prepared at LLOQ level (LLOQ QC) in three separate A&P runs. Based on the data shown in Tables 1 and 3, all LLOQ samples and LLOQ QC samples had good accuracy, ±109.1% of the nominal concentration and ±108.4% of the nominal concentration, respectively. This demonstrated that the LC–HRMS method can meet the stringent LLOQ criterion commonly required for a validated method.

By using an ion source equipped with the Jetstream Thermo Gradient Focusing Technology, the sensitivity of the LC–HRMS assay can be further improved at least ten fold to 0.5 ng/mL for both prednisolone and prednisone (Table 4), compared with the LLOQ of 5 ng/mL achieved with the Dual ESI source. In general, this level of sensitivity should be sufficient for supporting most pharmacokinetic and toxicokinetic studies. It should be noted that with the increased sensitivity of the Jetstream ion source, the detector may saturate at a lower concentration because of the larger number of ions introduced into the TOF. It was not an issue under the conditions used for the current assay. It is, however, important to carefully evaluate the dynamic range of a particular analyte during method development.

3.2.3. Selectivity and matrix effect

The selectivity and matrix effect were evaluated on six different lots of human plasma. The selectivity was evaluated by examining the extracted ion chromatograms of prednisone and prednisolone in double blank human plasma extracted samples. No interfering

Table 2
Mass accuracy of prednisone and prednisolone at different concentrations (exact masses of prednisone and prednisolone: *m/z* 359.1853 and 361.2010).

Prednisone				Prednisolone			
STD (ng/mL)	Mean peak area	Measured *m/z*	Mass accuracy (ppm)	STD (ng/mL)	Mean peak area	Measured *m/z*	Mass accuracy (ppm)
5.00	7143	359.1853	0.00	5.00	4449	361.1991	−5.26
10.0	15,095	359.1839	−3.90	10.0	10,830	361.2002	−2.21
25.0	34,845	359.1851	−0.56	25.0	24,915	361.2007	−0.83
100	139,137	359.1849	−1.11	100	98,381	361.2006	−1.11
400	608,050	359.1856	0.84	400	408,470	361.2010	0.00
1250	1,922,218	359.1837	−4.45	1250	1,248,370	361.2014	1.11
2000	2,960,454	359.1839	−3.90	2000	1,990,385	361.2000	−2.77
2500	3,795,191	359.1838	−4.18	2500	2,410,389	361.1997	−3.60

Table 3
Determination of plasma samples at LLOQ level (5.00 ng/mL) in six different lots of human plasma by LC–HRMS.

	Prednisone		Prednisolone	
	Calculated conc. (ng/mL)	%Dev	Calculated conc. (ng/mL)	%Dev
Lot 1	5.11	102.2	5.36	107.2
Lot 2	5.09	101.9	5.35	107.1
Lot 3	5.29	105.9	4.91	98.3
Lot 4	5.28	105.5	5.00	100.0
Lot 5	5.06	101.3	5.43	108.6
Lot 6	5.06	101.3	5.46	109.1

Table 4
Sensitivity of LC–HRMS in determining prednisone and prednisolone with ion source equipped with Jetstream Thermo Gradient Focusing Technology.

Prednisone				Prednisolone			
STD (ng/mL)	Mean peak area ratio	%Dev	%CV	STD (ng/mL)	Mean peak area ratio	%Dev	%CV
0.500[a]	0.0119	97.9	17.8	0.500[a]	0.0053	106.4	13.5
1.00[a]	0.0199	101.9	7.6	1.00[a]	0.0103	98.4	8.4
2.50[b]	0.0429	100.8	11.7	2.50[b]	0.0268	98.4	7.9
5.00[c]	0.0778	96.1	4.8	5.00[c]	0.0523	95.0	3.3
10.0[c]	0.1583	100.8	2.2	10.0[c]	0.1081	97.6	1.5
25.0[c]	0.3961	102.6	2.2	25.0[c]	0.2808	101.0	2.5
100[c]	1.5715	102.6	0.8	100[c]	1.1281	101.2	1.8
400[d]	6.4088	104.8	1.3	400[b]	4.7089	105.6	1.7
1250[c]	19.1011	100.0	1.5	1250[c]	14.3952	103.3	1.4
2500[c]	34.3320	89.9	1.3	2500[c]	28.1952	101.2	2.0
Number of replicates: *a* = 4, *b* = 3, *c* = 6, *d* = 5				Number of replicates: *a* = 4, *b* = 5, *c* = 6			

peak was observed at the retention times of prednisone and prednisolone and their internal standards (Fig. 3). The matrix effect was evaluated by comparing the peak areas of extracted blank samples spiked with analytes to those of neat solutions at 15 ng/mL, and IS's at 500 ng/mL. Negligible matrix effect was observed for the LC–HRMS method from all six lots of human plasma evaluated (0.94–1.01 for both analytes, and 0.97–1.09 for their internal standards). Taken together, they proved that endogenous compounds did not affect the performance of the LC–HRMS method despite full-scan data being acquired.

Table 5a
Comparison of the results for quantifying prednisone in human incurred samples by LC–HRMS and LC–MS/MS.

Sample ID	HRMS	SRM	%Diff	Sample ID	HRMS	SRM	%Diff
Pool 1-D 1-0.5h	5.39	5.90	**4.5**	Pool 2-D 1-0.5h	<LOQ	<LOQ	**NA**
Pool 1-D 1-1h	11.15	11.40	**1.1**	Pool 2-D 1-1h	10.94	10.48	**2.1**
Pool 1-D 1-1.5h	15.09	15.48	**1.3**	Pool 2-D 1-1.5h	14.73	15.62	**2.9**
Pool 1-D 1-2h	19.25	19.90	**1.7**	Pool 2-D 1-2h	20.59	21.72	**2.7**
Pool 1-D 1-3h	24.98	26.39	**2.7**	Pool 2-D 1-3h	26.32	27.97	**3.0**
Pool 1-D 1-4h	27.26	28.57	**2.4**	Pool 2-D 1-6h	16.14	17.75	**4.8**
Pool 1-D 1-6h	18.01	18.95	**2.5**	Pool 2-D 1-8h	11.75	12.07	**1.4**
Pool 1-D 1-8h	10.69	11.12	**2.0**	Pool 2-D 1-10h	7.51	7.28	**1.6**
Pool 1-D 1-10h	6.28	6.32	**0.3**	Pool 2-D 1-12h	<LOQ	<LOQ	**NA**
Pool 1-D 1-12h	<LOQ	<LOQ	**NA**	Pool 2-D 1-24h	<LOQ	<LOQ	**NA**
Pool 1-D 1-24h	<LOQ	<LOQ	**NA**	Pool 2-D 3-0h	<LOQ	<LOQ	**NA**
Pool 1-D 3-0h	<LOQ	<LOQ	**NA**	Pool 2-D 3-0.5h	<LOQ	<LOQ	**NA**
Pool 1-D 3-0.5h	<LOQ	<LOQ	**NA**	Pool 2-D 3-1.5h	11.69	11.67	**0.1**
Pool 1-D 3-1.5h	13.42	13.28	**0.5**	Pool 2-D 3-3h	27.35	29.18	**3.2**
Pool 1-D 3-3h	24.50	25.85	**2.7**	Pool 2-D 3-10h	6.73	6.69	**0.3**
Pool 1-D 3-10h	7.17	7.27	**0.7**				
Pool 1-D 3-12h	<LOQ	<LOQ	**NA**				

%Diff = absolute ((conc. by HRMS − mean conc.)/mean conc.) × 100. Mean conc. = (conc. by HRMS + conc. by SRM)/2.

Table 5b
Comparison of the results for quantifying prednisolone in human incurred samples by LC–HRMS and LC–MS/MS.

Sample ID	HRMS	SRM	%Diff	Sample ID	HRMS	SRM	%Diff
Pool 1-D 1-0.5h	103.80	108.41	**2.2**	Pool 2-D 1-0.5h	83.77	84.38	**0.4**
Pool 1-D 1-1h	138.56	140.96	**0.9**	Pool 2-D 1-1h	137.11	142.19	**1.8**
Pool 1-D 1-1.5h	159.28	158.79	**0.2**	Pool 2-D 1-1.5h	186.16	181.99	**1.1**
Pool 1-D 1-2h	195.32	192.94	**0.6**	Pool 2-D 1-2h	201.28	195.10	**1.6**
Pool 1-D 1-3h	169.01	167.77	**0.4**	Pool 2-D 1-3h	181.41	177.90	**1.0**
Pool 1-D 1-4h	146.98	143.90	**1.1**	Pool 2-D 1-6h	91.93	93.42	**0.8**
Pool 1-D 1-6h	89.17	87.17	**1.1**	Pool 2-D 1-8h	50.00	54.06	**3.9**
Pool 1-D 1-8h	57.11	54.04	**2.8**	Pool 2-D 1-10h	31.41	30.51	**1.5**
Pool 1-D 1-10h	30.55	30.37	**0.3**	Pool 2-D 1-12h	20.22	18.82	**3.6**
Pool 1-D 1-12h	19.72	18.12	**4.2**	Pool 2-D 1-24h	<LOQ	<LOQ	**NA**
Pool 1-D 1-24h	<LOQ	<LOQ	**NA**	Pool 2-D 3-0h	<LOQ	<LOQ	**NA**
Pool 1-D 3-0h	<LOQ	<LOQ	**NA**	Pool 2-D 3-0.5h	54.27	57.42	**2.8**
Pool 1-D 3-0.5h	81.44	79.77	**1.0**	Pool 2-D 3-1.5h	157.33	151.28	**2.0**
Pool 1-D 3-1.5h	165.85	169.82	**1.2**	Pool 2-D 3-3h	185.65	187.56	**0.5**
Pool 1-D 3-3h	184.00	183.52	**0.1**	Pool 2-D 3-10h	30.63	29.74	**1.5**
Pool 1-D 3-10h	35.88	34.05	**2.6**	Pool 2-D 3-12h	18.40	18.22	**0.5**
Pool 1-D 3-12h	21.23	20.70	**1.3**				

%Diff = Absolute ((conc. by HRMS − mean conc.)/mean conc.) * 100. Mean conc. = (conc. by HRMS + conc. by SRM)/2.

3.2.4. Incurred sample analysis

Incurred samples were pooled and the pooled samples were analyzed using the LC–HRMS and LC–MS/MS methods. The concentrations determined by the LC–MS/MS method served as references for evaluating performance of measuring the analytes concentrations in incurred samples by the LC–HRMS method. The results from the two methods were essentially identical for both analytes as shown in Tables 5a and 5b. This demonstrated that the LC–HRMS method can provide quantitative measurement of prednisone and prednisolone, with similar results to the LC–MS/MS method. It should be noted that a small amount of prednisone was observed in the pooled samples because prednisolone was converted back to prednisone *in vivo* [32].

3.3. Post-acquisition data-mining

One of the advantages of full scan HRMS is the feasibility of post-acquisition data-mining. In contrast to SRM, ions from a wide mass range were acquired and this data can be mined at a later time, without any need of re-processing or re-injecting the samples. This is illustrated by post-acquisition examination of two naturally occurring glucocorticoids: cortisone and cortisol (Fig. 1E

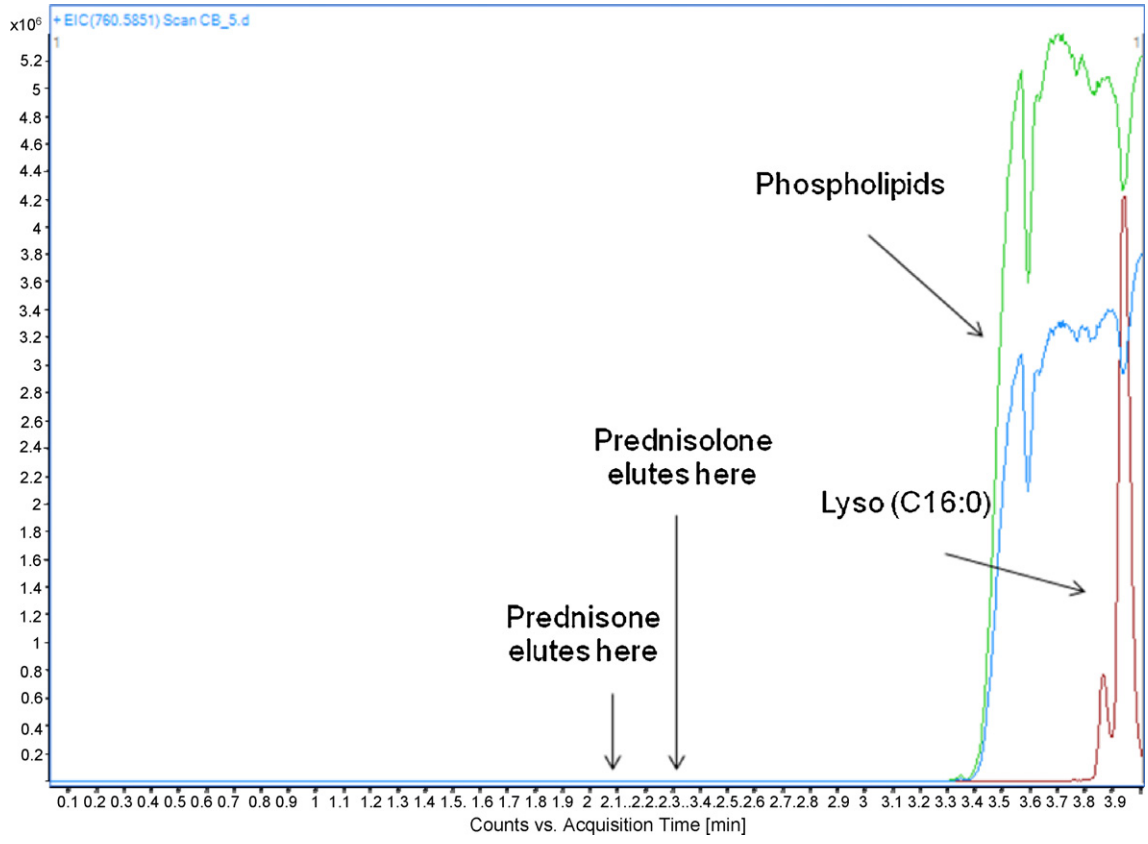

Fig. 4. Extracted ion chromatogram (EIC) of selected phospholipids in double blank human plasma sample by LC–HRMS. The blue trace was phosphatidycholine (PC) C16:0/C18:2 (exact mass of $[M+H]^+$ ion = 760.5851) while the green trace was phosphatidycholine (PC) C16:0/C18:1(exact mass of $[M+H]^+$ ion = 758.5094). The red trace was lysophosphatidycholine (Lyso PC) C16:0 (exact mass of $[M+H]^+$ ion = 496.3398).

and F, exact masses of 361.2010 and 363.2166). Cortisol is the pharmacologically active metabolite of cortisone. Their MS signals were acquired as part of the full scan data acquisition. By examining the peak areas of cortisone and cortisol in the EIC's of the six different lots of blank human plasma used in the matrix effect evaluation, we were able to obtain qualitative information on these two glucocorticoids in the different plasma lots, where peak areas of cortisol ranged from 41,101 to 134,006 while the peak area of cortisone ranged from 9355 to 13,807. It is interesting to note that the peak area of cortisone was quite consistent between different lots while the peak area of cortisol exhibited larger inter-lot variability. Besides accurate masses, the identity of the two glucocorticoids in the extracted samples was further confirmed by comparing the retention times of the analytes peaks in the EIC's of extracted plasma samples and neat solution of the standards.

In addition to monitoring biomarkers such as cortisol and cortisone, with the full-scan capability, it is feasible to monitor phospholipids post-data acquisition. This is illustrated in Fig. 4 in which EIC's from a lysophosphatidylcholine (lyso PC) and two phosphatidylcholines (PC's) are shown. There were no lyso PC's or PC's eluting at the retention times of the analytes, which confirmed the results from the matrix effect evaluation experiment described earlier. It is interesting to note that lysophosphatidylcholine (Lyso PC C16:0, exact mass 496.3398) was observed in spite of LLE being used as the extraction method. This is likely due to the use of acetonitrile in preparing the internal standard working solution which may permit the Lyso PC to be extracted into the organic layer [9]. Lyso PC's have been reported to elute earlier than the PC's [33] but in the present chromatographic conditions, it eluted in the same area of chromatogram as the PC's. This may be attributed to the particular combination of the LC column and mobile used for the LC–HRMS method. Another possibility could be that the observed peaks were from previous injections with another assay on the same column. Whatever the origin of the Lyso PC peak, this demonstrates the significant advantage of using LC–HRMS for targeted quantitative analysis as the technique allows retrospective querying of the acquired data.

It should be noted that when LLE (or SPE) is used as the extraction method, with their higher selectivity, it is likely that some of the biomarkers or metabolites are not extracted into the organic layer and therefore, are not available for post-acquisition analysis. Special care is therefore needed to interpret the qualitative data when LLE or SPE is used.

4. Conclusion

Here, we demonstrated that a full scan LC–HRMS assay meets the validation acceptance criteria in terms of accuracy, precision, selectivity, sensitivity, and matrix effect, commonly adopted for the LC–MS/MS approach. Furthermore, pooled incurred samples analyzed by both LC–HRMS and LC–MS/MS methods yielded essentially identical results. Taken together, it is feasible to use HRMS to support regulated bioanalysis. The LC–HRMS approach was also successfully used to obtain qualitative information on endogenous components such as phospholipids and naturally occurring glucocorticoids, via post-acquisition data-mining without the need of re-injecting/re-processing of the samples.

Additional work will be needed to fully implement and integrate HRMS in regulated bioanalysis. A thorough assessment of the effect of mass extraction window (MEW) and resolution on accuracy and precision of the method and further improvement in sensitivity and acquisition speed are some areas of focus for the technology and application. Another area that needs to be addressed is the amount of data generated from full-scan data acquisition. Thousands of samples are analyzed during the course of development of a drug candidate and the amount of data accumulated at the end will require careful consideration of storage space and retrieval of data for review. Finally, in-depth discussions with regulatory agencies will be needed to gain perspectives and feedback on this new technology platform, in particular with regard to post-acquisition data mining.

References

[1] M. Jemal, Biomed. Chromatogr. 14 (2000) 422.
[2] H.H. Maurer, Clin. Biochem. 38 (2005) 310.
[3] R.N. Xu, L. Fana, M.J. Riesera, T.A. El-Shourbagya, J. Pharm. Biomed. Anal. 44 (2007) 342.
[4] W.A. Korfmacher, Drug Discov. Today 10 (2005) 1357.
[5] S. Zhou, Q. Song, Y. Tang, N. Weng, Curr. Pharmaceut. Anal. 1 (2005) 3.
[6] N.R. Srinivas, R. Mullangi, Biomed. Chromatogr. 25 (2011) 65.
[7] I. Chu, A.A. Nomeir, Curr. Drug Metab. 7 (2006) 467.
[8] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, J. Chromatogr. B 877 (2009) 2198.
[9] M. Jemal, Z. Ouyang, Y. Xia, Biomed. Chromatogr. 24 (2010) 2.
[10] S. Ma, M. Zhu, Chem. Biol. Interact. 179 (2009) 25.
[11] M. Zhu, L. Ma, D. Zhang, K. Ray, W. Zhao, W.G. Humphreys, G. Skiles, M. Sanders, H. Zhang, Drug Metab. Dispos. 34 (2006) 1722.
[12] M. Zhu, L. Ma, H. Zhang, W.G. Humphreys, Anal. Chem. 79 (2007) 8333.
[13] H.H. Maurer, Ther. Drug Monit. 32 (2010) 324.
[14] H. Zhang, D. Zhang, K. Ray, M. Zhu, J. Mass Spectrom. 44 (2009) 999.
[15] N.R. Zhang, S. Yu, P. Tiller, S. Yeh, E. Mahan, W.B. Emary, Rapid Commun. Mass Spectrom. 23 (2009) 1085.
[16] M.W.F. Nielen, M.C. van Engelen, R. Zuiderent, R. Ramaker, Anal. Chim. Acta 586 (2007) 122.
[17] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Anal. Chim. Acta 673 (2010) 60.
[18] Y. Xia, J. Lau, T. Olah, M. Jemal, Rapid Commun. Mass Spectrom. 25 (2011) 2863–2878.
[19] M. Kellmann, H. Muenster, P. Zomer, H. Mol, J. Am. Soc. Mass Spectrom. 20 (2009) 1464.
[20] K.P. Bateman, M. Kellmann, H. Muenster, R. Papp, L. Taylor, J. Am. Soc. Mass Spectrom. 20 (2009) 1441.
[21] T. Rousu, J. Herttuainen, A. Tolonen, Rapid Commun. Mass Spectrom. 24 (2010) 939.
[22] T. Zhang, D.G. Watson, C. Azike, J.N. Tettey, A.T. Stearns, A.R. Binning, C.J. Payne, J. Chromatogr. B 857 (2007) 352.
[23] FDA, Guidance for Industry: Bioanalytical Method Validation, 2001.
[24] J.S. Jenkins, P.A. Sampson, Br. Med. J. 2 (1967) 205.
[25] R. Earla, S.H. Boddu, K. Cholkar, S. Hariharan, J. Jwala, A.K. Mitra, J. Pharm. Biomed. Anal. 52 (2010) 525.
[26] R. DiFrancescoa, V. Frerichsa, J. Donnellya, C. Haglera, J. Hochreitera, K.M. Tornatore, J. Chromatogr. B 859 (2007) 42.
[27] I.A. Ionita, D.M. Fast, F. Akhlaghi, J. Chromatogr. B 877 (2009) 765.
[28] V. Garga, W.J. Jusko, J. Chromatogr. 567 (1991) 39.
[29] R.L. Teng, L.Z. Benet, J. Chromatogr. 493 (1989) 421.
[30] G.R. Cannell, R.H. Mortimer, D.J. Maguire, R.S. Addison, J. Chromatogr. 563 (1991) 341.
[31] I.A. Ionita, F. Akhlaghi, Ann. Clin. Biochem. 47 (2010) 350.
[32] J.Q. Rose, A.M. Yurchak, W.J. Jusko, Biopharm. Drug Dispos. 1 (1980) 247.
[33] Y. Xia, M. Jemal, Rapid Commun. Mass Spectrom. 23 (2009) 2125.