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Determination of prednisolone in human adipose tissue incubation medium using LC–MS/MS to support the measurement of 11β-hydroxysteroid dehydrogenase activity☆

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ABSTRACT

A method for the determination of prednisolone in human adipose tissue incubation medium has been developed, validated and used to support studies designed to measure the activity of 11β-hydroxysteroid dehydrogenase in human adipose tissue. After incubation, samples (80 μL) were extracted using Oasis HLB μElute SPE plates and the resulting extracts were analyzed using reversed-phase chromatography coupled to an Applied Biosystems Sciex PE API-4000 mass spectrometer with a TurboIonSpray® interface (400 °C). The method was validated over the calibration range of 0.5–100 ng/mL. Intraday precision and accuracy were 6.1% R.S.D. or less and within 6.3%, respectively. Interday precision and accuracy were 4.2% R.S.D. or less and within 3.6%, respectively. Extraction recovery of prednisolone was greater than 84% over the range of low to high quality control sample concentrations. The validated assay was used to support studies designed to estimate *ex vivo* 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme activity in human adipose tissue.

1. Introduction

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is an enzyme involved in the extra adrenal production of cortisol, the principal circulating endogenous glucocorticoid in humans, from cortisone (Fig. 1). Preclinical experiments [1–3] have associated increased 11β-HSD1 activity with hyperglycemia, hyperlipidemia, insulin resistance and obesity, thereby supporting this enzyme as a potential therapeutic target. In humans, 11β-HSD1 is highly expressed in adipose and liver tissue and increased 11β-HSD1 expression has been associated with obesity [4–6].

The rate of *in vivo* conversion of cortisone to cortisol has previously been studied as a biomarker of 11β-HSD1 activity [7]. These experiments consisted of intravenous dosing of deuterated (d4) cortisol to human subjects and measurement of the rate of conversion to either deuterated (d3) cortisone or deuterated (d3) cortisol, before and after the oral dosing of carbenoxolone, a general inhibitor of both 11β-HSD1 and 11β-HSD2. The d3-cortisone or d3-cortisol could then be distinguished from respective endogenous glucocorticoids using LC–MS/MS. We sought to adapt this biomarker approach and apply it in an *ex vivo* fashion to adipose tissue samples biopsied from human subjects. These efforts were hampered due to the fact that isotopically labeled cortisone obtained from commercial sources was susceptible to isotopic exchange when in solution. Attempts to custom synthesize cortisone labeled at sites on the molecule not subject to exchange met with little success; all batches that were synthesized had a high proportion of either chemical or isotopic impurities.

Due to the difficulty of obtaining appropriate stable-labeled cortisone, prednisone was considered as an alternative substrate for measuring 11β-HSD1 activity. It was determined in initial experiments that prednisone was converted to prednisolone (Fig. 1) *in vitro* by 11β-HSD1 at a rate that was kinetically similar (data not shown) to the conversion of cortisone to cortisol, and that prednisolone could also be distinguished from endogenous glucocorticoids using LC–MS/MS. An experimental procedure was then developed for using prednisone as an *ex vivo* probe of 11β-HSD1. The development of the LC–MS/MS method used to support these studies and its application to support the profiling of enzyme activity in human adipose samples is described below.

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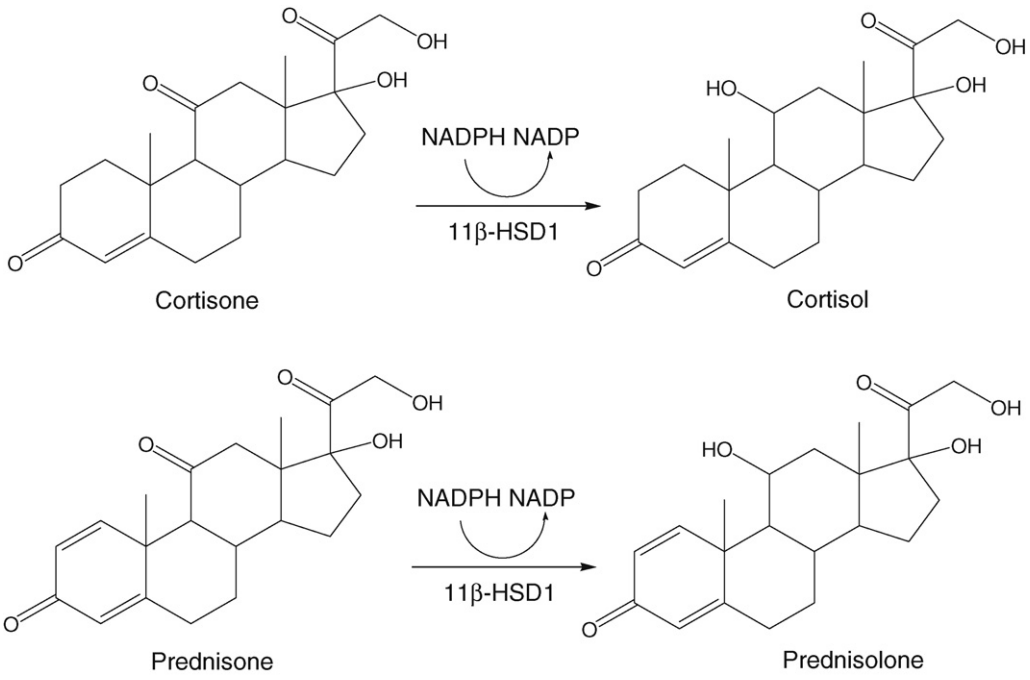

Fig. 1. Schemes showing conversion of cortisone to cortisol (top) and prednisone to prednisolone (bottom).

There are many recent LC–MS/MS methods which have been published which detail either single or simultaneous determination of different glucocorticoids, including prednisolone, from biological matrices. Methods for the determination of glucocorticoids have been developed for clinical applications [8–11], and are of special interest in the veterinary arena due to glucocorticoid use in the food industry [12–14] or in horse racing [15,16]. While LC–MS/MS methods for dosed glucocorticoids are numerous, few published methods are reported as fully validated to the current FDA criteria on bioanalytical methods validation [17]. In addition, there are few studies where fully validated LC–MS/MS methods have been applied to probe molecules used to support biomarker activities.

2. Experimental

2.1. Materials

Prednisolone was obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). The internal standard, d4-cortisol, was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Acetonitrile, methanol and water (HPLC grade) were obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid (Reagent grade) was from Aldrich, Inc. (St. Louis, MO, USA). Sodium phosphate monobasic was from J.T. Becker (Phillipsburg, NJ, USA). Dulbecco's Modified Eagle Medium was from Invitrogen Corporation (Carlsbad, CA, USA). All other reagents were ACS grade and were used as received. Oasis HLB μElute 96-well SPE plates were obtained from Waters Inc. (Milford, MA, USA).

2.2. Instrumentation

The LC–MS/MS system consisted of a Shimadzu system controller, two Shimadzu pumps (Columbia, MD, USA) and an API-4000 triple quadrupole mass spectrometer with a TurboIonSpray® interface (400 °C) from Applied Biosystems PE Sciex (Foster City, CA, USA). Data were collected and processed using Analyst® software (version 1.3) from Applied Biosystems. Samples were introduced to LC–MS/MS system through Leap CTC PAL Autosampler (Carrboro, NC, USA).

2.3. Chromatographic conditions

Extracted samples were separated by reversed-phase liquid chromatography on a Phenomenex (Torrance, CA, USA) Luna $C_{18}(2)$ 2.0 mm × 50 mm (5 μm) column preceded by a Varian precolumn filter (Walnut Creek, CA, USA). The mobile phases were water containing 0.02% formic acid (Mobile phase A) and methanol containing 0.02% formic acid (Mobile phase B). A gradient was performed from 50% to 85% Mobile phase B over 4 min at a flow rate of 0.35 mL/min.

2.4. MS/MS detection

Prednisolone and the internal standard d4-cortisol were dissolved and diluted in 50/50% (v/v) methanol/water to a concentration of 1 μg/mL. The neat solutions were directly infused separately into the API-4000 mass spectrometer using a syringe pump (Harvard Apparatus, Holliston, MA, USA) during the parameter optimization. Precursor ions for prednisolone and d4-cortisol were determined from mass spectra using the TurboIonSpray® source operating in the positive ionization mode. Quantitative Optimization of Analyst software (Applied Biosystems) was used in automatic optimization of the MS parameters followed by manual adjustment of source parameters to further maximize the response.

2.5. Adipose sample collection

Subcutaneous adipose tissue samples from volunteers were obtained under protocols that were reviewed and approved by Institutional Review Board. The tissues were provided by ZenBio Inc. (North Carolina). Following surgery, samples were flash frozen in liquid nitrogen and then stored at − 80 °C.

2.6. Preparation of human adipose tissue samples and control incubation medium

Control medium used for calibration was generated by incubating sections of frozen adipose tissue (~400 mg) in individual microfuge tubes in Dulbecco's Modified Eagle Medium (1.6 mL) on a shaking platform in a water bath for 30 min at 37 °C. Following the incubation, the tubes were centrifuged at 4 °C for 10 min at 20,800 × *g*. The supernatant was gently removed with a pipette and transferred to pre-chilled 50 mL centrifuge tubes followed by centrifugation at 2500 × *g* for 10 min at 4 °C to remove residual particulate material. Supernatants from all incubations performed using the same lot of tissue were pooled, mixed and aliquots were transferred into pre-chilled centrifuge tubes. They were stored at −60 to −80 °C and used as blank matrix for validation and sample analysis.

For analysis of the turnover of prednisone in human adipose samples, 50 mg aliquots of frozen tissue were used for the incubations which were carried out as described above, with the inclusion of 1 μM prednisone. Supernatant was collected and stored at − 60 to − 80 °C prior to the determination of prednisolone.

2.7. Preparation of standards and quality control samples

All standard solutions were prepared in 50/50% (v/v) methanol/water. The initial stock solution containing prednisolone (100 μg/mL) was diluted to give working standards containing 2, 4, 8, 32, 80, 200, 300, 400 and 10,000 ng/mL of prednisolone. Internal standard was prepared in 0.1 M sodium phosphate monobasic buffer in 99:1% (v/v) water:methanol at a concentration of 30 ng/mL. Standards of prednisolone in human adipose tissue incubation medium were prepared by adding 20 μL of each working standard to 80 μL of human adipose tissue incubation medium. The resulting incubation standards were used to quantitate clinical adipose tissue incubated samples containing prednisolone over the concentration range of 0.5–100 ng/mL.

Limit of quantitation (LOQ), low, middle, high and dilution quality control (QC) samples containing prednisolone at concentrations of 0.5, 1.5, 15, 80 and 120 ng/mL were prepared by diluting either 400 or 10,000 ng/mL stock solutions to 10 or 5 mL with control human adipose tissue incubation solution. Following preparation, 0.2-mL aliquots of quality control samples were transferred to separate 3.6-mL cryotubes (NUNC No. 379189), capped and stored at −60 to −80 °C. These quality control samples were used for validation of the method and analysis of human adipose tissue incubation medium.

2.8. Accuracy and precision in independent lots of matrix

To evaluate the accuracy and precision in independent sources of human adipose tissue incubation medium, six lots of human adipose tissue (from different patients) were incubated. Working standards (20 μL) containing 2, 4, 8, 32, 80, 200, 300, 400 and 10,000 ng/mL of prednisolone were spiked into the incubation solution (80 μL) from six different lots, respectively, resulting in six standard curves over the ranges of 0.5–100 ng/mL.

2.9. Extraction of incubation medium

An 80-μL aliquot of sample was pipetted into a Costar (Corning, NY, USA) 1.2-mL polypropylene tube in a 96-well format. A 100-μL aliquot of 30 ng/mL working internal standard solution was pipetted into each of the tubes containing the samples and the previously prepared standards. An additional aliquot of 0.1 M sodium phosphate monobasic buffer in 99:1% (v/v) water:methanol (100 μL) was added to tubes containing samples or quality controls (QCs) after which samples were vortexed.

Each well in an Oasis HLB μElute 96-well SPE plate was conditioned by sequential washes of 200 μL of methanol and water. The entire buffered sample was drawn through a well in the extraction plate using vacuum. The wells were each washed twice with 200 μL of 950:50:1% (v/v/v) water:methanol:formic acid and eluted with 80 μL of methanol into a 96-well collection plate. After elution, 40 μL of water was added to each well and the samples were mixed. The 96-well collection plate was capped with a polypropylene cover (Varian, Lake Forest, CA, USA) and transferred to an autosampler tray for injection (20 μL) onto the LC–MS/MS system.

2.10. Stability

Quality control samples ($n = 6$ at each concentration) were subjected to three freeze–thaw cycles consisting of storage at room temperature for at least 5 h, vortexing, and then refreezing at −60 to −80 °C for at least 12 h. After three freeze–thaw cycles the samples were analyzed using freshly spiked calibration standards. To evaluate bench-top stability, quality control samples ($n = 6$ at each concentration) were left on the bench-top at room temperature for 6 h prior to extraction. To determine the stability of extracted samples after storage in the autosampler tray, calibration standards ($n = 2$ at each concentration) and QC samples ($n = 6$ at each concentration) were extracted and analyzed. They were re-injected after storage in the autosampler tray at 8–12 °C for 3 days. Long-term stability was evaluated using quality control samples ($n = 4$ at each concentration) after storage in freezer at −60 to −80 °C for 156 days prior to extraction.

3. Results and discussion

3.1. MS/MS detection

Precursor ions for prednisolone and d4-cortisol were determined from mass spectra using the TurboIonSpray® source operating in the positive ionization mode. Under these conditions, the analytes yielded predominantly protonated molecules at *m*/*z* 361 and 367 for prednisolone and d4-cortisol, respectively. Optimized MS parameters are listed in Table 1. In a comparison using neat solution, it was observed that negative ionization produced less fragmentation than positive ionization for both prednisolone

Table 1
Optimized MS/MS parameters for prednisolone and d4-cortisol.

Analyte	Precursor/fragment	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)	Retention time (min)
Prednisolone	361.1/147.2	36	31	12	2.1
d4-cortisol	367.2/121.1	76	43	12	2.1
Prednisone	359.1/236.9	81	27	16	1.9
Cortisone	361.1/163.0	56	35	14	1.9
Cortisol	363.1/121.1	71	35	10	2.1

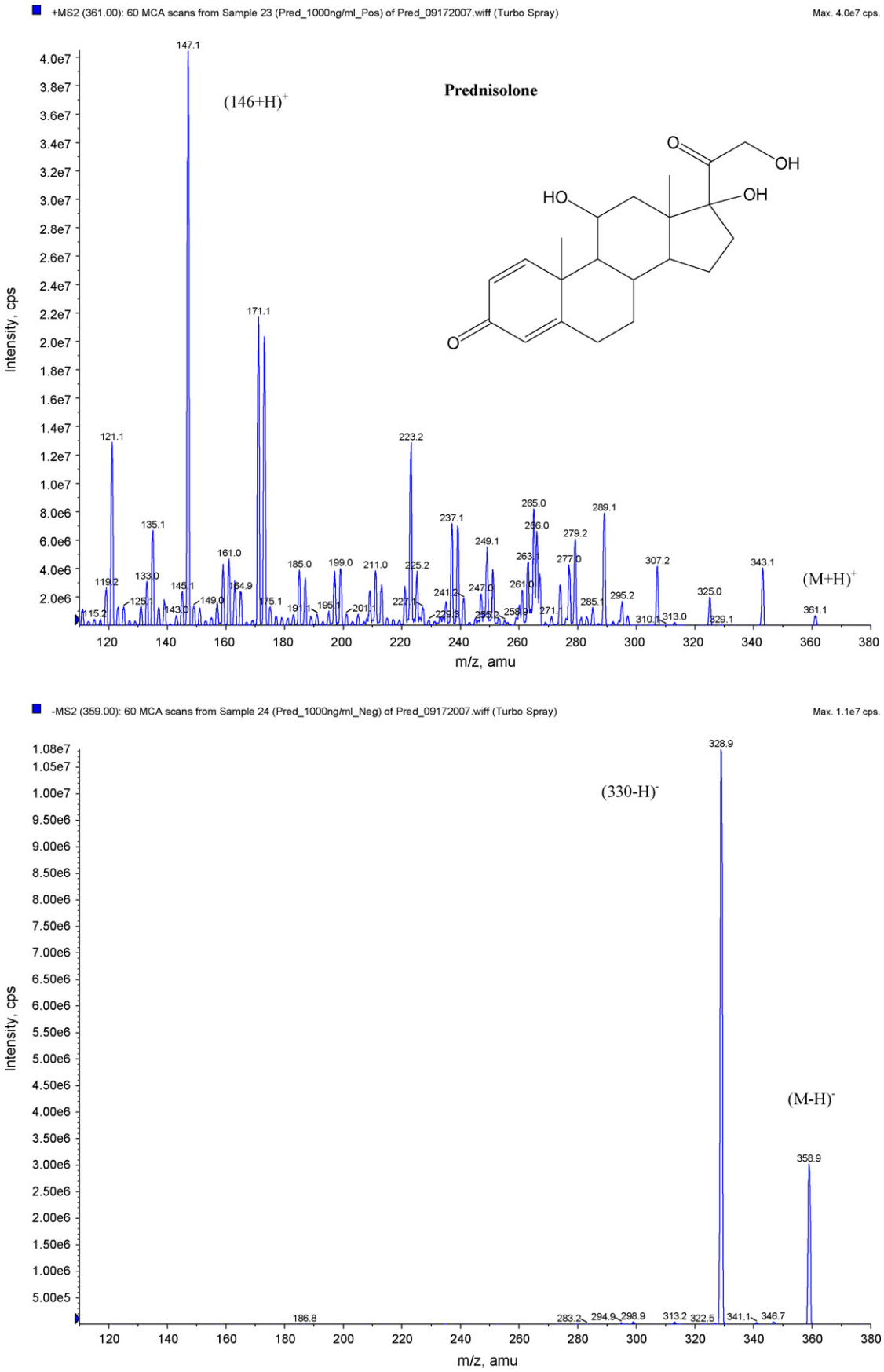

Fig. 2. Product ion mass spectra of the positive ion $(M+H)^+$ of prednisolone at *m*/*z* 361 (top panel) and negative ion $(M-H)^-$ of prednisolone at *m*/*z* 359 (bottom panel).

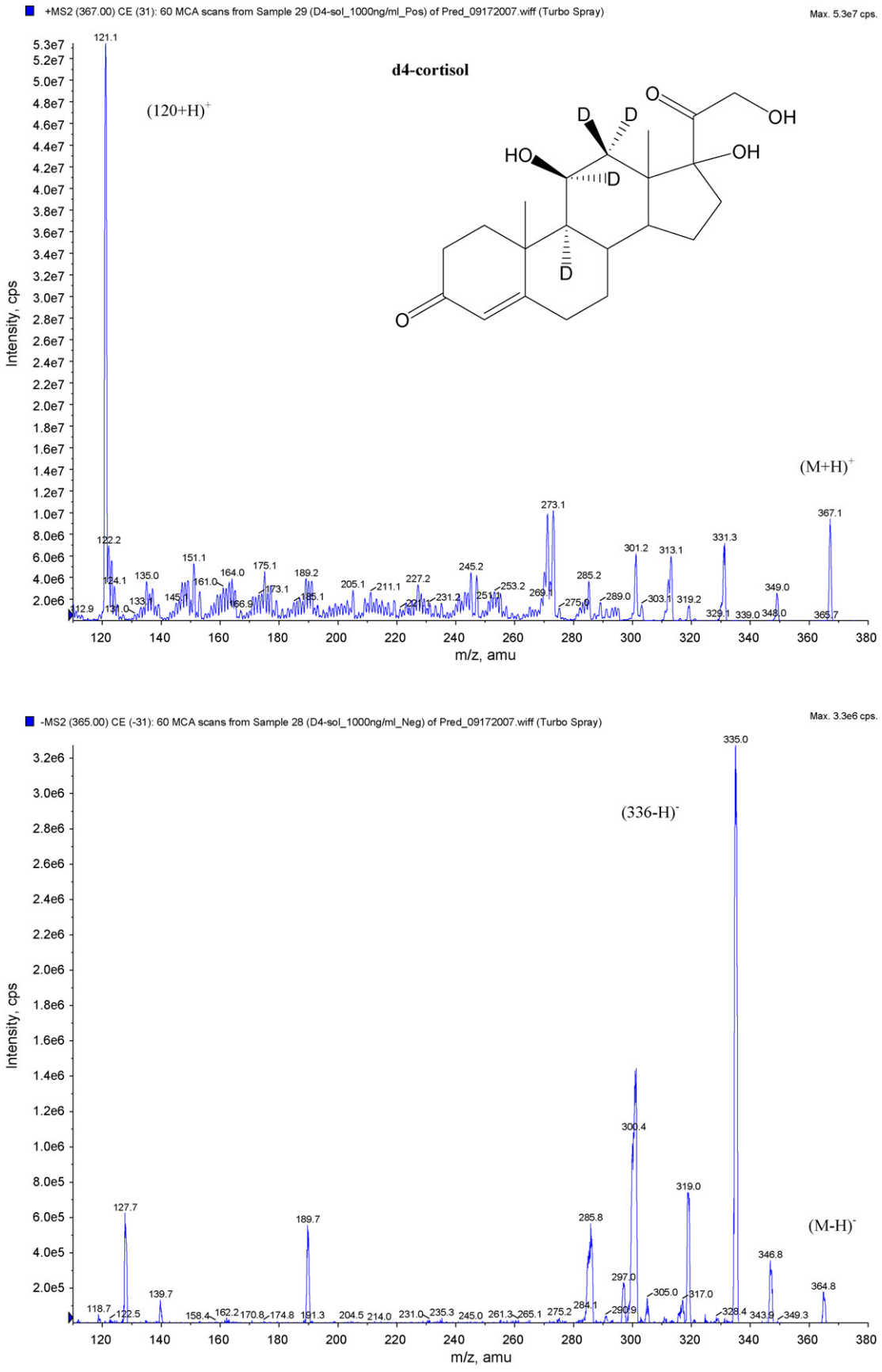

Fig. 3. Product ion mass spectra of the positive ion $(M+H)^+$ of d4-cortisol at m/z 367 (top panel) and negative ion $(M-H)^-$ of d4-cortisol at m/z 365 (bottom panel).

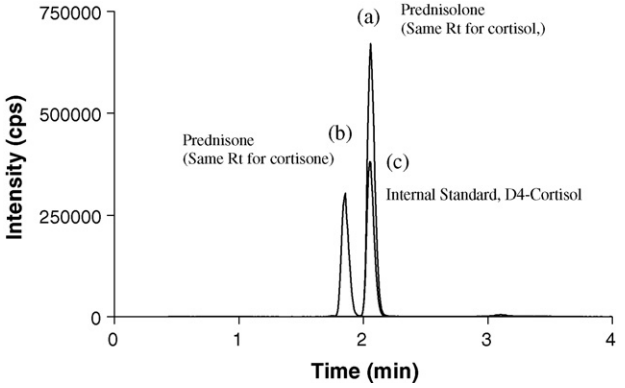

Fig. 4. Multiple reaction monitoring chromatograms from an injection of 50 ng/mL of (a) prednisolone, (b) prednisone, and (c) d4-cortisol.

and d4-cortisol (Figs. 2 and 3), which was in agreement with previous reports [18–20]. However, positive ion mode produced a much better signal-to-noise ratio (S/N) than negative ion mode when both were optimized for the glucocorticoids. The limit of detection (LOD) was about 10-fold better using positive ionization mode. The lower limit of quantitation (LLOQ) for prednisolone was 0.5 ng/mL (S/N of 134) and was sufficient for determining prednisolone concentrations observed in this study for adipose tissue incubation samples.

3.2. Cross-talk

Due to the presence of endogenous cortisone and cortisol in human adipose tissue and unconverted prednisone in the incubation medium, the method was optimized to allow chromatographic separation of prednisolone from prednisone and cortisone, using reverse phase chromatography. Using this method, a retention time (Rt) of 1.9 min was observed for both prednisone and cortisone while a Rt of 2.1 min was observed for prednisolone, cortisol and d4-cortisol (Fig. 4). There was no peak detected at the prednisolone retention time of 2.1 min when 100 ng/mL of prednisone, cortisone and d4-cortisol in neat solution were separately injected. There was "cross talk" observed from cortisol (about 0.2% of the cortisol area count) into the prednisolone channel when 100 ng/mL of cortisol in neat solution was injected. However, there was no measurable response into the prednisolone channel observed from endogenous cortisol when control human adipose tissue incubation samples were analyzed, probably indicating that endogenous cortisol in human adipose tissue samples was not high enough for 0.2% "cross-talk" to be detectable.

Table 2
Precision and accuracy data for determination of prednisolone (0.5–100 ng/mL) in six lots of human adipose tissue incubation medium.

Nominal (ng/mL)	Determined mean (ng/mL, $n=6$)	Accuracy[a] (%)	R.S.D.[b] (%)
0.5	0.51	101	10.3
1	0.98	97.9	6.74
2	1.99	99.3	4.78
8	7.73	96.6	2.44
20	19.7	98.6	4.78
50	50.6	101	3.02
75	76.5	102	2.42
100	103	103	2.53

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Relative standard deviation.

3.3. Intraday standard curve accuracy and precision in six lots of matrix

Separate assessments of intraday variability using six calibration curves prepared from six different lots (different patients) of control human adipose tissue incubation medium were performed. Accuracy and precision data are presented in Table 2. The intraday precision in six different lots of matrix, as measured by the relative standard deviation (%R.S.D.) was 10.3% or better at the LOQ (0.5 ng/mL) concentration and 6.7% or better for all other points on the calibration curves. Assay accuracy was found to be within 3.4% of nominal for all concentrations. The mean coefficient of variation for six calibration curves was 0.9959.

3.4. Intraday and interday accuracy and precision from QC samples

Intraday and interday accuracy and precision were obtained from experiments performed on three separate days, with two calibration curves and six replicates of quality control samples at each concentration on each day. Intraday and interday accuracy and precision from quality controls are shown in Table 3. The intraday relative standard deviation (%R.S.D.) from QC samples was ≤6.1%, while the accuracy was 95.9–106.3% of nominal at all concentrations including the LOQ quality control (0.5 ng/mL).

3.5. Stability

Stability of samples during multiple freeze–thaw cycles, after incubation at room temperature, after storage in the autosampler and re-injection, and long-term stability (−60 to −80 °C), was eval-

Table 3
Intraday and interday accuracy and precision of prednisolone quality control samples.

	LLOQ (0.5 ng/mL)	Low QC (1.5 ng/mL)	Middle QC (15 ng/mL)	High QC (80 ng/mL)	Dilution QC (120 ng/mL)
Intraday mean ($n=6$)	0.53	1.52	14.7	77.7	116
Accuracy[a] (%)	106	101	98.0	97.2	97.0
R.S.D.[b] (%)	4.91	1.85	1.89	1.78	0.57
Intraday mean ($n=6$)		1.48	14.3	75.1	
Accuracy[a] (%)		98.9	95.5	93.9	
R.S.D.[b] (%)		2.49	1.72	2.09	
Intraday mean ($n=6$)		1.59	14.7	78.5	
Accuracy[a] (%)		93.7	98.3	98.2	
R.S.D.[b] (%)		4.08	4.56	6.07	
Interday mean ($n=18$)		1.53	14.6	77.1	
Accuracy[a] (%)		102	97.3	96.4	
R.S.D.[b] (%)		4.18	3.14	4.16	

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Relative standard deviation.

Table 4
Stability of prednisolone.

Nominal concentration (ng/mL)	Bench-top stability at room temperature for 6 h			Storage at −60 to −80 °C for 156 days		
	Determined mean (ng/mL, $n=6$)	Mean %accuracy ($n=6$)	R.S.D. (%) ($n=6$)	Determined mean (ng/mL, $n=4$)	Mean %accuracy ($n=4$)	R.S.D. (%) ($n=4$)
1.5	1.52	100	2.06	1.48	98.9	4.28
15	14.7	98.2	1.38	14.7	97.7	1.74
80	77.0	96.3	0.99	77.6	97.0	0.67
Nominal concentration (ng/mL)	Stability after three freeze–thaw cycles			Storage in the autosampler at 8–12 °C for 3 days		
	Determined mean (ng/mL, $n=6$)	Mean %accuracy ($n=6$)	R.S.D. (%) ($n=6$)	Determined mean (ng/mL, $n=6$)	Mean %accuracy ($n=6$)	R.S.D. (%) ($n=6$)
1.5	1.53	102	6.01	1.48	98.7	3.78
15	14.3	95.5	2.85	15.2	101	3.19
80	78.6	98.3	5.36	80.4	100	1.71

uated and the results are summarized in Table 4. Prednisolone appears to be stable in human adipose tissue incubation medium after three freeze–thaw cycles (%R.S.D. ≤ 6.0% and accuracy within 102.1% of nominal) and was stable after storage on the bench-top at room temperature for 6 h prior to extraction (R.S.D. ≤ 2.1% and accuracy within 96.3% of nominal). The %R.S.D. was ≤3.8% and accuracy was within 98.7% of nominal after extracted prednisolone QC samples were stored in the autosampler tray at 8–12 °C for 3 days. Long-term stability was evaluated after prednisolone was stored at −60 to −80 °C for 156 days and analyzed against freshly prepared calibration curves. The %R.S.D. was ≤4.3% and accuracy was within 97.0% of nominal. These data suggested that prednisolone was stable in human adipose tissue incubation medium for 156 days under this storage condition.

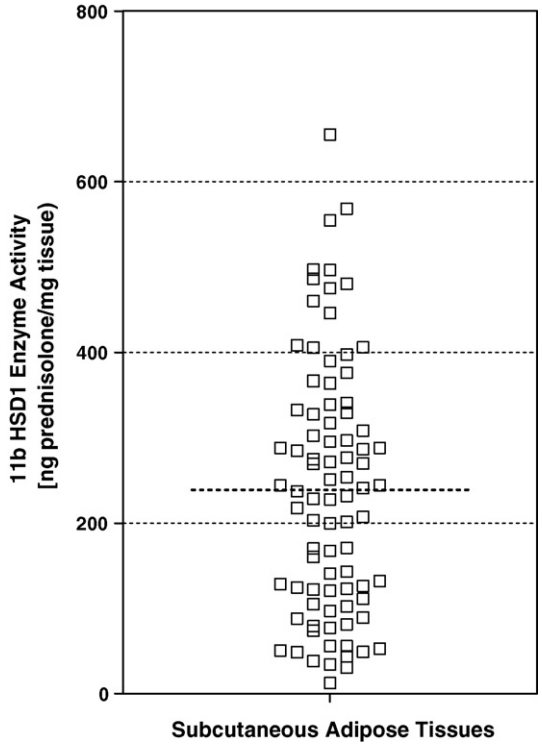

Fig. 5. Prednisolone concentrations measured after incubation of prednisone with adipose tissue samples obtained from 85 human subjects. Values ranged from 13 to 655 ng (35–1818 pMol)/mg of adipose tissue in the incubation. The mean prednisolone concentration was 239 ng (664 pMol)/mg of adipose (dotted line). Concentrations were corrected for the exact weight of the adipose tissue used.

3.6. Extraction recovery

Extraction recovery was evaluated for prednisolone using standard samples prepared at concentrations of 1, 20 and 75 ng/mL in control human adipose tissue incubation medium. Analyte recovery from the medium following extraction was determined by comparing the absolute peak areas of the standards spiked in human adipose incubation medium before extraction to control human adipose incubation medium extracted in the same manner then spiked post-extraction with a known quantity of prednisolone. Extraction recoveries for prednisolone were greater than 84% for all concentrations tested.

3.7. Human sample analysis

Two standard curves were processed for each batch, using human adipose tissue incubation medium containing prednisolone at concentrations of 0.5–100 ng/mL. Prednisolone concentrations were calculated from the equation $y = mx + b$, by weighted $(1/x^2)$ linear least square regression of the calibration line constructed from peak area ratios of prednisolone to internal standard versus nominal prednisolone concentration. These concentrations were then corrected for the quantity of adipose used in the incubation of patient samples.

Fig. 5 shows prednisolone concentrations determined by this method in adipose samples from 85 human subjects after incubation with prednisone. The concentration range for the prednisolone conversion product was from 13 to 655 ng (35–1818 pMol)/mg of adipose tissue. This result indicated that there was a wide range of endogenous 11β-HSD1 activities across subjects and justified the need for a robust and sensitive assay with a broad dynamic range. Calculation and comparison of relative 11β-HSD1 activities in different subject populations and the use of an 11β-HSD1 inhibitor to modulate this activity will be the subject of a future publication.

4. Conclusions

A μElute 96-well SPE extraction, LC–MS/MS method for the determination of prednisolone was developed and applied to the estimation of *ex vivo* 11β-HSD1 enzyme activity in human adipose tissue. Prestudy validation of the method was performed and the method was applied successfully to the analysis of adipose samples obtained from human subjects.

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