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Determination of corticosteroids in tissue samples by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for determination of corticosterone and 11-dehydrocorticosterone (11-DHC) levels in KKA^y mouse liver and adipose tissue, and hydrocortisone and cortisone levels in human adipose tissue has been developed. The corticosteroids were extracted from liver tissue with methanol/water and ethyl acetate for adipose tissue samples. Corticosterone and 11-DHC were separated with a methanol gradient and hydrocortisone and cortisone with an acetonitrile gradient containing trifluoroacetic acid on a reversed-phase column within 15 min. The corticosteroids were detected after electrospray ionization in positive mode with multiple reaction monitoring (MRM). The limits of quantification (LOQ) were estimated to be 15 nmol/kg liver and 1.6 nmol/kg adipose tissue for corticosterone and 5.4 nmol/kg liver and 0.92 nmol/kg adipose tissue for 11-DHC. The LOQ was estimated to be 0.2 nmol/kg adipose tissue for hydrocortisone and 0.4 nmol/kg adipose tissue for cortisone. The limits of detection (LOD) at 3 times S/N were estimated to be 0.07 nmol/kg adipose tissue for hydrocortisone 0.1 nmol/kg adipose tissue for cortisone. The variation of endogenous levels in KKA^y mouse from different animals (CV%) was high with mean liver tissue levels of 117 ± 25 (S.D.) nmol/kg for corticosterone and 62 ± 19 (S.D.) nmol/kg for 11-DHC ($n = 5$) and adipose tissue levels of 39 ± 20 (S.D.) nmol/kg for corticosterone and 2.4 ± 0.9 (S.D.) nmol/kg for 11-DHC ($n = 9$). Endogenous levels in human biopsy samples from adipose tissue were 12 ± 7.0 (S.D.) nmol/kg for hydrocortisone and 3.0 ± 1.6 (S.D.) nmol/kg for cortisone ($n = 16$). The new LC–MS/MS methods showed sufficient sensitivity and selectivity for determination of endogenous levels of corticosteroids in both KKA^y mouse liver and adipose tissue samples and human adipose tissue samples. The selectivity of the methods was verified by analysis of two different product-ions from each analyte.

Keywords: LC–MS/MS; Corticosterone; 11-Dehydrocorticosterone; Hydrocortisone; Cortisone; KKA^y mouse liver and adipose tissue; Human adipose tissue; Electrospray ionization

1. Introduction

Generally 11β-hydroxysteroid dehydrogenase1 (11β-HSD1) is thought to generate active glucocorticoid (corticosterone/hydrocortisone) from its inactive counterpart 11-DHC/cortisone [1]. Ricketts et al. have described the localization of 11β-HSD1 within human liver, adrenal, ovary, deciduas and adipose tissue [2]. The hyperglycaemia and hyperphagia of the KKA^y mouse appear to depend on glucocorticoids. KKA^y mouse has been used as a model for type 2 diabetes [3,4]. Therefore, we investigated the levels of endogenous corticosterone and 11-DHC in the target tissues, liver and adipose tissues from the KKA^y mice. In addition, there is an interest in determination of endogenous hydrocortisone and cortisone in human adipose tissue after administration of 11β-HSD inhibitors.

Traditionally, corticosteroids have been determined by fluorimetry [5], radioimmunoassay (RIA) [6], HPLC with ultraviolet absorption detection (HPLC-UV) [7,8] and gas chromatography–mass spectrometry (GC–MS) [9–11]. RIA offers high sensitivity and is used routinely but there is uncertainty in the selectivity of such assays [12]. Steroid hormones have been determined in muscle tissue

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samples by GC–MS that required derivatisation prior to gas chromatography [9]. LC–MS/MS has become the mainstay bioanalytical technique since sample preparation can be simplified without derivatisation, shorter chromatographic run time compared to GC and high sensitivity [13]. LC–MS offers advantages for determination of corticosteroids that are difficult to derivatize for GC–MS analysis and for determination of conjugated steroids (e.g., steroid sulfates and glucuronides) [14–16]. It can also be useful for compounds, which are less amenable for separation by GC [14]. Several ionization techniques for LC–MS operating at atmospheric pressure (API) have been used for steroids including electrospray ionization (ESI-MS) [17–19], atmospheric pressure chemical ionization (APCI) [15,20–22] and most recently atmospheric pressure photoionization (APPI) [23]. The choice of ionization technique is both compound- and instrument-dependent. The present work describes simple extraction procedures for the determination of corticosteroids in 0.1 g liver or 0.2 g adipose tissue together with instrumental conditions for selective and sensitive determination of corticosteroids. Validation of recovery, ion suppression, limit of quantification and selectivity are discussed.

2. Materials and methods

2.1. Chemicals

Corticosterone (4-pregnene-11β,21-diol-3,20-dione), 11-dehydrocorticosterone (4-pregnen-21-ol-3,11,20-trione), cortisone (4-pregnene-17α,21-diol-3,11,20-trione) and hydrocortisone (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) from (Sigma-Aldrich, Schnelldorf, Germany) and deuterium-labelled hydrocortisone (9,11,12,12-D4) from Cambridge Isotope Laboratories, Massachusetts, USA, were used.

Ammonia solution 25% pro analysi (Merck, Darmstadt, Germany), ethyl acetate pro analysi (Merck, Darmstadt, Germany), formic acid 98–100% pro analysi (Merck, Darmstadt, Germany), methanol (MeOH) LiChrosolv® gradient grade for liquid chromatography (Merck, Darmstadt, Germany), acetonitrile HPCL far UV (Labscan Dublin, Ireland), Milli-Q water, reagent grade deionised water (Milli-Q Plus: Millipore, Massachusetts, USA), *n*-heptane 95% HPLC (Lab-Scan, Dublin, Ireland) and trifluoroacetic acid Uvasol® for spectroscopy (Merck, Darmstadt, Germany) were used.

2.2. Tissue samples

KKA^y mouse samples, frozen liver and adipose tissue samples from untreated animals and frozen human subcutaneous adipose tissue were used for the determination of endogenous levels of corticosterone/11-DHC and hydrocortisone/cortisone and verification of the selectivity of the methods.

2.3. Homogenization of tissue samples

Tissue samples (liver and adipose) from KKA^y mouse were frozen in liquid nitrogen and stored at −70 °C. A piece of frozen mouse liver was weighed and homogenized by an Ultra-Turrax homogenizer in methanol (MeOH)/Milli-Q water (7:2, v/v) solution (5 ml/g liver) containing internal standard (50 nM cortisone) and adipose tissue was homogenized in ethyl acetate (5 ml/g adipose tissue) containing internal standard (5 nM cortisone). Both liver and adipose tissue homogenates were stored at −20 °C. Human adipose tissue samples were frozen and stored at −70 °C. A piece of frozen human adipose tissue was weighed and homogenized in ethyl acetate (5 ml/g adipose tissue) containing internal standard (10 nM hydrocortisone 9,11,12,12-D4). The samples were not thawed before homogenization with organic solvent to prevent further enzyme activity. Adipose tissue homogenates were stored at −20 °C.

2.4. Preparation of standards

Stock solutions of corticosteroids (corticosterone, 11-DHC, cortisone and hydrocortisone) were prepared in methanol. These stock solutions were diluted further with 25 or 50% MeOH (v/v) to make working solutions and stored at −20 °C. Aliquots of working solutions were spiked into matrices (pooled KKA^y mouse liver homogenate, pooled KKA^y mouse adipose homogenate and pooled human adipose tissue homogenate) to prepare calibration standards.

2.5. Sample preparation

Half a milliliter of the KKA^y mouse liver homogenate was shaken by a shaker model Mixer 5432 during 15 min in a 1.5-ml polypropylene tube (Eppendorf) and then centrifuged for 6 min at 20,200 × *g*. An aliquot of 100 μl supernatant was transferred to an HPLC vial containing 100 μl of 0.02% TFA in water and then 50 μl of the sample was injected into the LC–MS/MS system.

One milliliter of the KKA^y mouse adipose and/or human adipose homogenate was shaken in a 1.5 ml tube by a shaker model Mixer 5432 during 15 min and then centrifuged for 6 min at 20,200 × *g*. The organic phase was transferred to a new glass tube and evaporated by nitrogen gas at 40 °C to dryness. One milliliter heptane and 1 ml MeOH/water (7:2, v/v) solution was added and the tube was shaken by a shaker model IKA-Vibrax-VXR at 200 rpm during 15 min and then centrifuged at 2,060 × *g* for 10 min. The heptane phase containing triglycerides was aspirated to waste. The MeOH/water phase was evaporated by nitrogen gas at 40 °C to dryness and the residue was solved with 50 μl methanol. Then 150 μl of 0.02% TFA in water (v/v) was added and the tube was centrifuged at 2,060 × *g* for 10 min. One hundred microliters of the sample was injected into the LC–MS/MS system.

The spiked liver and adipose tissue homogenate standards were treated in the same way as the samples.

2.6. Instrumentation

The apparatus used were: homogenizer Ultra-Turrax T25 (Janker Kunkel Staufen, Germany), shakers Mixer 5432 (Eppendorf, Hamburg, Germany) and Typ VX2 and IKA-Vibrax-VXR (Janker Kunkel Staufen, Germany), centrifuges 5417R (Eppendorf, Hamburg, Germany) and GS-6R (Beckman, California, USA) and evaporator TurboVap LV (Zymark, Massachusetts, USA).

The HPLC system consisted of a HTS PAL autoinjector (CTC Analytics AG, Zwingen, Switzerland) and Hewlett Packard series 1100 LC-pumps (Agilent Technologies, Wilmington, DE, USA). A small molecule macrotrap, 3×8 mm (Michrom BioResources, California, USA) was coupled to the autoinjector six-port valve instead of a loop to concentrate and clean the sample.

The mass spectrometer was a Micromass Ultima Pt (Micromass, Manchester, UK) with a 4.0 version of MassLynx software.

2.7. Chromatography

Chromatographic separation for KKA^y mouse liver and adipose tissue samples were performed on a 5-μm Symmetry C8, 2.1×150 mm column (Waters, Massachusetts, USA). Mobile phase (A): 0.02% TFA in Milli-Q water (v/v) and mobile phase (B): 0.02% TFA in methanol (v/v) were used for elution. The gradient was from 50 to 70% (B) during 10 min, followed by 70 to 100% (B) during 0.5 min, continued for a minute at 100% (B) and then back to 50% (B) during 0.5 min followed by re-equilibration during 3 min with a flow rate of 0.3 ml/min. Human adipose tissue samples were separated on a 5-μm Luna C18(2), 2.0×150 mm column (Phenomenex, California, USA) with mobile phases consisting of (A): 0.02% TFA in Milli-Q water (v/v) and mobile phase (B): 0.02% TFA in acetonitrile (v/v). The gradient was from 25 to 40% (B) during 10 min followed by 40 to 100% (B) during 0.5 min continued during 1 min at 100% (B). Then back to 30% (B) during 0.5 min followed by re-equilibration for 4 min. The autosampler was washed with solvent 1 (1% ammonia in methanol, v/v) and solvent 2 (1% formic acid in Milli-Q water, v/v). The injection volumes were 50 μl for liver samples and 100 μl for adipose samples, respectively. The macrotrap column was washed with 0.25 ml of 1% formic acid in Milli-Q water before the valve was switched.

2.8. MS/MS

Detection was achieved by electrospray in positive ion mode with multiple reaction monitoring (MRM) for the transitions: m/z 347 → 121 for corticosterone, 345 → 121 for 11-DHC and 361 → 163 for cortisone, 363 → 121 for hydrocortisone and 367 → 349 for hydrocortisone (9,11,12,12-D4). Operating conditions were optimized by flow injection analysis. Two different product-ions were used to verify the selectivity for determination of corticosterone, 11-DHC, cortisone and hydrocortisone as shown in Table 1. The column effluent was diverted to the ion source from 3.5 to 10 min with the aid of a switching valve housed on the mass spectrometer.

2.9. Calculation

Spiked matrices were used for the calibration curves and unknown sample concentrations were calculated from the slope of peak area ratios of the corticosteroids and internal standard using a weighted least-square linear regression (weighting factor 1/concentration). Samples were homogenized in organic solvent using 5 ml/g liver and 5 ml/g adipose, respectively. One gram of matrix was assumed to be 1 ml. The concentration (nM) of unknown samples was determined from the calibration curve and then multiplied with the dilution factor 6 to get the concentration in the matrix (nmol/kg).

The concentration was calculated according to

$$\text{Concentration (nM)} = \frac{\text{peak areas ratio of the corticosteroid/IS}}{\text{slope}}$$

$$\text{Concentration (nmol/kg matrix)} = \text{concentration (nM)} \times \text{dilution factor}$$

Table 1
MRM transitions for analyte and IS

Analyte	Precursor ion (m/z)	Product ions (m/z)	Cone voltage (V)	Collision energy (eV)
Corticosterone	347	121	60	25
		311	60	16
11-Dehydrocorticosterone	345	121	60	23
		242	60	32
Hydrocortisone	363	121	35	18
		327	35	15
Cortisone	361	163	35	25
		121	35	25
Hydrocortisone (9,11,12,12-D4)	367	349	35	15

3. Results and discussion

3.1. Selectivity

Two different product-ions were used for quantification of corticosterone, 11-DHC, hydrocortisone and cortisone to verify the selectivity (Table 1). Detection was achieved by electrospray (positive ion mode) with multiple reaction monitoring (MRM). There was no significant difference in intensity for cone voltages between 35 and 60 V. Collision-induced dissociation (CID) mass spectra of corticosterone (collision energy 16 and 25 eV), 11-DHC (collision energy 23 and 32 eV), hydrocortisone (collision energy 15 and 18 eV) and cortisone (collision energy 25 eV) are shown in Fig. 1a–d. It is desirable to use isotope-labelled internal standards. However, only deuterium labelled hydrocortisone was commercially available. Cortisone was used as internal standard for determination of corticosterone and

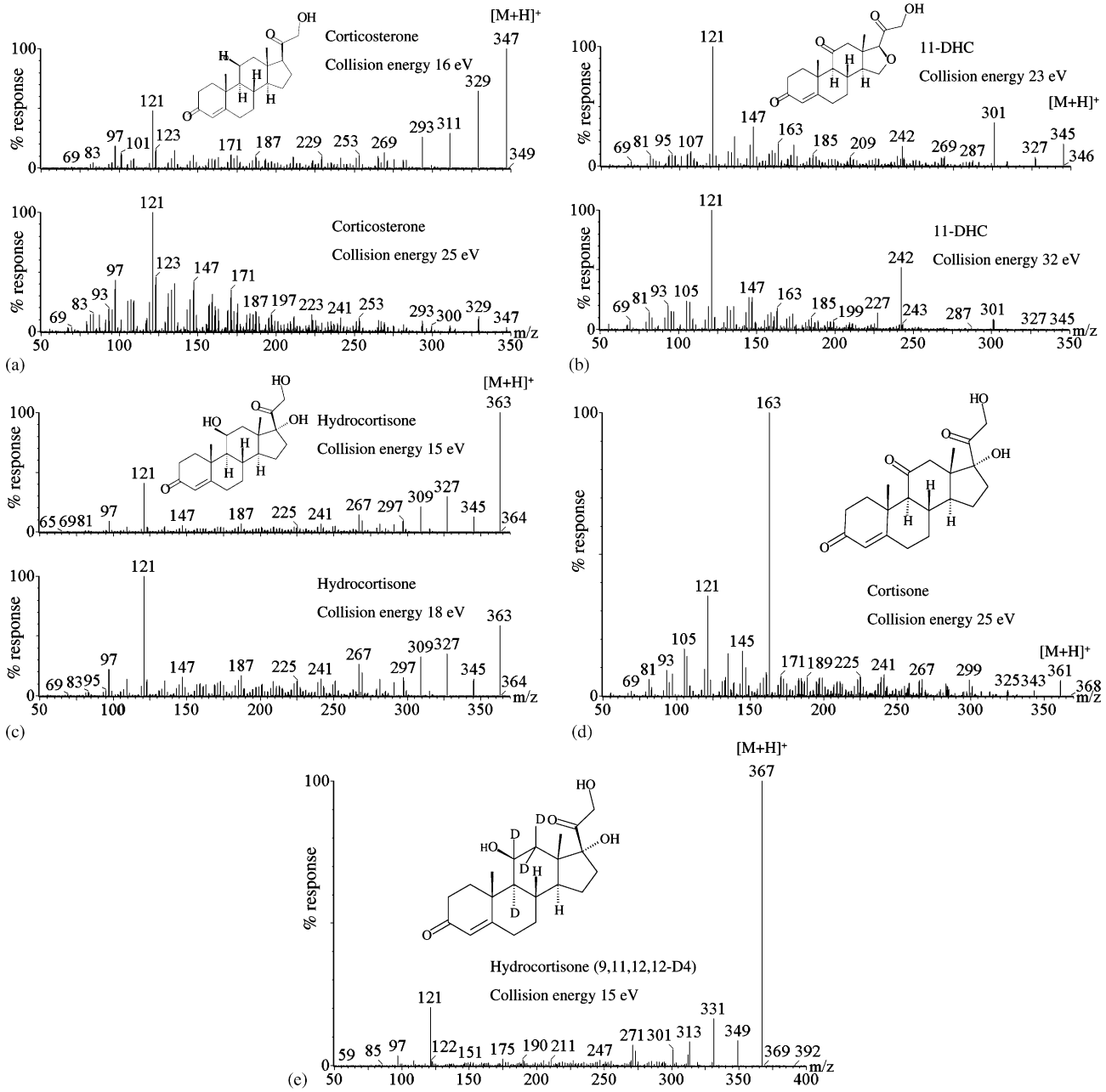

Fig. 1. Collision-induced dissociation (CID) mass spectra of (a) corticosterone in positive mode electrospray (cone voltage 60 V and collision energy of 16 eV above and 25 eV below), (b) 11-DHC in positive mode electrospray (cone voltage 60 V and collision energy of 23 eV above and 32 eV below), (c) hydrocortisone in positive mode electrospray (cone voltage 35 V and collision energy of 15 eV above and 18 eV below), (d) cortisone in positive mode electrospray (cone voltage 35 V and collision energy of 25 eV) and (e) hydrocortisone (9,11,12,12-D4) in positive mode electrospray (cone voltage 35 V and collision energy of 15 eV).

Table 2
Verification of the selectivity for quantification of corticosteroids in mouse liver, mouse adipose tissue and human adipose tissue samples by measurement of the concentration ratios determined from two MS/MS transitions

Matrix	Number (*n*)	Corticosterone 347 > 121/347 > 311 mean ± S.D.	11-DHC 345 > 121/345 > 242 mean ± S.D.	Hydrocortisone 363 > 121/363 > 327 mean ± S.D.	Cortisone 361 > 163/361 > 121 mean ± S.D.
Mouse liver	8	0.88 ± 0.067	1.1 ± 0.11	–	–
Mouse adipose	9	1.0 ± 0.038	1.1 ± 0.11	–	–
Human adipose	12	–	–	1.0 ± 0.078	0.9 ± 0.079

11-DHC in KKA^y mouse tissue samples, since this compound had similar chemical properties compared to the analytes. Deuterium-labelled hydrocortisone (9,11,12,12-D4) was used for determination of both hydrocortisone and cortisone in human adipose tissue samples. The recovery of the internal standards during sample preparation was similar compared to the analytes and no ion suppression was detected for none of the analytes or internal standards. The internal standard (hydrocortisone (9,11,12,12-D4)) was detected by the transition 367→349 (see Fig. 1e) instead of 367→121, due to interference at the transition 367→121 from human adipose tissue. The product-ion at *m*/*z* 121 originates from the A-ring of the steroids as discussed in detail by Williams et al. who used stable isotope labelling to elucidate the fragmentation mechanisms for CID of testosterone and testosterone analogs [24]. Similar concentrations of analytes were obtained using calibration curves with both product-ions in KKA^y mouse liver, adipose tissue and human adipose tissue samples. The concentration ratios determined from the two transitions were close to unity in KKA^y mouse liver, adipose and human adipose tissue samples as shown in Table 2, which indicates that the method was selective for those compounds. Typical chromatograms from KKA^y mouse liver and adipose tissue extracts monitored with two product-ions for detection of corticosterone and 11-DHC are shown in Figs. 2 and 3. Typical chromatograms from a human adipose tissue extract are shown in Fig. 4.

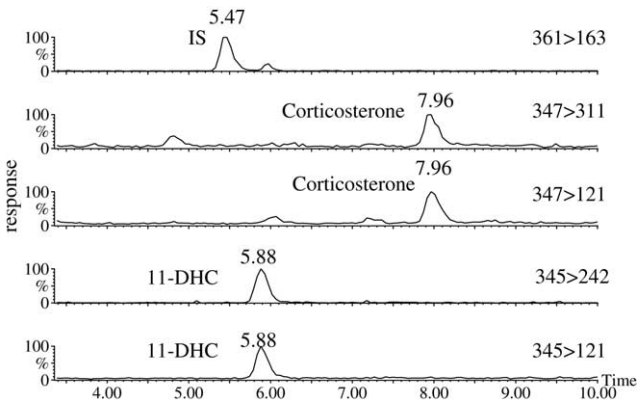

Fig. 2. Typical chromatogram from pooled KKA^y mouse liver tissue sample monitored with two different product-ions, for corticosterone (t_R 7.96 min, 122 nmol/kg), 11-DHC (t_R 5.88 min, 64.7 nmol/kg) and IS (t_R 5.47 min).

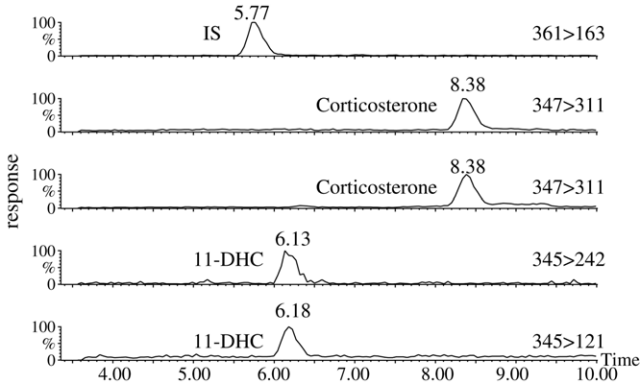

Fig. 3. Typical chromatogram from a KKA^y adipose tissue sample monitored with two different product-ions, for corticosterone (t_R 8.38 min, 37.2 nmol/kg), 11-DHC (t_R 6.13 and 6.18 min, 2.76 nmol/kg) and IS (t_R 5.77 min).

3.2. Linearity

Aliquots of working solutions of corticosteroids were added into matrices (pooled KKA^y mouse liver tissue homogenate, pooled KKA^y mouse adipose tissue homogenate or pooled human adipose tissue homogenate) to prepare calibration standards. The linearity of the method was investigated in the concentration ranges of 60–1200 nmol/kg for KKA^y mouse liver tissue sample, 6–600 nmol/kg for KKA^y mouse adipose tissue sample and 1.2–180 nmol/kg for human adipose tissue sample. The intercept was due to endogenous

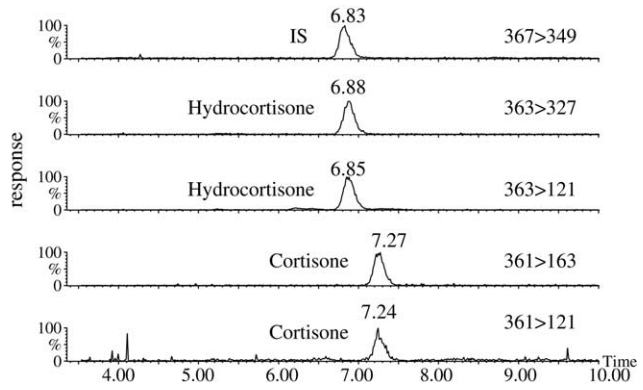

Fig. 4. Typical chromatogram from human biopsy sample from adipose tissue monitored with two different product-ions, for cortisone (t_R 7.24 and 7.27 min, 4.5 nmol/kg), hydrocortisone (t_R 6.85 and 6.88 min, 17 nmol/kg) and I.S. (t_R 6.83 min).

Table 3
Typical calibration curves for determination of corticosteroids in KKA^y mouse liver, KKA^y mouse adipose tissue and human adipose tissue samples (corticosterone 347 > 121, 11-DHC 345 > 121, hydrocortisone 363 > 121 and cortisone 361 > 163)

Matrix	Corticosteroids	Range (nmol/kg)	Calibration curve slope	r^2	Intercept
Mouse liver	Corticosterone	60–1200	1.179	0.9990	0.0254
Mouse liver	11-DHC	60–1200	2.319	0.9996	0.0284
Mouse adipose	Corticosterone	3.0–300	0.542	0.9641	8.510
Mouse adipose	11-DHC	3.0–300	0.974	0.9987	1.185
Mouse adipose	Corticosterone	6.0–600	1.103	0.9985	6.705
Mouse adipose	11-DHC	6.0–600	2.652	0.9996	1.174
Human adipose	Hydrocortisone	0.6–1200	6.787	0.9993	2.863
Human adipose	Cortisone	0.6–1200	6.443	0.9955	0.142
Human adipose	Hydrocortisone	1.2–180	8.409	0.9981	4.558
Human adipose	Cortisone	1.2–180	5.118	0.9996	0.174

levels of the analytes in the pooled homogenized matrices. For KKA^y mouse liver tissue sample, the coefficient of determination (r^2), using a weighting factor (1/concentration), was 0.9990 for corticosterone and 0.9996 for 11-DHC. For KKA^y mouse adipose tissue sample the r^2 was 0.9985 for corticosterone and 0.9996 for 11-DHC. The calibration curves for human adipose tissue sample showed that r^2 was 0.9981 for hydrocortisone and 0.9996 for cortisone (Table 3).

At another occasions the linearity of the methods was investigated in the concentration range of analytes in KKA^y mouse adipose matrix 3.0–300 nmol/kg and in human adipose matrix 0.6–1200 nmol/kg. The calibration curves showed that the r^2 was 0.9641 for corticosterone, 0.9987 for 11-DHC, 0.9993 for hydrocortisone and 0.9955 for cortisone (Table 3).

3.3. Recovery

The recoveries were determined from tissue homogenate and spiked tissue homogenates.

Tissue homogenates were mixed with standard solutions of the corticosteroids and extracted as described under sample preparation. Tissue extracts were prepared and a portion of the final extracts were mixed with standard solutions of the corticosteroids.

The absolute recovery was calculated from the peak areas according to

$$\text{Absolute recovery (\%) liver tissue} = 100 \times \frac{\text{spiked in homogenate} - \text{homogenate}}{\text{spiked in supernatant} - \text{homogenate}}$$

$$\text{Absolute recovery (\%) adipose tissue} = 100 \times \frac{\text{spiked in homogenate} - \text{homogenate}}{\text{spiked in final extract} - \text{homogenate}}$$

The absolute recoveries from spiked KKA^y mouse liver homogenate (300 nmol/kg) were 96% for corticosterone, 100% for 11-DHC and 100% for IS. The recoveries from spiked KKA^y mouse adipose homogenate (30 nmol/kg) were 99% for corticosterone, 93% for 11-DHC and 91% for IS. The recoveries from human adipose tissue homogenate were 100% (30 nmol/kg), 92% (60 nmol/kg) and 89% (180 nmol/kg) for hydrocortisone, 95% (30 nmol/kg), 88% (60 nmol/kg) and 86% (180 nmol/kg) for cortisone and 87% for IS (60 nmol/kg).

The slopes of the calibration curves with internal standard from the different matrices, KKA^y mouse liver tissue homogenate versus MeOH/water, KKA^y mouse adipose tissue homogenate versus ethyl acetate and human adipose tissue homogenate versus ethyl acetate, were compared. The slope ratios were close to 1 as shown in Table 4.

3.4. Limit of quantitation (LOQ) and limit of detection (LOD)

A 10 nM (corticosterone and 11-DHC) solvent standard, which corresponded to 60 nmol/kg liver, was extracted as described under sample preparation. The signal to noise (S/N) was calculated from the ratio between analyte peak signal to base line and peak-to-peak noise signal. The S/N was 12 for corticosterone and 33 for 11-DHC. An extract of 1 nM standard solution, which corresponded to 6 nmol/kg adipose tissue, gave an S/N ratio of 11 for corticosterone and 20 for 11-DHC. The LODs at an S/N ratio of 3 were estimated to be 15 nmol/kg liver and 1.6 nmol/kg adipose tissue for corticosterone and 5.4 nmol/kg liver and 0.92 nmol/kg adipose tissue for 11-DHC.

When human adipose tissue homogenate was spiked with a standard solution (1.2 nmol/kg adipose tissue) the S/N ratios were 65 for hydrocortisone and 24 for cortisone. A solvent standard 0.2 nM (hydrocortisone and cortisone), which corresponded to 1.2 nmol/kg adipose tissue, was extracted as described under sample preparation. The S/N was 54 for hydrocortisone and 33 for cortisone. The LOQs of the method at an S/N ratio of 10 were estimated to 0.2 nmol/kg adipose tissue for hydrocortisone 0.4 nmol/kg adipose tissue for cortisone. The LODs at 3 times S/N were estimated to be 0.07 nmol/kg adipose tissue for hydrocortisone 0.1 nmol/kg adipose tissue for cortisone.

Table 4
Comparison of calibration curve slopes with internal standard, from different matrices

Matrix	Corticosteroids	Calibration curve range (nmol/kg)	Slope	Slope ratio (homogenate/solvent)	r^2	Intercept
Mouse liver	Corticosterone	60–1200	1.746		0.9982	0.0441
MeOH/water	Corticosterone	60–1200	1.483		0.9996	−0.0006
	Corticosterone			1.18		
Mouse liver	11-DHC	60–1200	3.388		0.9981	0.0813
MeOH/water	11-DHC	60–1200	2.948		0.9999	−0.0006
	11-DHC			1.15		
Mouse adipose	Corticosterone	6.0–600	1.101		0.9984	7.763
Ethyl acetate	Corticosterone	6.0–600	1.176		0.9975	0.369
	Corticosterone			0.936		
Mouse adipose	11-DHC	6.0–600	2.652		0.9996	1.174
Ethyl acetate	11-DHC	6.0–600	2.723		0.9980	0.451
	11-DHC			0.974		
Human adipose	Hydrocortisone	0.15–75	12.957		0.9998	3.058
Ethyl acetate	Hydrocortisone	0.075–75	10.549		0.9998	0.000
	Hydrocortisone			1.23		
Human adipose	Cortisone	0.075–75	5.923		0.9964	0.182
Ethyl acetate	Cortisone	0.15–75	4.190		0.9975	−0.018
	Cortisone			1.41	0.9996	

The present method was developed on a Micromass Ultima Pt mass spectrometer where electrospray ionization gave better sensitivity compared to APCI. Addition of TFA to the mobile phase improved the sensitivity for ESI compared to other acidifying agents like formic acid. The choice of ionization technique is instrument-dependent and needs to be optimized for each model and ion-source construction. Recently developed photoionisation at atmospheric pressure appear to be an attractive alternative for ionization for steroids.

3.5. Precision and accuracy

Six separate sample preparations of one batch of KKA^y mouse liver tissue homogenate and one batch of adipose tissue homogenate containing IS were analyzed on each occasion. CV values were calculated from the analyzed concentration (nmol/kg) and ratio of concentration (corticosterone/11-DHC). The CVs for determination of liver samples were 3.0% for corticosterone, 1.6% for 11-DHC and 4.7% for the concentration ratio. The CVs for analysis of adipose samples were 11% for corticosterone, 15% for 11-DHC and 9.4% for the concentrations ratios (Table 5). The precision (% CV) and accuracy (% bias) for determination of human adipose tissue samples were evaluated from three batches of spiked human adipose tissue homogenate. Some pieces of frozen human subcutaneous adipose tissues were homogenized in ethyl acetate containing IS and then pooled. Aliquots of working solutions were spiked into the homogenate. The concentrations added to the pooled homogenate were 0, 12.6 and 120 nmol/kg adipose tissue for hydrocortisone and 2.4, 12.6 and 120 nmol/kg adipose tissue for cortisone. The intra-assay precision (% CV) varied from 1.7 to 5.2 for hydrocortisone and from 1.9 to 4.2 for cortisone (Table 5). The inter-assay precisions (% CV) between six different occasions were from 3.6 to 4.1 for hydrocortisone and from 4.8 to 8.1 for cortisone. The intra-assay accuracy values (% bias) were from −4.0 to 3.3 for hydrocortisone and from −13 to 2.0 for cortisone (Table 5). The inter-assay values (% bias) were from −3.5 to −6.4 for hydrocortisone and from −2.4 to −9.5 for cortisone.

3.6. Variation of endogenous level between animals/subjects

Four KKA^y mouse liver samples, 9 KKA^y mouse adipose tissue samples and 17 human adipose tissue samples were used for determination of endogenous steroid levels.

The mean endogenous levels of corticosteroids in KKA^y mouse tissue samples were: mouse liver values of 117 ± 25 (S.D.) nmol/kg for corticosterone and 62 ± 19 (S.D.) nmol/kg for 11-DHC (Table 6) and mouse adipose tissue values of 39 ± 20 (S.D.) nmol/kg for corticosterone and 2.4 ± 0.9 (S.D.) nmol/kg for 11-DHC (Table 7). Some of the 11-DHC levels were over the limit of detection but under the limit of quantification.

The mean endogenous levels of corticosteroids in human subcutaneous adipose tissue were 12 ± 7.0 (S.D.) nmol/kg for hydrocortisone and 3.0 ± 1.6 (S.D.) nmol/kg for cortisone (Table 8).

The results showed that the individual variations between animals/subjects of endogenous levels of corticosteroids were high.

3.7. Ion suppression

Similar peak areas of analytes were obtained from spiked KKA^y mouse liver and adipose homogenate compared to solvents standards. Consequently ion suppression was not significant.

Table 5
Intra-assay precision and bias for determination of corticosteroids in KKA^y mouse liver and adipose tissue homogenates and human adipose tissue homogenate

Matrix	Spiked conc. (nmol/kg)	Number (n)	Corticosteroids	Mean (nmol/kg)	SD	CV%	Bias%
Mouse liver	0	6	Corticosterone (MRM 347 > 121)	123	3.73	3.03	–
	0	6	11-DHC (MRM 345 > 121)	72.6	1.14	1.57	–
		6	Ratio, corticosterone/11-DHC	1.7	0.08	4.7	
Mouse adipose	0	6	Corticosterone (MRM 347 > 121)	45.9	4.81	10.5	–
	0	6	11-DHC (MRM 345 > 121)	2.83	0.43	15.2	–
		6	Ratio, corticosterone/11-DHC	16	1.5	9.4	
Human adipose	0	6	Hydrocortisone (MRM 363 > 121)	2.74	0.141	5.15	–
	+2.4	6	Cortisone (MRM 361 > 163)	2.26	0.06	2.68	−12.9
Human adipose	+12.6	6	Hydrocortisone (MRM 363 > 121)	15.4	0.524	3.41	−3.97
	+12.6	6	Cortisone (MRM 361 > 163)	11.7	0.493	4.21	−7.94
Human adipose	+120	6	Hydrocortisone (MRM 363 > 121)	124	2.13	1.72	3.33
	+120	6	Cortisone (MRM 361 > 163)	123	2.37	1.93	2.00

The accuracy was calculated according to: Bias (%) = 100 × (mean value of the back-calculated concentration/nominal concentration value) − 100.

Table 6
Endogenous levels of corticosterone and 11-DHC in KKA^y mouse liver tissue from different individuals

Samples from untreated animal	Corticosterone (nmol/kg)	11-DHC (nmol/kg)	Corticosterone/11-DHC ratio
Pool homogenate	122	64.8	1.9
ID 1	152	77.4	2.0
ID 2	101	49.1	2.1
ID 3	86.4	36.2	2.4
ID 5	122	80.4	1.5
Mean	117	61.6	2.0
S.D.	24.8	18.8	0.3
% CV	21.2	30.5	15.0

Post-column addition of analyte was used to study ion suppression effects from a human adipose sample. A 5 μM d4-hydrocortisone standard solution was added post-column at 10 μl/min to a T-coupling with the aid of an infusion pump equipped with a 0.25 ml syringe. No ion suppression occurred at the retention times of the corticosteroids.

A standard solution was spiked in matrices to investigate ion suppression effects. Fifteen microliters of a solution of 0.5 μM IS, 0.1 μM hydrocortisone and 0.1 μM cortisone was spiked in 135 μl matrix (homogenate extract, ethyl acetate extract or MeOH/0.02% TFA (25:75, v/v)). The area responses of analytes from matrices were used for the calculation of the recovery.

The absolute recoveries were calculated according to

$$\text{Ethyl acetate matrix effect}\,(\%) = 100 - 100 \times \frac{\text{spiked in extract}}{\text{spiked in 25\% MeOH 0.02\% TFA}}$$

$$\text{Homogenate matrix effect}\,(\%) = 100 - 100 \times \frac{\text{spiked in extract} - \text{homogenate extract}}{\text{spiked in 25\% MeOH 0.02\% TFA}}$$

Table 7
Endogenous levels of corticosterone and 11-DHC in KKA^y mouse adipose tissue from different individuals

Samples from untreated animal	Corticosterone (nmol/kg)	11-DHC (nmol/kg)	Corticosterone/11-DHC ratio
ID 2	20.2	5.06	4.0
ID 3	29.3	1.24[a]	24[b]
ID 4	37.5	2.76	14
ID 5	33.4	2.32	14
ID 6	56.0	3.42	16
ID 7	19.3	2.19	8.8
ID 8	49.6	2.77	18
ID 9	14.2	0.882[a]	16[b]
ID 10	73.4	3.46	21
Mean	39.1	2.38	16.5
S.D.	19.7	0.94	4.65
% CV	50.4	39.5	28.2

[a] The concentration was under limit of quantification.
[b] Ratio was not significant because of uncertain 11-DHC level.

Table 8
Endogenous levels of hydrocortisone and cortisone in human adipose tissue from different individuals

Sample	Hydrocortisone (nmol/kg)	Cortisone (nmol/kg)	Hydrocortisone/cortisone ratio
1	3.3	<LLOQ[a]	–
2	4.7	1.5	3.1
3	4.9	1.5	3.3
4	17	4.5	3.8
5	5.7	1.7	3.4
6	4.8	1.8	2.7
7	22	7.1	3.1
8	6.2	1.6	3.9
9	17	2.6	6.5
10	21	3.1	6.8
11	19	3.0	6.3
12	12	2.5	4.8
13	18	4.9	3.7
14	7.2	1.5	4.8
15	24	3.1	7.7
16	13	2.8	4.6
17	11	5.1	2.2
n	17	16	16
Mean	12.4	3.0	4.4
S.D.	7.01	1.62	1.64
% CV	56.5	54.0	37.3

[a] The measured value is 1.1 nmol/kg. It was estimated by extrapolation of standard curve below the LLOQ, 1.2 nmol/kg.

The matrix effects of human adipose tissue homogenate extract were 16% (hydrocortisone), 13% (cortisone) and 14% (IS) and for an ethyl acetate extract; 9.6% (hydrocortisone), 11% (cortisone) and 5.4% (IS).

Consequently ion suppressions were not significant for human adipose matrix.

3.8. Applicability

The applicability of the present methods was verified by determination of the endogenous levels of corticosterone and 11-DHC in KKA^{Y} mouse liver and adipose tissues and cortisone and hydrocortisone in human adipose tissue samples.

A small molecule macrotrap was coupled to the autoinjector six-port valve instead of a loop to concentrate and clean the sample. No deterioration of the macrotrap column was observed after analysis of 250 liver or adipose samples. Cortisone was used as an internal standard for the determination of corticosterone and 11-DHC in the mouse tissue samples while hydrocortisone (9,11,12,12-D4) was used as an internal standard for the human adipose tissue samples. Detection was achieved by electrospray in the positive ion mode with MRM for the transitions with two different product-ions of analytes. Higher response of product ion transitions: m/z 347 → 121 for corticosterone, 345 → 121 for 11-DHC and 361 → 163 for cortisone and 363 → 121 for hydrocortisone were used for determination of analytes in samples, after the selectivity was verified. No significant carry over (<0.3%) was observed and at least 90 samples could be analyzed with maintained sensitivity without cleaning of the ion-source. The method showed sufficient selectivity and sensitivity for quantification of endogenous levels of corticosterone and 11-DHC in liver and adipose tissue from KKA^{y} mice and hydrocortisone and cortisone in adipose tissue from humans but the variation between animals/subjects was high.

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