Journal of Chromatography B, 878 (2010) 2863–2869

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Measurement of cortisol, cortisone, prednisolone, dexamethasone and 11-deoxycortisol with ultra high performance liquid chromatography–tandem mass spectrometry: Application for plasma, plasma ultrafiltrate, urine and saliva in a routine laboratory

Brett C. McWhinney, Scott E. Briscoe, Jacobus P.J. Ungerer, Carel J. Pretorius*

Department of Chemical Pathology, Pathology QLD, Brisbane, Queensland, Australia

ARTICLE INFO

Article history:
Received 26 March 2010
Accepted 30 August 2010
Available online 9 September 2010

Keywords:
Tandem mass spectrometry
Ultra high performance liquid chromatography
Cortisol
Cortisone
Dexamethasone
Free hormones
11β-Hydroxysteroid dehydrogenase

ABSTRACT

We describe an ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC MS/MS) method suitable for a routine laboratory to determine endogenous and exogenous glucocorticoids in plasma, plasma ultrafiltrate, urine and saliva in a single analytical run. After addition of a multi-analyte internal standard, a standardised sample preparation procedure with solid phase extraction followed, before injecting into a tandem mass spectrometer with positive mode electron spray ionisation and multiple reactant monitoring acquisition. The chromatography time was 3 min. The limit of quantitation for cortisol and cortisone in plasma was 3.75 nmol/L and linearity extended to 2000 nmol/L. The limit of quantitation for cortisol in plasma ultrafiltrate and saliva was 0.6 nmol/L. The limit of quantitation for 11-deoxycortisol and prednisolone was 5 nmol/L and for dexamethasone 1 nmol/L. The intra-assay CV was <5% and the inter-assay CV <10% for all analytes in all matrices. Comparison with an immunoassay (IA) plasma cortisol method resulted in a regression equation of UHPLC = 0.79 × IA + 31.12 with $R^2 = 0.960$ ($p < 0.0001$). Comparison with a high performance liquid chromatography (HPLC) cortisol method yielded a regression equation of UHPLC = 1.06 × HPLC + 9.82, $R^2 = 0.992$ ($p < 0.0001$). The simultaneous measurement of endogenous and exogenous glucocorticoids contributed to patient care in cases with dexamethasone and metyrapone dynamic tests and unsuspected therapeutic glucocorticoid use.

1. Introduction

Cortisol is a steroid hormone produced by the adrenal gland under the influence of adrenocorticotrophic hormone (ACTH). Cortisol is transported in plasma bound to corticosteroid-binding globulin (CBG) and albumin. At the target tissue free cortisol diffuses across cell membranes and binds to the glucocorticoid α receptor (NR3C1). The cortisol–glucocorticoid receptor complex translocates to the nucleus and regulates gene expression by binding to the glucocorticoid response element of target genes [1]. The metabolic effects of cortisol are controlled by feedback regulation of the hypothalamic–pituitary axis secretion of ACTH and by the interconversion of cortisol and cortisone by 11β-hydroxysteroid dehydrogenase (11βHSD).

11βHSD type 2, which is expressed in the kidney, colon and salivary tissue, converts cortisol to cortisone to protect the mineralocorticoid receptor in these tissues from exposure to excess cortisol. In contrast 11βHSD type 1 is widely distributed in tissue and almost exclusively converts cortisone to cortisol in vivo, thereby facilitating glucocorticoid actions in tissue [2,3]. Urine free cortisone and the ratio of urine cortisol to cortisone are considered sensitive indices of 11βHSD type 2 activity [4]. The ratio of plasma cortisol to cortisone is a function of 11βHSD type 1 and alterations of this ratio may provide insight into pathological states such as the acute phase response in critically ill patients [5,6].

During critical illness free plasma cortisol provides a better indication of glucocorticoid status than total plasma cortisol, as changes in CBG and albumin can mask changes in glucocorticoid secretion if only total plasma cortisol levels are measured [7,8]. Several analytical methods for salivary cortisol have been published and it is

Abbreviations: UHPLC MS/MS, ultra high performance liquid chromatography–tandem mass spectrometry; HPLC, high performance liquid chromatography; 11βHSD, 11β-hydroxysteroid dehydrogenase; QC, quality control; IA, immuno-assay; CBG, corticosteroid-binding globulin; ACTH, adrenocorticotrophic hormone.

* Corresponding author at: Department of Chemical Pathology, Level 3, Block 7, Royal Brisbane and Women's Hospital, QLD 4029, Australia. Tel.: +61 7 36360083; fax: +61 7 36363417.
E-mail address: carel_pretorius@health.qld.gov.au (C.J. Pretorius).

1570-0232/$ – see front matter.
doi:10.1016/j.jchromb.2010.08.044

generally accepted to be representative of free plasma cortisol and a suitable sample for the investigation of altered glucocorticoid metabolism [9,10].

Synthetic glucocorticoids such as prednisolone and dexamethasone are commonly used in clinical practice both in therapeutic and in diagnostic settings [11]. The measurement of cortisol, cortisone and synthetic glucocorticoids in plasma and urine are potentially useful in the investigation and management of both over and under production of cortisol such as in cases of Cushing syndrome and Addison disease. Prednisolone or dexamethasone therapy suppresses endogenous glucocorticoid production and can be a cause of low serum cortisol. The ability to detect synthetic steroids in patient samples may prevent unnecessary investigations especially in unconscious or critically ill patients. Measurement of plasma dexamethasone after performing a dexamethasone suppression test to diagnose Cushing disease, may allow clinicians to assess compliance with the protocol. Metyrapone, an inhibitor of the 11β-hydroxylase enzyme, is used to assess the hypothalamic–pituitary–adrenal axis. Plasma levels of cortisol and the cortisol precursor, 11-deoxycortisol, are measured in this procedure [12].

A high performance liquid chromatography method (HPLC) with ultra violet detection has been used in our laboratory to simultaneously measure cortisol, cortisone, 11-deoxycortisol, prednisolone, methyl-prednisolone and dexamethasone in plasma and urine [13]. This HPLC method has a chromatography time of 32 min, requires a sample volume of 1 mL and has a limit of quantitation of 10 nmol/L for all analytes. Numerous liquid chromatography tandem mass spectrometry methods for urine free cortisol [14,15] and cortisone [16], plasma cortisol and cortisone [17], salivary cortisol and cortisone [10] and synthetic glucocorticoids [18,19] have been described, some of which are cited here.

Our aim was to develop an ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC MS/MS) method to measure a range of endogenous and exogenous glucocorticoids in different biological matrices with a simple, standardised sample preparation procedure and a short chromatography time. The method should be suitable for use in a high volume routine laboratory.

2. Experimental

2.1. Instrumentation

An Acquity Ultra Performance Liquid Chromatography system with a binary solvent manager, sample manager and sample organiser (Waters, Milford, USA) was coupled with a Micromass Quatro Premier XE mass spectrometer (Waters, Milford, USA). Chromatographic separation was achieved using an Acquity UPLC BEH C18 1.7 μm, 2.1 mm × 50 mm column with an in-line filter unit (Waters, Milford, USA). All data manipulation was carried out using the MassLynx V4.1 software (Waters, Milford, USA).

2.2. Chemicals and reagents

Methanol and acetonitrile (Merck, Darmstadt, Germany) were HPLC grade LiChrosolv®. Ammonium acetate and AnalaR® analytical grade formic acid were obtained from Sigma–Aldrich (St. Louis, USA). Hydrochloric acid (Ajax Finechem, Sydney, Australia) was UNIVAR® analytical grade. Sigma–Aldrich (St. Louis, USA) supplied all the chemical preparations of cortisol (hydrocortisone), cortisone, prednisolone, 11-deoxycortisol (Reichstein's substance S) and dexamethasone. The deuterated internal standards d4-dexamethasone, d2-11-deoxycortisol and d2-cortisone were purchased from CDN Isotopes (Scivac Pty Ltd., Sydney, Australia) and d4-cortisol from Cambridge Isotopes Laboratories (Novachem Pty Ltd., Victoria, Australia)

2.3. Sample preparation

Urine and saliva were collected into preservative free containers and plasma samples were separated at 2200 × *g* for 10 min. Ultrafiltrates of plasma free cortisol were prepared by equilibrating 500 μL of plasma at 37 °C for 15 min in Amicon Ultra-4 regenerated cellulose 30,000 molecular weight cut-off centrifugal filter devices (Millipore, Billerica, USA) before centrifugation at 3040 × *g* for 20 min at 37 °C. Aliquots were stored at −20 °C until analysis. Samples were thawed at room temperature before solid phase extraction was performed.

Total plasma cortisol samples (250 μL) were treated with 50 μL of 1 M HCl for 15 min at room temperature to displace cortisol and cortisone from binding proteins. To each sample (250 μL acidified plasma, 50 μL urine, 250 μL saliva or 250 μL ultrafiltrate) 100 μL of the multi-analyte internal standard and 500 μL H_2O was added before loading onto activated Oasis HLB 1 cc (30 mg) solid phase extraction cartridges (Waters, Milford, USA). The columns were sequentially washed with 1 mL of 20% methanol and then 1 mL of hexane. Samples were eluted with 1 mL 100% ethyl acetate, dried down at 50 °C and reconstituted in 100 μL of 45% methanol with 2 mmol/L ammonium acetate and 0.1% formic acid.

2.4. Calibrators and quality control procedures

A series of 10 calibrators were prepared in 20% methanol with a mixture of analytes – cortisol and cortisone 1–2000 nmol/L, prednisolone and 11-deoxycortisol 5–500 nmol/L and dexamethasone 0.5–50 nmol/L. A multi-analyte internal standard was prepared in 20% methanol with a concentration of 272 nmol L d4-cortisol, 272 nmol/L d2-cortisone, 141 nmol/L d2-11-deoxycortisol and 50.4 nmol/L d4-dexamethasone. We added the internal standard before the solid phase extraction to all samples, QC and calibrators.

Quality control (QC) materials with all the compounds of interest were not commercially available and we prepared in-house QC material from pooled patient samples spiked with known amounts of glucocorticoids. Aliquots were stored at −20 °C and were treated the same as the corresponding clinical samples. Urine QC1 consisted of pooled urine and QC2 was spiked to achieve concentrations of approximately 1000 nmol/L cortisol, cortisone and prednisolone. Plasma total QC1 was a pooled sample with added 11-deoxycortisol (15 nmol/L), dexamethasone (5 nmol/L) and prednisolone (30 nmol/L). Plasma total QC2 was spiked to obtain approximately 800 nmol/L cortisol, 400 nmol/L cortisone, 500 nmol L prednisolone, 200 nmol/L 11-deoxycortisol and 25 nmol/L dexamethasone. Plasma free QC1 was a pooled sample and plasma free QC2 was spiked to obtain approximately 80 nmol/L cortisol and cortisone. Saliva QC1 was a pooled sample and saliva QC2 was spiked with 20 nmol/L cortisol and cortisone.

2.5. Chromatographic and mass spectrometric conditions

The flow rate was 0.4 mL/min, sample injection volume 20 μL, column temperature 50 °C and the solvent gradients were as described in Table 1. The tandem mass spectrometer was operated in positive mode electron spray ionisation with the multiple reactant monitoring acquisition parameters presented in Table 2. The total analytical run time on the UHPLC MS/MS was 3 min.

2.6. Method validation

Ion suppression was evaluated by post-column infusion studies. Cortisol, cortisone, prednisolone, 11-deoxycortisol and

Table 1
Liquid chromatography solvent gradients for the UHPLC MS/MS method.

Time	%A	%B	Curve
0.00	55.0	45.0	Initial
0.50	55.0	45.0	6
2.00	50.0	50.0	6
2.10	55.0	45.0	6

Time measured in minutes, curve 6 refers to a linear change between initial and final conditions; A, 2 mmol/L ammonium acetate in water with 0.1% formic acid. B, 2 mmol/L ammonium acetate in methanol with 0.1% formic acid.

dexamethasone (1000 nmol/L) in 55:45 (v/v) mobile phase A:B, were infused post-column. The reduction in ion counts was used to detect any ion suppression following injection of ($n = 10$) urine, plasma and saliva samples with a low concentration analyte, charcoal stripped plasma and a water blank.

We tested assay linearity with polynomial regression according to CLSI document EP6-A. Method comparison was performed with a HPLC method [13] for cortisol, cortisone and 11-deoxycortisol, and with a cortisol immuno-assay (IA) performed according to the manufacturer's instructions on a Beckman Unicel DxI800 analyser (Beckman Coulter Diagnostics, Brae, USA). Accuracy of the UHPLC MS/MS cortisol measurement was estimated by analysing commercial cortisol calibrators (Abbott Laboratories, Illinois, USA) as plasma samples and comparing the results to the assigned values. The assigned cortisol values are traceable to BCR 192 and BCR 193 certified reference materials.

Table 2
Multiple Reactant Monitoring (MRM) and mass spectrometer conditions.

Compound	Transition	Cone voltage (V)	Collision energy (eV)
Cortisol quantifier	363.1 > 120.9	32	25
Cortisol qualifier	363.1 > 96.9	32	28
d4-Cortisol	367.1 > 120.9	32	25
Cortisone quantifier	361.1 > 163.0	33	25
Cortisone qualifier	361.1 > 121.0	33	32
d2-Cortisone	363.1 > 164.8	33	25
Prednisolone quantifier	361.0 > 146.9	18	20
Prednisolone qualifier	361.0 > 120.9	18	30
11-Deoxycortisol quantifier	347.1 > 96.9	32	25
11-Deoxycortisol qualifier	347.1 > 108.9	32	28
d2-11-Deoxycortisol	349.1 > 96.9	32	25
Dexamethasone quantifier	373.1 > 146.9	27	27
Dexamethasone qualifier	373.1 > 170.9	27	27
d4-Dexamethasone	377.1 > 148.8	27	27

Mass spectrometer conditions: capillary charge 0.6 kV, source temperature 120 °C, desolvation temperature 450 °C, desolvation gas flow 800 L/h, cone gas flow 50 L/h and collision cell gas flow 0.15 mL/min.

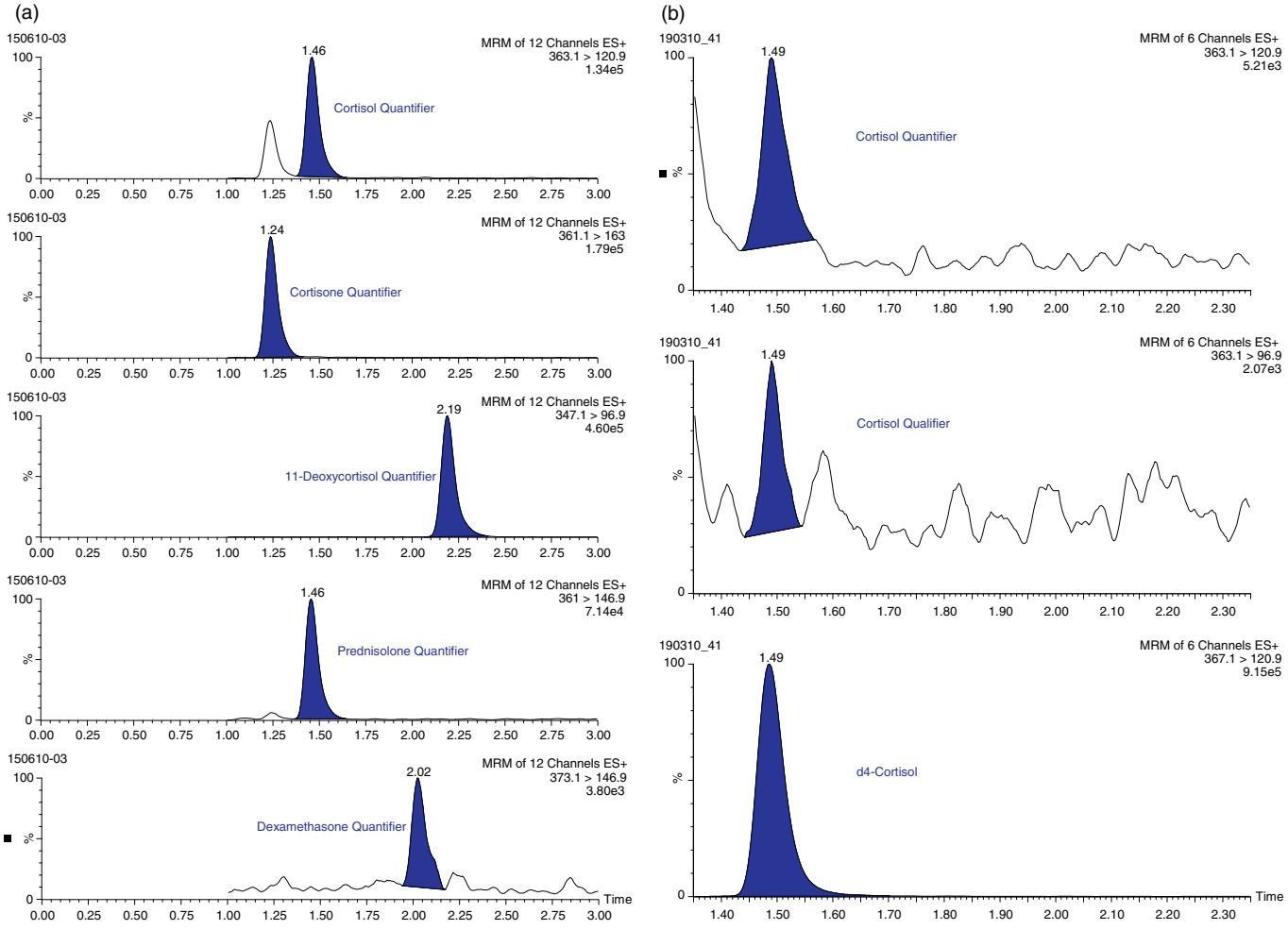

Fig. 1. Representative UHPLC MS/MS chromatograms of glucocorticoids. The chromatography time is presented on the *x*-axis and the *y*-axis is scaled to 100% of the ion count for the channel. The mass charge ratio of the parent and daughter ions, as well as the ion count for each channel is shown. (a) Standard with 25 nmol/L cortisol, cortisone, 11-deoxycortisol, prednisolone and 5 nmol/L dexamethasone. (b) Dilute plasma ultrafiltrate with 0.6 nmol/L cortisol. The signal to noise ratio was approximately 4:1. (c) Saliva sample with a cortisol of 2.1 nmol/L and a cortisone of 14.7 nmol/L. (d) Plasma ultrafiltrate with a cortisol of 6.3 nmol/L. (e) Plasma sample from a patient collected at 09:00 after administration of 1 mg dexamethasone at 23:00 the previous evening. Plasma cortisol was 133 nmol/L, cortisone 28 nmol/L and dexamethasone 9.0 nmol/L. (f) Urine from a patient on prednisolone treatment. Urine cortisol 17 nmol/L, cortisone 111 nmol/L and prednisolone 221 nmol/L.

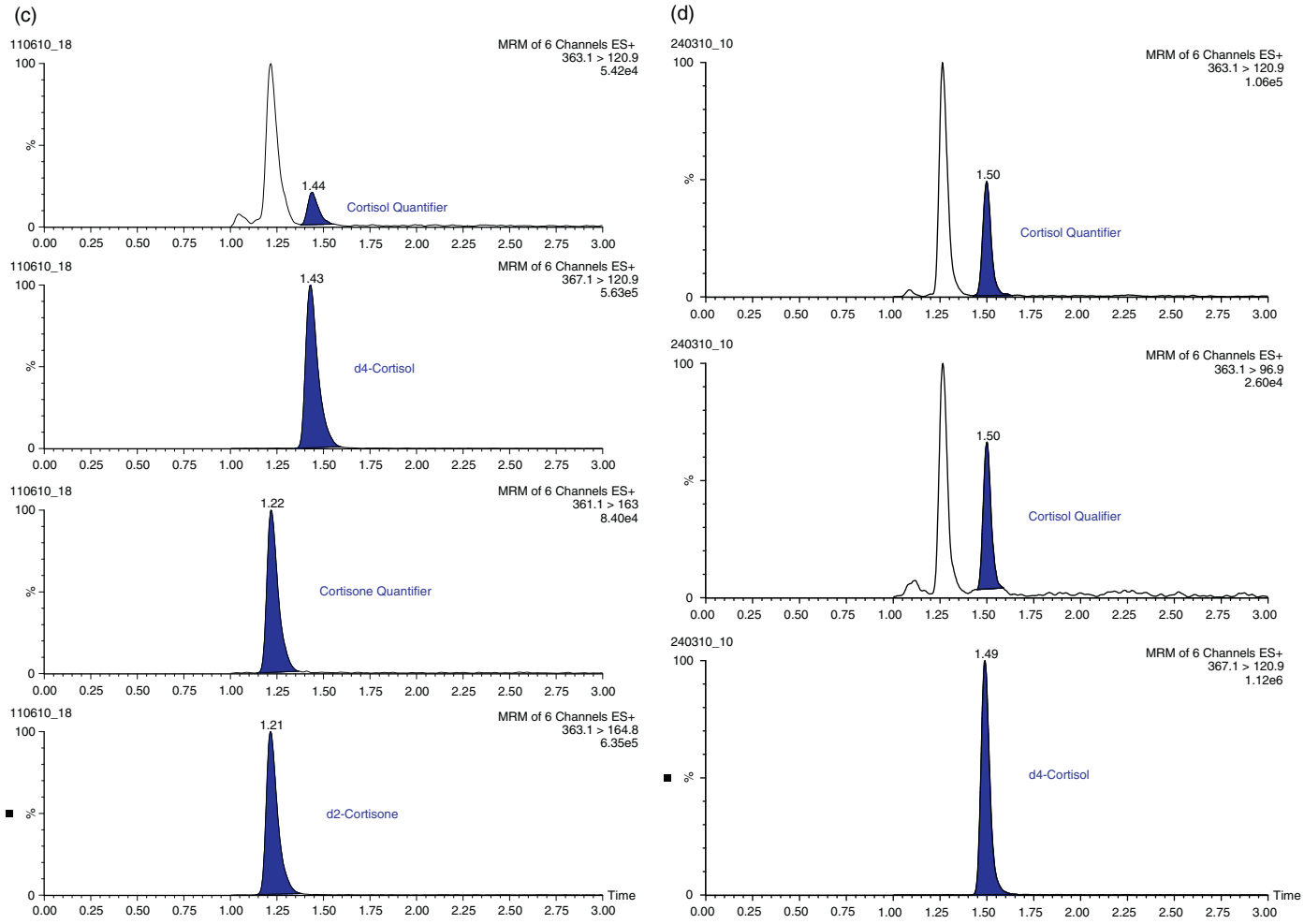

Fig. 1. (*Continued*)

Intra-assay CV was determined by 20 repeated measurements of the QC material in a single analytical run and inter-assay CV by repeat analysis of QC material over 20 analytical runs on consecutive days. The limit of quantitation for cortisol and other glucocorticoids in plasma and urine was defined as the lowest concentration that could be assayed with a CV of <10% determined on 10 replicates of a diluted plasma sample. The limit of quantitation for free plasma cortisol was defined as the lowest concentration that could be assayed with a CV of <15% on a diluted plasma ultrafiltrate.

To illustrate the clinical utility of the UHPLC method we determined glucocorticoids in four different groups of patients who presented for analysis to our laboratory. Firstly, we measured dexamethasone and cortisol in plasma collected at 09:00 from 5 males, median age 46.5 years (range 31.1–65.9) and 4 females, 49.7 years (23.7–54.3) after receiving 1 mg dexamethasone at 23:00 the previous day. Secondly, we evaluated the ratio of cortisol to cortisone, in plasma, urine and saliva in 23 patients in whom hypercortisolism was excluded on the basis of routine clinical and laboratory testing. This group consisted of 6 males, 47.5 years (27.5–65.9) and 17 females, 33.3 years (23.6–59.8). Thirdly, we investigated 10 male patients, 51.0 years (22.3–65.8) and 14 female patients, 52.7 years (26.3–67.5) who received 30 mg/kg metyrapone at 24:00 before having blood collected for cortisol and 11-deoxycortisol at 08:00 the following morning [20]. Lastly, we collected results from 1 male, aged 64.4 years, and 4 female patients, 43.1 years (32.1–48.2) who had urine cortisol measurements requested while on prednisolone.

2.7. Statistical analysis

Linearity and method comparison analysis were performed with Analyse-it version 2.21 software. Results were reported as median (range) and comparisons between groups were performed with the Friedman procedure.

3. Results/discussion

3.1. Method validation

The chromatographic separations of the respective analytes are depicted in Fig. 1a. Cortisone eluted at approximately 1.2 min, cortisol and prednisolone at approximately 1.5 min, dexamethasone after 2 min and 11-deoxycortisol at approximately 2.2 min. Chromatographic separation for cortisol and prednisolone was not required due to their different mass to charge ratios, cortisone was however resolved chromatographically from prednisolone. Minimal (<5%) ion suppression was observed for all analytes and with all the matrices investigated.

The limit of quantitation in plasma for cortisol and cortisone was 3.75 nmol/L (CV 7.4% and 5.6%, respectively), 5 nmol/L for 11-deoxycortisol (CV 8.3%) and prednisolone (CV 7.6%). The limit of quantitation for dexamethasone was 1.0 nmol/L (CV 9.5%). The limit of quantitation for plasma free cortisol was 0.6 nmol/L (CV 11.4% at 1.1 nmol/L and 10.0% at 0.6 nmol/L) with a signal to noise ratio of approximately 4:1 at this level (Fig. 1b). The intra-assay CV was

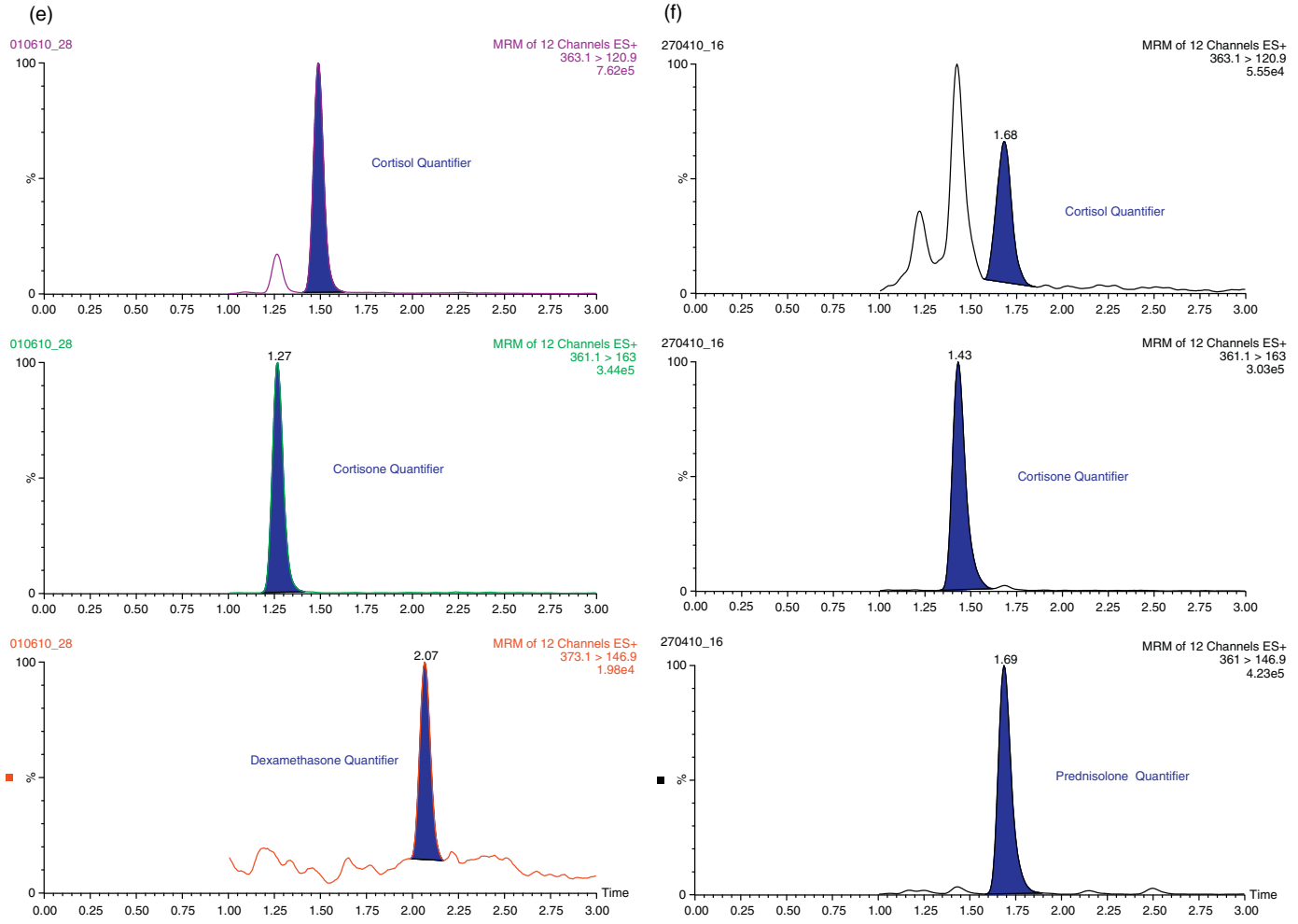

Fig. 1. (*Continued*)

<5% and the inter-assay CV <10% (Table 3) with plasma total cortisol demonstrating a CV of <5% in the clinically important range of approximately 77–730 nmol/L.

The UHPLC MS/MS method was linear up to 2000 nmol/L for cortisol and cortisone and between 5 and 500 nmol/L for 11-deoxycortisol, prednisolone and dexamethasone. The linearity for cortisol determined over 17 days resulted in a mean (SE) slope of 1.027 (0.002), intercept of 2.624 (0.946) and $R^2 = 0.999$ with <2.5% deviation from linearity at all concentrations with the exception of the 5 nmol/L standard (15% deviation).

3.2. Method comparison

The method comparison regression parameters are summarised in Table 4. The comparison between the UHPLC MS/MS and IA cortisol methods are presented in Fig. 2a and resulted in a regression equation of UHPLC = 0.79 × IA + 31.12 with $R^2 = 0.960$ ($p < 0.0001$). Cross-reactivity of IA methods with cortisone [21], prednisolone [22] and 11-deoxycortisol [13] have been described and this non-selectivity of the IA may partly explain the overestimation of plasma cortisol at high levels. The accuracy study (Fig. 2b) demonstrated a small positive bias for the UHPLC MS/MS cortisol method compared to the assigned values of the commercial calibrators with an average recovery of 108% ($n = 6$). This finding supported our opinion that the IA method overestimated plasma cortisol and not that the UHPLC MS/MS underestimated plasma cortisol.

Comparison of the UHPLC MS/MS and the HPLC methods showed good correlation for plasma 11-deoxycortisol and for cor-

Table 3
Imprecision results for urine, free plasma and total plasma glucocorticoid compounds as determined with UHPLC MS/MS.

	Intra-assay		Inter-assay	
	Mean (nmol/L)	CV (%)	Mean (nmol/L)	CV (%)
u-Cortisol	33.5	4.8	34.7	5.6
	1071	1.7	1080	3.5
u-Cortisone	152	3.5	157	3.8
	1120	2.1	1137	3.7
u-Prednisolone	1060	1.9	1044	3.4
pf-Cortisol	25.2	5.0	25.0	5.6
	76.8	3.6	76.9	3.0
pf-Cortisone	35.8	4.7	35.1	5.6
	85.7	3.1	85.8	3.8
p-Cortisol	77.6	3.4	77.5	4.6
	740	2.1	729	3.1
p-Cortisone	25.6	4.7	24.7	6.0
	541	2.1	525	3.8
p-11-Deoxycortisol	26.2	6.5	23.6	7.8
	312	3.1	308	6.9
p-Dexamethasone	25.4	4.7	22.5	9.7
	69.5	3.8	62.4	8.1
p-Prednisolone	533	2.3	515	4.2
s-Cortisol	2.1	7.8	ND	ND
	23.1	1.9	ND	ND
s-Cortisone	14.7	2.3	ND	ND
	34.5	1.9	ND	ND

u, urine; pf, plasma ultrafiltrate; p, plasma; s, saliva; ND, not done.
Intra-assay imprecision was determined by 20 repeated assays in a single analytical run and inter-assay imprecision by repeat analysis of QC material over 20 analytical runs on consecutive days.

Table 4
Method comparison with UHPLC MS/MS (linear regression).

	Comparative method	Slope (95% CI)	*p*, slope	Intercept (95% CI)	*p*, intercept	R^2 [a]	*n*
p-Cortisol	IA	0.79 (0.76–0.82)	<0.0001	31.12 (17.54–44.7)	<0.0001	0.960	155
p-Cortisol	HPLC	1.06 (1.03–1.08)	0.0023	9.82 (1.31–18.33)	0.0172	0.992	47
u-Cortisol	HPLC	1.13 (1.11–1.15)	<0.0001	−3.25 (−8.86 to 2.35)	0.2525	0.993	124
p-Cortisone	HPLC	0.90 (0.75–1.05)	0.1656	12.44 (6.38–18.51)	0.0002	0.814	47
u-Cortisone	HPLC	1.07 (1.02–1.13)	0.0074	21.12 (11.07–31.28)	<0.0001	0.942	124
p-11-Deoxycortisol	HPLC	0.98 (0.93–1.04)	0.5239	−3.22 (−9.35 to 2.92)	0.2958	0.973	43

u, urine; p, plasma; IA, immuno-assay.
If *p* value for the slope is <0.05 the null hypothesis that the slope = 1.0 is rejected.
If *p* value for the intercept is <0.05 the null hypothesis that the intercept = 0.0 is rejected.
[a] The 2-sided *p* value for all the Pearson correlation coefficients were <0.0001.

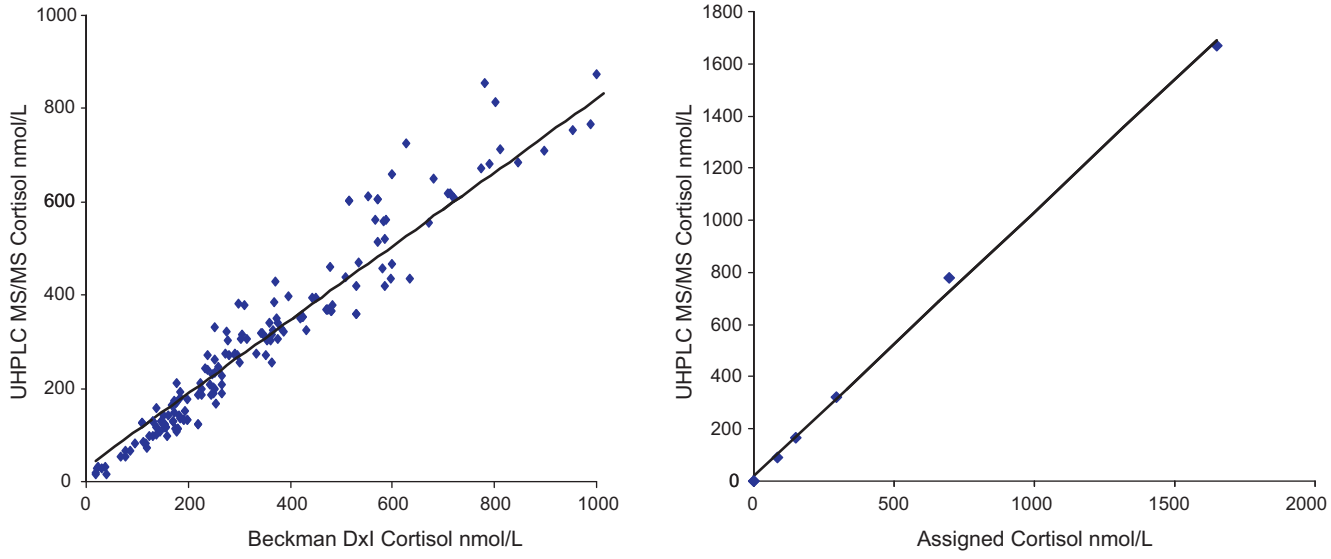

Fig. 2. (a) Comparison between the Beckman DxI cortisol immuno-assay (IA) and the UHPLC MS/MS method. $R^2 = 0.960$, UHPLC = 0.79 × IA + 31.12, $n = 155$. (b) Results of the accuracy study comparing the results of the UHPLC MS/MS method with the assigned values. The assigned values are traceable to certified reference materials BCR 192 and BCR 193. Results are the mean of two sets of duplicate results. $R^2 = 0.998$, UHPLC = 1.01 × assigned cortisol + 19.08, $n = 6$.

tisol in urine and plasma, with a $R^2 > 0.95$ ($p < 0.0001$). The UHPLC MS/MS method demonstrated a small but statistically significant positive bias for cortisol but not for 11-deoxycortisol. The positive bias for cortisol is of the same magnitude as demonstrated in the accuracy study and is not clinically significant. Comparison of cortisone measurements in urine and plasma demonstrated weaker correlation with $R^2 < 0.95$ ($p < 0.0001$).

We did not perform a method comparison with dexamethasone as all of the HPLC results were below the previously established limit of quantitation for this method of 10 nmol/L [13]. A comparison between saliva and plasma ultrafiltrate results were also not performed due to the inability of the HPLC method to accurately measure low concentrations of cortisol and cortisone. The improved analytical sensitivity of the UHPLC MS/MS method allowed measurement of dexamethasone in plasma and cortisol and cortisone in ultrafiltrates of plasma and saliva.

3.3. Clinical application

In samples submitted for routine analysis of cortisol the median (range) plasma total cortisol was 233 nmol/L (100–790), plasma total cortisone 54.4 nmol/L (31.1–105.6), plasma free cortisol 2.5 nmol/L (1.2–7.0), plasma free cortisone 3.4 nmol/L (2.2–7.0), salivary cortisol 2.7 nmol/L (1.0–8.1) and salivary cortisone 20.5 nmol/L (10.3–37.1). Representative chromatograms of saliva and plasma ultrafiltrates are presented in Fig. 1c and d.

The ratios of cortisol to cortisone in different sample types are illustrated in Fig. 3. The median (range) of the ratios of cortisol to cortisone in plasma 3.89 (2.36–8.54), plasma ultrafiltrate 0.68 (0.44–1.34), urine 0.27 (0.07–1.19) and saliva 0.15 (0.09–0.35) were significantly different from each other. Well-defined reference ranges for the free and ratios of the measured glucocorticoids

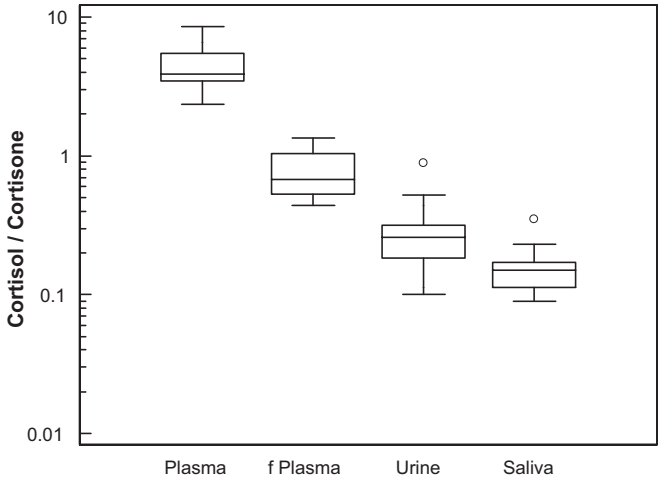

Fig. 3. Ratios of cortisol to cortisone in various sample types determined by UHPLC MS/MS. Results ($n = 23$) are represented as the median with the interquartile ranges and the error bars represent the 95 percentiles. Outliers are represented by open circles. The ratio of cortisol to cortisone was significantly different between all sample types. Friedman test: $n = 23$, F 226.7, degrees of freedom 3, $p < 0.001$, post-test all the variables were different from each other ($p < 0.05$).

in various sample types were not available for direct comparison, but Vogeser et al. [5] reported a plasma cortisol to cortisone ratio of 5.4, Taylor et al. [18] reported a ratio of approximately 0.5 in urine and Perogamvros et al. [10] a ratio of 0.2 in early morning saliva. These results, as well as ours, were obtained with small sample numbers and reference ranges in well-defined populations need to be developed.

In nine patients who had taken 1 mg dexamethasone the previous evening at 23:00, the median (range) morning (09:00) plasma total cortisol was 20.4 nmol/L (10.3–58.9), plasma free cortisol <0.6 nmol/L (<0.6–0.8) and dexamethasone was 4.5 nmol/L (2.7–9.0) (Fig. 1e).

In 13 patients who had an adequate response to metyrapone, defined as an 11-deoxycortisol >200 nmol/L, the median (range) of 11-deoxycortisol was 389 nmol/L (201–596) with a plasma UHPLC MS/MS cortisol of 49 nmol/L (24–298). In 11 patients with an inadequate response to metyrapone the 11-deoxycortisol was 128 nmol/L (<5–168) with a plasma UHPLC MS/MS cortisol of 49 nmol/L (7–171). In this latter group four patients had an IA cortisol >200 nmol/L that mistakenly suggested a failed procedure due to suboptimal metyrapone administration rather than impairment of the hypothalamic–pituitary–adrenal axis.

We detected prednisolone in the urine of five patients who underwent investigation for hypercortisolism since implementation of this method (Fig. 1f). The median urine prednisolone concentration was 402 nmol/L (221–14810) with a urine cortisol of 32 nmol/L (13–160). Prednisolone treatment was subsequently confirmed in all cases to be the cause.

The UHPLC MS/MS method has a 3 min chromatography time compared to the 32 min of the HPLC method, requires 250 μL plasma compared to 1 mL and has an improved limit of quantitation for all analytes. The ability to simultaneously determine prednisolone and dexamethasone assisted with the interpretation of unexpected low cortisol results as laboratories and clinicians are not always aware of these confounding medications at the time of sampling or analysis. The dexamethasone suppression test is a commonly performed procedure in the screening and diagnosis of Cushing syndrome and the ability to measure dexamethasone will increase the confidence in results where a lack of compliance may complicate interpretation. The simultaneous measurement of 11-deoxycortisol and cortisol with the metyrapone test also assisted with the interpretation of this diagnostic procedure. The ability to measure total and free cortisol and cortisone in plasma and urine provides an opportunity to investigate glucocorticoid metabolism in a range of pathological conditions [3].

4. Conclusion

The standardised sample preparation procedure, chromatography time, limit of quantitation and linear range make this method suitable for determination of endogenous and exogenous glucocorticoids in plasma, urine, saliva and plasma ultrafiltrates in a high throughput laboratory.

References

[1] T. Rhen, J.A. Cidlowski, New Engl. J. Med. 353 (2005) 1711.
[2] P.M. Stewart, Eur. J. Endocrinol. 149 (2003) 163.
[3] J.W. Tomlinson, E.A. Walker, I.J. Bujalska, N. Draper, G.G. Lavery, M.S. Cooper, M. Hewison, P.M. Stewart, Endocr. Rev. 25 (2004) 831.
[4] M. Palermo, C.H.L. Shackleton, F. Mantero, P.M. Stewart, Clin. Endocrinol. 45 (2003) 605.
[5] M. Vogeser, R. Zachoval, K. Jacob, Clin. Biochem. 34 (2001) 421.
[6] M. Vogeser, R. Zachoval, T.W. Felbinger, K. Jacob, Horm. Res. 58 (2002) 172.
[7] A.H. Hamrahian, T.S. Oseni, B.M. Arafah, New Engl. J. Med. 350 (2004) 1629.
[8] J.T. Ho, H. Al-Musalhi, M.J. Chapman, T. Quach, P.D. Thomas, C.J. Bagley, J.G. Lewis, D.J. Torpy, J. Clin. Endocrinol. Metab. 91 (2006) 105.
[9] E.F. De Palo, G. Antonelli, A. Benetazzo, M. Prearo, R. Gatti, Clin. Chim. Acta 405 (2009) 60.
[10] I. Perogamvros, L.J. Owen, J. Newell-Price, D.W. Ray, P.J. Trainer, B.G. Keevil, J. Chromatogr. B 877 (2009) 3771.
[11] J.M. Morton, S. Williamson, L.M. Kear, B.C. McWhinney, J. Potter, A.R. Glanville, J. Heart Lung Transplant. 25 (2006) 557.
[12] T.M. Fiad, J.M. Kirby, S.K. Cunningham, T.J. McKenna, Clin. Endocrinol. 40 (2008) 603.
[13] B.C. McWhinney, G. Ward, P.E. Hickman, Clin. Chem. 42 (1996) 979.
[14] S.J. McCann, S. Gillingwater, B.G. Keevil, Ann. Clin. Biochem. 42 (2005) 112.
[15] L. Wood, D.H. Ducroq, H.L. Fraser, S. Gillingwater, C. Evans, A.J. Pickett, D.W. Rees, R. John, A. Turkes, Ann. Clin. Biochem. 45 (2008) 380.
[16] R.L. Taylor, D. Machacek, R.J. Singh, Clin. Chem. 48 (2002) 1511.
[17] M.M. Kushnir, R. Neilson, W.L. Roberts, A.L. Rockwood, Clin. Biochem. 37 (2004) 357.
[18] R.L. Taylor, S.K. Grebe, R.J. Singh, Clin. Chem. 50 (2004) 2345.
[19] I.A. Ionita, D.M. Fast, F. Akhlaghi, J. Chromatogr. B 877 (2009) 765.
[20] J. Gibney, M.L. Healy, T.P. Smith, T.J. McKenna, J. Clin. Endocrinol. Metab. 93 (2008) 3763.
[21] S.K. Baid, N. Sinaii, M. Wade, D. Rubino, L.K. Nieman, J. Clin. Endocrinol. Metab. 92 (2007) 3102.
[22] N. Nishiyamaa, S. Makinoa, S. Suemarub, W. Nanamiyaa, K. Asabaa, T. Kanedaa, T. Mimotoa, T. Nishiokaa, T. Takaoa, K. Hashimotoa, Horm. Res. 54 (2000) 69.