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Short Communication

Quantitation of aldosterone in human plasma by ultra high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Aldosterone is a mineralocorticoid steroid hormone whose measurement in the clinical laboratory is principally performed for the investigation of primary hyperaldosteronism. Traditionally measurement of aldosterone has been performed by radioimmunoassay, however these assays lack specificity as they are prone to interference from structurally related steroid hormones. Herein, we have developed a novel, sensitive and specific method utilising solid phase extraction and quantitation of aldosterone from human plasma by UPLC–MS/MS. Standards, quality controls and samples (250 μL) were extracted using Oasis® HLB 96-well plates. Extract (30 μL) was injected onto a Krudcatcher UPLC In-Line Filter, 0.5 μm guard column, coupled to a Kinetex PFP, 100 mm × 2.1 mm, 2.6 μm column with methanolic mobile phase gradient elution. Eluant was connected to a Waters® Xevo TQS tandem mass spectrometer operating in electrospray negative mode. We detected multiple reaction monitoring (MRM) transitions of m/z 359.0 > 189.1 for aldosterone and 366.0 > 194.1 for d7-aldosterone respectively, which co-eluted at 2.65 min. Ion suppression was negligible. Mean recovery was 89.6%, limit of detection and lower limit of quantitation were 26 pmol/L and 30 pmol/L respectively. The assay was linear up to 3200 pmol/L (r^2 = 0.9999). Mean intra- and inter-assay imprecision and bias were all <10%. Comparison of the UPLC–MS/MS method with an immunoassay in routine clinical use in the UK yielded the equation UPLC–MS/MS = 0.789(RIA)–41.7, linear regression r^2 = 0.88, n = 54. We have developed a sensitive and specific method for the extraction and measurement of aldosterone from human plasma. The method features a simple 96-well plate solid phase extraction procedure, highly selective column chemistry and short chromatographic run times.

1. Introduction

Aldosterone is a mineralocorticoid steroid hormone produced by the zona glomerulosa of the adrenal cortex. The principle action of aldosterone is to regulate sodium reabsorption and potassium excretion in the renal collecting ducts and distal convoluted tubule. Control of synthesis and secretion of aldosterone is primarily exerted by the renin–angiotensin system (RAS), which is itself stimulated by a fall in systemic blood pressure and sodium depletion.

Clinically, measurement of aldosterone is important to identify patients with primary hyperaldosteronism. This condition is most commonly caused by autonomous aldosterone production by an adrenal adenoma (classical Conn's syndrome), or hyperplasia of one or both adrenal glands. Hyper-secretion of aldosterone typically presents clinically with drug resistant hypertension and, in up to 37% of cases, hypokalaemia [1]. The prevalence of primary hyperaldosteronism in patients with essential hypertension may be up to 10% [2], but due to non specific signs and symptoms the condition is frequently overlooked. Where clinical suspicion arises, screening is indicated as effective medical and surgical management options exist to control or cure the condition. The recommended initial screening test for primary hyperaldosteronism is assay of the plasma aldosterone to renin ratio (ARR) [2].

Measurement of aldosterone in human plasma in clinical laboratories has most frequently been performed using radio immunoassay (RIA) or enzyme labelled immunoassay [3]. The specificity of immunoassays is often poor due to antibody cross reactivity to other structurally related steroid hormones, which may result in falsely elevated concentrations. To overcome these limitations, in recent years there has been growing interest in the use of liquid chromatography tandem mass spectrometry (LC–MS/MS) for steroid hormone analysis in clinical laboratories. Recently, a small number of reports have been published detailing methods for the quantitation of aldosterone from human plasma by LC–MS/MS. Such methods utilise liquid–liquid extraction (LLE) with relatively long chromatographic run times [4–6], or require highly specialised automated extraction platforms [5] which may not be suitable for many clinical laboratories. Herein, we report the development of a highly sensitive and specific method for the

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measurement of aldosterone from human plasma by UPLC–MS/MS. The method utilises a simple 96-well plate solid phase extraction (SPE) technique, highly selective column chemistry and short chromatographic run times. The method provides a useful option for laboratories wishing to analyse aldosterone in routine clinical practice with relatively high throughput.

2. Methods

2.1. Materials

Oasis® HLB 96-well extraction plates were purchased from Waters (Hertfordshire, UK). LC–MS grade methanol was purchased from Greyhound (Birkenhead, UK). LC–MS grade acetonitrile, aldosterone, bilirubin and haemoglobin were purchased from Sigma (Dorset, UK). d7-aldosterone was purchased from Isosciences (Pennsylvania, USA). Polypropylene 2 mL 96-deep-well plates were purchased from Porvair Sciences (Surrey, UK), and 'Easy Pierce' heat sealing foil sheets from Thermo Scientific (Surrey, UK). Intralipid was purchased from Kabi Pharmacia (Uppsala, Sweden).

2.2. Calibration standards and quality control material

Stock solutions of aldosterone and d7-aldosterone were prepared by weighing pure aldosterone powder and solubilising in methanol. Calibration standards and quality controls (QCs) were prepared by spiking stock aldosterone solution into phosphate buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA). Final calibrator concentrations ranged from 0 to 3200 pmol/L. Three QC samples were prepared to achieve final concentrations of 150, 500 and 1500 pmol/L, representing clinically relevant aldosterone concentrations.

2.3. Sample preparation

Patient plasma samples were obtained using K^+EDTA or lithium heparin collection tubes. Deuterated d7-aldosterone was prepared to a final concentration of 60 μg/L in methanol, and 10 μL added to 250 μL standards, QC's and patient plasma samples. To all samples, 125 μL 0.1 mol/L zinc sulphate was added, and samples vortexed for 20 s. Following this, 250 μL methanol was added to each sample and the samples vortexed for 1 min, followed by centrifugation for 5 min at 8000 × *g*. Individual wells of an Oasis® HLB 96-well extraction plate were washed consecutively with 1 mL acetonitrile, 1 mL methanol, then 0.5 mL H_2O. The total volume of supernatants obtained following zinc sulphate and methanol precipitation were applied to individual wells of the extraction plate and washed with 0.5 mL H_2O then 0.5 mL 40% methanol. Bound material was eluted from the SPE adsorbent using 1 mL acetonitrile and collected into 2 mL 96-deep-well polypropylene plate. Acetonitrile was evaporated to dryness and wells re-constituted in 80 μL of 30:70 methanol:water (v/v). Finally, the plate was sealed with 'Easy Pierce' heat sealing foil, vortexed for 2 min and centrifuged for 5 min at 8000 × *g*.

2.4. Liquid chromatography

Chromatography was performed on a Waters® Acquity™ UPLC system. Extracted sample (30 μL) was injected directly from the 96-deep well plate onto a Krudcatcher UPLC In-Line Filter, 0.5 μm × 0.004″ (Phenomenex, Macclesfield, UK) guard column, coupled to a Kinetex PFP, 100 mm × 2.1 mm, 2.6 μm column (Phenomenex, Macclesfield, UK).

Mobile phase A contained laboratory grade de-ionised H_2O, and mobile phase B contained methanol. Initial conditions were 70:30 (v/v) mobile phase A:B. Following sample injection, elution was performed by means of a gradient from 30% to 65% mobile phase over 3 min, followed by 95% mobile phase B held for 30 s. Following this, the columns were re-equilibrated back to initial conditions and held for a further 1 min prior to the next sample injection. Total chromatographic run time was 4.5 min and total injection-to-injection run time was 5 min. The mobile phase flow rate was maintained at 0.45 mL/min, and the column maintained at 45 °C.

2.5. Tandem mass spectrometry

Eluate from the analytical column was injected directly into a Waters® Xevo TQS tandem mass spectrometer operating in the negative electrospray ionisation mode (Waters, Hertfordshire, UK). The instrument conditions were as follows: electrospray capillary voltage 2.8 kV, collision energy 20 eV, sample cone voltage 30 V and source offset 40 V. Desolvation gas flow and temperature were maintained at 500 L/h and 600 °C respectively, cone gas flow was maintained at 150 L/h, and the source temperature was 150 °C. Aldosterone and d7-aldosterone were detected in multiple reaction monitoring (MRM) mode with a dwell time of 0.125 s per channel. MRM transitions were m/z 359.0 > 189.1 (quantifier) and 359.0 > 297.0 (qualifier) for aldosterone, and 366.0 > 194.1 for d7-aldosterone. Collision gas flow was maintained at 0.15 mL/min. Resolution was 2.7 (low mass) and 14.7 (high mass) for both MS1 and MS2.

2.6. LC–MS/MS method validation

2.6.1. Ion suppression

Ion suppression/enhancement was assessed by continuous post-column infusion of d7-aldosterone (1 mg/L in 50:50 mobile phase *A*:*B*) directly into the mass spectrometer at a flow rate of 10 μL/min. Ten patient plasma samples were extracted and injected, as described in Sections 2.3 and 2.4, along with a methanol and H_2O blank. Ion suppression or enhancement was interpreted as a fall or increase in baseline count greater than 10% at the retention time of aldosterone.

2.6.2. Linearity

Linearity of the assay was assessed by repeat (n = 6) analysis of calibrators, with concentrations ranging from 0 to 3200 pmol/L. LC–MS/MS response was plotted against nominal concentration values using TargetLynx™ software (Waters, Hertfordshire, UK). In addition, patient plasma samples were assayed neat, diluted with PBS/BSA and assayed over a range of dilutions. Measured aldosterone concentrations were compared to expected concentrations. Linearity of the assay was confirmed by weighted linear regression with a correlation coefficient r^2 value > 0.99.

2.6.3. Accuracy and recovery

To confirm the utility of PBS/0.1% BSA matrix for preparation of standards and QCs, the following accuracy and recovery experiments were performed.

The method of standard additions was used with plasma and PBS/BSA-based spiked calibrators. Samples were spiked with a range of aldosterone concentrations from 50 to 2000 pmol/L. The PBS/BSA-based calibrators were deemed acceptable for use if calculated aldosterone concentrations were within 10% of plasma matrix following standard additions.

To calculate recovery, pure aldosterone stock solution was used to spike into six different patient plasma samples. These samples had endogenous aldosterone concentrations ranging from 104 to 222 pmol/L. The concentrations of aldosterone added to aliquots of each of these six plasma samples were 64, 353 and 1221 pmol/L. Each sample was assayed in triplicate and % recovery calculated from measured compared to expected concentrations. According

to validation guidelines issued by the US Food and Drug Administration [7], recovery values are acceptable if the mean measured value is within 15% of the true value.

2.6.4. Precision

Assay imprecision was assessed by spiking PBS/0.1% BSA matrix with 150, 500 and 1500 pmol/L of pure aldosterone. Samples were analysed repeatedly ($n = 15$) within a single analytical run to determine within batch imprecision, and analysed in separate batches ($n = 15$) over a period of two weeks to determine between batch imprecision. Precision was assessed as a function of the variation (%CV), and to assess bias, the percentage deviation from the target value was calculated from the difference between the mean observed and nominal concentrations. According to US FDA guidelines [7] precision and percentage deviation from the target value are deemed acceptable if <15%.

2.6.5. Lower limit of detection and quantitation

The lower limit of detection (LOD) was determined as the smallest detectable peak in extracted plasma above baseline noise (signal:noise ratio >3:1, peak to peak). The lower limit of quantitation (LLOQ) was determined using a pooled plasma sample with extremely low concentrations of aldosterone. Aliquots of this pooled sample were spiked with low concentrations of aldosterone. The aldosterone concentration in each sample was measured ($n = 15$) and the %CV and deviation from the theoretical target value determined. The LLOQ was assigned to the lowest concentration with both a CV and mean value from the theoretical target of <20% which is within US FDA validation guidelines [7].

2.6.6. Specificity

Solutions of a variety of natural and synthetic steroids were prepared in 50% (w/v) methanol/water and injected directly into the mass spectrometer without internal standard. Steroids were tested at excess concentration to allow easy identification of interfering peaks, as evidenced by signal at the retention times of aldosterone and d7-aldosterone. Steroids tested to a final concentration of 1 μmol/L were cortisol, testosterone, dehydroepiandrosterone sulphate, dehydroepiandrosterone, oestradiol, progesterone, cortisone, corticosterone, 17 hydroxyprogesterone, 11 deoxycortisol, 21 deoxycortisol, pregnenolone, 19 nortestosterone, epitestosterone and androstenedione. Synthetic steroids tested to a final concentration of 1 mg/L were fludrocortisone, dexamethasone, methylprednisolone, budesonide, prednisone, beclometasone, cyproterone, norethisterone, triamcinolone and prednisolone.

Additionally the effect of sample haemolysis, lipaemia and icterus was investigated by spiking patient plasma samples ($n = 5$) with increasing concentrations of haemoglobin (up to 10 g/L), triglyceride containing Intralipid (up to 20 mmol/L triglyceride) and bilirubin (up to 500 μmol/L). The % recovery was calculated from measured compared to expected aldosterone concentrations.

2.7. Method comparison

Patient plasma samples ($n = 54$) were analysed by both the UPLC–MS/MS method and an immunoassay in routine clinical use in the UK. Concentrations determined by each method were compared by Passing–Bablock and Bland–Altman analysis using the statistical analysis software Analyse-It™ (Analyse-It Software Ltd., Leeds, UK).

Additionally, our laboratory participates in the United Kingdom National External Quality Assessment Scheme (NEQAS). Plasma samples distributed by this scheme were analysed by the LC–MS/MS method, and calculated aldosterone concentrations compared to values obtained by RIA method users (principally the DPC 'Coat-A-Count' RIA) and other LC–MS/MS method users by Passing–Bablock and Bland–Altman analysis.

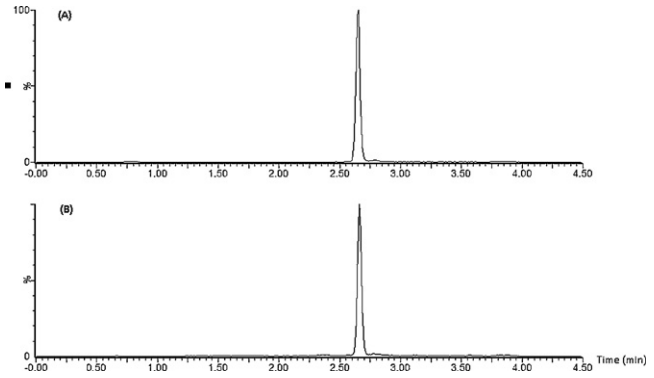

Fig. 1. Typical chromatograms for aldosterone and d7-aldosterone internal standard produced by the new UPLC–MS/MS method. (A) Chromatogram of the d7-aldosterone internal standard with a detector response of 1.13×106 cps. (B) Chromatogram of a patient sample with a detector response of 6.26×105 cps, yielding an aldosterone concentration of 1435.7 pmol/L. Both chromatograms demonstrate negligible interference in the immediate region of elution and both aldosterone and d7-aldosteone have a similar retention time of 2.65 min.

3. Results and discussion

3.1. Solid phase extraction and liquid chromatography

The SPE platform utilised in this method is a relatively simple 96-well plate extraction technique, which enables rapid, high throughput extraction of samples. This proved to be the most efficient SPE method we evaluated during the method development phase.

Of several different UPLC column chemistries trialled, the pentaflurophenyl functional group on the PFP column detailed in this method demonstrated optimal selectivity for aldosterone and achieved excellent chromatographic efficiency. The chromatographic retention time was 2.65 min for both aldosterone and d7-aldosterone, and chromatograms demonstrated clean discrete peak with no observed interference in the region of aldosterone and d7-aldosterone elution (Fig. 1). Additionally, this column achieved a short chromatographic run time with a total injection-to-injection run time of 5.0 min. This is an important consideration if the method is to be introduced into routine clinical practice as it enables higher throughput analysis of large numbers of samples. Finally, the PFP column demonstrated the most favourable ion suppression profiles compared to other C18 columns. Ion suppression/enhancement experiments using continuous post-column infusion of d7-aldosterone exhibited minimal signal interference (defined as a fall or increase in baseline count no greater than 10% at the retention time of aldosterone), following injection of extracted plasma samples into the mass spectrometer.

3.2. Method validation

Validation of the method was performed according to US FDA guidelines for industry [7], and validation data is summarised in Table 1.

Data averaged from six individual calibration curves demonstrated that the assay was linear up to at least 3200 pmol/L of aldosterone ($r^2 = 0.9999$; $y = 5^{-4}x + 9^{-5}$). In our experience the aldosterone concentration range utilised in the assay is large enough to cover the majority of patient samples, including those with proven hyperaldosteronism. Dilution of patient plasma samples with PBS/BSA diluent demonstrated acceptable measured aldosterone concentrations compared to expected concentrations

Table 1
Method validation data.

Linearity ($n = 6$)					
Linear range (pmol/L)			Mean r^2		
0–3200			0.9999		
Intra assay precision ($n = 15$)			Inter assay precision ($n = 15$)		
Target value (pmol/L)	Mean CV (%)	Mean bias (%)	Target value (pmol/L)	Mean CV (%)	Mean bias (%)
150	11.5	−11.9	150	12.6	−2.4
500	3.1	3.6	500	7.0	11.4
1500	2.5	5.2	1500	3.7	5.5
Recovery ($n = 6$)					
Target value (pmol/L)			Mean recovery (%)		
64			88.3		
353			92.1		
1221			88.5		
Limit of detection (pmol/L)					
26					
Limit of quantitation (pmol/L)					
30					

with mean linear regression value r^2 value >0.99. Samples with aldosterone concentrations above the top standard can be diluted to achieve a suitable concentration within the linear range of the assay.

Ion suppression experiments demonstrated that aldosterone eluted in a part of the chromatogram free from matrix effects. Comparison of plasma and PBS/BSA matrix by the method of standard additions demonstrated that the PBS/BSA matrix based calibrants were within the 10% target. Additionally, calculated % recovery of three different concentrations of spiked aldosterone into 6 different patient plasma samples yielded a mean recovery of 89.6% (range 88.3–92.1%) which is within acceptable limits according to US FDA guidelines. Taken together, these data confirm that PBS/BSA standards can be used to calibrate the assay.

The assay exhibited acceptable intra- and inter-assay precision and accuracy; mean co-efficients of variation (CV) for intra- and inter-assay precision were 5.7% (mean bias −1.0%), and 7.8% (mean bias 4.8%) respectively. The LOD was 26 pmol/L and the LLOQ was 30 pmol/L (CV% = 6.3, mean bias = −18%), which compares favourable to previously published methods which have reported LLOQ values of 50 pmol/L [6] and 69 pmol/L [5].

Assay of a wide range of natural and synthetic steroids demonstrated no detectable response above the lower limit of quantitation at the retention times of aldosterone and d7-aldosterone when injected into the mass spectrometer at excess concentration. There was no significant effect on aldosterone measurement in plasma samples spiked with high concentrations of bilirubin, haemoglobin or triglycerides.

Taken together, these data confirm the utility of the UPLC–MS/MS method to accurately, precisely and specifically measure aldosterone following SPE from human plasma.

3.3. Method comparison

Patient plasma samples ($n = 54$) were analysed by both the UPLC–MS/MS method and an immunoassay in routine clinical use in the UK. Passing and Bablock analysis yielded the equation LC–MS/MS = 0.789(RIA)–41.7, linear regression $r^2 = 0.88$, $n = 54$ (Fig. 2A). Bland–Altman analysis demonstrated a mean negative bias of −148.7 pmol/L (95% CI −205.3 to −92.1) for the UPLC–MS/MS method (Fig. 2B). These findings appear to be in agreement with previously published methods which have demonstrated significant negative bias in LC–MS/MS methods compared to immunoassay [4,6], although this is not a universal finding [5].

In addition, aldosterone concentrations in plasma samples distributed by UK-NEQAS were measured using our LC–MS/MS method and compared to RIA method users, and other LC–MS/MS users. Comparison of our LC–MS/MS method against RIA method users, yielded the Passing and Bablock equation LC–MS/MS = 0.950(RIA)–74.4, linear regression $r^2 = 0.96$ ($n = 21$),

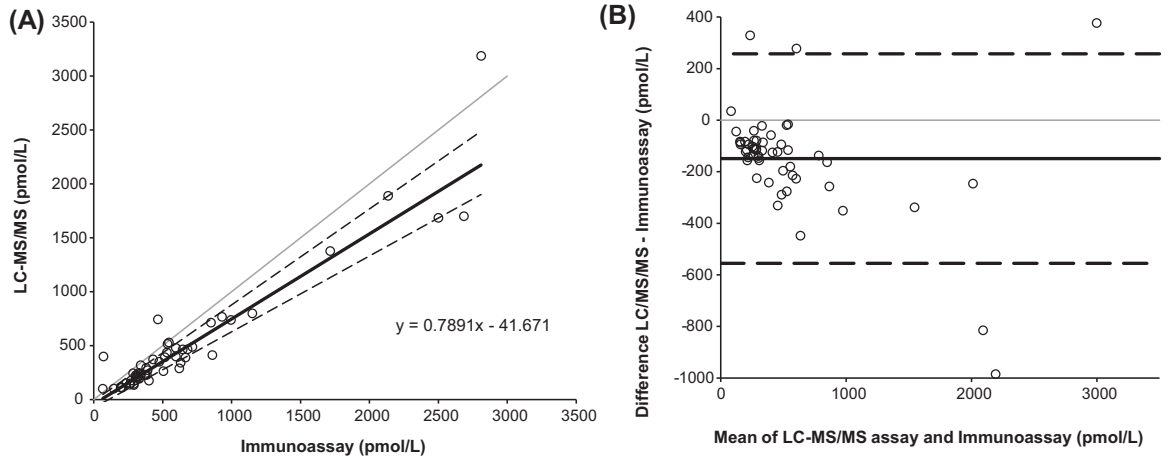

Fig. 2. Method comparison for aldosterone, between the LC–MS/MS method and an immunoassay in routine clinical use in the UK; (A) Passing–Bablock analysis yielded the equation LC–MS/MS = 0.789(RIA)–41.7, linear regression $r^2 = .88$, $n = 54$. (B) Bland–Altman analysis demonstrated a mean negative bias of −148.7 pmol/L (95% CI −205.3 to −92.1) for the UPLC–MS/MS method.

and Bland–Altman analysis demonstrated a mean negative bias of −109.6 pmol/L (95% CI −145.8 to −73.3) for our LC–MS/MS method. Comparison of our LC–MS/MS method against other LC–MS/MS method users, yielded the Passing and Bablock equation 'Our Method' = 1.000 ('other LC–MS/MS users') + 12.0, linear regression $r^2 = 0.96$ ($n = 31$), and Bland–Altman analysis demonstrated a mean positive bias of 15.0 pmol/L (95% CI −20.1 to 50.2) for our LC–MS/MS method.

4. Conclusions

We have developed a novel, sensitive and specific UPLC–MS/MS method for the quantitation of aldosterone from human plasma. Our intention was to simplify extraction conditions such that they could be used universally without the need for specialist automated extraction platforms. The method utilises a simple SPE technique, thus avoiding liquid–liquid extraction procedures which require transfer of organic solvent supernatants between tubes [4,6]. Additionally, we aimed to develop an assay with shorter chromatographic run times and higher throughput compared to previously published methods, enabling the assay to be used in routine clinical practice. This new method has a chromatographic run time of 5 min, which compares favourably to the longer run times (8–10 min) detailed in previous methods [4–6]. The highly selective column chemistry utilising the functional pentaflurophenyl group yields excellent chromatographic separation and ion suppression profiles. Finally, the method required smaller sample volumes than previous methods [4,6], and has a lower functional LLOQ [5,6].

The new UPLC–MS/MS method was compared to an immunoassay in routine clinical use in the UK. As expected, comparison of over 50 patient samples demonstrated a significant negative bias in the UPLC–MS/MS method, confirming the improved specificity of this method compared to immunoassay. Clinically, improved specificity is important to prevent over investigation of patients incorrectly diagnosed as having hyper aldosteronism. In addition our LC–MS/MS method exhibited similar performance in comparison to other LC–MS/MS users.

In conclusion, we have developed a novel, sensitive and specific method for the quantitation of aldosterone from human plasma. The method features a simple 96-well plate SPE procedure, highly selective column chemistry and short chromatographic run times.

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