


RESEARCH ARTICLE

Developing an ultra-performance liquid chromatography-tandem mass spectrometry for detecting aldosterone in human plasma

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Abstract

Background: Accurately measuring plasma aldosterone concentration is difficult but meaningful for primary aldosteronism (PA) diagnosis.

Methods: In this study, we developed an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for plasma aldosterone detection, evaluated its performance according to guidelines issued by CLSI, including detection limit, linearity, precision, and compared it with chemiluminescence immunoassay. Then, a reference range of plasma aldosterone in young people was established by using this method.

Results: The lower limit of quantitation (LOQ) was 10 pg/ml. The mean recovery rates of analyte added to serum were 100.07–102.05% in different concentrations. The linearity range was 20–2000 pg/ml. Inter-assay CVs were 2.20–3.97% at aldosterone concentrations of 65.66–854.75 pg/ml. The regression equation of UPLC-MS/MS (x) and chemiluminescence immunoassay (y) was $y = 1.002x + 65.854$ ($r = 0.9456$, $n = 237$). The reference range of plasma aldosterone detected by UPLC-MS/MS was 11.30–363.82 pg/ml in young people in South China, and there was no statistically significant difference in plasma aldosterone concentration between two genders.

Conclusion: In conclusion, UPLC-MS/MS can rapidly and accurately detect plasma aldosterone and is appropriate for clinical application.

KEYWORDS

aldosterone, performance, plasma, primary aldosteronism, UPLC-MS/MS

1 | INTRODUCTION

Autonomously excessive aldosterone secretion which is caused by aldosterone-producing adenoma and adrenal cortical hyperplasia¹

is the etiology of primary aldosteronism (PA). PA is mainly prevalent in secondary hypertension, which accounts for about 10–30% of refractory hypertension in different studies.^{2–5} Too much aldosterone causes not only high blood pressure through sodium and

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water retention but also organ damage through inflammation and fibrosis.¹ So, PA patients are more likely to suffer from cardiovascular complications,⁶ such as ventricular hypertrophy, cerebral infarction, arrhythmia, etc. Fortunately, surgical resection and aldosterone antagonists can effectively treat PA when the etiology is assured.⁷ Hence, plasma aldosterone measurement is significant for PA, even hypertension, diagnosis.

Aldosterone is extremely difficult to be accurately measured because of its trace and numerous analogs in plasma.⁸ At present, conventional tests based on radioimmunoassay and chemiluminescence immunoassay (CLIA) are unsatisfactory due to their defects in safety and accuracy,⁹ because radioimmunoassay uses radioisotope-labeled antibody to detect aldosterone, and CLIA does not distinguish structural analogs of aldosterone in plasma in some cases. Mass spectrum has great advantages in trace detection and anti-interference, so gas or liquid chromatography-tandem mass spectrometry (GC/LC-MS) has been applied in aldosterone detection.^{10,11} Sample needs complex derivatization before GC-MS detection, which makes it difficult for clinical application. Compared with GC-MS, LC-MS requires only simple sample pretreatment, therefore, aldosterone measurements based on LC-MS will be popular in clinical laboratories.

In this study, we developed an aldosterone detection method based on ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). We determined its methodological characteristics and a reference interval in young people by using this method. Besides, correlation and bias between this method and our daily-used CLIA method were also analyzed. Our results indicate UPLC-MS/MS has extremely high accuracy in aldosterone detection and requires simple sample pretreatment, which is suitable for clinical laboratory. Our study is one of the few studies on the detection of aldosterone by using UPLC-MS/MS.^{12,13} It lays foundation for clinical application of UPLC-MS/MS in aldosterone detection.

2 | MATERIALS AND METHODS

2.1 | Recruitment of healthy volunteers

This study was approved by the medical ethics committee of the hospital (batch number: [2018]054), and the agreement from every participant was obtained. A total of 237 healthy volunteers were recruited in the First Affiliated Hospital of Sun Yat-sen University. Inclusion criteria were (1) normal hypertension (systolic pressure: 90–140 mmHg; diastolic pressure: 60–90 mmHg, 1 mmHg = 0.133 kPa); (2) no obviously cardiac, liver, and renal dysfunction; (3) no drug intake in recent three months, including (a) diuretics, such as spironolactone, amiloride, aminophenylpyridine, etc.; (b) central nervous system drugs, such as clonidine, α -methyl dopa, etc.; (c) non-steroidal anti-inflammatory drugs; (d) anti-hypertensive drugs, such as angiotensin-converting enzyme inhibitors, angiotensin receptor

TABLE 1 Elution gradients

Gradient	Time (min)	Methanol (%)	Water (%)
1	0	30	70
2	0.5	30	70
3	3.5	70	30
4	4.5	95	5
5	5	95	5
6	5.5	30	70
7	8	30	70

antagonists, renin inhibitors, dihydropyridine calcium channel antagonists; (4) no smoking; (5) no pregnancy; (6) no recent surgery; (7) no hormone replacement therapy; (8) no family history of hypertension and diabetes.

2.2 | Sample collection

Before sample collection, participants were asked to have a full-night sleep (>8 h). After that, participants were asked to stand or walk for 2 h and seat for 15 min. Then, blood sample in upright position was collected between 9:00 and 10:00 AM. Specimen was collected in EDTA-K₂ anticoagulant tube. Plasma was separated by centrifugation at room temperature, 3000 g for 5 min. Then, plasma was split into Eppendorf tubes and store at -20°C before use. Another blood sample from the same volunteer was collected in a procoagulant tube at the same time to check the baseline characteristics, such as blood sodium and potassium concentrations, liver and kidney function, etc.

2.3 | Sample detection

Pretreatment was needed before UPLC-MS/MS detection. 15 μ l internal standard (10 ng/ml d4-aldosterone, Sigma-Aldrich) was added into 200 μ l plasma for each sample. Then, 200 μ l of 0.1 M zinc sulfate solution-methanol (v/v, 5:5) and 450 μ l of 0.05% phosphoric acid (v/v) were added. Sample was vortexed for 90 sec and centrifuged at 18,000 g for 10 min. 200 μ l methanol and pure water were respectively used to active SPE column (Oasis MAX, 30 μ m). Then, 625 μ l supernatant was added into the SPE column. The column was washed with 200 μ l of 0.05% phosphoric acid (v/v), 0.1% ammonia methanol-water (v/v, 1:9), and pure water, respectively. Aldosterone was washed by 50 μ l methanol-water solution (v/v, 7:3) before UPLC-MS/MS detection.

The UPLC-MS/MS system consisted of a Waters ACQUITY™ UPLC system and a Waters Xevo™ TQ-S tandem triple quadrupole mass spectrometer. 20 μ l eluent was injected into UPLC system. C18 column (CORTECS, 2.1*100 mm, 1.6 μ m particles) was used to separate. The mobile phase consisted of methanol and pure water. The flow rate was 0.3 ml/min. Elution gradients are shown in Table 1. Parameters for mass spectrometer setup and detected ion pairs are

shown in Tables 2 and 3. The ion source of the mass spectrometer worked in the negative ion model.

2.4 | Methodological evaluation

2.4.1 | Limit of detection (LOD) and limit of quantitation (LOQ)

Methodological evaluation and comparison were performed according to guidelines published by the Clinical and Laboratory Standards Institute (CLSI), including C50, C62-A, C28-A2, and CLIA'88. For the sensitivity evaluation, a series of low aldosterone concentration samples were repeatedly tested. LOD was defined as signal to noise ratio (S/N) >3 and coefficient of variation (CV) <20%. LOQ was defined as S/N >10 and CV <20%. Each sample was tested six times.

2.4.2 | Linearity

A series of aldosterone standards with different concentrations (20–2000 pg/ml) which contained internal standards were prepared. Each sample was tested twice, and average peak area ratio of aldosterone to internal standard was calculated. Linear regression analysis was done by using aldosterone standard concentrations (x-axis) and peak area ratios of aldosterone to internal standard (y-axis), and R^2 should be over 0.99.

2.4.3 | Recovery

50, 200, and 1000 pg/ml aldosterone were added into blood sample with known aldosterone concentration. Each sample was tested three times. Recovery concentration was calculated by the detected concentration subtracting the known concentration. Recovery rate

TABLE 2 Mass spectrometer setup parameters

Parameters	Value
Capillary voltage	2.5kV
Source temperature	150°C
Desolvation temperature	600°C

TABLE 3 Detected ion-pairs

Compound name	Parent (m/z)	Daughter (m/z)
ALD	359	189
ALD	359	297
d4-ALD	363	190

Abbreviation: ALD, aldosterone.

was defined as the percentage of the recovery concentration to the added concentration.

2.4.4 | Precision

A total of samples with different aldosterone concentrations (65.66, 234.14, and 854.75 pg/ml) were used. Each sample was measured twice at a time, 6 times a day for three consecutive days. CVs were calculated and compared with TEa (14.7%) given by CLIA'88. The CV of intra-assay precision should be no more than a quarter of TEa, and the CV of inter-assay precision should be no more than a third of TEa.

2.4.5 | Methodological comparison

A total of 237 samples were detected by UPLC-MS/MS and CLIA, respectively. Bias and regression coefficient were analyzed by Bland-Altman analysis.

2.5 | Reference range establishment

Aldosterone in plasma collected from 193 healthy volunteers (21–30 years) was measured by UPLC-MS/MS as previously described. Statistical difference was tested in different genders. Reference range was determined by percentile method or mean \pm 2 times standard deviation (SD).

2.6 | Statistical analysis

SPSS v23.0 (IBM) was used in the whole statistical analysis. Aldosterone concentrations were shown in mean \pm SD or indicated. CV was calculated by dividing SD by mean. Normal distribution was checked by Kolmogorov-Smirnov test. Outliers were excluded by Mahalanobis distance method. Data with normal distribution were analyzed by the Student *t* test and the Pearson correlation analysis to detect statistical difference and correlation between methods and genders. If the data were non-normal distribution, the Mann-Whitney *U* test and the Spearman correlation analysis were used. $p < 0.05$ was defined as statistically significant difference.

3 | RESULTS AND DISCUSSION

3.1 | Method validation

3.1.1 | Selectivity

First, we determined the selectivity of our method. A chromatogram graphical of aldosterone is shown in Figure 1. The chromatographic

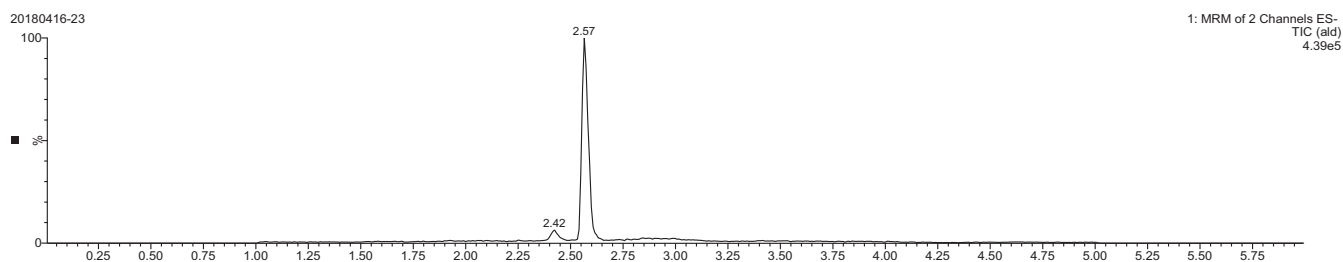


FIGURE 1 A chromatogram graphical of aldosterone

retention times of aldosterone and internal standard were about 2.5 min both, and no obvious abnormal peak was observed next to aldosterone and internal standard, which showed high selectivity of our method.

3.1.2 | LOD, LOQ, and linearity

After detecting a series of low aldosterone concentration samples, we found that the LOD for aldosterone detected by the developed UPLC-MS/MS method was 5 pg/ml, and the LOQ was 10 pg/ml. Then, we determined the linearity by detecting a series of aldosterone standards with different concentrations. We found the linearity range was 20–2000 pg/ml. The linear regression equation between the peak area ratio (y) and the aldosterone concentration (x) was $y = 0.0104861x + 0.00268166$ with an $R^2 = 0.9993$, which met the methodological requirement. Compared with previously reported MS methods for aldosterone detection, we found the LOQ of our method was comparable with previously published UPLC-MS/MS methods (10 and 10.83 pg/ml),^{12,13} while the linearity range was smaller than Tan et al.¹² reported method (10–5000 pg/ml) but larger than Hinchliffe et al.¹³ reported method (0–1153 pg/ml). Then, we compared our developed method with other reported LC-MS methods. Our method was comparable with them in LOD/LOQ, but had a larger linearity range and used less sample volume, which may be more suitable for clinical use.^{14,15}

3.1.3 | Recovery and precision tests

Recovery rates of the developed method were determined as 100.07%, 102.05%, and 101.95% after repeatedly measuring samples added with three different concentrations of aldosterone. For precision evaluation, results were shown in Table 4. The maximum intra-assay and inter-assay CVs were 2.75% and 3.97%, respectively, which appeared at the low aldosterone concentration. For medium and high aldosterone concentrations, intra-assay and inter-assay CVs were much less than those for the low concentration. Results of the recovery and precision tests all meet the requirements of the C62-A and CLIA'88 and are better than other reported LC-MS/MS methods,^{16–18} which means the developed method has a satisfactory repeatability and reproducibility.

TABLE 4 Results of precision evaluation

Sample	Times	Mean (pg/ml)	Intra-run CV (%)	Inter-run CV (%)
A	36	65.66	2.75	3.97
B	36	234.14	2.70	3.39
C	36	854.75	1.70	2.20

3.1.4 | Methodological comparison

For methodological comparison, the same batch of plasma samples were detected by the UPLC-MS/MS and CLIA methods respectively, and Mann-Whitney U test was used to compare the results, because both aldosterone concentrations detected by UPLC-MS/MS and CLIA were non-normal distribution. There was a statistically significant difference between the results of two methods ($p < 0.01$). The linear regression equation of UPLC-MS/MS (x) and CLIA (y) detected aldosterone concentrations was $y = 1.002x + 65.854$ with an $R^2 = 0.8941$. The average bias between aldosterone concentrations detected by these two methods was -66.07 pg/ml, which is consistent with previous findings that there is an obvious negative bias between LC-MS/MS methods and immunoassays.¹³ The SD of the bias was 31.27. There were 10 samples that exceeded the 95% consistency limit (Figure 2). These results indicate that the correlation between the two methods is good, although there is a fixed bias between them. These results lead us to believe that the developed UPLC-MS/MS method is sensitive and accurate, which might be suitable for clinical application.

3.2 | Reference range of UPLC-MS/MS detected aldosterone concentration

A total of 193 young volunteers (21–30 years old) met the inclusion criteria. Plasma aldosterone concentrations of the volunteers were detected by the UPLC-MS/MS method. The aldosterone concentrations were also non-normal distribution in this section. Three outliers were eliminated from the results based on the Mahalanobis distance method. 72 cases were male in the rest of cases. Before establishing the reference range, we examined the correlation between plasma aldosterone concentration and gender by using Mann-Whitney U test. We found that there was no correlation between them, which is in accordance with our

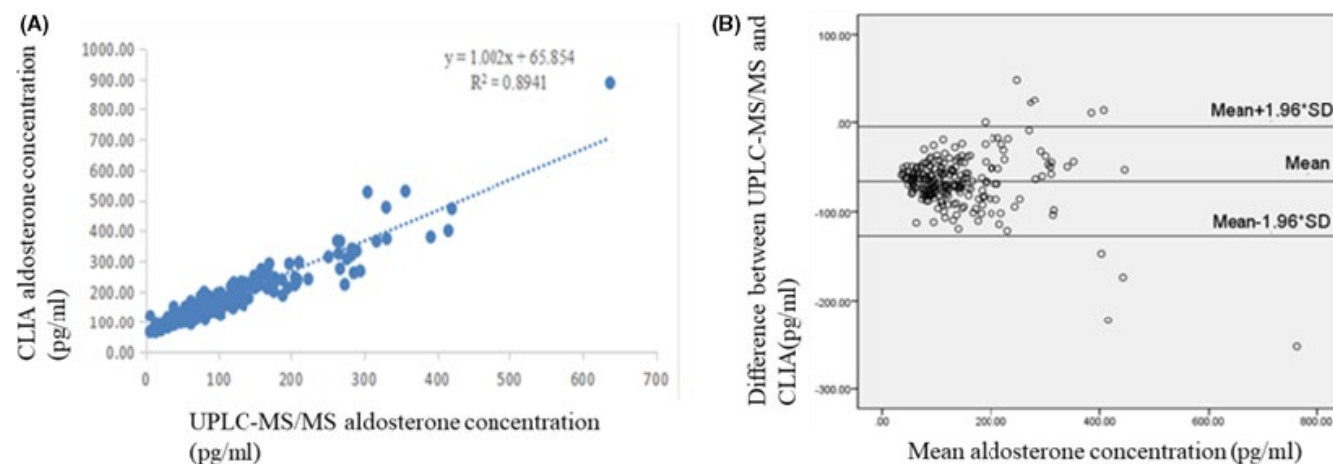


FIGURE 2 Methodological comparison between UPLC-MS/MS and CLIA. (A) Scatter plot of aldosterone concentrations was detected by two specified methods. Dotted line: the line of identity. (B) Bland-Altman plot with method difference expressed as absolute concentration. Solid line: -4.78 , -66.07 , and -127.36 pg/ml (from top to bottom)

previous study.¹⁹ So, we established a uniform reference range for both male and female. The reference range of UPLC-MS/MS-detected plasma aldosterone concentration was determined by 2.5% and 97.5% percentile values, which was 11.30–363.82 pg/ml. Although the lower limit of the reference range is similar to that reported in other studies, the upper limit of the reference range in our study is larger than that reported in other studies.^{12,20,21} We think it may be due to different sample sources and collecting method. Next, we will collect samples from other age groups, identify the correlation between aldosterone concentration and age, and establish a reference range in a wider age range.

4 | CONCLUSION

We developed an aldosterone detection method based on UPLC-MS/MS and evaluated its methodological characteristics. We think the aldosterone detection method based on UPLC-MS/MS is suitable for clinical application. We also used this method to establish a reference range of plasma aldosterone in young people.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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