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Determination of aldosterone in serum by liquid chromatography–tandem mass spectrometry

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Abstract

Measurement of serum aldosterone is clinically important in the diagnosis of hypertension. While isotope dilution gas chromatography–mass spectrometry (ID-GC–MS) provides reliable results, it requires derivatization and is lengthy and time-consuming. Detection by liquid chromatography–mass spectrometry (LC–MS) is a potentially superior method. The analysis utilizes 0.5 mL of serum. The samples were extracted with dichloromethane–ether. The extract was evaporated to dryness and aldosterone was analyzed by LC–MS/MS operating in the negative mode ESI after separation on a reversed-phase column. Aldosterone was also measured by RIA. The calibration curves for analysis of serum aldosterone exhibited consistent linearity and reproducibility in the range of 60–3000 pmol/L. Interassay CVs were 4.3–7.5% at aldosterone concentrations of 97–993 pmol/L. The lower limit of quantitation (LOQ) was 30 pmol/L (signal to noise ratio = 10). The mean recovery of the analyte added to serum ranged from 95 to 102%. The regression equation by LC–MS/MS (x) and RIA (y) method was: $y = 1.33x + 185$ ($r = 0.95$; $n = 124$). Sensitivity and specificity of the LC–MS/MS method for serum aldosterone offer advantages over GC–MS by eliminating derivatization. The novel method is rapid, reliable and simple to perform with a routine LC–MS/MS spectrometer. The sensitivity is adequate for patient samples. Aldosterone concentrations reported by nonextraction RIA were consistently higher than those produced by LC–MS/MS.

Keywords: Serum aldosterone; LC–MS/MS; ESI; Electrospray ionization mass spectrometry; API 3000; RIA

1. Introduction

Aldosterone is secreted by the zona glomerulosa of the adrenal cortex. It is the most important circulating mineralocorticoid and it plays a major role in sodium and potassium homeostasis [1]. Hyperaldosteronism is a cause of hypertension [2,3] and recent studies suggest that it is more common than earlier thought. Accurate measurements of renin and aldosterone concentrations are essential for correct diagnosis of conditions affecting the renin–angiotensin–aldosterone axis [4]. The serum concentrations of aldosterone are in the picomolar range and therefore sensitive aldosterone assays are required. Since the early 1970s radioimmunoassay (RIA) has been used to determine plasma aldosterone [5]. Initially, these assays comprised extraction and chromatography before the immunoassay, but these have gradually been replaced by simplified methods not using prefractionation of the sample. Recently, a rapid automated chemiluminescence immunoassay was reported [6,7]. Immunoassays use polyclonal rabbit antisera with various affinities and specificities. These immunoassays are prone to interference by cross-reacting steroids or other interfering substances and there is therefore an urgent need for improvement [7,8]. The high specificity of GC–MS and LC–MS assays provides a solution to the problem. Reference methods using LC–MS/MS or GC–MS have been proposed [9,10], but methods utilizing GC–MS require a laborious derivatization step before analysis. Previously published LC–MS/MS methods use either atmospheric-pressure chemical ionization (APCI) or photosparay ionization (APPI) together with multiple steroid profiling [9,11]. In order to avoid the time-consuming and labor-intensive sample processing and derivatization needed for GC, we developed a simple and rapid liquid chromatography–tandem MS (LC–MS/MS) method with electrospray ionization (ESI) for serum samples.

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2. Experimental

2.1. Materials

We purchased aldosterone (11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al) from Fluka and deuterium labeled internal standard (IS), aldosterone-d_7, was from IsoSciences. Stock solutions of aldosterone (400 μmol/L) and internal standard (5.5 mmol/L) in methanol were prepared by weighing. Working solutions of aldosterone (60–3000 pmol/L) in 350 mL/L methanol/water and of internal standard (0.1 μmol/L) in 500 mL/L methanol were prepared from stock solutions. Analytical HPLC columns were purchased from Waters. Methanol, dichlormethane, diethylether were purchased from Rathburn Chemicals Ltd. were of HPLC grade. All other chemicals were of analytical reagent grade.

2.2. Sample treatment

To 0.5 mL of patient or quality control serum 30 μL of 0.1 μmol/L d_7-IS in 500 mL/L methanol/water was added before extraction. The steroids were extracted for 3 min with 5 mL dichlormethane/ethylether (60/40) in a multi-tube vortexer. The phases were separated by centrifugation and the upper organic phase was transferred to a conical tube and dried under nitrogen. The residue was dissolved in 350 μL of 350 mL/L methanol/water.

2.3. Methods

Six calibrators containing 60, 150, 300, 750, 1500 and 3000 pmol/L of aldosterone were prepared in 350 mL/L methanol/water by dilution of the stock solution in methanol. To 0.5 mL of calibrator 30 μL of 0.1 μmol/L d_7-IS in 500 mL/L methanol/water was added. Forty microliters of sample extracts and calibrators were analyzed on an LC–MS/MS system comprising an API 3000 triple quadrupole mass spectrometer (PE Sciex) and an Agilent series 1100 HPLC system with a binary pump. Separation was performed on a Sunfire C18 column (2.1 mm × 50 mm, 3.5 μm; Waters) at 30 °C and a flow rate of 250 μL/min. The mobile phase was a linear gradient consisting of methanol and water. The gradient was: 0 min, 35% methanol; 1.5 min, 90% methanol; 2 min 90% methanol; 3 min 35% methanol; and 10 min 35% methanol. The column was directly connected to the electrospray ionization probe operating at 425 °C. The LC–MS/MS method was compared with a commercial RIA method without sample extraction (DiaSorin).

2.4. LC–MS/MS conditions

Aldosterone and IS were detected in the multiple-reaction monitoring mode of the tandem mass spectrometer with the following transitions: Aldosterone, *m/z* 359.2–189.4 and IS, *m/z* 366.2–193.4. Data were acquired and processed with the Analyst Software (Ver. 1.4, Sciex). All results were generated in negative-ion mode with the entrance potential at −10 V, the declustering potential at −63 V, and the collision cell exit potential at −15 V. The optimized focusing potentials were set at −375 V; the cell entrance potentials at −23 V; and the collision energy potential at −23 V as determined by manual tuning. Front-end electrospray settings for the MS/MS ionization source were as follows: curtain gas, 9; nebulizer gas, 8; GS2, 7000; CAD, 12, probe temperature 425 °C; and ionspray voltage −3750 V. For all MS/MS experiments, mass calibration and resolution adjustments on both the resolving quadrupoles were optimized using a polypropylene glycol solution with an infusion pump. Collisionally activated decomposition MS/MS was performed with nitrogen as collision gas.

2.5. LC–MS/MS assay validation

To study the linearity of the method, regression analysis of the six calibrators was carried out. The linearity of each standard curve was confirmed by plotting the peak area ratio of aldosterone to IS (y) versus aldosterone concentration (x). The unknown sample concentrations were calculated from the weighted ($1/x^2$) linear regression analysis of the standard curve.

Recovery of the sample preparation was determined by analyzing five different patient sera containing 0–194 pmol/L endogenous aldosterone spiked with 98 and 503 pmol/L aldosterone. The observed difference in concentrations was compared with the expected concentration. Accuracy of the measurement was also evaluated by a comparison of results ($n = 6$) obtained for a lyophilized human serum quality control material (DGKL-Referenzinstitut für Bioanalytik) with certified values determined by the ID-GC/MS reference method of Siekmann [10].

The degree of ion suppression attributed to the sample matrix was estimated in a separate set of experiments. Six sera were extracted as described, and the extracts were spiked with 300 pmol/L aldosterone and 5700 pmol/L IS. Additionally, the steroids were added to 350 mL/L methanol/water at the same concentrations. Ion suppression was calculated by comparing analyte peak areas obtained from pure methanolic solutions with those obtained from spiked serum samples after extraction.

3. Results

3.1. LC–MS/MS characteristics of aldosterone

The electrospray MS spectra obtained in the negative-ion mode by infusion of 3 μmol/L aldosterone is shown in Fig. 1. The first-quadruple (Q_1) scan shows a pronounced parent ion peak of aldosterone at *m/z* 359.2, which is the expected $(M - H)^-$ ion. The major daughter ions at *m/z* 189.4, 331.2 and 297.4 derived from fragmentation of the 359.2 peak (Fig. 1B).

Using the autotune algorithm provided in the system software, we optimized the instrument for transmission of the deprotonated molecular ion, *m/z* 359.2 and for maximum intensity of the selected fragment, *m/z* 189.4. Fig. 2A shows a LC–MS/MS chromatogram for a calibrator containing aldosterone at concentration of 60 pmol/L with a retention time of 5.2 min. Total run time was 10 min/sample. A signal-to-noise ratio of 38:1 was observed for a 60 pmol/L calibrator of

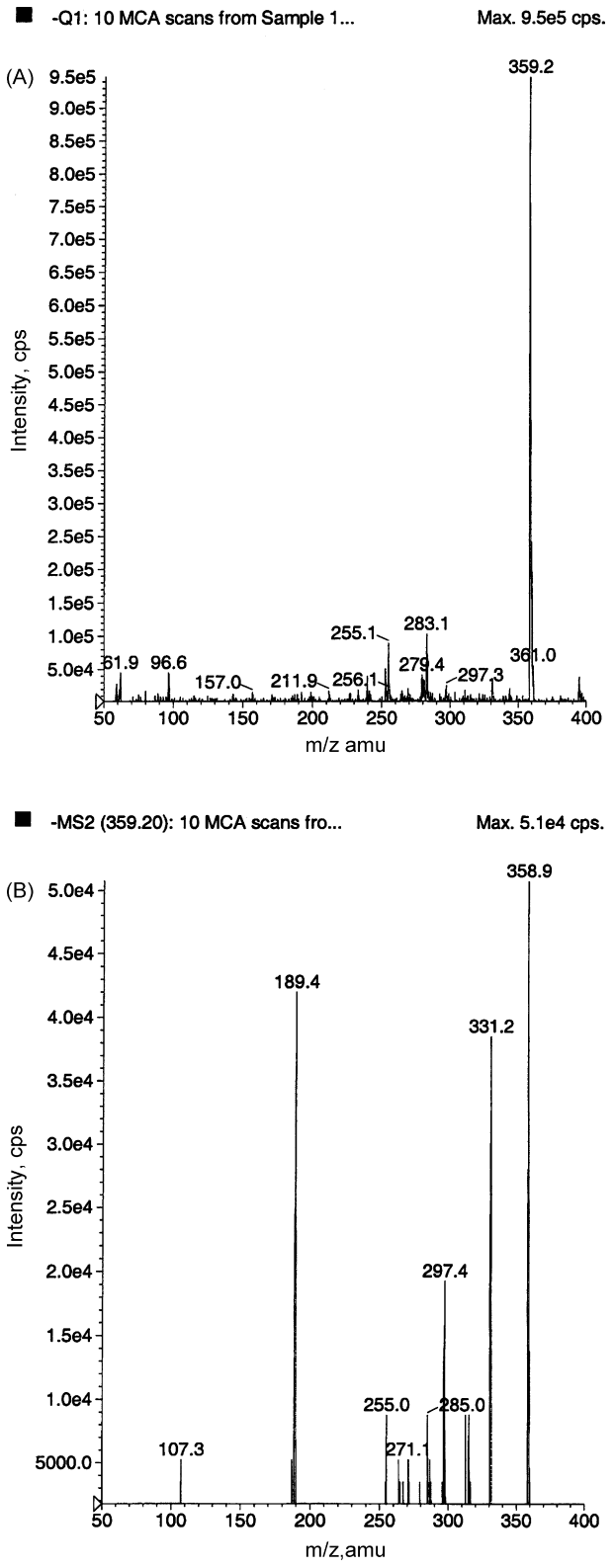

Fig. 1. Electrospray ionization mass spectrum (A) and product ion spectrum (B) for aldosterone. (A) Electrospray ionization mass spectrum for aldosterone in negative-ion mode (Q_1 scan). (B) Product ion spectrum for the aldosterone *m/z* 359 ion $(M-H)^-$. Aldosterone (3 μmol/L) in 700 mL/L methanol/water was infused at the electrospray tip of the quadrupole mass spectrometer.

Table 1
Within-run and between-run imprecision for serum aldosterone

	Within-run			Between-run		
Mean (pmol/L)	56.7	445	773	97	255	993
SD (pmol/L)	3.35	11.3	37.5	7.3	14.0	43
CV (%)	5.8	2.5	4.9	7.5	5.5	4.3
n	17	17	17	12	11	12

aldosterone. A typical chromatogram of aldosterone in patient serum is shown in Fig. 2B. The aldosterone concentration was 704 pmol/L. We separated the major interferences in the serum matrix from aldosterone by starting the gradient at a methanol concentration of 35%. Thus, most impurities elute before aldosterone which elutes as a sharp peak at 5.2 min (Fig. 2).

3.2. Precision

The within-assay CV calculated from 17 replicates for samples with the aldosterone concentrations of 57, 445 and 773 pmol/L were 2.5–5.8%. Total CVs were 4.3–7.5% for determinations on serum pools stored in frozen aliquots (Table 1).

3.3. Linearity

The LC–MS/MS method for analysis of aldosterone was linear at concentrations of 60–3000 pmol/L, with a mean correlation coefficient of 0.999 ($n = 15$). The lower limit of quantitation (LOQ) of the assay was 30 pmol/L based on a signal-to-noise ratio of 10.

3.4. Recovery

Mean recoveries of aldosterone were similar at the two concentrations tested. When aldosterone standards were added to four different sera the recoveries of aldosterone ranged from 88 to 109% (Table 2). As another accuracy assessment the quality control sample results ($n = 6$) were compared to those obtained by the ID-GC/MS reference method (10) and the mean deviation of our LC–MS/MS results was +0.7% with a range of −2.1% to 3.6% from the reference method values.

The matrix effect was assessed in serum samples spiked after extraction to assess the extent of variability of the MS/MS response (peak areas) for the same amount of aldosterone in six different serum extracts (Table 3). The peak areas for the same amount of aldosterone and IS spiked postextraction into different serum extracts varied from 74 to 100% for aldosterone and from 67 to 92% for IS in comparison with that of pure standard. Differences in recovery did not affect these results, since

Table 2
Mean recovery of aldosterone in four sera

Added analyte (pmol/L)	Recovery (% ± SD)
98 ($n = 4$)	95 ± 8.8
503 ($n = 4$)	102 ± 5.1
Mean ± SD	99.6 ± 7.2

analytes were spiked after extraction. Apparently, endogenous serum components co-eluting with aldosterone suppressed its ionization and result in a decreased peak area. This is compensated by a virtually identical suppression in peak area of the IS, 86% for the standard and 83% for the IS (Table 3).

Table 3
Signal suppression

	Signal, % of standard	
	Aldosterone	IS
Serum 1	92	86
Serum 2	84	83
Serum 3	74	67
Serum 4	79	78
Serum 5	88	89
Serum 6	100	92
Mean ± SD	86 ± 9.3	83 ± 9.0

3.5. Correlation between methods

The correlation between the LC–MS/MS (x) and the RIA assay (y) was determined with samples from 124 patients (Fig. 3A and B). Linear regression indicated fairly acceptable overall correlation (r) of 0.952: RIA = 1.33 LC–MS/MS + 185.

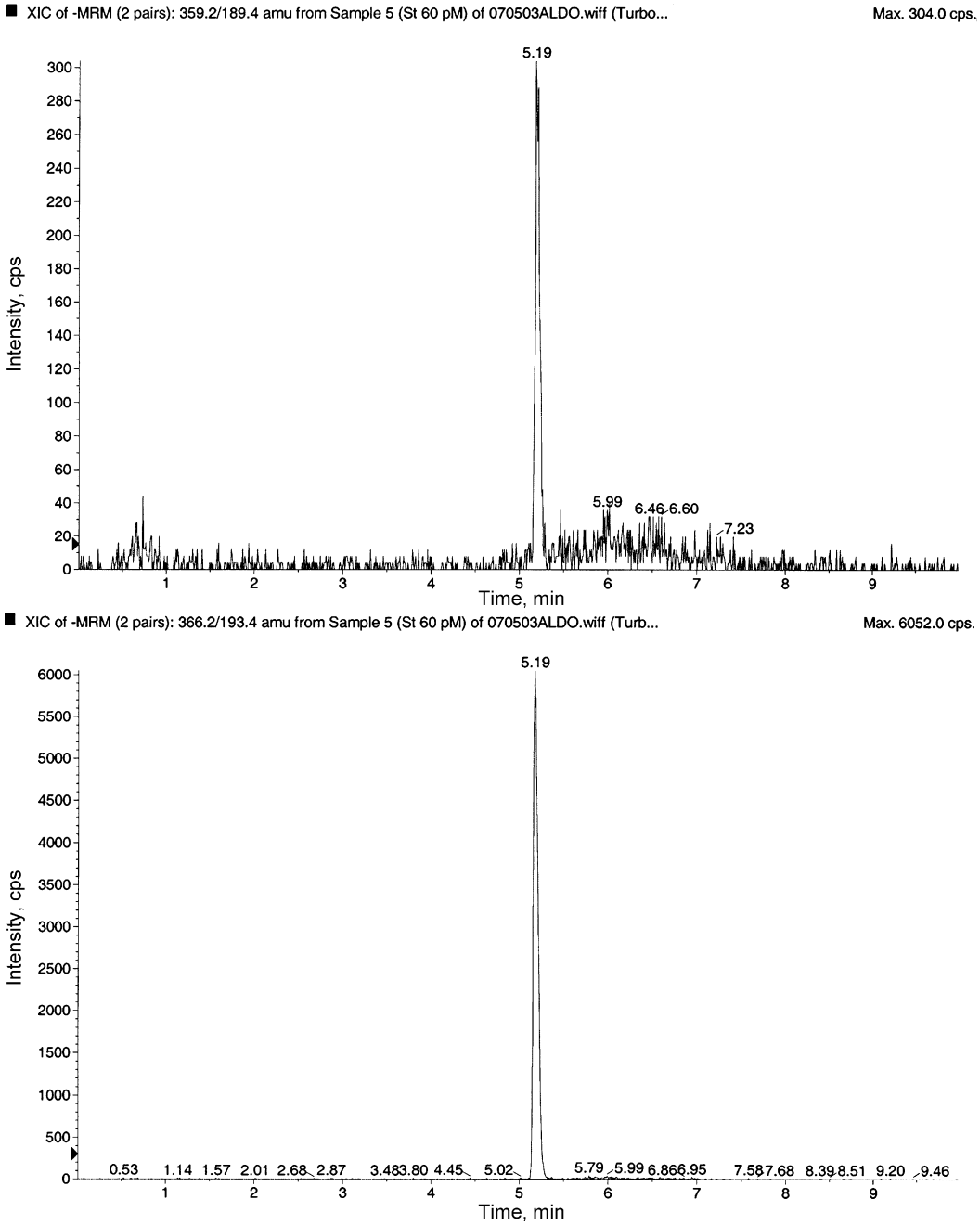

Fig. 2. LC–MS/MS chromatograms of (A) the 60 pmol/L calibrator and (B) a patient serum with 704 pmol/L aldosterone. Panels: Ion chromatogram following the fragmentation 359/189 of aldosterone (upper) and 366/193 of IS (lower).

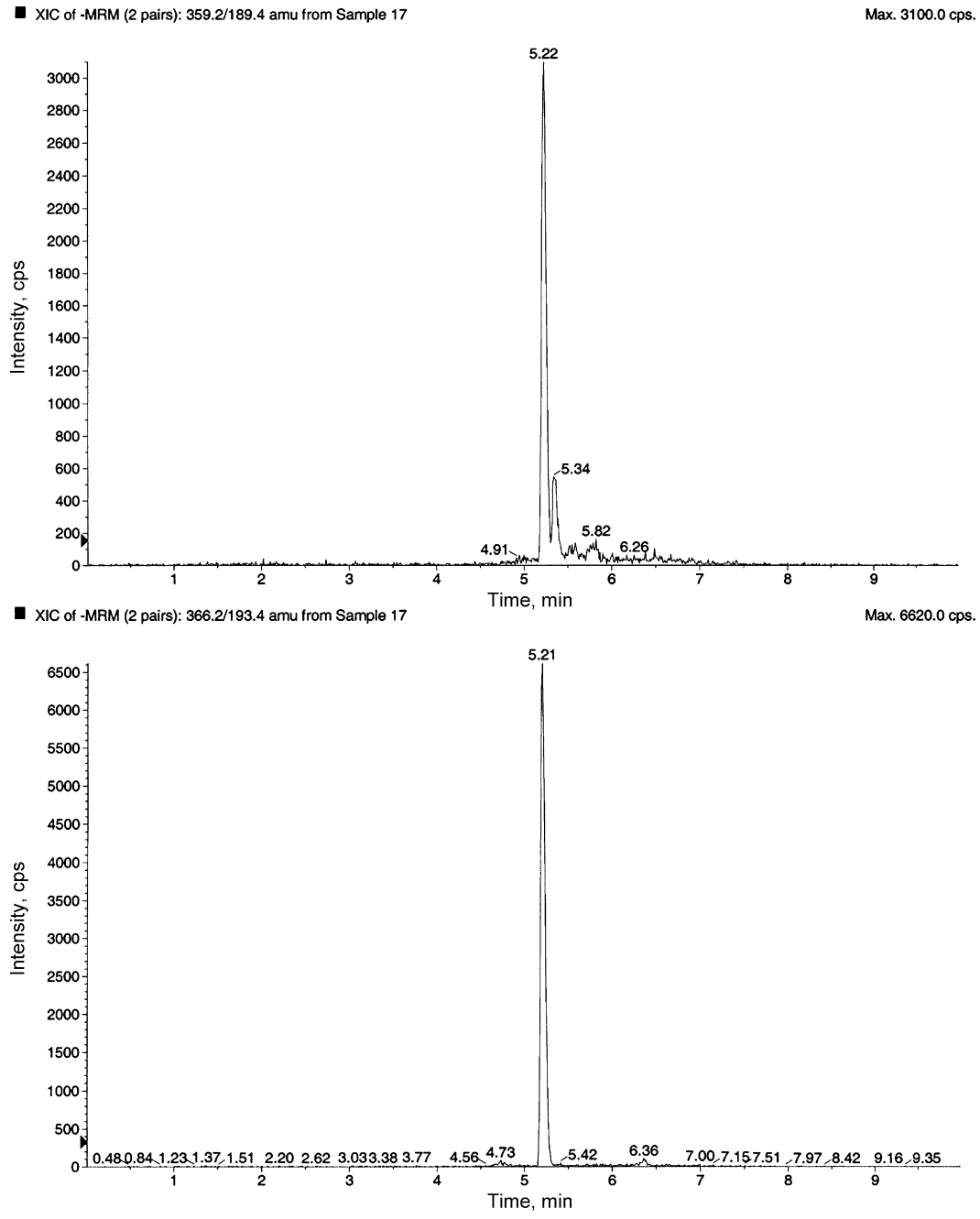

Fig. 2. (*Continued*).

However, the RIA gave on average 33% higher results than LC–MS.

4. Discussion

Recent reports have drawn much-needed attention to current problems with measurement of aldosterone [7,8]. The issue is very important in clinical practice because of the diagnosis of hyperaldosteronism. Schirpenbach et al. [7] have demonstrated marked differences in mean values obtained by the four different immunoassays. More than 100% differences were seen between commercial immunoassay results and an in-house RIA with extraction and chromatography. These results indicate a need for more accurate aldosterone assays. Our LC–MS/MS assay offers the sensitivity and accuracy required to assess abnormal aldosterone concentrations.

Gas chromatography with MS (GC–MS) is the accepted reference method for determination of steroid hormones and it has also been used for aldosterone determinations [10,12]. However, aldosterone needs to be derivatized before it can be analyzed. LC–MS/MS provides many of the advantages over GC–MS, but without the requirement for derivatization, sample preparation is much more simple. A reference method for aldosterone measurement using LC–MS/MS has been proposed [9] with the LOQ of 15 ng/L (42 pmol/L) obtained with a Sciex API III mass spectrometer and an APCI ion source. However, the IS used,

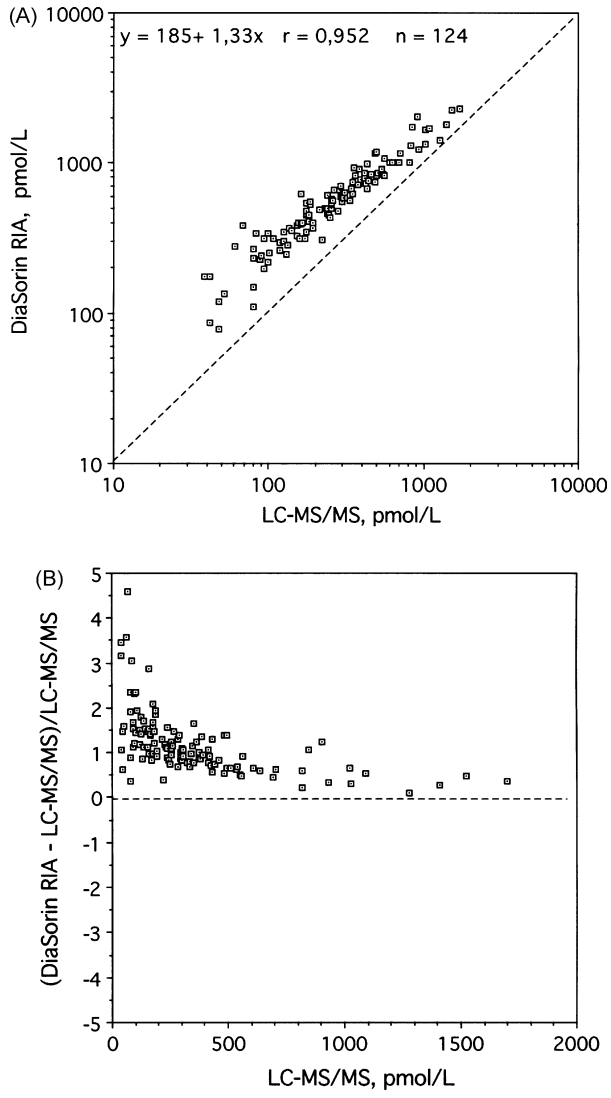

Fig. 3. (A) Correlation between results of aldosterone obtained by LC–MS/MS (x) and RIA (y). (B) Differential plot for the two methods. The dashed line shows the line of identity.

flumethasone, did not show the same retention time as aldosterone and so the matrix components could affect aldosterone and IS responses differently. Lately, aldosterone has been measured together with 11 other steroids with an API 5000 mass spectrometer and an APPI source [11] with a LOQ of 10 ng/L (28 pmol/L). Our LOQ of 30 pmol/L with an ESI source is in accordance with these results, all obtained in negative mode. This indicates that the more commonly used ESI ion source is more suitable than APPI for aldosterone since API 5000 is a more sensitive instrument than API 3000. Furthermore, rarely there is a clinical need to analyse aldosterone together with steroid profiling which only reduces sensitivity of the LC–MS/MS assay without providing any essential advantage for the diagnosis of hyperaldosteronism.

Ion suppression/enhancement is a major concern with mass spectrometric methods in biological samples. For this reason, the susceptibility of our methodology to ion suppression/enhancement was evaluated. Table 3 shows that there is 14–17% endogenous ion suppression with our methodology in the serum samples examined and the ion suppressions of aldosterone and IS are very similar in all individual sera tested. The retention times of aldosterone and IS are at 5.19–5.22 min (Fig. 2) and the analytes are well separated from major matrix components eluting at the start of the run which helps to minimize ion suppression.

In conclusion, to our knowledge this is the first study where aldosterone has been measured by LC–MS/MS using ESI in negative mode. Our method has a large linear range of 60–3000 pmol/L and the LOQ of 30 pmol/L, similar to those obtained with an APPI ion source and more advanced and expensive equipment [11]. Thus, the present assay is accurate, precise, and linear and based on its simplicity, it is suitable for routine analysis of aldosterone in clinical samples.

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