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Single assay measurement of Aldosterone-to-Renin ratio by Online SPE-UHPLC-MS/MS

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Mikaël LEVI[1], Nicola GRAY[2], Oneal JOSEPH[3], Ichiro HIRANO[1]
[1] SHIMADZU Corporation, MS Business Unit, Kyoto, Japan;
[2] SHIMADZU UK Limited, Milton Keynes, UK;
[3] Health Services Laboratory, London, UK.

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Introduction

Hypertension or high blood pressure is a highly prevalent disease. Secondary hypertension is caused by kidney or endocrine disorders. To define the cause of the secondary hypertension the measurement of aldosterone-to-renin ratio (ARR) is a useful tool, especially for screening of primary aldosteronism.
Aldosterone is classically measured in plasma. Renin, is quantified by mean of its activity, measuring the amount of angiotensin-I produced in a defined time range. Many reports on measurement of aldosterone or PRA by LC-MS/MS have been published but no method have been proposed to combine both in a single assay.
We present here a method that allow to measure low aldosterone levels as well as low PRA in a single sample with a simplified workflow for research purposes.

Methods and Materials

Principle of the Method

Renin enzymatic activity is measured by quantifying the amount of product, angiotensin-I (AT-I, DRVYIHPFHL), formed within a define time range. Renin substrate is angiotensinogen, endogenously present in the plasma samples. PRA is then given by the ratio of angiotensin-I concentration difference ($t1_h$-$t0_h$) on time range (1 h).
As angiotensin-I is naturally converted to angiotensin-II (AT-II, DRVYIHPF) by ACE (Angiotensin Converting Enzyme), this secondary reaction has to be inhibited, as well as other possible proteolytic enzymes, for accurate measurements. The buffer composition has been adjusted in this direction.
To check for efficient inhibition of ACE and other enzyme in individual samples, stable isotope labelled internal standards were selected and added to each samples. DRV*YI*HPFHL [V*=Val(U-$^{13}C_5$, ^{15}N), I*=Ile(U-$^{13}C_6$, ^{15}N)] for AT-I and DRVYI*HPF [I*=Ile(U-$^{13}C_6$, ^{15}N)] for AT-II, were used. As the labelling of these compounds are different, the transition of degradation product of AT-I ISTD into labelled AT-II can also be monitored without confusing with initial AT-II ISTD. In case of questionable results, it is then possible to check compound degradation signal in each sample.
Aldosterone is simultaneously measured in the sample (same method, same injection). $^2H_4$-Aldosterone was used as internal standard.

Sample Incubation and Preparation

Samples or Quality Controls were quickly thawed in a water bath at room temperature and then placed on ice until use.
Incubation buffer was prepared extemporaneously by mixing 10 mL of Tris buffer (1M Tris, 0.2M EDTA, buffered to pH 5.5 with acetic acid) with 100 µL of PMSF (phenylmethylsulfonyl fluoride, 100mM) and 100 µL of SBTI (Soybean Trypsin Inhibitor, 30 µg/mL).
In a polypropylene microtube, 20 µL of incubation buffer, 10 µL of ISTD solution (100 ng/mL of each) and 100 µL of sample were mixed. Samples were prepared in duplicate ($t0_h$ and $t1_h$). $T0_h$ sample was kept on ice while $t1_h$ sample was incubated at 37°C with gentle shaking. After 1 hour, all samples were precipitated with 150 µL of acetone, vortex mixed and centrifuged at 10000 rpm for 5 minutes at room temperature. Supernatant was then transferred to a polypropylene vial with integrated insert for analysis.

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Analytical Conditions

Samples were analysed by online SPE and UHPLC-MS/MS. The system using Nexera X2 and LCMS-8060 (Shimadzu Corporation, Kyoto, Japan) is shown in figure 1. Analytical conditions are described in tables 1, 2 and 3.

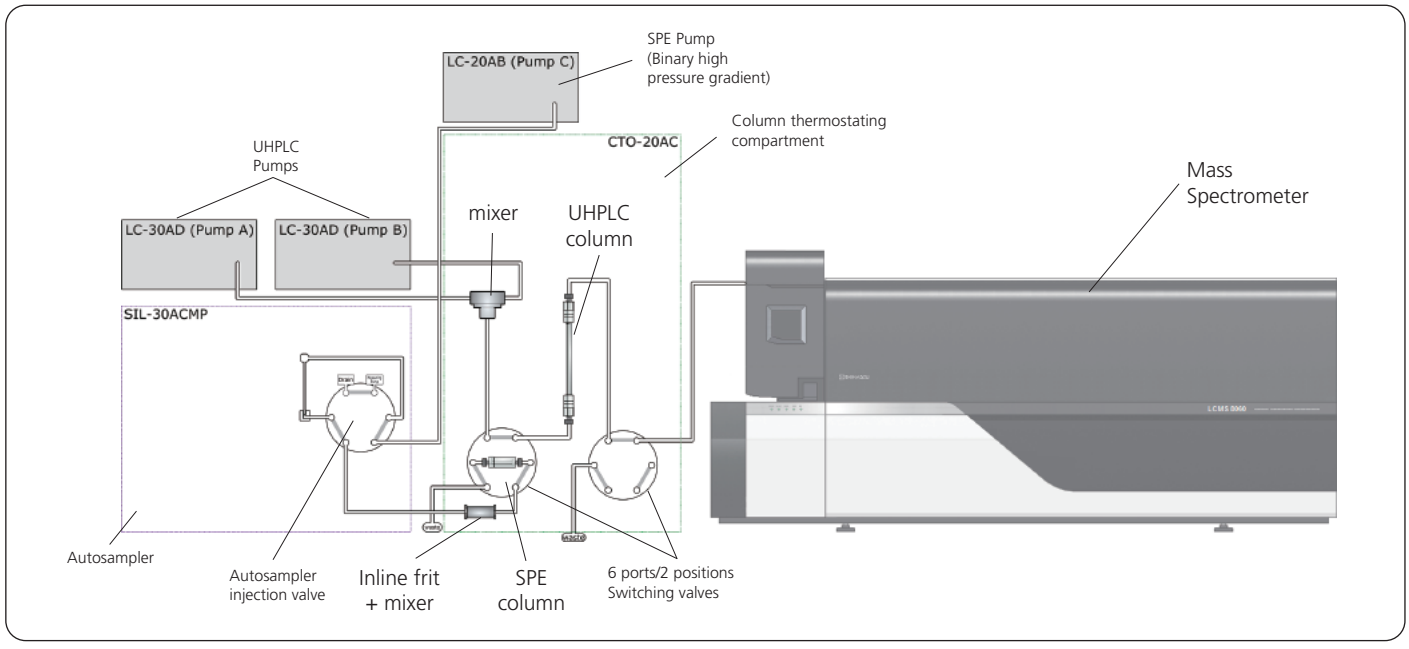

Figure 1 Overview of the analytical system

Table 1 Online SPE and UHPLC conditions

System	: Shimadzu Nexera X2
SPE Column	: Shim-Pack GISS C18 3 µm 10x2.1mm
UHPLC Column	: Shim-Pack GIST C18 2 µm 50x2.1mm
Temperature	: 50°C
SPE Mobile Phases	: (Loading) Water/Methanol 9/1 + 0.01% Formic Acid (Cleaning) Acetonitrile/Isopropanol 1/1 (v/v) + 0.5%formic acid
SPE Flow-rate	: 1.5 mL/min
UHPLC Mobile Phases	: (A) Water + 0.01% Formic Acid (B) Methanol
UHPLC Flow-rate	: 350 µL/min
Gradient	: 25%B (0-3min), 25-100%B (2.4 min), 25%B (1.6min)
Run Time	: 7 min
Needle Rinse	: R3 (MeOH/ACN/IPA/HCOOH 33/33/33/1) then R0 (Water/ACN 9/1)
Sample Temperature	: 5°C
Injection Volume	: 15 µL

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Table 2 MS parameters

System	: Shimadzu LCMS-8060 with heated ESI
Acquisition Mode	: Multiple Reaction Monitoring
Pause Time	: 1.5 ms
Polarity Switching	: 5 ms
Temperature	: DL 150°C - HB 300°C - Interface 400°C
Gas Flow	: Neb 3 L/min - Dry 5 L/min - Heating 15 L/min
CID Gas Pressure	: 300 kPa
Interface voltage	: +1.5 kV / - 4 kV

Table 3 MRM transitions

Compound	Ionization	MRM	Type	Dwell time (msec)	Comment
AT-I	Pos $[M+3H]^{3+}$	432.9 → 647.4 432.9 → 619.4	Quant Qual	59 59	b5 fragment ion a5 fragment ion
$^{13}C_{11}$,$^{15}N_2$ – AT-I	Pos $[M+3H]^{3+}$	437.3 → 660.3	Quant	59	b5 fragment ion
Aldosterone	Neg $[M-H]^-$	359.2 → 189.3 359.2 → 331.3	Quant Qual	130 130	---
D4-Aldosterone	Neg $[M-H]^-$	363.2 → 190.3	Quant	80	---
AT-II	Pos $[M+2H]^{2+}$	523.8 → 263.1	Quant	39	y2 fragment ion
$^{13}C_6$,^{15}N – AT-II	Pos $[M+2H]^{2+}$	527.6 → 263.1	Quant	39	y2 fragment ion
$^{13}C_{11}$,$^{15}N_2$ – AT-II	Pos $[M+2H]^{2+}$	530.6 → 263.1	Quant	39	y2 fragment ion. Degradation product of $^{13}C_{11}$,$^{15}N_2$ – AT-I

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Results

Calibration

Calibration standards were prepared in 0.1M Tris buffer with 0.1% BSA. The calibration range for AT-I was from 0.03 to 100 ng/mL. For aldosterone, it was ranging from 40 to 1500 pg/mL. Calibration standards were treated as samples without incubation. Figures 2 and 3 show calibration curves and standard chromatograms.

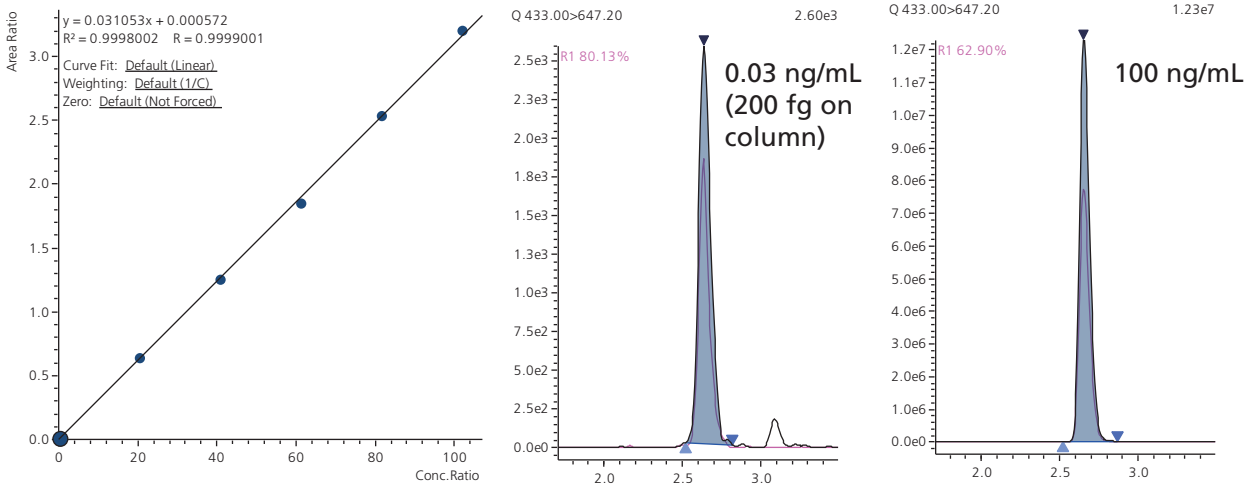

Figure 2 Angiotensin-I calibration curve and standard chromatograms

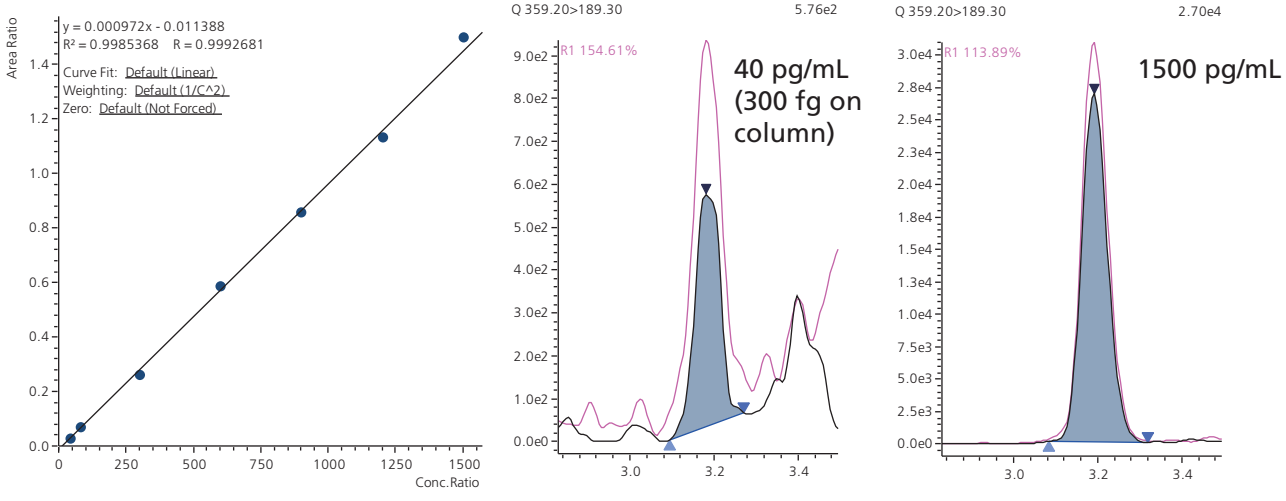

Figure 3 Aldosterone calibration curve and standard chromatograms

Recovery

Total recovery (extraction and matrix effect) was evaluated by comparing the signal of the internal standards in a neat standard to a plasma pool. Six individual samples were prepared and injected for each. Results are presented in table 4. The measured recovery were quite correct to enable an accurate and sensitive measurement.

Table 4 Recovery results

Sample Name	ISTD-AT-I : Area	D4-Aldosterone : Area	ISTD-AT-II : Area
Neat Standard	1772368	107781	3870759
	1807282	109471	3837293
	1706617	106726	3764526
	1828740	111658	3985407
	1774105	104522	3942174
	1758429	111482	3898811
Mean	1774590	108607	3883162
%RSD	2.4%	2.6%	2.0%
Plasma pool	1732915	85332	3822798
	1704835	84277	3866693
	1754041	85899	3897660
	1733457	75879	3726905
	1733954	81416	3741488
	1770662	79047	3765167
Mean	1738311	81975	3803452
%RSD	1.3%	4.8%	1.8%
Total Recovery	**98.0%**	**75.5%**	**97.9%**

Measurement of Controls

Bio-Rad Lyphochek® Hypertension Markers quality controls were assayed as eight individual samples at each level. Results obtained were compared with mean values provided on the certificate. While measured using RIA, values are in good accordance with our method. Results are presented in tables 5 and 6. For these samples, incubation time was extended to 3 hours).

Table 5 PRA results in Lyphochek Controls (n=8 per level)

Level	Mean (ng/mL)	%RSD	Measured PRA (ng/mL/hr)	Reference PRA (ng/mL/hr)	Accuracy
1	6.28	7.4%	1.72	1.80	95.7%
2	18.4	6.5%	5.25	5.60	93.7%
3	72.8	4.3%	20.7	28.5	72.5%

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Table 6 Aldosterone results in Lyphochek Controls (n=8 per level)

Level	Mean (pg/mL)	%RSD	Reference (pg/mL)	Accuracy
1	39.3	8.0%	39.0	101%
2	101	7.6%	86.0	118%
3	421	15.3%	420	100%

Conclusions

A single assay method for measurement of aldosterone-to-renin ratio was successfully set up thanks to high sensitivity LC-MS/MS instrumentation and fast polarity switching capabilities.
The method is fast and the workflow is simple thanks to the automated sample preparation by online SPE.
The sensitivity for AT-I is sufficient to measure PRA down to 0.03 ng/mL/hr, even with low incubation time of one hour. If necessary, the incubation time can then be extended to even lower the measurable PRA values.
Aldosterone LOQ is suitable for purpose.
The method will be useful to support research about primary aldosteronism or evaluation of new therapy.

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