Accuracy of Mass Spectrometry with the Ease of Immunoassay? LC-MS/MS Workflow improvements for Primary Aldosteronism Screening

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1 Background

Primary aldosteronism (PA) is the most common form of secondary hypertension and is responsible for 10% of hypertension in allcomers [1]. The two most common forms of PA are idiopathic adrenal hyperplasia (IAH) and unilateral aldosterone producing adenoma (APA). IAH is amenable to treatment with aldosterone antagonists (spironolactone, eplelrenone) and unilateral adrenalectomy for APA often leads to complete cure of PA. In addition to the all of the expected sequelae of hypertension (increased risk of: atherosclerosis, stroke, chronic renal impairment, coronary heart disease), patients with PA have risk of cardiac [2] and renal disease [3] that is out of proportion to their hypertension, indicating the specific pathogenetic role that aldosterone plays in PA. For this reason, identification and treatment of PA is important from the standpoint of public health.

The accepted manner of screening for primary aldosteronism is the collection of a concomitant plasma aldosterone and renin activity and to calculate their ratio. However, aldosterone is a difficult analyte owing to its low concentration and the presence of endogenous interferences [4]. Plasma renin is also technically challenging [5] and can be assessed either by the plasma renin activity (PRA) assay through the quantitation of angiotensin I (AngI), or by chemiluminescent sandwich assays directed at the renin molecule. However, the latter approach may not have significant correlation with PRA in the low renin state [6].

Both aldosterone and plasma renin activity determination have improved substantially from LC-MS/MS. For aldosterone, liquid-liquid [7, 8, 9] and supported liquid extraction methods [10, 11,

12] for aldosterone show much better intermethod comparison between one another [10, 12] than the comparison between IA methods (which may show 100 % bias) [13, 14] or the comparison between LC-MS/MS and IA [9, 12]. For plasma renin activity, both online and offline solid phase extraction (SPE) approaches have been developed and have been implemented into routine care [15, 16, 17]. These methods have better analytical sensitivity and specificity meaning that blank-subtraction is unnecessary [16, 18].

Despite the analytical advantages of LC-MS/MS determination of both aldosterone and renin over immunoassay, the attraction of walk-away automation using homogenous IA analyzers often wins out in the pragmatic and financially–driven laboratory environments in which we exist. It is therefore our mandate to make the quality LC-MS/MS accessible by improving workflows.

We present two proposed workflow improvements. First, we have developed a method for plasma renin activity and plasma aldosterone from the same sample preparation by sequential elutions off the same SPE plate and using mobile phase adjustments that have significantly enhanced aldosterone signal. Second, we present an exploration of using aldosterone:AngI as an alternative screening tool to aldosterone:PRA which could solve a number of standardization and technical problems with PA screening.

2 Methods

2.1 Aldosterone and PRA on the Same Sample Preparation

PRA was performed by LC-MS/MS as previously described [19] but without blank subtraction as we have demonstrated it to be unnecessary. In brief, AngI is generated from endogenous renin and angiotensinogen using 250 μL of patient sample buffered with 50 μL of 1M tris acetate containing 0.2M EDTA, 1 mM PMSF, and 109 nmol/L d7-aldosterone at pH=5.5. The final pH of the buffered sample is 6. This buffered plasma is incubated for 3 h at 37°C and the reaction is quenched with the addition of 10% formic acid containing stable isotopically labelled AngI. Aliquots of the "generate" are added to a Phenomenex Strata-X (Part No. 8E-S100-UGB) conditioned SPE plate, washed and then eluted with 0.25 mL of 100% methanol. Eluted material is analyzed by LC-MS/MS as

described [19].

Following the AngI elution, the SPE plate undergoes an additional elution with 2×700 µL of methyltertbutylether (MTBE) into a 96-well deepwell plate. The MTBE is then dried down under house air at ambient temperature in a 96-well dry-down manifold, reconstituted with 125 µL of 20:80 MeOH:H_2O analyzed as previously reported [10] but with lower concentration of ammonium acetate in mobile phases (0.25 nM).

Comparisons of PRA and aldosterone was performed against our current production methods (n=36 for PRA and n=61 for Aldosterone). Aldosterone analysis using the presented method was performed on EDTA plasma while the production method was performed on concomitantly collected EDTA plasma.

2.2 Statistical evaluation of aldosterone:AngI ratios

In 665 sequential specimens, one aliquot buffered AngI solution was immediately acidified and analyzed without 37°C incubation while the second aliquot was generated as per normal. PRA was compared statistically with [AngI] and test characteristics of the aldosterone:AngI ratio (AAR) was compared using aldosterone:PRA (ARR) as the surrogate for a gold-standard. Calculations were performed using the ROCR package of the R statistical programming language.

3 Results

3.1 Aldosterone and PRA–same sample preparation

Aldosterone by second elution from SPE on EDTA plasma compared very well to the current SLE method, performed on concomitantly collected red top serum. Regression equation was

$$[Aldo]_{\text{SPE}} = 0.99[Aldo]_{\text{SLE}} + 10.1 \text{ pmol/L}$$

weighted Deming Regression, $R^2 = 0.98$, $CI_{slope} = [0.95, 1.02]$ and $CI_{intercept} = [7.2, 14.5]$ pmol/L, median difference = +7.5 pmol/L. Comparison is shown in figure 1.

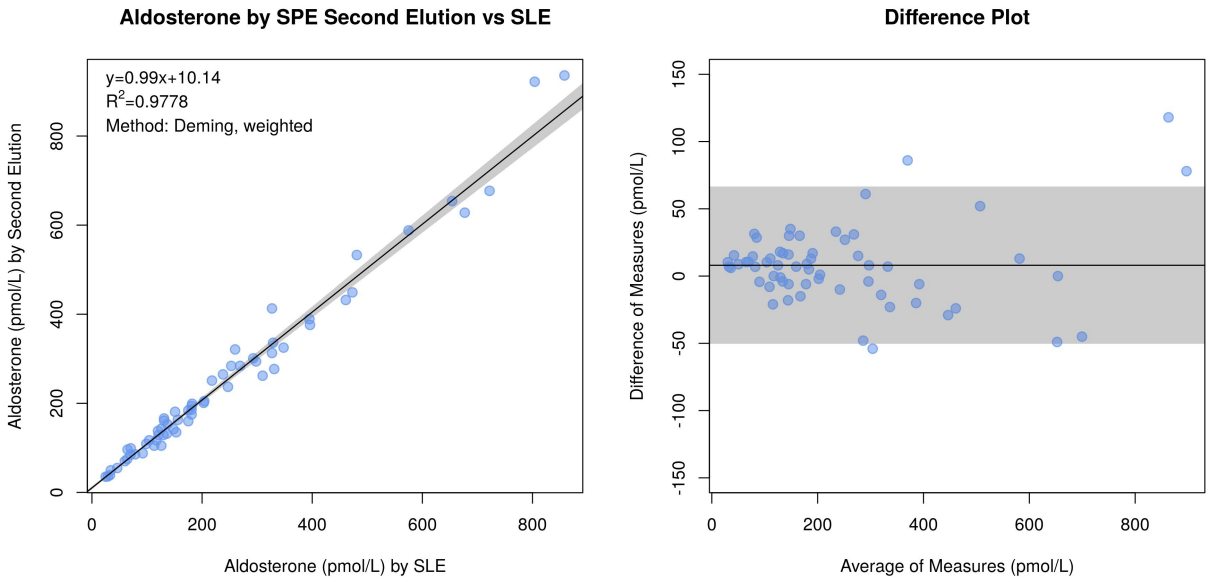

Figure 1: Regression comparison and difference plot between current production aldosterone method used for PA screening at St. Paul's Hospital vs exploratory method by second elution from the PRA SPE plate. Samples on the x-axis were performed on red top serum; those on the y-axis were performed on concomitantly collected EDTA plasma.

PRA as generated in the presence of the aldosterone IS showed a regression comparison of $PRA_{\text{modified}} = 0.94 \times PRA_{\text{current}} + 0.026$ ng/mL/h. Slope and intercept did not differ statistically from 1 and 0 respectively, $R^2 = 0.994$.

3.2 Statistical evaluation of aldosterone:AngI ratios

Comparison between [AngI] and PRA showed a relationship of

$$[AngI] = 0.04 \times PRA - 0.01 \text{ ng/mL}$$

The correlation was strongly significant $R^2 = 0.73, p < 10^{-10}$, for but demonstrated a large degree of scatter for the 620 specimens for which $PRA < 10$ ng/mL/h (figure 2). In the low renin speci-

mens ($PRA < 1$ ng/mL/h) wherein all PA is identified, this correlation deteriorated to $R^2 = 0.29$ but remained highly significant $p < 10^{-10}$. A large number (n=217) of the specimens had AngI concentrations below the limit of detection of the assay, in contrast to the 112 specimens that would have been reported as < 0.18 ng/mL/h by the PRA assay.

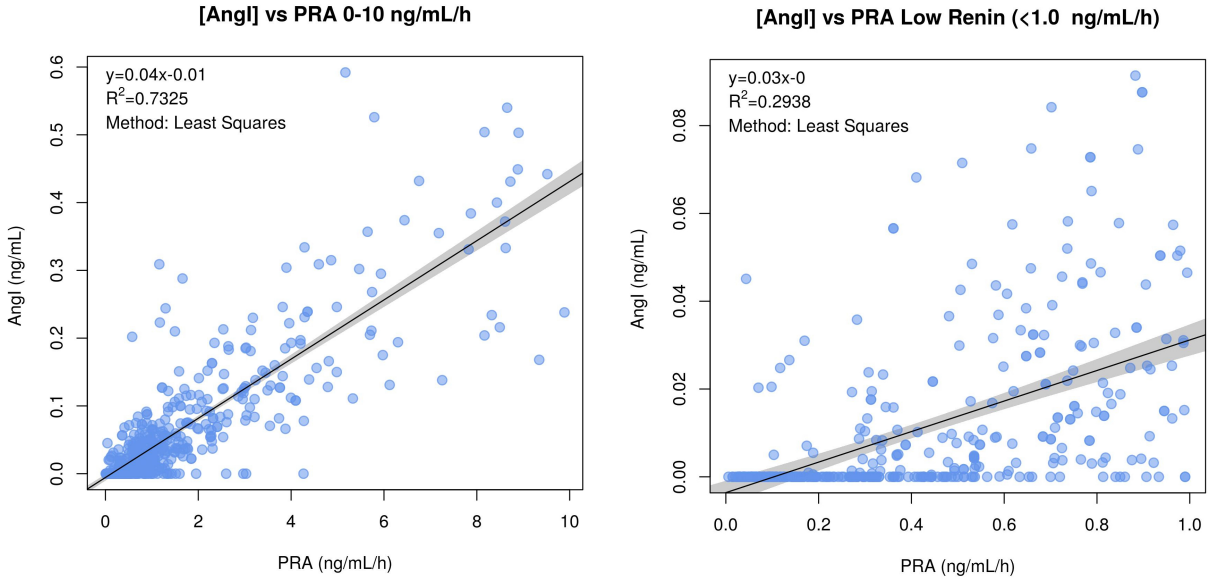

Figure 2: Comparisons of [AngI] and PRA in specimens between 0-10 ng/mL/h and in low PRA specimens.

The ability of the AAR to recapitulate the same screening information as the ARR was evaluated by ROC analysis. In the cohort of patients in whom aldosterone ≥ 200 pmol/L, i.e. those in whom PA was conceivably possible. The ROC area under curve was 0.89. The maximum diagnostic accuracy (i.e. maximum correct classification rate) was achieved at AAR of 17700 pmol/L:ng/ml but at this threshold sensitivity was only 70%. Greater than 95 % sensitivity (at 38% specificity) was achieved at 6370 pmol/L:ng/mL. See figure 3.

4 Conclusions

Aldosterone analysis on the same sample preparation as AngI for PRA has been achieved and represents a significant improvement in workflow and an opportunity for substantial savings. Re-

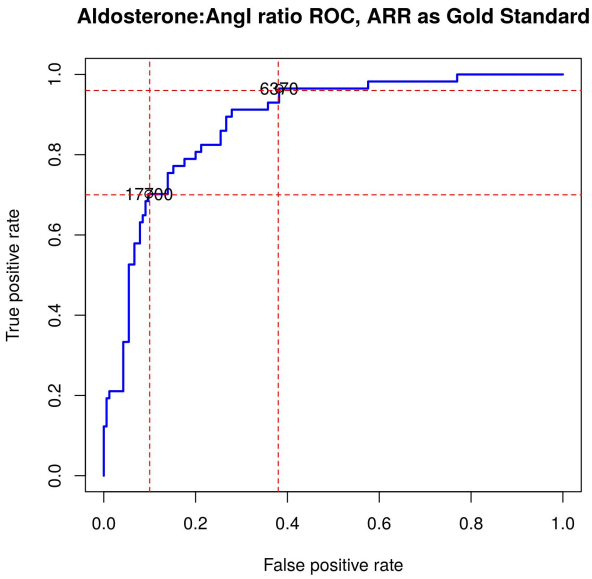

Figure 3: ROC curve for [AngI] using the AAR result as gold-standard.

gression characteristics against the current production method (SLE-LC-MS/MS) are excellent.

AngI concentrations in samples for which there was no special handling had higher correlation with PRA than reported for DRC ($R = 0.49$, $p < 10^{-10}$ vs $R = 0.14$, $p > 0.05$ [6]) in the low PRA range (< 1 ng/mL/h). However the AAR was unable to provide the same diagnostic information as the ARR when considering ARR the gold standard. We suggest that AAR and the aldosterone:AngII ratio should be analyzed in a cohort of subjects who go on to full diagnostic workup with saline suppression or fludrocortisone suppression, preferably with final patient outcomes. Analytical sensitivity of AngI may require improvement and preanalytical issues with direct AngI analysis certainly represent a potential vulnerability.

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