itive C-reactive protein, fibrinogen, and leukocyte count and the CYP2J2 genotype. The CAD status was included as main effect, and statistical adjustments were made for age, sex, and cardiovascular risk factors (body mass index, diabetes mellitus, hypertension, and smoking). None of these models revealed any statistically significant C-reactive protein, fibrinogen, and leukocyte count and the CYP2J2 genotype. The CAD status was included as main effect, and statistical adjustments were made for age, sex, and cardiovascular risk factors (body mass index, diabetes mellitus, hypertension, and smoking). None of these models revealed any statistically significant relationships between the CYP2J2 genotype and inflammatory markers (data not shown).

In contrast to a recent study (3), we could not confirm the −50G>T polymorphism as a risk factor for CAD or MI in our large cross-sectional LURIC cohort. In addition, we were not able to detect any relationship of this polymorphism with total mortality or cardiovascular mortality in this cohort at intermediate risk of death. Association studies of genetic polymorphisms often lead to discrepant results, especially if small numbers of cases and controls are examined. Therefore, the validity of genetic association studies using single nucleotide polymorphisms has recently been challenged (5). In many instances, positive associations seen in small studies have not been confirmed in subsequent studies of large cohorts.

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References


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A Liquid Chromatography-Mass Spectrometry Method for the Quantification of Urinary Albumin using a Novel 15N-Isotopically Labeled Albumin Internal Standard

To the Editor:

Recent studies suggest that microalbuminuria confers increased cardiovascular risk, even in nondiabetic and nonhypertensive persons (1–2). Clinically, the testing of microalbuminuria uses immunochemical methods. Controversy exists, however, regarding the accuracy of immunochemical and chromatographic methods for quantifying urine albumin (3). We earlier reported on liquid chromatography-mass spectrometry (LC-MS) for measurement of urinary albumin with bovine serum albumin (BSA) as an internal standard (4). We now report the preparation of 15N-labeled albumin as an internal standard and the validation of its use in an LC-MS assay for quantifying low concentrations of albumin in the urine of patients.

The Pichia pastoris strain GS115/His"Mut" (Invitrogen) was used to synthesize 15N-labeled human serum.

Table 1. Prevalence of CYP2J2 genotypes in CAD patients, CAD controls, and blood donor controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>All n = 4203, %</th>
<th>CAD patients n = 2547, %</th>
<th>CAD patients with MI n = 1350, %</th>
<th>CAD patients without MI n = 1197, %</th>
<th>CAD controls n = 696, %</th>
<th>Blood donor controls n = 960, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>3686 (87.7)</td>
<td>2225 (87.4)</td>
<td>1185 (87.8)</td>
<td>1040 (86.9)</td>
<td>618 (88.8)</td>
<td>843 (87.8)</td>
</tr>
<tr>
<td>GT</td>
<td>499 (11.9)</td>
<td>313 (12.3)</td>
<td>160 (11.9)</td>
<td>153 (12.8)</td>
<td>76 (10.9)</td>
<td>110 (11.5)</td>
</tr>
<tr>
<td>TT</td>
<td>18 (0.4)</td>
<td>9 (0.4)</td>
<td>5 (0.4)</td>
<td>4 (0.3)</td>
<td>2 (0.3)</td>
<td>7 (0.7)</td>
</tr>
</tbody>
</table>

χ² (additive mode) 0.276

a P value: CAD patients vs CAD controls.

b P value: CAD patients vs blood donor controls.

c P value: MI vs no MI in CAD patients.
albumin (HSA) [see details in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol53/issue3]. A Q-TOF Premier™ mass spectrometer (Waters/Micro-mass) was used to characterize the $^{15}$N-labeled HSA and study its fragmentation. Loo et al. (5) previously reported the generation of source-induced fragmentation of intact proteins in an electrospray ionization source of a mass spectrometer. They demonstrated production of b-series fragments consistent with the sequence of albumin from 10 different species. We confirmed these results with HSA, BSA, and $^{15}$N-labeled HSA on the Q-TOF Premier by raising the cone voltage from 40 to 80 volts. The highly charged molecular ions virtually disappear and are replaced by smaller charged fragment ions with charge states ranging from $+2$ to $+6$ (Fig. 1, A-C). These fragments are consistent with b-series fragment ions originating from the N-terminus of the HSA molecule.

To certify the purity of the $^{15}$N-labeled HSA, we compared the source-induced-fragmentation spectrum (specifically the $b_{24}$ ions) of labeled HSA with the coinciding ion obtained from commercial HSA (Fig. 1, D and E). For unlabeled HSA, the lowest expected mass for the isotope cluster of this ion was observed at the expected mass of $m/z = 684.6152$. For the labeled material, the signal at this mass vanished and a signal was observed at $m/z = 693.3276$, indicating virtually complete $^{15}$N-labeling of the 35 nitrogens in the $b_{24}$ fragment. Calculations indicated that each individual nitrogen in the protein was 98.95% $^{15}$N-labeled.

Because of low throughput, the Q-TOF mass spectrometer is not practical for routine clinical testing. Therefore, for routine clinical use, we validated the method using an API 5000™ triple quadrupole mass spectrometer (Applied Biosystems). Since tuning values of the API 5000 differ from those of the Q-TOF, the method was further optimized for the API 5000. The N-terminal fragment ions

Fig. 1. MS characterization of $^{15}$N-HSA.

(A), Q-TOF electrospray ionization spectrum at normal cone voltage (40V) showing a distribution of multiply-charged molecular ions during infusion of $^{15}$N-HSA [70 µg/L (1 fmol/µL)]. (B), increased cone voltage (80 V) induces fragmentation. (C), fragment ions are multiply charged b-fragments derived from the N-terminus of the albumin molecule. (D), isotope distribution for unlabeled HSA $b_{24}$. (E), isotope distribution for labeled $^{15}$N$_{35}$ HSA $b_{24}$.
corresponding to unlabeled b24^4+ (m/z = 685.1) and b24^3+ (m/z = 693.6) were used for quantification of HSA using ^15N-HSA as an internal standard: ^15N35-b24^4+ (m/z = 913.2) and ^15N35-b24^3+ (m/z = 924.1). The chromatographic conditions were the same as described earlier (4).

Our precision studies yielded intrassay CVs (n = 20) of 12.6% (17 mg/L), 10.1% (70 mg/L), and 4.0% (210 mg/L), and the interassay CVs (n = 10) were 12.2% (19 mg/L), 11.0% (80 mg/L), and 7.1% (230 mg/L). By running 20 replicate blanks we determined that the lowest analyte concentration indistinguishable from the blank was 2.5 mg/L. By running 20 replicates of a 5 mg/L sample, we established the limit of detection, defined as the lowest analyte concentration that can be distinguished from blank with >95% certainty, as 4.84 mg/L. The limit of quantification/functional sensitivity was set at 10.5 mg/L, corresponding to the lowest control patient samples with an interassay CV <20%.

Multiple calibration curves ranging from 4 to 625 mg/L were linear and were reproducible with the following linear regression equation: y = 0.01x + 0.20 (r^2 = 0.999).

The linearity of the assay was assessed by extracting 5 patient samples at the following serial dilutions: undiluted, 1:2, 1:4, and 1:8. The expected value for each sample was calculated based on the result obtained for the undiluted sample. The percentages of the expected results were 87%–121% (mean 98%) for urine specimens containing 80–207 mg/L albumin. In additional experiments, HSA was added at 3 concentrations (40, 80, and 170 mg/L) into patient samples (n = 5) and assayed in single determinations. For individual samples, calculated recoveries ranged from 93% to 117% (mean 105%). The comparison shows that the LC-MS method using ^15N-HSA is largely in agreement with the Hitachi method (y = 0.96x + 7.25, R^2 = 0.99), similar to when BSA was employed as the internal standard. In conclusion, we have developed a sensitive and specific LC-MS assay for detection and measurement of urinary albumin that employs ^15N-HSA as a unique and novel internal standard.

References

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Meeting Notice

The 2nd International Symposium on Integrated Biomarkers in Cardiovascular Diseases will be held at the Hilton Hotel in Berlin, Germany on June 21–23, 2007. The meeting is organized by the Charité Hospital-University of Medicine (Berlin, Germany) and the Giovanni Lorenzini Medical Foundation (Milan, Italy), under the auspices of the International Atherosclerosis Society, the German Cardiac Society, and the German HeartFailure Network.

The Meeting will be co-chaired by Rainer Dietz (President of the German Cardiac Society), Valentin Fuster (President of the World Heart Federation), and Rodolfo Paoletti (President, Fondazione Giovanni Lorenzini). The Director of the Symposium is Santica M. Marcovina (Director, Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington, Seattle, WA).

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