Selecting the Optimum Specimen for Assessing Slight Albuminuria, and a Strategy for Clinical Investigation: Novel Uses of Data on Biological Variation

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To investigate the optimum specimen for quantifying low, but abnormal, concentrations of albumin in urine, we assessed the analytical and biological components of variation in first morning, random untimed, and 24-h urine specimens from 11 apparently healthy individuals. The results were expressed in terms of albumin concentration, albumin/creatinine ratio, and albumin excretion rate. Analytical methods generally can meet the analytical goal of CV ≤ 18%. For reasons detailed herein, we prefer measurement of the albumin concentration in the first morning specimen. Expressing results as an albumin/creatinine ratio has little advantage. Albumin concentrations in first morning urines from 16 diabetic subjects showed larger intra-individual variation than for nondiabetic subjects but clearly fell into two groups: those with consistently normal albumin concentrations in urine and those with abnormal concentrations in some specimens. The intrinsic biological variation of the latter group means that the ideal 100% nosological specificity cannot be achieved with any cutoff point without inclusion of a large proportion of the former. Qualitative testing with a latex-agglutination technique also demonstrates this problem. Use of data on biological variation allows development of an appropriate clinical strategy to investigate diabetic patients.

Additional Keyphrases: variation, source of; diabetes; albumin/creatinine ratio; untimed vs 24-h urines; nephropathy; cutoff values

Assessment of abnormal, but low, concentrations of urinary albumin, considered an excellent means to evaluate early diabetic nephropathy (1–5), is clinically important because changes are reversible at the early stages of impaired renal function. In contrast, diabetics with frank albuminuria that can be detected by conventional urine dipstick tests already have progressive irreversible renal disease (6).

Many immunochemical methods have been described for quantifying low concentrations of urinary albumin (7–13). Several studies involving these techniques have shown that the urinary excretion of albumin by an individual varies markedly, predominantly in relation to changes in posture and the degree of physical activity and to the period of urine collection (14–19). Consequently, Mogensen (20) has advocated performing three estimations, of which two consecutive results must be positive, before classifying a diabetic as having albuminuria. He also stated (20) that short-term collections performed in the laboratory or clinic, timed overnight specimens, 24-h collections, or first (“early”) morning urine specimens were all suitable for this diagno-

sis. Besides the variety of urine specimen types possible, there are various ways in which the results can be reported: albumin concentration, albumin excretion rate, or albumin/creatinine ratio. However, the optimal specimen type and reporting format have not been well defined (21–23).

Data on biological variation can be used to (a) set desirable analytical goals for precision (24), (b) assess the utility of conventional population-based reference values (25), and (c) determine the significance of changes between serial results from an individual. We believe that such data can also be used to (d) determine which is the best specimen type to collect for analysis and (e) assess certain aspects of the clinical utility of newer tests, eliminating some of the need to perform large traditional clinical trials (26).

We selected a commercial radioimmunoassay with good precision and a low detection limit—the preferred performance characteristics for this study—and a direct assay of urinary creatinine, to investigate the components of analytical and biological variation in 24-h, untimed, and first morning urine specimens collected from 11 apparently healthy individuals, and we expressed the results in different ways. From these results, we selected the optimum specimen type—namely, first morning—and the best way to express results—namely, albumin concentration—and investigated the components of analytical and biological variation in 16 stable diabetic subjects.

Finally, because qualitative or semiquantitative tests such as the qualitative "AlbuScreen" latex-agglutination method (Cambridge Life Sciences, Cambridge, U.K.; now marketed as "AlbuSure") have much lower detection limits than the conventional dipstick methods and thus may be of value in outpatient clinics (27), we used this method to assay the specimens from the diabetic subjects and assessed some aspects of its potential clinical utility.

Methods

Analytical procedure. For the RIA, we used a double-antibody kit (from Pharmacia, Uppsala, Sweden) exactly according to the manufacturer's protocol. The counting and curve-fit procedures were done with a multi-head gamma counter (Model NE 1612 Turbo; Nuclear Enterprises Ltd., Edinburgh, Scotland); a four-parameter logistic curve-fit was used.

Biological variation. We recruited 11 apparently healthy laboratory staff members (six men and five women, ages 22–40 years) for the study; all were nondiabetic, according to the criteria of the World Health Organization (28). Each subject collected 10 first morning, 10 untimed, and five 24-h urine specimens during a six-week period characterized by no unusual physical activity. We also recruited 16 diabetic subjects (seven men and nine women, ages 11–66 years); 15 were insulin-dependent and one was not. The mean duration of diabetes was 11 years (range 5 to 19); none had clinically evident renal disease, and all excreted urines that were consistently negative or only trace positive by "Albus-" (Ames Division, Miles Laboratories, Stoke Fogen, U.K.). Each diabetic subject collected 10 first morning urine.

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All specimens were collected without preservative, the 24-h urine volumes were recorded, and aliquots of each specimen were stored at -20 °C. On the day of analysis, all specimens from a single subject were thawed at room temperature, and each was mixed thoroughly; the albumin concentration was determined with the Pharmacia RIA and urinary creatinine by direct alkaline picrate colorimetry (29) with the RA-1000 discrete analyzer (Technicon Instruments Corp., Tarrytown, NY 10591). To minimize analytical variation, we analyzed each set of specimens from an individual, in duplicate, in no specific sequence in the same analytical batch, and used reagents, standards, and quality-control materials from the same sources throughout.

A fresh aliquot of each diabetic specimen was also analyzed with the AlbuScreen kit, based upon latex-agglutination inhibition. In this assay, a positive result—no agglutination—is obtained with urinary albumin concentrations >30 mg/L, and agglutination denotes concentrations of albumin <30 mg/L.

Statistical analysis. The analytical (SDA)2 intra-individual (SDI)2, and interindividual (SDA)2 variances of albumin concentration, albumin excretion rate, and albumin/creatinine ratio were calculated by analysis of variance (30) for each type of specimen for diabetic and nondiabetic individuals. Student's unpaired t-test was used to compare the mean albumin concentrations for men and women, and the F-test was applied to assess the difference in intra-individual variances between these two groups.

Results and Discussion

Variation in Healthy Subjects

Table 1 shows the mean values, and estimated average analytical and intra- and interindividual variation for all of the healthy subjects, and separately for men and women, of albumin concentration and albumin/creatinine ratio both in first morning and random untimed urine specimens, and for these and albumin excretion rate in 24-h specimens.

Analytical variation. The analytical variances, estimated from the duplicate results for each specimen, were similar, irrespective of specimen type or mode of expression of results. For the range of albumin concentrations measured, all analytical CVs were <5% and contributed <1% to the total observed variances. A widely expressed view is that analytical variability should be equal to or less than half of the intra-individual biological variation (24); the analytical goal for the precision of urinary albumin assays is thus 18%.

Most currently used techniques can meet or surpass this analytical goal for precision; therefore, other criteria such as practicability and cost should be major influences on selection of appropriate methodology.

Urinary albumin concentration. The overall population mean albumin concentrations were similar in the three types of urine specimen. However, men had lower mean urinary albumin concentrations than women (Table 1), this difference being more significant in the random untimed urines (P < 0.01) than the first morning or 24-h specimens (P > 0.05). Because the difference between men and women is smallest for first morning urines, this specimen type is favored; moreover, reference values stratified according to gender would not be required.

Intra-individual variation. The first morning urine specimens displayed the smallest overall average intra-individual variance. Calculation of an albumin/creatinine ratio marginally reduced this variance in these, but increased it in both the untimed and 24-h specimens. Expression of the results of analyses of the 24-h specimens as albumin excretion rate did not lead to low intra-individual variation. Except for albumin concentration in the first morning urine, for which, interestingly, men and women had exactly the same average variation, the average intra-individual variation of all variables were greater for women than for men. This difference reached statistical significance for the untimed urine specimens; P was < 0.05 for albumin concentration and < 0.01 for the albumin/creatinine ratio.

Interindividual variation. First morning urine specimens also displayed the lowest interindividual variation of urinary albumin concentrations. Expressing the results as the albumin/creatinine ratio marginally increased the interindividual variance in first morning urines, and to a greater extent in the other two types of urine collection. The calculated interindividual variance of the random untimed urines was greatly diminished if men and women were assessed separately, reflecting the significant differences in mean albumin concentrations and interindividual variance in men and women.

Indices of Individuality and Heterogeneity

The index of individuality—that is, the square root of the ratio of intra- to interindividual variance—allows assessment of the utility of conventional population-based reference intervals (31). Such reference intervals, which are of particular importance when the test is used for screening or in making an initial diagnosis (when no previous data are available on an individual), are of particular value when the index is >1.4. However, significant heterogeneity among intra-individual variances, which will also impair the usefulness of reference intervals, may be masked by the simple use of the average intra-individual variance. Calculation of an index of heterogeneity—that is, the ratio of the observed CV of a set of individual variances (SDA)2 to the theoretical CV, [2/(n-1)]1/2, where n is the number of observations

Table 1. Mean Values, and Estimated Average Analytical (CVₐ), Intra-Individual (CVᵢ), and Interindividual (CVᵢ) Variances in Healthy Individuals

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Group</th>
<th>Mean</th>
<th>CVₐ %</th>
<th>CVᵢ %</th>
<th>CVᵢ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>First morning urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin concn, mg/L</td>
<td>All</td>
<td>8.13</td>
<td>3.6</td>
<td>86</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>4.42</td>
<td>3.0</td>
<td>49</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>12.74</td>
<td>3.3</td>
<td>79</td>
<td>33</td>
</tr>
<tr>
<td>Albumin/creatinine ratio, mg/mmol</td>
<td>All</td>
<td>1.10</td>
<td>3.8</td>
<td>103</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>0.47</td>
<td>4.8</td>
<td>37</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>1.89</td>
<td>3.0</td>
<td>88</td>
<td>46</td>
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<tr>
<td>Random untimed urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin concn, mg/L</td>
<td>All</td>
<td>7.84</td>
<td>3.9</td>
<td>61</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>5.54</td>
<td>3.2</td>
<td>49</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>10.71</td>
<td>3.9</td>
<td>60</td>
<td>40</td>
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<tr>
<td>Albumin/creatinine ratio, mg/mmol</td>
<td>All</td>
<td>1.00</td>
<td>4.7</td>
<td>85</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>0.51</td>
<td>3.3</td>
<td>49</td>
<td>45</td>
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<tr>
<td></td>
<td>Women</td>
<td>1.61</td>
<td>4.2</td>
<td>76</td>
<td>45</td>
</tr>
<tr>
<td>24-h urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin concn, mg/L</td>
<td>All</td>
<td>10.18</td>
<td>3.8</td>
<td>70</td>
<td>55</td>
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<tr>
<td></td>
<td>Men</td>
<td>7.01</td>
<td>3.2</td>
<td>52</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>14.15</td>
<td>3.7</td>
<td>69</td>
<td>43</td>
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</table>
per individual—allows assessment of the variability of the intra-individual variances of a group of individuals (32). If there were no heterogeneity of intra-individual variances, this ratio would be 1.00. The larger this ratio is, the more heterogeneity exists. Thus, ideally, the optimal specimen type to use is that with the highest index of individuality and the lowest index of heterogeneity.

Table 2 shows the indices of individuality and heterogeneity obtained for each of the specimen types studied. Although albumin concentration and albumin/creatinine ratio in the untimed urine specimens, and these and albumin excretion rate in the 24-h specimens, had relatively high indices of individuality, they exhibited high degrees of heterogeneity of intra-individual variances. Consequently, we believe that these specimens are unsuitable for use in screening programs. In contrast, the first morning urine specimens, when results are expressed as albumin concentration, displayed much less heterogeneity and had the lowest intra-individual biological variation. First morning urine is the most suitable specimen to use in initial diagnosis.

Use of the albumin/creatinine ratio offers no advantage. In all cases, in addition to the requirement to perform an additional assay, which would increase the random variation and increase costs, use of the ratio leads to greater heterogeneity of intra-individual variances, irrespective of urine specimen type.

Significance of Changes

Calculation of the critical difference—that is, the total variation required for a significant change to have occurred between two results from an individual patient—is of particular relevance when patients are monitored by evaluating results for serial specimens (33). Because the critical difference is due to analytical random variation and intra-individual biological variation, the specimen type should be chosen to minimize the critical difference—which can be expressed, for \( P < 0.05 \), as \( 2.77 \times (\text{CV}_A^2 + \text{CV}_P)^{1/2} \). Table 2 shows the critical differences, expressed as percentages of the mean values, for each of the urine specimen types. The difference required for a significant change between results is smallest for first morning urine specimens. Use of the albumin/creatinine ratio produces the smallest critical difference. However, as discussed earlier, there is greater heterogeneity of intra-individual variances than is found by using the albumin concentration. Thus, first morning urinary albumin concentration is the determination favored for serial monitoring of patients.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Index of individuality</th>
<th>Index of heterogeneity</th>
<th>Critical difference, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>First morning urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin concn</td>
<td>1.03</td>
<td>1.98</td>
<td>100</td>
</tr>
<tr>
<td>Albumin/creatinine ratio</td>
<td>0.77</td>
<td>3.31</td>
<td>87</td>
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<tr>
<td>Untimed urine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Albumin concn</td>
<td>1.41</td>
<td>3.89</td>
<td>238</td>
</tr>
<tr>
<td>Albumin/creatinine ratio</td>
<td>1.27</td>
<td>5.00</td>
<td>286</td>
</tr>
<tr>
<td>24-h urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin concn</td>
<td>1.15</td>
<td>3.03</td>
<td>169</td>
</tr>
<tr>
<td>Albumin/creatinine ratio</td>
<td>1.17</td>
<td>4.00</td>
<td>236</td>
</tr>
<tr>
<td>Albumin excretion rate</td>
<td>1.27</td>
<td>3.59</td>
<td>194</td>
</tr>
</tbody>
</table>

Variation in Diabetic Subjects

Figure 1 shows the mean and range of values for albumin concentration, and the albumin/creatinine ratio, for the optimum type of urine specimen (i.e., first morning) for each of the 16 diabetic subjects. Table 3 shows the calculated analytical, intra-, and interindividual variances. The average intra-individual variance of the diabetic subject group was larger than that for the healthy individuals, irrespective of whether results were expressed as albumin concentration or albumin/creatinine ratio. This increase in intra-individual variation in diabetic subjects as compared with apparently healthy individuals has also been recently noted for several analytes in serum (34).

Classification of an individual as having low but abnormal urinary albumin depends on the single numerical value applied as the cutoff point between health and disease. There is no current agreement concerning the optimum value for this cutoff point, most proposed values lying between 20 and 30 mg of albumin per liter. As shown in Figure 1, the diabetic subjects studied can be divided into two groups. There are 10 individuals whose albumin concentration, exactly as for healthy subjects, is always <30 mg/L in first morning urine specimens. These diabetic subjects would be considered to be at little risk of diabetic nephropathy. In contrast, there are six individuals who on some occasions would be classified as normal, but on others abnormal, that is, having a urinary albumin concentration >30 mg/L.

Consequences for Application of Urinary Albumin Assays

Of particular interest with regard to selection of cutoff points is the use of semiquantitative or qualitative screening tests. Figure 2 shows the results we obtained with the AlbuScreen method for the specimens collected from the diabetic subjects. The results obtained with this latex-agglutination method are stratified in concordance with the results obtained with RIA. Because the objective analytical goal for precision is a CV of 18%, we believe that semiquantitative or qualitative testing will be suitable as a first-line diagnostic test. However, the use of such tests does not overcome the problem that diabetic patients have large intra-individual biological variation. This is of great significance with respect to use of assays that measure low concentrations of urinary albumin. Ideally, screening tests for important treatable disease such as incipient diabetic nephropathy should have high (ideally 100%) nosological sensitivity, and any false positives that occur can be subsequently distinguished from the true positives by repeat testing or by performing an alternative test with different nosological characteristics. There is no single cutoff point that gives the desired sensitivity. At 30 mg of albumin per liter, all of the diabetic subjects with abnormal concentrations of albumin could be misclassified. At 20 mg of albumin per liter, in the worst-case situation, only one of those with abnormal albumin would always be correctly classified but, in addition, one of the subjects with normal albumin could be labeled incorrectly. At 10 mg of albumin per liter, three of the six diabetic subjects with abnormal concentrations could still be misclassified, while seven of the 10 normal diabetic subjects would be wrongly categorized.

We therefore advocate that the strategy diarragammed in Figure 3 be considered for wide adoption. After testing with Albustix or other similar procedure to detect frank albuminuria, those who are classified as negative are tested with AlbuScreen or other like qualitative procedure. A single
positive result will mean that the diabetic subject does have
an abnormal concentration of albumin, and appropriate
clinical intervention can be initiated. No healthy or diabetic
subject without disease ever has a urinary albumin concen-
tration exceeding 30 mg/L and consequently there are no
false-positive results and the test has 100% nosological
specificity. A single negative result does not exclude disease,
because, as we have shown, the biological variation of those
with abnormal concentrations of albumin is large, such that
these subjects do vary above and below the cutoff point of 30
mg of albumin per liter. Retesting of both groups will be
required. Quantitative analyses of albumin would have
advantage in the monitoring of individuals with an identi-
ified abnormality if it was borne in mind that the large intra-
individual biological variation enlarges the change neces-
sary for a significant difference between consecutive results.
It is in this situation that the determination of albumin/
creatinine ratio might be of greater value because the
critical difference between sequential results is smaller
than that of albumin alone (Table 3). We believe that qualitative
or semiquantitative assays would suffice for the retesting of
those with previous negative results. We suggest that use
of this strategy precludes setting the cutoff point of qualitative
or semiquantitative tests at <30 mg/L, because then the test

Table 3. Summary of Results for First Morning Urine Specimens from Diabetic Subjects

<table>
<thead>
<tr>
<th></th>
<th>Albumin/creatinine ratio, mg/mmol</th>
<th>Albumin, concn, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.25</td>
<td>14.35</td>
</tr>
<tr>
<td>CVa, %</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>CVb, %</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>CVa, %</td>
<td>75</td>
<td>76</td>
</tr>
<tr>
<td>Index of individuality</td>
<td>0.81</td>
<td>0.51</td>
</tr>
<tr>
<td>Index of heterogeneity</td>
<td>2.18</td>
<td>2.75</td>
</tr>
<tr>
<td>Critical difference, %</td>
<td>170</td>
<td>109</td>
</tr>
</tbody>
</table>

Outpatient Visit

Early Morning Spot Urine

Test with Albuscreen or similar procedure

POSITIVE

NEGATIVE

Frak albuminuria

Test with Albuscreen or other procedure

with cut-off at 30 mg/L

POSITIVE

NEGATIVE

Disease IS present

Disease MAY or MAY NOT be present

No objective evidence for clinical intervention

Objective evidence for clinical intervention

QUANTITATIVE RETEST

QUALITATIVE RETEST

Low result

High result

Decide whether action should be taken

CONTINUE

CEASE

Fig. 1. Parametric means and absolute ranges for (left) albumin concentration, and (right) albumin/creatinine ratio in first morning urine specimens from 16 diabetic subjects.

Fig. 2. Parametric means and absolute ranges for albumin concentration in first morning urine from 16 diabetic subjects, and results by the Albuscreen method.

Albuscreen-negative results (i.e., albumin <30 mg/L) were obtained for all specimens from nine individuals, the remaining subjects had positive (+) and negative (−) results as shown.

Fig. 3. Diagram of a possible clinical strategy for the optimum use of albumin concentration measured in first ("early") morning urine specimens for screening and monitoring early diabetic nephropathy.

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will have neither 100% sensitivity nor 100% specificity, and consequently, neither positive nor negative results will exclusively allow one to ascertain or exclude disease.

We have previously suggested (35, 36) that studies on biological variation, performed over relatively short periods of time with a small number of subjects, should be performed early in the evolution of any new test in clinical chemistry so as to define objective analytical goals, assess the utility of conventional population-based reference values, and determine the significance of changes between serial results. In this study, this concept has been amplified, and we have demonstrated the usefulness of data on biological variation in the selection of the best specimen to collect, and in the development of appropriate clinical strategies to ensure the optimum use of a clinical laboratory test.

We thank all those who provided urine specimens, Miss C. Boyle for organizing the collection of specimens from the laboratory staff, and Drs. W. Bennett, C. Forsyth, and R. Newton for recruiting their diabetic patients to the study. We acknowledge the help received from Cambridge Life Sciences and from Pharmacia in providing their reagents, either free of charge or at greatly reduced cost. We thank Tayside Health Board for their partial funding of this project.

References