min values with increasing hemolysis (4). Thus the choice of wavelength is critical in evaluating the effect of interfering substances.

References

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How Should We Measure Activity of Alanine Aminopeptidase in Urine?

To the Editor:

Alanine aminopeptidase [AAP; \( \alpha \)-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2] has proved to be a suitable urinary enzyme to use in detecting renal disorders (1), and numerous tests for determination of its activity have been recommended (2). L-Alanine-4-nitroanilide is the most widely accepted and most convenient substrate for determination of this enzyme (2). Meanwhile, three well-documented methods have been described in which this substrate was used (2-4). These methods differ in reaction temperature, buffer, buffer and substrate concentration, and especially in the sample, either native or gel-filtered urine. Whereas Matteenheimer et al. (2) and we (3) considered it necessary to remove enzyme inhibitors from urine by gel-filtration, Flandrois et al. (4) suggested, on the basis of their multivariate optimization study, new reaction conditions under which pre-treatment of urine samples would not be required for accurate AAP determination. Such a possibility would facilitate the automation of AAP measurement in clinical chemistry laboratories.

Enzyme inhibitors in urine vary considerably, both in healthy and diseased individuals (2). To show this effect and the influence of inhibitors on the AAP determination, we measured urinary AAP activities in native and gel-filtered (5) urine samples from 20 healthy adults and 20 renal-transplant recipients, using these three methods (2-4). We used the original conditions of Method I according to Matteenheimer et al. (2) at 25°C with 0.8 mmol/L L-alanine-4-nitroanilide and 70 mmol/L triethanolamine buffer, pH 7.6; of Method II according to Jung and Scholz (3) at 37°C with 2 mmol/L substrate and 50 mmol/L Tris HCl buffer, pH 7.8; and of Method III according to Flandrois et al. (4) at 30°C with 5.8 mmol/L substrate and 300 mmol/L Tris HCl buffer, pH 7.9.

We performed all assays at sample/final reaction volume ratios of 1/6.5 in the EPOS 5060 analyzer (Eppendorf Gerätebau Netheler & Hinz, Hamburg, F.R.G.). This final 0.154 dilution ratio of the samples was identical with the one Flandrois et al. (4) applied at one site of their optimization study.

We set the activity values measured in samples after gel filtration at 100% and calculated the percentages of the activities in untreated urine samples (Table 1). Except for Method III in renal-transplant recipients, the gel-filtered samples showed higher AAP activities than untreated samples. The activities in untreated urine samples compared with activities measured in gel-filtered samples, indicated in Table 1 as relative activities, were significantly lower in healthy persons than in renal-transplant recipients. We conclude that concentrations of enzyme inhibitors are higher in urine samples of healthy persons than in those of renal-transplant recipients, and that their inhibitory effects are diminished by increasing substrate concentration. However, the recommended substrate concentration of Flandrois et al. (4) does not entirely overcome the influence of inhibitors on the determination of AAP activity in healthy persons. Accurate determination of AAP is therefore not guaranteed when the final dilution ratio of the native urine samples in the reaction mixture is 0.154.

In a new series of experiments we measured AAP activities in urine of 20 healthy adults with Method III at various dilution ratios of the native urine samples in the reaction mixture and compared the activities with values measured in gel-filtered samples. The mean (and SD) percentage of activities in untreated samples in comparison with gel-filtered samples significantly increased, from 73.5% (9.2) at dilution ratio of samples of 0.154 to 90% (7.8), 94% (6.5), and 109% (7.3) at dilution ratios of samples of 0.076, 0.062, and 0.031, respectively. Thus, the influence of urinary enzyme inhibitors on AAP determination can be avoided only if activity determinations are performed, in addition to the reaction conditions recommended by Flandrois et al. (4), with higher dilutions of native urine samples in reaction mixture. Moreover, because use of highly diluted samples considerably diminishes the analytical sensitivity of the method, we recommend the use of gel-filtered urine samples for accurate AAP determination.

References

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Table 1. AAP Activity as Measured with Three Methods in Untreated and Gel-Filter ed Urine Samples from 20 Healthy Adults and 20 Renal-Transplant Recipients

<table>
<thead>
<tr>
<th></th>
<th>Untreated urine</th>
<th>Gel-filtered urine</th>
<th>Relative* activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/L, mean (and SD)</td>
<td>%, mean (and SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy adults</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method I</td>
<td>1.4 (0.8)</td>
<td>3.6 (2.3)*</td>
<td>46.7 (21.9)</td>
<td></td>
</tr>
<tr>
<td>Method II</td>
<td>6.5 (4.2)</td>
<td>11.9 (7.6)*</td>
<td>57.5 (19.5)</td>
<td></td>
</tr>
<tr>
<td>Method III</td>
<td>6.4 (4.0)</td>
<td>9.0 (5.6)*</td>
<td>77.5 (15.1)</td>
<td></td>
</tr>
<tr>
<td>Renal-transplant recipients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method I</td>
<td>5.7 (5.5)</td>
<td>7.7 (6.5)*</td>
<td>68.9 (20.8)*</td>
<td></td>
</tr>
<tr>
<td>Method II</td>
<td>22.6 (18.6)</td>
<td>27.1 (10.3)*</td>
<td>82.3 (15.6)*</td>
<td></td>
</tr>
<tr>
<td>Method III</td>
<td>19.0 (13.7)</td>
<td>19.3 (13.9)</td>
<td>101 (14.0)*</td>
<td></td>
</tr>
</tbody>
</table>

* Activity measured in untreated samples relative to the activity measured in gel-filtered samples. \( P < 0.001 \), significantly different (paired t-test) from activity measured in untreated urine samples. \( P < 0.01 \) and \( P < 0.001 \), significantly different (t-test) from relative activity obtained for healthy adults.

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Estimation of Sensitivity of Immunoassays

To the Editor:

McConway et al. (1) advocate use of the precision profile to determine the minimum detection limit (MDL) of immunoassays, arguing that the MDL (with 95% confidence) is the concentration corresponding to a CV of 22% read from the precision profile. They are correct in emphasizing the scatter of sample, rather than the zero-standard estimates in estimating the MDL, but readers should note that their argument is based on the incorrect use of statistics and that it makes some assumptions about immunoassays that are unlikely to be met in practice.

With regard to the statistics set out in the appendix to their paper, the errors in their argument are as follows:

1) “The standard error” (SE) referred to in the denominator of their equation 1 is unspecified, but would, in computing the t statistic as shown, be the SE of the difference in the means (X1 - X2). This should be calculated as the square root of the sum of the variances of X1 and X2, and not as shown, as the SE of X1. Their calculation incorrectly assumes that the estimate of the mean response at zero-analyte concentration is error-free, and will, therefore, underestimate the MDL.

2) The statistics X1, X2, and SD are in units of the response variable up to equation 2, but subsequently they are interpreted as being in concentration units. The nonlinear relationship between response variable and concentration implies that measures of scatter for these quantities are not easily transposed. It is misleading of the authors to introduce this transposition without making it explicit, and without demonstrating that it is valid to do so.

3) In calculating, albeit incorrectly, the CV at the MDL as being 22%, readers will note that this CV describes the scatter when test results are derived from single measurements. As the study described by the authors used duplicate measurements for each test result, the CVs reported in the precision profiles would apply to the scatter based on the mean of duplicate measurements. These two measures of scatter are not directly comparable, the latter being lower by a factor of √2.

In addition to these errors of commission, the authors omit to make clear the limitations of using the precision profile to determine MDL in the way they describe. The precision profile will give misleading estimates of the CV when the curve fit is poor, or where the zero standards are excluded from the curve fit and extrapolated CVs are presented in the profile. These pitfalls are well-recognized hazards in curve-fitting data from immunometric assays, particularly at low concentrations.

A reliable method of calculating the MDL has been presented by Rodbard (2), although this does not appear to be widely implemented in data-processing software. A simple but nonetheless valid approach has been proposed by Borth (3), who estimated the MDL (with 95% confidence) as the blank (zero) concentration + 2.5 S, where S is the within-assay standard deviation at concentrations approaching zero. Because S does not vary markedly across the low concentration range of interest, it can be measured directly from sample replicates, or from the precision profile if the precautions noted in the previous paragraph are observed.

There are, as Rodbard (2) has pointed out, many definitions of sensitivity in the literature that are conceptually and statistically unsound. The “22% CV” rule, as presented by McConway et al. (1), adds yet another. It would be unfortunate if immunoassayists were to be distracted by its apparent simplicity from using established and more-reliable methods of estimating the MDL.

References


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Ed. note: Seth says that, in May, he sent a copy of this letter to McConway, but there has been no response.

Criteria for Detection of Heterozygous Individuals with Lactate Dehydrogenase Subunit Deficiencies

To the Editor:

Maekawa et al. (1) stated that measurement of lactate dehydrogenase (EC 1.1.1.27, LD) isoenzymes in erythrocyte hemolysates is the most effective way to detect heterozygous individuals with LD-subunit deficiencies. They estimated the amount of each subunit in erythrocyte LD and calculated the ratio of H to M subunits (H/M). The criterion for detection of such heterozygous individuals was an H/M ratio of <2.3 for those with H-subunit deficiency and of >3.5 for M-subunit deficiency. This criterion was established by the method described by Shioya et al. (2), who used Cellogel membrane (Chemetron, Milan, Italy). In this method, activity bands were made visible by use of d,L-lactate and NAD+ as substrate, phenazine methosulfate (PMS) as intermediate, and tetrazolium salt (MTT) as the final hydrogen acceptor. Problems with this method include the fact that hemoglobin has a similar electrophoretic mobility to LD 3, and it interferes positively with the activity-based staining of LD 3. The cause of the latter interference is the nonspecific electron transfer activity of PMS in the reagent used for LD-activity staining.

Here, we propose a new criterion for detection of heterozygous individuals with LD-subunit deficiencies. We use Titan III membrane (Titan III Lipo)