were presumably due to factors other than warfarin. Even for the 13 hyperuricemic patients (uric acid > 0.48 mmol/L for men and 0.42 mmol/L for women) in our population, warfarin had no effect. In fact, uric acid increased during treatment in only one of these patients (Table 1).

Our results conflict with the report of Menon et al. (1), who found an increase in median uric acid plasma concentration from 0.34 mmol/L to 0.40 mmol/L in 12 patients started on warfarin therapy ($P < 0.01$). They also used the Wilcoxon signed-rank test and measured uric acid with the SMAc AutoAnalyzer. Their patients had clinical problems similar to ours, but were older (mean age 63 y) and included relatively more men (nine men, three women). It is not clear whether their subjects were enrolled prospectively or retrospectively.

Apart from possible influence of age- and sex-related differences, we do not understand why the results of the two studies differ. The lack of significant changes in uric acid in our study is probably not a false-negative result (type 2 error) because, given the mean (0.006 mmol/L) and variance (standard deviation 0.11 mmol/L) of the differences in the 40 paired samples, a statistically significant difference as small as 0.006 mmol/L would have been detected at the 0.05 level with a probability of approximately 90%.

Support for our finding that warfarin does not increase serum uric acid concentration comes from a randomized, placebo-controlled trial of warfarin therapy in 50 patients recovering from acute myocardial infarction in Norway (2). There were 39 men and 11 women in the study; their mean age was 61 y. Patients were excluded if they had a history of gout or diabetes or if they were on diuretics. After approximately 10 months of therapy (range six to 20 months), the mean serum uric acid values of the 23 anticoagulated patients were lower than those of the 27 controls (0.33 mmol/L vs 0.35 mmol/L, not significant by Student’s t-test).

Given the discrepancy between our results and those previously reported, a prospective study of consecutive hyperuricemic patients begun on warfarin will be necessary to resolve the question of risk of gout from warfarin therapy.

Donald L. Kaiser, DrPH, assisted with statistical analysis.

References

URINARY ALANINE AMINOPEPTIDASE ASSAY IMPROVED AS RESULT OF MULTIVARIATE RESPONSE-SURFACE ANALYSIS

Christine Flandrie,¹ Christiane Lahe,¹ Delphine Feldmann,² Jean Marc Gabastou,² Albane Gonnón,¹ and Irène Maire¹

Optimization of determination of alanine aminopeptidase in urine by univariate study led to a method involving pretreatment of urine with Sephadex G50. Re-examination of the optimization by multivariate study led us to recommend higher optimal concentrations: 5.8 mmol/L for the substrate and 300 mmol/L for the Tris buffer. Under these new conditions, pretreatment of urine was no longer necessary and the assay could be completely automated.

Additional Keyphrases: enzyme activity · assay optimization · simplex analysis

Alanine aminopeptidase [AAP; microsomal aminopeptidase, alpha aminocarboxypeptidase; EC 3.4.11.2] activity is localized in the brush-border membrane of the proximal renal tubule (1). Determination of AAP activity in urine is a useful indicator of acute rejections in kidney-transplant recipients (2, 3) and drug-induced nephrotoxicity (4–6). Several methods for measuring AAP activity in urine have been described (6, 7), including an optimized kinetic assay proposed by Jung and Scholz (8); but in all cases the presence of inhibitors (7) necessitated the pretreatment of urine by gel filtration (8, 9). Our intent here was to verify the optimization proposed by Jung and Scholz (8), which was based on a monovariate experimental procedure, by using multivariate response-surface analysis (10).

Materials and Methods

Assays were performed at two sites (site 1, Lyon; site 2, Paris), to verify each step of the optimization work. Previous exchange of control sera (Beckman Decision Levels 1 and 3) led to the same mean values being obtained at the two sites, whether a common manual procedure or automated adaptations were used.
Table 1. AAP Activity in 11 Urine Samples: Necessity for Pretreatment of Urine

<table>
<thead>
<tr>
<th>Untreated urine</th>
<th>Treated*</th>
<th>Diluted but untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAP, IU/L</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>27</td>
<td>22</td>
</tr>
<tr>
<td>61</td>
<td>84</td>
<td>77</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>6</td>
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<td>48</td>
<td>68</td>
<td>60</td>
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<tr>
<td>7</td>
<td>18</td>
<td>17</td>
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<tr>
<td>20</td>
<td>31</td>
<td>17</td>
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<tr>
<td>18</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>56</td>
<td>68</td>
<td>49</td>
</tr>
<tr>
<td>23</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>22</td>
<td>31</td>
<td>27</td>
</tr>
</tbody>
</table>

*According to Jung and Scholz (9).

Apparatus: At site 1, we used a discrete analyzer (Olli Type C 3000), dispenser Model 216, mixer Model 369, and incubator Model 354 (all from Kone Instruments, 91020 Evry, France). At site 2 we used an RA 1000 random-access analyzer (Technicon, 95331 Domont Cedex, France). At both sites we used PD 10 chromatographic columns (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Reagents: Tris buffer was from Boehringer, Mannheim, F.R.G.; l-alanine-4-nitroanilide hydrochloride and hydrochloric acid were from E. Merck GmbH, Darmstadt, F.R.G. Reagent I is Tris HCl buffer; reagent II is l-alanine-4-nitroanilide hydrochloride in Tris HCl buffer.

Procedures: Site 1: mix 0.50 mL of reagent I and 0.10 mL of sample to allow to equilibrate at 30 °C for 5 min. Then start the reaction by adding 50 μL of reagent II.

Site 2: mix 0.55 mL of reagent I and 0.06 mL of sample, allow to equilibrate at 30 °C for 5 min. Then start the reaction by adding 50 μL of reagent II.

At both sites, the increase in absorbance at 410 nm at 30 °C (caused by release of 4-nitroaniline) was then monitored continuously for 2 min. Each assay was performed in triplicate.

Samples: Urine specimens collected at 0900 h from healthy children and adults and from patients receiving nephrotoxic therapies (aminosides and anti-neoplastic drugs) were centrifuged and the pH was adjusted, if necessary, to between pH 6.5 and 7.5; samples were either used immediately or stored at 4 °C for less than 24 h.

Multivariate study: Three different factors of the reaction were studied: pH, Tris buffer, and substrate concentrations. Buffer concentrations tested ranged from 25 to 500 mmol/L (final concentration) and pH from 7.0 to 8.6; substrate concentrations tested ranged from 0.5 to 7.0 mmol/L (final concentration).

Data analysis: The data were analyzed by least-squares regression-analysis techniques (11, 12). The following second-order polynomial equation was used to fit the experimental data:

\[ y = b_0 + \sum_{i=1}^{p} b_i x_i + \sum_{i=j}^{p} b_{ij} x_i x_j + \sum_{i=j}^{p} b_{i} x_i^2 \]

In this equation, p is the number of factors tested (p = 3 or p = 2), y is the predicted response, and x_i are data calculated from buffer concentration, pH, and substrate concentration and scaled from -1 to +1 to minimize the effect of different variable ranges on the regression. We assessed the goodness or the lack of fit of the model by comparing the residual variance with the variance of the experimental error; by calculating the total variance, the \( F \)-test for significance of regression, and the coefficient of multiple determination \( R^2 \); and by visual analysis of the response residuals. We investigated the region of maximal expected activity: first, by applying a simplex maximization procedure (13) to the equation fitting the experimental data, and, second, by displaying our results. For displaying our results, we plotted theoretical activity as a function of various combinations of two variables, the remaining variable being held at the optimum selected value. We used two types of plots: a topographic contour plot of isactivity lines and a three-dimensional plot of activity. Multivariate analysis and simplex maximization programs have been written by C. Lahet for a microcomputer, Kontron ψ 980, using basic language.

Results

Urine pretreatment (by gel filtration of the centrifuged urine on Sephadex G 50) was necessary in the measuring conditions suggested by Jung and Scholz (9) (Table 1). Final dilution ratios for the samples were 0.154 for untreated urines, 0.066 for gel-filtered urines, and 0.039 for untreated but diluted urines. Higher dilution of the sample minimized but did not totally eliminate the influence of the inhibitors, but did result in a noticeable loss of sensitivity.

Table 2. Optimal Values of pH, Buffer, and Substrate Concentrations

<table>
<thead>
<tr>
<th>Subset</th>
<th>Site</th>
<th>Dilution ratio of the sample</th>
<th>Buffer concn, mmol/L (B)</th>
<th>pH (pH)</th>
<th>Substrate concn, mmol/L (B)</th>
<th>Buffer concn, mmol/L</th>
<th>Substrate concn, mmol/L</th>
<th>Calculated optima</th>
<th>Regress. statistics</th>
<th>Total variance corrected for the mean</th>
<th>F (regression)</th>
<th>t-value of interaction parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.086</td>
<td>25, 100, 600</td>
<td>7.0, 7.8, 8.6</td>
<td>0.5, 2.0, 3.5</td>
<td>200</td>
<td>7.6</td>
<td>3.0</td>
<td>0.94</td>
<td>98</td>
<td>956</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.086</td>
<td>110, 220, 350</td>
<td>7.0, 7.8, 8.6</td>
<td>1.0, 3.0</td>
<td>327</td>
<td>7.7</td>
<td>5.8</td>
<td>0.96</td>
<td>19</td>
<td>274</td>
<td>67</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.039</td>
<td>100, 250, 400</td>
<td>7.0, 7.8, 8.6</td>
<td>1.0, 4.0, 7.0</td>
<td>400</td>
<td>&gt;8.2</td>
<td>5.8</td>
<td>0.93</td>
<td>6</td>
<td>59</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.086</td>
<td>186, 325, 465</td>
<td>7.0, 7.8, 8.6</td>
<td>5.8</td>
<td>-a</td>
<td>7.7</td>
<td>-b</td>
<td>0.88</td>
<td>10</td>
<td>44</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>0.039</td>
<td>200, 350, 500</td>
<td>7.0, 7.8, 8.6</td>
<td>5.8</td>
<td>250</td>
<td>8.0</td>
<td>-b</td>
<td>0.86</td>
<td>15</td>
<td>59</td>
<td>8</td>
</tr>
</tbody>
</table>

*No maximum: see Discussion. *Not calculated; substrate concentrations set to 5.8 mmol/L.
Table 2 gives regression statistics. The initial ranges of variations for the three factors were chosen with regard to the optimal values previously determined by univariate study (8). The results of each multivariate study then led us to a better choice of the range of variations for the following experiments. We performed the first three subsets of experiments with the three factors of the reaction, using a $3 \times 3 \times 3$ or $4 \times 4 \times 4$ levels experimental design. The following experiments (subsets 4 and 5) were limited to two factors: pH and buffer concentration, the substrate concentration being set to the optimal value of 5.8 mmol/L. In the fourth subset, no optimal calculated concentration of the buffer was obtained; a minimax was observed (10) and the parameters b1 and b11 of the polynomial equation were not statistically significant (alpha > 0.05).

The two-variable isocontour plots and pseudo-three-dimensional plots are presented in Figures 1 and 2. They were limited to the most-representative experiments. We do not show subset 1 in Table 2, which led to calculated optimal concentrations outside the range of concentrations tested. The two-variable isocontour plots represent the complete range of variation of our parameters. Pseudo-three-dimensional plots allowed us to examine the reaction surface in another way, and represent either the total range of variation (Figure 1) or the area around the optimum found (Figure 2).

Choice of the optimal reaction conditions. The calculated optimal concentrations of the substrate were identical for the two sites: 5.8 mmol/L. For the other factors, the calculated values were different, even within one site. These apparent discrepancies may be easily explained by the presence of a broad plateau for Tris buffer concentrations ranging from at least 250 to 400 mmol/L and pH values from 7.7 to 8.4. For practical purposes (precipitation of the substrate when high buffer concentrations and a pH > 7.9 are used), the final compromise values chosen were the following: Tris 300 mmol/L, pH 7.9, and substrate 5.8 mmol/L.

The AAP activities of 57 treated (x) and untreated (y) urines as measured by our optimized method were identical. Calculated regression parameters of the linear model were as follows: slope 1.017, intercept $-0.753$, correlation coefficient 0.996.

Optimization conditions were verified by using an untreated urine and two different dilution ratios of this urine (0.066 and 0.039). The results obtained (not shown) led to the same optimal values.

Discussion

The study of Table 2 showed that $R^2$ values and $F$ values for regression were always significant (alpha < 0.05), showing that the postulated model fit the experimental data. The measurement process is highly precise: the variance of the pure experimental error is ≤ 6. For all subsets except subset 1, the residual variances are small enough (residual variance ≤ 19). In a practical sense (12), we therefore accepted the regression model as adequately representing the experimentally observed data except for subset 1, where high residual variance showed a lack of fit of the model to the data; this subset will not be discussed further. The total variance reflects the variation of the AAP activity with the variation of the factors tested, so its great value in the first subset shows a wide variation of AAP activity outside the optimal area. In contrast, its small value in subset 4 reflects the weak variation of the AAP activity in the optimal area.

The coefficient of interaction between buffer concentration and pH is negative but not very significant in the optimal area. The interaction coefficients between buffer and substrate concentrations or pH and substrate concentrations were positive and highly statistically significant (alpha < 0.01). This finding demonstrates the interdependence of these factors, which explains the difference obtained with the monovariate study and underlines the need of multivariate experiments in such cases.

The study of isoactivity contour plots and pseudo-three-dimensional plots for subset 2 (Figure 1) reveals a great increase of AAP activity with increasing concentrations of substrate and buffer and pH values up to, respectively, 4.5
mmol/L, 250 mmol/L, and 7.7, and a smoother variation of AAP activity above these values, the most important factor being the substrate concentration. This weak variation, demonstrated by the small values for the total variance in subsets 4 and 5 (Table 2), is confirmed by pseudo-three-dimensional plots showing a broad plateau in the optimal area (Figure 2).

We confirmed the need of pretreatment of urine in the method of Jung and Schols (8) (Table 1). But after multivariate optimization of the method, this pretreatment became unnecessary—that is, better optimization overcomes the inhibitors' influence.

Our re-assessment of optimal conditions when untreated urine is used confirmed the previously reported optimal condition for treated urines.

We conclude that multivariate studies are required if a method involving interdependent reaction parameters is to be truly optimized. For measurement of AAP activity this procedure allows us to determine a broad plateau optimum area for the three parameters studied. In these areas the method is more resistant to concentration variations and inhibitors' influence. This improved procedure leads to a great practical advantage: urine pretreatment is no more a prerequisite to accurate AAP determination, and complete automation of the AAP measurement is then possible.

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References

More-Sensitive Enzyme-Multiplied Immunoassay Technique for Procainamide and N-Acetylprocainamide in Plasma, Serum, and Urine

Paul R. Henry and Rohini A. Dhruv

A commercially available (Syva Co.) enzyme-multiplied immunoassay technique (EMIT) for the quantitative determination of procainamide (PA) and N-acetylprocainamide (NAPA) was modified to allow automated quantitative analysis of approximately 100 samples per day, in a working range of 0.1 to 2.0 μg/mL. Such a test was needed to evaluate the pharmacokinetic characteristics of controlled-release dosage forms characterized by long half-lives at low plasma concentration. Analytical recovery of PA and NAPA from serum, plasma, and urine was satisfactory, but at extreme ratios for PA:NAPA the accuracy of determining the lower-concentration component became unsatisfactory. In fact, however, we found no such ratios in 5400 clinical samples assayed by this procedure.

Additional Keyphrases: pharmacokinetics · controlled-release medication · anti-arrhythmic drugs

The drug procainamide (PA), used in the treatment of individuals with cardiac arrhythmia, is therapeutically effective at concentrations of 4 to 6 μg/mL in plasma (1). Its major metabolite, N-acetylprocainamide (NAPA), is equally effective against cardiac arrhythmias. Toxic effects are observed when the total concentration of PA and NAPA exceeds 15 μg/mL (2). Thus, determination of concentrations of PA as well as NAPA in plasma has been essential in individuals being treated with PA, and an enzyme-multiplied immunoassay technique (EMIT) is a well-accepted method for this purpose (3, 4).

Controlled-release formulations of PA, currently under development, are expected to maintain therapeutic concentrations of the drug in plasma for prolonged periods when administered to patients. However, typical of this type of formulation are prolonged periods during which the concen-