Instability of Calibration Curves of Liquid-Chromatographic Techniques with Electrochemical Detection: Role of the Detector, Jacques Massé, Pierre Leclerc, and Marcel Pouliot (Service de biochimie, Hôpital du Saint-Sacrement, Québec, Canada, G1S 4L8)

Many factors are known to affect the stability of calibration curves of liquid-chromatographic (LC) techniques, including variability in the extraction procedures and decreasing efficiency of the column as it becomes fouled by impurities. We have evaluated the contribution of the electrochemical (EC) detector to the instability of the calibration curve of a method for determining morphine in serum (1).

We used an amperometric detector (Model 460; Waters Chromatography Division, Millipore Corp., Milford, MA 01757) with a glassy carbon electrode. The standard curves were obtained by plotting morphine/internal standard peak-height ratios against morphine concentrations. Within 20 days, without interruption of the LC system, we obtained 16 calibration curves on different days. Before each run, we also injected unextracted aqueous solutions of morphine and internal standard (IS).

In the first few days of that period, the slope of the calibration curve decreased to about one half of its initial value, then stabilized, and finally increased slightly over the last four days. The intercept was not significantly different from zero. With daily injections of unextracted aqueous solutions, we found a gradual decrease of the response of the detector. The peak heights for an identical quantity of morphine injected decreased by a factor of three. For the IS, the decrease was less important. Cleaning the working electrode restored the initial sensitivity, indicating that the electrode was the source of the decrease in sensitivity.

A correlation study between the slope of the calibration curve and the response of the detector to morphine revealed a coefficient of determination \( r^2 \) of 0.58, indicating an important contribution of the response of the detector to the variability of the slope.

We concluded that the stability of calibration curves of LC-EC techniques should be established in the evaluation of a new technique, an aspect frequently overlooked in papers presenting LC-EC methods. Moreover, we concluded that instability can in good part be attributed to variability of the response of the detector as the working electrode surface is being fouled by impurities or reaction products.

Reference

New Sensitive Method for Determining Cysteine Proteinase Inhibitors, Ladislav Fukal and Evert Kasaffrek (Dept. of Biochem., Inst. of Chem. Technol., 166 28 Prague 6, Czechoslovakia; and Inst. of Pharm. and Biochem., 130 60 Prague 3, Czechoslovakia)

Cysteine proteinase inhibitors (CPI) have been isolated from plasma and other body fluids and from various tissues, and extensively studied (1). These inhibitors inactivate several different cysteine proteinases, including cathepsins, papain (EC 3.4.22.2), and calpain (EC 3.4.22.17). Thus their physiological function appears to involve regulation of cysteine proteinases. Many authors have mentioned a connection between a change in CPI concentration and certain diseases (2–6).

Usually, inhibitory activity is assayed by using papain with either the protein substrate or the synthetic substrate N\(_2\)-benzoyl-arginine-p-nitroanilide (7). Recently, we synthesized new substrates (8): N-succinyll-Ala-Cys (S-benzyl)-p-nitroanilide (I), N-succinyll-Leu-Cys (S-benzyl)-p-nitroanilide (II), and N-succinyll-Leu-Cys (S-benzyl)-p-nitroanilide (III). We compared these substrates in a procedure involving papain activity determination, with the following results:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of p-nitroaniline release by papain</th>
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<tbody>
<tr>
<td>Bz-Arg-pDNA</td>
<td>1</td>
</tr>
<tr>
<td>I</td>
<td>53</td>
</tr>
<tr>
<td>II</td>
<td>97</td>
</tr>
<tr>
<td>III</td>
<td>552</td>
</tr>
</tbody>
</table>

Evidently, with these new substrates the sensitivity of papain determination is manyfold that with the conventional substrate.

Details of the final reaction mixture: temperature 35 °C, pH 7.4, and, per liter, 0.1 mmol of papain, 0.5 mmol of substrate, 10 mmol of cysteine hydrochloride, 2 mmol of EDTA, and 66.7 g of dimethylformamide.

Determination procedure: The enzyme reaction takes place in a spectrophotometer cuvette. Pre-incubate 2.7 mL of 0.2 mol/L phosphate buffer containing the cysteine hydrochloride and EDTA, plus 0.1 mL of the solution of papain in distilled water, for 10 min at 35 °C. Then add 0.2 mL of substrate solution in dimethylformamide and record the increase in absorbance at 410 nm.

Papain assay with the above new p-nitroanilides makes possible the more-sensitive determination of CPI such as kininogens and cystatins in plasma, synovial fluids, tumor cells, leukocytes, semen, sex glands, cataract lenses, gingival tissue, and brain. These p-nitroanilides also are suitable substrates for a sensitive assay of some human cysteine proteinases. Such studies are in progress.

References

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