Comparison of Methods for Measurement of Na+/Li+ Countertransport Across the Erythrocyte Membrane

To the Editor:

About 30% of patients with insulin-dependent diabetes mellitus develop diabetic nephropathy. Since diabetic nephropathy contributes to a large extent to the high mortality of these patients, a risk marker for the development of this condition is desirable. Increase in Na+/Li+ countertransport across the red cell membrane has been suggested as such an early marker [1-4], although this was not uniformly confirmed [5-7]. In this study, the three main methods to load erythrocytes with Li+ were compared and tested for their measuring error and intrasubject variation of V_max and K_0.5 for Na+.

The "classic" LiCl loading [8, 9] is the most "physiological" and noninvasive method. However, the loading procedure takes 3 h, which precludes the use of this method as a standard procedure. The Li2CO3 loading as described by Elving et al. [6] has the advantage that it takes only 30 min to load the cells with Li+. The Li+ enters the cell via the HCO3-/Cl- exchanger as a LiCO3 ion in exchange for a Cl- ion, which explains the fast Li+ loading. This method has been reported to give plots to which Michaelis-Menten kinetics apply [10]. The nystatin method was developed by Canessa et al. [11] because at 150 mmol/L Na+ (the highest concentration that can be used at an osmolarity of 300 mosmol/L), the extracellular binding site for Na+ is often not saturated. With nystatin, an antifungal drug that penetrates the plasma membrane, the intracellular and extracellular osmolarity can be raised to 600 mosmol/L, so extracellular concentrations of Na+ up to 300 mmol/L can be used. Because of this, K_0.5 values can in principle be measured more accurately than with the other methods. We found, however, that even at this high Na+ concentrations the V_max of diabetic patients is often hardly reached.

Participants in this study were four male patients with insulin-dependent diabetes with diabetic nephropathy and four male healthy volunteers. The subjects had fasted overnight before a blood sample was taken.

The efflux media for the erythrocytes loaded with Li+ by using the LiCl or Li2CO3 methods contained 0-150 mmol/L NaCl, 150-0 mmol/L choline chloride, 1 mmol/L MgCl2, 10 mmol/L Tris-3-(N-morpholino)propanesulfonic acid (MOPS) buffer pH 7.4, 10 mmol/L glucose, and 0.1 mmol/L ouabain. Na+ concentrations were 0, 10, 20, 40, 60, 80, 100, 120, and 150 mmol/L. The sum of the concentrations of Na+ and choline was always 150 mmol/L. The efflux media for the erythrocytes loaded with Li+ by using the nystatin method contained 0-300 mmol/L NaCl and 300-0 mmol/L choline chloride; the rest of the medium was the same. Used Na+ concentrations were 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, and 300 mmol/L. Here the sum of the concentration of Na+ and choline was always 300 mmol/L. Li+ concentrations were measured by atomic absorption spectrometry (Model 4100; Perkin-Elmer, Norwalk, CT). The V_max and K_0.5 values were determined or extrapolated with the computer program Graphpad Inplot version 4.0 (Graphpad software, San Diego, CA). A rectangular hyperbola (binding isotherm) was fitted through the data. The following equation was used: V = A*(Na+)/[(B+Na+)/]; A = V_max B = K_0.5.

From each subject a blood sample was taken twice, with a time interval of 1 month. The data were analyzed and the V_max and the K_0.5 for Na+ were determined. R^2 (coefficient of determination), a marker for the fit of the curve to Michaelis-Menten kinetics, was calculated. When the LiCl method was used, 25% of the R^2 values were <0.7, which indicates a poor fit to Michaelis-Menten kinetics. There were no curves including Hill plots that fitted better. The nystatin method generated only one R^2 value <0.7, and with the Li2CO3 method all R^2 values were >0.7. It is clear that the data obtained with the Li2CO3 or nystatin method fit better to Michaelis-Menten kinetics than the data obtained with the LiCl method. This was a reason for us to continue with the methods involving Li2CO3 and nystatin.

Next, the CV of the measuring error of the data obtained with the Li2CO3 or nystatin Li+ loading methods was analyzed (with a paired t-test). At one day the same sample of blood was analyzed twice (Table 1). It was assumed that the difference was negligible between the "month-to-month variation within one sample" and the measuring error. It was further assumed that differences between two methods were statistically independent from each other, both between subjects and between months. For both methods the CV of the measuring error of the K_0.5 for Na+ did not significantly differ from the measuring error of the V_max (nystatin P = 0.21; Li2CO3 P = 0.29). When the two methods are compared, there seems to be no important difference in the CV of the measuring error of the values for V_max or K_0.5. For the V_max the difference between the values obtained with the nystatin and Li2CO3 method was 0.6% (SD = 6.3, P = 0.79) and for the K_0.5 for Na+ the difference was -5.3% (SD = 13.1, P = 0.29).

In addition, the intrasubject variation was examined (Table 1). A blood sample was taken three times, with intervals of 1 month. The samples were analyzed with both the Li2CO3 and nystatin Li+ loading methods. For both methods the intrasubject variation for the K_0.5 for Na+ was large. Table 1 shows that for all methods the V_max is more constant than the K_0.5. For the nystatin, method comparison of the values for the CVs of V_max and K_0.5 for Na+ gives a difference of 22.7% (SD = 19.9, P = 0.015) and for the Li2CO3 method the difference was 12.6% (SD = 15.0, P = 0.05). From Table 1 it seems as if the variation of the V_max values obtained from one individual is smaller when the nystatin method is used, although this trend could not be supported statistically (P = 0.09).

Until recently the Na+/Li+ countertransport activity was measured
as the Li⁺ efflux rate in medium containing 150 mmol/L Na⁺ after subtraction of the efflux rate in Na⁺-free medium. LiCl was used to load erythrocytes. As a discriminatory value, 400 μmol Li⁺/(h*L red blood cells (RBC)) was used. Subjects with a countertransport above this value were described as at risk. The values we found for the mean Na⁺/Li⁺ countertransport at 150 mmol/L Na⁺ for the healthy individuals, when measured with the LiCl loading [mean 225 μmol Li⁺/(h*L RBC)]⁻¹, are below this cutoff value. The values obtained with the Li₂CO₃ method or the nystatin method are higher, although <400 μmol Li⁺/(h*L RBC) [260 and 312 μmol Li⁺/(h*L RBC)]. For the whole group (diabetic and nondiabetic patients) the values for V₁₅₀ are significantly different when two of the three methods are compared. The V₁₅₀ however, has been criticized as a marker because it was reported that at 150 mmol/L Na⁺ the V₃₃₉ was often not reached. Our results confirm this. Measurement of V₃₃₉ and K₉₅ for Na⁺ could give more reliable values. Moreover, as Rutherford et al. [12] already mentioned, any differences in V₁₅₀ observed can be due to differences in either V₃₃₉ or K₉₅. In addition, changes in both the K₉₅ and the V₃₃₉ have been reported [13,14]. If V₃₃₉ and K₉₅ are to be used as risk markers, new cutoff values for abnormal Na⁺/Li⁺ countertransport activity have to be established.

Both the Li₂CO₃ and nystatin loading methods result in higher values for K₉₅ and V₃₃₉ than those obtained with the LiCl method (P <0.001). The reason for this is unknown. One possible explanation could be that loading with Cl⁻ means exchange of HCO₃⁻ for Cl⁻ via the band 3 anion transporter. This could lead to pH changes in the erythrocyte, which could influence Na⁺/Li⁺ countertransport. Elving et al., however, reported that after the washing cycles the intracellular HCO₃⁻ and Cl⁻ concentrations were identical in cells loaded with either Li₂CO₃ or LiCl [16]. Another possibility is that the optimal internal Li⁺ concentration is not reached in all experiments by this method.

Besch et al. [15] already compared the LiCl method with the Li₂CO₃ method. In contrast to the present study, they found no difference in values for V₃₃₉ or K₉₅. But they concluded that the Li₂CO₃ method was to be preferred because this method takes considerably less time. Zerbini et al. [16] compared the LiCl method with the nystatin method and they concluded that at 150 mmol/L Na⁺ the maximum activity is not always reached, and that therefore the nystatin loading method is to be preferred. But none of these studies compared all three methods or studied the measuring error and intrasubject variation of the values obtained.

Hardman and Lant [17] and Wierzbicki [18] raised the question whether nystatin will be removed by washing. The fact that the mean values of our results obtained with both the nystatin method and Li₂CO₃ method do not differ significantly from those obtained with the nystatin method (P > 0.21 and 0.08). The values for V₃₃₉ and K₉₅ for Na⁺ obtained with the LiCl method differed significantly from both the nystatin and Li₂CO₃ method; P <0.001 for the V₃₃₉ and P = 0.016 for K₉₅ for Na⁺ when nystatin and LiCl are compared, P <0.001 for both the V₃₃₉ and the K₉₅ when Li₂CO₃ and LiCl are compared.

Table 1. Measuring error and intrasubject variation of K₉₅ and V₃₃₉ when the nystatin loading or Li₂CO₃ loading method is used.

<table>
<thead>
<tr>
<th>Method</th>
<th>Value</th>
<th>Mean (Median)</th>
<th>SD</th>
<th>(95% confidence Interval)</th>
<th>Measuring error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V₃₃₉</td>
<td>540</td>
<td>68</td>
<td>8.1²</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>K₉₅</td>
<td>74</td>
<td>24</td>
<td>30.8²</td>
<td>10</td>
</tr>
<tr>
<td>Nystatin</td>
<td>V₃₃₉</td>
<td>378</td>
<td>70</td>
<td>16.6</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>V₃₃₉</td>
<td>533</td>
<td>127</td>
<td>18.4²</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>K₉₅</td>
<td>89</td>
<td>32</td>
<td>31.0²</td>
<td>16</td>
</tr>
<tr>
<td>Li₂CO₃</td>
<td>V₃₃₉</td>
<td>339</td>
<td>43</td>
<td>11.7</td>
<td>11</td>
</tr>
</tbody>
</table>

The P-values for the differences between the CVs were: " vs $P = 0.015; " vs $P = 0.21; "$ vs $P = 0.049; " vs $P = 0.09; "$ vs $P = 0.79; "$ vs $P = 0.98; "$ vs $P = 0.29. The mean values for V₃₃₉, K₉₅, and V₃₃₉, and V₃₃₉, were 392, 43, and 311 respectively, when the LiCl method was used. The V₃₃₉ values and the values for K₉₅ for Na⁺ obtained with the Li₂CO₃ method did not differ significantly from those obtained with the nystatin method (P > 0.21 and 0.08). The values for V₃₃₉ and K₉₅ for Na⁺ obtained with the LiCl method differed significantly from both the nystatin and Li₂CO₃ method; P <0.001 for the V₃₃₉ and P = 0.016 for K₉₅ for Na⁺ when nystatin and LiCl are compared, P <0.001 for both the V₃₃₉ and the K₉₅ when Li₂CO₃ and LiCl are compared.
This could be due to changes in binding of Na\(^+\), but may also reflect changes in translocation rate of the transporter. But because of the large fluctuations of the $K_{0.5}$ for Na\(^+\) within one subject over time (intervals of 1 month), it is doubtful whether the $K_{0.5}$ can be used to identify patients at risk for development of diabetic nephropathy.

To conclude, in this study the effect of different Li\(^+\) loading methods (LiCl, Li\(_2\)CO\(_3\), or nystatin) on the reproducibility of the $K_{0.5}$ and $V_{\text{max}}$ values for Na\(^+\)/Li\(^+\) exchange were compared. The LiCl method generates Na\(^+\)/Li\(^+\) exchange activities that often do not apply to Michaelis-Menten kinetics. Our results suggest that both the nystatin method and the Li\(_2\)CO\(_3\) method are preferred over the LiCl method.

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**Antioxidant Activity of the Stilbene Astringin, Newly Extracted from Vitis vinifera Cell Cultures**

To the Editor:

Numerous epidemiological studies in France have shown a strong neg-
ative correlation between moderate red wine consumption and the inci-
dence of cardiovascular disease [1, 2]. Compared with other alcoholic 
beverages, red wine contains a much higher content of phenolic constitu-
ents. Frankel et al. [3] have shown that total phenolic compounds 
extracted from red wine inhibit the oxidation of human low-density li-
poprotein (LDL) in vitro, which may provide an explanation for the “French paradox.” In fact, increasing evidence suggests that oxidized LDL might be responsible for promoting atherogenesis.

Miller and Rice-Evans [4] examined a variety of red wines for total antioxidant activity and, based on the data of Frankel et al. [5] for the composition of wine, suggested that unidentified compounds must con-
tribute to this total.

With the help of Vitis vinifera cell suspension obtained from Gamay 
Teinturier vine-plant, we isolated and characterized stilbenes (cis- and trans-piceid and trans-resveratrol) and anthocyanins (malvidin-3-O-β-
glucoside and peonidin-3-O-β-glucoside) by spectrometric methods. Furthermore, we found evidence of production of astringin (a stilbene), which has never been reported as a constituent of Vitis vinifera and of 
wines [6, 7]. Here, we report our study the antioxidant potency of these compounds isolated from Vitis vinifera cells by measuring the inhibi-
tion of lipid peroxidation induced by Cu\(^{2+}\) (IC\(_{50}\) = concentration at which one-half of the induced peroxidation is inhibited) in fresh human LDL preparation; we determined this by measuring the production of thiobar-
bituric acid-reactive substances [8]. The lack of interference from the 
coloration of anthocyanins is verified by HPLC assays.

The IC\(_{50}\) of trans-resveratrol (Table 1) is of similar magnitude to that