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 Vmax and K0.5 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 intra- 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, K0.5 values can in principle be measured more accurately than with the other methods. We found, however, that even at this high Na+ concentrations the Vmax 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 Vmax and K0.5 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 = Vmax B = K0.5.

From each subject a blood sample was taken twice, with a time interval of 1 month. The data were analyzed and the Vmax and the K0.5 for Na+ were determined. R2 (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 R2 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 R2 value <0.7, and with the Li2CO3 method all R2 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 K0.5 for Na+ did not significantly differ from the measuring error of the Vmax (nystatin P = 0.21; Li2CO3 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 K0.5 for Na+ was large. Table 1 shows that for all methods the Vmax is more constant than the K0.5. For the nystatin method comparison of the values for the CVs of Vmax and K0.5 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 Vmax 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⁺/Li⁺
free medium. LiCl was used to load erythrocytes. As a discriminatory value, 400 μmol Li⁺/(h*L 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)] were below this cutoff value. The values obtained with the Li₂CO₃
method or the nystatin method are used as risk markers, new cutoff values for abnormal Na⁺/Li⁺ counter-
transport activity have to be established.

Both the Li₂CO₃ and nystatin loading methods result in higher values for K₀₅ for Na⁺ 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 erthrocyte, which could influence Na⁺/Li⁺ counter-
transport. Elving et al., however, reported that after 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.

Borch 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 V_max or K₀₅. 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 NaCl 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 Wierzbički [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 and Li₂CO₃ method do not differ indicates that the erythrocyte membranes are not damaged when nystatin is used. The same authors as well as Thomas et al. [19] questioned whether the data obtained with the LiCl method is to be fit to Michaelis-Menten kinetics. We
found that both the data obtained with the Li₂CO₃ and the nystatin loading procedure do fit to Michaelis-
Menten kinetics. On the other hand, the data obtained with the LiCl method showed more variation.

Rutherford et al. reported that changes in K₀₅ for Na⁺ rather than in V_max are the explanation for increased V₁₅₀ Na⁺/Li⁺ countertrans-
port in insulin-dependent diabetes mellitus patients with nephropathy.

Table 1. Measuring error and intrasubject variation of K₀₅ and V_max 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 (95% confidence Interval)</th>
<th>Measuring error</th>
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<tr>
<td></td>
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<td></td>
<td>SD (95% confidence Interval)</td>
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<tr>
<td>V_max</td>
<td>540</td>
<td>68</td>
<td>8.1* (3.4-12.8)</td>
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<td></td>
<td>(457)</td>
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<tr>
<td>K₀₅</td>
<td>74</td>
<td>24</td>
<td>30.8* (15.1-45.1)</td>
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</tr>
<tr>
<td></td>
<td>(76)</td>
<td></td>
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<tr>
<td>Nystatin</td>
<td>V₃₅₀</td>
<td>70</td>
<td>16.6 (8.5-23.4)</td>
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<td></td>
<td>(324)</td>
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<tr>
<td>V_max</td>
<td>533</td>
<td>127</td>
<td>18.4* (7.6-29.2)</td>
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<tr>
<td></td>
<td>(455)</td>
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<tr>
<td>K₀₅</td>
<td>89</td>
<td>32</td>
<td>31.0* (19.4-42.6)</td>
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<td></td>
<td>(76)</td>
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<tr>
<td>Li₂CO₃</td>
<td>V₃₅₀</td>
<td>339</td>
<td>11.7 (4.9-18.5)</td>
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</tr>
<tr>
<td></td>
<td>(292)</td>
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</table>

The P-values for the differences between the CVs were: * vs $P = 0.015$; $P = 0.021$; $P = 0.049$; $P = 0.08$; $P = 0.09$; $P = 0.79$;
* vs $P = 0.98$; $P = 0.09$. The mean values for V_max, K₀₅ for Na⁺, and V₃₅₀ were 392, 43, and 311 respectively, when the LiCl method was used. The V_max
values and the values for K₀₅ 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 V_max and K₀₅ for Na⁺ obtained with the LiCl method differed significantly from both the nystatin and Li₂CO₃ method; $P = 0.001$ for the V_max and $P = 0.016$ for K₀₅ for Na⁺ when nystatin and LiCl are compared, $P < 0.001$ for both the V_max and the K₀₅ when Li₂CO₃ and LiCl are compared.
We are grateful to the Dutch Diabe
tover the LiCl method.

Menten kinetics. Our results suggest
that often do not apply to Michaelis-
ates Na'/Li'1 ' exchange activities
both the nystatin method and
9. Canessa M, Adragna W, Solomon HS, Connolly
fect of different Li"1 "  loading methods
reproducibility of the Ka5 and
vals of 1 month), it is doubtful
ify patients at risk for development
within one subject over time (inter-
changes in translocation rate of the
[14].
8. Canessa M. Kinetic properties of Na/H ex-
6. Elving LD, Wetzels JFM, De Pont JJHHM, Ber-
5. Crompton CH, Balfe JW, Balfe JA, Chatzilias A,
4. Manglli R, Bending JJ, Scott G, Li LK, Gupta A,
3. Fujita J, Tsuda K, Seno M, Obayashi H, Fukui I,
2. Rutherford PA, Thomas TH, Carr SJ, Taylor R,
1. Fujita J. Teinturier vine-plant, we isolated
unidentified compounds must con-
tribute to this total.

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1) is of similar magnitude to that
concentration at which

tion of lipid peroxidation induced by
ation of phenolic constitu-
provide an explanation for the
beverages, red wine contains a much
beverages, red wine contains a much

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-
itive correlation between moderate red wine consumption and the in-
cease 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 ex-
tracted from red wine inhibit the oxidation of human low-density li-
oprotein (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 atherosclerosis.

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-

ic acid 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 Cu21 (IC50 = 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 IC50 of trans-resveratrol (Table
1) is of similar magnitude to that

References
1. Fujita J, Tsuda K, Seno M, Obayashi H, Fukui I, Seno Y. Erythrocyte sodium-lithium coun-
tertransport activity as a marker of predisposition to hypertension and diabetic nephropathy in NONIDM. Diabetes Care 1994:17:977-82.
2. Rutherford PA, Thomas TH, Carr SJ, Taylor R, Wilkinson R. Changes in erythrocyte sodium-
3. Krolewski AS, Canessa M, Warram JH, Laffel LMB, Christlieb AR, Knowler WC, Rand LJ. Pre-
disposition to essential hypertension and sus-
140-5.
4. Anglij RJ, Bending JJ, Scott G, Li LK, Gupta A, Viberti GC. Increased sodium-lithium coun-
5. Crompton CH, Balfe JW, Balfe JA, Chatzilias A, Daneman D. Sodium-lithium transport in ado-
6. Elving LD, Wetzels JFM, De Pont JJHHM, Ber-
den JHM. Increased erythrocyte sodium-lithium countertransport: a useful marker for diabetic
countertransport activity are not inherited in diabetic nephropathy. Diabetologia 1990:33:
619-24.
8. Canessa M. Kinetic properties of Na/H ex-
change and Li/Na, Na/Na, and Na/Li ex-
changes of human red cells. Methods Enzymol
9. Canessa M, Adragna W, Solomon HS, Connolly
TM, Tosteson DC. Increased sodium-lithium countertransport in red cells of patients with
302:772-6.
10. Rutherford PA, Thomas TH, Carr SJ, Taylor R,
Wilkinson R. Kinetics of sodium-lithium coun-
tertransport activity in patients with uncompli-
11. Canessa M, Zerbini G, Laffel LMB. Sodium activa-
tion kinetics of red blood cell Na'/Li'1 ' coun-
12. Rutherford PA, Thomas TH, Wilkinson R. Eryth-
rocyte sodium-lithium countertransport: cli-
ically useful, pathophysiological instructive or just phenomenology? Clin Sci 1992:82:341-
52.
13. Canessa M. Erythrocyte sodium-lithium coun-
tertransport: another link between essential hypertension and diabetes. Curr Opin Nephrol
14. Rutherford PA, Thomas TH, Wilkinson R. In-
creased erythrocyte sodium-lithium counter-
transport activity in essential hypertension is
15. Besch W, Schlager D, Brahm J, Kohnert KD.
Validation of red cell sodium-lithium counter-
transport measurement—Influence of different loading conditions. Eur J Clin Chem Clin Bio-
dium-lithium countertransport has low affinity for sodium in hyperinsulinemic hypertensive
17. Hardman T, Lant A. Measurement of sodium-
27:315.
19. Thomas TH, West IC, Rutherford PA. Measure-
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