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### Comparison of Methods for Measurement of $\text{Na}^+/\text{Li}^+$ 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  $\text{Na}^+/\text{Li}^+$  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  $\text{Li}^+$  were compared and tested for their measuring error and intrasubject variation of  $V_{\text{max}}$  and  $K_{0.5}$  for  $\text{Na}^+$ .

The "classic"  $\text{LiCl}$  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  $\text{Li}_2\text{CO}_3$  loading as described by Elving et al. [6] has the advantage that it takes only 30 min to load the cells with  $\text{Li}^+$ . The  $\text{Li}^+$  enters the cell via the  $\text{HCO}_3^-/\text{Cl}^-$  exchanger as a  $\text{LiCO}_3^-$  ion in exchange for a  $\text{Cl}^-$  ion, which explains the fast  $\text{Li}^+$  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  $\text{Na}^+$  (the highest concentration that can be used at an osmolarity of 300 mosmol/L), the extracellular binding site for  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  concentrations the  $V_{\text{max}}$  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  $\text{Li}^+$  by using the  $\text{LiCl}$  or  $\text{Li}_2\text{CO}_3$  methods contained 0-150 mmol/L  $\text{NaCl}$ , 150-0 mmol/L choline chloride, 1 mmol/L  $\text{MgCl}_2$ , 10 mmol/L Tris-3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer pH 7.4, 10 mmol/L glucose, and 0.1 mmol/L ouabain.  $\text{Na}^+$  concentrations were 0, 10, 20, 40, 60, 80, 100, 120, and 150 mmol/L. The sum of the concentrations of  $\text{Na}^+$  and choline was always 150 mmol/L. The efflux media for the erythrocytes loaded with  $\text{Li}^+$  by using the nystatin method contained 0-300 mmol/L  $\text{NaCl}$  and 300-0 mmol/L choline chloride; the rest of the medium was the same. Used  $\text{Na}^+$  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  $\text{Na}^+$  and choline was always 300 mmol/L.  $\text{Li}^+$  concentrations were measured by atomic absorption spectrometry (Model 4100; Perkin-Elmer, Norwalk, CT). The  $V_{\text{max}}$  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:  $V = A*[\text{Na}^+]/(B+[\text{Na}^+])$ ;  $A = V_{\text{max}}$ ,  $B = K_{0.5}$ .

From each subject a blood sample was taken twice, with a time interval of 1 month. The data were analyzed and the  $V_{\text{max}}$  and the  $K_{0.5}$  for  $\text{Na}^+$  were determined.  $R^2$  (coefficient of determination), a marker for the fit of the curve to Michaelis-Menten kinetics, was calculated. When the  $\text{LiCl}$  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  $\text{Li}_2\text{CO}_3$  method all  $R^2$  values were  $>0.7$ . It is clear that the data obtained with the

$\text{Li}_2\text{CO}_3$  or nystatin method fit better to Michaelis-Menten kinetics than the data obtained with the  $\text{LiCl}$  method. This was a reason for us to continue with the methods involving  $\text{Li}_2\text{CO}_3$  and nystatin.

Next, the CV of the measuring error of the data obtained with the  $\text{Li}_2\text{CO}_3$  or nystatin  $\text{Li}^+$  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  $\text{Na}^+$  did not significantly differ from the measuring error of the  $V_{\text{max}}$  (nystatin  $P = 0.21$ ;  $\text{Li}_2\text{CO}_3$   $P = 0.29$ ). When the two methods are compared, there seems to be no important difference in the CV of the measuring error of the values for  $V_{\text{max}}$  or  $K_{0.5}$ . For the  $V_{\text{max}}$  the difference between the values obtained with the nystatin and  $\text{Li}_2\text{CO}_3$  method was 0.6% (SD = 6.3,  $P = 0.79$ ) and for the  $K_{0.5}$  for  $\text{Na}^+$  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  $\text{Li}_2\text{CO}_3$  and nystatin  $\text{Li}^+$  loading methods. For both methods the intrasubject variation for the  $K_{0.5}$  for  $\text{Na}^+$  was large. Table 1 shows that for all methods the  $V_{\text{max}}$  is more constant than the  $K_{0.5}$ . For the nystatin, method comparison of the values for the CVs of  $V_{\text{max}}$  and  $K_{0.5}$  for  $\text{Na}^+$  gives a difference of 22.7% (SD = 19.9,  $P = 0.015$ ) and for the  $\text{Li}_2\text{CO}_3$  method the difference was 12.6% (SD = 15.0,  $P = 0.05$ ). From Table 1 it seems as if the variation of the  $V_{\text{max}}$  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  $\text{Na}^+/\text{Li}^+$  countertransport activity was measured

**Table 1. Measuring error and intrasubject variation of  $K_{0.5}$  and  $V_{max}$  when the nystatin loading or  $Li_2CO_3$  loading method is used.**

Method	Value	Mean (Median)	Intrasubject variation		Measuring error	
			SD	(95% confidence interval)	SD	(95% confidence interval)
Nystatin	$V_{max}$	540 (457)	68	8.1 <sup>a</sup> (3.4–12.8)	57	7.7 <sup>h</sup> (3.9–11.5)
	$K_{0.5}$	74 (76)	24	30.8 <sup>c</sup> (15.1–45.1)	10	9.1 <sup>d</sup> (4.0–14.2)
	$V_{150}$	378 (324)	70	16.6 (8.5–23.4)	27	7.1 <sup>b</sup> (2.3–11.9)
	$V_{max}$	533 (455)	127	18.4 <sup>e</sup> (7.6–29.2)	38	7.1 <sup>f</sup> (3.7–10.5)
	$K_{0.5}$	89 (76)	32	31.0 <sup>g</sup> (19.4–42.6)	16	14.3 <sup>h</sup> (6.8–21.8)
$Li_2CO_3$	$V_{150}$	339 (292)	43	11.7 (4.9–18.5)	11	4.4 (1.6–7.2)

The *P*-values for the differences between the CVs were: <sup>a</sup> vs <sup>c</sup> *P* = 0.015; <sup>b</sup> vs <sup>d</sup> *P* = 0.21; <sup>e</sup> vs <sup>g</sup> *P* = 0.049; <sup>f</sup> vs <sup>h</sup> *P* = 0.09; <sup>a</sup> vs <sup>c</sup> *P* = 0.09; <sup>b</sup> vs <sup>f</sup> *P* = 0.79; <sup>c</sup> vs <sup>g</sup> *P* = 0.98; <sup>d</sup> vs <sup>h</sup> *P* = 0.29. The mean values for  $V_{max}$ ,  $K_{0.5}$  for  $Na^+$ , and  $V_{150}$  were 392, 43, and 311 respectively, when the LiCl method was used. The  $V_{max}$  values and the values for  $K_{0.5}$  for  $Na^+$  obtained with the  $Li_2CO_3$  method did not differ significantly from those obtained with the nystatin method (*P* = 0.21 and 0.08). The values for  $V_{max}$  and  $K_{0.5}$  for  $Na^+$  obtained with the LiCl method differed significantly from both the nystatin and  $Li_2CO_3$  method; *P* < 0.001 for the  $V_{max}$  and *P* = 0.016 for  $K_{0.5}$  for  $Na^+$  when nystatin and LiCl are compared, *P* < 0.001 for both the  $V_{max}$  and the  $K_{0.5}$  when  $Li_2CO_3$  and LiCl are compared.

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  $\mu\text{mol } Li^+ / [h^*L \text{ red blood cells (RBC)}]$  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  $\mu\text{mol } Li^{++} / (h^*L \text{ RBC})^{-1}$ ], are below this cutoff value. The values obtained with the  $Li_2CO_3$  method or the nystatin method are higher, although <400  $\mu\text{mol } Li^+ / (h^*L \text{ RBC})$  [260 and 312  $\mu\text{mol } Li^+ / (h^*L \text{ RBC})$ ]. For the whole group (diabetic and nondiabetic patients) the values for  $V_{150}$  are significantly different when two of the three methods are compared. The  $V_{150}$ , however, has been criticized as a marker because it was reported that at 150 mmol/L  $Na^+$  the  $V_{max}$  was often not reached. Our results confirm this. Measurement of  $V_{max}$  and  $K_{0.5}$  for  $Na^+$  could give more reliable values. Moreover, as Rutherford et al. [12] already mentioned, any differences in  $V_{150}$  observed can be due to differences in either  $V_{max}$  or  $K_{0.5}$ . In addition, changes in both the  $K_{0.5}$

and the  $V_{max}$  have been reported [13, 14]. If  $V_{max}$  and  $K_{0.5}$  are to be used as risk markers, new cutoff values for abnormal  $Na^+ / Li^+$  countertransport activity have to be established.

Both the  $Li_2CO_3$  and nystatin loading methods result in higher values for  $K_{0.5}$  and  $V_{max}$  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  $HCO_3^-$  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  $HCO_3^-$  and  $Cl^-$  concentrations were identical in cells loaded with either  $Li_2CO_3$  or LiCl [6]. 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  $Li_2CO_3$  method. In contrast to the present study, they found no difference in values for  $V_{max}$  or  $K_{0.5}$ . But they concluded that the  $Li_2CO_3$  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 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  $Li_2CO_3$  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 by Zerbini et al. [16] fit to Michaelis–Menten kinetics. We found that both the data obtained with the  $Li_2CO_3$  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  $K_{0.5}$  for  $Na^+$  rather than in  $V_{max}$  are the explanation for increased  $V_{150}$   $Na^+ / Li^+$  countertransport in insulin-dependent diabetes mellitus patients with nephropathy

[14]. This could be due to changes in binding of  $\text{Na}^+$ , but may also reflect changes in translocation rate of the transporter. But because of the large fluctuations of the  $K_{0.5}$  for  $\text{Na}^+$  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  $\text{Li}^+$  loading methods ( $\text{LiCl}$ ,  $\text{Li}_2\text{CO}_3$ , or nystatin) on the reproducibility of the  $K_{0.5}$  and  $V_{\text{max}}$  values for  $\text{Na}^+/\text{Li}^+$  exchange were compared. The  $\text{LiCl}$  method generates  $\text{Na}^+/\text{Li}^+$  exchange activities that often do not apply to Michaelis-Menten kinetics. Our results suggest that both the nystatin method and the  $\text{Li}_2\text{CO}_3$  method are preferred over the  $\text{LiCl}$  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 negative correlation between moderate red wine consumption and the incidence of cardiovascular disease [1, 2]. Compared with other alcoholic beverages, red wine contains a much higher content of phenolic constituents. Frankel et al. [3] have shown that total phenolic compounds extracted from red wine inhibit the oxidation of human low-density lipoprotein (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 contribute 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*- $\beta$ -glucoside and peonidin-3-*O*- $\beta$ -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 inhibition of lipid peroxidation induced by  $\text{Cu}^{2+}$  ( $\text{IC}_{50}$  = concentration at which one-half of the induced peroxidation is inhibited) in fresh human LDL preparation; we determined this by measuring the production of thiobarbituric acid-reactive substances [8]. The lack of interference from the coloration of anthocyanins is verified by HPLC assays.

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