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 Li_2CO_3 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 HCO_3^-/Cl^- exchanger as a $LiCO_3^-$ 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, $K_{0.5}$ 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 Li_2CO_3 methods contained 0–150 mmol/L NaCl, 150–0 mmol/L choline chloride, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer pH 7.4, 10 mmol/L glucose, and 0.1 mmol/L ouabain. 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 Li_2CO_3 method all R^2 values were >0.7. It is clear that the data obtained with the Li_2CO_3 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 Li_2CO_3 and nystatin.

Next, the CV of the measuring error of the data obtained with the Li_2CO_3 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 $P = 0.21$; Li_2CO_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_{max} or $K_{0.5}$. For the V_{max} the difference between the values obtained with the nystatin and Li_2CO_3 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 Li_2CO_3 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 Li_2CO_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_{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

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

The *P*-values for the differences between the CVs were: [a] vs [c] $P = 0.015$; [b] vs [d] $P = 0.21$; [e] vs [g] $P = 0.049$; [f] vs [h] $P = 0.09$; [a] vs [e] $P = 0.09$; [b] vs [f] $P = 0.79$; [c] vs [g] $P = 0.98$; [d] vs [h] $P = 0.29$. 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 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 Li_2CO_3 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 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 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_2CO_3, or nystatin) on the reproducibility of the $K_{0.5}$ and V_{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_2CO_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 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*-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 inhibition 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 thiobarbituric 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

Table 1. IC_{50} values obtained with phenolic substances.

	IC_{50}, mean ± SD, μmol/L[a]
Stilbenes	
Astringin	3.3 ± 0.9
Astringinin	1.9 ± 0.2
cis-Resveratrol	19.0 ± 2.0
trans-Resveratrol	2.6 ± 0.7
cis-Piceid	16.6 ± 1.5
trans-Piceid	19.3 ± 2.5
Anthocyanins	
Malvidin-3-*O*-β-glucoside	3.8 ± 0.8
Peonidin-3-*O*-β-glucoside	3.2 ± 0.7

[a] IC_{50} = 50% inhibition concentration (obtained from inhibition curve). n = 3 for each compound. (Trolox was used as reference: IC_{50} 5.0 ± 0.4 μmol/L.)

cis-Resveratrol and astringinin are obtained by enzymatic hydrolysis of *cis*-piceid and astringin, respectively.

previously reported *[9]*. The activity of the *cis*-isomer is 7 times weaker, probably because of its nonplanar conformation.

Astringin (3-OH-*trans*-piceid) is 6 times more potent than *trans*-piceid, which confirms the major importance of catechol structure in antioxidant effect, as described for flavonoids. On the other hand, the conjugation of an OH group with a sugar decreased the antioxidant potential, according to the IC_{50} values of *trans*-resveratrol vs *trans*-piceid and astringinin vs astringin. The antioxidant activities of anthocyanins are similar to those of *trans*-resveratrol and astringin.

These data support previous work *[3]* suggesting that an array of phenolic substances in red wine may, because of their antioxidant properties, exert a protective effect against atherogenesis during a long period of moderate intake of red wine. We know little about the absorption, in vivo metabolism, and pharmacokinetics of phenols in wine. Consequently, we plan to produce ^{13}C-labeled versions of these compounds to study both their absorption in human volunteers and their plasma antioxidant activity.

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Microalbumin and Freezing

To the Editor:

"Microalbumin" (MA; albumin present in very small quantities) has assumed an increased role in the early diagnosis, assessment, and treatment of diabetic kidney disease *[1–5]*. The measurement is reliably carried out by several methods, but the practical issue of MA stability during urine storage for extended periods remains controversial.

MA in urine stored at 4 °C is stable for at least 1 week and as long as 8 weeks *[6, 7]*. Freezing the urine, however, has been reported by some to cause falsely low values *[8, 9]*; others have reported no decrease in urines stored frozen for 4–6 months *[6, 10]*, albeit with sporadic decreases in some urines *[6]*. This is an important issue in cases when transport to a reference laboratory can take several days and storage at 4 °C is impractical. Townsend et al. have reported that the simple act of mixing the urine after thawing can prevent falsely low values for MA *[11]*.

We measured MA by immunonephelometry with an Array 360 analyzer (Beckman Instruments, Brea, CA) calibrated once every 2 weeks as recommended by the manufacturer; no drift was detected over that period. Repeated assay of Beckman Level I (mean albumin 10.3 mg/L) control gave an interassay CV of 3.2% (n = 68). Fresh, unfrozen urines were centrifuged for 10 min at 3000*g* to remove debris. Frozen urines were stored uncentrifuged at −20 °C. After thawing, they were shaken (3–4 hand inversions) or not, centrifuged as above, and then analyzed. All determinations were carried out at room temperature.

Preliminary experiments showed that one or two freeze–thaw cycles resulted in the loss of MA concentration in most urine samples if they were not shaken after thawing. For another 23 samples, mixing after either a 1- or 3-week freeze resulted in all retaining the MA concentrations they had before freezing.

To confirm these results, we divided each of a subsequent 39 samples into three aliquots: The first aliquot from each sample (group 1) was used to measure MA in unfrozen urines; those in group 2 were frozen for a week, thawed, not mixed before centrifugation, and analyzed for MA; and those in group 3 were frozen for a week as in group 2, but were mixed before centrifuging and measuring MA. Most of the MA values in group 2 were falsely low: 77% (30 of 39) were at least 30% lower than the MA measured in the corresponding unfrozen aliquots—even after only one freeze–thaw cycle. For these 30 urines, the mean MA concentration was 7.3 mg/L, vs 11.8 mg/L for group 1, a 38% difference. Of the 39 samples in group 3, the MA concentration in 37 was the same as for the previously unfrozen aliquots (within the imprecision of the method, 3.2%); in the other two, the MA concentration decreased by ~50%: from 13.6 to 7.2 mg/L (patient 1) and from 11.3 to 6.2 mg/L (patient 2).

We rechecked in triplicate the original unfrozen urine from these two patients and found no difference from the original MA values reported for group 1. Similarly, reassay of the two samples in group 3 confirmed the decreased MA. Resuspending the latter samples by vigorous vortex-type mixing, recentrifuging, and reassaying again showed the same decreases in MA. We also resuspended the pellets from both samples in 1 mL of urine and measured these directly for MA. The MA concentration was not significantly higher in these resuspended specimens than in the original supernatants. We were thus unable to account for the "missing" MA.

Study of subsequent samples from these two patients along with three controls gave the following results. Although the MA in frozen urine from patient 1 did not return to the unfrozen sample values even with mixing (MA was still decreasedby 50%, as before), the MA results for patient 2, as well as for all of the controls, were, on mixing, the same as in the corresponding previously unfrozen samples (within experimental error). Adequate mixing seems essential to retain MA concentration after freezing and thawing. However, an unpredictable but permanent loss can occur in a few samples, for which we postulate that the antigen is distorted in such a way that the antibody may not detect the entire complement of MA present.

We conclude that in most cases (but not all) urine can be safely frozen for at least 3 weeks without alteration in MA, provided that, after thawing, the sample is well mixed (3–4 hand inversions) before centrifugation. Adequate mixing, or lack of it, may explain the reported discrepancies. As previously noted *[6]*, MA is truly decreased in a small proportion of frozen urines, and restoration to the MA concentration in the unfrozen sample may not be possible, even with mixing. We suggest attaching a note to the MA result for a previously frozen specimen indicating that, where the clinical situation warrants, the result should be confirmed with an MA measurement on a fresh, unfrozen specimen.

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Handling of Blood Samples for Determination of Thalidomide

To the Editor:

There is a growing interest in using thalidomide to treat various immunological diseases. Clinical trials or use may include monitoring its plasma or blood concentrations. Thalidomide degrades in aqueous media, the rate of hydrolysis depending on pH and temperature. Thus, the proper handling of blood or plasma samples is crucial. Having developed HPLC methods for determining thalidomide in blood *[1, 2]*, we wish to comment on three recently published protocols for sample handling.

Boughton et al. *[3]* collected blood in heparin tubes and centrifuged within 15 min. Aliquots of plasma were then transferred to tubes containing twice the volume of 1 mol/L HCl. With this treatment, thalidomide was stable for several weeks even at room temperature. Lyon et al. *[4]* proposed immersing blood samples in an ice-slush filled cup to

take to a clinical laboratory for centrifugation. The plasma aliquots are then transported and stored at −25 °C until analysis. With this procedure, the fraction of thalidomide remaining after 30 days was calculated to 0.90. This method *[4]* was adapted from an HPLC assay for thalidomide *[1]* in blood developed in our laboratory; in our method, however, an equal volume of citrate buffer is immediately added to the blood samples, which are then frozen. Huupponen and Pyykkö *[5]*, finally, recommend that blood samples must be handled refrigerated and the plasma separated promptly. They did not address the issue of degradation of thalidomide later during storage of the samples.

Lyon et al. *[4]* chose not to use our protocol. The reasons were stated to be potential inaccuracy because of variable ratios of liquid buffer:blood volume when collection tubes are partially filled and variation in thalidomide extraction because of buffer-induced hemolysis. In addition they concluded that rapid sample cooling alone would sufficiently preserve the thalidomide and that acidification would not significantly improve the stability of samples stored at low temperature.

We respond as follows: The correct ratio of buffer:blood volume does not depend on filling the collection tubes; rather, it is assured by pipetting an aliquot of blood into an extraction tube containing a known amount of buffer. The person in charge of sampling thus needs some laboratory skill; however, the procedure proposed by Lyon et al. requires having a clinical laboratory in the vicinity, so in neither case can sampling be performed without access to skilled personnel. As for the risk of variable degrees of hemolysis, all blood:buffer samples are completely hemolyzed by freezing and thawing, and reproducible extraction yields have been documented *[1, 2]*.

Moreover, the implications of monitoring whole-blood concentrations of thalidomide must be discussed briefly. Thalidomide is not extensively distributed to blood cells, the erythrocyte:plasma concentration ratio being ~0.8 in normal human blood (unpublished). Over the hematocrit range 0.25–0.45, the resulting blood:plasma concentration ratio would theoretically vary from 0.95 to 0.90, and the error in using a mean (experimentally observed) conversion factor of 0.92 would be negligible. Also, the binding of thalidomide to plasma proteins (albumin) is quite low, with a mean value for the enantiomers of 60% (unpublished). Distribution to blood cells and plasma protein binding should therefore not be important determinants for the diffusion of thalidomide to its sites of action. We thus question whether monitoring free (or even total) plasma concentrations instead of whole-blood concentrations would improve correlations to pharmacological effects sufficiently to warrant the necessary extra work-up (with its associated stability problems), except possibly in patients with very abnormal blood chemistry because of renal or hepatic disease.

Boughton et al. *[3]* confirm the observation that acidification of thalidomide samples improves the stability. However, by waiting as much as 15 min before acidification, as much as 4% of the thalidomide could be lost, assuming a degradation half-life of 4 h at 37 °C *[2]*. The reason why acidification as reported by Lyon et al. *[4]* did not improve the stability of thalidomide in plasma samples was probably insufficient lowering of pH (to only 6.0). As a result, Lyon et al. acknowledged they had a serious problem with degradation of quality-control materials. In contrast, when the pH of the blood:buffer mixture was 5.1 *[1]*, we could find no degradation of thalidomide (1 mg/L) in blood:citrate buffer after 75 days at −25 °C (unpublished).

If one wishes to measure the concentrations of the separate enantiomers of thalidomide, then the three procedures described *[3-5]* would probably all be unacceptable, because enantiomeric inversion is twice as fast as hydrolysis in blood *[2]* as well as in plasma (unpublished). Concomitant with the loss of total thalidomide, the change affecting the enantiomeric ratio would be even greater. In contrast, there was no detectable racemization of thalidomide enantiomers in blood:buffer mixtures stored for 100 days at −25 °C *[2]*.

Because we have received inquiries about the handling of samples for clinical trials, and because of the problems with the above methods *[3–5]*, we describe our sample handling procedure in detail here.

1) Add 2.00 mL of 25 mmol/L citrate buffer (pH 1.5) to 10-mL glass-stoppered extraction tubes.

2) Collect blood in anticoagulated (with heparin) evacuated tubes.

3) Without delay, transfer 2.00 mL of blood to each extraction tube, and mix immediately.

4) As soon as possible, freeze and store at −25 °C.

5) Analyze all samples within 75 days.

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Two of the authors referred to respond:

To the Editor:

Eriksson and Björkman have highlighted their recommended method for blood acidification and storage

before thalidomide determination. The recent reports they cite used two strategies to minimize specimen degradation: acidification and rapid cooling, either alone or in combination. All authors agree that special handling is required.

Contrary to the Eriksson et al. study with rat blood *[1]*, our previous report *[2]* did not recommend dilution of whole-blood specimens with acidic buffer for human clinical trials for several reasons:

1) Our study addressed the assay of plasma thalidomide. In our experience, mixing blood and acidic buffers promoted hemolysis, which increased the variability in thalidomide recovery from plasma. Our study demonstrated that acidification of plasma to pH 6.0 did not stabilize thalidomide at −25 °C; consequently, we advocated that frozen specimens be analyzed within 1 month to minimize degradation.

2) In multicenter clinical trials, specimen collection frequently occurs in an area away from the laboratory, resulting in delays before specimen centrifugation or handling. To immediately reduce the degradation of thalidomide, we advocate rapidly cooling specimens in ice-slush before transporting the blood specimen to the laboratory for centrifugation and frozen storage of plasma *[2]*. We still maintain that specimen cooling is the technically simplest method of reducing the rate of thalidomide degradation for a short time to allow transportation and centrifugation.

If a clinical trial requires the measurement of whole-blood thalidomide, immediate acidification of whole blood could be accomplished by collecting blood into evacuated test tubes already containing a volume of acidic buffer. Partial filling of the test tubes in this approach would lead to variability in the blood:buffer ratio, a source of preanalytical variation. Eriksson and Björkman suggest mixing equal volumes of blood and buffer in the laboratory. This acidification method would be accurate and precise but would necessitate immediate processing to avoid the loss of as much as 4% of the thalidomide within 15 min, as they have noted.

3) Acidification to pH <6.0 may offer long-term aqueous thalidomide stability. Clearly, our study *[2]* and that of Eriksson et al. *[1]* demonstrate the instability of thalidomide in plasma at −25 °C at pH 6.0 or pH 7.4, respectively. Boughton et al. demonstrate that acidification improves short-term stability *[3]*. Contrary to earlier reports suggesting that aqueous thalidomide is stable at pH 6.0 *[4, 5]*, we agree with Eriksson and Björkman's suggestion that a pH <6.0 may be required to confer stability.

Eriksson and Björkman's statement above that whole-blood thalidomide specimens at pH 5.1 and −25 °C have long-term stability is important. In our opinion, it suggests that a combination of rapid specimen cooling and prompt acidification to pH 5.1 (or less) and frozen storage of the blood or plasma could provide for both short-term and long-term stability and allow for longer intervals between specimen collection and thalidomide determination in clinical trials.

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Nonisotopic Method for Precise Detection of $(CAG)_n$ Repeats

To the Editor:

Muglia et al. *[1]* recently reported a sensitive method for silver nitrate staining of PCR products separated by polyacrylamide gel electrophoresis for the diagnosis of Huntington disease. We use silver staining *[2]* of PCR products to detect expanded CAG trinucleotide repeats in other diseases as well, specifically, spinal bulbar muscular atrophy (expanded repeat length range 40–62), spinal cerebellar ataxia type I (40–82), and dentatorubral-pallidoluysian atrophy (49–75). The primers and PCR reaction mixtures described by Watkins et al. *[3]* can be used with the following thermal conditions for each gene amplification: 2 min of denaturation at 96 °C; followed by 30 cycles of 96 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min; and finally 10 min at 72 °C for final extension. For more-accurate sizing of PCR products on the gel, we use a 50-bp ladder from Pharmacia (Uppsala, Sweden; cat. no. 27–4005). In view of the need for precise diagnostics procedures for the dynamic trinucleotide repeat expansions *[4]*, we recommend using these easy methods.

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Undisclosed Radioactivity in Specimens: How Much of a Problem?

To the Editor:

Our institution, a clinical reference laboratory, receives specimens from a wide geographic location. In September 1996, a waste-disposal contractor alerted us to the presence of residual radioactivity in a 30-gallon (~135-L) drum of what was supposed to be nonradioactive laboratory waste. Because clinical laboratories are regulated regarding the receipt, use, and disposal of radioactive material, we sought the source. Investigation revealed that the radiation originated from a urine specimen container, specifically from a specimen sent for catecholamine testing. During an evaluation for a possible pheochromocytoma, the patient involved had undergone nuclear imaging with radiolabeled *m*-iodobenzyl guanidine (MIBG) before urine collection for biochemical studies (MIBG is often used in nuclear medicine to evaluate suspected pheochromocytomas *[1]*). The isotope involved was ^{131}I, which has an 8-day half-life. In spite of the short half-life, residual radioactivity was detected >30 days after the isotope's arrival in the laboratory.

After considering the implications of this case, we began to screen for radioactivity specimens received for catecholamine analysis, because this group included patients who were likely to be evaluated with nuclear imaging procedures. Initial screening was performed with a hand-held radiation monitor (TBM-6SP; Technical Associates, Canoga Park, CA), and those specimens identified as radioactive were quantified with a gamma counter (Genesys 6000; Laboratory Technologies, Roselle, IL). Between October and January, ~1% of the specimens screened (8 of 928) were found to be radioactive. These specimens had been received from multiple locations and contained between 0.2 and 1160 μCi/L. Total radioactivity in a single 24-h collection was as much as 0.4 mCi. The health risks to laboratory personnel from inadvertent exposure to the low amounts of radiation described here are minimal. However, associated regulatory and licensing issues are of considerable concern.

Low-level radioactive waste issues are discussed in references such as Hill *[2]*. Nuclear Regulatory Commission (NRC) regulation 10 CFR 20.2003(b) states that excreta from individuals undergoing medical diagnosis or therapy with radioactive materials are exempt from regulation if discharged directly into the sanitary sewer. However, the subject of radioactive specimens forwarded to the clinical laboratory is not commonly addressed in the literature, particularly specimens containing undisclosed radiation and shipped to distant laboratories. Regulations concerning laboratories vary widely by state and by type of licensure. Many states are licensed directly through the NRC. In our case, the laboratory has a "radioactive material license" from the State of Utah, which allows the presence of several radionuclides on site, including ^{131}I, up to a total of 3 mCi. Licenses do not necessarily include ^{131}I, and each laboratory experiencing problems similar to that described here needs to determine what is allowed under its specific licensing regulations. Analysis of low-level radioactive specimens is permissible as long as the amounts are in compliance with licensing regulations. Liquid specimens containing low-level radioactivity can be discarded into the sewer, but the containers should be considered solid radioactive waste and treated accordingly (10 CFR 20.2003). Decay in storage is an authorized method for radioactive waste disposal.

A separate issue involves Department of Transportation (DOT) regulations concerning shipment of radioactive materials. If patients' specimens are included under DOT regulations concerning shipment of "limited quantities" of radioactive materials (specifically 49 CFR 173.421 and related paragraphs), then certain conditions must be met, including appropriate packaging and minimal labeling requirements *[3]*. Above specified radiation values, more-stringent packaging and labeling requirements apply.

A number of questions remain, although, clearly, undisclosed radiation has the potential to be a significant problem for the clinical laboratory. When a radioactive specimen is discovered, the laboratory needs to verify that it is licensed to have radioactive material of that nature on-site. Clinical laboratories should be aware that specimens are shipped from patients who have undergone nuclear medicine procedures; these specimens may contain undisclosed low-level radiation, which may cause problems with waste disposal and related regulatory policies.

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Rapid Qualitative TSH Test to Screen for Primary Hypothyroidism

To the Editor:

We evaluated a qualitative, solid-phase two-site immunochromato-

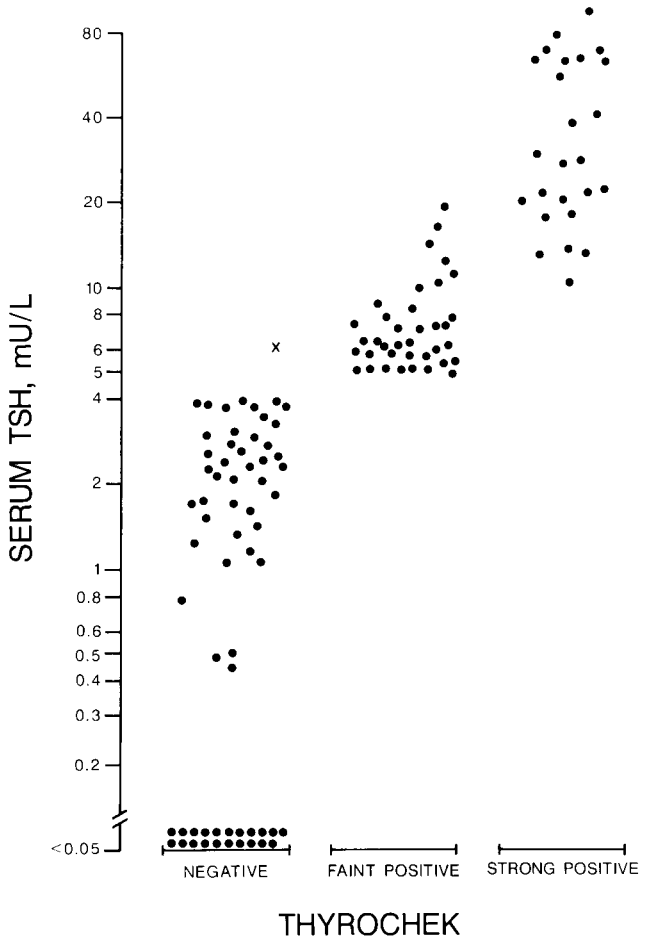

Fig. 1. ThyroChek result compared with serum TSH by IMx measurement.

graphic assay (ThyroChek; Franklin Diagnostics, Cedar Knolls, NJ) for identifying serum samples that contain thyrotropin (TSH) in a concentration >5 mU/L. The test is intended for use as a screening method for primary hypothyroidism. Reagents were provided to us by the manufacturer. The test is performed by adding 4 drops of serum (~0.1 mL) to the well of the cassette. The serum is adsorbed onto the nitrocellulose membrane, exposed to the mobile-phase antibody to intact TSH that is labeled with colloidal gold, and then passes through the site at which capture antibodies (anti-TSHβ) are attached. The presence of TSH >5 mU/L creates a pink line that is read at 10 min after adding the sample.

We evaluated the test by using serum samples that had been assayed for TSH previously by the Abbott IMx Ultrasensitive hTSH II method. The samples were obtained with the patients' approval according to the ethical standards of our institution and had been stored frozen for up to 6 months. Our normal range for this assay is 0.4–4.0 mU/L; the within-assay and between-assay CVs are <8% *[1]*. The samples were divided into four categories: suppressed, TSH <0.05 mU/L, n = 23; normal, TSH 0.4–4.0 mU/L, n = 41; subclinical hypothyroidism, TSH 5–10 mU/L, n = 30; and hypothyroidism, TSH >10 mU/L, n = 31. The samples were randomized, tested in groups of 5 to 10 samples, and the reader of the ThyroChek test was blinded to the clinical status and immunoassay results. The ThyroChek results for each category were (positive/negative): suppressed 0/23, normal 0/41, subclinical hypothyroidism 29/1, and hypothyroidism 31/0. On the basis of these data, the sensitivity of the assay is 98% (60/61) and the specificity is 100% (64/64). Several samples of each category tested repeatedly (up to 4 times) gave the same result.

Samples with TSH concentrations >20 mU/L gave very intense lines that were readily seen. Samples with TSH concentrations of 5–10 mU/L gave a faint response that was more easily recognized after the experience of performing several assays. The results are shown in Fig. 1.

Screening for mild thyroid failure in older patients has been recommended as a cost-effective procedure *[2]*. The ThyroChek assay appears to be useful as a qualitative screening test for primary hypothyroidism.

This work was supported by VA Medical Research Funds.

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Pitfalls in Discriminating Sulfhemoglobin from Methemoglobin

To the Editor:

Zoppi et al. *[1]* recently described a case of sulfhemoglobinemia. The exact cause of the dyshemoglobinemia in this patient was not clearly mentioned, but it was probably drug-induced. The patient was improperly treated with methylene blue, on the basis of an analysis of whole blood by CO-oximetry in which the presence of methemoglobin was re-

ported. The authors did not specify the type of CO-oximeter used for the initial analysis. However, from the tabulated data the reader is apt to conclude that the measurement was done on an IL 482 instrument (Instrumentation Laboratory, Milan, Italy).

The IL 282 and IL 482 CO-oximeters have not been constructed for determination of sulfhemoglobin. Nevertheless, these instruments can give a strong indication of the presence of sulfhemoglobin in a blood sample on the basis of a combined positive result for methemoglobin and a negative value for carboxyhemoglobin. This bizarre phenomenon was first observed by Zwart et al. and published in an evaluation report of the IL 282 CO-oximeter in 1981 *[2]*. In the IL 282 and IL 482 operator's manuals, it is mentioned that these instruments were not constructed for the determination of sulfhemoglobin and that sulfhemoglobin may interfere with the determination of methemoglobin. The wording of the statement, however, may be unclear: "The spectral absorbance of sulfhemoglobin is similar to that of methemoglobin and the same limitations would apply. Sulfhemoglobin is not commonly found in blood, however, making such interference rare." We would favor wording such as: "The spectral absorbance of sulfhemoglobin is similar to that of methemoglobin. Therefore, the presence of sulfhemoglobin in a blood sample will cause a positive interference or false-positive result for methemoglobin. The presence of sulfhemoglobin in a blood sample is suspected whenever a methemoglobin result is displayed ≥10%, combined with a negative carboxyhemoglobin value." IL now markets the model 682 CO-oximeter, which will detect and actually indicate sulfhemoglobin concentrations >1.5%, making this clarification in the new instrument's manual unnecessary. However, manufacturers could help users if they clearly mention all known clinically relevant limitations in instrument manuals and update the manuals with all essential information published in the scientific literature.

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Hair Iron Content: Possible Marker to Complement Monitoring Therapy of Iron Deficiency in Patients with Chronic Inflammatory Bowel Disease?

To the Editor:

Bissé et al. *[1]* suggest that measurement of the concentration of iron in hair might prove useful in assessing iron status. They state: "To our knowledge, however, no attempt has been made to include the measurement of hair iron in an evaluation of body iron."

Older references are sometimes difficult to locate, but may be very important. In 1945 Flesch and Rothman found that trichosiderin is a red hair pigment that contains iron *[2]*. This finding suggests to some of us who were interested in iron metabolism that the use of hair iron concentrations would not be a satisfactory indicator of patient iron status. However, several investigators did investigate the iron content of hair as an index of iron status. In 1956, Green and Duffield *[3]* measured the iron content of the hair of patients with pulmonary tuberculosis, chronic rheumatoid arthritis, diabetes, pernicious anemia, hemochromatosis, and, notably, 53 patients with iron deficiency. In their investigations they took into account the color of the hair of their patients. Nonetheless, they concluded that "The results of this study would justify the conclusion that the iron content of scalp hair gives no indication as to the state of the body iron stores in an individual patient. While serial analyses of hair samples suggested that in some individuals the iron content of the hair fell when they became iron depleted and rose as iron stores were restored, this was not a consistent observation." Later, Lovric and Pepper *[4]* quantified the iron of various segments of hair shafts of children, comparing the hair iron of 22 controls, 60 children with iron deficiency, and 9 children with iron overload. They concluded ". . . no significant differences in iron levels could be elicited in the three groups of children studied."

The data of Bissé et al. are interesting, but in view of the extensive earlier observations, it seems to me unlikely that hair iron concentrations will prove useful in assessing body iron stores.

Supported by National Institutes of Health grants HL25552 and RR00833, and the Stein Endowment Fund.

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Two of the authors of the paper referred to above reply:

To the Editor:

We appreciate the interest expressed by Beutler in our recent publication *[1]*, particularly as the references

[2–4] quoted in his letter were unknown to us.

We believe that the topic is so important that it deserves a wider base than that published in the quoted references.

Regarding the hair pigment trichosiderin, Flesch and Rothman *[2]* found this substance only in red-haired subjects. The structure as well as the biological function of trichosiderin have not yet been elucidated. In our study we examined no red-haired individuals. But if one considers the world population at risk of iron deficiency as a whole, the proportion of red-haired individuals is small. Despite some differences in hair iron concentrations that could be related to hair color *[5]*, it is speculation to regard trichosiderin as a potential hindrance in the clinical use of hair iron concentration. Moreover, Creason et al. *[5]* found that vanadium and iron in hair were significantly correlated. This finding is interesting because vanadium is bound, as is iron, to transferrin in blood.

The main problem in measuring trace elements in hair is the absence of a scientifically based reference procedure, and hair iron values differ considerably among laboratories. Before examining hair iron in patients and in healthy subjects, the iron status of all subjects should first be clearly described and staged by the currently established tests. The quality management of the data is also very important. Unfortunately the authors quoted by Beutler have used only serum iron for the evaluation of iron status. Therefore they could neither clearly characterize the groups nor distinguish between iron-deficiency anemia and the anemia related to infections, inflammation, or malignancy. This and the analytical problems may partially explain the inconsistent trends observed *[3, 4]*.

The low ferritin concentrations found in our patients clearly indicate that the organism was not basically influenced by the inflammation. Thus our results allow no general statement for patients with chronic bowel diseases or patients with other inflammatory diseases.

Extended studies that include different groups of patients as well as healthy individuals must be performed before we can obtain deeper insight into the metabolic pathway of hair iron. The iron status must be evaluated by using transferrin receptor and zinc protoporphyrin in addition to the conventional tests of iron status.

Until solid experimental data are available, it is premature to reach final conclusions on the clinical value of hair iron concentration.

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The author of the letter responds:

To the Editor:

It is not uncommon for investigators to incline toward the view that any studies performed >10 years ago must be flawed because of the primitive nature of science before current technology was available. However, hematologists were able to diagnose iron deficiency quite accurately even 40 years ago *[1]* without the advantage of serum ferritin concentrations. Hematologists such as Green and Duffield, and Lovric and Pepper were well qualified to make the distinction between the anemia of iron deficiency and that of inflammation, and I do not believe that their findings should be dismissed lightly merely because they were performed many years ago.

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Ernest Beutler

Equivalence of Critical Error Calculations and Process Capability Index C_{pk}

To the Editor:

The concept of process capability has been used by the manufacturing industry to quantify the relation between product specifications and the measured process performance *[1]*. Various ratios and indices have been developed to describe this relation. We have previously reported the application of the simplest of these, C_p (the capability index or capability ratio), to the selection of quality-control (QC) algorithms appropriate to the specification limits and analytical imprecision *[2]*. C_p is defined as (USL − LSL)/6σ, where USL and LSL are the upper and lower specification limits of an analytical process, and σ is the standard deviation of the process.

In contrast to the approach taken by us, the use of medically important critical systematic error (ΔSE_C) and critical random error (ΔRE_C) calculations for the selection of QC algorithms has been promulgated *[3–5]*. Westgard and Burnett *[6]* have also described the relation between C_p and ΔSE_C and have shown that, assuming zero bias, C_p can be directly related to ΔSE_C by equation:

$$\Delta SE_C = 3C_p - z \qquad (1)$$

where z is a factor for a one-tailed test of significance (usually set at 1.65 for 95% confidence, assuming a gaussian distribution).

A limitation of the use of C_p is that this particular capability index does not consider any bias present within the analytical process. C_{pk} is a related capability index used by the manufacturing industry that considers bias as well as imprecision *[1]* and is defined as:

$$C_{pk} = \min\left[\frac{\mu - LSL}{3\sigma}, \frac{USL - \mu}{3\sigma}\right]$$

where USL, LSL, and σ are as for C_p, and μ is the mean of the process.

We have now derived the mathematical relation between the process capability index, C_{pk}, and medically important critical errors, ΔSE_C and ΔRE_C, for cases of nonzero bias.

If the bias is nonzero and the specification limits are symmetrical about the "true value" ($\overline{X}_0$), then:

$$USL = \overline{X}_0 + TE_a$$

and

$$LSL = \overline{X}_0 - TE_a$$

Therefore, $C_{pk} =$

$$\min\left[\frac{TE_a + \mu - \overline{X}_0}{3\sigma}, \frac{TE_a + \overline{X}_0 - \mu}{3\sigma}\right]$$

or

$$C_{pk} = \left[\frac{TE_a - |\mu - \overline{X}_0|}{3\sigma}\right]$$

$$= \left[\frac{TE_a - bias}{3\sigma}\right]$$

From ref. *6*:

$$\Delta SE_c = \frac{TE_a - bias}{\sigma} - z$$

By substitution:

$$\Delta SE_C = 3C_{pk} - z \qquad (2)$$

Similarly:

$$\Delta RE_C = 3C_{pk}/z_2 \qquad (3)$$

where z is as defined above, and z_2 is a factor for a two-tailed test of significance (usually set at 1.96 for 95% confidence, assuming a gaussian distribution). The previously derived relation *[6]* between ΔSE_C and C_p shown in Eq. 1 can now be seen to be a limiting value of Eq. 2 for the special case of zero bias.

We have found that implementation of a QC strategy based on capability indices may be conceptually easier to understand than one based on ΔSE_C and ΔRE_C *[2]*. By rearranging Eqs. 2 and 3, we can derive:

$$C_{pk} = \frac{\Delta SE_C + z}{3} \qquad (4)$$

and

$$C_{pk} = \frac{\Delta RE_C \times z_2}{3} \qquad (5)$$

This form of the relation may facilitate the educational and training process for those laboratories that choose this perspective. Conversely, for those laboratories that have been using QC strategies based on process capabilities, a simple pathway is now offered to convert their strategies to the more widely adopted approach of using critical error parameters.

There exists ready availability of computational aids for the selection of QC algorithms based on critical errors, such as the "QC Validator" software program *[7]* and the use of QC selection grids *[8]*. As Eqs. 2 through 5 permit interconversion of C_{pk} with ΔSE_C and ΔRE_C, there appears to be no need to develop parallel sets of tools based on C_{pk}.

The demonstration of the mathematical equivalence of ΔSE_C, ΔRE_C, and C_{pk} now permits a unification of those approaches based around process capability *[2]* and those around medically important critical errors *[3–5]*. Finally, the equivalence between approaches based on critical error and C_p *[6]*, and now also C_{pk}, means that there is available a common language between clinical chemistry and quality professionals in the manufacturing industry.

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