Bias plots for each organ type showed the same pattern of scatter as the overall bias plots.

The greatest differences between the two methods are chromatographic. The retention times are quite different, with original retention times of 5.14 and 6.90 min for CsA and CsD, respectively, vs times of 9.39 and 11.17 min for CsC and CsA. In particular, CsD elutes after the CsA peak, whereas CsC elutes before CsA; thus, the internal standard is not in the same position with respect to the CsA peak in the two methods. This reversal of position can be a serious drawback when switching from CsD to CsC as internal standard, because it is very simple to misidentify the peaks and miscalculate results if the chromatograms are not carefully labeled.

The extended time necessary for running the assay with CsC also slows turnaround time. However, a 14-min run time was necessary for the CsC method, because slow-moving "junk" peaks interfered by coeluting with CsA if shorter times were used.

Although the original procedure also readily detects CsA concentrations below the lower limit of claimed linearity, the CsC method does not detect CsA <25 μg/L and so is actually less sensitive at low concentrations—probably because later-eluting peaks on a chromatogram tend to be broader and therefore less well-defined than faster-eluting peaks.

In the course of this study of the modified method, we did not find any known substances that coeluted with CsA, suggesting that this method is fairly specific for CsA. The excellent correlation between the two methods suggests that the CsC method is as specific for CsA as is the original method of Moyer et al.

Currently, many institutions are switching from HPLC methods to monoclonal immunoassays for measuring CsA. There are many reasons for this, including smaller sample requirements and simpler methods calling for much less technical expertise. Probably the strongest incentive for switching to an immunoassay is the greatly reduced turnaround time. The extended run time necessary with the CsC method will aggravate this factor. Nonetheless, institutions that plan to continue to assay CsA by HPLC will need to convert to CsC as an internal standard when their stock of CsD runs out. As we have demonstrated, the conversion will require few changes in the actual procedure; the major changes will involve extension of the analytical time and careful identification of the new elution peaks.

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HPLC Micro-Method for Determining 8-Aminolevulinic Acid in Plasma

To the Editor:

Several investigators (1–3) report highly sensitive fluorometric methods for determining 8-aminolevulinic acid (ALA) in urine. These methods combine the precolumn derivatization of ALA with acetylacetone and formaldehyde and HPLC with a reversed-phase column. In the present study, we further improved this fluorometric HPLC method and applied it to the measurement of ALA in plasma with samples obtained from 32 healthy men.

All chemicals used were of analytical grade. Acetylacetone reagent was prepared by mixing 15 mL of acetylacetone, 10 mL of ethanol, and 75 mL of distilled water. Formaldehyde solution (100 g/L) was prepared by 3.7-fold dilution of the chemical reagent (370 g/L) with distilled water and stored in the dark. The working ALA standard (100 μg/L) was made by diluting ALA stock solution (100 mg/L) with distilled water. The fluorescent derivatization of ALA was performed as follows: Mix 3.5 mL of the acetylacetone reagent, 0.05 mL of plasma sample, and 0.45 mL of the formaldehyde solution with a vibrator mixer for ~3 s, and heat this mixture (total 4 mL) for 10 min at 100 °C on the aluminum block. Without delay place the test tube in an ice-cold bath. Filter a small volume of this reaction mixture through a disposable HPLC filter, then store the filtrate in the dark until analysis. When the filtrate is left in the dark at room temperature (23–25 °C), the fluorescence derivative of ALA is stable for >8 h.

The HPLC analysis was carried out with the following apparatus (all from Shimadzu, Ltd., Kyoto, Japan): Model LC-6A HPLC; column, Shim-pack CLC-ODS, 150 × 4.6 mm, 5-μm particle size, reversed-phase; detector, fluorescence HPLC monitor (Model RF-535) with excitation/emission wavelengths of 370/460 nm, range × 4, sensitivity high. The mobile phase was methanol/water/glacial acetic acid (500/500/10 by vol); flow rate, 0.7 mL/min; column temp, 40 °C, maintained with a column oven; chart speed, 5 mm/min; data processor, Shimadzu C-R3A; injection volume, 20 μL.

In the fluorescent derivatization of ALA as an application of the Hantzsch reaction, the concentration of formaldehyde in the reaction mixture (total reaction volume, 4 mL) particularly affects the formation of the fluorescent ALA derivative. The optimum concentration of formaldehyde used ranged from 7.5 to 12 g/L in the reaction mixture, indicating that the final concentration of formaldehyde (3.5 g/L) used by Okayama et al. (3) is not the optimum condition for the fluorescent ALA derivatization. In the present study, we used a final concentration of 11.25 g/L, which corresponds to using 0.45 mL of the formaldehyde solution in the 4-mL reaction mixture. When the reaction mixture was heated for 10 min at 100 °C, the fluorescence intensity of the ALA derivative reached a maximum and remained constant until 30 min of heating; therefore, we used 10 min as the optimum heating time.

Fig. 1. Fluorescence HPLC chromatograms of ALA derivative (A) from a LA standard (100 μg/L in water) and (B) from a normal plasma (ALA 20.7 μg/L).

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Figure 1 shows chromatograms of a 100 μg/L ALA standard and of plasma ALA (30.7 μg/L) from a healthy man. The fluorescence derivative of ALA was completely separated from other fluorescent substances in plasma. The retention time of the ALA derivative was 6.9 min under these HPLC conditions.

The within-run variation (CV) of the present HPLC method was ~5% for aqueous ALA, 100 μg/L (n = 5). The detection limit for ALA in plasma was 3 μg/L (23 nmol/L), which corresponds to 1.15 pmol of ALA in the 50-μL plasma sample. Analytical recovery of ALA added to several human plasma samples (n = 6) ranged from 78% to 89% (mean 83.5%).

We determined plasma ALA from 32 healthy men with the fluorometric HPLC method. The mean ± SD of plasma ALA concentration obtained was 8.2 ± 4.7 μg/L (range 3.7–24.8 μg/L).

In conclusion, the fluorometric HPLC method for determining ALA in plasma is highly sensitive, specific, and simple. The concentration of plasma ALA can be measured in a 50-μL sample.

References

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Skeletal Muscle Phosphate Uptake during Euglycemic–Hyperinsulinemic Clamp

To the Editor:
Plasma phosphate (P_i) concentration is vulnerable to P_i shifts between intra- and extracellular compartments. Hypophosphatemia can result from cellular P_i uptake in response to glucose and insulin (1, 2). There is indirect evidence that liver and, especially, skeletal muscle are the principal sites of P_i uptake (3, 4). P_i uptake has been thought to be driven by incorporation of P_i into organic phosphates pools (3), which would tend to lower cellular [P_i] (2). However, muscle cell [P_i] (measured by 31P magnetic resonance spectroscopy) increases during oral glucose loading (5) and insulin–glucose infusion (6), suggesting that if muscle P_i uptake does occur, it must be driven by a primary increase in cell sarcolemmal P_i transport (7), which is consistent with older in vitro work (8). However, P_i uptake by forearm muscle is reportedly reduced by oral glucose loading (4). Here, we report the results of a study of P_i balance across forearm muscle during euglycemic–hyperinsulinemic clamp.

The study was approved by the local hospital ethical committee. After an overnight fast, seven apparently healthy, unmedicated subjects (6 men, 1 woman), ages 25–41 years, with a body mass index (weight/height², in kg/m²) of 19–24 participated in the study. As described elsewhere (6, 9), cannulas were inserted retrogradely into an antecubital vein draining deep tissues and into a vein draining the contralateral hand, which was warmed to provide arterialized blood samples. Three baseline samples were taken over 40 min before a primed infusion of insulin (35 mIU·min⁻¹·m⁻²) was started, and further samples were taken at 15, 30, 60, 90, and 120 min. Arterialized venous plasma [glucose] was measured every 5 min with a Beckman Model 2 glucose analyzer (Beckman Instruments, High Wycombe, Bucks, UK) and clamped at the fasting concentration. Insulin was measured by radioimmunoassay and P_i was measured on an American Monitor (Indianapolis, IN) parallel analyzer. Blood flow was measured by strain-gauge plethysmography, and net P_i flux into muscle was calculated as the product of the flow and the arteriovenous [P_i] difference. Results are presented as mean ± SE. Other aspects of these studies were published elsewhere (9).

Plasma [glucose] was constant for 120 min, whereas plasma [insulin] increased within 16 min to a plateau of 50–55 mIU/L, which was maintained until t = 120 min (9). Arterial and venous [P_i] both decreased over the first hour and reached a plateau that was stable over the second hour (Figure 1A). Thus the rate of change of plasma [P_i] (Figure 1B) was significantly negative between 15 and 30 min before decreasing to zero. This rate can be taken as a measure of the rate of net P_i flux out of the extracellular fluid, because renal P_i output shows only a small decrease during insulin clamp (B. W. Morris, personal communication) and oral glucose loading (10) and therefore reflects the cellular uptake of P_i (10).

Linear-regression analysis of the relationship over 120 min between the rate of P_i uptake (interpolated to the midpoint of each interval) and the rate of change of plasma [P_i] gives a slope of -0.25 (r = 0.6, significantly different from zero at P <0.05). This result can be interpreted as follows: If all muscle behaved like forearm muscle, and if this uptake mechanism were the sole cause of the decrease in plasma [P_i], and if extracellular fluid and muscle represent ~20% and 40% respectively, of body weight, and if there are M liters of muscle water per liter of forearm, then the predicted regression slope = [1000/10 × 60] × M × (20/40). This is satisfied if M = 0.3, which (while we have no measurement of this) seems reasonable.

In a previously reported study of the hypophosphatemia of oral glucose loading, the rise and fall in the rate of change of plasma [P_i], which was sim-

Fig. 1. Arterial and venous plasma [P_i] (A) and rate of P_i uptake by forearm muscle (B)
A: Plasma [P_i] in arterial (C) and venous (B) blood during insulin–glucose infusion. Values are significantly different from basal values at t = 60, 75, and 90 min (all P <0.05 by paired t-test). B: P_i uptake by forearm muscle (B); at t = 15–30 min, values are significantly greater than zero (P <0.05 by unpaired t-test). Rate of change of plasma [P_i] (C) calculated from successive pairs of mean arterial and plasma [P_i] (assigned to the midpoint of each sampling interval); significantly different from zero at t = 22.5 min (P <0.01 by paired t-test). The broken line shows rate of change of plasma [P_i] in a previously reported study of oral glucose loading (10) for comparison.