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Editor's Note: The above recommendations mirror those in our Information for Authors. We think they are worth repeating, in view of the high frequency with which we ask authors to observe them.

Monitoring Cyclosporine by HPLC with Cyclosporin C as Internal Standard

To the Editor:

Cyclosporin A (CsA, cyclosporine) is currently the immunosuppressant drug of choice for organ-transplant patients. Monitoring the concentration of CsA is often critical to patient management because of the adverse side effects and extremely individual metabolic variability of this drug. HPLC has long been considered the "gold standard" methodology for monitoring CsA concentrations. Cyclosporin D (CsD), the form of cyclosporine traditionally used as the internal standard for HPLC assays, has become unavailable since Sandoz stopped supplying it (personal communication, Kathleen Roskaz, Inventory Control, Sandoz Research Institute, East Hanover, NJ) and as stocks have run out. Here we show that cyclosporin C (CsC) can be used as an alternative internal standard with some modifications of the original procedure of Moyer et al. (1).

The HPLC we used to determine CsA was a Perkin-Elmer (Norwalk, CT) system with a Supelco (Bellefonte, PA) 5-cm LC-1 column and ultraviolet detection at 214 nm. This is an isocratic, reversed-phase system. The modifications made to the original procedure were: (a) changing the mobile phase from acetonitrile/methanol/water (33/3/4 by vol) to acetonitrile/water (46/54 by vol); (b) slowing the flow rate from 1.4 to 1.2 mL/min; (c) extending the run time from 11 to 14 min; and (d) substituting CsC for CsD.

After modifying the procedure to use CsC as the internal standard, we ran precision and linearity studies, as well as a correlation study with patients' samples. Between- and within-run precisions were determined at two concentrations of CsA-supplemented whole-blood controls. Between-run (n = 14) mean CsA concentrations were 174.3 and 512.4 μg/L, and the corresponding CVs were 4.7% and 4.0%. For the within-run (n = 9) precision study, mean CsA concentrations of 169.6 and 496.1 μg/L yielded CVs of 5.1% and 4.9%, respectively.

To determine the linearity and sensitivity of the revised procedure, we prepared blank whole-blood samples, then added CsA at concentrations ranging from 12.5 to 2000 μg/L (the range of linearity of the method of Moyer et al.). We found that the results of the modified method varied linearly with concentration in the range of 25–1000 μg/L, with a correlation between theoretical and actual values (r) of 1.00 and a regression equation of y = 0.10x − 4.37 μg/L. Unlike the original method, the modified method did not detect the 12.5 μg/L sample.

To study the correlation between the two methods, we assayed a series of samples from pediatric liver (n = 45) and kidney (n = 29) transplant patients and adult heart (n = 55) transplant patients. Any samples arriving in the laboratory for routine CsA determination that exceeded 4 mL of EDTA-treated whole blood were assayed by both methods. Figure 1 shows the regression and bias plots of results from those samples with CsA concentrations <400 μg/L. We present these data (n = 119) instead of those for all of the patients' samples (n = 129) to more clearly demonstrate the arrangement of data in the concentration range that includes most of the CsA values. The overall correlation for patients' samples was y = 0.989x + 1.66 μg/L (r = 0.981); for the 0–400 μg/L data shown in Figure 1, r = 0.982. As shown, the CsC method demonstrates neither a negative nor a positive bias in comparison with the original method. The scatter in the bias plots appears to be about equal on both sides of the line.

When we considered the data from each organ type separately, the regression equation for the liver-transplant patients was y = 1.064x − 14.5 μg/L (r = 0.989), for the renal patients was y = 0.948x + 6.39 μg/L (r = 0.989), and for the heart patients was y = 0.860x + 21.8 μg/L (r = 0.954).

Fig. 1. Regression (left) and bias (right) plots for patients' samples with CsA values <400 μg/L, used for comparison of CsA values determined by HPLC with CsD vs CsC as internal standard.

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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 chromatographs are not carefully labeled. The extended time necessary for running the assay with CsC also slows turnaround time. However, a 15-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 chromatograph 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 δ-Aminolevulinic Acid in Plasma

To the Editor

Several investigators (1-3) report highly sensitive fluorometric methods for determining δ-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 let 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 x 4.6 mm, 5-µm particle size, reversed-phase; detector, fluorescence HPLC monitor (Model RF-635) 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 ALA standard (100 µg/L in water) and (B) from a normal plasma (ALA 20.7 µg/L).

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