g/L each), followed by 700 μL of 1.5 mol/L Tris-HCl buffer containing 20 g/L Na₂EDTA, pH 8.6, to adsorb 5-S-CD onto the alumina. The mixture was immediately shaken for 5 min on an EM-36 microtube mixer (Taito, Koshigaya, Japan). After centrifugation the aqueous layer was removed by aspiration, and the alumina was washed twice with 1 mL of water. Then, 500 μL of 0.1 mol/L potassium phosphate buffer, pH 4.0, was added to the tube, the mixture was shaken for 2 min, the aqueous layer was removed by aspiration, and the alumina remaining was washed with 1 mL of water. 5-S-CD was then eluted with 100 μL of 0.4 mol/L HClO₄ by shaking for 2 min. After centrifugation, 30 μL of supernatant was injected into the chromatograph. 5-S-CD was quantified from peak-height ratios between 5-S-CD and Me-CD for sample and standard. The detection limit for 5-S-CD in serum was <0.1 nmol/L.

Figure 1 shows HPLC chromatograms of a pooled serum sample from melanoma patients processed with the previous and present methods. Washing the alumina with a phosphate buffer, pH 4.0, eliminated the peak for 3,4-dihydroxyphenylacetic acid (DOPAC).

A standard curve, constructed by calculating peak-height ratios between 5-S-CD and internal standard (Me-CD) vs the amount of 5-S-CD added to serum, showed linearity (r = 1.00) for 5-S-CD concentrations from 1 to 1000 nmol/L. Between-run (n = 10) mean 5-S-CD concentrations were 7.06 and 53.2 nmol/L; the corresponding CVs were 7.1% and 4.2%. For the within-run (n = 10) precision study, a mean concentration of 5.51 nmol/L yielded a CV of 3.9%.

To determine analytical recovery, we added 5-S-CD to a pooled serum sample having a 5-S-CD concentration of 4.5 nmol/L to increase the concentration by 10 and 100 nmol/L, respectively (n = 5 each). The increase (mean ± SD) found after this addition was 13.5 ± 0.46 (89% ± 3.0%), and 95.3 ± 1.48 nmol/L (91% ± 1.5%), respectively. Absolute recoveries of 10 nmol of 5-S-CD and 30 nmol of Me-CD were 78% (SD 5.6%) and 76% (SD 4.9%), respectively. We found that the correlation between the method we described earlier (5) (c) and the present method (γ) with concentrations in the range of 1.5–75 nmol/L was y = 0.980x – 0.252 nmol/L (r = 1.00, n = 28).

Further improvement was achieved by using Me-CD, which differs from 5-S-CD only in the methyl group, instead of α-methyl-DOPA as the internal standard. From its structure, Me-CD would be expected to have chemical characteristics more resembling 5-S-CD than would α-methyl-DOPA and, accordingly, the precision of the analysis would be improved because of the close similarity in chemical properties. Moreover, in the present method, we cannot use α-methyl-DOPA as internal standard, because an unknown phosphate compound deriving from a serum component overlaps with α-methyl-DOPA in the chromatogram.

Low concentrations of 5-S-cystey-nil adducts of dopamine (DA) and DOPAC have been detected in DA-rich brain regions in several mammalian species (6,7). By the present method, recoveries of 5-S-cystey-nil-DA and 5-S-cystey-nil-DOPAC relative to 5-S-CD were 98% and 52%, respectively. 5-S-Cystey-nil-DA and 5-S-cystey-nil-DOPAC were eluted at 18 and 52 min, respectively, in the HPLC chromatograms.

Phenylboronate affinity gel can also be used to extract 5-S-CD from serum (8) with high selectivity. However, this method is complicated and requires large amounts of samples, e.g., 3 mL of serum, whereas our method is simple and requires only 500 μL of serum. One can pretreat 10 serum samples within 60 min before injecting them into a chromatograph. We usually pretreat two batches of 10 samples each per day. These 20 samples can be analyzed by HPLC in <20 h when an autosampler is used.

Fig. 1. Typical HPLC chromatograms of a pooled serum sample from melanoma patients processed with (A) the previous method and (B) the present method.

Peaks: 1, 5-S-CD; 2, α-methyl-DOPA; 3, Me-CD; 4, DOPAC; 5, an unknown phosphate compound deriving from a serum component. We automatically inject a 30-μL aliquot of an analytical sample into the HPLC system every 45 min. The sensitivity was set to 2.6 nA full-scale.

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Kazumasa Wakanatsu
Shosuke Ito
Fujita Health Univ.
School of Health Sci.
Toyoake
Aichi 470-11, Japan

1 Author for correspondence.

Free Triiodothyronine in Hypothyroidism and in Nonthyroidal Illness

To the Editor:

In a recent Letter (1), Mullinger and Gates conclude that the FT₃ Amerlex (Kodak Clinical Diagnostic, Amersham, UK) is more suitable than the FT₃ Amerlex MAB to estimate free triiodothyronine if this analyte (FT₃) is to be used as a prognostic factor for severely ill patients. However, determination of FT₃ is used primarily to assess thyroid function, not a patient’s chance to survive a critical illness. In that indication, we find FT₃ Amerlex MAB to be more discriminant. We measured FT₃ by the two methods in 35 samples from 18 hypothyroid patients [group A: low free thyroxine (FT₄)], high
thyrotropin (TSH) and in 34 samples from 34 patients hospitalized for a nonthyroidal illness, whose thyroid function was normal on the basis of normal values for TSH and FT₄ (group B). Serum TSH was measured with IRMA RIA Gnost (Behringwerke, Marburg, Germany) (normal reference range, 0.1–4.5 mIU/L) and FT₄ with the FT₄ Amerlex MAB kit (10.3–25.6 pmol/L). This method for measuring FT₄ has been well validated in nonthyroidal illness (2, 3). Normal reference intervals for FT₃, determined by the two kits in 68 euthyroid subjects (normal TSH and FT₄) without serious extrathyroidal illness were 3.2–7.1 pmol/L (mean ± 2SD) for FT₃ Amerlex and 4.1–8.0 pmol/L for FT₃ Amerlex MAB, a range slightly different from that determined by the manufacturer on a larger sample of subjects (3.4–7.2 pmol/L).

Thirty (86%) of the samples drawn in group A had a low FT₃ when measured by Amerlex and 34 (97%) were low by Amerlex MAB. This difference in sensitivity is small, and not significant considering the small number of control subjects and hypothyroid patients. In this group, the two FT₃ estimates were significantly correlated (r = 0.69; Fig. 1A).

In contrast, 14 of the 34 hospitalized patients (44%) with an extrathyroidal illness and normal TSH and FT₄ had a low FT₃ (0.5–2.5 pmol/L) by the FT₃ Amerlex (specificity vs hypothyroidism in this population: 56%). By the FT₃ Amerlex MAB, FT₃ estimates were normal in 13 of these patients and slightly increased in 1. The two FT₃ estimates were not correlated in this group (Fig. 1B), indicating that the Amerlex FT₃ estimate does not reflect thyroid function in sick patients.

Thus Mullinger and Gates are right when stating that the FT₃ estimate by FT₃ Amerlex MAB will lose its prognostic significance in severely ill patients, but FT₃ Amerlex MAB gives a better estimate of thyroid function. Our results also confirm that FT₃ is less sensitive than FT₄ with regard to the diagnosis of hypothyroidism, because some patients of group A with a low FT₄ had normal FT₃ estimates by both methods, particularly when the manufacturer’s range for FT₃ Amerlex MAB was used.

References

Pierre J. Bergmann¹,² Liliane Van Tricht²
Labs. of ¹Clin. Chem. and ²Nuclear Med.
Brugmann University Hospital
Université Libre de Bruxelles
4 Place Van Gehuchten
1020 Brussels, Belgium

* Corresponding author.

Colorimetric Quantification of Urinary Iron During Deferoxamine Therapy

To the Editor:

The concentration of iron in urine is of interest in monitoring deferoxamine therapy for acute iron poisoning (1) or iron overload (2). Atomic absorption spectrophotometry (AAS), the Reference Method for iron measurement (3), is usually not available in most routine laboratories. Routine automated methods for measuring serum iron with use of a chromogen do not detect iron in these samples (1), because the complex of deferoxamine and Fe²⁺ is not dissociated. Several types of pretreatment of urine samples to dissociate the deferoxamine–Fe²⁺ complex have been described (1, 4).

Given the need for a sensitive, (semi-)automated procedure to monitor deferoxamine therapy in urine, we combined a ferrozine-based method for iron measurement (kit 1127683/691; Boehringer Mannheim, Mannheim, Germany), implemented on an automated analyzer (Hitachi 747; Hitachi, Tokyo, Japan), with a pretreatment. We optimized and compared two types of pretreatment: thioglycolate–trichloroacetate (1) and ascorbate–citrate (4).

To measure the recovery of iron, we added FeCl₃ to normal urine with or without equimolar concentrations of deferoxamine. Urine samples of patients with proteinuria or in deferoxamine therapy were collected. Each sample was thoroughly mixed, and aliquots were stored at 4°C and analyzed within 4 h.

In the thioglycolate–trichloroacetate pretreatment, the reagent (0.29 mol/L thioglycolic acid and 0.61 mol/L trichloroacetate in water) is added to an equal volume of fresh urine. In the ascorbate–citrate pretreatment, 3.5 volumes of reagent (0.48 mol/L ascorbate and 0.57 mol/L citrate in water) is added to 1 volume of fresh urine. These pretreatments are done at room temperature, and the pretreated samples are analyzed after 15–60 min on the automated analyzer. A control urine sample is processed the same way for a reagent blank.

We tested the linearity of the procedures from 0.41 to 1000 μmol/L iron. In the absence of deferoxamine, recovery of added iron (95% at 1000 μmol/L) and linearity (r² = 0.9999) were excellent without pretreatment. In urine containing both deferoxamine and iron (Fig. 1), iron could not be detected without pretreatment of the samples. Pretreatment with thioglycolate–trichloroacetate yielded poor recovery (19%) and linearity (r² = 0.81). Pretreatment with ascorbate–citrate was superior in both recovery (92%) and linearity (r² = 0.9996) and was thus adopted for further experiments and routine analysis. The ascorbate–citrate reagent solution was stable for at least 3 days at 4°C, although values for the reagent blank tended to increase. The reagent can be stored for longer periods at −18°C.

Because the molar absorptivity of the Fe²⁺–ferrozine complex (ε = 28 000 L·mol⁻¹·cm⁻¹) (5) exceeds that of the Fe³⁺–tripryridyltriazine complex (ε = 22 600) (5), the detection limit for chelated iron was 0.8 μmol/L. This analytical sensitivity, although inferior to that of AAS (0.08 μmol/L (3)), allows our procedure to be used in the deferoxamine–iron excretion test (positive result: chelated iron excre-