The Ciba Corning FT₄ assay is a one-step (analog) method (2). All FT₄ analog methods depend on the principle that there is negligible binding of analog to normal albumin. However, it has been shown that such methods exhibit significant binding of analog to the abnormal albumin present in patients who have FH and that this interferes with the assay (3).

In the four FH patients studied here no interference was present with the Ciba Corning FT₄ assay.

Although results for a larger group of subjects would be desirable, FH is very uncommon. Given that the results for these four patients were consistent, we conclude that the analog FT₄ method manufactured by Ciba Corning is not affected by the abnormal T₄-binding albumin in patients with FH.

References

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Fluoxetine, an Antidepressant, and Norfluoxetine, its Metabolite, Determined by HPLC with a C₁₈ Column and Ultraviolet Detection

To the Editor:

Fluoxetine hydrochloride (Prozac®; Eli Lilly Co., Indianapolis, IN) and its N-demethylated metabolite norfluoxetine are antidepressant drugs. Their activity is based on the selective inhibition of serotonin recapture in the presynaptic neurons of the central nervous system (1, 2). Fluoxetine and norfluoxetine can be detected by gas chromatography and electron capture (3, 4) but this technique is too difficult for many laboratories. Therefore, HPLC determinations of these drugs have been developed (5-10). But they are still difficult to perform or are subject to interferences with several drugs such as benzodiazepines, imipramine, and amitriptyline (5, 6, 10).

An easier technique with less interference is described here.

In this method, HPLC with ultraviolet detection, we used a 125 × 4 mm C₁₈ analytical column (Ecotube Nucleosil; 5-μm particles; Bischoff, Leonberg, Germany) with a 1-cm C₁₈ precolumn (10-μm particles; Bischoff). The mobile phase consisted of 630 mL of distilled water, 370 mL of acetonitrile, 0.400 mL of diethylamine, and 25 mL of PicB5 (water-methanol-1-pentanesulfonic acid).

To prepare standard curves, we supplemented drug-free human serum samples with methanolic solutions of fluoxetine and norfluoxetine to final concentrations of 50, 100, 200, and 400 μg/L. For checking accuracy, we prepared samples containing fluoxetine and (or) norfluoxetine at −50 μg/L by mixing appropriate working solutions of fluoxetine and norfluoxetine with drug-free serum.

Blood samples were collected about 18 h after a single dose of fluoxetine. The samples were drawn into heparinized tubes and centrifuged (2100 × g, 10 min) within 2 h after collection. Plasma was separated and stored at −20 °C until use.

Samples (1 mL) of serum or plasma were transferred to borosilicate stabilized glass tubes, and 30 μL of methanolic trimipramine was added to each tube, followed by 200 μL of NaOH (0.33 mol/L). The samples were shaken for 5 s and then further extracted by shaking with 7 mL of n-hexane/isomyl alcohol (985/15 by vol) for 20 min. After centrifugation (5 min, 2100 × g, 20 °C), we collected the organic phase and adjusted it to acidic pH by adding 200 μL of HCl (0.1 mol/L). We then shook the samples for 1 min, discarded the organic phase, and injected 30 μL of the aqueous phase (containing fluoxetine and norfluoxetine) into the HPLC column. The chromatographic conditions were: flow rate 1.7 mL/min, column temperature 55 °C, and detection wavelength 230 nm.

Figure 1a shows the chromatogram of an extract obtained from drug-free human serum, supplemented with 200 μg/L each of fluoxetine and norfluoxetine and 200 μg/L of trimipramine, the internal standard. The retention times of fluoxetine, norfluoxetine, and trimipramine were respectively 5.6, 7.2, and 8.4 min. Analytical recoveries of the added substances ranged from 75% for norfluoxetine to 88% for fluoxetine and 90% for trimipramine.

The sensitivity (defined as signal/noise ratio = 2) is 10 μg/L in our conditions. Thus our detection limit is lower than the previously published ones, 15 to 30 μg/L (5, 8). The calibration is linear from 25 to 800 μg/L for both fluoxetine and norfluoxetine, with excellent correlation as indicated by linear regression of the peak ratio for the drug to the internal standard (y) vs drug concentration (x): y = 0.005367x + 0.004956x + 0.0180697 (r = 0.9970) for norfluoxetine. The CVs within-run were 7% for fluoxetine and 10% for norfluoxetine at concentrations of 200 μg/L.

A chromatogram of a patient's plasma extract is shown in Figure 1b. To study whether drugs commonly used in psychiatry (eight benzodiazepines and eight antidepressants) interfered, we first directly injected the different products into the HPLC. Then we injected extracts obtained after supplementing blood samples with the drugs.

No interference was found with the benzodiazepines tested (alprazolam, bromazepam, clorazepate dipotassi- um, diazepam, flunitrazepam, loraze-
oxetine concentrations and antidepressant response. Ther Drug Monit 1989;11:165–70.


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immunobilization of Barley Oxalate Oxidase onto Alkylamine Glass for Determining Urinary Oxalate

To the Editor:

Enzymatic colorimetric determination of urinary oxalate by oxalate oxidase is simple, sensitive, and specific, but is expensive because of the high cost of the enzyme (1). The immobilization of the enzyme onto an insoluble support permits its reuse and thereby reduces the cost of the procedure. Bais et al. (2) reported immobilization of barley oxalate oxidase onto nylon tubing and used it in a continuous-flow system for urinary oxalate determination. We describe herein immobilization of oxalate oxidase onto alkylamine glass beads and its application in discrete assays for urinary oxalate.

Zirconia-coated alkylamine glass beads (pore diameter 55 nm) were a gift from Corning Glass Works, Corning, NY, and barley oxalate oxidase (EC 1.2.3.4) was purchased from Sigma Chemical Co. (St. Louis, MO).

The glass beads were activated (3) by addition of 25 g/L glutaraldehyde (1.0 L/100 g beads) with stirring for 2 h at 30 ± 5°C. The excess glutaraldehyde was decanted and the beads were then washed with 0.05 mol/L sodium phosphate buffer (pH 7.0). Barley oxalate oxidase was added to activated beads (1.5 g/100 g beads) and kept at 4°C for 24 h for coupling to occur. Unbound enzyme was washed off with reaction buffer (0.01 mol/L sodium succinate buffer, pH 4.0) until no enzyme activity was detected in the washings. The enzyme retained 74% of the initial activity after immobilization, with a conjugation yield of 0.42 mg/g support.

Compared with the native enzyme, the conjugated enzyme showed greater stability in the cold, had a higher optimum reaction pH, and was more resistant to inhibition by NaCl. The reaction time at 30°C was decreased (Table 1).

The assay of immobilized oxalate oxidase was carried out in a 25-mL conical flask wrapped with black paper. The reaction mixture, 1.9 mL of 0.1 mol/L sodium succinate buffer, pH 4.0, and 200 mg of beads with bound enzyme were preincubated at 30°C for 2 min in a temperature-controlled water bath-shaker. The reaction was started by adding 0.1 mL of oxalic acid (0.5 mmol/L). After 2 min of incubation at 30°C, 1 mL of color reagent was added and kept at 30°C for 7 min to develop the color. The reaction mixture was decanted into a cuvette. Absorbance was read in a Spectronic-20 spectrometer ( Milton Roy, Rochester, NY) at a wavelength of 520 nm, and the amount of H2O2 generated was determined from the calibration curve for H2O2. The color reagent—50 mg of 4-aminophenazone, 100 mg of solid phenol, and 1 mg of horseradish peroxidase (KZ 1.0) per 100 mL of 0.4 mol/L sodium phosphate buffer, pH 7.0—was stored in an amber bottle at 4°C. Fresh reagent was prepared every week.

Table 1. Comparative Characteristics of Soluble and Immobilized Barley Oxalate Oxidase

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Soluble enzyme (6, 7)</th>
<th>Immobilized enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>3.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Temp. for max. activity, °C</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Thermal stability at 75 °C, %</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Eₐ, cal/mol</td>
<td>9140</td>
<td>11 480</td>
</tr>
<tr>
<td>Time for max. activity at 30 °C, min</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Kₘ for oxalate, mol/L</td>
<td>4.2 × 10⁻⁴</td>
<td>3.7 × 10⁻⁴</td>
</tr>
<tr>
<td>% inhibition by NaCl (1 mmol/L)</td>
<td>57</td>
<td>18</td>
</tr>
</tbody>
</table>

Standard assay conditions were used for each determination. To study the thermal stability, we heated the enzyme at 75°C for 40 min, cooled it, and assayed it at 30°C.

References