Urine drug testing is used in many applications, including preemployment screening, human performance testing, neonatal care, probation monitoring, and emergency room care. Rapid, cost-effective immunochemical techniques such as enzyme immunoassays are effective tools for excluding the presence of various drugs; however, they may lack the specificity necessary for accurate identification. Confirmatory techniques such as HPLC, gas chromatography–mass spectrometry (GC–MS), and thin-layer chromatography are often used to validate presumptive immunochemical findings.

Immunochemical cross-reactivity causes obvious problems for the clinical laboratory. Eliminating false positive screening results would increase accuracy, efficiency, and cost-effectiveness and improve patient management. The current study was designed to isolate, identify, and quantify the compound(s) responsible for false positive methadone results, using enzyme immunoassay reagents from Diagnostic Reagents, Inc. Many screening panels include a test for methadone, a narcotic agonist used in narcotic maintenance treatment, especially for heroin addiction. Methadone is extensively metabolized by hydroxylation and dealkylation of the nitrogen methyl groups; these dealkylated metabolites are unstable and spontaneously cyclize to form 2-ethylene-1,5-dimethyl-3,3-diphenylpyrrolidinone (EDDP), which may further N-demethylate (1). In maintenance subjects, urine concentrations for methadone and EDDP range from 1 to 50 mg/L (2).

Verapamil, a calcium channel-blocking agent used for the treatment of hypertension and cardiac arrhythmias, is also extensively metabolized. Metabolic pathways include N-demethylation, O-demethylation, and cleavage of the C—N—C bonds (3). Chemical structures of methadone, verapamil, and several verapamil metabolites are presented in Fig. 1 (3).

Enzyme immunoassay reagents were purchased from Diagnostic Reagents, Inc. (lot nos. 5E059, 5M112, 6M174, and 6E083, cat. no. 0597). Verapamil and norverapamil were gifts from Searle; the other metabolites were gifts from Knoll AG. Methadone, sodium phosphate, sodium hydroxide, sodium carbonate, and 1-heptanesulfonic acid were acquired from Sigma Chemical. Methanol, acetic acid, isopropanol, phosphoric acid, acetonitrile, and dichloromethane were purchased from Fisher Scientific; deuterium-labeled internal standards were purchased from Radian; and triethylamine and isopropyl ether were purchased from Aldrich Chemical. The solid-phase extraction cartridges, Spe-ed Scan ABN cat. no. 2774, were purchased from Applied Separations.

The immunoassay for methadone was performed according to the manufacturer's protocol on an Olympus AU5000 analyzer (Olympus America). Assay parameters were as follows: 15 μL of sample, 125 μL of Antibody Reagent A, 125 μL of Enzyme Reagent E, 340 nm, and 37 °C (4) with a single point calibration at 300 μg/L urine. Samples with an absorbance rate change greater than or equal to the calibrator were analyzed by GC–MS for confirmation.

Into appropriately labeled 16 × 125-mm culture tubes were placed 4 mL of 0.1 mol/L phosphate buffer, pH 6, internal standard (100 ng of d₃-methadone), and a 1 mL aliquot of urine. Calibrators at concentrations of 200, 300, and 1000 μg/L were prepared and extracted concurrently. The concentration of methadone was calculated with a least-squares regression equation for the three calibrators, using the area-response ratio for the methadone quantitation ion to its deuterated analog.

Samples were extracted on solid-phase columns previously activated by the sequential addition of 2 mL each of methanol, water, and phosphate buffer. Buffered urine was then added to the column, and the liquid was pulled through at ~1 mL/min under reduced pressure. The columns were washed with 3 mL of phosphate buffer and 1 mL of 1 N acetic acid and dried for 10 min under reduced pressure. Potentially interfering substances were removed by adding 5 mL of dichloromethane/isopropanol (2:1, by volume). The columns were again dried under reduced pressure for 2 min.

Methadone was eluted from the column with 3 mL of 2% triethylamine in isopropanol (by volume). The eluant was evaporated to dryness under nitrogen at 70 °C, reconstituted with 50 μL of reagent alcohol, and transferred to autosampler vials for analysis.

Analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph fitted with a 15-m DB5-MS capillary column with a 0.25 mm i.d. and a 0.25-μm film thickness. The injection port was maintained at 275 °C; the transfer line temperature was 280 °C. Helium was used as a carrier gas at 45 cm/s, maintained in a constant flow mode by electronic pressure control. The initial oven temperature was 100 °C; the temperature was programmed to change at 15°/min to a final temperature of 320 °C. One microliter of sample was injected in a splitless mode (1 min splitless time), using an HP-7673A autosampler. The mass spectrometer was an HP-5970 operated in electron impact–ion selective mode. The ions monitored for methadone were 294 (the quantitation ion), 223, and 309.

The ions monitored for d₃-methadone were 297 (the quantitation ion) and 312. Each ion was monitored for 30 m sec. The electron multiplier voltage was 2000 V. The method would also confirm and quantitate EDDP, if present. Assay performance characteristics for GC–MS confirmation included a limit of detection of 50 μg/L, a limit of quantitation of 75 μg/L, and an upper limit of linearity of 1500 μg/L.

For the identification of verapamil and its metabolites, the mass spectrometer was operated in scan mode, scanning from mass 40 to mass 550 at 0.84 scans/s; the GC program was also different, starting at an initial temperature of 45 °C (1 min hold), then ramping at 20°/min to a final temperature of 330 °C.
Six random urine samples that were shown by GC–MS to contain verapamil and its metabolites were analyzed according to a modification of the HPLC procedure described by Kuwada et al. (5). An AC 8 column was used instead of a C 18 column, and the mobile phase was two parts aqueous ion-pair buffer to one part of acetonitrile to maintain selectivity. The results are shown in Table 1.

Thirty-six urine samples that screened positive for methadone by the Diagnostic Reagents immunoassay kit were included in this study. Of these, 12 (33%) were confirmed positive for the presence of methadone, with an administrative reporting concentration of 300 µg/L. One sample was confirmed for methadone at a concentration of 91 µg/L. Seven of these 12 methadone-confirmed positive samples were examined for the presence of verapamil and its metabolites; none contained any verapamil-related compounds. Verapamil and its metabolites were identified in 20 samples (56%). These samples were negative for methadone at the limit of detection of the assay; in addition, no EDDP was detected. One sample was negative for both methadone and verapamil-related compounds, and the remaining two were negative for methadone but unavailable for verapamil confirmation.

Seven verapamil compounds were examined for cross-reactivity with the methadone immunochemical assay. These compounds were chosen because they have been shown to be the major species excreted in human urine (3). To achieve an immunochemical response equivalent to 300 µg methadone/L required 83 mg PR-25/L, 80 mg D-617/L, 30 mg PR-23/L, and 20 mg verapamil/L. Norverapamil, D-620, and PR-22 exhibited minimal or no cross-reactivity with the assay at concentrations up to 200 mg/L.

As can be seen from Fig. 1, compounds retaining the methyl group on the nitrogen exhibit the greatest cross-reactivity. Demethylation of the oxygen atom apparently reduces reactivity only slightly, as can be seen by examining verapamil and PR-23. Scission of the C—N—C bond will not reduce cross-reactivity as long as the nitrogen is still methylated, as seen with D-617 and PR-25.

Cross-reactivity data for verapamil and its metabolites would seem to indicate that there were insufficient concentrations of the compounds measured to account for the positive immunochemical result for methadone. Verapamil, the compound exhibiting the greatest cross-reactivity, is only present in concentrations of up to ~5 mg/L. Several explanations for this apparent discrepancy can be postulated. There are at least eight other metabolites of verapamil that were not included in this study, several of which contain a methylated nitrogen (3, 6). Eichelbaum et al. stated that they were unable to isolate any O-dealkylated metabolites from the urine without enzy-

---

Table 1. Concentration (mg/L) of various verapamil-related compounds in six human urine samples.

<table>
<thead>
<tr>
<th>Compound</th>
<th>A (mg/L)</th>
<th>B (mg/L)</th>
<th>C (mg/L)</th>
<th>D (mg/L)</th>
<th>E (mg/L)</th>
<th>F (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>0.25</td>
<td>5.3</td>
<td>0.3</td>
<td>0.9</td>
<td>5.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Norverapamil</td>
<td>1.4</td>
<td>6.9</td>
<td>3.1</td>
<td>4.3</td>
<td>9.9</td>
<td>3.0</td>
</tr>
<tr>
<td>D-617</td>
<td>20</td>
<td>12</td>
<td>15</td>
<td>36</td>
<td>31</td>
<td>18</td>
</tr>
<tr>
<td>D-620</td>
<td>7.2</td>
<td>5.0</td>
<td>5.3</td>
<td>17.8</td>
<td>12.3</td>
<td>3.7</td>
</tr>
<tr>
<td>PR-25</td>
<td>5.8</td>
<td>NDa</td>
<td>ND</td>
<td>ND</td>
<td>22.4</td>
<td>ND</td>
</tr>
<tr>
<td>PR-23</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>16.8</td>
<td>ND</td>
</tr>
<tr>
<td>PR-22</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, none detected.
matic hydrolysis (3); our findings are consistent with this observation (except for patient E). Our analysis did not include hydrolytic cleavage of glucuronide/sulfate conjugates, and may, therefore, have greatly underestimated the concentration of these compounds. Many conjugates of pharmaceutically active compounds exhibit substantial cross-reactivity with immunochemical assays. Another possibility is that the cross-reactivity is caused, in part, by a combination of analytes. Each sample examined by HPLC contained multiple metabolites, and although the concentrations of the individual metabolites were not sufficient to exceed the 300 μg/L threshold response, the combined presence of several compounds might account for the immunochemical response observed.

Although the apparently low concentration of verapamil and its metabolites detected in the false-positive specimens has yet to be definitively explained, it was demonstrated that these compounds do cross-react with the Diagnostic Reagents antibodies for methadone. Furthermore, the results of the study reiterate two important points. First, the results underscore that immunochemical results should only be used for excluding the presence of a particular drug from urine. Second, if positive identification is necessary, an alternative, nonimmunological method should be used.

The authors are indebted to Knoll AG for the verapamil compounds and to Daniel Lichtenwalner for assistance in the preparation of Fig. 1.

References

Simple, Rapid Nonradioactive Method to Detect the Three Most Prevalent Hereditary Fructose Intolerance Mutations, Catherine Costa, Jean Marc Costa, Jean-François Deleuze, Alain Legrand, Michelle Hadchouel, and Christiane Baussan (1 Laboratoire de Biochimie 1, Hôpital de Bicêtre, AP-HP 78 avenue du Général Leclerc, Le Kremlin Bicêtre; 2 Laboratoire de Biologie Moléculaire, Hôpital American de Paris; 3 U 347 INSERM IFR 21, Département de Pédiatrie, CHU de Bicêtre, France; * author for correspondence; fax 33 (1) 45 21 35 74)

Hereditary fructose intolerance (HFI) is an autosomal recessive disorder, the true incidence of which is not known but may be estimated as high as 1 of 20 000 (1, 2).

It is caused by any of several molecular abnormalities of the human aldolase B gene (3). Twenty-one mutations have been reported worldwide (2). Three mutations (A149P, A174D, and N334K) of the aldolase B gene account for 95% of the defective alleles in Europe (2, 4). They are investigated by various methods, including restriction enzyme digestion, direct sequencing, use of an amplification refractory mutation system, and allele-specific oligonucleotide hybridization (4). Unidentified HFI patients are at high risk if inadvertently given an infusion of fructose, sorbitol, or inverted sugar (5, 6). Three methods are used for diagnosis. The measurement of blood variables in response to intravenous fructose tolerance test and assay for aldolase B activity on liver biopsy samples are both invasive methods and do not allow heterozygote diagnosis (7). The third method, allele-specific oligonucleotide hybridization (4), circumvents these issues but is a radioimmunoassay restricted to certified laboratories. We have developed a simple and rapid method for detecting simultaneously the three most prevalent mutations in Europe by PCR-mediated, site-directed mutagenesis and restriction analysis. We investigated the diagnostic value of this method in patients from independent families.

Thirty-five HFI patients from 30 independent families were studied. HFI presented with acute symptoms (hypoglycemia, vomiting, and liver failure) before 6 months of age in 12 patients. In a second group of 23 patients, HFI presented with symptoms related to chronic exposure (hepatomegaly, growth delay, and aversion toward foods containing fructose) between 1 and 6 years of age. Diagnosis was assayed by conventional methods: intravenous fructose tolerance tests or aldolase B activity assays in liver biopsy samples.

Genomic DNA was obtained from peripheral blood leukocytes from 100 μL of blood by a method derived from the technique described by Higuchi (8). The PCR was used to amplify DNA sequences. Primers corresponding to exons 5 and 7 were derived from the genomic sequence. For the A149P mutation, the forward primer used was 5′-AGC GCT GTG CTC AGT ACA AGA A-3′ (ol149A), and the reverse primer was 5′-GTG AGG GAA CGC CCT GGC TCG CTT-3′ (ol149B). For the A174D mutation, the forward primer 5′-CAA CGC CCT GCC TCG CTG CG-3′ (ol149A) contained a mismatch (T:A) three bases upstream from the 3′ OH end to introduce a new site, TaqI (TCGA), only when mutant DNA is amplified; the reverse primer was 5′-CTA CTG GCT TGT GGG ACC AAA TCT-3′. For the N334K mutation, the forward primer used was 5′-CTT ACC AAA GAA ATG CTC AGA A-3′ (ol334A), and the reverse primer was 5′-AGT AGG TAT AGC AGG CTT GCA A-3′ (ol334B). Each PCR was carried out with 10 μL of the cell lysate by using AmpliTaq DNA polymerase (Perkin-Elmer). Each reaction was in a final volume of 50 μL containing 200 μmol/L of each deoxynucleotide triphosphate (Pharmacia LKB), 1.5 mmol/L MgCl2, 1.25 U AmpliTaq, and 0.2 μmol/L of each primer. Thirty PCR amplification cycles were performed after denaturation at 95 °C for 5 min as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s,