A solid-phase enzyme immunoassay (EIA) involving microtiter plates was modified for analysis of cocaine in sweat. Sweat was collected with the PharmChek™ sweat patch and drugs were eluted from the collection pad of the patch. The sweat contained primarily parent cocaine. The assay was determined to have cross-reactivity for cocaine of 102% relative to 100% for the benzoylecgonine (BE) calibrators and for cocaethylene of 148%. The optimum cutoff concentration for this modified assay, determined by receiver-operating characteristic curve analysis, was 10 μg/L cocaine or BE equivalents. At this concentration the assay had 94.5% sensitivity and 99.1% specificity vs gas chromatography–mass spectrometry (GC-MS) as an acceptable indicator of the true clinical state. The positive predictive value at a prevalence of 50% was 99%. Threshold analysis for positives suggested that the 95% confidence interval for a positive result by the EIA was between 12.5 and 15 μg/L and that quality-control samples at 5 and 15 μg/L could be run with each batch to certify the precision around the cutoff. All positive samples must be confirmed by GC-MS. The sensitivity and specificity of the overall analysis system (immunoassay screen and GC-MS confirmation) was 86% and 97%, with known cocaine dosing of volunteers as the acceptable indicator of the true clinical state.

INDEXING TERMS: sensitivity • specificity • predictive value • benzoylecgonine • ecgonine methyl ester • gas chromatography–mass spectrometry • ROC curves

Drugs are excreted in sweat and, for basic drugs, sudoriferous excretion may be a significant route of elimination. Henderson and Wilson reported that >15% of a methadone dose was eliminated in sweat when sweat production was increased to 1 L/h by exercise [1]. Parent drug is the predominant analyte found in sweat [2, 3]. Cocaine and ecgonine methyl ester (EME) predominate in sweat after cocaine administration.5 Cone et al. [2] found that cocaine appeared in sweat within the first 4–8 h after administration of cocaine in controlled studies and peaked within the first 24–48 h. The cocaine concentrations in sweat are consistent with ~1–2% of the dose excreted. In this study a microtiter plate enzyme immunoassay (EIA) for cocaine metabolite was modified to detect cocaine in sweat.

Drugs in sweat were collected by subjects wearing a Band-aid®-like PharmChek™ sweat patch [2, 3]. Water (monomer and dimer forms), oxygen, carbon dioxide, and other gases pass freely through the polyurethane adhesive Tegaderm™ covering of the patch but molecules larger than vapor-phase isopropanol are excluded by the molecular pore structure (~2 nm) of the plastic membrane. In most cases parent cocaine persisted on the PharmChek sweat patch throughout the period of wear (7 days). Occasionally cocaine degraded to benzoylecgonine (BE) during the period of wear; however, cocaine use was still detectable when the patch collection pad was eluted with solvent. Any use of cocaine during patch wear was accompanied by the appearance of cocaine in sweat and the accumulation of increasing amounts of drug on the collection pad of the patch.

An immunoassay for screening for drugs in sweat must cross-react with the parent drugs and with the lipophilic metabolites excreted in sweat. It must have a dynamic range in the concentration range encountered with the sweat patch eluate and, for qualitative screening tests, have a cutoff concentration appropriate for this application. In this study receiver-operating characteristic (ROC) curve analysis [4–6] was used to choose a cutoff calibration that optimized diagnostic sensitivity, specificity, and predictive value. Gas chromatography–mass spectrometry (GC-MS) analysis was used as the measure of presence of the drug [5]. In addition, the clinical sensitivity and specificity of the complete system (immunoassay screen and GC-MS confirmation) for detection of cocaine in sweat was calculated from known dose studies [6].

5 Nonstandard abbreviations: EME, ecgonine methyl ester; EIA, enzyme immunoassay; BE, benzoylecgonine; ROC, receiver-operating characteristic; GC-MS, gas chromatography–mass spectrometry; LOD, limit of detection; SIM, selected-ion monitoring; and LOQ, limit of quantification.
Materials and Methods

SPECIMEN COLLECTION
Sweat was collected from human subjects by using the PharmChek sweat patch (Sudormed, Santa Ana, CA), which was worn on the skin by the subject for 7 days. The PharmChek sweat patch is a nonocclusive dressing consisting of a medical-grade cellulose blotter paper collection pad, covered by a thin (0.024 mm) polyurethane and acrylate adhesives membrane (Tegaderm transparent dressing; 3M, St. Paul, MN). A nine-digit serial number is printed underneath the polyurethane for use in chain of custody. The water component of sweat, vaporized by body heat, passes through the polyurethane; solids, salts, and drugs excreted in the sweat are trapped on the collection pad, which has a surface area of ~14 cm² and collects a minimum of 300 μL/day of insensible perspiration in a 22 °C environment. Exercise, higher temperatures, or other factors that increase sweating increase the amount collected. Patches were worn by nondrug users, by known drug users in controlled administration experiments, and by suspected drug users in field studies. All human-subject studies were approved in advance by the respective institution's responsible committee.

At the end of the wear period, the collection pad was removed with disposable tweezers and placed in a 5-mL capped tube. The cellulose collection pad was eluted with 2.5 mL of 0.2 mol/L (pH 5.0) acetate buffer with methanol (25:75, by vol). Pads and elution buffer were mixed for 30 min on a slow-speed reciprocating shaker at 150–200 cycles per minute. Aliquots of 50 μL were screened by immunoassay; aliquots of 1 mL were analyzed for cocaine and cocaine metabolites by GC-MS. Eluates were refrigerated for up to 10 days to batch specimens. Specimens found to be positive by GC-MS were frozen at −5 °C to −15 °C and stored.

IMMUNOASSAY
The STC cocaine metabolite microplate EIA (STC Diagnostics, Bethlehem, PA) is a solid-phase competitive immunoassay involving horseradish peroxidase labeled with a cocaine derivative. To each well 50 μL of patch eluate, control, or calibrator is added, along with labeled enzyme, and allowed to incubate for 30 min at room temperature. After competition to bind to an antibody that is fixed to the surface of the well, the wells are washed six times with distilled water, substrate (3,3',5,5'-tetramethylbenzidine) is added, and the color produced after a 30-min incubation (stopped with 100 μL of 1 mol/L sulfuric acid) is measured at 450 nm and at 630 nm as a reference according to the dual-wavelength instructions of the manufacturer (Bio-Tek Instruments, Winooski, VT). Dual-wavelength readings significantly reduce optical interference caused by scratched or fingerprinted microplates because the scratches or fingerprints similarly reduce the amount of light at both wavelengths, whereas the substrate changes affect the absorbance at only one wavelength. The absorbance is inversely proportional to the quantity of cocaine and cocaine metabolites in the specimen. The calibrators and controls in the kit consist of sweat pad elution buffer with BE added at 0, 5, 10, and 50 μg/L. Supplemented patch controls at 5 and 15 μg/L are provided separately or made up by the laboratory. Control patches are supplemented with working solutions of BE in methanol and dried at ambient conditions for 1 h.

The limit of detection (LOD) was defined from the signal-to-noise ratio at the zero-drug concentration as the mean zero absorbance (A₀) minus the noise times three (LOD = A₀ – 3 SD) [7]. The LOD was determined by obtaining the absorbance values for 24 negative patches and calculating the SD and 3 SD of the absorbance. The apparent BE concentration at the calculated LOD absorbance is the LOD of the assay.

The analytical sensitivity of the EIA around the cutoff concentration was assessed by analyzing replicate supplemented samples at concentrations of 2.5, 5, 10, 12.5, and 17.5 μg/L BE. The percent of positive responses was determined for each concentration. A positive response was an absorbance lower than the mean absorbance for the cutoff calibrator, which was determined for 10 replicate calibrator samples assayed after the calibration curve and before the supplemented samples. Samples (n = 72) were run on 7 different days at two different authors' laboratories at each concentration.

Cross-reactivity was determined by analyzing suspected cross-reactants at concentrations equal to the cutoff concentration, and at 10 times and 100 times the cutoff concentration. In addition, a series of common drugs was tested at concentrations of 10 000 μg/L (Table 1). The percent cross-reactivity was calculated as follows:

% cross-reactivity = apparent BE concentration from calibration curve added concentration of compound × 100.

The percent displacement in the immunoassay measured from the negative calibrator was calculated from the following:

<table>
<thead>
<tr>
<th>Table 1. Common substances with no cross-reactivity in the STC microtiter plate EIA for cocaine in sweat at concentrations of 10 000 μg/L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprazolam</td>
</tr>
<tr>
<td>Amobarbital</td>
</tr>
<tr>
<td>Amphetamine</td>
</tr>
<tr>
<td>Butabarbital</td>
</tr>
<tr>
<td>Butabital</td>
</tr>
<tr>
<td>Clidiazepoxide</td>
</tr>
<tr>
<td>Clonazepam</td>
</tr>
<tr>
<td>Clorazepate</td>
</tr>
<tr>
<td>Codeine</td>
</tr>
<tr>
<td>Demoxepam</td>
</tr>
<tr>
<td>Dextromethorphan</td>
</tr>
<tr>
<td>Diazepam</td>
</tr>
<tr>
<td>Ephedrine</td>
</tr>
<tr>
<td>Fenoprofen</td>
</tr>
<tr>
<td>Flurazepam</td>
</tr>
<tr>
<td>Gemfibrazi</td>
</tr>
<tr>
<td>Hydromorphone</td>
</tr>
<tr>
<td>Hydroxyzalpalam</td>
</tr>
</tbody>
</table>
For within-run and total precision a total of 280 collection pads were supplemented with BE. Forty pads at each concentration were prepared at concentrations of 2.5, 5, 7.5, 10, 15, 17.5, and 50 μg/L. BE in 100 μL of methanol working solution was dropped onto the pad and the pads were allowed to dry at ambient conditions for 1 h. Two supplemented pads from each concentration were eluted and analyzed by EIA every day for 20 days. Within-run and total precision were evaluated for the immunoassay according to calculations based on the NCCLS EP5-T2 [8].

GC-MS
Cocaine and cocaine metabolites were extracted from patch eluate buffer with solid-phase columns. Controls and calibrators consisted of 0, 4, 10, and 12 μg/L each of cocaine, BE, and EME in eluate buffer. Internal standards, consisting of 15 μg/L each of cocaine, BE, and EME, were added to each sample before extraction. Samples (1 mL) of eluent buffer were extracted in 200-μg Clean-Screen extraction columns under reduced pressure and eluted with methylene chloride:2-propanol (8:2 by vol) with 20 g/L ammonium hydroxide.

BE and EME were derivatized by using hexafluoroisopropanol and pentafluoropropionic anhydride and analyzed by GC-MS with selective-ion monitoring (SIM) [Hewlett-Packard (Palo Alto, CA) HP 5890 GC with 12 m × 0.18 mm DB-1 capillary, splitless, injection port, 250°C, program: 90°C, 20°C/min to 130°C, 35°C/min to 270°C (1.5 min), and 30°C/min to 300°C (1 min)]. The detector was a mass-selective detector in SIM mode monitoring the following ions (m/z): cocaine 182, 185, 272, 303, and 306; BE 318, 321, 334, 439, and 442; and EME 182, 185, 314, 345, and 348. Cocaine was analyzed in this same chromatographic assay in its undervatitized form. Retention times were cocaine 7.2 min, BE propyl derivative 6.5 min, and EME propyl derivative 4.5 min. Quantification was achieved by using the ratios of the m/z 182/185 and 318/321 ions for the drugs and the deuterated internal standards compared with those ratios for the external standard calibration curve. The limit of quantification (LOQ) was cocaine 4 μg/L, BE 2 μg/L, and EME 3 μg/L, and the LOD was cocaine 3 μg/L, BE 2 μg/L, and EME 2 μg/L.

ROC CURVE ANALYSIS
The number of true positives (TP), false negatives (FN), false positives (FP), and true negatives (TN) was determined for six putative cutoff concentrations (at 2.5, 5, 10, 15, 20, and 30 μg/L BE equivalents) by comparison of the immunoassay result with the result by GC-MS. A sample was considered a true positive if both the immunoassay and the GC-MS results were in concordance, i.e., both positive for cocaine (cocaethylene present above the putative cutoff concentration), or a true negative if both were negative for cocaine. Samples for which the immunoassay was positive (absorbance below the mean of the cutoff calibrator absorbance) but the GC-MS result revealed cocaine concentrations negative or below the LOQ were defined as false positives. Samples for which the immunoassay result was negative (absorbance above the mean of the cutoff calibrator) but the GC-MS result showed cocaine concentrations above the LOQ of the assay were defined as false negatives. Sensitivity and specificity were calculated according to the following formulas [9]: sensitivity = TP/(TP + FN); specificity = TN/(TN + FP).

Sensitivity was plotted vs 1 specificity for the six possible cutoff concentrations to obtain ROC curves [4]. Positive predictive value was calculated from the following formula:

positive predictive value =

\[
\text{sensitivity × prevalence} \div \left[ (1 - \text{specificity}) \times (1 - \text{prevalence}) \right] + (\text{specificity} \times \text{prevalence})
\]

Results

ANALYTICAL PRECISION AND ACCURACY
The LOD, calculated as 3 SD of the zero calibrators [7], was 0.76, 0.58, and 0.95 μg/L on 3 different days. From this the LOD for the STC Cocaine Metabolite Microtiterplate EIA was <1 μg/L. BE equivalents.

Cross-reactivity of the EIA at 10 μg/L was 143% for cocaethylene, 102% for cocaine, 20% for egonine, and 18% for EME relative to 100% for the BE calibrators. None of the 38 common drugs or chemicals tested at 10,000 μg/L showed any cross-reactivity in the assay (Table 1).

The analytical sensitivity of the EIA around the cutoff concentration was assessed by analyzing replicate supplemented samples at concentrations of 2.5, 5, 10, 12.5, and 17.5 μg/L. The percent positive response graphed for each concentration yielded the threshold response graph shown in Fig. 1. The slope of the curve is the qualitative response of the EIA. From this, one can see that the 95% confidence limits for a positive response lie between 12.5 and 15 μg/L. A sample containing exactly 10 μg/L cocaine and (or) cocaine metabolites has a 55% probability of being screened positive.

The intraassay precision (mean CV) of the absorbance averaged over 4 days (n = 15 each day) was 7.3% at 10 μg/L, 6.9% at 2.5 μg/L, 8.1% at 5 μg/L, 11.9% at 12.5 μg/L, 9.2% at 15 μg/L, and 8.8% at 17.5 μg/L. The interassay precision of the percent displacement over 20 days was 13.6% at 10 μg/L, 25.5% at 2.5 μg/L, 17.7% at 5 μg/L, 12.3% at 15 μg/L, and 10.6% at 17.5 μg/L.
Diagnostic Accuracy

As seen in Fig. 2, for 501 specimens from 290 subjects, with GC-MS as an acceptable indicator of the true clinical state (a "gold standard" reference for true presence or absence of cocaine), the false-positive rate rose rapidly below a cutoff of 5 μg/L and the false-negative rate rose rapidly above a cutoff of 10 μg/L. From the ROC curve (Fig. 3), the maximum sensitivity and specificity were obtained at a cutoff of 10 μg/L. At a cutoff concentration of 10 μg/L, the sensitivity of the immunoassay was 260/275 = 94.5% and the specificity was 224/226 = 99.1%. For these values the predictive value for a positive immunoassay result for cocaine and cocaine metabolites in sweat at 50% prevalence was 99%. This value is in the range reported for positive predictive values of commercially available laboratory immunoassay tests (RIA and EIA) for cocaine metabolites in urine [10], which ranged from 91.9% to 97.8% at a prevalence of 50%.

At the 10 μg/L cutoff, there were only two false-positive results for the 226 negative samples. For these two samples, the EIA gave a positive response but the GC-MS did not detect cocaine. In both of these samples, although there was no cocaine, there was a significant amount of BE. For these two instances, the humidity and pH conditions of the skin and the time of residence on the patch may have allowed hydrolysis of the cocaine present to BE. If the GC-MS results for BE are taken into consideration, there would be no false positives at a cutoff of 10 μg/L. Reanalyzing the data with the criteria for a positive GC-MS result of cocaine >4 μg/L or BE >2 μg/L once again resulted in the choice of 10 μg/L as the best cutoff for the EIA, with a sensitivity of 94.5% and a specificity of 100%.

Discussion

Validation of an immunoassay for use with alternative specimens requires that the immunoassay cross-react with the analytes found in the biological fluid, have a dynamic range within the concentrations of drug found in the fluid, and be accurate in detection of drug present. Accuracy for a qualitative test is validated by demonstrating that the cutoff concentration has the required diagnostic sensitivity and specificity to yield a useful predictive value in the populations encountered in screening situations. Obviously, in the absence of traditional or regulatory cutoff values, diagnostic sensitivity and specificity considerations should direct the choice of a cutoff. In this study ROC curves, obtained by using GC-MS for cocaine as the gold standard or reference as to whether the drug was present or not, indicated either 5 μg/L or 10 μg/L as the best cutoff. The latter cutoff was the more conservative choice, since it minimized the false positives at the expense of slightly increased false negatives.

However, no immunoassay-positive result would be reported without confirmation. The complete system, sweat-collection patch, immunoassay, and GC-MS, was evaluated by using known dosing of cocaine from controlled dosing studies [2, 3]. The concordance of positive and negative GC-MS results for cocaine on sweat-patch specimens with known drug doses given to patch-wearing subjects is shown in Fig. 4. From the data in Fig. 4, the clinical sensitivity of the overall system is 56/65 = 86% and the clinical specificity is 38/39 = 97%. The predictive value of a positive result reported for cocaine in sweat with this
system would be 96.8% at 50% prevalence. The data in Fig. 4 were obtained from 104 patch specimens from 38 subjects who participated in these controlled dosing studies. Drug doses given were 10 and 25 mg of cocaine administered intravenously; 32, 50, and 126 mg of cocaine administered intranasally; and 32 and 42 mg of cocaine smoked. These doses are typical of street drug doses used in a single hit. However, in actual drug abuse, the cocaine user does not typically stop after a single hit but continues to take additional hits as long as cocaine is available. Therefore, these results indicate the ability of the sweat collection and analysis system to detect cocaine in sweat after minimal cocaine use.

References