Technical Note

Automated Homogeneous Immunoassay Analysis of Cotinine in Urine

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Abstract

A study was conducted to evaluate the performance comparison of a homogeneous enzyme immunoassay (EIA) designed to detect cotinine in urine and carbon monoxide (CO) breath measurements to determine smoking status. The clinical comparison was done using urine and breath specimens from 218 volunteers. Urine samples were analyzed by immunoassay and confirmed by gas chromatography–mass spectrometry (GC–MS). Breath carbon monoxide was determined by a commercial analyzer. Using cutoffs of 10 ppm for CO and 500 ng/mL for urinary cotinine, the relative sensitivity/specificity was 93.6%/74.0%. The positive predictive value was 86.8%, and the negative predictive value was 86.5%. However, comparison of the EIA to GC–MS showed a sensitivity/specificity of 96.2%/98.4% and a positive predictive value of 99.3%. The EIA was also evaluated non-clinically for precision, stability, recovery, and interferences. In addition, the non-clinical evaluation demonstrated coefficients of variation from 0.37 to 1.09% across cotinine concentrations ranging from 0 to 5000 ng/mL. The assay was found to be highly specific for cotinine and cross-reacted to a limited degree with 3-hydroxycotinine. Finally, multiple freeze-thaw cycles of urines containing cotinine showed no degradation of the drug in the specimen when tested in the EIA. Thus, the EIA tested is a rapid, lab-based test that can reliably determine cotinine levels and their relation to smoking status.

Introduction

The health hazards associated with smoking, both active and passive, have led to widespread smoking cessation efforts in the workplace and other public settings (1,2). Therefore, an accurate and reliable method for validating smoking status is desirable. Exposure to tobacco smoke has been measured by a variety of methods including carbon monoxide (CO) monitoring and assays specific for nicotine metabolites. CO is a major component of tobacco smoke, and the relatively low CO levels found naturally in the body rise significantly upon inhalation of tobacco smoke (3). Nicotine can be ingested through a variety of vehicles, including smoke inhalation and chewing tobacco. Nicotine has a relatively short half-life (24–84 min), making it an unreliable indicator of smoking status. However, it is rapidly metabolized in the circulatory system to a number of metabolites. In a 24-h urine, 10% of a nicotine dose is excreted as cotinine, 35% as hydroxycotinine (3-OH-cotinine is the most abundant nicotine metabolite), and approximately 4% as nicotine-1'-N-oxide, with only 5% excreted unchanged. The excretion of nicotine is enhanced by acidification of the urine, whereas cotinine excretion is less affected by pH changes (3). Of nicotine metabolites, cotinine has a relatively long half-life (10–40 h) and is much more useful for identifying smokers. The assay of cotinine as the target analyte has been shown previously to be more sensitive and specific than CO monitoring for measuring smoking status (4). However, these comparisons were done using laborious radioimmunoassays.

The OTI AUTO-LYTE® Cotinine EIA is a homogeneous enzyme immunoassay used for the analysis of cotinine in urine. The assay is based on competition between drug in the sample and drug labeled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH) for antibody binding sites. Enzyme activity decreases upon binding to the antibody; free drug in the sample is proportional to enzyme activity. Active enzyme converts oxidized nicotinamide adenine dinucleotide (NAD) to NADH, resulting in an absorbance change that is measured spectrophotometrically at 340 nm (5). Using a large clinical analyzer, sample analysis is complete in less than 30 s. Therefore, the goal of this study was to evaluate this new method to determine smoking status and method robustness.

Materials

All chemicals used in the immunoassay were obtained from Sigma Chemical Co. (St. Louis, MO) or the Aldrich Chemical Co. (Milwaukee, WI) for reagents. Immunoassay kits were provided by OraSure Technologies (Bethlehem, PA). The CO2 monitor (Spirometric CMD-1) was purchased from Spirometrics Medical Equipment Co. (Auburn, ME). The key components in the immunoassay kit are the antibody and enzyme conjugate (glu-
cose-6-phosphate dehydrogenase labeled with trans-4-carboxy cotinine). The antibodies used were from sheep immunized with trans-4-carboxy cotinine conjugated to a hapten carrier. The assay's enzyme conjugate was prepared by covalently coupling trans-4-carboxy cotinine to glucose-6-phosphate dehydrogenase according to methods described elsewhere (5). The assay must be run on an analyzer capable of precisely timing and measuring reagents into reaction cuvettes. The assay relies on enzyme modulation which results from antibodies binding in proportion to the amount of free cotinine in a sample (5). In the presence of substrate, the enzyme will catalytically convert NADH to NAD which is measured spectrophotometrically at 340 nm. Although it may be possible to perform the assay on any spectrophotometer, it is recommended that a qualified clinical analyzer be used. For general applications, 125 μL of antibody reagent is mixed with 3 μL of sample, after which an additional 125 μL of enzyme conjugate is added. Immediately, readings are taken in 30-s intervals for up to 7 min. The change in NADH to NAD over the time period is plotted, and unknowns are compared to values from calibrators. In the present study, these calibrators were prepared in pooled negative human urine at concentrations of 0, 100, 250, 500, 1000, 2500, and 5000 ng/mL of cotinine.

For gas chromatographic–mass spectrometric (GC–MS) analysis, cotinine and cotinine-d3 were purchased from Radian (Austin, TX). Solid-phase extraction columns (SPE) were manufactured by United Chemical Technologies (catalog no. ZSDAU020, Bristol, PA). All other solvents and chemicals were high-performance liquid chromatography (HPLC) or A.C.S. grade.

Methods

This randomized, blinded, multi-site study was convened to compare the performance of the OTI AUTO-LYTE Cotinine EIA (urine) with the Spirometrics CMD-1/CO-Monitor in distinguishing smokers from non-smokers in a healthy adult population (75% smokers). In addition, the OTI Cotinine EIA was used semiquantitatively and compared with GC–MS and self-reports to determine smoking status.

Clinical study design

In all, 218 matching breath and urine specimens were collected and tested. Of these, 160 (73%) were from self-reported cigarette smokers. Of the self-reported cigarette smokers, six were also cigar smokers, one was a pipe smoker, and five used chewing tobacco and/or snuff. No specimens were obtained for nicotine gum or patch users. Specimens were donated by volunteers from the normal blood/plasma donor pools at the Biological Specialty Corporation (BSC). BSC is an independent blood and plasma donor facility located in Reading, PA.

Upon entrance into the study, each volunteer completed a medical history questionnaire and informed consent. If a volunteer qualified, clinical site personnel measured the CO level in his/her breath using the Spirometrics CMD-1/CO-Monitor and collected oral fluid and urine samples. All sample collections were made within 30 min of the CO measurement. Each urine specimen was tested individually for the presence of cotinine using the OTI AUTO-LYTE Cotinine EIA run on a Hitachi 747 Autoanalyzer. All urine samples were confirmed using GC–MS. The performance of the OTI Cotinine EIA was evaluated based on comparison to CO-Monitor or GC–MS results.

Urine was collected from each volunteer within 30 min of the CO breath test. A minimum of 10 mL urine was collected in a plastic sample bottle coded with the volunteer's ID number. Specimens were refrigerated immediately after collection and during transport. Urine specimens were tested for the presence of cotinine by EIA using a Hitachi 747 Autoanalyzer. Various cutoff levels for the immunoassay were tested. ROC analysis was performed to select the optimum cutoff.

CO procedure

Clinical site personnel administered the breath CO test using the CMD-1/CO Monitor according to the manufacturer's instructions. After the first measurement, the T-valve was removed, and the wand was waved back and forth in the air several times to allow all excess CO to escape from the sensor. The T-valve was then replaced and a second measurement taken. If the second measurement differed by > 20% from the first one, then the sensor was cleared again and a third measurement taken. All (two or three) measured CO levels were recorded. The average of the two valid measurements was used.

Non-clinical immunoassay studies

The EIA was also evaluated for non-clinical performance. These parameters included precision, cross-reactivity, reagent stability, effects of potential interferants, and freeze/thaw of samples.

Precision

The precision of the test was determined by running 40 replicates of 5 patient specimens (pooled and unpooled) that contained low to high concentrations of cotinine over a 5-day period. Intra-assay precision was calculated using data from the first day, and interassay precision was calculated from all five days.

Analytical specificity

The OTI AUTO-LYTE Cotinine EIA was designed to detect cotinine in urine. Structurally related compounds were also tested for cross-reactivity at concentrations up to 10,000 ng/mL. This included structurally related analogues of nicotine as well as other metabolites. The percent cross-reactivity was determined by comparison to a standard curve of cotinine calibrators. A series of non-structurally related compounds were also tested at 10,000 ng/mL in spiked human urine.

Recovery

Because the assay is semiquantitative, the percent recovery of spiked cotinine in urine from non-smokers was determined. Five urine samples of approximately 10 mL each were collected from self-reported non-smokers and spiked with 0, 250-, 500-, and 1000-ng/mL concentrations of cotinine. The samples were analyzed in duplicate with the OTI AUTO-LYTE Cotinine EIA. The percent recovery of cotinine (% RC) in each sample was calculated using the following formula:

\[
\% \text{RC} = \frac{X - Y}{Z} (100\%)
\]
where \( x \) = calculated cotinine concentration (ng/mL) for spiked sample; \( y \) = calculated cotinine concentration (ng/mL) for blank urine; and \( z \) = cotinine concentration for spike.

**Freeze-thaw study**

The effect of multiple freeze-thaw cycles on urine specimens containing cotinine was also determined. Two positive clinical specimens containing a low and high concentration of cotinine were used.

Duplicate 5-mL aliquots of each specimen were kept refrigerated into three separate plastic tube sets each marked “Cycle 1”, “Cycle 2”, and “Cycle 3”. All six tubes were frozen (-10 to -20°C) at 23 h, the “Cycle 3” tubes were removed from the freezer and thawed at room temperature (20-27°C) for 2 h, then returned to the freezer. At 22 h, the “Cycle 3” and “Cycle 2” tubes were removed from the freezer and thawed at room temperature for 2 hours, then returned to the freezer. At 65 h, the “Cycle 3”, “Cycle 2”, and “Cycle 1” tubes were removed from the freezer and thawed at room temperature. Finally, all six of the freeze-thaw samples and the two refrigerated samples were tested on Hitachi 747 (\( n = 5 \)).

**Interference study—Hitachi 747**

The effects of the potential interferants ascorbic acid, pH, specific gravity, protein, hemoglobin, bilirubin, and glucose on the OTI AUTO-LYTE Cotinine Assay were evaluated. Table 1 outlines the levels and conditions for each of the interferants tested. Stock solutions of (-)-cotinine were prepared in methanol at two concentrations (\( 1 \times 10^5 \) and \( 1 \times 10^6 \) ng/mL). The two stock solutions were used to spike 25-mL portions of each interferant solution as follows: 25 \( \mu \)L of \( 10^6 \) ng/mL cotinine to obtain 100 ng/mL cotinine, 18.8 \( \mu \)L of \( 10^5 \) ng/mL cotinine to obtain 750 ng/mL cotinine, and 37.5 \( \mu \)L of \( 10^5 \) ng/mL cotinine to obtain 1500 ng/mL cotinine.

Ascorbic acid, protein-human albumin, hemoglobin, bilirubin, or glucose was added to 100 mL of cotinine negative normal human urine at the target concentrations for each potential interferant. Cotinine stock solutions were spiked into 25-mL portions of each potential interferant solution to make stock solutions containing 100, 750, and 1500 ng/mL cotinine. The remaining portions of specific gravity solutions were not spiked (cotinine-unspiked). The cotinine-spiked specific gravity solutions and Cotinine stock solutions were spiked into 25-mL portions of each of the pH solutions to make interferant solutions containing 100, 750, and 1500 ng/mL cotinine. The remaining portions of pH solutions were not spiked (cotinine-unspiked). The cotinine-spiked pH solutions and cotinine-unspiked pH solutions were run on Hitachi 747 (\( n = 5 \)) according to the assay’s package insert.

A sample was considered unaffected by the interferant tested if the result was < 15% changed from the control containing no interferant.

**GC-MS**

Cotinine and cotinine-\( d_3 \) were purchased from Radian. Solid-phase extraction columns (SPE) were manufactured by United Chemical Technologies (catalog #ZSDAU020). All other solvents and chemicals were HPLC or A.C.S. grade.

Standards were prepared at concentrations of 0, 10, 50, 100, 250, and 500 ng/mL of cotinine in blank urine. Deuterated cotinine was used as the internal standard at a concentration of 100 ng/mL. Following buffering of 1 mL of urine with 2 mL of pH 6 phosphate buffer (0.1M), the samples were added to SPE columns that had been rinsed with 3 mL methanol, 3 mL deionized water, and 2 mL pH 6 phosphate buffer (0.1M) in a vacuum manifold. The columns were then washed with 3 mL deionized water, 2 mL 0.1M HCl, and 3 mL methanol followed by drying for 5 min. The cotinine was then eluted with 3 mL of methylene chloride/isopropanol/ammonium hydroxide (78:20:2). The solvent was evaporated to dryness under nitrogen at 40-50°C. The dried extract was reconstituted with 50 \( \mu \)L of ethyl acetate and transferred to an auto-sampler vial.

One microliter was injected into a Hewlett-Packard 5890 GC coupled to a 5970 MS in the splitless mode using selective ion monitoring. The injection port was set at 250°C. The initial oven temperature was 100°C ramping up to 300°C at a rate of 30°C/min with a final hold of 0.5 min. The interface was set at 280°C.

Ions monitored were \( m/z \) 176, \( m/z \) 98, \( m/z \) 119, and \( m/z \) 118 for cotinine and \( m/z \) 179, \( m/z \) 122, \( m/z \) 101, and \( m/z \) 121 for cotinine-\( d_3 \). Quantitative ions were \( m/z \) 176 and \( m/z \) 179.
Quantitation was based on a five-point standard curve using peak-area ratios. Qualitative criteria for retention time and ion ratios conformed to standard criteria.

Results

A total of 218 urine specimens from self-reported smokers (160)/non-smokers (58) were assayed for cotinine using the OTI AUTO-LYTE Cotinine EIA on a Hitachi 747 Autoanalyzer. In addition, each subject’s CO was measured within 30 min of urine collection. All specimens were confirmed for free cotinine in urine by GC-MS. Receiver Operating Characteristic Plot analysis based on 10 ppm CO and various concentrations of cotinine for the EIA suggest 500 ng/mL as the optimal cutoff for the immunoassay (Figure 1).

In addition, clinical sensitivity was calculated for carbon monoxide (CO) versus cigarettes per day (CPD), enzyme immunoassay (EIA) versus CPD, EIA versus GC-MS, CO versus GC-MS, and EIA versus CO. Figure 2 summarizes these comparisons that show that best sensitivity/specificity is achieved when EIA and GC-MS are used. Ranges for the concentrations seen among the smokers varied. Sorting the data, it was found that subjects self-admitting to 0, 1–10, 11–20, 21–40, or > 40 cigarettes per day had cotinine values ranging from 0 to 2025; 3 to 3604; 40 to 3745; 144 to 3261; and 1643 to 2225, respectively.

Non-clinical evaluation of EIA precision demonstrated coefficients of variation for intra-assay of 0.37–0.75% and for interassay of 0.66–1.09% over 5 days using calibrators ranging from 0 to 5000 ng/mL. These results were determined using the signal reporting units from the Hitachi 747 Autoanalyzer.

Analytical specificity was determined by spiking various ubiquitous, medicinal, and structurally related compounds into drug-free, normal human urine. The assay did not cross-react with any of the ubiquitous and non-structurally related compounds tested (Table II). The only compound that cross-reacted to any degree was 3-hydroxycotinine, whereas no cross-reactivity was seen to parent nicotine (Figure 3). In addition, none of the materials tested for interference affected the immunoassay. This includes the various levels of ascorbic acid, protein, hemoglobin, bilirubin, and glucose. Finally, urine adjusted to pH of 4–8 or specific gravity of 1.000–1.030 did not affect the immunoassay.

The assay is not considered quantitative because of the antibody’s potential to cross-react with various compounds. Therefore, the term semiquantitative is more applicable. Even though the assay is semiquantitative, recovery studies with cotinine spiked in urine were performed. The assay recovered 64–167% when tested with spiked urine containing 250–1000 ng/mL cotinine. This is not unexpected given the sigmoidal response curve for the immunoassay.

Finally, the stability of cotinine and its metabolites were assessed using low- and high-positive urine specimens in the EIA that were frozen/
thawed up to three cycles. No degradation of cotinine or its metabolites was seen in the EIA results. Results were within 5% of the original result suggesting urine specimens containing cotinine are easily stored, frozen, and reanalyzed.

Discussion

Methods to distinguish smokers from non-smokers have included chemical and biochemical techniques such as immunoassay, HPLC, GC–MS, and chemical colorimetric techniques. Applications for determining smoking status have included epidemiological studies, insurance risk assessment, and cessation assessment (6). In any of these situations determination of smoking status is performed to assess health risk. Therefore, the method used should be rapid, specific, and easy to perform.

In this report, a homogeneous EIA is evaluated and compared to breath determinations of carbon monoxide and urine confirmation by GC–MS. Determination of CO is common, but not very practical for testing large populations. In addition, CO may be elevated because of a variety of conditions (1). In the current study, volunteers were tested after admission of smoking status. The data shown in Figure 1 suggest that many of them smoked close to the time of sampling, given the high levels of CO that were found.

In comparison, GC–MS is routinely used for confirmation testing for other drugs of abuse. It is a highly specific technique and considered the "Gold Standard" method. However, GC–MS (like CO determinations) is not able to rapidly test large populations and is even more labor and cost intensive.

Thus, a homogeneous EIA that can be used on numerous analyzers is ideal. As presented, the EIA method used on a Hitachi 747 Autoanalyzer is precise, specific, and unaffected by common medicines. Clinical sensitivity and specificity were greater than 90% when comparing EIA and GC–MS. This further supports the accuracy of the EIA.

Finally, for subjects who reported smoking more than one cigarette per day, the sensitivity/specificity of the EIA was 93/93%, and for CO, it was 82.6/85%. The positive predictive value for the EIA was 97.4%, showing the EIA to be highly accurate within the population tested.

In conclusion, the determination of smoking status has been studied in numerous body fluids including urine, blood, plasma, and saliva (7). Good correlation has been seen with determination of cotinine in blood and plasma (7). In comparison, urine is less predictable because it varies depending upon multiple factors but may be non-invasively collected (1,2,7,8). The study presented suggests the EIA evaluated provides a rapid, accurate, laboratory result which agrees well with GC–MS. The percent agreement between these methods is in agreement with previous studies which suggest cotinine to be the best indicator of smoking status but lacked a practical method such as the homogeneous EIA presented (2).

References


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