Assessment of a Simple Colorimetric Procedure to Determine Smoking Status in Diabetic Subjects

George Phillipou,1 Stephen K. James, Christopher J. Seaborn, and Patrick J. Phillips

The performance of a colorimetric assay for nicotine metabolites to validate self-reported smoking classification (nonsmoker, ex-smoker, and smoker) was assessed in a group of diabetic patients (n = 201). Comparison of the results with those of cotinine immunoassay (ELISA), by comparing respective areas under receiver operating characteristic curves, established the superiority of the cotinine immunoassay method. Adjusting the urinary concentrations of nicotine metabolites for creatinine excretion significantly lowered test performance. The sensitivity and specificity for the assay of nicotine metabolites to discriminate smoking classification within the diabetic patients at a threshold of ≥ 28 μmol/L “cotinine carboxylic acid equivalents” were 68.4% and 98.6%, respectively; the corresponding sensitivity and specificity for urinary cotinine at a cutoff of ≥ 0.5 μmol/L were 94.7% and 100%. The low sensitivity of the colorimetric urinary nicotine metabolites assay precludes its application as an objective assessment of smoking status in our patient population.

Indexing Terms: cotinine/urine/ELISA compared/ROC curves

There is strong evidence that smoking is a major independent risk factor for cardiovascular disease and mortality in diabetes (1), as well as being related to microvascular complications (2). Measurement of biochemical tobacco markers in prospective or epidemiological studies of diabetes mellitus should therefore provide several advantages, including confirmation of self-reported information and a quantitative estimate of the actual intake of cigarette smoke.

Although nicotine (see Fig. 1) is the major constituent of cigarette smoke, usually its principal metabolite cotinine is measured in plasma or urine by either chromatographic or immunoassay techniques (3). A simple colorimetric assay, based on the König reaction of urinary nicotine metabolites, has also been described (4, 5), with sensitivity and specificity similar to that for cotinine immunoassays (5, 6).

The main purpose of this study was to assess the validity of the colorimetric assay quantifying urinary nicotine metabolites as a verification of the smoking status of diabetic patients, the majority of whom had coexisting medical disorders (hypertension, cardiovascular disease) and were taking a variety of medications.

Fig. 1. Chemical structures for nicotine (1), cotinine (2), trans-3'-hydroxycotinine (3), cotinine carboxylic acid (4), and nicotinic acid (5).

Materials and Methods

Urine specimens. All new patients referred to the Diabetes Clinic are routinely assessed for renal complications by measuring their albumin excretion rate on a timed overnight urine specimen. Urinary creatinine concentration is also routinely measured at time of specimen receipt. For the present study, 252 of these urine specimens, which had been subsequently stored at −20°C for periods of 1–16 weeks, were available. The study met the precepts of our hospital ethics committee.

Smoking status. For 201 patients a self-reported smoking history was available either in an initial interview summary or from information in the clinical case notes. Patients were classified as nonsmokers, ex-smokers, or smokers. The ex-smokers group comprised not only individuals who had recently stopped smoking (within 1–2 months) but also others who had not smoked for many years.

Colorimetric assay of urinary nicotine metabolites. Cotinine carboxylic acid (CCA; Fig. 1), nicotine, cotinine, barbituric acid, and Chloramine-T were purchased from Sigma–Aldrich (Castle Hill, Australia).

The procedure used was essentially that reported by Peach et al. (4), except that CCA, rather than cotinine, was chosen as the calibration standard—being cheaper, available in stable crystalline form, and structurally closer to trans-3'-hydroxycotinine (Fig. 1), the main urinary metabolite of nicotine (7). In brief, the colorimetric method is as follows: Add 250 μL of urine, standard, or quality-control material, in duplicate, to a test tube, followed by 100 μL of acetate buffer (4.0 mol/L, pH 4.7) and allow the mixture to stand at room temperature for 15 min. Then in rapid succession, add 50 μL of aqueous potassium cyanide (100 g/L), 50 μL of aqueous Chloramine-T (100 g/L), and 250 μL of barbituric acid solution (10 g/L, in an equivalence mixture of water and ace
Table 1. Characteristics of diabetic patients (n = 201) and concentrations of urinary tobacco markers.

<table>
<thead>
<tr>
<th></th>
<th>Non-smokers</th>
<th>Ex-smokers</th>
<th>Smokers</th>
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<tbody>
<tr>
<td>No.</td>
<td>120</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>% of total</td>
<td>59.7</td>
<td>21.4</td>
<td>18.9</td>
</tr>
<tr>
<td>Men, %</td>
<td>45.8</td>
<td>69.8</td>
<td>55.3*</td>
</tr>
<tr>
<td>Age, years</td>
<td>62 (41–76)</td>
<td>61 (47–72)</td>
<td>51 (47–72)b</td>
</tr>
<tr>
<td>Nicotine metabolites, μmol/L</td>
<td>11 (5–20)</td>
<td>11 (5–21)</td>
<td>38 (15–68)b</td>
</tr>
<tr>
<td>Nicotine metabolites/creatinine ratio, mmol/mol</td>
<td>2.1 (0.9–4)</td>
<td>1.5 (0.7–4.2)</td>
<td>6.7 (1.5–13.1)b</td>
</tr>
<tr>
<td>Cotinine, μmol/L</td>
<td>0.014 (0–0.09)</td>
<td>0.033 (0–0.16)</td>
<td>5.09 (2.48–5.6)b</td>
</tr>
<tr>
<td>Cotinine/creatinine ratio, mmol/mol</td>
<td>0.002 (0–0.015)</td>
<td>0.007 (0–0.017)</td>
<td>0.80 (0.158–2.14)b</td>
</tr>
</tbody>
</table>

*χ² = 7.4, P = 0.025.

bSignificantly different from non- or ex-smokers: P < 0.0001 (Mann–Whitney U test).

tone); vortex-mix; and let the samples stand at room temperature in the dark for 15 min. Vortex-mix the samples again, dispense 300 μL into duplicate wells of a microtitre plate, and record the absorbance at 490 nm with background subtraction at 630 nm (we used an EL311 microplate reader from Bio-Tek Instruments, Winooski, VT).

The concentration of nicotine metabolites was determined by reference to a standard curve prepared by asessing CCA at 0, 6.25, 12.5, 25, 50, and 100 μmol/L. The results therefore refer to “μmol/L CCA equivalents.” The intraassay variation (n = 20) was CV 6.7% at 70 μmol/L, and the quality-control urine (n = 19) had intra- and interassay CVs of 5.3% and 6.1%, respectively (mean, 77 μmol/L). The relative responses of nicotine, CCA, and nicotinic acid with respect to cotinine (100%) were 46%, 141%, and 24%, respectively.

Quantitation of urinary cotinine and albumin. We also used the competitive ELISA, Cofi-Traq, purchased from Serex (Tenafly, NJ), to measure urinary concentrations of cotinine. The assay, which has a stated minimum detection limit of 0.045 μmol/L cotinine, was used according to the manufacturer’s instructions. Sample concentrations calculated as exceeding the highest cotinine standard (5.68 μmol/L) were reported as ≥5.68 μmol/L.

Results

Comparison of self-reported smoking history and smoking status based on a urinary cotinine ≥0.5 μmol/L established five misclassifications: two false negatives—self-reported smokers with cotinine <0.5 μmol/L—and three false positives—self-reported “non” smokers with cotinine ≥0.5 μmol/L. All three false positives were from the ex-smokers group and each had a urinary cotinine concentration ≥5.68 μmol/L. The two negatives were one patient who had described herself as a “social” smoker (cotinine 0.17 μmol/L) and another (cotinine 0.18 μmol/L) who had been hospitalized for about a week when the urine specimen was taken.

Patients’ particulars and analyte values are summarized in Table 1. The relative proportion of patients in the three categories is similar to that recently reported for a larger sample (n = 3006) of people with diabetes (8). The data also indicate that current smokers are significantly younger than nonsmokers and ex-smokers, and that a difference exists between the categories for men and women—mainly a higher proportion of male ex-smokers.

Comparison of the sensitivities and specificities of the urinary nicotine metabolites and cotinine assays, corrected and uncorrected for creatinine excretion, is based on the areas under the receiver operating characteristic (ROC) curves (9) and is summarized in Table 2. The results establish that urinary cotinine determined by immunoassay is a significantly better discriminator of smoking status than is colorimetric estimation of nicotine metabolites, and that adjustment for creatinine excretion lowers diagnostic performance.

Figure 2 shows the sensitivity, specificity, and efficiency over a continuum of cutoff values for nicotine

Table 2. ROC curve analysis for assays of cotinine and nicotine metabolites.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Area under ROC curve ± SE</th>
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<tbody>
<tr>
<td>Cotinine, μmol/L</td>
<td>0.9990 ± 0.001a</td>
</tr>
<tr>
<td>Nicotine metabolites, μmol/L</td>
<td>0.9168 ± 0.030b</td>
</tr>
<tr>
<td>Nicotine metabolites/creatinine ratio, mmol/mol</td>
<td>0.8349 ± 0.044</td>
</tr>
<tr>
<td>Cotinine/creatinine ratio, mmol/mol</td>
<td>0.9974 ± 0.002</td>
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aP = 0.003 vs nicotine metabolites.

bP = 0.046 vs nicotine metabolites/creatinine ratio.

![](Fig. 2. Sensitivity (●), specificity (X), and efficiency (―) of different nicotine metabolites cutoff concentrations to discriminate a urinary cotinine ≥0.5 μmol/L.)

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metabolites to discriminate corresponding urinary cotinine concentration of ≥0.5 μmol/L in a larger sample (n = 282) that included subjects of unknown smoking status and for which the calculated smoking prevalence, based on a urinary cotinine ≥0.5 μmol/L, was 17.9%. The area under the ROC curve was 0.9569 ± 0.017 for the urinary cotinine determination, compared with 0.8882 ± 0.033 (P = 0.026) for the nicotine metabolites/creatinine ratio.

Discussion

In the original description of the colorimetric nicotine metabolites assay by Peach et al. (4), sensitivity and specificity were reported as 100% and 91%, respectively, for urine specimens collected from 78 nonsmoking and 103 smoking male volunteers. Subsequently, Barlow et al. (5), using a slightly modified procedure, cited a sensitivity and specificity of 93% and 96.6%, respectively, in 511 pregnant women (128 self-reported smokers) at 32–36 weeks of gestation. More recently, Bruckert et al. (10) compared three tobacco markers—urinary cotinine, thiocyanate, and nicotine metabolites—in 250 hyperlipidemic patients (143 smokers) and concluded that the nicotine metabolites assay can “accurately evaluate smoking status.” However, no performance characteristics for the three assays were provided.

In the present study, the sensitivity and specificity of the assay of urinary nicotine metabolites to characterize smoking status (cotinine ≥0.5 μmol/L) in the diabetic subjects (Table 1) were 68.4% and 98.6%, respectively, at a threshold value of ≥28 μmol/L. The sensitivity within our group of patients is therefore appreciably lower than previously found in healthy subjects (4–6). Unlike previous findings (5, 6), the cotinine immunoassay had significantly better performance to discriminate smoking status than did the colorimetric nicotine metabolites method (Table 2). Furthermore, adjusting the concentration of the nicotine metabolites for creatinine significantly decreased, rather than increased (4), assay performance (Table 2).

The colorimetric procedure, based on the König reaction, is not specific for nicotine or its metabolites but is a reaction common to most substituted pyridines. Furthermore, as stated above, the relative response, even among similar compounds (e.g., in Fig. 1), is quite varied. Therefore, both intra- and interindividual variations in overall nicotine metabolism may significantly affect the concentrations of urinary nicotine metab-

lites. Similarly, when metabolic pathways are affected, e.g., by certain medical disorders and certain medications, the discriminatory thresholds established in healthy subjects to characterize smoking status may no longer be accurate.

Validation of self-reported smoking is primarily aimed at assessing under-reporting (false negatives). Many studies, however, have found minimal misclassification in this direction, and the accuracy of self-reported status parallels that established by biochemical methods (11). The low sensitivity found in this study for the colorimetric assay of nicotine metabolites precludes its application as an objective measure of smoking status. We do not know the reasons for this low sensitivity, but it may be related to the pathophysiology of diabetes mellitus or to the medications used to treat diabetes and associated conditions.

References