Assessment of simple colorimetric procedures to determine smoking status of diabetic subjects

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The performance of a simple colorimetric assay for urinary nicotine metabolites to assess smoking status in diabetic subjects (n = 251) was investigated. Several variations of the colorimetric assay and a qualitative extraction procedure were evaluated in comparison with a cotinine immunoassay as the “gold standard.” Among these, the best overall performance was achieved with the qualitative test (sensitivity 95%; specificity 100%). The quantitative measurement of total nicotine metabolites performed less well (sensitivity 92%; specificity 97%) but could be improved by incorporating a blank extraction (sensitivity 98%; specificity 98%). Allowance for diuresis appeared to offer no advantage over the other methods. These results support previous findings regarding the use of these colorimetric procedures in nondiabetic subjects and, contrary to other recent observations, their performance was not impaired in diabetic patients.

Smoking is the major cause of preventable morbidity and mortality in the Western world; recent evidence indicates that about half of regular cigarette smokers will die from their smoking [1]. Because smokers often fail to admit to their addiction and because the efficiency with which smokers smoke their cigarettes varies greatly between individuals (Ellard GA & Wang DY, unpublished results), epidemiological studies of smoking-related diseases would be greatly facilitated if reliable methods could be established for identifying smokers and assessing their relative daily intakes of tobacco-derived compounds.

The most widely used specific marker for monitoring tobacco smoking has been the nicotine metabolite cotinine, determined in plasma or saliva by either RIA, gas chromatography, or GC/MS [2]. More than 10 years ago, however, one of us (G.A.E.) described a simple, inexpensive qualitative colorimetric urine test for assessing smoking status based on the König reaction, in which pink-red chromophores formed from nicotine and its metabolites by condensation with diethylthiobarbituric acid (DETB)4 were extracted into ethyl acetate [3]. According to the self-reported smoking status of the participating subjects, this method had a sensitivity of 100% and a specificity of 97%. The relative nicotine intakes of confirmed smokers were then assessed by quantifying the ratios of “total nicotine metabolites” to creatinine (TNM/C ratio method). Nicotine metabolites were estimated by using barbituric acid as the condensing reagent and a cotinine calibrator; creatinine was quantified by the alkaline picrate method [3].

We have subsequently used both the qualitative DETB extraction method and manual or automated quantitative TNM/C ratio methods to analyze >8000 urine samples in a series of smoking-related investigations [4]. In one of these investigations [5], parallel cotinine estimations undertaken with a capillary GC/MS method [6] showed that the sensitivity and specificity of the qualitative DETB procedure were 97% and 99%, respectively.

Interestingly, no other workers appear to have attempted to use the simple DETB extraction method to identify smokers, or to evaluate its specificity and sensitivity. Instead they appear to have automatically assumed that variants of the quantitative TNM/C procedure would be superior [7–9]. Furthermore, one of these groups [9] presented evidence suggesting that such methods were markedly inferior to cotinine-based procedures in diabetic patients and that adjusting urinary nicotine metabolite concentrations for creatinine lowered test performance.

The main purpose of this study was to assess the efficiency of the qualitative DETB extraction procedure and four alternative quantitative variants of the TNM method for identifying smokers in a population of dia-

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4 Nonstandard abbreviations: DETB, diethylthiobarbituric acid; TNM/C, ratio of total nicotine metabolites to creatinine; ACR, albumin/creatinine ratio.
abetic patients. For comparison, we made parallel cotinine determinations as the “gold standard.”

Materials and Methods

Urine samples were collected from 113 self-reported smokers and 138 self-reported nonsmokers attending the diabetic clinic at the Ealing Hospital. Given the overall self-reported prevalence of smoking among the patients of only 13%, the subjects were deliberately selected to produce similar numbers of smokers and nonsmokers. The patients’ ages ranged from 21 to 84 (mean 57) years for the nonsmokers and 19–83 (mean 53) years for the smokers. The samples were deep-frozen until analysis. All the analyses were undertaken “blind,” without reference to the self-reported smoking status of the patients. All samples were collected in accordance with the ethical code at Ealing Hospital.

The apparent smoking status of the patients was determined by the qualitative DETB extraction method as previously described [3], except that all the volumes used were reduced to 40% of that originally used. The apparent concentrations of TNM in the samples were also determined by assaying 200-μL aliquots with barbituric acid as the condensing reagent and quantifying results by comparison with a 56.8 μmol/L (10 mg/L) cotinine calibrator [3]. Creatinine concentrations were determined with use of alkaline picrate by the Jaffe reaction.

To investigate whether it might be advantageous to allow for the contribution of the natural color of the urine samples to apparent TNM concentrations, we also measured the absorbance (at 506 nm, the λ_{max} of the chromophore derived from cotinine) for 280-μL aliquots of acetone/water (5:9 by vol) in place of the potassium cyanide, Chloramine-T, and barbituric acid reagents. Intrassay CVs for the determination of TNM concentrations were 4.4% at 65.3 μmol/L and 2.4% at 27.5 μmol/L (n = 20); interassay CVs were 3.5% at 68.7 μmol/L and 6.6% at 27.8 μmol/L (n = 10).

After the colorimetric analyses were complete, urinary cotinine concentrations were determined by RIA, essentially as described by Knight et al. [10]. Aliquots (10 μL) of the samples or cotinine calibrators (concentration range 0.14–4.26 μmol/L) were incubated overnight with cotinine antibody and radiolabeled cotinine. Bound and free fractions were separated by using a polyethylene glycol-assisted second-antibody technique. The radioactivity of the bound fraction was counted and cotinine concentrations were estimated by using a 4-parameter logistic curve-fitting program. Within- and between-batch imprecision was 5.1% and 7%, respectively. The influence of diuresis on urinary cotinine concentrations was allowed for with the procedure described by Thompson et al. [11], in which log_{10} cotinine and log_{10} creatinine concentrations of the urine samples from the smokers were regressed against each other; the “adjusted” cotinine concentrations were those that would have been anticipated if the urinary cotinine concentration of any given sample had been equal to the geometric mean creatinine concentration.

Results

The distributions of urinary cotinine concentrations according to self-reported smoking status are shown in Fig. 1. The corresponding distributions when cotinine concentrations were adjusted to allow for the influence of diuresis are also shown.

Cutoff points for both adjusted and unadjusted urinary cotinine concentrations were at ~1.14 μmol/L (200 μg/L). Comparing the smoking status classifications that were based on either adjusted or unadjusted cotinine concentrations showed only two discrepancies. One patient, with unadjusted and adjusted cotinine concentrations of 1.35 and 1.0 μmol/L, respectively, was regarded as borderline and hence unclassifiable; the other patient, with values of 1.1 and 3.1 μmol/L, respectively, was considered to be a smoker. Hence, the biochemically (cotinine gold-standard) defined study population consisted of 130 nonsmokers and 120 smokers, 102 women and 148 men. Among the smokers the correlation between urinary cotinine and creatinine concentrations was highly significant (r = 0.39 for log-transformed data; P < 0.001).

Comparing the urinary cotinine findings with the patient’s self-reported smoking status showed 126 confirmed nonsmokers, 108 confirmed smokers, 4 “social” smokers, and 12 “deceivers.” The social smokers were self-reported smokers who had urinary cotinine concentrations below the 1.14 μmol/L cutoff point; their reported cigarette consumptions ranged from 1 to 4 per day. The deceivers were self-reported nonsmokers with cotinine concentrations of >1.14 μmol/L (range 1.5–67 μmol/L, geometric mean 12 μmol/L); the overall deception rate among the professed nonsmokers was thus 12 of 138, or 9%.

The urinary cotinine concentrations of the 108 confirmed smokers ranged from 1.14 to 77 μmol/L (median 18.8 μmol/L, geometric mean 15.9 μmol/L) and were similar in women and men (medians of 17.6 and 21.0 μmol/L, respectively).

The performance of the qualitative DETB extraction method as judged by the cotinine-based smoking status of the patients is summarized in the first section of Table 1. None of the 130 urine samples from the nonsmokers gave ethyl acetate extracts with a definite pink or red color (100% specificity): 127 gave essentially colorless extracts, and 2 gave extracts with a characteristically different blue/purple hue. One, with a concomitant cotinine concentration of 0.1 μmol/L, gave a blue/purple extract that was questionably pinkish and therefore was read as “doubtful.”

Among the 120 urine samples from cotinine-defined smokers, 3 were read as negative. One from a self-reported nonsmoker with a urinary cotinine concentration of 1.5 μmol/L gave a seemingly colorless extract. The other 2 samples from self-reported smokers gave blue/
purple extracts, which may have obscured the pink colors (colors given by relatively low concentrations of nicotine metabolites; the cotinine concentrations in these urines were 3.0 and 4.2 μmol/L). Three other urine samples from the biochemically defined smokers gave questionably pinkish extracts and were read as “doubtful”: One with a concomitant cotinine concentration of 5.7 μmol/L gave a predominantly peach-colored extract, whereas the 2 oth-

| Table 1. Comparative sensitivities and specificities of potential methods for smoker classification. |
|---------------------------------|-------|-------|-------|
|                                  | Cutoff| Sensitivity | Specificity | AUROC* |
| Qualitative DETB                |       | (n = 120a) |    |       |
| TNM, μmol/L                    | 11.7  | 95        | 100  | 0.983 |
| TNM — blank, μmol/L           | 6.6   | 92        | 97   | 0.991 |
| TNM/creatinine, mmol/mol       | 1.70  | 98        | 98   | 0.991 |
| TNM — blank/creatinine, mmol/mol | 1.14  | 95        | 98   | 0.986 |

a Cotinine-validated smokers.
b Cotinine-validated nonsmokers.
c Area under ROC curve.
ers had low concomitant cotinine concentrations (1.1 and 3.9 μmol/L). By contrast, the cotinine concentrations in the remaining 114 samples that gave definitely positive DETB results ranged from 1.8 to 76.5 μmol/L (geometric mean 16.8 μmol/L). Thus, the overall sensitivity of the qualitative DETB extraction method (counting doubtful results as negative) was 95%, 114 of 120.

The performance of classification strategies based on alternative quantitative measures are summarized in the remainder of Table 1. Attempting to classify subjects on the basis of urinary TNM concentrations was arguably less successful. It could, however, be improved by allowing for the presence of natural components in the urine absorbing at 506 nm (i.e., subtracting the blank reading) and, to a lesser extent, by ratioing to creatinine. However, attempting to allow for both the absorption of natural components and effects of diuresis by calculating (TNM – blank)/creatinine ratios did not result in an improved classification. The areas under the ROC curves for the different potential classification methods ranged from 0.983 to 0.991 and did not differ significantly among themselves (all \( P > 0.05 \)).

The correlations, amongst the smokers, between each of the quantitative procedures and the urinary cotinine determination, both with and without adjustment for diuresis, were determined by using Spearman Rank analysis and are expressed in Table 2. The strongest relationships with the cotinine (gold standard) estimations were obtained by correcting the TNM measurements (TNM – blank) for natural urinary components (Fig. 2). The relationship between TNM and cotinine worsened when cotinine was corrected for diuresis, whereas that of TNM/creatinine improved when cotinine was concomitantly adjusted for diuresis (Table 2).

The mean and median reported daily cigarette consumptions of both sexes of smokers were identical (12.5 ± 8.2 for women and 10 for men). However, the reported cigarette consumptions were extremely “digitalized,” with three-quarters of the smokers reporting smoking 5, 10, 15, 20, 30, or 40 cigarettes a day.

Median apparent TNM concentrations were essentially identical in both women and men, and were 3.4 μmol/L in nonsmokers and 47.7 μmol/L in smokers. However, because the creatinine concentrations in urine samples from women were lower than those from men [mean values of 6.2 (SE 0.35) vs 9.6 (SE 0.5) mmol/L], the mean TNM/C ratios were higher in the women [mean ratios for nonsmoking women 0.80 vs 0.58 mmol/mol for men, and for smoking women 10.5 (SE 1.3) vs 6.4 (SE 0.4) mmol/mol for men]. The corresponding median TNM/C ratios for smokers were 9.4 mmol/mol for the women and 6.0 mmol/mol for the men.

The elimination of nicotine metabolites by smokers did not appear to be influenced by early renal nephropathy [identified by increased albumin/creatinine ratios (ACRs)]. Thus the correlation coefficient (\( r \)) for the regression of log TNM vs log creatinine for the smokers with ACRs >4 was 0.77—compared with 0.59 for smokers

| Table 2. Regression analysis of nicotine metabolite methods. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | TNM             | TNM/creatinine  | TNM – blank     | TNM – blank/creatinine |
| Cotinine        | 0.89            | 0.57            | 0.89            | 0.59             |
| Adjusted cotinine | 0.78            | 0.68            | 0.85            | 0.69             |

All significant at \( P < 0.0001 \).
without microalbuminuria (ACRs <3). Consequently, among the smokers, the variance of TNM/C ratios (mean 7.5, SD 5.1) was significantly less (P <0.001) than that of TNM concentrations (mean 63, SD 55).

Discussion
The ages of the patients in our study were similar to those investigated by Phillipou et al. [9]. The cut-off urinary cotinine concentration (1.14 μmol/L, or 200 μg/L) we used to classify smokers was similar to that found (1–2 μmol/L) by Barlow et al. [7, 8], who used basically the same cotinine RIA [10] as we did here. Phillipou et al. [9], using an ELISA procedure, chose a cut-off value of 0.5 μmol/L. The appropriate cut-off will be influenced by the specificity of the method for determining cotinine. Thus in the study of De Waard et al. [6], who used a specific gas-chromatographic method with a detection limit of 90 nmol/L, the most appropriate cut-off was a urinary cotinine concentration of 0.40 μmol/L. In accordance with these conclusions, Jarvis et al. [12], who routinely determine cotinine by gas chromatography, recommended a cut-off of 0.28 μmol/L, and the gas-chromatographic results of Wall et al. [13] gave a cut-off of 0.22 μmol/L. By contrast, the results obtained by Wald et al. [14], using an earlier RIA method [15], suggested a considerably higher cut-off value (~0.73 μmol/L).

The median urinary cotinine concentration of the confirmed smokers in our study (18.8 μmol/L) was virtually identical to that found by Puhakainen et al. [8] among confirmed male smokers (19 μmol/L), but much higher than that (5.5 μmol/L) found by Barlow et al. [7] among pregnant smokers. The fact that we found similar urinary cotinine concentrations among both sexes in our diabetic smokers strengthens their suggestion [7] that the metabolism of cotinine may be accelerated during pregnancy. In keeping with the greater analytical specificity of the GC/MS method, used by De Waard et al. [6], the median urinary cotinine concentration in their (nonpregnant) female smokers (5.1 μmol/L) was considerably lower than that found in our study (18.8 μmol/L).

Based on the cotinine concentrations of the urine samples from the 250 subjects in our study, the specificity of the rapid and simple qualitative DETB extraction method was 100% and the sensitivity was 95%. These estimates are very similar to those obtained (99% and 97%, respectively) by comparing parallel GC/MS cotinine determinations and qualitative DETB urine test results in two recent interlocking investigations of lung cancer risk in a cohort of Dutch women [5, 6]. Thus the DETB test clearly performs excellently not only in normal, healthy subjects but also in diabetic patients.

The results presented in Table 1 show that if smoking classification were based on the quantitative estimations of TNM, as has been proposed by Barlow et al. [7], Puhakainen et al. [8], and Phillipou et al. [9], poorer discrimination would be expected (specificity 97%, sensitivity 92%).

Although exact comparisons between our findings and those of Barlow and Puhakainen and their colleagues are not possible, the three sets of results seem to be in general agreement. Thus median apparent urinary TNM concentrations in smokers and nonsmokers were respectively 43.3 and 7.9 μmol/L in the study by Barlow et al. (a 5.5-fold difference), 47.7 and 3.4 μmol/L in our study (a 14-fold difference), and 66.3 and 2.9 μmol/L in the study by Puhakainen et al. (a 23-fold difference). Barlow et al. chose a cutoff of 19 μmol/L; Puhakainen et al. [8] did not specifically state their cutoff value, but it was probably similar to that suggested by our analysis (11.7 μmol/L). Both studies chose cutoffs that would give cotinine-based specificities of 100%. A comparison of the results they obtained with their cotinine RIA and TNM estimations indicates that the plate reader modification of Barlow et al. [7] had a sensitivity of 95%, whereas that of the automated analyzer version of Puhakainen et al. [8] was virtually 100%.

By contrast, markedly inferior results were obtained by Phillipou et al. [9]. Their median apparent TNM concentrations in smokers and nonsmokers were 53.6 and 15.5 μmol/L, respectively, a difference of only 3.5-fold. It was therefore not surprising that their method performed so badly. Their use of a cutoff of 28 μmol/L to achieve a specificity of 98.6% yielded a sensitivity of only 68%.

One possible reason for their disappointing results may have been that they measured absorbance at 490 nm (with blank correction at 630 nm) instead of at ~508 nm, the absorption maximum for the chromophores derived from cotinine and other nicotine metabolites when barbiturate is used as the condensing reagent. This would have increased the relative contribution of natural (essentially yellow) urinary components to the final absorbance readings, giving increased absolute values and reducing the discrimination between smokers’ and nonsmokers’ urine samples. The benefit of using an appropriate blank correction is supported by the observation that the best correlations with cotinine measurements were given when TNM concentrations were corrected for the absorption of natural urinary components (Table 2). Allowing for the absorbance of the urine by using the blanking procedure might be worth exploring further, especially given the ease of its performance with a microtiter plate reader system similar to that devised by Barlow et al. [7].

Equally, the deliberate enrichment of our sample cohort to give approximately equal numbers of smokers and nonsmokers, albeit with the aim of increasing sample numbers with concentrations near the assay cutoff, may in itself have given rise to apparently superior sensitivity and specificity data. In comparison, in the study by Phillipou et al., the prevalence of self-reported smokers was only 19%.

The TNM/C ratios obtained among the diabetic patients in this study were in broad agreement with our findings in previous studies in essentially healthy subjects. Among the diabetic nonsmokers, TNM/C ratios
averaged 0.6 mmol/mol among the men and 0.8 mmol/mol among the women—values closely similar to previous mean values of 0.5 mmol/mol among men [3] and 0.7 mmol/mol for women [5, 16]. The nicotine intakes of our diabetic smokers also seemed generally similar to those of the healthy volunteers participating in previous investigations. Thus among the diabetic men, TNM/C ratios averaged 6.4 (SE 0.4) mmol/mol, which may be compared with a previous mean estimate of 7.1 mmol/mol [17]. The mean TNM/C value for the female diabetic smokers (10.5, SE 1.3, median 9.4 mmol/mol) appeared to be rather higher than that found in previous studies (mean 6.7, SE 0.4, median 6.1 mmol/mol) in the controls in the Dutch study [5] and median 6.9 mmol/mol among the pregnant smokers in the Scottish investigation [16], but this might have been a consequence of the relatively small number [30] of female diabetic smokers in our current study.

Despite the similarities of results with those from previous studies, the reported average daily cigarette consumption of our diabetic patients in the present study (12.5) was considerably less than that (18 per day) for all British smokers, as estimated from mean annual cigarette sales and prevalence of cigarette smokers [18, 19]. Given that the mean nicotine intakes of the diabetic patients were clearly not below average, perhaps their knowledge of the particularly harmful effects of smoking in diabetes had encouraged them to underreport their cigarette consumption. A similar phenomenon was seen in a recent study conducted among pregnant smokers [16].

In conclusion, contrary to the report of Phillippou et al. [9], quantitative variants of the König reaction with barbituric acid as the condensing reagent performed as well among our diabetic patients as among other previously studied groups. Thus there was no evidence in our investigation to support their suggestion that the pathophysiology of diabetes mellitus or the medications used to treat diabetes adversely influences the colorimetric assessment of smoking status. Apparent renal impairment as indicated by increased microalbumin/creatinine ratios did not diminish the influence of diuresis on the excretion of nicotine and its metabolites; thus, contrary to the evidence they presented, the variance of the urinary concentrations of TNM in smokers was reduced by rationing to creatinine. Furthermore, the evidence obtained in the current study (Table 1) suggests that, for screening patients in terms of their smoking status, the simple and rapid qualitative DETB method performs as well as any of the more technically demanding alternative quantitative König-based colorimetric procedures that might be envisaged for assessing smoking status. Only where sensitivity was deemed to be more important than specificity would estimation by the best quantitative procedure (TNM – blank) be preferable.

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References