Assessment of Smoking Status among Workers Using an Improved Colorimetric Method

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Abstract: To monitor smoking status among workers, we improved the colorimetric method to detect cotinine and other nicotine metabolites in urine. In adding ethanol in the reaction mixture, the quantitative measurable time defined as the duration with more than 95% of the peak absorbance, extended to 80 min in contrast to 16 min in the original method. As the analytical condition, a aliquot of urine sample (0.5 ml) was mixed with 0.5 ml of ethanol, with 0.2 ml of 4 M acetate buffer (pH: 4.7), with 0.1 ml of 150 mM KCN, with 0.1 ml of 0.44 M chloramin and then with 0.5 ml of 78 mM barbituric acid. A linear relationship was observed between cotinine concentration up to 80 µM and absorbance at 508 nm (r=0.998, p<0.01). Mean levels of nicotine metabolites among non smokers and smokers were 4.9 and 47.4 µM cotinine equivalent, respectively. Sensitivity and specificity were 96.1% and 96.7%, respectively for nicotine metabolites concentration among workers (n=385) when adopting 6.9 µM cotinine equivalent as a cut off value and the area under the ROC curve was 0.982. This method can be applicable to quantitative detection of smoking status.

Key words: Smoking status, Monitoring, Nicotine metabolites, Urine, Colorimetric method

Introduction

Smoking is one of the major risk factors for all causes of death, as well as cancer and cardiovascular diseases1, 2. Although continuous declining trends have been observed among Japanese men, the prevalence of smoking is still higher than in Western countries3. Health Japan 21, a national movement for health promotion in Japan, aims to reduce the prevalence of smoking, not only to avoid passive smoking4.

Questionnaires have been widely used to assess smoking status in the observational studies. However, to evaluate a smoking cessation program, self-reported status is not necessarily reliable5. Carbon monoxide in expired air and nicotine metabolites in saliva or urine samples have been used to evaluate smoking status quantitatively6. However, since the concentration of carbon monoxide decreases shortly after the last smoke, the determination of the concentration of nicotine metabolites is a more reliable indicator of smoking status than the concentration of carbon monoxide7.

Nicotine is metabolized into several metabolites and excreted into urine. Determination methods for cotinine, one of the major metabolites, have been developed using high performance liquid chromatography8, gas chromatography9 and radio-immuno-assay10. Although they were specific and sensitive, they are expensive and time consuming and not suitable for routine measurements. Colorimetric methods also have been developed to assess smoking status by detecting cotinine and other nicotine metabolites as cotinine equivalent using barbituric acid. It showed favorable sensitivity and specificity to distinguish smokers from non-smokers11, 12. However, since absorption density in the reaction mixture was attenuated immediately, automated analytical procedure was developed for routine
measurements\(^{13}\).

In this study, we improved the reaction condition to keep the height of optical density long enough that we can determine large number of urine samples using simple equipments. We applied this method to assess smoking status among workers and examined sensitivity and specificity.

**Materials and Methods**

**Subjects**

All workers in an electronics manufacturing factory were enrolled (n=410). Three hundred eighty five workers participated in this survey with written informed consent (men: 256, women: 129), with a response rate of 94.1%. Smoking status and number of cigarettes per day were assessed using a self-administered questionnaire at the annual health check-up in June 2000. Prevalence of smoking was 65.6% for men and 14.7% for women. Average number of cigarettes among smokers was 17.0 ± 6.5. Casual urine samples were also collected and stored in a freezer (–20°C) until analysis.

**Reagents and Standard Solution**

Cotinine was purchased from Wako Pure Chemicals (Osaka, Japan). Other reagents were purchased from Kanto Chemicals (Tokyo, Japan). We weighed 17.6 mg of cotinine and dissolved it in 100 ml of deionized water (1,000 µM) and then standardized by the commercial standard solution (1,000 µM) purchased from Sigma (Illinois, USA). We stored these stock solutions at 5°C until analysis.

**Original analytical procedure of nicotine metabolites**

Following to Peach\(^ {11}\) et al., we took 1 ml of 50 µM cotinine standard solution in a glass tube, added 0.2 ml of 4 M acetate buffer (pH: 4.7) and then mixed vigorously. As a reference, we used 1 ml of deionized water. Every 15 s, we added 0.1 ml of 0.15 M KCN, 0.1 ml of 0.44 M chloramin and 0.5 ml of 78 mM barbituric acid, respectively. Soon after we mixed the solution, we measured the optical density at 508 nm using a double beam spectrophotometer (Model U-2001, Hitachi, Tokyo) with a standard cell at 25°C and recorded the changes in the density with time. We defined quantitatively measurable time (QMT) as the period with the absorption at more than 95% of the maximal absorption. Urinary concentration of nicotine metabolites was expressed as cotinine equivalent using cotinine as a standard substance. Urinary concentrations of creatinine were determined by the method of Jaffe using a creatinine test kit (Wako, Osaka, Japan). Creatinine adjusted levels were calculated by dividing the cotinine concentration by the urinary creatinine levels.

**Modification of the reaction condition**

Instead of using deionized water in the aqueous standard solution (50 µM, 1 ml) in the original method, we used 1 ml of 20%, 40%, 60% and 80% aqueous solutions (V/V) of ethanol and examined the effectiveness to extend the QMT. We also examined the KCN concentration to reduce the amount of KCN used.

**Determination of nicotine metabolites in urine samples**

An aliquot of urine sample (0.5 ml) was mixed with 0.5 ml of ethanol, with 0.2 ml of 4 M acetate buffer (pH: 4.7), with 0.1 ml of 1.5 M KCN, with 0.1 ml of 0.44 M chloramin and then with 0.5 ml of 78 mM barbituric acid. As a reference, 0.5 ml of urine sample was diluted with 1.4 ml of deionized water. After 100 min left at room temperature, optical density was measured at 508 nm. Since values obtained by the colorimetric method were sums of nicotine metabolites, we expressed them as the cotinine equivalent concentrations using cotinine as a standard.

**Statistical analysis**

SPSS for Windows (SPSS Corp.) was used for statistical analysis. Sensitivities and specificities were calculated based on the area under receiver operating characteristics (ROC) curves for determining smokers from non-smokers. And then we compared the usefulness of urine concentrations of nicotine metabolites and the creatinine adjusted values\(^ {14}\).

**Results and Discussion**

We examined the changes in absorbance with the addition of ethanol in the reaction mixture for the cotinine standard solution (50 µM). Table 1 shows the QMT with the various concentrations of ethanol in the standard mixture. When the ethanol concentration was higher, QMT became longer. In the original method, absorbance reached a peak in 13 min and decreased rapidly thereafter. QMT of the original method was 16 min. Using 1 ml of 21% ethanol, although it took longer to reach a peak (33 min), QMT became longer than that under the original method (41 min). Using 51% ethanol, it took the longest to reach the peak (64 min). QMT became the longest with 57% ethanol (98 min). However, the reaction mixture went turbid when the concentration of ethanol exceeded 34%. The extending effect of QMT may be in part due to hydrophobicity of the reaction mixture since a similar effect was observed with methanol, propanol and glycerol while QMT extension was not as long as in ethanol.
(data not shown). Hereafter, we adopted the mixture of 0.5 ml of ethanol and 0.5 ml of aqueous cotinine standard in the reaction mixture (21% ethanol in the reaction mixture).

When the concentration of KCN decreased from the original condition (1.5 M) to 15 mM, time profile showed similar changes to those under the addition of ethanol. Time to get maximum absorbance became longer as the KCN concentration decreased (11 min and 27 min at 1.5 M and 150 mM KCN, respectively). QMT also became longer (17 min and 42 min at 1.5 M and 150 mM KCN, respectively). However, absorbance became lower in the concentration of less than 15 mM. Figure 1 show the changes in the time profile of the absorbance according to different conditions of ethanol and KCN in the reaction mixture. When we used 0.1 ml of 150 mM KCN (C) without adding ethanol, QMT become much longer than in the original method (A). With adding 0.5 ml of ethanol, QMT was also longer when we used 150 mM KCN (D) than when we used 1.5 M KCN (B). Thus, we adopted 150 mM for KCN concentration.

A linear relationship was observed from 5 μM to 80 μM (r=0.9998, p<0.001, n=10) using the final condition. In concentrations of higher than 80 μM, although the relationship was not linear, determination could be conducted up to 260 μM when we used a non linear analytical curve. Within-day variation of the determination was 1.0% (n=5) and day-to-day variation in consecutive 5 d was 5.2% (n=5). Detection limit was 0.15 μM cotinine equivalent (signal to noise ratio=2). Sample recovery of the urine sample of smokers was 109% (n=5).

Figure 2 shows the distribution of urinary concentration of nicotine metabolites (expressed as μM cotinine equivalent) among workers divided by the self-reported smoking habits. Mean levels of nicotine metabolites among non smokers and smokers were 4.9 and 47.4 μM cotinine equivalent, respectively. We performed ROC analysis for the levels of urinary nicotine metabolites and the creatinine-adjusted value for discriminating smoker from non-smoker. Sensitivity and specificity were 96.1% and 96.7%, respectively for cotinine concentration among workers (n=385) when adopting 6.9 μM cotinine equivalent as a cut off value and the area under the ROC curve was 0.982. Numbers of cigarettes between classified and misclassified smokers (17.0 and 16.5, respectively) and percentages of women were also similar for the two groups (7.3% and 9.5%, respectively).

However, sensitivity and specificity for creatinine-adjusted cotinine level were 95.0% and 95.3%, respectively and the
area under ROC curve was 0.976. Thus, creatinine adjustment did not improve the discrimination in agreement with a previous report\textsuperscript{10}. The crude value seems to be appropriate for identification of smokers.

A significant correlation coefficient (r=0.246, p<0.01) was also observed between the number of cigarette per day and concentrations of nicotine metabolites among smokers (n=177) in similar to the original method\textsuperscript{11}). However, the urinary concentration of nicotine metabolites was not appropriate to estimate the amount of nicotine intake since the concentrations were overlapped considerably among light smokers and heavy smokers (46.5 ± 36.5 and 59.0 ± 39.3, respectively). This simple colorimetric method can be used to monitor smoking status among healthy workers, but it may not be useful among the patients taking drugs since this method may detect various unrelated substances\textsuperscript{90}.

Conclusion

We modified a simple colorimetric determination method for urinary nicotine metabolites. The sensitivity and specificity of this method was 96.1\% and 96.7\%, respectively. This method could be applicable to quantitative identification of smoking status among healthy workers.

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References