Quantification of Cotinine in Plasma and Saliva by Liquid Chromatography

Dwayne A. Machacek and Nai-Siang Jiang

Measurement of cotinine, a nicotine metabolite, has been studied as a method for monitoring smoking behavior and determining smoking status. We describe a specific, sensitive method for quantifying it in plasma and saliva by reversed-phase paired-ion liquid chromatography and detection by absorbance at 257 nm. The cotinine is extracted with methylene chloride, and 2-phenylimidazole is the internal standard. Cotinine peak heights are linearly related to the amount on the column from 0 to 500 ng. The mean (± SD) concentration of cotinine in plasma of 31 passively exposed nonsmokers was 2.1 ± 1.6 µg/L (range, 0–7.9 µg/L). The regression of saliva cotinine concentration (y) on plasma cotinine concentration (x) at 0, 24, and 48 h in 10 smokers who refrained from smoking for 48 h was y (µg/L) = 1.155x (µg/L) + 0.245 (r = 0.986). The efficiency of cotinine as a biological marker was determined at 0, 24, and 48 h of smoking abstinence. Within-run CVs were 3.5% (n = 5) and day-to-day CVs 4.4% (n = 6) at 150 µg/L.

Additional Keyphrases: nicotine・tobacco smoking・chromatography・reversed-phase ion-pair・phenylimidazole as internal standard・measurements in passively exposed nonsmokers・cutoff value

Biochemical markers have been used to supplement information about smoking behavior obtained through questionnaires (1, 2) or from direct observation or physical signs of tobacco use at examination or interview. These markers include nicotine or cotinine (its primary metabolite) (3–5), carboxyhemoglobin (6, 7), thiocyanate (2, 8, 9), and carbon monoxide in expired air (10, 11). The rapid metabolism of nicotine makes it less than ideal as a biochemical marker. However, cotinine has a longer biological half-life than nicotine and usually is present in higher concentrations in both blood and saliva. In a comparison study (7) of cotinine, carboxyhemoglobin, and thiocyanate concentrations in blood from 187 cigarette smokers and 181 nonsmokers, cotinine appeared to be the best biochemical marker for segregating smokers from nonsmokers.

The quantitative analysis of cotinine in physiological fluids can be achieved with gas–liquid chromatography (12, 13), radioimmunoassay (14, 15), and liquid chromatography (16–20). This paper describes a liquid-chromatographic method for measuring cotinine in both saliva and plasma. After extraction with an organic solvent, the components of the extract are resolved by chromatography on a column specifically designed for ion-pair applications. Use of an automated sample injector with this column provides improved sensitivity and increased sample capacity as compared with earlier liquid-chromatographic methods (16–20).

We have used this chromatographic method to measure cotinine in smokers and passively exposed nonsmokers. The half-life of cotinine has been determined by monitoring it in both plasma and saliva over a 48-h period. The efficiency of cotinine as a biological marker was determined in volunteers who had low daily cigarette consumption and who were willing to abstain from smoking for 48 h.

Materials and Methods

Reagents. Caffeine and (−)-cotinine were purchased from Sigma Chemical Co., St. Louis, MO 63178; (−)-nicotine and 2-phenylimidazole were from Aldrich Chemical Co., Milwaukee, WI 53201. The methanol, acetonitrile, and methylene chloride were from Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442. Sodium heptanesulfonate was from Eastman, Rochester, NY 14650. Potassium phosphate monobasic (ACS grade) was purchased from Fisher Scientific, Fair Lawn, NJ 07410.

Lavender-stoppered 10-mL blood collection tubes (Vacutainer) containing K3EDTA were from Becton Dickinson Immunodiagnostics, Rutherford, NJ 07070.

Apparatus. The chromatographic equipment consisted of a 255 × 4.6 (i.d.) mm Altex Ultrasphere-IP, C18 reversed-phase column, 5-µm particle size, from Beckman Instruments, Inc., Berkeley, CA 94710; a Model 7335 column inlet filter from Rheodyne, Inc., Cotati, CA 94928; and a Model 6000A chromatographic pump and WISP 710B automatic sample injector from Waters Associates, Inc., Milford, MA 01757. The ultraviolet absorbance detector, purchased from Kratos Analytical Instruments, Ramsey, NJ 07446, was a Spectraflow Model 773 with a deuterium lamp and a flow cell consisting of a 12-µL cuvette with an 8-mm path.

Procedures. The cotinine stock standard, 5.0 g/L in methanol, was refrigerated at 4 ºC until used (stable for three weeks). Before analysis, the cotinine standard was diluted with a mixture of acetonitrile and 50 mmol/L potassium phosphate buffer, pH 4.8 (25/75 by vol). A stock solution of 2-phenylimidazole (550 mg/L in methanol) was stored at 4 ºC (stable for three months). The chromatographic mobile phase, prepared daily, consisted of 170 mL of acetonitrile, 830 mL of 0.05 mol/L potassium phosphate, and 0.8 mmol of heptanesulfonic acid. This mixture was adjusted to pH 4.8 at room temperature and degassed before use.

The assay protocol was as follows. Acidify 1 mL of saliva or plasma with 70 µL of 1.5 mol/L H2SO4. Add 100 µL of 3.12 µmol/L 2-phenylimidazole (1:1000 dilution of the stock solution), and extract with 5 mL of methylene chloride in a 15-mL conical screw-capped tube by gentle tumbling on a rotating mixer for 10 min. The emulsion that may form during the extraction is broken up in an ultrasonic water bath. Separate the aqueous and organic phases by centrifugation.

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gation (10 min, 1000 x g); remove the methylene chloride fraction (lower phase) with a glass transfer pipette and discard it. To the remaining aqueous phase, add 70 mL of 3.6 mol/L KOH, and extract with 8 mL of methylene chloride (rotating mixer, 20 min). Centrifuge the samples for 10 min at 1000 x g. Remove and discard the aqueous layer. Wash the organic phase with 1 mL of 0.1 mol/L NaOH. Pipette 5 mL of the washed methylene chloride into a 15-mL conical glass tube, warm to 45 °C, and gently evaporate the solvent under nitrogen. The sample residues can be stored at -20 °C until analyzed.

For analysis, reconstitute each sample residue with 100 μL of the chromatographic mobile phase, transfer this to a limited-volume insert, and place the insert in an auto- sampler vial from which 50 μL is injected into the chromatographic system. The chromatographic flow rate is 1.0 mL/min. Cotinine is detected by absorbance at 257 nm and identified by retention time (4.5 min). The amount of cotinine in the sample is calculated from the peak on the strip-chart recorder relative to the peak for a known mass of cotinine standard. Sample extraction efficiency is calculated by dividing the peak height of the internal standard in the unknown sample by the peak height of the internal standard in the calibration standard.

Subjects. Our nonsmokers were 17 men and 14 women, ages 27–49 years, who were not exposed to tobacco smokers in their work area or principal living quarters. No attempt was made to restrict access of these subjects to coffee break areas or public meeting places, where some passive exposure to tobacco smoke was likely. The 10-mL venipuncture samples were collected into Vacutainer Tubes and kept on ice until centrifuged; 5 mL of the plasma was stored at -20 °C until analyzed. Each of the 18 smokers was questioned about their daily cigarette consumption and then the 10-mL blood sample was collected.

For the saliva and plasma cotinine comparison and for the biological half-life study, we selected from among the volunteers two men and eight women (ages 19–35 years) who generally smoked three to 20 cigarettes per day. Nine of these admitted they smoked daily; one (subject no. 2) described herself as an intermittent smoker who was not smoking at the time but agreed to smoke the night before the study was initiated. We asked these 10 subjects not to smoke for the 48 h after the baseline samples were collected. Both plasma and saliva samples were collected at baseline and 24 and 48 h. Each volunteer was instructed to collect a 3-mL saliva sample in a clean glass jar and bring it directly to the laboratory. Each sample was transferred to a glass conical tube and centrifuged at 1500 x g for 10 min. The clear supernate was transferred to a glass vial and stored at -20 °C until analyzed for cotinine. The blood samples were collected and processed as described above.

Results and Discussion

Cotinine is extracted from plasma or saliva in a single-tube extraction procedure. The initial extraction with methylene chloride at acidic pH removes nonpolar compounds that elute from the reversed-phase column after the internal standard. Eliminating these compounds shortens analysis time and also allows automated injection of cotinine extracts. 2-Phenylimidazole was chosen as the internal standard for three reasons: its extraction efficiencies from plasma and saliva are similar to those of cotinine in our methylene chloride extraction procedure; it absorbs well at 257 nm, which is close to the absorbance maximum for cotinine (262 nm); and its retention time on the analytical column, less than 12 min, permits rapid analysis.

Using an ion-pairing column at pH 4.8 delays the elution of the internal standard, thereby improving the resolution of cotinine from caffeine (Figure 1). In none of our samples was the caffeine peak large enough to interfere with quantification of cotinine.

The calibration curve for cotinine was linear from 0 to 500 ng injected onto the column. The smallest amount detectable was 200 pg per injection, which corresponds to 0.5 μg/L in plasma or saliva when the prescribed extraction procedure is used. The analytical recovery of cotinine added to a nonsmokers' plasma pool was 92.3%. The CVs within-run and day-to-day were 3.5% (n = 5) and 4.4% (n = 6), respectively, at 150 μg/L. The sensitivity of the assay sufficed to measure cotinine in plasma, both of active tobacco smokers and of nonsmokers who had been exposed to tobacco smoke.

The cotinine concentrations in plasma and saliva shown in Table 1 gave the regression line y (μg/L) = 1.55x (μg/L) + 0.245 (r = 0.986), x being the value for plasma, y for saliva. This degree of correlation indicates that either blood or saliva could be used for monitoring smoking behavior.

The biological half-life of cotinine in plasma and saliva was calculated from the cotinine values obtained for these 10 same subjects by the expression C24 = C0 (0.5) 24/t1/2, in which C0 and C24 represent the baseline and 24-h plasma or saliva cotinine values in μg/L and t1/2 is the biological half-life in hours. For the second 24-h period, C48 = C24 (0.5) 24/t1/2, in which C48 represents the final cotinine value. The two
Table 1. Cotinine Concentrations (µg/L) In Plasma and Saliva of 10 Cigarette Smokers Who Did Not Smoke for 48 Hours

<table>
<thead>
<tr>
<th>Usual daily cigarette consumption</th>
<th>Baseline</th>
<th>24 h</th>
<th>48 h</th>
<th>Mean cotinine half-life, h*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Saliva</td>
<td>Plasma</td>
<td>Saliva</td>
</tr>
<tr>
<td>10-12</td>
<td>110.2</td>
<td>102.0</td>
<td>56.8</td>
<td>64.0</td>
</tr>
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<td>6-7</td>
<td>13.0</td>
<td>13.7</td>
<td>4.8</td>
<td>6.6</td>
</tr>
<tr>
<td>7-8</td>
<td>148.9</td>
<td>189.5</td>
<td>64.6</td>
<td>92.8</td>
</tr>
<tr>
<td>10-15</td>
<td>160.0</td>
<td>194.8</td>
<td>41.0</td>
<td>42.1</td>
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<td>15</td>
<td>44.7</td>
<td>66.4</td>
<td>23.7</td>
<td>27.0</td>
</tr>
<tr>
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<td>131.8</td>
<td>131.8</td>
<td>50.7</td>
<td>62.3</td>
</tr>
<tr>
<td>5</td>
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</tr>
<tr>
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<td>159.6</td>
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<tr>
<td>10-20</td>
<td>198.4</td>
<td></td>
<td>85.4</td>
<td>104.0</td>
</tr>
</tbody>
</table>

*Overall means (±SD): from values for plasma, 20.2 ± 5.4 h (n = 18); from values for saliva, 18.8 ± 7.0 h (n = 15). **Was not smoking regularly, but smoked three cigarettes the night before baseline measurement. ***Insufficient sample for cotinine analysis.

plasma and two saliva half-lives for each subject were averaged (Table 1). The high half-life value for subject 7, 49.9 h, represents the half-lives of 17.4 h on day 1, 66 h on day 2. We believe that this subject smoked on day 2. Without the values for concentrations in plasma and saliva at day 2, subject 7 would have a mean half-life of 19.6 h (range, 13.7 to 29.2 h), which in fair agreement with the report by Lynch (21) of a mean terminal half-life for 47 subjects of 14.6 h (range, 7.1–28.0 h) and that by Benowitz et al. (22) of a mean half-life in 12 subjects (who had abstained from smoking for three days) of 19.7 (SD 6.5) h.

In our 31 nonsmoking subjects, the mean plasma cotinine concentration was 2.1 (SD 1.6) µg/L (range, 0–7.9 µg/L). The highest value was 7.9 µg/L; the two next highest values were 4.3 and 3.9 µg/L. We could detect no cotinine in three subjects.

The mean plasma cotinine concentration in 10 cigarette smokers who consumed an average of 10 cigarettes per day was 137.7 (SD 78.2) µg/L (range, 44.7–290.0 µg/L). In eight smokers who then increased their consumption to an average of 20 cigarettes per day, the mean was 302.0 (SD 118.2) µg/L (range, 181.1–524.3 µg/L). The correlation coefficient was 0.447. Differences in individual smoking habits (such as whether or not to inhale the smoke) and in metabolic rates would contribute to the wide range of cotinine concentrations and the poor correlation between cotinine concentrations and number of cigarettes smoked.

The smoking cessation study was used to determine the terminal half-life of cotinine and the effectiveness of using cotinine as a biological marker of smoking. We deliberately included some subjects whose cigarette consumption was less than 10 cigarettes per day. To determine if we could identify smokers correctly after 48 h of smoking abstinence, we chose a plasma cotinine value of 7.9 µg/L (>3 SD above our nonsmoker mean, 2.1 µg/L, and the highest value observed among our 31 nonsmokers) as the cutoff value. Any subject whose cotinine concentration exceeded this value would be considered a smoker. All values were >7.9 µg/L in the initial baseline samples. At 24 h, all smokers who smoked daily were correctly identified, but subject 2 was classified as a nonsmoker. At 48 h, concentrations in plasma and saliva were <7.9 µg/L in subjects 2 and 3.

Subject 2 was included in this study to illustrate that intermittent smokers may be difficult to identify correctly by using cotinine as the marker. The time elapsed between smoking and sample collection also is an important factor and should be considered before one uses cotinine as a marker of tobacco smoking.

References


