Anabasine and Anatabine as Biomarkers for Tobacco Use during Nicotine Replacement Therapy

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Abstract
In this study we determined urine concentration of the tobacco alkaloids anabasine and anatabine, nicotine and its metabolites cotinine, and nornicotine in 99 cigarette smokers and 205 smokeless tobacco users. We also investigated the possibility that anabasine and anatabine can be used as biomarkers for tobacco use during nicotine replacement therapy.

Urine samples and data on self-reported tobacco use were obtained from subjects enrolled in tobacco cessation programs. Urine concentrations of tobacco alkaloids and metabolites were measured and correlated with self-reported tobacco use. Concentrations of anabasine and anatabine were used to validate abstinence in smokeless tobacco users who used nicotine gum as part of the therapy.

Correlations of alkaloid concentration with self-reported tobacco use before treatment ranged from fair to poor. In subjects abstaining from smokeless tobacco but using nicotine gum, anabasine and anatabine levels were below the cut-point of 2 ng/ml despite high concentrations of nicotine and cotinine resulting from nicotine gum use.

Anabasine and anatabine concentrations in urine can be used to validate abstinence or measure the extent of tobacco use in persons undergoing nicotine replacement therapy.

Introduction
More than 4000 compounds have been identified in tobacco smoke, and at least 50 of these have been found to be carcinogenic (1, 2). Epidemiological studies in smokers indicate a dose-response relationship between the number of cigarettes smoked per day and the risk of developing certain smoking-related diseases (3). The alkaloid nicotine is the major pharmacologically active substance in tobacco (4). There is good evidence that most smokers are dependent on nicotine and that the severity of tobacco dependence may be related to the level of nicotine intake. Consequently, determining exposure to specific substances in tobacco and tobacco smoke is useful in epidemiological studies exploring relationships between exposure to particular toxic substances and development of disease, in assessing the outcome of tobacco dependence treatment programs, and in assessing the risks of potentially less harmful or nonaddictive tobacco products.

A major methodological issue is measuring exposure. Self-report measures, such as the number of cigarettes smoked per day, do not take into account individual differences in smoking behavior or consumption of tobacco products that may differ in their delivery of toxic substances. To validate self-reports of subjects in tobacco-dependence treatment studies, it is desirable to have a biochemical measure of tobacco use for determining treatment outcome (5–10). The most widely used biochemical measure of tobacco use is cotinine, the proximate metabolite of nicotine, which can be measured in blood, saliva, or urine (5, 6, 8, 10). Cotinine is quite specific for use of tobacco or for use of nicotine-containing medications. Small amounts of nicotine are found in some foods, but nicotine derived from dietary sources is insignificant compared with the amounts derived from tobacco use. Cotinine also has the advantage that it has a long half-life compared with nicotine (11). Cotinine concentrations do not fluctuate greatly during the day, and levels in blood are much higher than those of nicotine, thus facilitating its measurement. Thiocyanate (a metabolite of hydrogen cyanide) in serum, carboxyhemoglobin in blood, or expired carbon monoxide have been used to detect smoking, but these biomarkers have significant dietary and environmental sources, and are less specific and less sensitive for detecting smoking than nicotine or its metabolites (5, 6, 8, 10).

Although an excellent biomarker for tobacco use, cotinine is not a valid marker in persons undergoing treatment with nicotine medications such as gum, transdermal patches, nasal sprays, or inhalers. Carbon monoxide (expired CO or carboxyhemoglobin) and thiocyanate may be used to detect heavy

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smoking but, being products of combustion, are not applicable for detecting smokeless tobacco use. Substances that are present in tobacco, measurable in biological fluids, but not derived metabolically from nicotine would be valuable for validating tobacco abstinence in persons undergoing NRT.\(^3\)

Tobacco contains alkaloids, structurally related to nicotine (Fig. 1), that are not likely to be present in foods or to have other sources of exposure, and are not present in nicotine-containing medications. Nicotine in tobacco (12) and its metabolite cotinine (13) are nearly optically pure \(S\)-isomers, whereas tobacco contains substantial (4–45%) amounts of the \(R\)-isomers of the minor alkaloids nornicotine, anabasine, and anatabine (14). In this paper, we report concentrations of the tobacco alkaloids anabasine and anatabine in the urine of cigarette smokers and smokeless tobacco users, and describe the application of these measures to validate abstinence in smokeless tobacco users undergoing therapy with nicotine gum. Concentrations of nicotine and its metabolites nornicotine and cotinine, as well as anabasine and anatabine, in urine of a large population of cigarette smokers and smokeless tobacco users are also reported.

Materials and Methods

Subjects and Clinical Protocols. Subjects were persons enrolled in clinical trials for cessation of tobacco use. Study 1 (Pacific Research Institute) and study 2 (University of Minnesota) were programs for cessation of smokeless tobacco use that used nicotine gum as part of the therapy. Study 3 (University of California San Francisco) was a smoking cessation program using behavioral therapy and nicotine gum. Informed consent was obtained from all of the subjects. The studies involving human subjects were approved by the respective Institutional Review Boards.

Urine samples were obtained before treatment and, for studies 1 and 2, urine specimens were also obtained at follow-up. Urine samples were acidified with sodium bisulfate and stored frozen until analysis. Determination of anabasine and anatabine concentrations in urine was used as an outcome measure in studies 1 and 2.

In study 1 (15), 100 smokeless tobacco users were recruited to a four-session cessation program. All of the subjects were medically screened and randomly assigned to receive either 2 mg of nicotine gum (Nicorette) or a placebo gum, as an adjunct to the behavioral group treatment program. Both subjects and group leaders were blind as to the condition of the subject. Ninety-seven males and 3 females entered the program, and 76% completed the 6-week treatment program and attended the four group counseling sessions. Concentrations of anabasine and anatabine were determined in urine of all of the subjects before treatment, and in urine of 76 subjects completing the study.

Study 2 examined the effects of nicotine gum versus placebo by group behavioral treatment versus minimal contact treatment (16). Smokeless tobacco users (210) who were willing to quit were randomly assigned to one of the following: (a) behavioral treatment plus 2 mg of nicotine gum (\(n = 55\)); (b) behavioral treatment and placebo gum (\(n = 50\)); (c) minimal contact treatment and 2 mg of nicotine gum (\(n = 51\)); or (d) minimal contact and placebo gum (\(n = 54\)). Participants were asked to chew either active nicotine or placebo gum for a period of 8 weeks. At the end of this treatment period, subjects were given the option to receive another box of free gum. Participants assigned to the group behavioral treatment participated in eight sessions over the course of 10 weeks. Those individuals assigned to the minimal contact condition met four times for individual sessions with the nurse over the 10-week period. Nonuse of tobacco was determined by self-report. Concentrations of anabasine and anatabine were determined in the urines of the 105 subjects who were assigned to nicotine gum treatment before treatment and in urine of 103 of these subjects on follow-up, having completed the study. Urine samples from 118 subjects using nicotine gum in another study with a similar protocol (16) were analyzed to verify that nicotine gum does not contain significant amounts of anabasine or anatabine.

Study 3 urine samples (99 persons) were obtained from cigarette smokers before beginning smoking cessation programs. Of these, 52 were from a study of early versus late quitting (17). The subjects were 59% female and 91% Caucasian, who smoked >10 cigarettes per day. Subjects were between 21 and 60 years of age. The remaining 47 subjects were from a study of cognitive behavioral therapy versus psycho-educational therapy. All of the participants received NRT. Subjects were 52% women and 88% Caucasian who smoked >20 cigarettes per day.

Urine samples from 35 nonsmokers were obtained to determine the specificity of anabasine and anatabine for tobacco use. The subjects were persons who provided a urine specimen before beginning a study of the effects of a low dose of nicotine in smokers or were laboratory personnel. Nonsmoking status was determined either by plasma cotinine being <15 ng/ml (10) or by being obtained from laboratory personnel known to be nonsmokers. Of these, 49% were female and 49% were Caucasian.

Analysis of Urine Samples. Concentrations of nicotine and cotinine in urine were determined (limit of quantitation, 10 ng/ml) using gas chromatography with nitrogen-phosphorus detection by a modification of a method published previously (18). The structural analogs of nicotine and cotinine, 5-meth-
nicotine and 1-methyl-5-(2-pyridyl)-pyrrolidinone (orthocotinine), were used as internal standards. A procedure for simultaneous extraction of nicotine and cotinine was used (19).

Concentrations of anabasine, anatabine, and nornicotine (limit of quantitation, 1 ng/ml) were determined by gas chromatography-mass spectrometry (20).

**Data Analysis.** The ratio of nicotine:cotinine was determined for each individual subject, and the mean values for smokers and smokeless tobacco users were computed from these ratios. Analyses of differences in means comparing smokeless tobacco users and cigarette smokers were performed by t tests.

The criterion for tobacco use was set at a concentration of >2 ng/ml in urine for both anabasine and anatabine. The basis for this criterion was inspection of urine concentration data from a group of people who were known not to use tobacco.

Sensitivity and specificity of anabasine and anatabine concentrations for detecting tobacco use were determined as described by Browner et al. (21). Sensitivity was determined in 179 persons enrolled in smokeless tobacco cessation programs (studies 1 and 2).

Sensitivity (expressed as percent) is defined as 100 × a/(a + c), where a is the number of subjects who continued to use tobacco (true positives), and c is the number of tobacco users with concentrations of anabasine and anatabine below the cutoff of 2 ng/ml (false negatives). Self-reported tobacco use was considered to be accurate, because there would be no incentive to falsely report continued tobacco use.

Specificity was determined in 35 persons who did not use any form of tobacco. Specificity (expressed as percent) is defined as 100 × b/(b + d), where d is the number of nontobacco users with urine concentrations of anabasine and anatabine below the cutoff of 2 ng/ml (true negatives), and b is the number of nontobacco users with anabasine and anatabine concentrations >2 ng/ml (false positives).

**Results**

Concentrations of nicotine, cotinine, and the minor alkaloids anabasine, anatabine, and nornicotine in urine of smokers and smokeless tobacco users before beginning tobacco cessation programs are given in Table 1. Mean nicotine concentrations ranged from 1310 to 1960 ng/ml, and were significantly lower in smokeless tobacco users than in cigarette smokers. Mean cotinine concentrations ranged from 1790 to 2420 ng/ml, and were significantly higher in smokeless tobacco users than in cigarette smokers. The ratio of nicotine: cotinine in urine of smokeless tobacco users (subjects from studies 1 and 2 combined) and cigarette smokers averaged 0.67 and 1.24, respectively. The difference between the two groups was significant, $P < 0.005$. Because some investigators (22) have reported cotinine concentrations normalized to creatinine concentrations, levels expressed as ng/mg creatinine are also reported in Table 1.

Mean nornicotine concentrations in urine of all of the tobacco users were similar (range = 107–127 ng/ml), as were anabasine concentrations (range = 22–24 ng/ml). Mean anatabine concentrations in urine of cigarette smokers (22 ng/ml) were about half those found in urine of smokeless tobacco users (41–45 ng/ml).

To test the specificity of the alkaloids for tobacco use, we measured concentrations of nornicotine, anabasine, and anatabine in the urine of 35 nonsmokers (confirmed by cotinine analysis), who reported that they did not use other forms of tobacco and did not use nicotine-containing medications. Concentrations of nornicotine, anabasine, and anatabine were below the limit of quantitation (1 ng/ml) in all but 3 of the subjects. Of these 3, the urine of one subject contained 3.4 ng/ml anabasine, with anatabine and nornicotine below the limit of quantitation. Another of the 3 contained 4.4 ng/ml nornicotine and 1.7 ng/ml anatabine, with anatabine below the limit of quantitation. The urine of the third subject contained 12.2 ng/ml nornicotine, 2.42 ng/ml anabasine, and 1.84 ng/ml anatabine. Whether these results were because of a low level of tobacco use or exposure, or whether because of small amounts of substances interfering with the assay is unknown, although the subject whose urine contained measurable amounts of all three alkaloids might indicate some prior tobacco use. However, using the criterion of both anabasine and anatabine concentrations being >2 ng/ml to classify a person as using tobacco, none of the 3 persons would have been classified as having used tobacco, and the specificity for detecting tobacco use is 100% (21).

Concentrations of anabasine and anatabine were used to validate cessation of smokeless tobacco use in studies 1 and 2. Subjects were considered to have relapsed if concentrations of both anabasine and anatabine were >2 ng/ml. The results are presented in Table 2. In study 1, concordance between self-reported abstinence and urinary concentrations of both anabasine and anatabine <2 ng/ml was 100%. In study 2, the concordance was 79%. The absence of measurable amounts of either anabasine or anatabine in the urine of many subjects ($n =

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### Table 1. Mean concentrations of tobacco alkaloids in urine of smokers and smokeless tobacco users

<table>
<thead>
<tr>
<th>Smokeless tobacco study 1</th>
<th>100</th>
<th>nc/ml (SD)</th>
<th>an/Mg Creatinine (SD)</th>
<th>Range</th>
<th>Concentration</th>
<th>Mean ns/ml (SD)</th>
<th>Mean ns/ml (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anabasine</strong></td>
<td></td>
<td>24 (31)</td>
<td>41 (51)</td>
<td>0.20</td>
<td>&lt;2</td>
<td>107 (107)</td>
<td>1130 (1170)</td>
</tr>
<tr>
<td><strong>Anatabine</strong></td>
<td></td>
<td>19 (20)</td>
<td>34 (41)</td>
<td>0.16</td>
<td>&lt;2</td>
<td>87 (78)</td>
<td>1590 (2700)</td>
</tr>
<tr>
<td><strong>Nornicotine</strong></td>
<td></td>
<td>0.106</td>
<td>0.239</td>
<td>0.22</td>
<td>&lt;2</td>
<td>3.4–4.4</td>
<td>0.19–18.00</td>
</tr>
<tr>
<td><strong>Cotinine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>264–9470</td>
<td>187–22,100</td>
</tr>
</tbody>
</table>

**Smokeless tobacco study 2**

<table>
<thead>
<tr>
<th>105</th>
<th>nc/ml</th>
<th>an/Mg Creatinine (SD)</th>
<th>Range</th>
<th>Concentration</th>
<th>Mean ns/ml (SD)</th>
<th>Mean ns/ml (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anabasine</strong></td>
<td></td>
<td>23 (30)</td>
<td>45 (61)</td>
<td>0.20</td>
<td>&lt;2</td>
<td>127 (105)</td>
</tr>
<tr>
<td><strong>Anatabine</strong></td>
<td></td>
<td>16 (16)</td>
<td>32 (33)</td>
<td>0.16</td>
<td>&lt;2</td>
<td>102 (93)</td>
</tr>
<tr>
<td><strong>Nornicotine</strong></td>
<td></td>
<td>0.100</td>
<td>0.118</td>
<td>0.14</td>
<td>&lt;2</td>
<td>3.4–5.13</td>
</tr>
<tr>
<td><strong>Cotinine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>254–5920</td>
</tr>
</tbody>
</table>

**Cigarette smokers study 3**

<table>
<thead>
<tr>
<th>99</th>
<th>nc/ml</th>
<th>an/Mg Creatinine (SD)</th>
<th>Range</th>
<th>Concentration</th>
<th>Mean ns/ml (SD)</th>
<th>Mean ns/ml (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anabasine</strong></td>
<td></td>
<td>22 (23)</td>
<td>22 (24)</td>
<td>0.10</td>
<td>&lt;2</td>
<td>113 (103)</td>
</tr>
<tr>
<td><strong>Anatabine</strong></td>
<td></td>
<td>19 (14)</td>
<td>20 (17)</td>
<td>0.10</td>
<td>&lt;2</td>
<td>101 (64)</td>
</tr>
<tr>
<td><strong>Nornicotine</strong></td>
<td></td>
<td>0.08–4</td>
<td>0.83</td>
<td>0.33</td>
<td>&lt;2</td>
<td>3.3–302</td>
</tr>
</tbody>
</table>

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*ng/ml = ng/mg creatinine; a = number of true smokers; b = number of true non-smokers; c = number of false smokers; d = number of false non-smokers.*

* a Urine concentrations before beginning tobacco cessation programs.
* b $P < 0.005$ comparing smokeless tobacco versus cigarettes.
* c $P < 0.05$ comparing smokeless tobacco versus cigarettes.
Table 2  Urine anabasine and anatabine concentrations as outcome measures in smokeless tobacco cessation studies employing nicotine gum*

<table>
<thead>
<tr>
<th></th>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>76</td>
<td>103</td>
</tr>
<tr>
<td>Claiming abstinence</td>
<td>45</td>
<td>89</td>
</tr>
<tr>
<td>Validated abstinence</td>
<td>45 (100%)</td>
<td>70 (79%)</td>
</tr>
<tr>
<td>Number of deceivers</td>
<td>0 (0%)</td>
<td>19 (21%)</td>
</tr>
<tr>
<td>Number reporting relapse</td>
<td>31</td>
<td>14</td>
</tr>
<tr>
<td>Number of false negatives</td>
<td>7 (23%)</td>
<td>5 (36%)</td>
</tr>
</tbody>
</table>

* Subjects were considered to be using tobacco if concentrations of both anabasine and anatabine in urine were >2 ng/ml.

Discussion

Urinary concentrations of anabasine, anatabine, nor nicot ine, nicotine, and cotinine were determined in 99 cigarette smokers (study 3), and were compared with concentrations of these alkaloids in 205 smokeless tobacco users (studies 1 and 2) before initiating treatment (Table 1). The sums of concentrations of nicotine and its metabolite cotinine in the urine of cigarette smokers and smokeless tobacco users were similar, suggesting similar levels of nicotine absorption. The lower ratio of nicotine/cotinine in smokeless tobacco users (0.67) compared with cigarette smokers (1.24) is most likely a result of more nicotine being swallowed by smokeless tobacco users, which then undergoes presystemic metabolism to cotinine in the liver (24). Concentrations of anabasine and anatabine in urine of all tobacco users were much lower than concentrations of nicotine, as expected, because of much lower levels in tobacco (23). Cotinine levels were high because it is a major nicotine metabolite. Nor nicot ine, both a minor alkaloid found in tobacco and a minor metabolite of nicotine, was present at levels higher than those of anabasine and anatabine, but much lower than nicotine and cotinine.

Interestingly, concentrations of anatabine were on average 2-fold higher in urine of the smokeless tobacco users as compared with cigarette smokers, despite similar nicotine and cotinine levels, and although smokeless tobacco products contain considerably lower levels of anabasine than cigarette tobacco, 0.084 mg/gram versus 0.27 mg/gram (23). A likely explanation is that anatabine is decomposed to a much greater extent than

is nicotine in burning tobacco, resulting in lesser absorption by cigarette smokers than by smokeless tobacco users. It should also be pointed out that anabasine, anatabine, and nor nicot ine, being secondary amines, are capable of being converted to nitrosamines by reaction with nitrogen oxides or nitrite in vivo (25). N'-nitrosoanabasine and N'-nitrosornonicot ine are carcinogenic in animal models (26). For this reason, urine nornicotine concentrations, which have not been reported previously for a large population of tobacco users, are included in Table 1.

Objective outcome measures to validate self-reports of abstinence in tobacco cessation programs are needed (5–10). In addition, methods for quantitating tobacco consumption are needed in studies for evaluating potential harm reduction. The nicotine metabolite cotinine, measured in blood, saliva, or urine, is the most widely used biomarker for tobacco use (5). However, cotinine or other nicotine metabolites are not applicable for assessing tobacco use in persons undergoing NRT. The objective of our studies was to evaluate the use of urine concentrations of the minor tobacco alkaloids anabasine and anatabine as outcome measures for persons undergoing NRT. These alkaloids should not be present in nicotine-containing medications, and, indeed, we found that subjects abstaining from smokeless tobacco and using nicotine gum did not excrete measurable amounts of anabasine or anatabine.

Urinary levels of anabasine and anatabine were evaluated as outcome measures for smokeless tobacco cessation in two studies that used nicotine gum as part of the treatment. There was generally good concordance between self-reported tobacco abstinence, and urine concentrations of anabasine and anatabine being below the cutoff (2 ng/ml; Table 2). In study 1, concordance was 100% of the 45 subjects reporting abstinence, and in study 2 it was 79% of the 89 subjects who reported abstinence.

Concordance with self-reported relapse and urine measures of anabasine and anatabine was also evaluated (Table 2). In study 1, there were 7 false negatives, with 23% of the 31 subjects reporting relapse, and in study 2 there were 5 false negatives, which was 36% of the 14 subjects who reported relapse. The finding of false negatives is presumably because of infrequent or low-level tobacco use, with enough time elapsed between relapse and obtaining a sample for concentrations to drop below the limit of quantitation of the assay. The half-lives of anabasine and anatabine, based on urinary excretion data, were found to be 16 h and 10 h, respectively (23).

Correlations of anabasine, anatabine, and cotinine in urine of subjects before beginning tobacco cessation programs were correlated with their self-reported tobacco use (Table 3). Correlations ranged from fair to poor.

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better with cigarette consumption than they were for smokeless tobacco consumption. Presumably, this is because of less variability in the systemically absorbed dose of nicotine from a cigarette than from a dip or tin of tobacco. This could be because the dips are of different sizes, there are different concentrations of nicotine in different smokeless tobacco products, the products are used differently by different people (i.e., used for different duration of time or held in the mouth differently), and/or because of differences in saliva pH, which affect nicotine absorption. However, for smokeless tobacco, it has been reported that frequency and duration of tobacco use, rather than amount, appear to be better indicators of nicotine/cotinine exposure (28). Consequently, if frequency and duration measures had been obtained in the present study, correlations may have been better.

In a previous study, we found generally good correlations between nicotine intake from tobacco (determined from blood nicotine concentrations and nicotine clearance data; Ref. 29) and urine concentrations of nicotine, cotinine, anabasine, and anatabine. Data from that study are also shown in Table 3 and in Fig. 2 (23). Consequently, the measurement of tobacco alkaloids or their metabolites as biomarkers is advantageous for estimating the amount of tobacco consumed (11, 23). In tobacco cessation studies, it may be useful to have a quantitative estimate of tobacco consumption; for example, to assess potential harm reduction in persons who cut down on tobacco use but cannot quit. Our studies have demonstrated that urine levels of anabasine and anatabine can be used to assess tobacco consumption during NRT.

Acknowledgments
We thank Patricia Buley, Sandra Tinetti, and the staff of the General Clinical Research Center for assistance in conducting the clinical study; Gang Liang, Irving Fong, and Minjiang Duan for carrying out the gas chromatography-mass spectrometry analyses; Gunnard Modin for statistical analysis; and Kaye Welch for editorial assistance.
References

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