We describe a rapid, sensitive method for the routine simultaneous determination of nicotine and cotinine in 1 mL of plasma. Extraction in 10-mL screw-capped Teflon tubes with methylene chloride after deproteinization with trichloroacetic acid eliminated emulsion formation. The extract, after evaporation and reconstitution in 30 μL of mobile phase, is injected into a reversed-phase C-18 ion-pair column of an isocratic high-performance liquid-chromatographic unit. Absorbance is monitored at 256 nm. The mobile phase is a citrate-phosphate (30 mmol each per liter) buffer mixture containing 50 mL of acetonitrile and 1 mmol of sodium heptanesulfonate per liter. 2-Phenylimidazole is the internal standard. The detection limit is 1 μg/L for nicotine and 3 μg/L for cotinine. The standard curve is linear from 0 to 700 μg/L for both compounds. The average CV for nicotine in the concentration range 0-100 μg/L is 6.5%, and that for cotinine in the concentration range 50-700 μg/L is 4%.

Additional Keyphrases: smoking · chromatography, reversed phase

For the last three decades there has been considerable interest in having a simple, rapid, sensitive, and specific method for quantifying nicotine and its major metabolite, cotinine, in biological specimens (1). Techniques that have been used include gas chromatography (GC) (2-6), GC-mass spectrometry (7), radioimmunoassay (8, 9), high-performance liquid chromatography (HPLC) (10-13), and enzyme-immunoassay (14). In most of the published procedures separate methods are used for the two substances. But with only a few GC methods (2, 6) can both substances be simultaneously assayed. The main reason for this is that cotinine requires a much higher temperature than nicotine for elution from a packed GC column (3). Kogan et al. (6) used temperature programming from 150 to 200 °C for simultaneous assay of the two compounds, and used a packed column; yet the cotinine peak tailed. The use of capillary GC columns—which are more stable, inert, and allow greater resolution than packed GC columns, when used in conjunction with an alkali flame ionization detector—has led to very sensitive assays for the two compounds (2, 5). However, the poor long-term stability of the detector and of the capillary column limits the use of this GC method (2, 5).

HPLC methods (Table 1) for quantification of the two analytes have been reported (10-13). But none of these methods has been properly developed or modified for the routine assay of the two analytes in a small volume of human plasma. Perhaps the stringent demands of the assay (4) have precluded development of a routine, sensitive, and specific HPLC method. Only Machacek and Jiang (13) have used HPLC to measure the range for cotinine in plasma and saliva from smokers.

The many liquid-liquid extraction methods reported for extracting these analytes nicely exploit the different solubilities of the free bases in organic solvents. Nicotine is more readily soluble in diethyl ether (3) and cyclohexane (14) than is cotinine, and the latter is much more soluble in n-butanol (3) than is nicotine. Both the free bases are appreciably soluble in methylene chloride (5, 6). Methylene chloride is thus considered the most efficient solvent for the simultaneous extraction of both the free bases from biological specimens. In many of the liquid-liquid extraction methods, the use of methylene chloride to extract nicotine and cotinine from the alkalized plasma invariably is accompanied by formation of an emulsion (2, 5, 13), which makes the extraction messy, cumbersome, and time consuming, and leads to the poor extraction efficiencies.

We have developed a simple procedure for simultaneously extracting nicotine and cotinine from plasma with methylene chloride, with no emulsion formation, and a simple, isocratic, reversed-phase HPLC method for simultaneously measuring nicotine and cotinine in 1 mL of plasma.

Materials and Methods

Reagents

Nicotine hydrogen (+) bitartrate (99% pure; BDH Chemicals, Poole, U.K.) was from Gallard-Schlesinger, Long Island, NY; cotinine from Sigma Chemical Co., St. Louis, MO; and 2-phenylimidazole from Aldrich Chemical Co., Milwaukee, WI. Trichloroacetic acid (reagent grade), potassium phosphate dibasic (ACS grade), and citric acid monohydrate (ACS grade) were from Matheson, Coleman & Bell, Norwood, OH; "HPLC-grade" water, acetonitrile, methanol, and methylene chloride from Fisher Scientific, Fair Lawn, NJ; potassium hydroxide (reagent grade) and hydrochloric acid (reagent grade) from J. T. Baker Chemical Co., Phillipsburg, NJ; and sodium heptanesulfonate and nicotine (98% pure) from Eastman Kodak Co., Rochester, NY.

The mobile phase is a mixture of citrate and dibasic phosphate (30 mmol each per liter) containing 1 mmol of sodium heptanesulfonate and 50 mL of acetonitrile per liter. Its pH is 6.1.

All solutions used in the extraction and dilution of standards—such as 5 mol/L potassium hydroxide, 100 g/L trichloroacetic acid solution, and 50 mmol/L hydrochloric acid—were in "HPLC-grade" water. (This grade of water was used throughout.)

Nicotine and cotinine free bases are inconvenient to handle, especially in attempting to prepare standard solutions accurately. Nicotine solutions are markedly unstable in the presence of air (15). Indeed, we found that after a couple of months, a commercially obtained nicotine sample that was frequently exposed to the atmosphere had less than 30% of its original concentration. Therefore, we used nico-
Table 1. Summary of Earlier HPLC Assay Methods for Nicotine (Nic) and Cotinine (Cot)

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Mobile phase</th>
<th>Flow, ml/min</th>
<th>Detection λ, nm</th>
<th>Int. std</th>
<th>Specimen &amp; analyte</th>
<th>Detection limit, µg/L</th>
<th>Remarks</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18 Bondapak</td>
<td>Ethyl acetate/isopropanol: ammonia (80:20:0.4)</td>
<td>0.75</td>
<td>260</td>
<td>Desipramine</td>
<td>Urine, 3 mL, Nic &amp; Cot</td>
<td>5</td>
<td>Cot used very impure</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Dioxane/isopropanol: ammonia (80:3:0.4)</td>
<td>1.0</td>
<td>254</td>
<td>—</td>
<td>Urine, dog plasma, 5 mL, Nic &amp; Cot</td>
<td>2</td>
<td>XAD-2 resin extraction, recovery 80%.</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Water:methanol:acetic acid</td>
<td>1.5</td>
<td>—</td>
<td>Plasma, urine, 2 mL, 14C-Nic &amp; 14C-Cot</td>
<td>1</td>
<td>Fractions collected and quantified from DPM values. Pharmacokinetic and metabolic studies</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphate buffer: acetonitrile (83:17) contains 0.8 mmol of heptanesulfonate per liter, pH 4.8</td>
<td>1.0</td>
<td>257</td>
<td>2-Phenylimidazole</td>
<td>Plasma or saliva, 1 mL, Cot</td>
<td>1</td>
<td>—</td>
<td>13</td>
</tr>
</tbody>
</table>

Tine hydrogen bitartrate and cotinine perchlorate for preparing standard solutions.

To prepare cotinine perchlorate, add 0.5 mL of a 11.65 mol/L solution of perchloric acid to 0.5 g of commercial cotinine in 15 mL of reagent-grade isopropanol. Filter the off-white, impure cotinine perchlorate and recrystallize it from methanol, using decolorizing charcoal. Dry the shining, colorless, needle-shaped crystals overnight in a vacuum desiccator. We determined the purity and composition of the compound from its elemental analysis, melting point, and nuclear magnetic resonance spectrum. The cotinine perchlorate we prepared is a mono-perchlorate and is an anhydrous compound (molecular formula C_{10}H_{13}N_{2}C_{1}O_{5}). Analytical data: %C found 43.39, calcd 43.26; %H found 4.69, calcd 4.72; %N found 10.01, calcd 10.09; %Cl found 12.71, calcd 12.77; and melting point 214 °C [a value of 217.5–218.5 °C was quoted for another sample from this same preparation by Jacob Peyton, San Francisco General Hospital, CA (personal communication)].

Standard Solutions

Prepare separate stock solutions of each compound, in a concentration of 200 mg (of free base) per liter. The nicotine solution contains 153.6 mg of nicotine hydrogen bitartrate and the cotinine solution contains 78.79 mg of cotinine mono-perchlorate per 250 mL of water. These stock solutions are stable for at least one year at 4 °C. Prepare working standard solutions for nicotine (10 to 120 µg/L) and cotinine (20 to 600 µg/L) by diluting the stock standards with either water or normal human plasma that has been stripped of all steroids and possible drugs by stirring with neutral charcoal (50 g per liter of plasma) overnight and filtering. The stripped plasma is completely free of nicotine and cotinine (as we determined in 10 repeated analyses). Store the working standards in 1.2-mL aliquots in centrifuge tubes at −20 °C until assay. The working standards are stable for at least a year at −20 °C.

Internal Standard

Prepare the 1 g/L stock solution of 2-phenylimidazole in methanol/water (10/90 by vol). Prepare the 600 µg/L working standard by diluting 600 µL of stock standard to 1 L in methanol/dilute (50 mmol/L) hydrochloric acid (10/90 by vol). Prepare working standard solutions freshly once a month.

Instrumentation

To obtain the ultraviolet absorption spectra, we used a Model DU 60 scanning spectrophotometer (Beckman Instruments, Arlington Heights, IL) and matched quartz cells with a 10-cm pathlength.

The HPLC unit consisted of a pump (Model 655-A; Hitachi, Tokyo, Japan), a variable-wavelength ultraviolet detector (Model 655-A23, Hitachi) with a deuterium lamp, and an 11-µL flow-cell (pathlength 5 mm). We used a 15 × 0.2 cm column of ODS Hypersil (3-µm particle size, from Shandon Inc., Pittsburgh, PA), an injector with a 200-µL loop (Model 7125; Rheodyne, Cotati, CA), and a dual-channel integrator (Model D-2000, Hitachi). The flow rate for the mobile phase was 0.3 mL/min, the column pressure 20.7 MPa (3000 psi).

Procedure

We used translucent 10-mL, screw-capped Teflon tubes (Nalgene FEP Oak Ridge type, from VWR Scientific, Chicago, IL) in our extraction work. We soaked the Teflon tubes overnight in alcoholic potassium hydroxide, washed them in tap water, and then cleaned them in an automatic dishwasher; finally, we rinsed the tubes with ethanol three times and dried them in an oven at 100 °C. We soaked the Teflon caps for 2 h each in 2 mol/L nitric acid and Alconox detergent, then hand washed them with tap water; finally, we rinsed them with distilled water and ethanol, and air dried them at room temperature.

Label one Teflon tube for each standard, unknown, and quality-control specimen; then pipet into each tube 1 mL of sample, 100 µL of the internal standard (2-phenylimidazole), and 1 mL of 100 g/L trichloroacetic acid solution. Cap the tubes tightly, vortex-mix for 30 s, and centrifuge them at high speed for 20 min. Decant the clear supernate into a second set of clean, 10-mL, screw-capped Teflon tubes. To this protein-free plasma extract, add 0.5 mL of a 5 mol/L potassium hydroxide solution and 6 mL of methylene chloride. Cap the tubes, agitate for 30 min in a horizontal
shaker, and centrifuge to separate the phases. Aspirate the aqueous (top) layer and add 3.0 mL of 0.5 mol/L hydrochloric acid solution to the organic phase and vortex-mix for 30 s. Separate the phases by centrifugation and use a Pasteur pipet to remove and discard the organic (lower) layer. To the acidic aqueous solution remaining in the Teflon tube, add 0.5 mL of 5 mol/L potassium hydroxide solution and 5 mL of methylene chloride and vortex-mix for 30 s. Separate the phases by centrifugation, aspirate the aqueous (top) layer, add 200 μL methanolic hydrochloric acid (10 mmol HCl in methanol) to the remaining solution, mix gently, and evaporate the organic solvent under nitrogen in a water bath at 40 °C. Wash the sides of the Teflon tube with 200 μL of methanolic hydrochloric acid and evaporate the solution. Reconstitute the residue in 30 μL of mobile phase and inject all of it into the HPLC column. Before reconstitution, the dried samples can be capped and kept stored at 4 °C for up to three days without loss or breakdown.

Results and Discussion

Much has been reported on the difficulties of the gas-chromatographic assay of nicotine (4, 5; little has been reported on the HPLC method. The extent of contamination in the nicotine assay from extraneous sources largely depends on the particular method used. GC and GC–mass-spectrometric methods seem to be more prone to extraneous contamination than does the HPLC method. We know, from our experience with this HPLC work in assaying more than 600 samples, that extraneous contamination is manageable with proper precautions. Redistilling commercial solvents, use of special hoods, and installation of air-filter units are all unnecessary. Nevertheless, all reagents must be individually checked for contamination before use for each assay. We observed precautions throughout the work to eliminate casual contamination from various other sources such as glassware, water, plastic pipet tips, injection syringes, injector ports, nitrogen, and air. Smoking was strictly forbidden in the laboratory and surrounding hallways. The technician must be a nonsmoker.

Earlier reports on the ultraviolet absorption spectra of nicotine (17–19) and cotinine (20) appeared in 1910, 1913, 1940, and 1957. There is very little information on the molar absorbances at the ultraviolet maxima. Figure 1 shows the ultraviolet spectra of solutions of the inorganic salt of nicotine, cotinine, and 2-phenylimidazole. We used inorganic salts of nicotine and cotinine here rather than their organic salts (i.e., nicotine bitartrate, or cotinine fumarate) because the organic anions interfere. We used commercial nicotine from Eastman Kodak (98% pure) in preparing its hydrochloride salt solution. We opened the nicotine container and performed the initial dilution with hydrochloric acid in a nitrogen air bag. Further dilutions were done under normal laboratory atmosphere. This nicotine standard compared remarkably well with the standard based on nicotine hydrogen bitartrate. We prepared pure cotinine perchlorate to obtain its ultraviolet spectrum (see Materials and Methods section). Nicotine has a maximum at 254 nm, cotinine has a maximum at 260 nm, and 2-phenylimidazole has a broad maximum, centered at 250 nm. The molar absorbance of nicotine at 254 nm in the methanolic hydrochloric acid is 4597. The molar absorbance of cotinine perchlorate in methanol/water mixture (80/20 by vol) at its maximum at 260 nm is 2262. Maximum absorbance for nicotine, as measured in the Hitachi ultraviolet detector, was at 256 nm. Because greater sensitivity is needed in measuring nicotine concentrations than cotinine concentrations when the biological specimen is serum or plasma, we chose to monitor ultraviolet absorbance at 256 nm.

We standardized the assay every day, using a series of standards containing, per liter, 10 to 120 μg of nicotine and 50 to 600 μg of cotinine. Aqueous and plasma-based standards were used randomly. The extraction efficiency for aqueous standards was uniformly better than for plasma-based standards, probably owing to entrapment and consequent loss of a small amount of nicotine and cotinine in the protein precipitate of plasma-based standards and the better decantation possible with the homogeneous aqueous standards–trichloroacetic acid solution. The average (n = 8) extraction efficiency for a 20 μg/L nicotine standard in plasma was 87%, and that for a similar 120 μg/L cotinine standard was 84%. Extraction efficiencies were determined by comparing the peak heights of extracted standards with those of the unextracted ones. For quantification we used peak-height measurements. A graph was drawn, peak-height ratios vs the concentration of nicotine or cotinine. For each compound, the line passed through the origin, unlike in the GC methods (6, 15). This HPLC method is linear from 0 to 700 μg/L for each analyte. Analytical recovery for both nicotine and cotinine was assessed by supplementing charcoal-stripped plasma and assaying. Mean (and SEM) recovery (n = 8) for samples of nicotine in plasma in concentrations of 10, 30, 60, and 90 μg/L were 95 (7), 97 (3.4), 95 (3.4), 95 (6.5), and 96 (6.2) percent, respectively. Corresponding values (n = 8) for cotinine samples in concentrations of 60, 150, 300, and 450 μg/L were 95 (6.1), 96 (4.7), 97 (5.8), and 98 (4.4) percent. Table 2 shows the precision data for the two compounds in the assay.

Figure 2 shows representative chromatograms obtained during the assay. All peaks are baseline-resolved and symmetrical, and they enabled accurate quantification of nicotine and cotinine. With use of 1 mL of aqueous standards,
the sensitivity of the assay is 1 \( \mu \text{g/L} \) for nicotine and 3 \( \mu \text{g/L} \) for cotinine. The signal-to-noise ratio of the baseline for the method is less than 0.3 \( \mu \text{g/L} \) under ordinary assay conditions (attenuation 2). Nicotine peak heights were always about 40% greater than those for cotinine for solutions of identical concentrations (extracted or unextracted), indicating reproducible extraction efficiencies for the two analytes throughout the study. Our experimental observations are in contrast to a literature report in which the peak height of 37 ng of nicotine was found to be less than two-thirds the peak height of 19 ng of cotinine (13). The lower sensitivity they observed for nicotine may be ascribable to the impure standard used or to poor extraction efficiency in their extraction method. In any case, there is no other published HPLC chromatogram of a nicotine-cotinine standard, extracted or unextracted. Even in some of the reported GC work (5, 6), the importance of using stable salts of the free bases as standards in this assay is unrecogized.

There is a large extraneous peak with a retention time of about 5.75 min in many of the chromatograms, except for those of the aqueous and stripped-plasma standards. The retention time of the peak matches that of caffeine. This caffeine peak is present in the chromatograms of extracts of many smokers' plasma, as obtained by either gas-liquid chromatography or liquid chromatography (5, 13). In many cases the peak is much larger than the earlier-eluting cotinine peak. Good resolution of the two peaks is important for accurate quantification of cotinine. A higher pH (~6.1) of the mobile phase ensures resolution of the two peaks. There is another extraneous peak, which appears just before the nicotine peak in chromatograms of sera of all smokers. Once again, the resolution of these two peaks is important for accurate quantification of nicotine. The two peaks are resolved by adjusting the percentage composition of the organic content of the mobile phase, its pH, and the sodium heptanesulfonate concentration.

Nicotine and cotinine are moderately polar compounds. Use of ion-pair chromatography for separation of the two compounds is documented (11, 13). Our rationale for the choice of reversed-phase ion-pair chromatography is threefold: (a) aqueous salt solutions of the two compounds can be directly injected into the column; (b) by changing the pH of the mobile phase, the retention times of the components can be easily altered; and (c) again, the retention times of the compounds can be easily changed by increasing or decreasing the concentration of the organic solvent. Any bonded reversed-phase column (C-8, C-18, or CN) can be used to resolve the compounds. Our initial choice of the 3-\( \mu \text{m} \) C-18 end-capped reversed-phase (Shandon) column of dimensions 15 \( \times \) 0.2 cm was to maximize the assay's sensitivity for nicotine. It is well known (21) that a fourfold increase in sensitivity is possible when a mini-bore column (internal diameter 0.2 cm) is used, as compared with the usual dimensions (15 \( \times \) 0.46 cm). We obtained the same sensitivity by using a column of the latter size from a different manufacturer (Dynamax 15 \( \times \) 0.46 cm column packed with 3-\( \mu \text{m} \) Microsorb C-18; Rainin Instrument Co., Woburn, MA). However, there is an economic advantage in using a mini-bore column. The flow rate required is 0.3 mL/min for a mini-bore column. For the larger column (Rainin) a flow rate of 1.5 mL/min and a higher proportion of acetonitrile (10%) are necessary.

Earlier HPLC work (12, 13) on the assay shows the influence of pH of the mobile phase on the separation of the compounds. Kyerematen et al. (12) obtained very good separation of the compounds (Table 1) by using a C-18 \( \mu \)Bondapak column and a mobile phase of pH 6.26. Machacek and Jiang (13), in their work (Table 1) on quantifying cotinine in plasma, used an ion-pair Altex UltraSphere column with a mobile phase of pH 4.8, but did not get good resolution of the two peaks. We observed interesting changes with increasing pH of the mobile phase (see Figure 3). The retention time of the nicotine peak increased with a gradual increase in pH from 3.0 to 7.3. But, in contrast, there was very little change in the retention time for the cotinine peak with increase in pH of the mobile phase. At pH ~6.3, nicotine is eluted after cotinine, and the nicotine peak begins to tail, while the cotinine peak sharpens. The optimum pH range is 6.1 to 6.5 for good resolution of the two compounds. The separation of the peaks is also influenced by the proportions of heptanesulfonate and acetonitrile in the mobile phase. When a mini-bore column is used, care should be exercised in limiting the amount of heptane sulfonate (counter ion) used, for the amount of packing material is limited. Rarely, nicotine and cotinine peaks were co-eluted. When this happened, we adjusted (usually lower-

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**Table 2. Precision Data for the Nicotine–Cotinine Assay**

<table>
<thead>
<tr>
<th></th>
<th>Mean (and SD)*</th>
<th>Mean (and SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intersassay</td>
<td>Intra-assay</td>
</tr>
<tr>
<td></td>
<td>CV, %</td>
<td>CV, %</td>
</tr>
<tr>
<td>Nicotine</td>
<td>10.2 (1.1)</td>
<td>10.0 (0.9)</td>
</tr>
<tr>
<td></td>
<td>19.8 (1.6)</td>
<td>21.2 (1.4)</td>
</tr>
<tr>
<td></td>
<td>60.3 (5.1)</td>
<td>58.8 (2.4)</td>
</tr>
<tr>
<td>Cotinine</td>
<td>50.6 (2.8)</td>
<td>48.1 (2.8)</td>
</tr>
<tr>
<td></td>
<td>120.4 (6.0)</td>
<td>124.4 (4.1)</td>
</tr>
<tr>
<td></td>
<td>315.0 (7.4)</td>
<td>308.0 (7.0)</td>
</tr>
</tbody>
</table>

*\( n = 10 \).
ing) the mobile-phase pH and the peaks could be separated.

The extraction method used in this assay is time consuming, but not any more so than in others (2, 3). The significant improvement of the method is that no emulsion is formed, the extraction is simple, and "clean" chromatograms are obtained. Thirty samples can be extracted in about 5 h and injected in about 6 h. Assay time, per sample, is about 10 min, less than half the time required for the GC capillary-column method (2). Extracted samples can be covered with Parafilm and stored refrigerated for one or two days before injection. There was no evidence of any breakdown of either nicotine or cotinine under these conditions. This confirms the view that the salt forms of these bases are more stable and are to be preferred. Back extraction of the free bases into acid from methylene chloride ensures cleaner chromatograms and avoids carryover of steroids and other impurities from one injection to the next. Such clean extraction of the sample can minimize the maintenance of the capillary or packed columns and the alkali bead detector of the GC methods necessary (2, 5). The higher temperatures used in GC methods may contribute to greater debris and possible decomposition of the analytes. The ambient-temperature HPLC method is superior to GC methods, because the analytes are not subjected to any temperature effects.

Many different types of compounds have been used as internal standards in GC (2, 5, 6), GC–mass-spectrometric assays (7), and the few HPLC (10, 13) methods. Multiple internal standards have been used in some assays (2, 3, 5). In a few of the GC assays, separate internal standards, very similar in structure to the two analytes, have been used, and they seem to have improved the sensitivity and precision of the assays (3)—but these compounds are not commercially available. We find 2-phenylimidazole to be a good internal standard in our assay (13). The extraction efficiency of the

internal standard is consistently about 95%. The first step (30-min shaking, for extraction into methylene chloride) is critical and ensures good extraction of the three compounds.

To minimize the loss of the analytes by adsorption onto glass and to increase the sensitivity of the assay, silanize the glassware used in extraction (6, 15). An alternative method is to use the 10-mL Teflon tubes with Teflon screw caps, and indeed this novel use of all-Teflon tubes may be more economical in the long run.

We determined nicotine and cotinine in 120 smokers' plasma samples, obtained 10 min after the cessation of smoking cigarettes with various nicotine contents. The mean concentration of nicotine was 14 μg/L, the range 5–46 μg/L. The mean cotinine concentration was 300 μg/L, the range 180–460 μg/L. These values agree with values reported by GC (2, 6) and HPLC (13) methods.

We gratefully acknowledge the help of Drs. Ovide and Cindy Pomerleau of our department for providing patients' samples, support, and encouragement.

References