High-Performance Liquid-Chromatographic Method Compared with a Modified Radioimmunoassay of Cotinine in Plasma

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Cotinine is a sensitive and specific biochemical marker of exposure to cigarette smoke. We describe a simple solid-phase extraction of cotinine from plasma before quantification by HPLC. Extraction recovery was 97.9% ± 11.0% for plasma concentrations of 5–400 μg/L. Baseline separation of cotinine and caffeine was achieved within 11 min of injection onto a C18 reversed-phase column. The mobile phase was citric acid/dibasic potassium phosphate (30 mmol/L each, pH 6.0) containing 100 mL of acetonitrile per liter. Within-day and day-to-day precision (CV) were 4.7% and 8.4%, respectively. We also describe a modification of the Nicotine Metabolite RIA kit (Diagnostic Products Corp.) for quantifying cotinine in plasma. Recovery of cotinine from supplemented plasma was within 10% of the expected value with this RIA kit. Interassay precision averaged 8.1% for samples in the range 50–400 μg/L; intra-assay precision averaged 3.6% at 230 μg/L and 8.7% at 53 μg/L. Correlation between the two methods was RIA = 1.13 HPLC + 14.8 (n = 128, r = 0.957, P < 0.001). Both methods are technically simple to perform.
Evaluation of the effects of human exposure to active and passive cigarette smoke requires a sensitive and specific quantitative biochemical marker. Over the last 20 years numerous compounds have been used for this, e.g., carboxyhemoglobin, thiocyanate, and the hydroxy-ethylvaline adduct of hemoglobin (1, 2). These compounds, however, have been criticized for lack of specificity and falsely increased results because of in vivo interferences (3–6). Recently, nicotine and one of its major metabolites, cotinine (Figure 1), have been accepted as the best indices by which a clinical history of smoking may be validated and correlated to clinical and biochemical effects (7–12).

Nicotine has a very short half-life, <2 h (13), and plasma concentrations tend to fluctuate considerably throughout the smoking day. Only a small percentage of inhaled nicotine is excreted in urine (13). Cotinine, however, has a long half-life of ~19 h (14). Considerable attention has been paid to measuring concentrations of urinary cotinine (15–17), but random (untimed) urine samples require standardization to creatinine content (18), and the completeness of 24-h collections may be unreliable. Conversely, plasma concentrations remain relatively constant throughout the smoking day (14), do not exhibit the lag time effect of urine samples, and for clinical studies provide a much better indication of exposure to cigarette smoke.

Numerous high-performance liquid-chromatographic (HPLC) methods have been published for determining cotinine in plasma and urine (19–23). The sample preparation, however, involved tedious organic extraction (19, 20, 22, 23). We have developed a simple method for solid-phase extraction of cotinine from plasma before quantification by HPLC and used the method to evaluate a commercially available Nicotine Metabolite RIA kit (by Diagnostic Products Corp., from Intermedico, Markham, Canada) modified for determining cotinine in plasma.

![Diagram of nicotine metabolism](image)

**Materials and Methods**

**Reagents**

(–)-Cotinine was from Aldrich Chemical Co., Milwauk ee, WI; citric acid monohydrate and 2-propanol were from BDH Chemicals, Toronto, Canada; and acetonitril (ACS grade), methanol (ACS grade), and chloroform (HPLC grade) were from Fisher Scientific, Fair Lawn NJ. C_{18} solid-phase extraction cartridges (Sep-Pak Classic 300 mg) were purchased from Waters Associates Milford, MA. Nicotine Metabolite RIA kits were purchased from Diagnostic Products Corp.

**Patients’ Samples**

As part of a large study to investigate the effects of maternal smoking during pregnancy, 10 mL of blood was collected by venipuncture into lithium-heparin Vacutainer Tubes (Becton Dickinson, Mississauga, Canada). Samples were kept on ice for ~30 min before the plasma was separated and stored at –70 °C until analysis.

**Quality-Control Material**

Pooled plasma from nonsmokers, supplemented with cotinine to three different concentrations, and plasma obtained from a heavy smoker were aliquoted and stored frozen at –70 °C.

**HPLC Analysis**

**Instrumentation:** HPLC analysis was performed with a Series 4 Liquid Chromatograph equipped with an LCI-100 Laboratory Computing Integrator (Perkin Elmer Corp., Instrument Division, Norwalk, CT). Sample injection was carried out with an AS-100 HPLC Autosampler (Bio-Rad Labs., Richmond, CA). The mobile phase was citric acid/dibasic potassium phosphate (30 mmol/L each, pH 6.0) containing 100 mL of acetonitrile per liter. Cotinine was isocratically eluted at a rate of 1.0 mL/min from a Supelcosil LC-18DB 15 × 0.46 cm column (3-μm particle size) fitted with a 2 × 0.46 cm guard column (5-μm particle size; both from Supelco Oakville, Canada). Absorbance of the column eluent was monitored at 262 nm with a Lambda-Max Model 480 LC spectrophotometer (Waters Associates) equipped with a deuterium lamp and a flow cell consisting of a 14-μL cuvet (10-mm pathlength).

**Standards:** Plasma standards were prepared by supplementing pooled plasma from nonsmokers to obtain concentrations of 0, 25, 50, 100, 200, and 400 μg of cotinine per liter. Before being supplemented, the pooled plasma was analyzed by HPLC to confirm the absence of endogenous cotinine. Plasma standards were stored in 1.2-mL aliquots at –70 °C. Aqueous standards were prepared fresh daily from a stock solution (5 mg/L) of cotinine.

**Sample extraction:** Plasma standards, quality-control material, and patients’ samples were extracted in groups of nine as follows: Sep-Paks were conditioned by washing sequentially with 5 mL of methanol and 3 mL of distilled water; 1.0-mL samples were drawn into
1-mL tuberculin syringes and loaded onto the Sep-Pak. The Sep-Paks were then washed with 1 mL of water. Cotinine was extracted with three 1-mL aliquots of chloroform:2-propanol (95:5 by vol). The eluent was evaporated to dryness under nitrogen at 37 °C, reconstituted with 200 μL of water, filtered through 0.45-μm (pore-size) filters, and refrigerated until HPLC analysis. Reconstituted samples were stable for one week at −20 °C.

Radioimmunoassay

The RIA is a polyethylene glycol-accelerated double-antibody liquid-phase procedure. The primary antiserum is raised in rabbits by using cotinine–protein conjugate. 125I-labeled cotinine with a specific activity of ~3500 Ci/g is used as the tracer. Antibody and tracer were used undiluted because these titers yielded zero-binding values (%B0) of 40–50%. RIAs were performed according to the manufacturer’s instructions with slight modification: we prepared additional standards by diluting the 100 and 500 μL standards with the 0 standard to yield values of 0, 50, 100, 250, and 500 μg/L. Plasma was substituted for urine in the procedure. The total batch size was 100 tubes. In brief, we incubated 50 μL of sample, standard, or quality control for 30 min with 100 μL of 125I-labeled cotinine and 100 μL of polyclonal rabbit anti-nicotine-metabolite antiserum. Next we added precipitating solution (1.0 mL) of goat anti-rabbit gamma globulin and dilute polyethylene glycol and incubated the mixture for 10 min. The samples were centrifuged and decanted, and the radioactivity of the bound fraction was counted with a Model 1260 Multi-Gamma II gamma counter (LKB Wallac, Turku, Finland) with spline data reduction.

The manufacturer’s determined cross-reactivity of the assay is 1.5% for nicotine at 1000 μg/L and 15.2% for 3-hydroxycotinine at 1000 μg/L. No cross-reactivity has been found for commonly used analgesics, narcotics, opiates, barbiturates, or caffeine.

Statistical Analysis

For comparative statistical analysis of aqueous vs extracted cotinine standard curves, we used MYSTAT (SYSTAT Inc., Evanston, IL) to perform multiple linear-regression analysis of variance. We evaluated the correlation between RIA and HPLC methods by using linear regression (Lotus 1-2-3, Version 2.2; Lotus Development Corp., Cambridge, MA).

Results and Discussion

Several chromatographic methods have been published recently for quantifying cotinine in plasma (19, 20). We modified these methods to allow baseline separation of cotinine from caffeine, the major potentially interfering compound in plasma, within 11 min without the need for an ion-pairing reagent (Figure 2). Because cotinine retention times are minimally affected by changes in pH (19), we chose a mobile phase with pH 6 to maximize the separation of cotinine and caffeine.

Previously reported HPLC analyses for plasma cotinine have involved tedious organic extractions (19, 20). Our method involves simple solid-phase extraction with C18 Sep-Paks and 1.0 mL of plasma. A 1-mL water wash before elution of cotinine is sufficient to remove most of the water-soluble components of plasma and results in a clean chromatogram (Figure 3). Of all the extraction solvents tested (dichloromethane, tetrahydrofuran, carbon tetrachloride, chloroform, ethyl acetate, cyclohexane, cyclohexanone, 2-propanol, methanol, ethanol, acetonitrile, n-heptane, acetone), chloroform:2-propanol (95:5 by vol) gave the best combination of clean chromatography and good recovery. Recovery was improved by ~25% by eluting with three 1-mL aliquots rather than one 3-mL aliquot.

Recovery of cotinine from supplemented plasma was 97.9% ± 11.0% over the concentration range of 5–400 μg/L. Samples were batched in sets of nine, with one technologist extracting 54 samples per day. Most previously reported HPLC methods have involved 2-phenylimidazole as an internal standard. We obtained poor peak shape and inconsistent recoveries of 2-phenylimidazole in our solid-phase extraction and chromatographic conditions. Therefore, we evaluated methods of external standardization. Analysis of variance of peak areas for aqueous vs extracted standard curves showed no significant difference between the curves obtained over 19 analysis days (F = 0.891, P = 0.346). The y-intercept was not significantly different from zero (t = −1.057, P = 0.292); therefore, we calculated results for all patients’ samples and quality controls by using the
aqueous standard curve of each day with a forced-zero intercept.

The within-day precision (CV) of this method was 4.7% at 164 μg/L; the day-to-day precision is summarized in Table 1. The detection limit was 10 μg/L (or 5 ng on the column). Hemolysis and lipemia had no effect on the recovery of cotinine.

Although this HPLC technique offers a substantial improvement over previously reported methods, it is not practical for processing large numbers of samples. Diagnostic Products Corp. has recently introduced an RIA for quantifying nicotine metabolites in urine. We modified this kit by diluting the standards supplied to produce a standard curve appropriate for plasma (0–500 μg/L). Recovery of cotinine from supplemented plasma samples was within 10% of the expected value over the range 20–400 μg/L. The interassay precision of the RIA technique is shown in Table 1. Intra-assay precision was 3.6% at 230 μg/L and 8.7% at 53 μg/L. Standards, samples, and quality controls may be processed in 100-tube batches.

We assayed plasma samples of smokers, using both the HPLC and RIA methodologies. Correlation between the two techniques was as follows: RIA = 1.13 HPLC + 14.8 (r = 0.987, P < 0.001, n = 128). The higher RIA values may reflect binding of the polyclonal antibodies of the immunoassay with other metabolites in the patient’s plasma. This was not a problem with plasma samples we analyzed by RIA because fewer metabolites are present in plasma than in urine and the major cross-reacting metabolite, 3-hydroxycotinine, is in lower concentrations than in urine (24). In the 128 samples analyzed by both techniques, we identified no interfering substances. Although the RIA kit was designed for quantifying high concentrations of cotinine (500–2000 μg/L) in urine, antibody/antigen dilution studies for the plasma analytical range (0–500 μg/L) indicate that optimal zero binding (%B₀ = 40–50%) is obtained when the tracer and antibody are used undiluted (data not shown).

Cotinine is a sensitive and specific biochemical marker of exposure to cigarette smoke. We developed a rapid sample-preparation technique for quantifying cotinine in plasma by HPLC and modified a RIA kit for nicotine metabolites to analyze serum samples that gave precise and accurate determinations of cotinine in plasma. The choice of methods depends on the availability of instrumentation in individual laboratories as well as the number of samples to be processed.

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References
 Improvement of a Direct Spectrophotometric Assay for Routine Determination of Superoxide Dismutase Activity

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The growing interest in measuring superoxide dismutase (EC 1.15.1.1) in many diseases calls for useful routine assays. For this purpose, the direct spectrophotometric method of Marklund (J Biol Chem 1976;251:7504–7) was improved to offer an alternative to the imprecise, indirect assays currently used. The decay of O$_2^-$ (from KO$_2$) at pH 9.5 was monitored as the decrease in ΔA (ΔA = A$_{250nm}$ - A$_{300nm}$). Superoxide dismutase was determined from the pseudo-first-order rate constant of O$_2^-$ dismutation. The precision of the assay was improved by increasing the concentration of O$_2^-$ and expanding the interval for measurements of O$_2^-$ concentrations to 4–16 μmol/L. Other assay characteristics, including temperature, were also optimized. In hemolysate the assay had a within-day CV of 5.5–13% and a between-day CV of 4%. Mn-superoxide dismutase and some superoxide dismutase mimics are inhibited at alkaline pH. Therefore, the method is primarily recommended for Cu,Zn-superoxide dismutase.

Additional Keywords: kinetic enzyme assay - oxygen radicals

Antioxidant enzymes are of vital importance in an organism's defense against oxidative stress. The most important are the superoxide dismutases (SODs; EC 1.15.1.1), which remove O$_2^-$ according to the following reaction (1, 2):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Three isoenzymes of SOD have been described in eukaryotes. The predominating form, Cu,Zn-SOD, and a minor fraction, Mn-SOD (3), are found in the cytoplasm and in the mitochondrial matrix, respectively. The least prevalent is a Cu,Zn-SOD that differs from the intracellular form by being a tetrameric glycoprotein; found mainly in the extracellular fluids, it therefore is named extracellular SOD (EC-SOD) (4, 5).

The growing interest in the role of oxidative stress and the antioxidant defense in many diseases (6) calls for the development of reliable methods suitable for routine testing in clinics. Of the three isoenzymes, analysis for Cu,Zn-SOD appears to be the most important (7, 8), and several methods for this have been published (9, 10). Most are indirect methods, based on the principle that O$_2^-$, typically generated by xanthine oxidase, reduces an indicator, e.g., cytochrome c or nitroblue tetrazolium. If SOD is present, the rate of elimination of O$_2^-$ is enhanced with a correspondingly decreased or completely prevented reduction of the indicator. However, when testing crude biological material such as blood, tissue homogenates, or fluids, the