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Development and validation of sensitive method for determination of serum cotinine in smokers and nonsmokers by liquid chromatography/atmospheric pressure ionization tandem mass spectrometry

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We describe a sensitive and specific method for measuring cotinine in serum by HPLC coupled to an atmospheric pressure chemical ionization tandem mass spectrometer. This method can analyze 100 samples/day on a routine basis, and its limit of detection of 50 ng/L makes it applicable to the analysis of samples from nonsmokers potentially exposed to environmental tobacco smoke. Analytical accuracy has been demonstrated from the analysis of NIST cotinine standards and from comparative analyses by both the current method and gas chromatography/high-resolution mass spectrometry. Precision has been examined through the repetitive analysis of a series of bench and blind QC materials. This method has been applied to the analysis of cotinine in serum samples collected as part of the Third National Health and Nutrition Examination Survey (NHANES III).

Tobacco use is regarded as the greatest single preventable cause of premature mortality in the US. A recent study concluded that 418 690 deaths during 1990 in the US, or ~1 of every 5 deaths, could be attributed to the active use of tobacco products, particularly cigarettes *[1]*. Cigarette smoking is recognized as a major risk factor for lung cancer and for cancers at a variety of other sites, chronic obstructive pulmonary disease (including emphysema), cardiovascular disease, and a variety of respiratory infections. The national annual cost of the premature mortality attributable to active smoking has been estimated to include $\$12 \times 10^9$ to $\$35 \times 10^9$ in direct healthcare costs and an additional $\$27 \times 10^9$ to $\$35 \times 10^9$ in lost earnings *[2, 3]*.

Furthermore, the adverse effects of smoking are not necessarily limited to active users of tobacco products. In recent years, concern about the health risks experienced by nonsmokers who are involuntarily exposed to tobacco smoke, e.g., fetuses exposed in utero to nicotine and other compounds from maternal smoking or nonsmokers through passive smoking, i.e., exposure to environmental tobacco smoke (ETS),[2] has increased. ETS is similar, but not identical, to the mainstream smoke inhaled during active smoking, and many of the hazardous substances known to be present in mainstream smoke are also present in ETS. In fact, because of differences in combustion and aging, some of these substances are actually more prevalent in ETS than in mainstream smoke. Strong evidence indicates that ETS represents a serious and

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[2] Nonstandard abbreviations: ETS, environmental tobacco smoke; NHANES, National Health and Nutrition Examination Survey; APCI MS/MS, atmospheric pressure chemical ionization tandem mass spectrometry; MRM, multiple reaction monitoring; GC, gas chromatography; LOD, limit of detection; EIA, enzyme-linked immunoassay; ISTD, internal standard.

substantial public health problem in the US *[4]*. ETS has been classified as a Group A (known human) carcinogen by the Environmental Protection Agency, is believed to be responsible for ~3000 lung cancer deaths per year among nonsmokers, and has been causally linked to many other health problems as well *[4]*. Because of these serious health concerns, an assessment of exposure to tobacco products was included in the design of the Third National Health and Nutrition Examination Survey (NHANES III). The objective of these analyses was to determine the extent of both active tobacco use and ETS exposure in a statistically representative sample of the entire US population *[5]*.

In general, both active smoking and exposure to ETS have been assessed either by interview or by biochemical analysis. In addition, ETS exposure has sometimes been inferred from environmental air analysis. Biochemical measurements of appropriate markers are often a useful adjunct to interviews in the classification of smokers and other active users of tobacco products. Such measurements are particularly valuable in assessing exposure to ETS because individuals may differ in their awareness of the extent and duration of such exposures. Markers of exposure to cigarette smoke include carbon monoxide (carboxyhemoglobin), thiocyanate ion, nicotine, and cotinine, a primary metabolite of nicotine.

At present, cotinine (Fig. 1) is generally regarded as the best marker for monitoring tobacco exposure in either actively or passively exposed individuals *[6]*. Nicotine, the most tobacco-specific component of cigarette smoke that is present in relatively abundant amounts (~1–2 mg/cigarette), is clearly absorbed and is measurable in both active and passive smokers *[7, 8]*. However, nicotine is rapidly metabolized and has a half-life of only 1–2 h, so it is rather poorly suited to monitoring chronic exposure. By contrast, cotinine has a much longer half-life of ~18–20 h, making it more appropriate for use as an exposure marker *[7]*. Cotinine can be reliably measured in blood, saliva, and urine, and all three sources are generally regarded as acceptable for monitoring nicotine exposure in people *[6, 9, 10]*. For the quantitative assessment of both active and passive smoking in the NHANES III population, we selected serum cotinine.

Many methods have been described for measuring cotinine in blood *[10-30]*. These assays are generally based on GLC with flame ionization, nitrogen–phosphorus, or mass spectral detection, HPLC with UV detection, or immunoassays. Such methods have been shown to be reliable for quantitating serum cotinine concentrations >10–15 μg/L, the range normally encountered in active smokers *[9, 10, 31]*, and in some cases, these methods may also be used for measuring concentrations in the range of ~2–10 μg/L, which is consistent with relatively substantial passive exposure to ETS. However, some of these methods are not sensitive or selective enough to reliably measure serum cotinine at the lower passive exposure concentrations, which may extend to well below 1 μg/L. Because our objective for NHANES III was to monitor the entire range of nicotine exposure, from that of active smokers to those who are only minimally exposed to ETS, we needed a procedure for these analyses that provided both excellent sensitivity and high selectivity. In addition, because of the large number of samples involved in this study, the new method needed to be capable of maintaining high sample throughput over an extended period of time.

To meet these requirements for a very sensitive and selective method of analyzing serum cotinine that is also capable of continual high throughput, we developed a new method based on HPLC linked to atmospheric pressure chemical ionization tandem mass spectrometry (APCI MS/MS). This method, which has been in routine operation in our laboratory for >4 years, combines the sensitivity and selectivity for serum cotinine assays re-

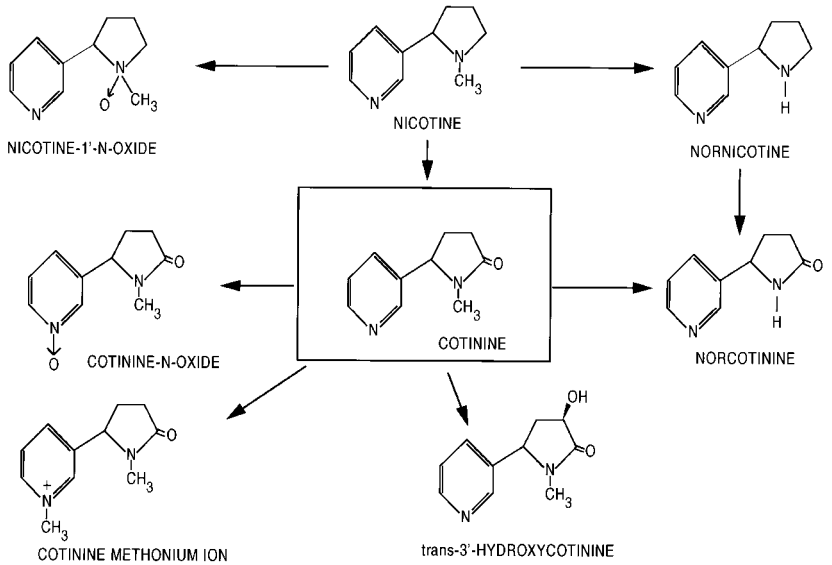

Fig. 1. Partial metabolic scheme for nicotine in humans.

quired for NHANES III and similar studies with the rapid analysis and robust performance needed for such large-scale surveys *[32]*.

Materials and Methods

Standards and reagents. Native cotinine [(−)-cotinine, 98%] was purchased from Aldrich Chemical Co., and *N*-methyl trideuterated cotinine was obtained from Cambridge Isotopes Lab., Andover, MA (DLM-1819, lot no. F-0549, 98%). High-purity cotinine perchlorate (>99%) was a gift from NIST; its preparation has been described *[33]*. 5-Methylcotinine was a generous gift from Peyton Jacob, University of California at San Francisco. [*G*-^{3}H]Cotinine, obtained from NEN Life Science Products, was further purified by solvent extraction, SCX-chromatography, and HPLC before use. Its estimated specific activity was then 17.5 Ci/mol. Trichloroacetic acid (99%) and ammonium acetate (99.999%) were also purchased from Aldrich, whereas potassium hydroxide (85–90% reagent) was obtained from Fisher Scientific. Water was either prepared from deionized water that was further processed with the use of an ORGANICpure water system (Barnstead) or was HPLC-grade water from Burdick and Jackson Labs., distributed by American Scientific Products. All other solvents were also high-purity HPLC grade from Burdick and Jackson unless otherwise indicated. Toluene was Microsolve VLSI (GC 99.9%) grade, and both methylene chloride and methanol were Burdick and Jackson GC^2 grade. LC/MS nebulizer gas was zero-grade air, whereas the barrier and collision gases were nitrogen and argon, respectively. All gases were ultrahigh-purity grades.

Calibration standards. One complete set of cotinine calibration standards was prepared for use throughout the entire NHANES study. All glassware was washed and silanized before use. A group of 12 standards with cotinine concentrations ranging from 0 to 25 μg/L (serum equivalents) and an internal standard (ISTD), cotinine-D_3, at 5 μg/L were prepared in toluene. The stock ISTD solution used for supplementing samples was prepared in isopropanol, 40 mL/L in toluene. Standards were analyzed to confirm their suitability and then aliquoted into washed, silanized glass ampules, flame-sealed, and stored at −20 °C until used. Generally, a new set of ampules was opened every 2 weeks.

QC materials. Four serum pools were prepared for use in this study. Two were bench QC pools with nominal cotinine concentrations, determined during characterization assays, of ~202 and ~1.84 μg/L; these were used in high and low cotinine sample assays, respectively. Two aliquots of the bench QC pools at the appropriate concentration were included in each assay, one at the beginning of the assay and the other at the end. The other two pools were blind QC materials. After the blind pools were prepared, we aliquoted them into vials with labels indistinguishable from those of standard NHANES sample vials and randomly inserted them into assays according to a computer-generated protocol. The high and low blind QC pools had nominal cotinine concentrations (as determined during the initial characterization screening) of ~212 and ~0.262 μg/L, respectively. The number of blind QC samples varied for each assay, but averaged between 2 and 3 vials per assay of 50 samples. In addition, we required that at least one blind QC sample be present in every assay of 50 samples; when at least one sample was not designated at random by the assay-generating software, an additional blind QC position was inserted manually.

Preparation of materials. Although background contamination from environmental cotinine is far less of a problem than is commonly observed with nicotine, very small amounts of cotinine are present in ETS in both the gas and particulate phases *[34–36]*, and background cotinine from environmental sources can potentially interfere in very sensitive analyses. Therefore, the careful and rigorous control of possible interference sources is very important in these assays. We sought to minimize background interferences by first permanently banning any smoking in the entire building in which the CDC cotinine analysis laboratories are located; this ban went into effect more than a year before any NHANES analyses were carried out. In addition, all analysts working on any sample-handling aspect of this project were required to be nonsmokers. The initial sample extraction was carried out in reusable 10-mL Oak Ridge-type Teflon tubes that were cleaned by hand with Liqui-Nox laboratory detergent (Alconox, New York, NY), sonicated, thoroughly rinsed with deionized water, and then dried and sent to the central CDC automated glassware washing facility to be cleaned again. On return, the tubes were rinsed once more with methanol, air-dried, and stored in sealed containers over desiccant until used. The samples used for predesignated high and low cotinine concentration assays were kept separate at all times and were periodically spot-checked for any evidence of residual contamination. Glassware that contacted samples was disposable and was precleaned and silanized before use. This glassware was stored in sealed packages protected from the environment until it was used and then discarded. All prepared aqueous reagents were made up in the laboratory according to designated schedules with the use of ORGANICpure water and protected from atmospheric exposure at all times except when they were being used. All prepared reagents and pure solvents were routinely tested for evidence of cotinine contamination.

Instrumentation. Analyses were conducted with a PE Sciex API III atmospheric pressure ionization triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments) with heated nebulizer interface installed. The mass spectrometer was interfaced with a short length of fused-silica tubing (~0.10 mm × 0.5 m) to a Perkin-Elmer base-

deactivated C_{18} column (4.6 mm × 3 cm; 3-μm particle size) mounted in a Hewlett-Packard Model 1090L liquid chromatograph equipped with an autosampler. The entire system from sample injection to data acquisition was computer-controlled with standard Sciex software.

Sample preparation. The range of serum cotinine concentrations can encompass >4 orders of magnitude when both smokers and nonsmokers are included. Therefore, all NHANES samples were first prescreened by an enzyme-linked immunoassay (EIA) for cotinine (STC Diagnostics, Bethlehem, PA) and placed into one of two categories, high or low, on the basis of a nominal cutoff concentration of ~25 μg/L as measured by the EIA. Individual LC/MS/MS assays of 50 samples each were then built from these preclassified groups. Each NHANES assay included 2 water blanks, 2 bench QC pools at the appropriate concentration, and 46 unknown samples, including a variable number of blind QC pools. Because the blind samples were indistinguishable to the analysts from normal study samples, they were treated as unknowns and were passed through the preliminary EIA screening procedure and subsequently allocated into LC/MS/MS assays like all other samples.

Extraction and cleanup. Normally, 100 samples were prepared and analyzed each day in 2 assays of 50 samples each. Each run consisted of a water blank in positions 1 and 50, a bench QC sample in positions 2 and 49, and a variable number of blind QC samples interspersed among the unknowns in positions 3–48. All unknown and blind QC samples carried coded identifications that were not known to the analysts. Separate bench and blind QC pools with cotinine concentrations in the appropriate range were selected for use with high and low assays.

In each run, printed labels were applied to a set of silanized culture tubes (13 × 100 mm), samples were thawed and carefully mixed, and 10 μL of the ISTD and the appropriate sample volume were placed in each tube. Undiluted serum samples (1 mL) were used for the low concentration assays, whereas samples classified as high concentration were assayed as 1:20 dilutions (50 μL of serum + 950 μL of water) or greater, as necessary. The tubes were capped and placed on an Eberbach orbital shaker (Eberbach, Ann Arbor, MI) at low speed for 20–30 min to allow the ISTD to equilibrate with the sample.

We then added 1 mL of 100 g/L trichloroacetic acid to each tube with an automated pipetter. The tubes were recapped, vortex-mixed for 30 s on a VWR MultiTube Vortexer (VWR Scientific), and centrifuged at ~1900*g* for 20 min. The trichloroacetic acid supernatants were transferred to labeled Oak Ridge-type Teflon tubes (16 × 80 mm), and 0.5 mL of 5 mol/L KOH was added with an Eppendorf Repeater pipet to each sample and mixed briefly. We then added 6 mL of methylene chloride to each tube with the use of an automated pipet, capped the tubes with Teflon screw-caps, and mixed them vigorously for 30 min on the MultiTube Vortexer. After vortex-mixing, the samples were briefly centrifuged again, and the upper (aqueous) layers were carefully removed by a water aspirator.

A set of sodium sulfate columns containing ~1.2 g of high-purity Na_2SO_4 in 10-mL polypropylene columns (prepared by Varian Sample Preparation Products) were placed in support racks and rinsed with ~4 mL of methylene chloride. The methylene chloride layers from the samples were then passed through the washed columns and collected in clean, labeled silanized glass tubes (13 × 100 mm). The tubes were placed in a Savant Model SC200 SpeedVac (Savant Instruments) equipped with an RT4104 trap and a VN100 VaporNet and taken just to dryness. We added 200 μL of methylene chloride to each residue, swirling gently to ensure that the bottom region of the tube was well-mixed, and transferred the contents to prerinsed, glass-lined autosampler microvials. The vials were placed uncapped in a bench-top hood, and the solvent was allowed to evaporate at room temperature overnight. The next morning, 20 μL of toluene was added to each sample, and the vials were capped, briefly vortex-mixed, and placed in autosampler cartridges for analysis.

Sample analysis. We injected 10 μL of the sample into the HPLC, which was operated isocratically at 1 mL/min with a mobile phase of methanol/ammonium acetate, 2 mmol/L (80:20 by vol). Mass spectrometric detection was carried out by positive-ion APCI with corona discharge ionization with the Sciex API III heated nebulizer interface. The temperature of the nebulizer probe was maintained at 500 °C, and we used multiple reaction monitoring (MRM) with argon as the collision gas at a nominal thickness of 700×10^{12} molecules/cm^2. We used the *m/z* 177→80 transition ion of native cotinine, in a ratio to the corresponding *m/z* 180→80 ion of the ISTD for quantification; we also monitored the somewhat weaker *m/z* 177→98 ion of native cotinine for confirmation. Dwell times were normally set at 100 ms. The retention time for cotinine in this system was <1 min, and the total assay time per sample was ~2 min.

Standards analysis. The complete set of 12 standards was evaluated four times each day; twice before and twice after the sample analyses. Before any samples were measured, the standards set was assayed twice, from 0 to 25 μg/L and then from 25 to 0 μg/L. The 24 results from this series were then immediately integrated and evaluated on-line on the Macintosh controller with the use of a custom Pascal program that checked instrument sensitivity from the mean ISTD area counts, checked the two blank (0 μg/L) standards to confirm the absence of contamination, and compared the back-calculated concentration for each standard with the expected value. The program printed the results of this preliminary evaluation accompanied by a suitability recommendation on the basis of preprogrammed acceptable limits for each param-

eter. Only after acceptable instrument performance had been documented in this way were the day's sample analyses begun. After the analysis of unknowns, the standard set was again assayed twice in both the forward and reverse directions. Thus, a total of 48 standards were analyzed each day along with the unknown samples.

Data analysis. Sciex system software (MacQuan) was used to carry out the initial peak localization and integration for each sample; then, all sample identification, MRM ion areas, and retention time data were transferred as ASCII files to SAS data sets (Statistical Analysis System, Cary, NC) where they were merged electronically with the sample cleanup and related information that had been entered earlier into a sample database. All data processing and further analyses were then conducted off-line with the use of SAS procedures. The calibration curve for this assay was slightly nonlinear throughout the concentration range from 0 to 25 μg/L; therefore, all quantitations were derived as part of a SAS program with the use of a moving 5-point regression technique as described in *Results and Discussion*.

Additional analyses. For comparisons with gas chromatography (GC)/low-resolution MS, we used a Hewlett-Packard MSD consisting of an HP 5890 gas chromatograph interfaced to an HP5970B mass-selective detector. Analyses were made by splitless injection of 2 μL of the sample in toluene onto a 0.2 mm × 12 m Ultra-1 column (Hewlett-Packard). Selected-ion monitoring was used, and quantitation was based on the area ratios of the native and ISTD molecular ions at *m/z* 176 and 179, respectively. High-resolution GC/MS was carried out by injection of 1 μL of the sample in toluene onto a 0.25 mm × 30 m DB-1701 column (J&W Scientific) mounted in an HP5890 gas chromatograph, which was interfaced to a VG Autospec mass spectrometer operated at 10 000 resolution. Two ions were monitored in these assays at *m/z* 176.0950 and 179.1138 for native cotinine and the ISTD, respectively.

Results and Discussion

Analytical scheme. As mentioned above, the range of serum cotinine concentrations that we needed to accommodate in this study encompassed several orders of magnitude. Therefore, we carried out a preliminary EIA screen on each sample. From the results of this preliminary evaluation, we classified each sample as belonging to either the low or high category, with a cutoff concentration of 25 μg/L, which is the concentration of the highest calibration standard used in the LC/MS/MS analyses. The highest serum cotinine concentration that would be expected in a nonsmoker exposed to ETS is ~10–13 μg/L *[37, 38]*, whereas most active smokers have serum cotinine concentrations considerably >25 μg/L. Therefore, most of the low-group samples are presumed to be from nonsmokers, whereas the high-group samples include the active smokers in the population. Approximately 30% of the NHANES III, Phase 1 samples were screened into the high category, with the remaining majority being low samples. After this preliminary evaluation by EIA, the samples were categorized and then extracted, concentrated, and analyzed in batch mode as described previously.

Calibration curve. A representative calibration curve is given in Fig. 2. This curve is based on 302 replicate analyses of the standard set (3624 standards). New curves were generated whenever a substantial change was made to the instrument (e.g., repairs, preventive maintenance service, or a new state file), and the appropriate calibration was applied to all samples analyzed within that time interval. Although not readily apparent in Fig. 2, these curves are slightly curvilinear over the three orders of magnitude covered, as indicated by a Fowlis–Scott test of

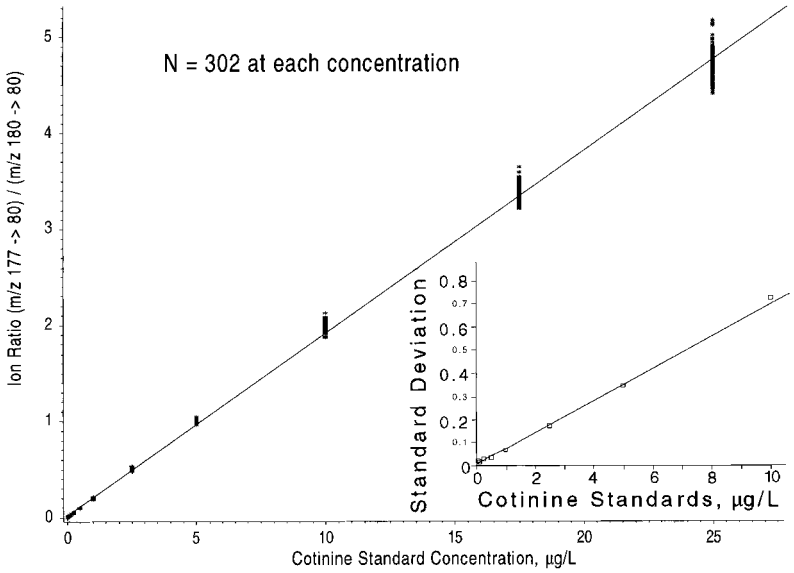

Fig. 2. Representative cotinine calibration curve by LC/MS/MS.

The *inset* is a plot of the SD for back-calculated concentrations vs the actual standard concentration over the range of 0–10 μg/L.

linearity *[39]* and by the overestimation of back-calculated values in the lowest concentration ranges when a single, linear calibration was used. To correct for this behavior, we used a moving 5-point regression for calibration. This approach was favored by the large number of standards included in each calibration series, which helped to compensate for the smaller number of concentration points (i.e., 5) used for each regression curve.

For the moving regression analysis, the calibration curve is subdivided into eight overlapping 5-point regions with concentration ranges of 0–0.25, 0.025–0.5, 0.05–1 μg/L, and so forth; a regression analysis is carried out for each region, generating a set of 8 separate regression equations. When unknowns are analyzed, the ion ratio for the unknown is calculated and compared with the mean ratio at the midpoint of each of the 8 regression curves. The curve with a midpoint ratio closest to that of the unknown sample's ratio is then selected for quantitation. The entire processing, selection, and quantitation algorithm is embedded in a SAS program and proceeds automatically.

The inset to Fig. 2 shows the SD for the back-calculated values from the lower-concentration standards plotted vs the standard concentration. The limiting SD (S_0) calculated from these data (i.e., the value of S as the native cotinine concentration approaches 0) was 0.0085. Thus a limit of detection (LOD), defined as $3 \times S_0$ *[40]*, of ~26 ng/L can be estimated from these data. This value reflects only the instrumental analysis, however, and does not address other contributions from the entire assay. Therefore, we calculated the method LOD from the long-term SD of the blank *[41, 42]*. The mean ± SD of the water blank was 0.0225 ± 0.016886 μg/L (n = 455), from which we calculated an estimated method LOD of 50 ng/L.

The high sensitivity (low detection limit) of this method is important, since our primary interest is in monitoring ETS exposure among nonsmokers. Analytes such as pyridine with relatively high gas-phase basicities tend to be measured with very high sensitivities by atmospheric pressure ionization instruments because of the favorable proton transfer kinetics from the hydronium ion reagents *[43]*, and cotinine was found to be ionized and detected very efficiently in this system. Previous methods for measuring cotinine in serum by immunoassays, HPLC, or GC, including GC/MS procedures, have generally been limited to concentrations of a few micrograms per liter or greater. GC with nitrogen–phosphorus detection is potentially capable of detection limits on the order of a few hundred nanograms per liter *[10–14]*, but the specificity of that GC is much lower than that of MS/MS, and isotopically labeled ISTDs cannot be used. Conversely, the use of deuterated cotinine as an ISTD and the clean ion chromatograms obtained from tandem MS analysis contributed to the low detection limits that are attainable with our method.

The importance of this enhanced sensitivity was evident in the results from Phase 1 of NHANES III, in which the geometric mean serum cotinine concentration among nonsmoking adults was 0.205 μg/L *[32]*. More than one-half of the ~10 500 people examined in Phase 1 had serum cotinine concentrations <1 μg/L *[32]*; thus, a substantial amount of information would have been lost in this study if a less sensitive analytical technique had been used.

Ion chromatograms. MRM ion chromatograms from a typical serum sample are given in Fig. 3. In each case, the response has been normalized by the system software to 100%. Quantitation in these assays is based on the ratio of the area counts from Ion A (177→80) to that of the ISTD ion (Ion B, 180→80), whereas the confirmation ratio is determined from the ratio of Ion C (177→98) to Ion A. As noted above, these assays typically produce very clean MRM chromatograms with relatively few interferences. This reduction in the chemical noise of the assay also makes a substantial contribution to the sensitivity of the analysis. The calculated cotinine concentration of the serum sample in Fig. 3 was 0.72 μg/L.

Recoveries. Preliminary evaluations showed good agreement between recoveries estimated from the ISTD area count for each sample normalized to the mean ISTD area count for that day's standards vs recoveries estimated by adding 5-methylcotinine as an additional external standard. Therefore, we routinely estimated recoveries from the ISTD area counts. Overall, recovery for the water blanks and for the (diluted) high cotinine samples estimated in this way averaged ~70% through the entire procedure. Some cotinine is lost during drying of the extracts, although losses of cotinine from methylene chloride extracts are much less than those of the more volatile nicotine, and such losses tend to be minimized when centrifugal vacuum evaporation is used and care is taken not to overdry the sample.

For the samples with lower cotinine concentrations, from which 1 mL of undiluted serum was taken for analysis, the recoveries were somewhat lower, averaging ~60%. This difference probably reflected additional losses in the protein pellet at the trichloroacetic acid precipitation step. This interpretation is in agreement with preliminary experiments in which serum pools supplemented and equilibrated with [*G*-^{3}H]cotinine were extracted by this procedure. About 10–15% of the radioactivity was retained in the pellet in those studies, and this residual activity was not appreciably recovered by additional extractions of the pellet. Benowitz et al. *[38]* have reported that ~2.6% of plasma cotinine is bound to protein. Although the precipitation of serum proteins may have contributed to somewhat lower cotinine recoveries, it completely eliminated problems with emulsions, which was essential to maintaining the high daily throughput of this method. In all cases, native cotinine losses during sample preparation are associated with compensatory

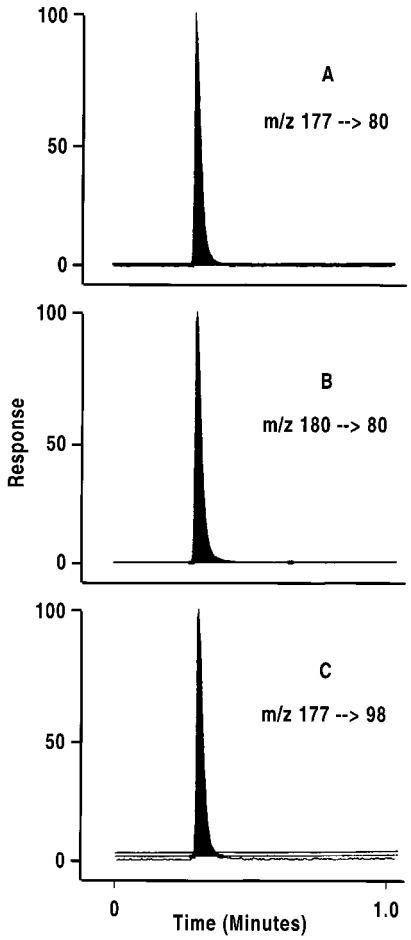

Fig. 3. MRM ion chromatograms for a representative serum sample. The calculated cotinine concentration for this sample was 0.72 μg/L.

losses of the ISTD and thus should not alter the sample quantitation on the basis of the ratio of the two analytes.

Confirmation ratio. Two native cotinine ion-transition pairs are monitored in this method. Quantitation is based on the 177→80/180→80 ratio with the primary progeny ions being used for both the native and labeled cotinine. In addition, a confirmation ratio is calculated for each sample with the ratio of the two progeny ions that are recorded for native cotinine (i.e., 177→98/177→80). Fig. 4 is a plot of this ratio as a function of ($\log_{10}$) cotinine concentration for 21 439 standards (1949 analysis sets) at concentrations ranging from 0.025 to 25 μg/L. These standards were analyzed over a period of 26 months.

Unlike confirmation ratios that are based on elemental isotopic distributions, MRM confirmation ratios are not fundamental and may vary as a result of small differences in the analytical conditions, particularly as they pertain to the operation of Q2 (the collision cell). However, when the analytical conditions remain fixed, this variation is relatively limited, and within-day CVs tend to be reasonably small (~3.5–4%). Therefore, each confirmation ratio for individual samples was evaluated relative to the value calculated from that day's four sets of standards.

Because the *m/z* 177→98 transition ion pair is less abundant than the 177→80 ion pair, the confirmation ratio becomes unreliable at the lowest cotinine concentration values. As indicated in Fig. 4, the ratio remained relatively uniform down to a concentration of ~250 ng/L, but tended to diverge downward from the expected value at the lowest concentrations—probably a result of the very low ion counts for *m/z* 98 at these low concentrations. Consequently, the confirmation ratio tends to be less valuable at sample concentrations <~250 ng/L.

Accuracy evaluations. The accuracy of this method was evaluated in part by analyzing a series of aqueous standards prepared by weight from a pure cotinine perchlorate stock. Standards were prepared with expected concentrations (corrected for perchlorate) ranging from 6.2 μg/L to 120 ng/L. Aliquots (1 mL) of these standards were then taken for analysis in the same manner as unknown serum samples. As indicated in Table 1, in each case the calculated concentrations for these samples agreed well with the expected values. The accuracy of this method was also evaluated by the analysis of aliquots of NIST Reference Material 8444 (cotinine in freeze-dried human urine). The

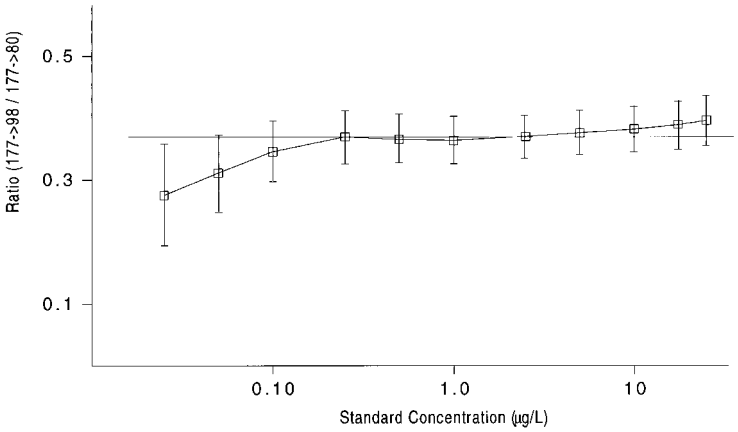

Fig. 4. Ratio of the confirmation ion (*m/z* 177→98) to the quantitation ion (*m/z* 177→80) measured in 1949 sets of standards with concentrations ranging from 0.025 to 25 μg/L. The *error bars* represent 1 SD.

Table 1. NIST cotinine perchlorate samples.

Sample	Expected, μg/L	Observed (mean ± SD), μg/L	Obs/Exp, %
1	6.21	6.22 ± 0.25	100
2	3.10	2.93 ± 0.31	95
3	1.24	1.31 ± 0.08	106
4	0.62	0.61 ± 0.03	98
5	0.41	0.45 ± 0.02	110
6	0.25	0.25 ± 0.01	100
7	0.12	0.13 ± 0.01	108

Cotinine solutions in water at known concentrations were prepared by using NIST cotinine perchlorate and analyzed by our standard LC/APCI MS/MS method. n = 3 for each.

urine reference material was used because no serum-based materials were available. In the RM 8444 set, only the blank sample with a recommended concentration of 0.8 ± 0.3 ng/g had a target within the low concentration range of primary interest to us. Our result for the analysis of this blank sample was 0.898 ± 0.048 ng/g (n = 4). These materials were also analyzed as routine unknowns according to our regular procedures.

Supplemented serum analyses. Cotinine perchlorate was also used to supplement a serum sample at four different nominal concentrations ranging from ~0.5 μg/L to 230 μg/L. The serum sample used in this case was from a nonsmoker who had no known source of ETS exposure during the 5 days before donation. After the serum was supplemented, it was well-mixed and then aliquoted into vials with coded labels and frozen. On four separate occasions over an interval of several months, vials were selected for analysis by a chemist with knowledge of the codes, but with no other involvement in this study, and analyzed as true unknowns by our method. The results from this series of analyses are given in Table 2.

Comparisons with GC/MS. To further evaluate the performance of our LC/MS/MS method, we carried out split-sample assays with a group of serum samples by both LC/MS/MS and GC/MS. As indicated in Fig. 5A, very good agreement was observed throughout a range of concentrations for a set of serum samples analyzed first by LC/MS/MS and then reanalyzed by capillary GC/low-resolution MS. Because of the relatively limited sensitivity of the low-resolution GC/MS analysis, this comparison was restricted to samples with a serum cotinine concentration ≥10 μg/L. For comparisons at the very low concentration range of primary interest to us, it was necessary to use high-resolution GC/MS. The results from split-sample analyses of 28 serum samples by both LC/MS/MS and by high-resolution GC/MS at 10 000 resolving power is given in Fig. 5B. These data were in very close agreement down to the detection limit with results obtained by the LC/MS/MS method and provided further assurance of the accuracy of our identification of cotinine in the LC/MS/MS assays, even at very low sample concentrations. Although LC/MS/MS and high-resolution capillary GC/MS are both mass spectrometric procedures with chromatographic inlets, the nature and extent of the chromatographic separation is different in the two techniques, and high-resolution MS is regarded as the most selective analytical technique available for analyses of this type. Thus we believe that the close agreement in results obtained by these two approaches when analyzing common samples provides strong confirmation for the analytical validity of our LC/MS/MS method.

Table 2. Replicate analyses of a supplemented serum sample.

Sample	Target concentration,[a] μg/L	Observed (mean ± SD),[b] μg/L	CV, %	n
A	0.57	0.63 ± 0.087	13.8%	8
B	1.15	1.02 ± 0.08	7.8	5[c]
C	11.5	11.8 ± 0.99	8.4	8
D	229	231 ± 9.7	4.2	8

[a] Serum from a nonmoker with no known recent exposure to ETS was supplemented with cotinine perchlorate to yield four target concentration levels. Aliquots were then analyzed as blind unknowns on a periodic basis.

[b] Values are from aliquots of these samples measured on four separate occasions.

[c] Assayed on three occasions only.

Assay precision. As noted in *Materials and Methods*, both bench and blind QC materials were routinely included in each assay of 50 samples. Results from the analysis of the first 200 assays of the low-sample bench QC from the NHANES III, Phase 1 series *[32]*, with a target value of 1.84 μg/L based on an initial set of 20 characterization assays, were 1.88 ± 0.122 μg/L (mean ± SD), with a CV of 6.46%. Each sample run also contained a variable number of blind QC samples that were unknown to the analysts as QC samples. The target value for this pool on the basis of the initial set of characterization assays was 0.262 μg/L, and the result obtained from the NHANES III, Phase 1 series, was 0.268 ± 0.031, with a CV of 11.6% (n = 624). The high assays also contained both bench and blind QC samples that were inserted into the assays in a similar manner. The results observed for these materials in NHANES III, Phase 1, were 207 ± 12.7 μg/L, with a CV of 6.14% (n = 136) for the bench QC sample and 212 ± 14.8 μg/L, with a CV of 6.98% (n = 233) for the blind QC pool. Thus the long-term precision for these analyses was ~6% in each case except for the very low-concentration blind QC pool, which had a CV of ~12%.

Interferences. No endogenous substances in serum that could interfere in these analyses have yet been identified. To interfere, a substance would need to be extractable from serum by methylene chloride, be relatively nonvolatile, have the same retention time as cotinine during HPLC, be ionizable by APCI under our conditions of analysis, have a molecular mass of 176 ± 1, and fragment in Q2 to form a product ion at *m/z* 80. In addition, any

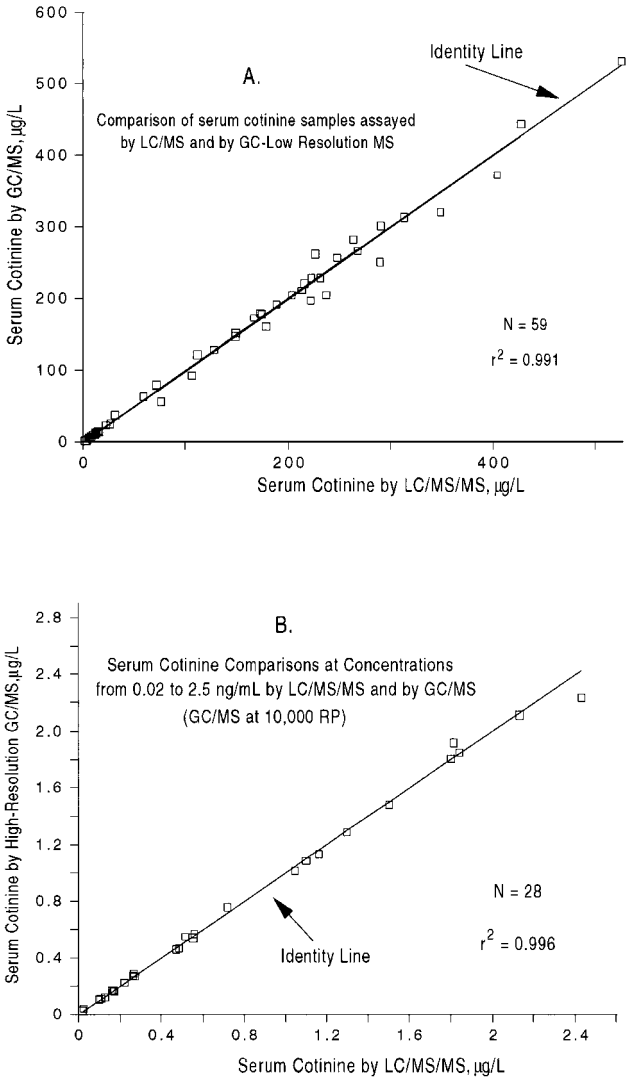

Fig. 5. Comparison of the analysis of serum samples by HPLC APCI MS/MS with either capillary GC/low-resolution MS (*A*) or capillary GC/high-resolution MS at 10 000 resolving power (*B*).

significant interference should be detectable by a shift in the confirmation ratio for that sample. Both ascorbic acid and serotonin have a molecular mass of 176 and are present in blood; however, analysis of standards confirmed that neither of these compounds would interfere in this analysis. At this time, the only substance known to be potentially capable of interfering in our assay is the pharmaceutical Pemoline (Cylert™, Abbott Labs.), a central nervous system stimulant; it can be resolved from cotinine if necessary by slightly altering the chromatographic conditions.

Cotinine stability. Because it is often necessary to store serum samples for an extended time before the analysis, the stability of cotinine during storage is an important consideration. Several investigators have reported that cotinine in serum is quite stable during storage *[10]*, and our results are in agreement. Fig. 6 shows the results from the analysis of two serum pools that were held either at room temperature or at 37 °C for up to 6 weeks. In all cases, the serum cotinine concentration remained essentially constant throughout the period of analysis. Fig. 7, which shows the monthly mean values observed for the low-concentration bench and blind QC pools over a period of 20 months, illustrates that both pools maintained a constant concentration during this period. The same results have been observed with the high-concentration QC pools. After >4 years, we have seen no evidence of instability in any of these serum pools during storage at −60 °C.

In conclusion, this method for the analysis of cotinine in serum by LC/APCI MS/MS combines the very high sensitivity and analytical specificity of APCI MS/MS in MRM mode with the ruggedness and speed of HPLC. This method has enabled us to undertake the analysis of large numbers of samples in a semiautomated manner while maintaining high sensitivity with a concomitant high degree of confidence in the correct identification of

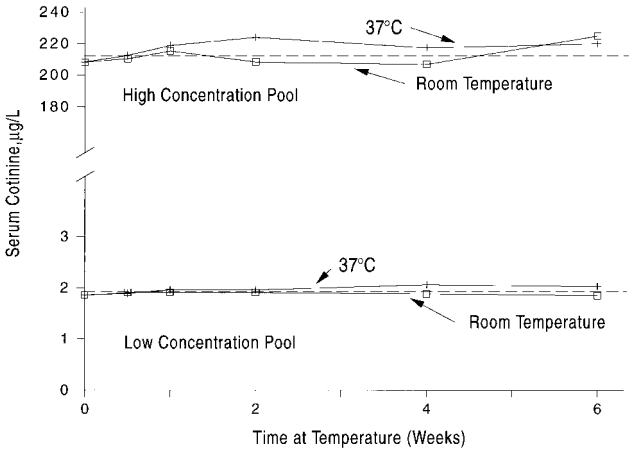

Fig. 6. Stability of two serum pools at different concentrations of serum cotinine and stored either at room temperature (~24 °C) or at 37 °C. All samples were analyzed by the standard LC/MS/MS method.

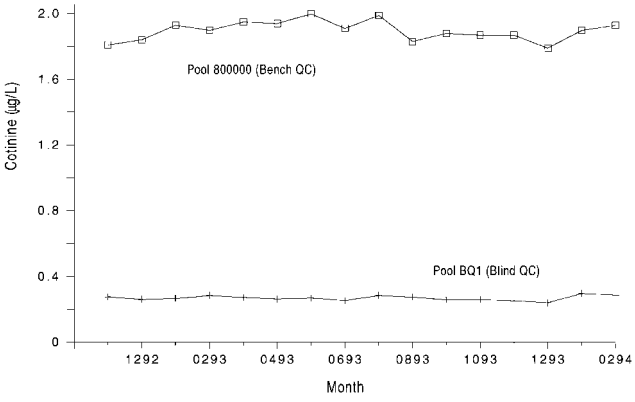

Fig. 7. Monthly means for the low-concentration bench and blind QC pools over 20 months.

the analyte. Over the past 4 years, >32 000 samples have been analyzed on a single instrument by this method. The sensitivity, accuracy, and ruggedness of this method make it very well-suited to the demands of high-sample-volume epidemiologic investigations of ETS exposure in selected populations.

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