Exposure to Environmental Tobacco Smoke Measured by Cotinine $^{125}$I-Radioimmunoassay

George J. Knight, Glenn E. Palomaki, Dale H. Lee, and James E. Haddow

We describe a polyclonal-antiserum-based $^{125}$I-radioimmunoassay for cotinine that is suitable for measuring nonsmokers' passive exposure to tobacco smoke in the environment. The standard curve ranged from 0.25 to 12.0 μg/L, with an estimated lower limit of sensitivity of 0.2 μg/L (95% B/B₀ = 0.2 μg/L; 50% B/B₀ = 4.0 μg/L). The median within-assay CVs for patients' samples with cotinine values from 0.4 to 1.3, 1.4 to 2.4, 2.5 to 4.6, and 4.7 to 15.6 μg/L were 13.9%, 7.2%, 5.1%, and 5.7%, respectively. Between-assay CVs for two quality-control sera with average values of 1.53 and 3.68 μg/L were 14.3% and 7.8%, respectively. Analytical recovery of cotinine from smokers' sera diluted in zero calibrate ranged from 91% to 116%. Cotinine values measured on 79 paired sera and urines from nonsmokers showed significant correlation with self-reported exposure to environmental tobacco smoke (r = 0.49, P < 0.001 for sera; r = 0.57, P < 0.001 for urine). The log of the values for serum and urine cotinine were also significantly correlated (r = 0.85, P < 0.001). Evidently, polyclonal antiserum can be used to develop a cotinine assay for measuring exposure to environmental tobacco smoke that compares well with that described for monoclonal-based assays.

Cotinine, the major metabolite of nicotine, is now commonly measured in serum, urine, and saliva to quantify tobacco smoke intake and to confirm smoking status (8–7), and is increasingly used to assess uptake of environmental tobacco smoke (ETS) by nonsmokers as well (8–13). Cotinine has been measured by "high performance" liquid chromatography (14, 15), gas chromatography (16–18), radioimmunoassay (19, 20), and enzyme immunoassay (21). Immunoassays have been described involving monoclonal or polyclonal antiserum (19–21). Polyclonal-based cotinine immunoassays, as those involving an $^{125}$I tracer, can be less sensitive and more imprecise than comparable monoclonal-based assays because of the presence of "bridge" antibodies produced in response to the amide group, which links cotinine to the carrier protein immunogen (20, 22, 23). In $^{125}$I-based assays, the $^{125}$I-labeled cotinine/tyramine radioligand contains the same amide linking group and, as a consequence, the tracer reacts more strongly with the cotinine antiserum than does cotinine itself, resulting in an assay with a shallow dose-response curve and decreased sensitivity.

We have previously described (20) an improved $^{125}$I-based radioimmunoassay suitable for measuring cotinine in smokers, in which we used polyclonal antiserum depleted of "bridge" antibodies by absorption with a nicotine derivative covalently bonded to the carrier protein by an amide bond. The resulting cotinine assay developed is comparable in sensitivity, precision, and slope of the dose response curve to other $^{125}$I-based radioimmunoassays. We report here a radioimmunoassay protocol involving use of this absorbed antiserum, which is suitable for measuring cotinine in serum and urine of nonsmokers exposed to ETS. We also provide data validating the clinical reliability of the assay.

Materials and Methods

Reagents

Reagent chemicals were identical to those previously described (20). Goat anti-rabbit IgG antiserum was obtained from Pel-Freez, Rogers, AR.

Procedures

All procedures were identical to those previously described (20), with the two following exceptions:

Radioligand iodination: Iodinate and chromatograph N-(p-hydroxyphenethyl)-trans-cotinine carboxamide as previously described, but use 2.0 mCi of Na$^{125}$I to increase the estimated specific activity of the tracer to 300 kCi/mol.

Calibrators: Prepare calibrators, ranging from 0.25 to 12.0 μg/L, by dissolving cotinine picroate (ours was a gift from Dr. Peyton Jacob III, San Francisco General Hosp. Med. Center, San Francisco, CA) in serum pooled from sera obtained from nonsmoking individuals not exposed to ETS. To ensure that cotinine is not present in the calibrator serum pool, select only those nonsmokers' sera giving the highest count when each individual serum is run as a $B₀$ tube. As a further check on the calibrator diluent, compare the count in $B₀$ tubes from pre- and post-dialysis aliquots of the pool. Dialyze a 10-mL aliquot of the serum pool for seven days against 4 L of phosphate-buffered saline (per liter, 0.01 mol of potassium phosphate and 0.15 mol of sodium chloride, pH 7.4) to remove low-molecular-mass substances. The counts on the two aliquots should agree within 1%, indicating that the cotinine concentration in the calibrator pool is close to zero.

Cotinine assay, serum: In a 12 × 75 mm polystyrene tube, add 100 μL of calibrator or unknown to 800 μL of Tris pH 7.4 buffer (per liter, 150 mmol of sodium chloride, 10 mmol of Tris, and 1 g of gelatin) containing 80 000 counts/min of tracer. Add 100 μL of rabbit anti-cotinine antibody diluted 300-fold in Tris buffer, and vortex-mix. Incubate overnight (16 to 24 h) at room temperature. In the morning, add 100 μL of goat anti-rabbit antiserum diluted 25-fold in Tris buffer. Incubate at room temperature for 2 h. Add 1.0 mL of 80 g/L polyethylene glycol to each tube and thoroughly vortex-mix. Centrifuge at 3000 × g for 30 min, decant the supernate, and count its radioactivity in a gamma counter. Express all counts/min as a percentage of the radioactivity of the zero cotinine calibrator ($B/B₀$) after correcting counts for nonspecific binding. Fit calibrators to four-parameter logistic log model.

Cotinine assay, urine: Use the same assay protocol as for serum but decrease sample volume to 10 μL. Add 100 μL of the cotinine-free serum diluent to ensure that the matrix composition of the sample tube resembles that of the calibrators. The 10-μL urine sample represents only 1% of...
the final reaction volume of 1.0 mL, which minimizes the effect of varying urine composition on assay results.

**Linearity.** Serially dilute urine samples with cotinine concentrations <10 μg/L, using the zero standard, and assay. Compare assay results with those predicted, using the cotinine value obtained on the undiluted urine as the reference value.

**Recovery.** Measure cotinine in serum samples from smokers, using the previously described assay (20). Dilute these sera 50-fold with the zero calibrator and assay in the system described above. Compare assay values with those predicted by dividing the initial cotinine value by 50.

**Creatinine determinations:** Determine creatinine in urine samples with diagnostic kit no. 555 from Sigma Chemical Co., St. Louis, MO, using package-insert instructions.

**Study subjects:** Having given informed consent, a total of 79 nonsmoking individuals from four locations participated: (a) employees of a local hospital with smoking restricted to designated areas, (b) students of a local college with smoking allowed in common areas, (c) government employees at a workplace where smoking is permitted, and (d) employees at our own institution, where smoking is prohibited. Study subjects were asked to estimate their exposure to ETS for the current day and for the previous one, two, and three days. A 10-mL specimen of blood and a urine specimen were obtained from each participant. Serum was separated within 2 h and stored, along with the urine, at -20°C. Samples were thawed just before assay.

**Results**

**Analytical Variables**

**Assay standard curve:** Figure 1 shows a typical standard curve. The lower limit of sensitivity, as defined by 90% B/B₀ is about 0.4 μg/L; although values as low as 0.20 μg/L are distinguishable from the zero calibrator (95% B/B₀ = 0.18 μg/L).

**Precision within assay:** This was assessed by comparing the means of replicates of 145 nonsmokers’ sera from four separate assays. Median within-assay coefficients of variation (CVs) ranged from 13.9% to 5.7% for cotinine concentrations from 0.4 μg/L to 15.6 μg/L.

**Precision between assays:** Internal quality-control sera with average values of 1.53 μg/L and 3.68 μg/L yielded CVs of 14.3% and 7.8%, respectively, in 17 separate assays.

**Linearity:** Figure 2 compares the actual assay values obtained on six serially diluted urine samples with those predicted from the value obtained for the undiluted urine samples. Linearity was satisfactory over the range 0.4 to 10.0 μg/L.

**Analytical recovery:** Table 1 lists cotinine values obtained by direct assay of six smokers’ sera after 50-fold dilution in zero calibrate. These values are compared with those predicted by dividing the value obtained on the undiluted smokers’ sera by 50. Recoveries ranged from 91% to 116% (mean 99.8%).

**Clinical Studies**

Among the 79 individuals studied, average cotinine concentrations in serum increase significantly as self-reported exposure to environmental tobacco smoke increases (Spearman rank correlation test on ungrouped data, r = 0.48, P <0.001), although each quartile of exposure overlaps (Figure 3). Average values for serum cotinine for each quartile of exposure are 0.92, 0.92, 1.14, and 1.36 μg/L, respectively. Average values for urine cotinine (uncorrected for creatinine) show a similar correlation (Spearman rank correlation test on ungrouped data, r = 0.57, P <0.001). Average urinary cotinine concentrations for

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<th>Table 1. Cotinine Assay Values Obtained for 50-Fold-Diluted Sera from Smokers Compared with Expected Values</th>
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<td>Cotinine value, undiluted sera</td>
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<td><strong>Av. recovery</strong></td>
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**Fig. 1.** Typical curve for cotinine
95% B/B₀ = 0.18 μg/L; 90% B/B₀ = 0.36 μg/L; 80% B/B₀ = 0.88 μg/L; 50% B/B₀ = 4.17 μg/L; 20% B/B₀ = 19.3 μg/L.

**Fig. 2.** Linearity of diluted urine samples
Closed circles represent urine cotinine values from six undiluted samples. Open circles represent the urine cotinine values for 1:2, 1:4, and 1:8 dilutions of the original samples.
Discussion

Clearly, an $^{125}$I-based radioimmunoassay used (20) for measuring cotinine associated with active smoking can be modified to measure cotinine found in the serum and urine of nonsmokers exposed to environmental tobacco smoke. The assay's lower sensitivity is 0.2 to 0.4 µg/L, with between-assay CVs ranging from approximately 8% to 15%, in that portion of the standard curve where values for cotinine found in ETS exposure are typically found. The slope of the standard curve is approximately 14% per doubling dose over the range 1.0 to 8.0 µg/L.

The sera of study subjects exposed to ETS contains possible interfering substances as a result of tobacco smoke intake (e.g., nicotine) that would be absent from the sera of nonexposed individuals. Therefore, in place of standard recovery studies, in which chemically pure cotinine is added to nonsmokers' serum, we diluted smokers' sera for which cotinine values were known and compared the result with that expected. With this approach, potential interfering substances are present in the same proportion after dilution as are found in the active smokers' sera. The assayed values of the diluted sera agreed well with those predicted by calculation.

In a recent report, $^{125}$I-based radioimmunoassays involving polyclonal antisera were less sensitive and produced shallower dose–response curves than those obtained by using monoclonal antibodies (22). However, in this comparison polyclonal antisera were used that contained bridge antibodies and, as we have reported previously (20), use of such antisera results in an inferior assay. The authors also used a $^{125}$I tracer of low specific activity (47 kCi/mol vs 300 kCi/mol in the present study), which further diminished the sensitivity of the polyclonal based assay. In the assay reported here antisera depleted of bridge antibody was used, and results compare favorably with those reported when monoclonal antisera was used. The slope of the dose–response curve for this assay, as defined by the change in percent binding per doubling dose, is 14% for amounts of cotinine ranging from 0.1 to 0.8 ng per tube, vs 15% over the range from 0.1 to 1.0 ng per tube for the monoclonal assay, as estimated from the published standard curve (22). About 0.40 ng of cotinine is required to produce 50% inhibition of the zero standard for the present assay vs an average of 0.55 ng for the monoclonal assay. These data indicate that polyclonal antisera can produce an assay equivalent to that obtained by using monoclonal antisera if interfering bridge antibodies are first removed. These results also suggest that alternative approaches to overcoming the “bridge” effect, such as linking
the hapten to an iodinatable derivative or solid phase with a chemical bond different from that used for linking the hapten to the carrier protein immunogen (23), may prove successful for cotinine.

We have previously reported that the cotinine concentration in an untreated urine specimen shows significant correlation with self-assessed ETS exposure; our present clinical studies corroborate these findings. We have also reported that cotinine as measured by the present assay in the serum of nonsmoking mothers in the second trimester of pregnancy is inversely correlated with birthweight (13). In the present study, serum cotinine correlated significantly with self-reported exposure. Furthermore, serum and urine cotinine concentrations were highly correlated, indicating that either serum or an untreated urine specimen can reliably be used to estimate exposure to ETS. These findings agree with those of others (3, 12), who conclude that the choice of body fluid for epidemiological studies can respond to the needs of the study design rather than any inherent advantage of measuring cotinine in particular body fluids.

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References

Nonenzymic Glycation of Human Immunoglobulins Does Not Impair Their Immunoreactivity
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Diabetic patients have an increased proportion of their immunoglobulins nonenzymically glycated. To investigate the possibility that this may contribute to increased susceptibility to infection, we compared the immunoreactivity of glycated and nonglycated human immunoglobulins against rubella and hepatitis; streptococcal exoenzyme and infectious mononucleosis; human lymphocytotoxic antigens (HLA); and Varicella zoster in terms of antigen-antibody binding, cell agglutination, cytotoxicity, and complement-fixation properties, respectively. We found no evidence to support the supposition that glycated immunoglobulins are functionally impaired.

Diabetic patients are generally—though not universally—recognized to have impaired resistance to infection (1). Two recent reports lend support to the speculation that