

## **DEVELOPMENT AND CHARACTERIZATION OF AN ELISA FOR COTININE IN BIOLOGICAL FLUIDS**

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*Cotinine is a major metabolite of nicotine and serves as an important biomarker of tobacco smoke exposure. To monitor exposure to tobacco smoke or nicotine, a sensitive enzyme-linked immunosorbent assay (ELISA) for cotinine was developed. The test had an I-50 of between 0.5 and 1.0 ng/ml for cotinine and about 500-fold less affinity for nico-*

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tine. Few matrix effects were not detectable in human saliva, although relatively small matrix effects (1-50 for cotinine, 2.8 ng/ml) were observed in human serum and urine. The test accurately measured the levels of cotinine in NIST standards in freeze-dried human urine derivative material ( $r = .9999$ ) indicating its reliability for measurement of cotinine. The test readily detected low levels (5-500 ng/ml) of cotinine in human saliva and serum samples. Also, the levels of cotinine in plasma and urine samples from smoke-exposed mice and rats could be rapidly analyzed for cotinine. This ELISA is therefore a sensitive and accurate test for the determination of cotinine in plasma, serum, saliva, and urine samples from humans and animals, and can be successfully used for monitoring and quantifying exposure to tobacco smoke or nicotine.

## INTRODUCTION

Active and passive exposure to tobacco smoke is associated with a number of health effects in exposed populations. While a vast amount of data on health-related effects of smoking has accumulated, it has been generally difficult to accurately quantify the tobacco smoke exposure in individuals, especially passive smokers. Various biomarkers, such as blood levels of nicotine and its metabolites, isocyanate, carboxyhemoglobin, and urinary mutagens, have been employed to assess tobacco smoke exposures. Nicotine is generally regarded as a specific biomarker of tobacco smoke exposure as it is an important, pharmacologically active constituent of tobacco and is found only in tobacco plants at significant concentrations. Unfortunately, its plasma half-life is fairly short and it is found in relatively low concentrations in the blood and urine of active and passive smokers (Kyerematen et al., 1990), thus limiting its utility as a biomarker. In experimental animals it is especially difficult to accurately assess smoke exposure not only because the nicotine concentrations are low, but also because the sample size is very small.

Cotinine, a major metabolite of nicotine, possesses many characteristics that make it a very useful biomarker for monitoring tobacco smoke exposures. Its steady-state levels in plasma are severalfold higher than that of nicotine, reaching as high as 300-500 ng/ml or higher in the plasma of smokers, and the steady-state levels of cotinine in human plasma are directly related to nicotine intake (Rosa et al., 1992). Additionally, its plasma half-life is relatively longer, about 18 h in humans, which means that it functions as an integrative biomarker of nicotine exposure. Whereas detectable plasma and urinary levels of nicotine likely indicate recent (less than 6 h) exposure to tobacco smoke, cotinine levels in the plasma and urine of smokers remain high for at least 24 h after cessation of smoking (Kyerematen et al., 1990). Also, cotinine levels in urine tend to be higher than plasma levels of nicotine and persist for longer periods. These characteristics make cotinine a useful biomarker of tobacco smoke exposure (Watts et al., 1990).

As part of an effort to characterize animal models for studying the inhalation toxicity of tobacco smoke, we have been interested in developing a rapid, sensitive, and inexpensive routine method to quantify the expo-

sure of small laboratory rodents to cigarette smoke under a variety of conditions. Immunoassay, especially enzyme-linked immunosorbent assay (ELISA), is one of the few analytical techniques that can readily detect circulating concentrations of nicotine, cotinine, or *trans*-3'-hydroxycotinine (Benkirane et al., 1991; Langone et al., 1973; Kyerematen et al., 1990).

In this report we detail the development and characterization of a highly sensitive ELISA for cotinine and outline the characteristics of this ELISA and its application to the detection of cotinine in blood, saliva, and urine specimens from humans and laboratory animals.

## MATERIALS AND METHODS

### Animals

New Zealand White rabbits were used in the raising of anti-cotinine antibody. Samples of plasma and urine were obtained from rats and mice following exposure to tobacco smoke as described earlier (Gairola, 1986). These samples were collected as part of an ongoing study and held frozen at  $-80^{\circ}\text{C}$  for 1–2 yr. All animal experiments were performed according to the protocols approved by the University of Kentucky Institutional Animal Care and Use Committee operated under PHS Animal Welfare Assurance A3336-01.

### Human Subjects

Human serum specimens from verified tobacco cigarette smokers and nonsmokers were obtained from the Department of Oral Health Science, College of Dentistry, University of Kentucky. These samples had been collected for an unrelated study (Bridges et al., 1990) and had been kept frozen at  $-20^{\circ}\text{C}$  for 1–2 yr. These serum samples were collected from a group of 170 smoking and 170 nonsmoking healthy male volunteers. They were all recruited from the University of Kentucky and the local community. Subjects were excluded if on medication or if they used tobacco in any form other than cigarettes. The samples were collected by venipuncture from fasted individuals in the early morning. The smoking group consumed cigarettes ad libitum 5 min prior to sampling. A batch of 20 samples from each group (smokers and nonsmokers) was randomly chosen for the evaluation of the cotinine ELISA.

Saliva specimens collected from teen-age Kentucky public school students were obtained from the Health, Physical Education and Recreation Department, University of Kentucky. These samples had been part of an unrelated study concerning adolescent tobacco use (Noland et al., 1988, 1990) and had been stored at  $-20^{\circ}\text{C}$  for 1–2 yr. Samples from 471 male and female volunteer subjects were chosen from a total group of 1067 individuals for further analysis. A questionnaire was used to determine whether each subject was a tobacco smoker or nonsmoker. A batch of 20 samples

from each group, smokers and nonsmokers, was obtained for the evaluation of the cotinine ELISA.

A series of human urine samples from nonsmokers was provided by the University of Kentucky Medical Center Clinical Toxicology Laboratories. These samples were collected for clinical evaluation and were released to us once the specimen had been cleared by the clinical toxicology laboratory. A pool consisting of four of these urine samples was used for preparation of standard curves in the matrix studies.

### ELISA Procedures

The cotinine ELISA developed here was similar in format to those reported by Stanley and co-workers (1991). An anti-cotinine antiserum was raised in rabbits inoculated with a cotinine-protein complex (Stanley, 1992). This anti-cotinine antiserum (used without further purification) was noncovalently coated (Voller et al., 1976) on flat-bottom microtiter wells (Costar Corp., Cambridge, Mass.). A carboxyl derivative of cotinine (Stanley, 1992) was linked to horseradish peroxidase (HRP) (Zymed Labs, So. San Francisco) to give rise to a covalently bound cotinine-HRP conjugate (Wie and Hammock, 1982). Authentic cotinine (Sigma Chemical Co., St. Louis, Mo.) and analogs standards were prepared in methanol and diluted to appropriate concentrations in assay buffer (0.1 M potassium phosphate-buffered saline, pH 7.4, with 0.1% bovine serum albumin) or biological fluids. Nicotine N-oxide, cotinine N-oxide, *trans*-3'-hydroxycotinine, *cis*-3'-hydroxycotinine, N-methylcotinine, and dimethylcotinine were kindly provided by J. Donald deBethizy (RJR-Nabisco, Winston-Salem, N.C.). Cotinine-containing lyophilized human urine reference material was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, Md.).

All assays were performed at room temperature. The assay was started by adding 20  $\mu$ l of the standard, test, or control samples to each well, along with 180  $\mu$ l of cotinine-HRP conjugate solution. After an incubation period of 1 h, the wells were washed with wash buffer (0.01 M phosphate buffer, pH 7.4, with 0.05% Tween-20), and 150  $\mu$ l tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry, Gaithersburg, Md.) was added to each well. The optical density (OD) of each well was read at a wavelength of 650 nm with an automated microplate reader (EL310 Microplate Autoreader, Bio-Tek Inc., Winooski, Vt.) approximately 60 min after addition of the substrate.

During the assay, the presence of unbound cotinine in the standard or test sample competitively prevented the binding of the cotinine-HRP complex to the antibody present in the antiserum. Since the reaction of TMB substrate with HRP was responsible for the color (blue) production in the ELISA, the apparent concentration of cotinine in the sample was inversely related to the OD of the well. Apparent cotinine concentrations in biological specimens were calculated based on standard curves that were run in duplicate with each individual assay.

## RESULTS

Standard inhibition curves were constructed to determine the sensitivity and specificity of the cotinine antiserum using various metabolites of nicotine and related compounds (Fig. 1). Cotinine inhibited the ELISA with an I-50 (analyte concentration at half-maximal inhibition) of less than 1.0 ng/ml, and exhibited at least 10-fold lower affinity for *trans*-3'-hydroxycotinine, *cis*-3'-hydroxycotinine, and dimethylcotinine. Other tested congeners showed minimal activity, since concentrations several hundred times higher were needed to inhibit the test (Table 1).

Matrix or background effects can severely limit the usefulness of ELISA tests by changing their sensitivity. We therefore determined the shifts in I-50 values when the standard curves were prepared in three different matrices: human saliva, serum, and urine. Standards prepared in serum and saliva showed virtually no matrix effects, while the apparent I-50 for samples prepared in human urine was increased to about 2.8 ng/ml (Fig. 2).

To further validate the test, its performance was evaluated using cotinine standards obtained from NIST. A high correlation ( $r = .9999$ ) between the NIST standards and the apparent cotinine concentrations measured by the ELISA was observed (Fig. 3), suggesting a very good fit between actual cotinine levels in biological samples and apparent cotinine levels as measured by this assay.

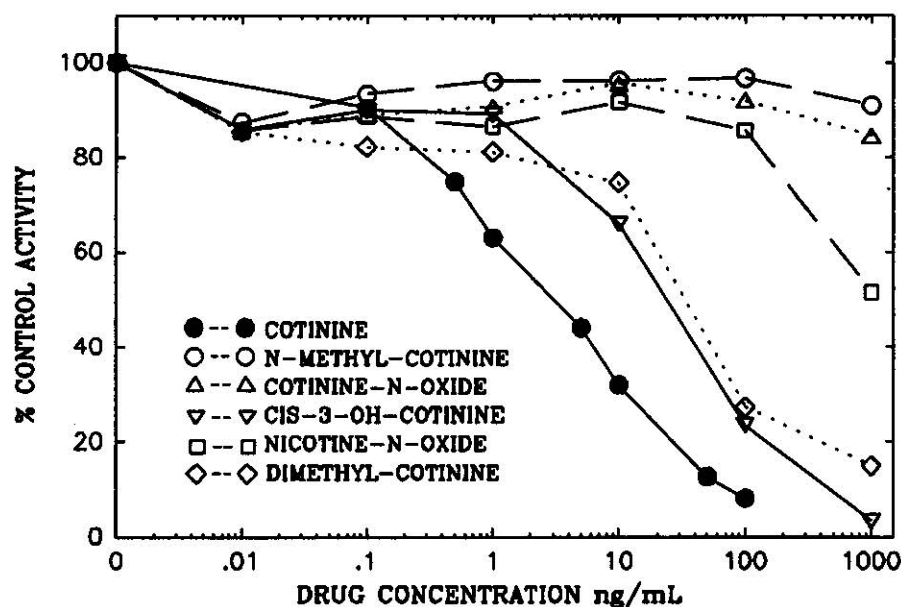
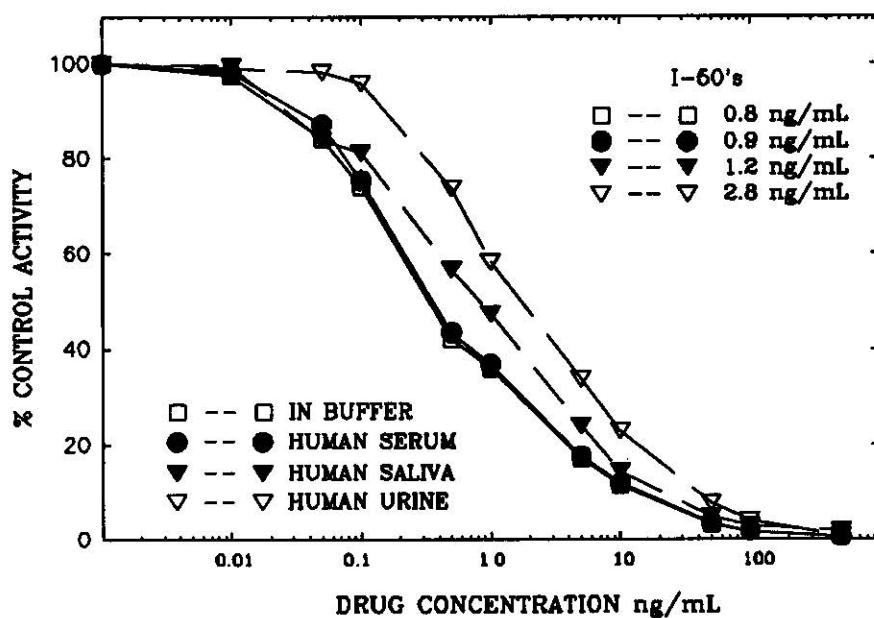


FIGURE 1. Cross-reactivity of cotinine antiserum by ELISA. ELISA activity for anti-cotinine antibody plotted as a function of added compound. Half-maximal inhibition occurs at around 1.0 ng/ml for cotinine. *cis*-3'-Hydroxycotinine and dimethylcotinine each showed cross-reactivity to the cotinine antibody.

**TABLE 1.** Cross-Reactivity of the Cotinine ELISA for Several Cotinine Analogs is Expressed as the I-50 (Analyte Concentration at Half-Maximal Inhibition)

Compound	I-50 (ng/ml)
Cotinine	1
<i>trans</i> -3'-Hydroxycotinine	12
<i>cis</i> -3'-Hydroxycotinine	15
Dimethylcotinine	20
Nicotine	700
Nicotine <i>N</i> -oxide	1000
Cotinine <i>N</i> -glucuronide	>1000
Cotinine <i>N</i> -oxide	>1000
<i>N</i> -Methylcotinine	>1000
Nicotinamide	>1000
Nicotinic acid	>1000
Nikethamide	>1000

Note. The cotinine antibody is relatively specific for cotinine and three related compounds.



**FIGURE 2.** Sample matrix effects of cotinine ELISA standard curve. There was an increase of the inhibition of the cotinine ELISA by cotinine in the presence of buffer (no matrix) and 10  $\mu$ l (matrix volume) of human serum, saliva, and urine.

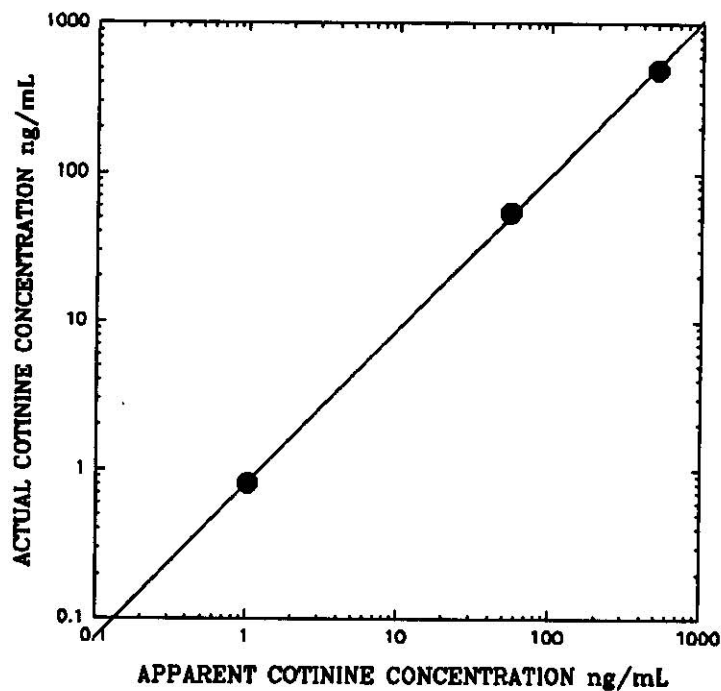


FIGURE 3. Cotinine ELISA compared quantitatively with NIST standards in human urine. There was excellent correlation between actual cotinine concentration in NIST standards and apparent cotinine concentration in these standards ( $r = .9999$ ).

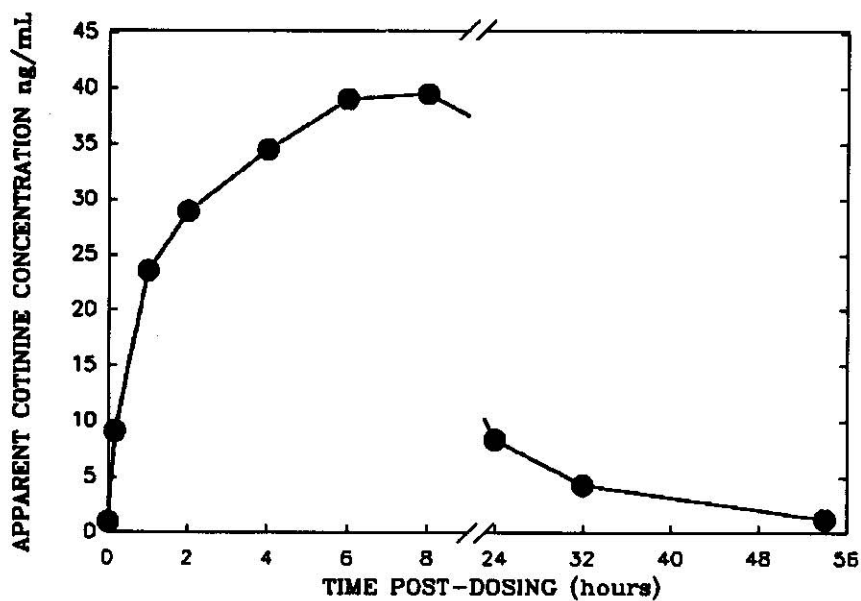


FIGURE 4. Detection of cotinine in rat plasma after a 0.5 mg/kg iv dose of nicotine. Plasma levels of cotinine after administration of 0.5 mg/kg of nicotine iv to a single rat increased to about 8 h after dosing, followed by a slow decline.



After standardization of the assay as already described, various experiments were performed to determine its utility in measuring the levels of cotinine in plasma and urine from rodents experimentally exposed to nicotine or tobacco smoke. Blood samples were collected from rats given a single intravenous (iv) infusion of 0.5 mg nicotine/kg body weight and analyzed for cotinine using our ELISA. Peak plasma cotinine levels of about 40 ng/ml were detected at 8 h, after which the nicotine levels dropped rapidly, returning to baseline levels of less than 2 ng/ml by 54 h postdosing (Fig. 4). The experiment showed that plasma levels of cotinine in rat plasma are readily detected by this ELISA for up to 30 h after iv administration of nicotine.

In the next series of experiments, plasma samples were collected from three control and three smoke-exposed mice and rats and analyzed for cotinine. The data clearly differentiated between the exposed and unexposed groups of animals (Table 2). Similarly, urine samples from approximately 40 smoke-exposed and unexposed mice were analyzed for cotinine. The mean concentration of apparent cotinine in urine samples from exposed mice was 595 ng/ml, with a range of 240–890 ng/ml, while the 40 samples from unexposed animals averaged 8.96 ng/ml. These data clearly demonstrated the potential of this assay in monitoring the exposure of animals to tobacco smoke under experimental conditions.

This ELISA was next used for measuring the serum and salivary cotinine levels of human smokers and nonsmokers, and was found to readily distinguish between these groups. A series of human serum samples was analyzed in a blind study by this ELISA. The cotinine levels of the serum samples from smokers averaged  $307 \pm 125.1$  ng/ml, while those from the nonsmokers averaged  $6.74 \pm 1.39$  ng/ml (Fig. 5). In related experiments saliva samples were collected from smoking and nonsmoking volunteers for analysis. Again, the cotinine ELISA effectively distinguished between smokers (average  $328.7 \pm 111.8$  ng/ml) and nonsmokers (average  $5.22 \pm 2.14$  ng/ml) (Fig. 6).

## DISCUSSION

We have developed an ELISA for cotinine that detects this major metabolite of nicotine with an apparent I-50 of about 1 ng/ml or less. This test is relatively specific for cotinine in that it has a better than 10-fold apparent higher affinity for cotinine than for *trans*-3'-hydroxycotinine, *cis*-

TABLE 2. Cotinine Concentrations in Plasma of Rats and Mice Exposed to Mainstream Tobacco Smoke

Animal Groups	Plasma cotinine levels (ng/ml)	
	Rats	Mice
Room controls	$1.01 \pm 0.20$	$2.01 \pm 0.58$
Sham controls	$1.85 \pm 1.00$	$2.12 \pm 0.27$
Smoke-exposed	$49.0 \pm 22.4$	$95.5 \pm 14.2$



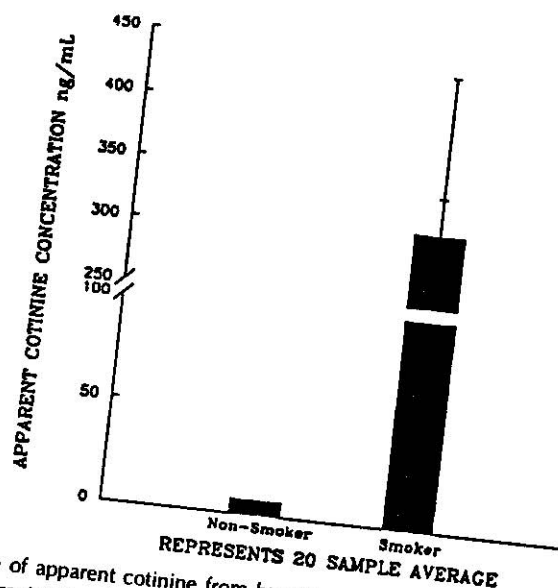


FIGURE 5. Detection of apparent cotinine from human serum samples using cotinine ELISA. The left-hand bar shows apparent serum levels of cotinine in 20 nonsmokers, while the right-hand bar shows apparent serum levels of cotinine in 20 smokers.

3'-hydroxycotinine, and dimethylcotinine. The test is essentially unreactive with nicotine and nicotine N'-oxide since it has 500–1000 times less affinity for these substances than for cotinine. This test also accurately quantified NIST cotinine standards in urine with a correlation coefficient of  $r = .9999$ , a very acceptable level of accuracy for an enzyme immunoassay.

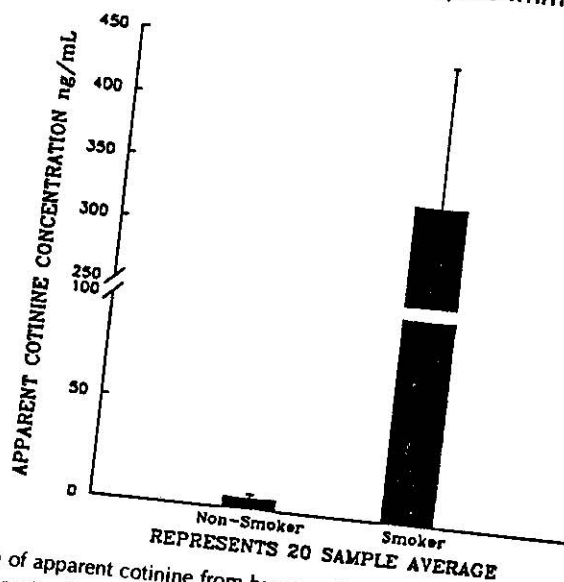


FIGURE 6. Detection of apparent cotinine from human saliva specimens using cotinine ELISA. The left-hand bar shows apparent saliva levels of cotinine in 20 nonsmokers, while the right-hand bar shows apparent saliva levels of cotinine in 20 smokers.

In addition to accuracy and sensitivity, this test is relatively resistant to interfering materials in biological samples. When this test was run in the presence of 10  $\mu$ l human saliva or serum the apparent I-50 for cotinine was not significantly affected, and when the test was run in the presence of human urine the I-50 was reduced from 1.0 to about 2.8 ng/ml, a relatively modest matrix effect. Human urine samples even from nonsmokers are likely to contain small amounts of cotinine, and as such some of the I-50 shift observed in human urine tests could likely be due to the presence of traces of cotinine.

The apparent resistance to matrix effects or interfering substances in matrix is a very useful characteristic of this ELISA. Immunoassay-based tests are generally not entirely free of matrix effects, and the utility of an immunoassay depends largely on its sensitivity for the analyte of choice and its ability to distinguish between the analyte and extraneous material. This is especially so for tests such as a cotinine ELISA, which may be required to detect very small levels of cotinine as in small experimental animal models or epidemiological studies of environmental tobacco smoke exposure.

The results of the rat experiment showing an increase and a subsequent decrease following infusion of a bolus dose of nicotine further demonstrated the utility of this assay for pharmacokinetic studies.

Studies of human salivary and serum samples again demonstrated the utility of this test in distinguishing between smokers and nonsmokers. Forty- and 60-fold differences in the apparent cotinine levels of saliva and serum samples, respectively, in smokers and nonsmokers clearly established the usefulness of this assay for validating the smoking status of subjects in large epidemiological studies. Although both saliva and serum samples are useful for assessing the exposure to tobacco smoke or nicotine (Watts et al., 1990), low background binding and the relative ease of collecting saliva samples make it the fluid of choice for routine analyses of cotinine levels.

Urinary analysis is also useful; however, the problem with urine is that its volume, pH, flow, and renal function are unpredictable variables (Watts et al., 1990). Correction using creatine content of urine has been suggested (Hoffmann and Brunemann, 1983), but it provides only a partial solution as creatine excretion rates are quite variable between individuals. For this reason blood was considered the fluid of choice by Watts et al. (1990), followed closely by saliva, which was considered acceptable, and good correlations were reported between serum and saliva levels for results from the same subject (Benowitz, 1983).

These data are entirely consistent with what is known of the metabolism and disposition of cotinine in humans (Bjercke et al., 1986; Kyerematen et al., 1990; Watts et al., 1990). Cotinine is the major metabolite found in plasma and its plasma half-life, at approximately 17.2 h, is relatively long. Cotinine is therefore a highly effective biological marker of tobacco smoke and nicotine exposure. Additionally, there appears to be an approximately 1:1 correlation between plasma and salivary levels of cotinine (Watts et

al., 1990). Therefore, because of the very low matrix or background effect in plasma and saliva with this ELISA, both of these biological fluids make good substrates for distinguishing between smokers and nonsmokers when using cotinine as the biological marker.

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