!!!DO NOT REMOVE THIS COPY!!!

Please

183

W.A. Rees[1,2], S. Kwiatkowski[4], S.D. Stanley[5], D.E. Granstrom[2], J-M. Yang[4], C.G. Gairola[1,3], D. Drake[4], J. Glowczyk[4], W.E. Woods[2], and T. Tobin[1,2]

DEVELOPMENT AND CHARACTERIZATION OF AN ELISA FOR *trans*-3-HYDROXYCOTININE, A BIOMARKER FOR MAINSTREAM AND SIDESTREAM SMOKE EXPOSURE.

REFERENCE: Rees, W. A., Kwiatkowski, S., Stanley, S. D., Granstrom, D. E., Yang, J-M., Gairola, C. G., Drake, D., Glowczyk, J., Woods, W. E., and Tobin, T., "The Development and Characterization of an ELISA for *trans*-3-hydroxycotinine, a Biomarker for Mainstream and Sidestream Smoke Exposure," *Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment—Fifth Volume, ASTM STP 1306*, David A. Bengtson and Diane S. Henshel, Eds., American Society for Testing and Materials, 1996.

Abstract: *trans*-3-Hydroxycotinine is the major urinary metabolite of nicotine in man and can serve as an important biomarker of tobacco smoke exposure. A sensitive ELISA test for *trans*-3-hydroxycotinine was developed with an I-50 for this nicotine biomarker of between 1.0-3.0 ng/ml. This ELISA test has about 10 fold less affinity for cotinine and 1000-fold less affinity for nicotine and other nicotine metabolites. No matrix effects were detectable in human saliva and relatively small matrix effects (I-50 for *trans*-3-hydroxycotinine about 25 ng/ml) in urine was observed. The assay readily detected levels of apparent *trans*-3-hydroxycotinine in urine samples from smoke-exposed mice and rats. This ELISA is therefore a sensitive test for the determination of *trans*-3-hydroxycotinine in plasma, saliva, and urine samples from humans and animals, and can be used to monitor exposure to tobacco smoke or nicotine.

Keywords: Enzyme-Linked Immunosorbant Assay, nicotine, *trans*-3-hydroxycotinine, cotinine, metabolite

Active and passive exposure to tobacco smoke is associated with a number of health effects in exposed populations. While considerable data on health-related effects of smoking has accumulated, it has been difficult to accurately [illegible] tobacco smoke exposure in individuals, especially passive smokers. Various biomarkers, e.g., blood levels of nicotine and its metabolites, isocyanide, carboxyhemoglobin and urinary mutagens, etc., have been employed in attempts to assess tobacco smoke exposures. In this regard, nicotine is generally seen as a specific biomarker of tobacco smoke exposure as it is an important,

The Graduate Center for Toxicology[1], The Maxwell Gluck Equine Research Center[2], Tobacco and Health Research Institute[3], Dept. of Plastic Surgery[4], University of Kentucky, Lexington, Kentucky 40546-0099

Dept. of Chemistry University of Warsaw, Poland[4]

Truesdail Laboratories, Inc.[5], Tustin, California

Duke University Medical Center[6], Durham, N.C.

Leave

pharmacologically active constituent of tobacco and is found at significant concentrations only in tobacco plants.

On the other hand, nicotine's plasma half-life is short, and it is found only in relatively low concentrations in the blood and urine of active and passive smokers (Kyerematen et al., 1990). 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 generally small. What is required is a highly sensitive detection method for a non-invasive biomarker of passive smoke or nicotine exposure.

trans-3-Hydroxycotinine, the major urinary metabolite of nicotine, possesses many characteristics required of a biomarker for monitoring tobacco smoke exposures. Its steady state levels in plasma are several-fold higher than those of nicotine, reaching concentrations as high as 115 to 160 ng/ml or higher in the plasma of smokers (Kyerematen et al., 1991); additionally, the steady state levels of *trans*-3-hydroxycotinine in human plasma are directly related to nicotine intake (Neurath et al., 1988). Another advantage is that its plasma half-life is relatively long (6 hours) (Neurath et al., 1988), which means that it can serve as an integrative biomarker of nicotine exposure. A further advantage is that *trans*-3-hydroxycotinine levels in human urine tend to be substantially higher than plasma levels of nicotine and persist for longer periods. These characteristics make *trans*-3-hydroxycotinine a potentially very useful biomarker of tobacco smoke exposure in humans (Watts et al., 1990).

As part of an effort to characterize animal models for studying the inhalation toxicity of tobacco smoke, a rapid, sensitive and inexpensive routine method to quantify the exposure of humans and animals to cigarette smoke was developed. Immunoassay, specifically Enzyme-Linked Immunosorbent Assay (ELISA), is one of the few analytical techniques that can readily detect circulating concentrations of cotinine or *trans*-3-hydroxycotinine (Benkirane, et al., 1991; Langone et al., 1973; Kyerematen et al., 1990). In this regard, an ELISA which detected low levels of cotinine in humans and animal fluids was previously developed.

This report details the development and characterization of a highly sensitive ELISA for *trans*-3-hydroxycotinine; we outline the characteristics of this ELISA, and its application to the detection of this agent in saliva specimens from man and plasma and urine specimens from laboratory animals. This *trans*-3-hydroxycotinine test may be used as an alternative to or in conjunction with the cotinine ELISA allowing for more accurate quantification of tobacco smoke exposure.

Published as #183 from the Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky.

Published as Kentucky Agricultural Experimental Station Article #92-4-167 with approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station.

Supported by grants entitled "Detection of Nicotine and Cotinine by Enzyme-Linked Immunosorbant Assay" from the Kentucky Tobacco Research Board.

MATERIALS AND METHODS

Animals:

New Zealand White rabbits 3-4 years old were used to raise the anti-*trans*-3-hydroxycotinine antibody. Samples of plasma and urine were obtained from rats and mice following exposure to tobacco smoke as previously described (Gairola, 1986). These samples were collected as part of an ongoing study and held frozen at 4°C prior to analysis. 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. These rabitts were fed Agway Rabbit Chow *ad lib* under 8 hours of light at 62°F.

Human Subjects:

Saliva specimens collected from the Department of Plastic Surgery, University of Kentucky were used to evaluate the assay. These samples were collected as part of a previous study concerning patient pre- and post-operative surgery health status and had been stored at 4°C for 2 years. Blind samples from 30 volunteer subjects were chosen from a total group of approximately 50 individuals for analysis. The status of the individual (smoker or non-smoker) remained unknown until the assay results were completed. A series of human urine samples from non-smokers was provided by the University of Kentucky Medical Center Clinical Toxicology Laboratories. These samples were collected for clinical evaluation and were released for analysis once the specimen had been cleared by the clinical toxicology laboratory. Four of these urine samples were pooled and the pooled sample was used for the preparation of standard curves in the matrix studies.

Hapten Synthesis and Conjugation

The *trans*-3-hydroxycotinine hapten possessing a carboxylic group in the β-position of the pyridine ring was synthesized as follows. Methyl 5-formylnicotinate was synthesized according to the procedure given by Wenkert (1970) and purified by silica-gel chromatography. This aldehyde was used in the preparation of N-methyl-5-carboxymethylpyridil nitrone and applied afterwards in the 1,3-dipolarcycloaddition to methyl acrylate. The mixture of the diastereoisomeric isoxazolidines obtained was then reduced over Nickel-Raney catalyst yielding a mixture of (±)-*cis* and (±)- *trans*-3-pyrrolidone derivatives. The separation of single diastereoisomeric racemates was reached via silica-gel chromatography. The less polar (±) -*trans*-isomer structure was assigned by comparison of its hydrogen nuclear magnetic resonance (H-NMR) spectrum with the data given in the paper of Dagne (1972) for the natural *trans*-3-hydroxycotinine. The H-NMR spectrum of the (±) *cis* isomer corresponded well with the data given in the same paper for *cis*-3-hydroxycotinine.

The resulting compound was then covalently linked to both bovine serum albumin (BSA) and horseradish peroxidase (HRP)(Zymed Labs, So. San Francisco, CA). The coupling of the carboxyl compound to the different proteins was as follows. The *trans*-3-hydroxycotinine derivative (0.3 mg) was dissolved in 0.3 ml anhydrous dimethylformamide (DMF) at 0°C with 2μl of triethylamine (Reagent 1). Reagent 2 consisted of 5μl isobutyl chloroformate per milliliter DMF (precooled to 0°C). Next, 100μl of Reagent 2 was added to Reagent 1, mixed well and allowed to stand at 0°C for 15 minutes. The protein of choice (BSA or HRP) (0.8 mg dissolved in 1.2 ml H_2O plus 0.18 ml of 50 mM Na_2CO_3) was added to the above mixture and sealed with parafilm. The reaction mixture was rotated overnight at 4°C and then dialyzed against phosphate buffered saline extensively. A portion of the purified derivative-protein mixture was taken and stored at 4°C ready for use (Wie and Hammock, 1982).

ELISA Procedures:

The *trans*-3-hydroxycotinine ELISA developed here was similar in format to those reported by Stanley and co-workers (1991). A dilute solution of Protein-A (200ul@200mg/ml)(Genzyme, Boston,MA.) was first applied to the bottom of microtiter wells (Costar Corp., Cambridge, MA), then the *trans*-3-hydroxycotinine antiserum (100ul)(used without further purification) was non-covalently coated (Voller et al., 1976) to the Protein-A coated wells. Authentic *trans*-3-hydroxycotinine and analog standards were prepared in methanol and diluted to appropriate concentrations in assay buffer (0.1M potassium phosphate buffered saline, pH 7.4 with 0.1% bovine serum albumin) or biological fluids. *trans*-3-hydroxycotinine, nicotine-N-oxide, cotinine-N-oxide, cis-3'-hydroxycotinine, and dimethylcotinine were kindly provided by J. Donald deBethizy (RJR-Nabisco, Winston-Salem, N.C.).

All assays were performed at room temperature. The assay was started by adding 20 μl of the standard, test, or control samples to each well, along with 180 μl of *trans*-3-hydroxycotinine-HRP conjugate solution. After an incubation period of 1 hr, the wells were washed with wash buffer (0.01M phosphate buffer, pH 7.4 with 0.05% Tween-20) and 150 μl of KY Blue ELISA substrate (ELISA Technologies, Lexington, KY.) were 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 30 min after addition of the substrate.

During the assay, the presence of unbound *trans*-3-hydroxycotinine in the standard or test sample competitively prevented the binding of the *trans*-3-hydroxycotinine-HRP complex to the antibody present in the antiserum. Since the reaction of KY Blue substrate with HRP was responsible for the color (blue) production in the ELISA, the apparent concentration of *trans*-3-hydroxycotinine in the sample was inversely related to the OD_{650} of the well. Apparent *trans*-3-hydroxycotinine concentrations in biological specimens were calculated based on standard curves which were run in duplicate with each individual assay.

RESULTS

Figure 1 illustrates the standard inhibition curves that were constructed to determine the sensitivity and specificity of the *trans*-3-hydroxycotinine antiserum using various metabolites of nicotine and related compounds. *trans*-3-Hydroxyotinine inhibited the ELISA with an I-50 (analyte concentration at half-maximal inhibition) of about 3.0 ng/ml, and this ELISA test also exhibited a 10 fold lower affinity for cotinine and *cis*-3-hydroxycotinine. Other tested congeners (dimethyl-cotinine, nicotine, nicotine N-oxide, nicotimamide, nicotinic acid, nikethamide, niacinamide) showed minimal cross-reactivity, and several hundred fold higher concentrations were needed to inhibit the test.

Matrix or background effects can severely limit the usefulness of ELISA tests by changing their sensitivity. Therefore the apparent shifts in I-50 values were determined when the standard curves were prepared in different biological matrices, i.e., rat plasma and urine; human saliva and urine. Standards prepared in human saliva showed virtually no matrix effects (Fig. 2) while rat plasma exhibited minimal matrix effects (Fig. 4). While the apparent I-50 for samples prepared in human urine increased about 10-fold (Fig. 2), the samples prepared in rat urine increased about 20-fold (Figure 3).

The ability of this test to detect apparent *trans*-3-hydroxycotinine was evaluated in urine samples collected from smoke-

s similar in
. A dilute
) was first
Cambridge, MA),
thout further
976) to the
and analog
riate
buffered
cal fluids.
le, cis-3'-
l by J. Donald

ssay was
samples to
P conjugate
re washed with
en-20) and 150
on, KY.) were
was read at a
EL310
mately 30 min

droxycotinine
binding of
esent in the
P was
the apparent
inversely
otinine
ed on standard
say.

that were
f the *trans*-3-
otine and
ISA with an
about 3.0
affinity for
(dimethyl-
nic acid,
and several
the test.

usefulness of
pparent shifts
re prepared in
human saliva
ally no matrix
effects (Fig.
urine
rat urine

rom smoke-

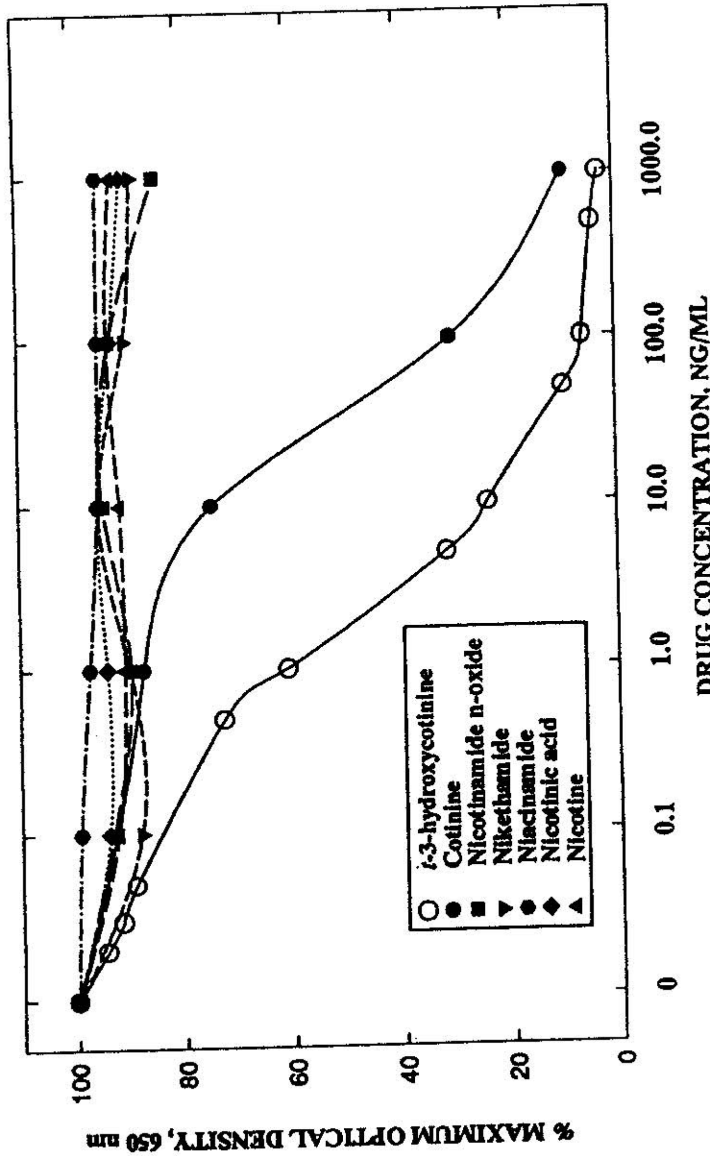

Figure 1: Cross-reactivity of *trans*-3-hydroxycotinine antiserum by ELISA. ELISA activity for anti-*trans*-3-hydroxycotinine antibody was plotted as a function of added compound. Half-maximal inhibition occurred around 2.0-3.0 ng/ml for *trans*-3-hydroxycotinine.

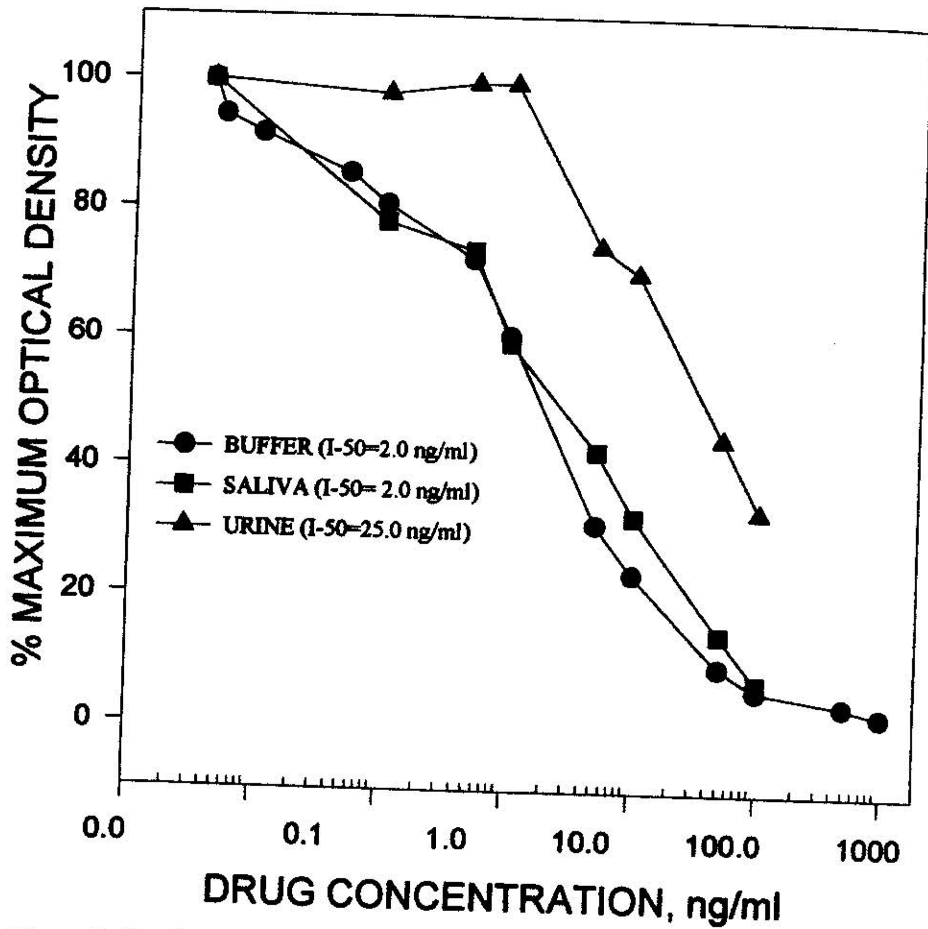

Figure 2: Sample matrix effects of *trans*-3-hydroxycotinine ELISA standard curve. The symbols show an increase of the inhibition of the ELISA by *trans*-3-hydroxycotinine in the presence of buffer, and 20ul of human saliva or urine.

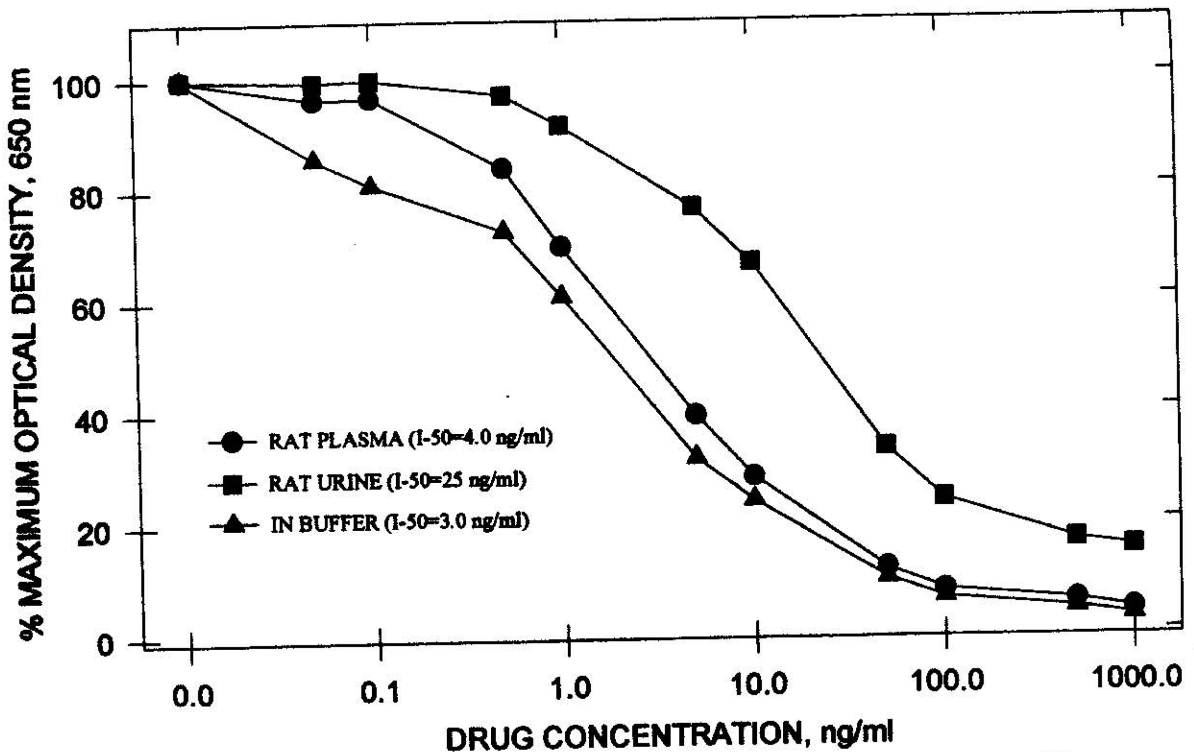

Figure 3: Sample matrix effects of *trans*-3-hydroxycotinine ELISA standard curve. The symbols show an increase in the inhibition of the ELISA by *trans*-3-hydroxycotinine in the presence of buffer and 20ul of rat plasma or urine.

exposed rats. The test clearly differentiated between the smoke exposed and unexposed groups of animals (P>.05) (Fig. 4).

This ELISA was next used to estimate the salivary concentrations of apparent *trans*-3-hydroxycotinine in human smokers and nonsmokers. The test was found to readily distinguish between these groups (P>.05). A series of 30 human saliva samples were analyzed in a blind study utilizing this test. The *trans*-3-hydroxycotinine levels of the saliva samples from smokers averaged 386.9 ng/ml, while those from the non-smokers averaged 3.6 ng/ml (Fig. 5).

Similarly, 40 urine samples from approximately 20 smoke-exposed mice (A.M. and P.M. collection) and 20 unexposed mice (A.M. and P.M. collection) were analyzed for *trans*-3-hydroxycotinine. Again, not only did this test clearly differentiate between the two groups of mice, but also between mainstream and sidestream smoke-exposed mice (P>.05). The mean concentration of apparent *trans*-3-hydroxycotinine in (P.M.) urine samples from smoke-exposed mice was 3760.4 ng/ml, with a range of 1054.2 ng/ml to 8720.8 ng/ml, while the samples from unexposed animals averaged 34.0 ng/ml (Figure 6).

DISCUSSION

An ELISA for *trans*-3-hydroxycotinine was developed that detected this major urinary metabolite of nicotine with an apparent I-50 of about 3.0 ng/ml. This test was relatively specific for *trans*-3-hydroxycotinine in that it has a 10 fold higher apparent affinity for *trans*-3-hydroxycotinine than for cotinine or *cis*-3-hydroxycotinine; in human urine the *trans* form predominates over the *cis* form (>98%). This test is essentially unreactive with nicotine and its other metabolites since this antibody has 1,000 times less affinity for these agents than *trans*-3-hydroxycotinine.

In addition to its sensitivity, this test is relatively resistant to interfering materials in biological samples. When this test was run in the presence of 20 µl of human saliva, the apparent I-50 for *trans*-3-hydroxycotinine was not significantly affected; when the test was run in the presence of human urine, the I-50 was reduced about 10-fold. Urine samples of many species affectively contain substances that cause some background interference. It has been our experience with other ELISA's that these background effects are best controlled by sample dilution.

This apparent resistance to matrix effects or interfering substances in matrix is a useful characteristic of this ELISA. No immunoassay based test is entirely free of matrix effects, and the utility of an immunoassay is determined largely by its sensitivity for the analyte of choice, and its ability to distinguish between the analyte and extraneous material. This is especially true for tests such as a *trans*-3-hydroxycotinine ELISA, which may be required to detect very low levels of *trans*-3-hydroxycotinine as in small experimental animal (mouse) models or in epidemiological studies on environmental tobacco smoke.

The ELISA results from the whole body smoked-exposed rat urine samples (6 smoked and 2 control) showed low levels of *trans*-3-hydroxycotinine and high levels of cotinine. In humans, the *trans*-3-hydroxycotinine metabolite has been suggested as a better biomarker of tobacco smoke exposure because it is more abundant in smokers than the cotinine metabolite, due to the production in humans of a *trans*-3-hydroxycotinine glucuronide. In rats, however, there are two other metabolites that are longer lived than cotinine, cotinine-N-oxide and 2'-hydroxydemethylcotinine (Kyerematen et. al. 1991). Our results are

consistent with those of Kyerematen in that they also found less *trans*-3-hydroxycotinine than cotinine in the rats after tobacco smoke exposure.

Also, from an analytical standpoint there have been reports indicating procedural problems when extracting the *trans*-3-hydroxycotinine metabolite due to its high water solubility making the detection of *trans*-3-hydroxycotinine difficult by HPLC or GC/MS. This problem may be resolved by using this ELISA assay which requires no extraction procedure.

Experiments using smoked-exposed mice further established the

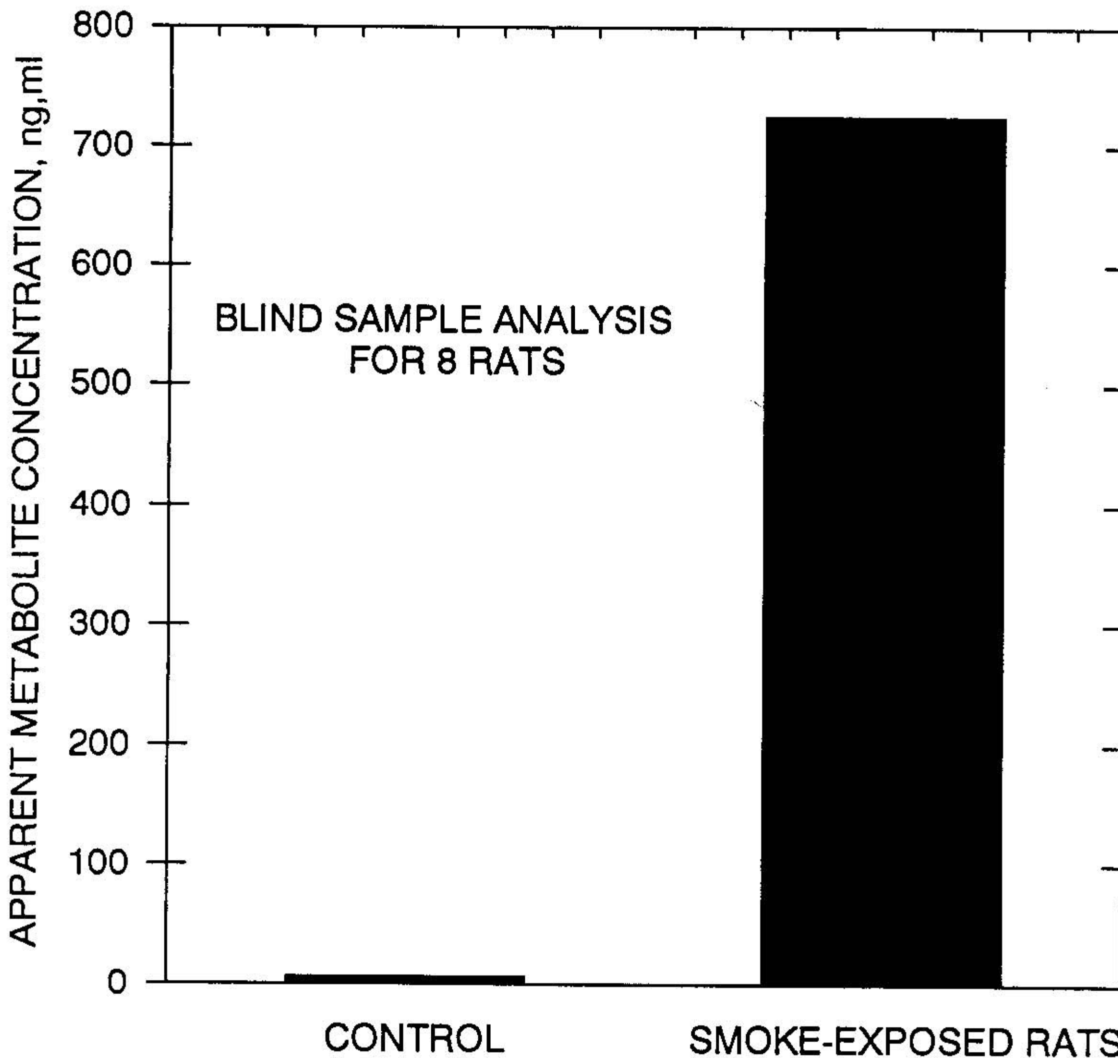

Figure 4: Detection of apparent *trans*-3-hydroxycotinine metabolite from rat urine specimens using the *trans*-3-hydroxycotinine ELISA. The right hand bar indicates the apparent urine levels of *trans*-3-hydroxycotinine in six smoke-exposed rats, while the left hand bar indicates the apparent urine levels in two control rats.

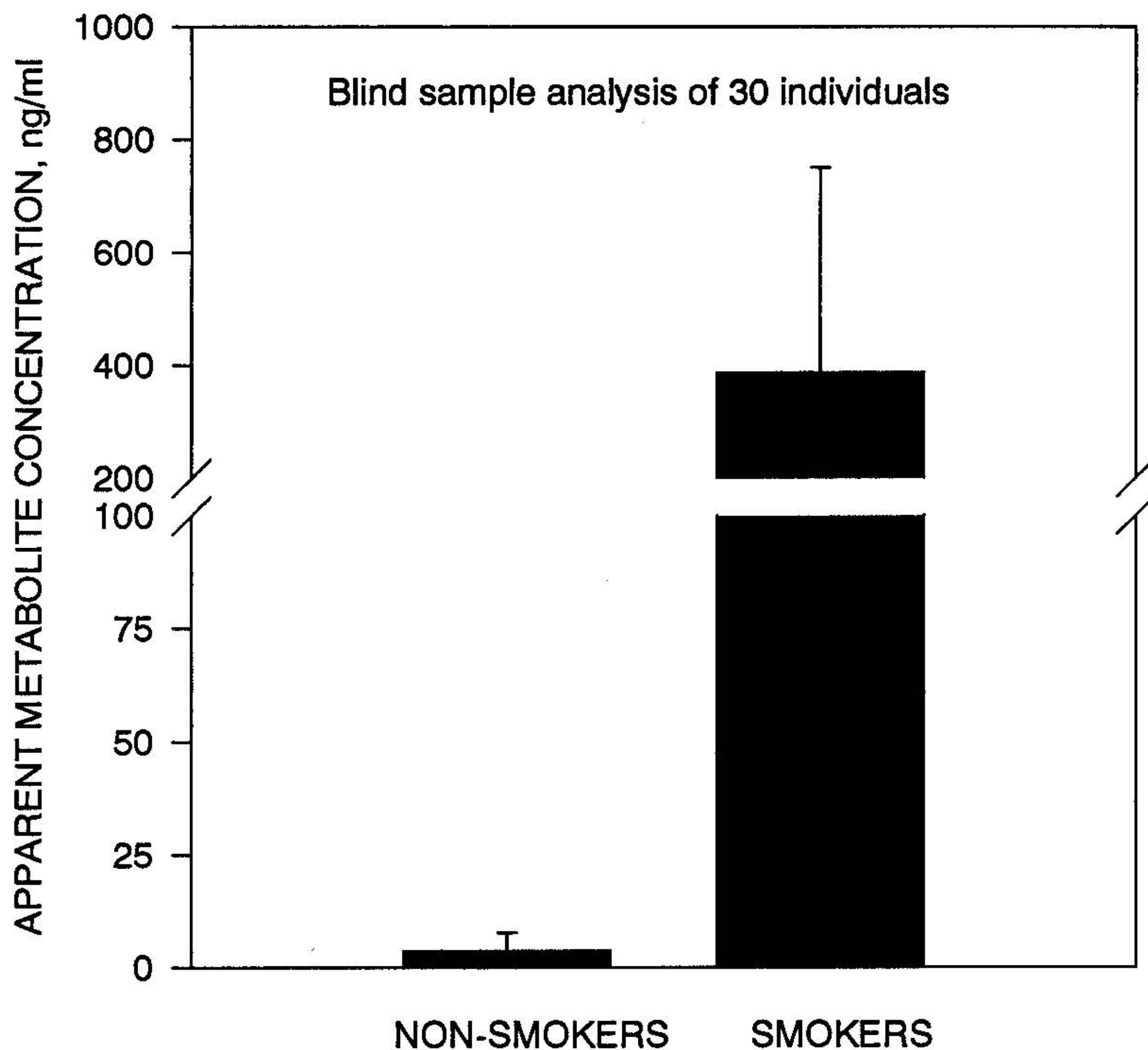

Figure 5: Detection of apparent *trans*-3-hydroxycotinine from human saliva specimens using the *trans*-3-hydroxycotinine ELISA. The left hand bar shows the apparent saliva levels of *trans*-3-hydroxycotinine in nonsmokers, while the right hand bar shows the apparent saliva levels in smokers.

DETECTION OF *trans*-3-HYDROXYCOTININE EQUIVALENTS IN MOUSE URINE FROM MAINSTREAM AND SIDESTREAM SMOKE-EXPOSED MICE

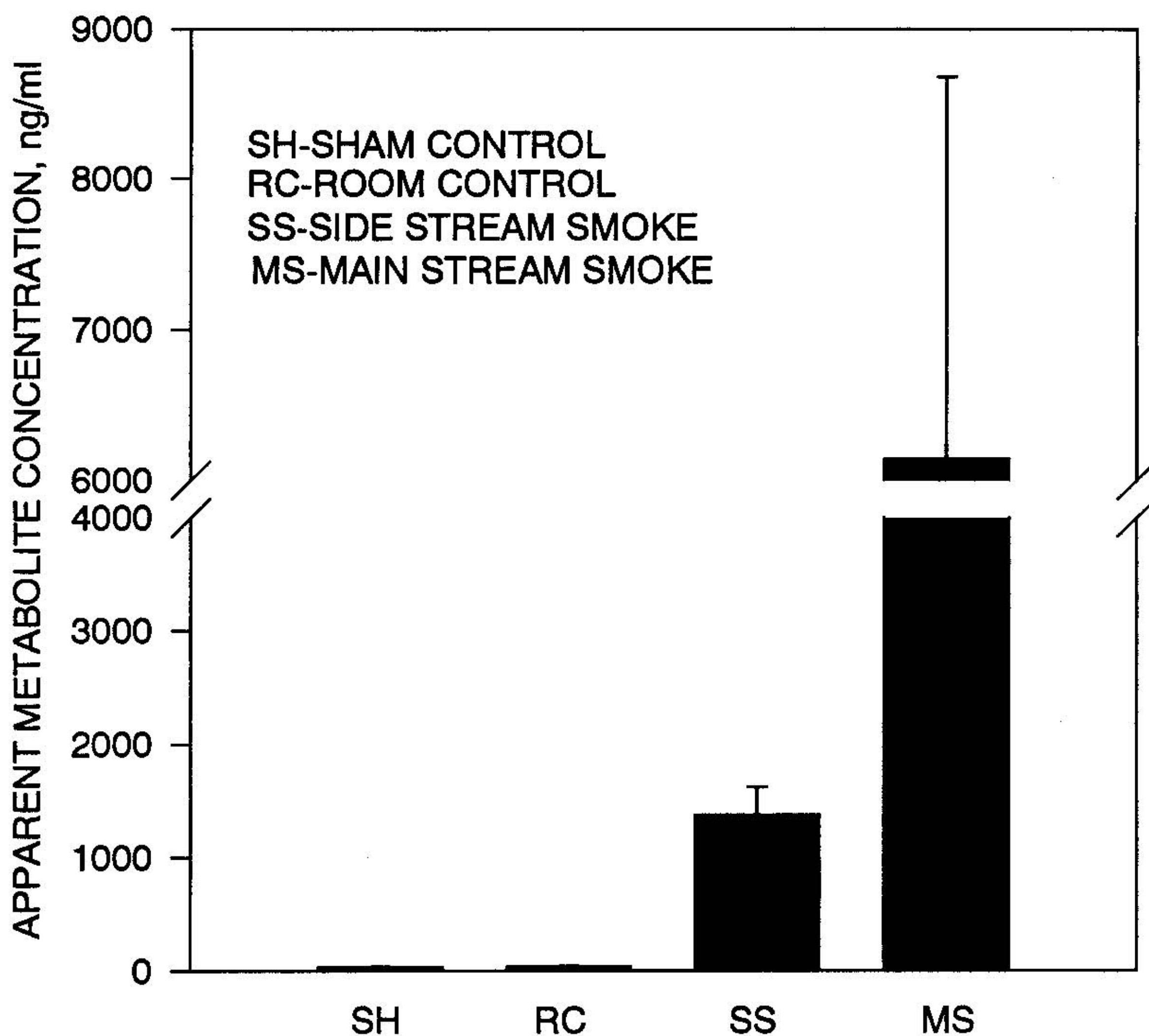

Figure 6: Detection of apparent *trans*-3-hydroxycotinine in mouse urine using the *trans*-3-hydroxycotinine ELISA. The bars indicate (from right to left) the route of administration of smoke to four groups of five rats per group e.g. main-stream(MS), side-stream(SS), or control (sham and room) mice.

sensitivity and utility of this *trans*-3-hydroxycotinine ELISA. To our knowledge this is the first study which suggests that there is a *trans*-3-hydroxycotinine metabolite produced in mice. After analysis with both the cotinine and *trans*-3-hydroxycotinine ELISA's, we found that the apparent levels of *trans*-3-hydroxycotinine were higher than those of cotinine. In this case, cotinine may have artificially raised the *trans*-3-hydroxycotinine levels due to cross-reaction; however, because *trans*-3-hydroxycotinine was found in greater amounts compared to cotinine, our work strongly suggests that this metabolite exists in mice exposed to tobacco smoke. We were also impressed by the assay's ability to discern between the two routes of administration of the smoke e.g. mainstream smoke exposure and sidestream smoke exposure. These data clearly suggest the potential for application of this assay when assessing animal models for tobacco smoke exposure.

Finally, the ability of this test to detect *trans*-3-hydroxycotinine in saliva samples from human smokers and nonsmokers was evaluated. This test appeared to be remarkably sensitive when it was used to distinguish between salivary samples from self-declared smokers and non-smokers. As shown in Figure 6, the saliva levels of *trans*-3-hydroxycotinine in samples from about 30 smokers averaged 386.9 ng/ml of apparent *trans*-3-hydroxycotinine, while those from non-smokers averaged about 3.6 ng/ml, close to a one hundred fold difference in apparent *trans*-3-hydroxycotinine levels.

The very low backgrounds found in salivary samples and the relative ease and non-invasiveness of collection of this sample makes salivary sampling a very attractive testing mode. Beyond this, recent opinion in this field suggests that saliva or serum are likely to yield the most useful data concerning relative exposure to nicotine (Watts et al., 1990). In this regard, the problem with urine is that volume, pH, urine flow and renal function are unpredictable variables (Watts et al., 1990) and correcting for creatine content, a suggested maneuver (Hoffmann and Brunemann, 1983), is only a partial solution; This is because creatine excretion rates are quite variable between individuals.

These data are entirely consistent with what is known of the metabolism and disposition of *trans*-3-hydroxycotinine in man (Bjercke et al., 1986; Kyerematen et al., 1990; Watts et al., 1990). *trans*-3-Hydroxycotinine is the major metabolite found in plasma and its plasma half-life, at approximately 6 hours, is relatively long. *trans*-3-Hydroxycotinine is therefore a highly effective biological marker of nicotine exposure. Because of the very low matrix or background effect in urine and saliva when using this ELISA, both of these biological fluids provide excellent means to distinguish between smokers and non-smokers when using *trans*-3-hydroxycotinine as a biological marker. An additional useful feature of this assay concerns the difficult extraction procedure currently used in isolating the *trans*-3-hydroxycotinine metabolite from biological fluids. Simply stated ELISA's need no sample preparation which allow for quick and easy metabolite identification. We also have raised questions regarding the production of the *trans*-3-hydroxycotinine metabolite in mouse, and we hope to pursue this issue in the future utilizing the high sensitivity of this ELISA.

REFERENCES

Benkirane, S., A. Nicolas, M.-M. Galteau, and G. Siest. 1991. Highly sensitive immuno-assays for the determination of cotinine in serum and saliva: Comparison between RIA and an avidin-biotin ELISA. *Eur J Clin Chem Clin Biochem.* 29:405-410.

Benowitz, N.L. 1983. *The use of biologic fluid samples in assessing*

tobacco smoke consumption. Measurement in analysis and treatment of smoking behavior. Eds. J. Grabowski and C.S. Bell. NIDA Research Monograph #48. U.S. Government Printing Office, Washington, D.C.

Bjercke, R., G. Cook, N. Rychlik, H. Gjika, H. Van Vunakis, and J. Langone. 1986. Stereospecific monoclonal antibodies to nicotine and cotinine and their use in enzyme-linked immunosorbent assay. *J Immunol Meth*. 90:203-213.

Bridges, R.B., J.G. Combs, J.W. Humble, J.A. Turbek, S.R. Rehm, and N.J. Haley. 1990. Population characterisitcs and cigarette yield as determinants of smoke exposure. *Pharmacol Biochem Behav*. 37(1):17-28.

Dange E. and Castagnoli N., (1972) Structure of hydroxycoyinine. a nicotine metabolite *J. Med. Chem.*, 15, 356-360.

Gairola, C.G. 1986. Free lung cell response of mice and rats to mainstream cigarette smoke exposure. *Toxicaol Appl Pharmacol*. 84:567-575.

Hoffmann, D., and K.D. Brunemann. 1983. Endogenous formation of N-nitrosoproline in cigarette smokers. *Cancer Res*. 43:5570-5574.

Kyerematen, G.A., M.L. Morgan, B. Chattopadhyay, J.D. deBethizy, and E.S. Vesell. 1990. Disposition of nicotine and eight metabolites in smokers and nonsmokers: Identification in smokers of two metatolites that are longer lived than cotinine. *Clin Pharm Therap*. 48(6):641-651.

Kyerematen, G.A., E.S. Vesell. 1991. Metabolism of Nicotine. *Drug Metabolism Reviews*. 23(1&2): 3-41.

Langone, J.J., H.B. Gjika, and H. Van Vunakis. 1973. Nicotine and its metabolites. Radioimmunoassays for nicotine and cotinine. *Biochem*. 12(24):5025-5030.

Neurath, G.B., M. Dunger, O. Krenz, and F.G. Pein. 1988. trans-3'-Hydroxycotinine: A main metabolite in smokers. *Klin Wochenschr*. 66(Suppl XI): 2-4.

Noland, M.P., R.J. Kryscio, R.S. Riggs, L.H. Linville, L.J. Perritt, and T.C. Tucker. 1988. Saliva cotinine and thiocyanate: Chemical indicators of smokeless tobacco and cigarette use in adolescents. *J Behav Med*. 11(5):423-433.

Noland, M.P., R.J. Kryscio, R.S. Riggs, L.H. Linville, L.J. Perritt, and T.C. Tucker. 1990. Use of snuff, chewing tobacco, and cigarettes among adolescents in a tobacco-producing area. *Addict Behav*. 15(6):517-530.

Rosa, M., R. Pacifici, I. Altieri, S. Pichini, G. Ottaviani, P. Zuccaro. 1992. How the steady-state cotinine concentration in cigarette smokers is directly related to nicotine intake. *Clin Pharmacol Therap*. 52:324-329.

Stanley, S.D. 1992. Development and validation of immunoassay-based tests for environmental tobacco smoke exposure. Ph.D. diss., University of Kentucky, Lexington.

Stanley, S.D., A. Jeganathan, T. Wood, P. Henry, S. Turner, W.E. Woods, M.L. Green, H-H. Tai, D. Watt, J. Blake, and T. Tobin. 1991. Morphine and etorphine: Detection by ELISA in equine urine. *J Anal Tox*. 15:305-310.

Voller, A., D.W. Bidwell, and A. Bartlett. 1976. The enzyme linked immunosorbent assay (ELISA). *Bull Wld Health Organ*. 53:55-56.

Watts, R., J.J. Langone, G. Knight, and J. Lewtas. 1990. Cotinine analytical workshop report: Consideration on analytical methods for determining cotinine in human body fluids as a measure of passive exposure to tobacco smoke. *Envir Hlth Persp.* 84:173-182.

Wenkert,E., Dave, K.G., Dainis, I., Reynolds, G.D. (1970) General methods of synthesis of indole alkaloids, VII. Attempted approach to the Iboga-Voacanoga series. *Austral. J. Chem.*, 23, 73.

Wie, S., and B.D. Hammock. 1982. The use enzyme linked immunosobent assay (ELISA) for the determination of Triton-X nonionic detergents. *Anal Biochem.* 125:168-176.