ABSTRACT: We extend our earlier work on the influence of environmental availability of nicotine by increasing the concentrations of oral nicotine solutions and by using gas chromatography-mass spectrometry (GC-MS) applications to examine nicotine and its primary metabolite cotinine in the blood serum. Sprague Dawley rats were chronically exposed to nicotine solutions (5 μg/ml and 8 μg/ml) through drinking bottles in their home cage. At the end of oral nicotine exposure, blood serum was analyzed by GC-MS for nicotine and cotinine content. Intake results supported previous studies showing rats will readily and voluntarily ingest oral nicotine, even at relatively high concentrations. Further, GC-MS results indicated nicotine in the majority of spiked and unspiked samples, but with wide variations in nicotine serum counts. Although GC techniques need to be refined, this study is the first to establish that nicotine concentrations delivered via a truly voluntary oral route results in pharmacologically detectable levels of the drug in biological samples and this knowledge advances our understanding and future application of the oral nicotine model.

Nicotine is the main substance with psychoactive properties found in tobacco products (Gutkin, Dehaene, & Changeux, 2006; Bolliger et al., 2000) that creates great potential for addiction by altering reward systems and relevant psychomotor and cognitive processes in the brain (Besson et al., 2007). Once ingested, nicotine breaks down into a number of metabolites, including cotinine which is the major nicotine metabolite. Within the human body the half-life of cotinine is approximately 17 hours, whereas the half-life of nicotine is only about two hours (Wall, Johnson, Jacob, & Benowitz, 1998). Cotinine concentration in various body fluids is considered to be among the most useful markers of nicotine exposure currently available (Swan, Habina, Means, Jobe, & Esposito, 1993). Due to the long life of cotinine in biological samples, it has been used as an indicator of recent tobacco smoke or nicotine exposure (Sepkovic & Haley, 1985).

Many techniques have been developed to detect nicotine and/or cotinine in different biological matrices. Thin layer chromatography (TLC) has been used for the detection of nicotine and cotinine in human urine by a solid-liquid extraction and this technique has been applied in the sampling of urine from children who were exposed to environmental tobacco smoke (Tyrpien et al., 2000). TLC techniques have also shown that nicotine is more efficient than cotinine at passing the blood-brain barrier in rats by measuring the nicotine and cotinine levels in brain tissue (Riah, Courriere, Dousset, Todeschi, & Labat, 1998).

Another technique, enzyme-linked immunosorbent assay (ELISA) has been used to test a vaccination against nicotine during continued nicotine administration in rats and the effects on nicotine distribution to the brain (Hieda, Keyler, Ennifar,
Fattom, & Pentel, 2000). ELISA was also used to study the detection of nicotine and cotinine in urine, serum, and saliva of active and passive human smokers (Ziegler, Kauczok, Dietz, Reith, & Schmidt, 2000; Langone, Cook, Bjercke, & Lifschitz, 1988; Eramo et al., 2000; Benkirane, Nicolas, Galteau, & Siest, 1991).

Gas chromatography (GC) coupled with mass spectrometry (MS) is often considered the gold standard in substance detection, including drugs of abuse. This assay method has been employed for the simultaneous quantification of nicotine and cotinine in urine of passive and active smokers (Man, Gam, Ismail, Lajis, & Awang, 2006; Heinrich-Ramm, Wegner, Garde, & Baur, 2002) and for direct nicotine delivery in nonhumans (Jung, Chung, Chung, Lee, & Shim, 1999). Several extraction methods exist to quantify nicotine and cotinine in plasma or serum by GC-MS using ion trap detection in plasma or serum of rats and humans (e.g., Cognard & Staub, 2003; Jacob, Wu, Yu, & Benowitz, 2000; Jung, et al.).

In previous studies, we demonstrated that nicotine can be administered chronically and voluntarily in the drinking water of rats and that the environmental availability of the oral nicotine influences ingestion (Biondolillo & Pearce, 2007; Boyett, Pearce, & Biondolillo, 2007). Though there is systemic absorption of pharmacologically significant amounts of nicotine when delivered orally, this route relies on the relatively slow process of gastric absorption and first-pass metabolism before becoming available in the bloodstream. Hence, questions arise concerning both the speed with which nicotine becomes available and the amount of nicotine ultimately available to the central nervous system, important issues for the development of dependence (for a review see Warnakulasuriya, Sutherland, & Scully, 2005). In this study, we used GC-MS applications to examine nicotine and cotinine levels in the serum of rats exposed to nicotine through our oral model.

**MATERIALS AND METHODS**

**Animals**
Forty-eight Sprague-Dawley rats (20 male; 28 female: Harlan Company, Indianapolis, Ind., USA) served as subjects. Rats were approximately 25 days old at the beginning of the study and housed in sex-matched pairs within clear polycarbonate cages (Allentown Caging) fitted with Cell-Sorb Plus bedding topped with stainless steel wire lids. The subjects were on a 12:12 light: dark cycle with the lights on from 1300 through 0100. While in the cages, the rats had free access to Purina Lab Diet 5012 and water at all times. The colony room was maintained at 20-22 °C. The Arkansas State University Institutional Animal Care and Use Committee approved all procedures.

**Administration of oral nicotine**
Centrifuge tubes (50 ml) fitted with rubber stoppers and stainless steel drinking tubes were used for the drinking bottles. All rats had six bottles placed on the top of the wire lids through which they could access the drinking tubes. As a counter measure, the bottles were presented in varying sequences atop each cage with all like solutions in consecutive pairs. For all subjects bottle positions remained constant throughout the study to increase the likelihood that rats could discriminate between nicotine solutions and water. Nicotine solutions were made by mixing nicotine base (Sigma Aldrich) in tap water at concentrations of 5 μg/ml and 8 μg/ml nicotine base/water. Solutions were mixed approximately once a week and stored in amber bottles. The rats received two bottles of the 5 μg/ml mixture, two bottles of the 8 μg/ml mixture, and two bottles of tap water. All subjects received the six bottle arrangement. Each bottle was identified by cage, subject(s), content and order of presentation on the lid of the home cage. As in previous studies from our lab (Biondolillo & Pearce, 2007; Biondolillo, Pearce, Louder, McMickle, under review), bottles were filled with nicotine solution or water and the mass of intake for each drinking bottle was measured daily. Approximately 23 hours later bottles were removed, weighed again, and a difference score was calculated by subtracting removal bottle weight from placement weight (g), a procedure considered to be a reliable measure of fluid intake (Stolerman & Kumar, 1972). This score was used to reflect intake from individual bottles in the previous 24 hour period. Body weights were collected every third day. Bedding and food were changed and replaced once a week. All subjects were exposed to all liquids for 23 hours out a of 24 hour period. During the 1 hour of nonexposure intake data were recorded and cages were cleaned. This arrangement was presented for 42 consecutive days for 6 pairs of subjects, and 63 consecutive days for 13 pairs of subjects as well as for 10 subjects that were separated from their home cage partner and housed individually for 2 weeks at the end of the study. Considering that all rats had entered the stage of young adulthood and the age variations did not necessarily reflect different developmental stages, such as adolescence (Spear, 2000), intake data were combined...
during analysis for same-sex rats sacrificed at the different time points.

The experimental design was part of a larger study examining chronic intake of oral nicotine in maternally and nonmaternally exposed rats, the results of which are not reported here. For this aspect of the study, we were blind to maternal exposure assignment, female rats were not pregnant and nicotine intake was examined only during the 23 hour exposure period immediately previous to termination and blood collection.

Chemicals
Nicotine and cotinine standards and analytical grade methanol, dichloromethane, petroleum ether and ethyl ether were purchased from Sigma (St. Louis, MO, USA).

Standards and Controls
Solutions of reference standards were prepared in methanol and these standards were used to create calibration curves as a control. Serum standards were prepared by adding known amounts of the nicotine standard or cotinine standard to serum of nicotine exposed rats. Referred to as spiked samples, this approach allowed for matrix matching to unspiked samples.

Instruments and chromatographic conditions
A Varian CP3800 (Walnut Creek, CA, USA) gas chromatograph equipped with a Saturn 2200 ion-trap detector performed the gas chromatographic analyses. Helium was used as the carrier gas with a head pressure of 5 psi, and the flow rate was 1ml/min. A Restek capillary column of 60mRtxiTM-5ms x .53 mm ID was used, coated with a 0.25 μm film. Instrument settings were based on Elobeid’s (2006) initial measurements of nicotine in tobacco smoke but chromatographic conditions were modified to suit elution of the compound in serum. The column temperature was programmed from an initial temperature of 45 °C held for 2 minutes, increased to 110 °C at 35 °C/min, then increased to 200 °C at 25°C/min held for 2 min, and finally to 280 °C at 12 °C/min and held for 3.88 min. The injection port temperature was 250 °C. Injections were made in splitless mode (1:1, redundant) using the CP-8400 Varian autosampler. The ion-trap was operated in electron ionization with methanol as the liquid reagent. The transfer line, manifold and trap temperatures were 150 °C, 80 °C, and 250 °C, respectively. Instrument control and data acquisition were carried out using the Varian Workspace. For quantification NICO m/z 84 and COT m/z 98 were used. The complete identifications for nicotine and cotinine were made with the mass spectrometers based off mass charge ratios and the associated retention times, 10.05 min for nicotine and 12.69 min for cotinine.

Biological sample collection and preparation
Rats were killed by decapitation and blood was collected in 2 ml tubes and centrifuged for 15 minutes at 10,000 rpm. The serum was collected and stored at -20 °C until analysis.

Sample preparation
Serum was collected from each rat and approximately 2 ml were reserved to be prepared as spiked and unspiked samples for GC-MS analyses. In spiked samples, 1 ml of serum was spiked with nicotine and/or cotinine standards for a 500 ppb dilution. Standards were not added to 1 ml unspiked samples. A solvent solution was made using 1 ml of dichloromethane and 1 ml of 1:1 petroleum ether: ethyl ether. A solution was made by combining a 2:1 ratio of solvent to serum and samples were shaken and allowed to settle at room temperature. The clear organic layer was collected, put into a new test tube and evaporated under a nitrogen stream at room temperature. One ml of methanol was added to the test tube to suspend the nicotine. The mixture was then transferred to an amber vial for GC-MS analysis.

RESULTS

Intake
Consumption of the two nicotine solutions by sex is plotted in Figure 1 as a mean intake score for the 23 hours preceding blood collection. As evident from these data, male and female pairs ingested both the 5 μg/ml and the 8 μg/ml nicotine solutions. Male pairs (N = 7) drank similar amounts of the 5 μg/ml and 8 μg/ml nicotine solutions (Ms = 23.75 and 21.51), but female pairs (N = 12) drank less of the 5 μg/ml and more of the 8 μg/ml nicotine solution (Ms = 17.65 and 24.44). Intake data were analyzed as a series of ANOVAs with the software program SPSS. The amount of nicotine solution consumed by pairs was analyzed statistically with solution (5 μg/ml vs. 8 μg/ml) as a within subjects factor and sex (male vs. female) as a between subjects factor but this analysis revealed no significant solution x sex interactions, F(1, 17) = 1.33, p = 0.26, nor main effects for sex or
solution $F(1, 17) = .22$ and .34, ns. Figure 2 illustrates intake data for 10 individually housed rats (6 males and 4 females), an arrangement that allowed for description of nicotine ingestion by body weight. Once corrected for body weight, solution x sex interactions were not significant and there was not a main effect for sex, $F(1, 8) = 2.47$ and 1.11, ns, but this analysis exposed a main effect for solution $F(1, 8) = 5.44, p = .048$. Adjustments for body weight revealed subjects drank significantly more of the 8 μg/ml nicotine solution compared to the 5 μg/ml solution ($M_s = .40$ and .16). Also depicted in Figure 2 is the enormous amount of variability in the amounts of the two nicotine solutions ingested by both male and female rats, an indication that some rats were very high and others very low consumers of the nicotine solutions.

**Fig 1.** Mean consumption ± standard error of two nicotine solutions, 5 μg/ml and 8 μg/ml by male and female rats housed in pairs and presented with 2 bottles of each nicotine solution and 2 bottles of water in the home cage. The male group contained 20 rats and the female group contained 28 rats. Bars reflect mean consumption measured in grams of nicotine solution during the 23 hours prior to termination.

**Fig 2.** Mean ingestion ± standard deviation of 5 μg/ml and 8 μg/ml nicotine solutions by male (N = 6) and female (N = 4) rats housed individually. Bars reflect mean nicotine consumption measured in milligrams per kilogram body weight during the 23 hours prior to termination.

**Gas Chromatography-Mass Spectrometry**

Spiked and unspiked serum samples from 41 of the 48 rats were prepared and analyzed by GC-MS. Reasons for the exclusion of serum from 7 rats included low serum collection and in one case mislabeling of the vials so that proper identification of two subjects was not possible. Nicotine was found in 28 of 41 (68.29%) spiked samples and 21 of 40 (52.50%) unspiked samples, with wide variations in peak areas on chromatograms, an indication of variations in serum concentration. GC-MS results indicated the nicotine retention time was $10.05 \pm 0.06$ min in spiked and $10.06 \pm 0.07$ mins in unspiked samples. Detection of cotinine was less successful as it was found in only 18 out of 41 (43.90%) spiked and 2 out of 40 (5.00%) unspiked samples. Cotinine retention time was $12.69 \pm 0.08$ min and $12.61 \pm 0.001$ min in spiked and unspiked samples, respectively. See Figure 3 for the chromatographic separation of nicotine and cotinine in serum samples from one of the subjects in which cotinine was detected. Figure 4 depicts nicotine peaks in spiked and unspiked serum samples from the same subject.
Alternative routes of nicotine delivery, including oral administration, hold promise as aides to smoking cessation in humans (Martinet, Bohadana, & Fagerström, 2006, 2007; Rose, Herskovic, Trilling, & Jarvik, 1985; Schneider, Jarvik, & Forsythe, 1984) and these results offer important contributions to this field. First, they demonstrate that both male and female rats will readily consume higher concentrations of oral nicotine than previously reported. Previous studies showed rats tend to consume relatively small amounts of nicotine in concentrations exceeding 1 µg/ml (Flynn, Webster, & Ksir, 1989) and modifications such as water deprivation (LeHouezec, Martin, Cohen, & Molimard, 1989; Lang, Latiff, McQueen, & Singer, 1977), food deprivation (Lang et al.), or sweetening of nicotine solutions (Smith & Roberts, 1995) have been used to encourage consumption. Using a nicotine bitartrate solution of 3 µg/ml, we reported increased consumption by adolescent female rats with no manipulation other than increasing the availability of nicotine, a result deemed the multiple bottle effect (Biondolillo & Pearce, 2007). The current study is the first to report consumption of a 5 µg/ml or 8 µg/ml nicotine freebase solution in both male and female rats. Considering the consumption levels reported here, perhaps the threshold for nicotine tolerance/avoidance has not yet been reached, at least not with the approach provided by the multiple bottle procedure.

Second, previous published studies both in our lab (Boyett et al., 2007) and others (Dadmarz & Vogel, 2003) have examined individual differences in intake of oral nicotine and report high, moderate and low consumers. In unpublished studies, we have also consistently observed sex differences in nicotine consumption, with females drinking more nicotine when adjusted for body weight. Similar findings were reported in periadolescent mice (Klein, Stine, Vandenbergh, Whetzel, & Kamens, 2004) where sex differences in nicotine consumption, but not serum cotinine levels, suggested differences in nicotine pharmacokinetics. Klein and colleagues contended that female mice may more rapidly metabolize nicotine or perhaps eliminate cotinine more slowly compared to male mice. Although sex differences in the consumption data were not observed in our study, this may be due to the small number of subjects as the observed power on these analyses was considerably low, falling under 30% for analyses on the solution x sex interaction effects and the main effect of sex. We contend that increasing the number of subjects and extending the application of this method in future
studies will offer a comparative tool to understand the influences on voluntary nicotine use as individual, sex, and pharmacokinetic differences are also observed in human tobacco users (Benowitz & Jacob, 1997; Shiffman, 1989, 1991).

Third, these results provide evidence that despite the oral route using the relatively slow process of gastric absorption and first-pass metabolism, systemic absorption of nicotine may result in pharmacologically detectable amounts when delivered orally, and at least in certain individuals, nicotine remains available in the bloodstream for up to 2 hours following the last exposure.

Although the techniques need to be refined, our study is the first to report determination of nicotine in serum by GC-MS in rats orally exposed to this drug. We adapted the methods from other reported protocols on nicotine delivered via other routes of administration (Cognard & Staub, 2003; Elobeid, Chai, Clarke, Hannigan, & Russ, 2005; Elobeid, 2006; Man, et al., 2006) yet additional modifications are needed. For instance, glassware cleaning procedures may need to be improved as loss of nicotine by adsorption on glassware has been reported (Davis, 1986; Teeuwen, Aalders, & Van Rossum, 1989). Further, when conducting chemical analyses on biological samples, it is rare to get 100% yield, and such high percentage outcomes often reflect other problems; although in regard to detection in spiked samples the aim is to get 90%, a yield considered standard in chemometric outcomes, this goal may be unattainable under certain conditions. Inherent variability in serum sample analysis exists in terms of the performance of the sample within the injector and the loading of the column with volatiles derived from the sample (Robyn Hannigan, personal communication). Also, there were times during the project when the GC or MS did not operate as expected and concerns of the autosampler no longer injecting sample were considered, as were the age of the samples and decay of nicotine or cotinine when samples were prepared but the analyses delayed. Although it is unclear why nicotine and cotinine were not detected in a higher percentage of the spiked samples, we anticipate that as we refine these procedures in subsequent studies, either the detection of nicotine and cotinine will be more consistent, or an explanation for the variability will be discovered.

We hope to improve this method in order to routinely detect nicotine and cotinine in serum and brain samples. Such information may help us to better understand nicotine dependence by addressing concerns about both the speed with which nicotine becomes available and the amount of nicotine ultimately available to the central nervous system. The next logical step is to examine particular brain regions for nicotine, nicotine metabolites, or changes in neurotransmitters or neurotransmitter receptors following exposure.

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REFERENCES


Cognard, E., & Staub, C. (2003). Determination of nicotine and its major metabolite cotinine in plasma or serum by gas chromatography-mass spectrometry using ion-trap
VOLUNTARY CONSUMPTION CONCENTRATED ORAL NICOTINE
detection. Clinical Chemistry Laboratory Methods, 41(12), 1599-1607.


