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Original Investigation

Evaluation of the Effect of Ammonia on Nicotine Pharmacokinetics Using Rapid Arterial Sampling

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Abstract

Introduction: The nicotine bolus theory states that the dependence-producing potential of cigarettes relates to a rapid increase in nicotine at brain receptor sites. It has been suggested that ammonia, a compound typically found in tobacco products, further increases the amount of nicotine absorbed and its absorption rate. The aim of this study was to determine whether different ammonia yields in cigarettes affected the rate or amount of nicotine absorption from the lungs to arterial circulation.

Methods: 34 adult smokers received 3 separate puffs from each of 2 test cigarettes with different ammonia yields (ammonia in smoke: 10.1 μg per cigarette vs. 18.9 μg per cigarette), followed by rapid radial arterial blood sampling (maximum one sample per second) with 30 min between puffs. Arterial blood samples were assayed for nicotine by liquid chromatography tandem mass spectrometry. Pharmacokinetic modeling was performed and the two test cigarettes were assessed for bioequivalence.

Results: No significant differences were found in area under the curve, C_{max}, or T_{max} and the 2 test cigarettes were found to be bioequivalent based on 2 one-sided tests at a significance level of 5%. In addition, the zero-order rate constant (k_0) obtained from the initial slope of the curves and the model-dependent first-order rate constant (k_a) were not significantly different.

Conclusions: This study provides strong evidence that the different ammonia yields of the test cigarettes had no impact on nicotine pharmacokinetics; thus, the ammonia did not increase the rate or amount of nicotine absorption from a puff of cigarette smoke.

Introduction

Evidence obtained from scientific literature, as well as from public health reports, demonstrates that cigarette smoking is addictive and that nicotine is a primary mediator of this addictive potential (Benowitz, 1999; Le Foll & Goldberg, 2006; Stolerman & Jarvis, 1995). A concern of the public health community is that higher rates and amounts of unprotonated nicotine reaching nicotine acetylcholine receptors enhance the dependence potential (U.S. Department of Health and Human Services [U.S. DHHS], 1988; Willems, Rambali, Vleeming, Opperhuizen, & van Amsterdam, 2006). The "nicotine bolus theory" proposes that inhaled nicotine in tobacco smoke is delivered to the brain in a very rapid manner; theoretically, within a few seconds of smoke inhalation (Gourlay & Benowitz, 1997; Royal College of Physicians [RCP], 2000; Russell & Feyeraband, 1978; U.S. DHHS, 1988). This rapid bolus of nicotine to the brain after a puff is thought to produce intensive stimulation of the dopamine reward pathway in the brain (Henningfield & Keenan, 1993; Russell & Feyeraband, 1978), which in turn perpetuates smoking.

Reports suggest the peak arterial blood concentration of nicotine is as high as 100 ng/ml within the first few seconds of smoke inhalation during cigarette smoking (Henningfield, Stapleton, Benowitz, Grayson, & London, 1993), but others indicate much lower levels and more extended timeframes (Gourlay & Benowitz, 1997; Moreyra, Lacy, Wilson, Kumar, & Kostis, 1992; Rose, Behm, Westman, & Coleman, 1999). Rose et al. (1999) sampled arterial blood every 5 s during cigarette smoking (average nicotine 0.12 ± 0.04 mg per puff, average interpuff interval 66.5 ± 31.3 s) or intravenous nicotine delivery (0.12 ± 0.04 mg per infusion) and found mean arterial nicotine concentrations of 10–50 ng/ml. A subsequent positron emission tomography (PET) study demonstrated that the time to reach 90% of peak brain levels of nicotine in smokers after one puff averaged 126 s (Rose et al., 2006). Another PET study with [^{11}C]nicotine showed that after a single puff, the time to rise from 20% to 80% of the maximum nicotine brain concentration ranged from 11 to 69 s with significant intersubject

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variability (Berridge et al., 2010). However, these authors did not directly measure peak nicotine levels in the brain.

The U.S. Food and Drug Administration (U.S. FDA) reported that ammonia-releasing compounds are added to cigarettes during manufacturing to enhance nicotine delivery and increase the rate of nicotine absorption (U.S. FDA, 1995). Researchers have hypothesized that ammonia compounds are added to elevate the pH of mainstream cigarette smoke, thus increasing the bioavailability of nicotine through facilitation of nicotine conversion from a salt to the "free-base" form (Henningfield, Pankow, & Garrett, 2004; Pankow, 2001; Pankow et al., 1997; Willems et al., 2006). In fact, ammonia-forming compounds are used as flavorants and processing agents in cigarette manufacturing (Dixon, Lambing, & Seeman, 2000) and are also inherent to tobacco leaves. Much of the ammonia added to tobacco blend components during manufacture is lost during subsequent processing and thus is unavailable for effects on nicotine absorption (Seeman, 2007). Studies of the chemical and physical properties of nicotine in the presence of ammonia in mainstream smoke aerosol are not consistent and have been described elsewhere (Dixon et al., 2000; Pankow, 2001; Seeman, 2007; Seeman et al., 2004; Seeman & Carchman, 2008). In any case, these in vitro smoke chemistry evaluations do not provide direct experimental evidence as to whether increasing levels of ammonia in tobacco or tobacco smoke ultimately lead to an increase in the rate or amount of nicotine absorption in the respiratory tract.

To our knowledge, there have been only two human in vivo studies looking at the effect of ammonia on nicotine absorption while smoking a cigarette (Armitage, Dixon, Frost, Mariner, & Sinclair, 2004; van Amsterdam et al., 2011). Armitage et al. (2004) found no difference in nicotine retention between two ammoniated cigarettes (containing diammonium phosphate [DAP] or urea; the cigarettes contained 26 and 38 μg ammonia per cigarette in mainstream smoke, respectively) and a control cigarette (containing nonammoniated reconstituted tobacco sheet [RTS]; mainstream smoke ammonia level 16 μg per cigarette) under two inhalation conditions (75 or 500 ml inhalation volumes). In addition, there were no significant differences in plasma nicotine profiles sampled from venous blood after smoking the three cigarette types. In a recent study of two commercial cigarette brands with a 3.8-fold difference in ammonium content, van Amsterdam et al. (2011) showed no difference in venous nicotine levels after correction for inhaled puff volume. However, there remains a paucity of direct experimental evidence that examines whether ammonia affects the rate or amount of nicotine absorption in the respiratory tract, and many researchers have indicated the need for pharmacokinetic studies preferably examining arterial blood (Callicutt et al., 2006; Dixon et al., 2000; Henningfield et al., 2004; Seeman, 2007; Willems et al., 2006).

We designed a pharmacokinetic (PK) study to determine the concentrations of nicotine in arterial blood after cigarette smoke inhalation, with the assumption that arterial blood levels represent the amount and time course of nicotine availability to the brain. Using a specialized apparatus, which enabled both automated smoke delivery and rapid multiple blood sampling (McKinney et al., 2010), we were able to examine the disposition of nicotine in arterial blood within seconds of delivery to the lungs. The objectives of the current study were to evaluate the primary pharmacokinetic parameters (C_{max}, T_{max}, area under the curve [AUC], and absorption rate) of nicotine in arterial blood and to assess the effect of ammonia on pharmacokinetic parameters of nicotine in arterial blood after single puff inhalation of cigarette smoke.

Methods

Subjects and Study Design

This research study was a double-blind, two-treatment, multiple measurements randomized pharmacokinetic study conducted at the Center for Drug Studies, School of Pharmacy, at Virginia Commonwealth University in Richmond, VA. This study was conducted in accordance with Good Clinical Practices, as contained in the U.S. Code of Federal Regulations (Title 21, Parts 50 and 56) and was approved by Western Institutional Review Board (Seattle, WA) prior to participant recruitment. All participants provided written consent to participate in the study.

Fifty-three volunteers from the metropolitan Richmond, VA, area were prescreened to select 34 subjects to be enrolled in the study to ensure the study was adequately powered (80%) to test for bioequivalence. Participants were healthy adult male and female smokers (recruited by posted flyers and mail advertisements) between the ages of 21 and 65 years. They had a smoking history of 10–30 cigarettes/day for at least 12 months prior to study initiation of manufactured non-menthol cigarettes with a tar range of 7–12 mg tar per cigarette. Participants were excluded from the study for any history or evidence of cardiovascular, endocrine (diabetes and thyroid), respiratory (asthma and chronic obstructive pulmonary disease), oncologic, hematologic, neurologic, psychiatric, or other serious medical conditions. Females who were pregnant, lactating, or intending to become pregnant during the course of the study were also excluded. Participants who used any other type of nicotine-containing product other than manufactured cigarettes or had a history of drug or alcohol abuse prior to the study were also excluded. No alcohol-containing food or beverages were permitted for 48 hr prior to check-in.

Written informed consent was obtained from each participant following explanation of the study procedures and any possible risks of participation. Screening of participants was completed within 1 month of the start of the study. All participants were required to provide a medical history, undergo a physical examination (including vital signs), and provide blood and urine samples for screening of inclusion and exclusion criteria.

Experimental procedures began with check-in to the investigation site the evening of the first day. Each participant underwent a training session to learn to inhale cigarette smoke through a mouthpiece in a controlled manner within the defined flow rate of inhalation. The participants were then confined to the clinic overnight, with no smoking allowed after 11:00 p.m.

The following morning, approximately 1 hr after a light breakfast, the Allen (1929) test for integrity of the radial and ulnar arteries was performed. Upon passing the Allen's test, radial arterial puncture and insertion of an arterial catheter (20 gauge; 4.45 cm) were performed by an experienced hospital technician under the supervision of the study physician. Biomonitoring detectors for pulse rate, blood oxygen level, electrocardiogram, and laser Doppler blood flow in the finger of the

catheterized wrist were attached, and the participant was seated in a comfortable chair.

Measurements from each of two types of test cigarettes were made three times (separate cigarettes for each measurement), with a 30-min washout period between measurements. This yielded a total of six sets of measurements from each participant. All six test cigarette puffs were administered in a randomized sequence.

A custom-designed system (McKinney et al., 2010) was utilized both to deliver cigarette smoke and to collect arterial blood samples in a rapid automated fashion (Figure 1). The smoke delivery apparatus allowed a precisely controlled amount of cigarette smoke to be inhaled by the participant at a constant rate. The apparatus consisted of a calibrated smoking machine and a tubing system with a flow meter that controlled the total amount of smoke and air inhaled by the participant. Each cigarette was presmoked for three puffs by the smoking machine (2 s, 35 cc puff at 1-min intervals). The study measurements were always made from the fourth puff of each test cigarette to ensure the highest possible uniformity in smoke delivery. Thirty seconds after a baseline blood sample was taken, the participants were instructed to inhale the fourth puff of cigarette smoke, which was mixed with clean air entering through an open valve in the mouthpiece of the smoke delivery apparatus. The participants were instructed to continue inhalation of air at a constant rate (150–250 ml/s), using a visual rate indicator as biofeedback, until a cutoff was reached (900 ml air or 7 s total inhalation time), and then, the flow was switched off. The overall amount of cigarette smoke and air inhaled by the participant did not exceed 35 and 900 ml, respectively. The puff volume of smoke inhaled was within the range of reported values for puff volumes in the typical smoker population (Adams, Lee, Rawbone, & Guz, 1983; Zacny, Stitzer, Brown, Yingling, & Griffiths, 1987). Exhaled smoke was immediately vented out of the test room by a vacuum hose directly above the participant.

Once inhalation of cigarette smoke and air crossed a minimal 10 ml threshold, the apparatus triggered rapid automated arterial blood sampling of approximately 1 ml of blood in increments of 1–4 s by a programmed fraction collector. Blood samples were collected after each puff at 0 (start of inhalation), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 34, 38, 42, 46, 50, 54, 58, and 62 s. Figure 2 shows a schematic of the experimental design. Between each of the six puff inhalations and arterial blood collection runs, there was a 30-min washout period with no smoking. The arterial catheter was flushed every 10 min with heparinized saline to maintain patency. For each subsequent puff, a new test cigarette was used for generating the cigarette smoke. No more than 300 ml of blood was collected during the entire study.

After the arterial blood sampling was completed for all six puffs and the catheter was removed, participants were asked to smoke one of each of the two test cigarettes ad libitum in a randomized order approximately 30 min apart and evaluate the smoking experience using a modified Cigarette Evaluation Scale (CES) questionnaire (Westman, Levin, & Rose, 1992). All participants completed an exit examination administered by the study physician no earlier than 1 hr after catheter removal before being approved for checkout.

Test Cigarettes

The test cigarettes were manufactured in a small-scale manufacturing plant at Philip Morris USA Research Center, following the same processes and specifications as large-scale commercial cigarette production. The compositions of the two test cigarettes are described in detail in Table 1. The cigarette designated "Reference" was designed to be similar in composition to commercially marketed cigarettes in the 7–12 mg tar per cigarette range. This cigarette contained 80% inclusion of a research blend (consisting of 15% burley tobacco, 15% oriental tobacco,

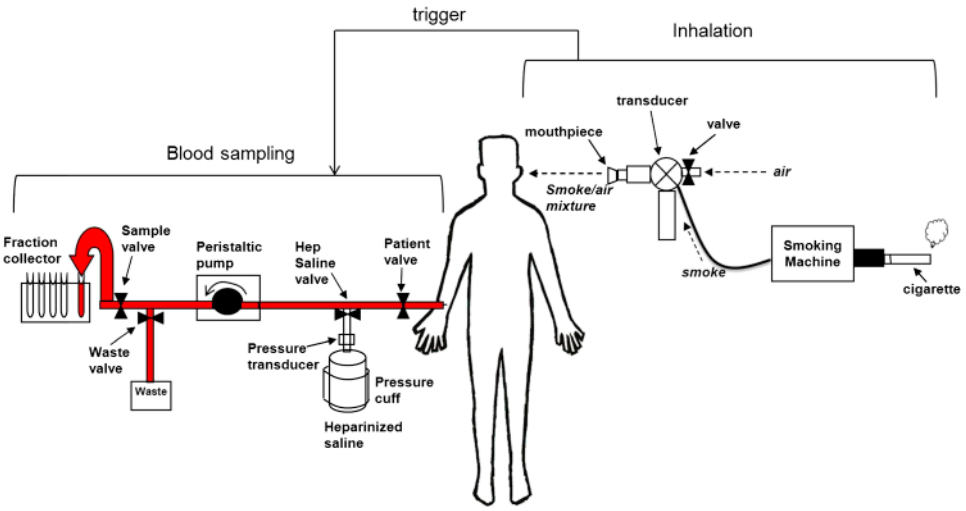

Figure 1. Schematic diagram of the blood sampling and smoking machine/inhalation components. All equipment is computer controlled with LabVIEW 7.1.1 programming. The fraction collector is fully enclosed in a plexiglass box fitted with a carbon filter fan to prevent escape of aerosolized blood. The heparinized saline bag resides in a manually controlled pressure cuff, and the pressure is monitored by an in-line pressure transducer. The smoking machine is computer controlled to deliver a specific volume of smoke in a specific duration to the inhalation device, which contains a flow-through transducer to detect start of inhalation. A biomonitoring system (not shown) measures physiological parameters as well as records the pressure reading from the heparinized saline cuff and the change in air velocity across the mouthpiece as the participant begins to inhale, triggering the blood sampling machine (reprinted from McKinney et al., 2010).

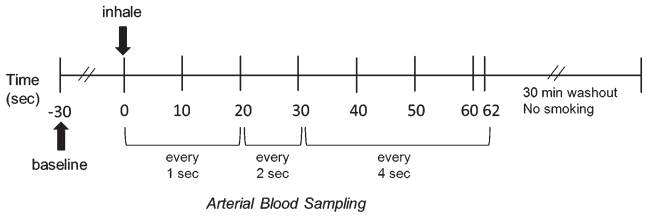

Figure 2. Experimental design. Participants inhaled three separate puffs from each of the two test cigarette types for a total of six puffs over the course of the study. Puff presentation was randomized for each participant. Blood sampling was automated to collect 1 ml samples at the intervals indicated.

38% bright tobacco, 12% stem, and 20% expanded tobacco) and 20% inclusion of tobacco sheet material containing DAP (Davis & Nielsen, 1999). The "Reference" cigarette yielded a level of ammonia in smoke of approximately 18 μg/cigarette (Table 1). Typical ammonia levels in this tar category (as measured in a market map of Philip Morris and other brand cigarettes and the 2R4F reference cigarette) range from 9.4 ± 0.9 to 18.7 ± 2.4 μg/cigarette (Counts, Hsu, & Tewes, 2006), and thus, we chose our two test cigarettes to be representative of this tar category. The cigarette designated "Low Ammonia" contained the same research blend consisting of 15% burley tobacco, 15% oriental tobacco, 38% bright tobacco, 12% stem, and 20% expanded tobacco, with no DAP inclusion, and yielded an ammonia level in smoke of approximately 10 μg/cigarette. The two test cigarettes had similar cigarette designs as far as filter, cigarette paper, and flavor system, with only slight differences in smoke chemistry (Table 1), which could be attributed to the tobacco sheet material. Cigarette analysis was performed in an ISO-accredited product testing laboratory at Philip Morris USA Research Center (Richmond, VA).

Nicotine Analytical Methods

Immediately after collection of all blood samples from each puff, the samples were centrifuged in a refrigerated centrifuge (4 °C) at 3,000 rpm (1,500*g*) for 10 min. Plasma was harvested into Teflon-capped silanized tubes, labeled, and stored in a −70 °C freezer until analysis. The analytical method for the determination of nicotine in human plasma consisted of a two-step liquid–liquid extraction followed by LC/MS/MS (Byrd, Davis, & Ogden, 2005). Internal standard, base, and ethyl ether were added to human plasma and nicotine along with the internal standard extracted. The aqueous phase was frozen and discarded. Nicotine was back-extracted from the solvent phase using formic acid in water. The aqueous phase was frozen and the solvent phase discarded. The aqueous phase was then allowed to melt and 10 μl injected into the LC/MS/MS. The method was validated for extraction recovery, selectivity, matrix effects, conformance to the calibration model, limit of quantification, accuracy, and precision. All validation parameters were within acceptance criteria established by the FDA Guidance for Industry (Bioanalytical Method Validation). The recovery of nicotine at 2.5, 10, and 75 ng/ml was 126, 103, and 102%, respectively. The calibration curves were found to be linear over the range of 2.5–75 ng/ml. Six blank plasma samples from different individuals were free of interferences at the retention time of nicotine and the internal standard. The overall inter-run precision (measured as percent relative *SD*, %RSD) was 13.3%, 9.0%, 9.2%, and 5.6% for the 2.5, 4, 20, and 60 ng/ml controls, respectively. The percent differences from nominal concentrations for the 2.5, 4, 20, and 60 ng/ml controls were 4.4%, 14.3%, 3.4%, and 7.0%, respectively.

Table 1. Cigarette Composition and Mainstream Smoke Chemistry for the Two Test Cigarettes

	Low ammonia (no DAP)	Reference (+DAP)
Cigarette weight (g)	0.894 (0.018)	0.898 (0.008)
Circumference (mm)	24.78 (0.04)	24.71 (0.05)
Cigarette RTD (mm H_2O)	117 (6.09)	117 (6.10)
Filter ventilation (%)	26 (2.91)	24 (1.80)
Tobacco blend (%)	Burley 15 Oriental 15 Bright 38 Stem 12 Expanded tobacco 20	Same blend as low with 20% of total blend replaced with tobacco sheet material to yield: Burley 12 Oriental 12 Bright 30.4 Stem 9.6 Expanded tobacco 16 Sheet material 20
FTC tar (mg/cig)	9.2 (0.35)	9.7 (0.30)
FTC nicotine (mg/cig)	0.81 (0.04)	0.74 (0.01)
FTC carbon monoxide (mg/cig)	9.8 (0.31)	10.2 (0.25)
Ammonia in smoke (μg/cig)	10.1 (0.3)	18.9 (1.7)
FTC puffs/cig	8 (0.4)	7 (0.1)

Note. . Values are reported as mean (*SD*). DAP = diammonium phosphate; FTC = Federal Trade Commission; RTD = resistance to draw.

Pharmacokinetic Analysis

Pharmacokinetic modeling was conducted using WinNonlin version 5.0.1 software (Pharsight Corporation, Mountain View, CA). A one-compartment model with first-order absorption and first-order disposition appeared to provide the best fit, using the Akaike's information criterion and residual analysis. The AUC was calculated using the linear trapezoidal rule. The maximum nicotine concentration (C_{max}) and time to reach maximum concentration (T_{max}) were identified visually. Some participants exhibited multiple small peaks in the concentration time curve in which case the peak with the highest value was defined as the C_{max}, whereas the first peak was defined as the C_{peak1} with the corresponding T_{peak1}. The conventional first-order absorption rate constant (k_a) was estimated using WinNonlin.

In addition, preliminary examination of only the initial absorption phase of nicotine indicated linearity for most but not all participants. Due to our specific interest in this initial absorption of nicotine from the lungs into the arterial circulation, a visual assessment was followed by a linear regression analysis performed in Microsoft Excel on datapoints beyond the postinhalation initial lag time. The number of datapoints included in the analysis was increased continually as long as the straight line of fit maintained linearity. The point at which the r^2 value decreased below .95 for the consecutive datapoints was used for data truncation in the calculation. The slope of the resulting linear regression represented the zero-order input rate constant (k_0; Gibaldi & Perrier, 1982). Any individual observations without a minimum of three linear datapoints or which never reached an r^2 value of .95 were excluded from the overall k_0 mean.

Statistical Analysis

To assess whether the two test cigarettes were biologically equivalent with respect to the rate and amount of the nicotine delivered, we applied commonly accepted methods for testing bioequivalence (U.S. FDA, 2000). According to these criteria, two products or preparations are considered to be bioequivalent, relevant to the measured biologically active ingredient (i.e., nicotine) if the 90% *CI* of the means of the relevant metric (e.g., $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, C_{max}) of the test formulation falls within the range of 80%–125% of the reference formulation. The log transformation is recommended because the pharmacokinetic parameters usually display a log-normal distribution (Chow & Liu, 2009).

A linear mixed model analysis was performed for each of the primary PK variables. A random intercept term was included in this model to account for intra-subject variability, and an autoregressive covariance pattern was used to model the correlations among observations repeated over time on each individual. Missing data were treated as missing at random (none of the missing data were due to nonquantifiability or limits of quantification). Diagnostics were performed to assess normality and other model assumptions using the residuals from the normal mixed models. This is especially important because the distribution of T_{max} is often skewed. In our data, the assumption of normality held for all metrics. However, since logarithmic transformation is recommended in the literature, for completeness, the results are presented for both the log-transformed and the original variables. In the case of k_a, the diagnostics showed lack of homogeneity, and there was one outlier. To account for this, the analyses were repeated without the outlier and weighted by the inverse of the *SE*s of the regression fit that produced the estimates of k_a.

From the mixed effects model, linear contrasts were used to obtain a difference between the two types of test cigarettes based on the least squares estimates, and the corresponding 90% *CI*s were directly obtained. The washout (carryover effect) was tested by analysis of variance (ANOVA) using the baseline measurements obtained before each administration of a test cigarette. Bioequivalence was determined using Schuirmann's two one-sided tests procedure (Schuirmann, 1987). Assuming the cigarette with the smaller mean to be the reference, the 20% lower and upper bounds for bioequivalence were obtained. The two cigarettes were deemed bioequivalent if the estimated 90% *CI* of the "test" product was completely contained within the lower and upper bioequivalence bounds relative to the "reference" product. This method is an extension of the average bioequivalence (ABE) because the estimates of the mean and the variance for each metric were obtained from a repeated measures analysis.

The pharmacokinetics and statistical analysis were completed prior to unblinding of study conditions.

Cigarette Smoking Experience Evaluation

The CES questionnaire (Westman et al., 1992) measures the experience of cigarette smoking by asking participants to provide a number of ratings such as, "How did smoking affect you on a 7-point scale (1 = *not at all*; 7 = *extremely*) for the following: satisfaction, good taste, calming, concentration, feeling awake, reducing hunger, reducing irritability, craving reduction, nausea, dizziness, intensity of smoke in nose and throat, and intensity of smoke in lungs." In addition, the question "How likely would you be to smoke this cigarette again in the future?" was asked. Descriptive statistics (minimum, maximum, mean, *SE*) were applied, and these data were also assessed for differences.

Results

Study Participants

Overall, of the 34 participants enrolled, 28 participants (14 males and 14 females) had evaluable data from the study. The remaining six participants did not complete the study for the following reasons: One participant failed the Allen's test, three participants had arterial line failure after catheterization, and two participants were discontinued from dosing due to mechanical issues. The average age of the participants was 36 years (range 21–56 years). The participants' self-reported mean cigarette consumption was 19 cigarettes/day (range 10–30 cigarettes/day) for an average of 18 years (range 2–44 years). The mean score on the Fagerström Test for Nicotine Dependence (Heatherton, Kozlowski, Frecker, & Fagerström, 1991) was 5 (range 1–7).

Nicotine Pharmacokinetics

Of a possible 168 total sets of data collected, the final dataset used for statistical analysis (all parameters except k_0) consisted of 144 sets (completed puffs) from 28 participants. For the overall k_0 mean, 102 sets of data were included based on the criteria described earlier. Nonevaluable data (missing observations)

resulted from inability to catheterize, catheter positioning, insufficient blood flow, or mechanical issues. Figure 3 illustrates the overall group mean nicotine concentration–time profiles in arterial blood following inhalation of the two test cigarettes (three puffs from each test cigarette). No significant differences were found in AUC, C_{max}, or T_{max} (Table 2), and the two test cigarettes were found to be bioequivalent, relevant to these parameters.

A visual inspection of the plasma concentration time curves showed that the model fits were not significantly influenced by small multiple peaks that occurred later in the sampling interval for some individuals. A comparison of the model-dependent measure k_a for the two test cigarettes resulted in nonbioequivalence at a significance level of 5% (Table 2). The *SE*s of the estimates from the model were different for different puffs. There was also one very large outlier among the estimates of k_a. When the statistical analysis was repeated excluding the outlier and the estimates were weighted by the inverse of the *SE*s, there was bioequivalence between the two test cigarettes for k_a (Table 2).

As stated earlier, preliminary examination of the initial absorption phase of nicotine indicated linearity for most but not all participants. This was similar to what was seen in a pilot study reported earlier (McKinney et al., 2010), indicating an initial zero-order absorption rate process. There was no statistical difference in the zero-order rate constant (k_0), representing the initial slope of the curves. Analysis of the data for these 102 observations indicated that the zero-order process accounted for about 90% of the initial rate to the C_{peak1} originally calculated from compartmental modeling. Similarly, the C_{peak1} and the time to C_{peak1} (T_{peak1}) were also bioequivalent for the two cigarette types.

Due to design constraints and normal manufacturing variability, the two test cigarettes yielded slightly different amounts of nicotine. When the data were adjusted to account for this difference by multiplying the data for the reference cigarette by a factor of 0.81/0.74 (where 0.81 is the nicotine yield for the low ammonia cigarette and 0.74 is the nicotine yield for the reference cigarette), the two test cigarettes remained bioequivalent in terms of AUC, C_{max}, and k_0 (Table 2). T_{max} and k_a were not adjusted for nicotine yields because they are independent of the total amount absorbed.

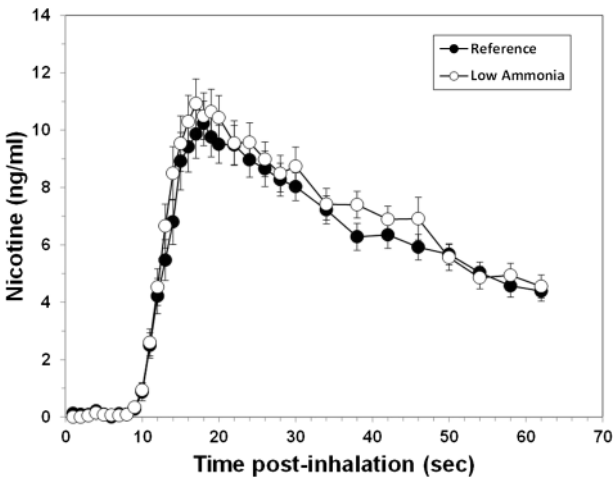

Figure 3. Nicotine pharmacokinetics. The graph shows mean plasma nicotine concentration after smoke inhalation from either the reference (18.9 μg per cigarette) test cigarette or the low ammonia (10.1 μg per cigarette) test cigarette (each point is the average of three puffs for all participants). Start time of inhalation = 0 s. Arrival time of smoke in the mouth = 4 s. The *SE*s of the mean at each timepoint ranged from 0.05 to 1.00.

A lag time (representing the time from the beginning of inhalation to the start of the rising slope of the nicotine plasma concentration curve) with a mean of 12 s (range = 7–24 s) was consistently observed. By the beginning of the next blood sampling session, the nicotine concentration–time curves were at or close to baseline; we confirmed with an ANOVA that a 30-min washout time between puffs was sufficient to eliminate any carryover effect.

Cigarette Smoking Experience Evaluation

There were no statistically significant differences in the CESs between the two test cigarettes (data not shown). Thus, there was no evidence of any pharmacodynamic differences between the two cigarette types.

Safety Evaluation

There were no serious adverse events reported during the study. All adverse events reported during the study were listed as mild in severity. The most commonly reported adverse events were redness, tenderness, and discomfort at the catheterization site and were determined by the investigator to be procedure related but not study product related. During the study, the biomonitoring system collected data from the participants. At no time during the study did these readings for any participant exceed the upper and lower safety limits determined by the study physician.

Discussion

Due to a lack of direct evidence regarding ammonia's effects on nicotine pharmacokinetics in the smoker, the public health community specifically calls for this type of research, that is, "fundamental chemical and biological research on the behavioral and physiological consequences (including smoker uptake) of treating tobacco products with ammonia, ammonia precursors, and other chemical bases" (Henningfield et al., 2004). The current study was designed to address this gap and allow direct measurement of nicotine from arterial blood. Our rapid automated blood sampling system allowed precise measurements of pharmacokinetic parameters of nicotine from two test cigarettes of differing smoke ammonia yields with extremely high time resolution (one sample per second; McKinney et al., 2010).

The reported results provide strong evidence that the different ammonia yields of the two test cigarettes had no impact on nicotine pharmacokinetics. We found no difference in nicotine's rate of absorption (k_0) and peak concentration (C_{max}) in the arterial blood. The cigarette with the higher level of ammonia in smoke, which had a similar ammonia level to a commercially available, 7–12 mg tar range cigarette (Federal Trade Commission), showed a trend toward a lower rate of absorption and total dose. That could be explained by the slightly lower nicotine delivery from the cigarette due to

Table 2. Summary Statistics and Bioequivalence Results

PK variable	Mean (*SD*) Reference$_3$	Mean (*SD*) Low NH_3	90% *CI* for the difference	90% *CI* for the difference in logarithms	Bioequivalence[a]
AUC (ng.s.ml^{-1})	349 (205)	386 (207)	−67.18, −5.88	−0.22, −0.02`	Yes
C_{max} (ng.ml^{-1})	12.3 (7.6)	13.3 (7.7)	0.24, 2.18	−0.16, 0.00	Yes
T_{max} (s)	23 (8)	21 (8)	−0.67, 3.51	−0.00, 0.14	Yes
C_{peak1} (ng.ml^{-1})	10.8 (7.3)	11.8 (7.4)	−2.06, 0.11	−0.16, 0.01	Yes
T_{peak1} (s)	17 (3)	16 (3)	−0.03, 1.67	−0.02, 0.13	Yes
k_0 (ng.s^{-1}.ml^{-1})	2.9 (1.5)	3.1 (1.5)	−0.55, 0.14	−0.17, 0.00	Yes
k_a (s^{-1})	0.5 (0.6)	0.7 (0.6)	−0.39,0.08	−0.36,0.00	No
$k_{a(adj)}$[b] (s^{-1})	0.4 (0.2)	0.5 (0.2)	0.00, 0.09	−0.21, 0.02	Yes
Corrected for nicotine					
AUC[c] (ng.s.ml^{-1})	383 (214)	385 (215)	−33.97, 28.59	−0.13, 0.07	Yes
C_{max}[c] (ng.ml^{-1})	13.5 (7.9)	13.2 (8.0)	−1.01, 1.51	−0.07, 0.09	Yes
C_{peak1}[c] (ng.ml^{-1})	11.8 (7.6)	11.7 (7.7)	−1.04, 1.25	−0.07, 0.10	Yes
k_0[c] (ng.s^{-1}.ml^{-1})	3.2 (1.5)	3.1 (1.6)	−0.27, 0.45	−0.08, 0.10	Yes

Note. AUC = area under the curve; PK = pharmacokinetic.

[a]Bioequivalence is suggested when 20% of the low ammonia mean (−ve and +ve) is within the 90% *CI*. In the log scale, the 90% *CI* for the difference should be completely contained within (−0.233, 0.182). A significance level of 5% is used.

[b]Based on the data without the outlier and estimates weighted by the inverse of the *SE*.

[c]PK variables corrected for difference in nicotine between the two test cigarettes. T_{max}, T_{peak1}, and k_a were not corrected because they are dose independent.

the manufacturing process (see below). However, even when the data were corrected for this difference in nicotine yield, there were still no significant differences in nicotine pharmacokinetics between the two test cigarettes. Hence on a puff-by-puff basis, our data do not support the theory that an increase in ammonia delivery in cigarette smoke corresponds to an increase in the rate or amount of nicotine absorption into the blood. This is in line with Armitage et al. (2004), who found that retention of nicotine in the respiratory tract was unchanged after addition of ammonia-forming compounds such as DAP or urea to the RTS. In addition, they found no significant differences in the venous plasma nicotine profiles for the untreated versus ammoniated cigarette types. Our value for k_a was higher in the low ammonia condition, indicating faster absorption rate, which would appear to contradict the theory that higher levels of ammonia in cigarette smoke would increase the rate of nicotine absorption. One reason for this might be that, unlike the other measures, the k_a values are estimates from a nonlinear model fit.

In mainstream tobacco smoke, only the free-base form of nicotine (and not the protonated form) can volatilize from the smoke particles to the gas phase of the inhaled smoke (Pankow, Tavakoli, Luo, & Isabelle, 2003). Thus, some researchers suggest that the magnitude and rate of deposition of nicotine in the lungs from smoking are dependent upon the fraction of nicotine in free-base form. With a novel experimental approach employing a Teflon bag for smoke collection to which excess ammonia was added, Pankow et al. (2003) determined that the fraction of free-base nicotine was significant in commercial cigarettes and translated to high deposition rates in the respiratory tract. However, Lauterbach et al. recently reported that the use of free-base nicotine measurements alone (or as a ratio to total smoke nicotine) to determine if ammonia compounds have been used to treat tobacco is inappropriate. They stated that the main drivers of free-base nicotine in total particulate matter (TPM) are cigarette design features and acidic compounds in smoke formed from pyrolysis, which cause differences in smoke pH (Lauterbach, Bao, Joza, & Rickert, 2010). Their research demonstrated that when cigarettes are smoked under conditions mimicking human smoking, the concentrations of nicotine in free-base form in the TPM and the mainstream smoke gas–vapor phase are physiologically insignificant (Lauterbach et al., 2010). Thus, other scientists also doubt the relationship between ammonia compounds as tobacco additives and the amounts of free-base nicotine in smoke (Seeman & Carchman, 2008).

The only difference between our two cigarettes was the level of DAP-containing sheet material in the reference cigarette compared with its absence in the low ammonia cigarette. The processing was the same for both cigarettes except for the inclusion of the sheet material. All other cigarette design features were held constant as well. Thus, we believe that no other cigarette design parameters contributed to changes in smoke alkalinity aside from the difference in ammonia levels. The inclusion of sheet material had an effect on final smoke chemistry, primarily on nicotine and water yields. From a practical standpoint, it was impossible to match nicotine and tar yields in the low ammonia blend and the reference blend due to the difference in water content. However, the variation in tar and nicotine in our two test cigarettes was similar to the inherent manufacturing variation in commercially marketed cigarettes over a typical 6-month period. We accepted these differences as a necessary outcome of achieving two cigarettes with different ammonia yields and therefore corrected for nicotine yields in our final calculations.

The nicotine bolus theory proposed in the literature states that nicotine is rapidly absorbed from the lungs after smoking, resulting in very high peak concentrations in arterial blood with a transit time to the brain of just a few seconds (Gourlay &

Benowitz, 1997; Henningfield & Keenan, 1993; RCP, 2000; Russell & Feyeraband, 1978; U.S. DHHS, 1988). In our study, a considerable lag time for nicotine's first appearance in arterial blood following each puff of smoke inhalation was observed in all instances (mean = 12 s, range = 7–24 s). This lag time agrees with the findings of Berridge et al. (2010), who reported that a nicotine bolus (as measured by counts of [^{11}C]nicotine) arrived at the arterial sampling site at approximately 10–15 s after start of inhalation. Our time to peak concentration (T_{max}) as determined by the PK model had a mean value of 21–23 s, while our observed T_{peak1} averaged 16–17 s, both similar to the 20-s time to peak found by Rose et al. (1999). Distribution of nicotine into lung tissues, which act as a depot for nicotine, could account for this slower release of nicotine into the arterial circulation (Rose et al., 1999). It is important to consider that Rose's sampling frequency was every 5 s, and thus, his time to peak value could be up to 5 s longer than reported. The faster sampling rates in our study and Berridge's study yielded a higher precision in determination of time to peak values.

We did see multiple peaks (ranging from 2 to 20 s apart) in some of the concentration–time curves for some puffs in some of the participants and thus chose to measure not only C_{max} but C_{peak1} as well. The linear input rate k_0 was based on the datapoints leading up to this first peak (C_{peak1}), which was in a few cases not necessarily the highest peak. These multiple peaks could be a function of blood recirculation within the body. Zubieta-Calleja, Zubieta-Castillo, Paulev, and Zubieta-Calleja (2005) reported that circulation time from pulmonary alveoli to finger was approximately 15–16 s, but normal variation exists, depending on age, gender, and central blood volume. Alternately, there could be absorption of nicotine from the upper bronchi of the lungs into venous circulation concurrent with arterial absorption from the alveoli. This venous nicotine would then recirculate through the lung and appear some short time later in the arterial blood samples. However, we did not see this phenomenon in every participant nor in every puff from a single participant, so it may have another possible explanation, including sampling variability. It is important to remember that although we attempted to control inhalation parameters, there was still a range (150–250 ml/s) within which the participants were required to inhale, suggesting that there may in fact be some differences within participants with respect to relative distribution of the smoke bolus within the respiratory tract, that is, from bronchi (venous absorption) to alveoli (arterial absorption).

In order to standardize the experimental conditions for all participants and eliminate any confounding due to individual behavioral differences, the present study was designed to have tightly controlled smoke delivery and inhalation parameters. Hence, this experimental setup did not directly mimic normal smoking behavior, but it did allow a direct comparison of the effect of ammonia on the absorption of nicotine. Although only a single puff was delivered each time, we have no evidence to believe that the potential influence of ammonia on nicotine pharmacokinetics would change after smoking an entire cigarette. In addition, smoke delivered to participants from the smoking machine would have aged slightly over the time it took to reach the mouthpiece; however, all puffs were delivered in exactly the same timeframe, and the process was the same for both test cigarettes. We performed an ANOVA on the baseline measurements to verify washout success between puffs and found that there was no significant carryover effect. This study did not address the kinetics of nicotine in different populations of smokers (e.g., dependent versus nondependent) in which Rose et al. (2010) have shown a significant difference in rate of nicotine accumulation. We have no reason to believe that the influence or lack thereof of different levels of ammonia in the cigarette on nicotine rate and amount of absorption would be different in dependent and nondependent smokers.

The key findings of the present study demonstrate that there were no differences in peak arterial nicotine concentration (C_{max}), time to peak (T_{max}), amount of nicotine (AUC), or rate of absorption (k_0) for the two test cigarettes with different smoke ammonia yields. Thus, the two test cigarettes were found to be bioequivalent under the parameters used in the present study. This study directly contradicts the hypothesis that an increase in ammonia levels in smoke increases nicotine's absorption into the arterial blood. A recent study by van Amsterdam et al. (2011) supports our conclusions, finding that a higher level of ammonium salts in commercial cigarettes does not lead to higher nicotine absorption, as measured in venous blood samples. It is once again important to note that the present study examined nicotine in arterial blood only on a puff-by-puff basis under controlled inhalation conditions. However, we have no indication from the literature or from mechanistic points of view (Seeman, 2007; Seeman et al., 2004; Seeman & Carchman, 2008) to believe that ammonia levels in the cigarettes would differentially influence nicotine absorption in subsequent puffs. Ideally, further studies should be done in this area to test variations in ammonia levels in commercially marketed cigarettes. In summary, in this study, we found that ammonia levels in the cigarettes do not influence the absorption of nicotine as measured by arterial blood concentrations.

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Declaration of Interests

None declared.

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