APPENDIX A6.1.3.17 NICOTINE - FACTSHEET

	HPHC	BoExp assessed by PMI
IUPAC name	3-[(2S)-1-methylpyrrolidin-2-yl]pyridine	a) Urinary NEQ (Nicotine equivalent), i.e., the molar sum of: 1) nicotine 2) nicotine-N-glucuronide 3) cotinine 4) cotinine-N-glucuronide 5) trans-3'-hydroxycotinine 6) trans-3'-hydroxycotinine-O-glucuronide b) Plasma nicotine c) Plasma cotinine
Usual abbreviation	-	a) NEQ, NE b) N/A c) N/A
Other names	Nicotine, (S)-nicotine	-
CAS number	54-11-5	1) 54-11-5 2) 152306-59-7 3) 486-56-6 4) 139427-57-9 5) 34834-67-8 6) 132929-88-5
Formula and molecular weight	$C_{10}H_{14}N_2$ MW: 162.23	1) $C_{10}H_{14}N_2$ MW: 162.232 2) $C_{16}H_{22}N_2O_6$ MW: 338.356 3) $C_{10}H_{12}N_2O$ MW: 176.218 4) $C_{16}H_{20}N_2O_7$ MW: 352.341 5) $C_{10}H_{12}N_2O_2$ MW: 192.217 6) $C_{16}H_{20}N_2O_8$ MW: 368.34

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TABLE OF CONTENTS

APPENDIX A6.1.3.17 NICOTINE - FACTSHEET 1
Main characteristics 3
ADME properties 4
Exposure levels in smokers and non-smokers 8
NEQ 8
List of abbreviations 12
References 13

TABLE OF TABLES

Table 1 Representative urinary nicotine equivalents levels in smokers and non-smokers 11
Table 2 Representative urinary nicotine equivalents levels after smoking abstinence 11

TABLE OF FIGURES

Figure 1 Pathways of nicotine metabolism 6
Figure 2 Quantitative scheme of nicotine metabolism, based on estimates of average excretion of metabolites as percent of total urinary nicotine (reader is advised to interpret them with caution) 8

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Main characteristics

Chemical class	Alkaloid
HPHC lists	FDA-93 [1]; FDA-18 [2]; HC [3, 4]; PMI-58; WHO-39 [5]
Source in cigarettes	Amount in MSS (3R4F): 0.707 mg (ISO); 1.90 mg (HCI) [6] Smoke phase: particulate phase [7] Presence in tobacco / pyrosynthesis (T°): transfer below 300°C [8] 70% of the nicotine in tobacco is recovered intact after smoking, (in mainstream and side stream smoke, and on the butt) [9]. The rest is converted to other substances (ending up mostly in the sidestream smoke) [9, 10].
Other sources	Nicotine is the principal alkaloid found in tobacco (about 95% of the total alkaloid content in tobacco) [11]. It is most frequently encountered in tobacco products for smoking, chewing, snuffing and tobacco "without smoking" (smokeless tobacco). It was formerly used as an insecticide (now rare and prohibited). It is used as an adjunct to smoking cessation programs (gums, patches), called nicotine replacement therapies (NRT) [12]. Nicotine is also found in low levels in vegetables such as potatoes, tomatoes, and eggplants [13, 14].
Toxicity class (FDA) [1]	RDT, AD
Toxicity – CA	-
Toxicity – RT	-
Toxicity – CT	-
Toxicity – RDT	A causal relationship between maternal smoking and congenital malformation has been shown in humans, however, overall data provide support that nicotine makes a relatively modest contribution to the effects of tobacco use on fetal growth when compared with the larger contribution of the combination of both nicotine and products of combustion in cigarette smoke [15, 16]. Overall, fetal birth weight seems to be only moderately affected by a dose of 6 mg/kg per day of nicotine bitartrate, and has no effect at lower doses, as shown mainly in rodent studies [16]. Animal studies with nicotine show adverse effects on the developing central nervous system of rats [17] and nicotine effects on the brain have been postulated to be involved in the pathophysiology of Sudden Infant Death Syndrome [18]. The California EPA has listed nicotine as a developmental toxicant since 1990 [15, 19].

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Toxicity – AD	Nicotine is an addictive drug. Addiction develops as a neurobiologic adaptation to chronic nicotine exposure [15]. The pharmacologic reasons for nicotine use are enhancement of mood, either directly or through relief of withdrawal symptoms, and augmentation of mental or physical functions [20]. The pharmacologic basis of nicotine addiction can be seen as a combination of positive reinforcements, such as response to negative affect [21].
Toxicity - others	Nicotine is extremely toxic and can be lethal at doses of 60 mg (for an adult) [22]. More than 200,000 incidents involving ingestion of tobacco products, 89% of which involved children below 6 years old were reported by the American Association of Poison Control Centers (AAPCC) [23] in a period covering twenty-seven years of annual reports (from 1983-2009) [24]. All other available toxicity data can be found on the Website Aggregated Computational Toxicology Resource (ACToR) [25].

ADME properties

Absorption and distribution [11, 12, 26] - Nicotine base is well absorbed through skin, making it an occupational risk for tobacco harvesters (green tobacco sickness) [27]. After inhalation, nicotine enters the blood where, at pH 7.4, it is about 70% ionized. Binding to plasma proteins is less than 5%. Studies showed that, after intravenous (IV) administration, the distribution of ^{14}C-labeled nicotine is immediate, reaching the brain of mice within 1 min after injection [26-28]. Similar findings based on positron emission tomography of the brain, were seen after injection of ^{11}C-nicotine in monkeys [26]. Nicotine inhaled in tobacco smoke enters the blood almost as rapidly as after rapid IV injections. Because of delivery into the lung, peak nicotine levels may be higher and lag time between smoking and entry into the brain shorter than after IV injection [26]. After smoking, the action of nicotine on the brain is expected to occur quickly. Rapid onset of effects after a puff is believed to provide optimal reinforcement for the development of drug dependence. The effect of nicotine declines as it is distributed to other tissues. The drug is distributed extensively to body tissues. The distribution half-life, which describes the movement of nicotine from the blood and other rapidly perfused tissues, such as the brain, to other body tissues, is about 8-9 min [26, 29]. Distribution kinetics, rather than elimination kinetics (or plasma half-life; about 2 hr), determine the time course of the CNS actions of nicotine after smoking a single cigarette. Consistent with a half-life in plasma of about 2 hours, accumulation of nicotine over 6 to 8 hours during regular smoking and persistence of significant levels of nicotine in the blood for 6 to 8 hours after cessation of smoking, i.e., overnight, has been observed [26, 27]. Using the more sensitive urinary excretion to assess half-life has shown terminal half-life of around 11 h [30]. This longer half-life is probably reflecting the release of nicotine from body tissues [30]. Based on a half-life of 2 h for nicotine, accumulation is predicted over 6-8h of regular smoking, and persistence of significant levels for 6-8h after smoking abstinence [26]. Thus, cigarette smoking represents a situation where the smoker is exposed to

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significant concentrations and possibly pharmacological effects of nicotine for 24 hours a day [26].

Metabolism [27] - Nicotine is a tertiary amine which is composed of a pyridine and a pyrrolidine ring. Its metabolism has been largely studied and is schematized in Figure 1 [11, 27]. Nicotine undergoes a large first pass effect during which the liver metabolizes 80% to 90% of the inhaled amount [31]. To a smaller extent, the lung is also able to metabolize nicotine, as suggested by one study in dogs [32]. The major metabolite of nicotine is cotinine; while nicotine-1'-N-oxide is a minor metabolite (only 4-7% of the nicotine absorbed by smokers). The transformation of nicotine into cotinine involves two steps: 1) CYP2A6 activation, and; 2) oxidation by a cytoplasmic aldehyde oxidase. Nicotine-1'-N-oxide is not further metabolized, except by reduction back to nicotine in the intestines.

In addition to oxidation of the pyrrolidine ring, nicotine is metabolized by two non-oxidative pathways, methylation and glucuronidation. About 3-5% of nicotine is converted to nicotine glucuronide.

Cotinine is extensively metabolized to trans-3'-hydroxycotinine. The most abundant metabolite in mice is trans-3'-hydroxycotinine, accounting for almost 40%, whereas cotinine itself accounts for only about 15% of the dose of nicotine [12]. It is also the main nicotine metabolite detected in smokers' urine [27]. Cotinine levels in various biological fluids are widely used to estimate intake of nicotine in tobacco users. The usefulness of cotinine as a quantitative marker of nicotine intake is limited by individual variability in percentage conversion of nicotine to cotinine and in the rate of elimination of cotinine. Since it accounts for a much greater percentage of nicotine, trans-3'-hydroxycotinine measurement, either alone or in combination with measurement of other metabolites, may be a superior quantitative marker of nicotine intake than cotinine alone [12].

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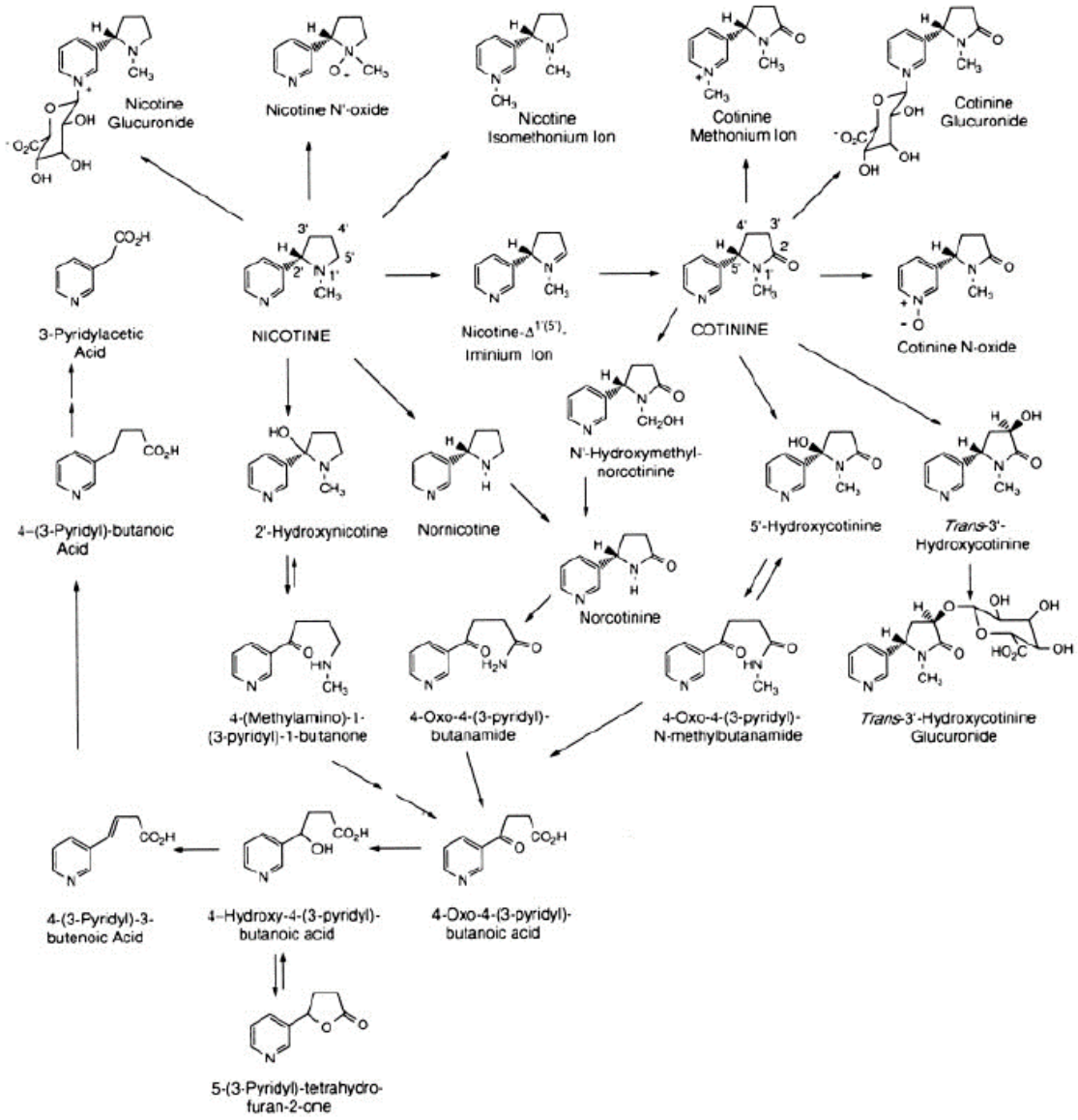


Figure 1 Pathways of nicotine metabolism [11]

Figure by Hukkanen J, Jacob P, 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. Pharmacol Rev. 2005; 57(1):79-115. Reprinted with permission of the publisher. Copyright © 2016 by the American Society for Pharmacology and Experimental Therapeutics.

The total clearance rate of nicotine is of about 1200 ml min^{-1}. The metabolism of cotinine is much slower, with a total clearance averaging 45 ml min^{-1}. Trans-3'-hydroxycotinine total clearance is also rather slow, about 82 ml min^{-1}. Intravenous infusion of nicotine in abstinent adult smokers has shown half-lives of 2.3 h for nicotine [33], 17.5 h for cotinine [33] and 6.6 h for trans-3'-hydroxycotinine [34].

Nicotine metabolism is influenced by a number of factors, such as those that influence liver blood flow (e.g., diet, or meals, or sleep). Some products influence the activity of

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CYP2A6, such as grapefruit juice, and possibly menthol. A number of medications also influence CYP2A6 activity, either by inducing it or by inhibiting it [35]. Polymorphism of CYP2A6 and other enzymes also has some influence on the clearance of nicotine [11]. Age, gender and ethnicity also influence nicotine metabolism, lower in elderly, higher in women, higher during pregnancy, higher in whites than in blacks (possibly influenced by a higher consumption of menthol cigarettes by blacks), higher in Latino-Americans and whites than in Chinese-Americans [36]. Smoking itself influences the rate of metabolism of nicotine, with a lower clearance in smokers than non-smokers [27]. β-nicotyrine, a minor tobacco alkaloid was shown to effectively inhibit CYP2A6 in vitro [37], and could contribute to the decreased metabolism of nicotine in smokers [27]. On the other hand, other studies have reported a higher rate of nicotine metabolism in smokers, as claimed, for example, by Kyerematen et al. [38]

Because cotinine, the major proximate metabolite of nicotine, is further metabolized to trans-3′-hydroxycotinine essentially exclusively by CYP2A6, the ratio of 3'-hydroxycotinine over cotinine (also referred as nicotine metabolism ratio) can be used an indicator of CYP2A6 enzymatic activity [39]. Such ratio is similar in blood, plasma, and saliva, whereas in urine, it did not consistently provide similar NMRs [40]. Urine NMRs has nevertheless been suggested as a reasonably good proxy for blood, plasma, and saliva NMRs, based on the correlations between these variables [40].

Excretion [27] - Although 70-80% of nicotine is metabolized via the cotinine pathway, only 10-15% of nicotine absorbed by smokers appears in the urine as free (unconjugated) cotinine [41]. At least six primary metabolites of cotinine have been reported in humans: *trans*-3′-hydroxycotinine, 5′-hydroxycotinine (also termed allohydroxycotinine), which exists in tautomeric equilibrium with the open chain derivative 4-oxo-4-(3-pyridyl)-N-methylbutanamide, cotinine N-oxide, cotinine methonium ion, cotinine glucuronide, and norcotinine (demethylcotinine). Trans-3′-hydroxycotinine is the main nicotine metabolite detected in smokers' urine, accounting for 40-60% of the dose, together with its glucuronide conjugate. Quantitative scheme of nicotine metabolism with estimated relative excretion of the different metabolism is shown in Figure 2 [11, 27]. Nicotine-N-glucuronide, cotinine, cotinine-N-glucuronide, trans-3'-hydroxycotinine, and trans-3'-hydroxycotinine-O-glucuronide are usually assessed, and are referred to as "nicotine equivalents" (NEQ). Other profiles of urinary nicotine metabolites have been more recently developed to assess the nicotine dose, such as "Nicotine + 8" [42], "Nicotine + 9" [43] or "Nicotine + 10" [44] (i.e. nicotine and eight or ten of its metabolites, respectively), however, they have not yet demonstrated added value when compared to the measurement of NEQ.

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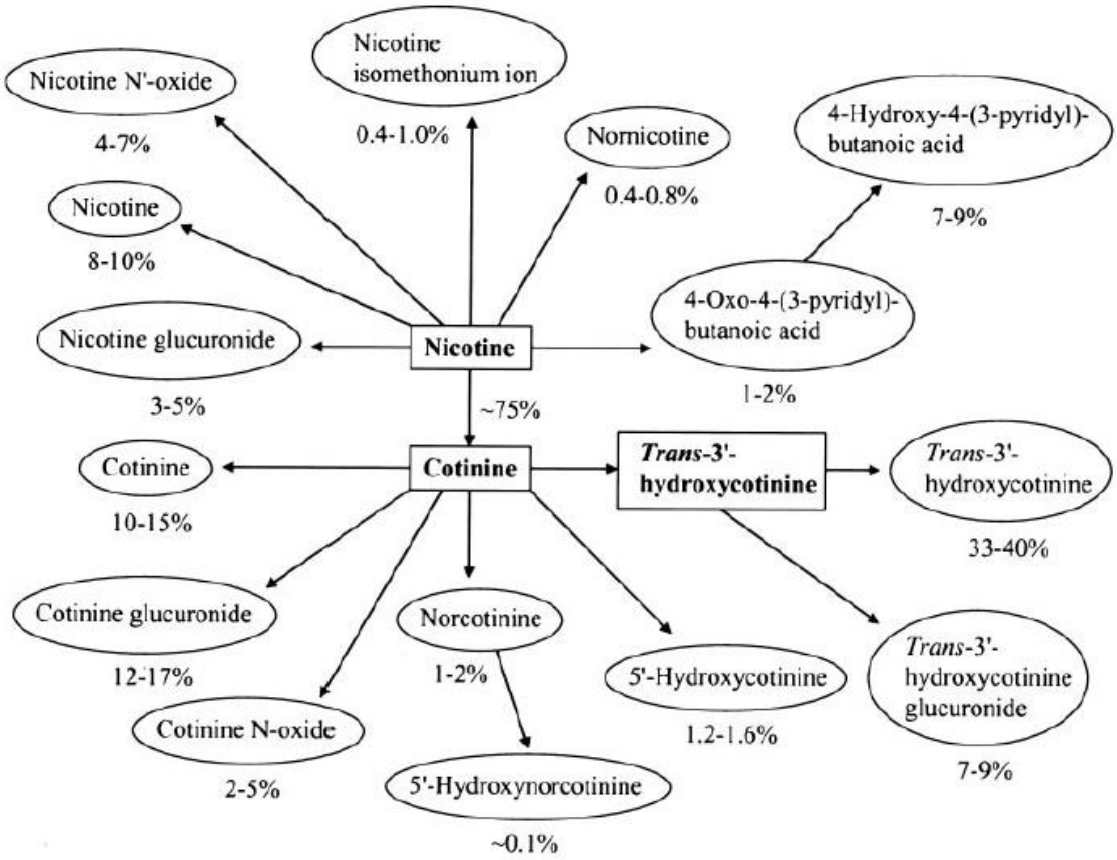


Figure 2 Quantitative scheme of nicotine metabolism, based on estimates of average excretion of metabolites as percent of total urinary nicotine (reader is advised to interpret them with caution) [11]

Figure by Hukkanen J, Jacob P, 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. Pharmacol Rev. 2005; 57(1):79-115. Reprinted with permission of the publisher. Copyright © 2016 by the American Society for Pharmacology and Experimental Therapeutics.

Exposure levels in smokers and non-smokers

NEQ

In non-smokers	Ranges: NA [45]
In smokers	Ranges: 70.4–154 µmol/24hr [11.42-24.98 mg/24h] [45]

In smokers, typical levels of NEQ range from 70.4 to 154 µmol/24hr (i.e. 11.42-24.98 mg/24h) ([45] and Table 1). They reach very low levels (i.e. 0.00 to 2.21 mg/24h) after 5 to 8 days of smoking cessation (Table 2).

Because the almost exclusive source of nicotine is tobacco or NRT products, the presence of its metabolite cotinine in plasma or urine is used to confirm the smoking status (or NRT use) of a person. A commonly used test is to semi-quantitatively determine cotinine in urine, because of its simplicity and non-invasiveness (dipstick), which can distinguish smokers from non-smokers in a semi-quantitative manner (with cut-off values of around

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200 ng/mL of cotinine). Other common techniques are based on gas chromatography (GC) and high-performance liquid chromatography (HPLC) [46], or liquid chromatography-tandem mass spectrometry (LC-MS/MS). Cut-off values have been established to distinguish active tobacco-products users from passively exposed persons [46]. The most widely cited cotinine cut-off points of 14 ng/ml for serum and saliva as well as 50 ng/ml for urine were provided by Jarvis et al. [47]. Benowitz et al. have proposed optimal serum cotinine cut-off points of 3.08 and 2.99 ng/ml for adults and adolescents, respectively, and estimated an optimal urine cotinine cut-off point of 15 ng/ml. These values take into account differences between racial/ethnic groups [36]. Different cut-off values have been suggested in different studies [46, 48-50], ranging from 31.5 to 550 ng/mL, however, most cotinine tests in urine use cut-off values of 200 ng/mL, in order to misclassify someone as a smoker (false positive), when he/she actually is a non-smoker and might have been exposed to passive smoke.

Because of the short plasma half-life of nicotine (around 2 h), its use as a biomarker of exposure is not recommended for general use [27]. Nicotine concentrations in plasma are not significantly correlated with either cotinine or trans-3'-hydroxycotinine concentrations in plasma, however, cotinine and trans-3'-hydroxycotinine concentrations in plasma are significantly correlated [34, 51]. Cotinine, which is also very specific for use of tobacco or NRT products, has the advantages of a longer half-life (16 h), but has the limitation of individual variability in percentage conversion of nicotine to cotinine and in rate of elimination of cotinine itself [51]. Cotinine concentrations in plasma are linearly and directly related to nicotine intake and show little variation for up to 2 h after the last exposure to nicotine [52]. Nicotine concentrations in blood have, however, been extensively used as a biomarker to assess changes in smoking topography in experimental studies and in pharmacokinetic studies (reviewed in [51]).

Assuming that a smoker reaches a steady state concentration of nicotine, the daily amount of nicotine uptake is equal to the amount of nicotine and metabolites excreted in the 24h-urine, which are, as described above, well accounted for by measuring NEQ [51] (Table 1). Due to the half-lives of the nicotine metabolites, the 24-h urinary NEQ essentially represents an about two-day moving average of nicotine dose, which can present some issue in clinical setting, as the exposure from the day prior to entering the clinic is unknown [53]. Levels at baseline are thus not controlled, unless a confinement controlled period is planned before the beginning of the actual exposure study.

In order to obtain NEQ values, the following formula should be applied:

$$\begin{aligned} \text{NEQ [g]} = {} & (\text{free nicotine [µmol/L]} + \text{nicotine-glucuronide [µmol/L]} + \\ & \text{free cotinine [µmol/L]} + \text{cotinine-glucuronide [µmol/L]} + \\ & \text{free } \textit{trans}\text{-3'-hydroxycotinine [µmol/L]} + \textit{trans}\text{-3'-} \\ & \text{hydroxycotinine-glucuronide [µmol/L])} * 162.2 \text{ [µg/µmol]} \\ & * \text{urine volume (L)} / 1000 \text{ [µg/mg]} \end{aligned}$$

All concentrations must be in µmol/L, thus conversion factors must thus be applied for these metabolites, as described below:

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- Free nicotine: the molecular weight is 162.232 g/mol. Therefore to convert nicotine from ng/mL to nmol/L, the result in ng/mL is multiplied by 6.164.
- Nicotine glucuronide: the molecular weight is 338.356 g/mol. Therefore to convert nicotine from ng/mL to nmol/L, the result in ng/mL is multiplied by 2.955.
- Cotinine: the molecular weight is 176.218 g/mol. Therefore to transform cotinine from ng/mL to nmol/L, the result in ng/mL will be multiplied by 5.675.
- Cotinine-glucuronide: the molecular weight is 352.341 g/mol. Therefore to transform cotinine from ng/mL to nmol/L, the result in ng/mL will be multiplied by 2.838.
- Trans-3'hydroxycotinine: the molecular weight is 192.217 g/mol. Therefore to transform trans-3' hydroxycotinine from ng/mL to nmol/L, the result in ng/mL is multiplied by 5.202.
- Trans-3'-hydroxycotinine-glucuronide: the molecular weight is 368.34 g/mol. Therefore to transform trans-3' hydroxycotinine from ng/mL to nmol/L, the result in ng/mL is multiplied by 2.715.

Like for the NEQ levels in urine, the levels of nicotine or cotinine in plasma largely depend on the smoking behaviour, with the puff volume, depth of inhalation, extent of dilution with room air and the rate and intensity of puffing that vary between smokers [27]. The time of measurement will also impact the variability of measurements. For smokers, steady-state levels are more likely reached in the afternoon than in the morning, when nicotine levels have declined during the night (a usually smoking abstinent period) [51].

Because of the short nicotine plasma elimination half-life (around 2 h), measurements will also largely depend on when the last cigarette was smoked. It is unusual to measure nicotine in arterial bloods, but experiments have shown up to tenfold arterial/venous nicotine concentration ratios, ranging more typically between 2 and 3 [27], indicating distribution of nicotine in the tissues. During the day, blood or plasma nicotine concentrations in cigarette smokers typically range between 10 and 37 ng/mL, with peak concentrations between 19 and 50 ng/mL [27].

Cotinine concentrations in serum have been significantly associated with self-reported exposure to environmental tobacco smoke (reviewed in [51]). Cotinine concentrations in serum show less circadian fluctuations than nicotine, and correlate well with the amount of cigarettes smoked per day. Typical blood cotinine concentrations in smokers range between 136 to 385 ng/mL [51].

Concentrations of cotinine and other nicotine metabolites are considerably higher in urine than in plasma or saliva, making chemical analysis easier, however, saliva is a very practical matrix to sample, and can be of great interest for epidemiological studies [54-58]. Levels of nicotine measured in saliva were shown to be in good correlation with the nicotine measured in the used filters and the NEQ levels in urine [59], as well as with the number of cigarettes smoked per day, even though this correlation was moderate [60, 61].

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Table 1 Representative urinary nicotine equivalents levels in smokers and non-smokers

Author (year)	Levels in smokers	Levels in non-smokers (or ex-smokers)	Units; variability
Scherer (2006) [62]	18.1 ± 10.9	NM	mg/24h; mean ± SD
Zedler (2006) [63]	64.51 (0.56, 182.34) [10.466 (0.091, 29.581)]	< 0.03 (0.01, 1.22) [0.005 (0.002, 0.198)]	nmol/mg creat [mg/g creat]; median (min, max)
Scherer (2007) [64]	11.4 ± 8.1	NM	mg/24h; mean ± SD
Mendes (2008) [65]	22.2 ± 7.9	NM	mg/24h; mean ± SD
Lowe (2009) [66]	9.42 ± 5.98 (≤ 10 cpd) 24.91 ± 7.25 (≥ 20 cpd)	0.05 ± 0.08	mg/24h; mean ± SD
Lowe (2009) [66]	6947 ± 4336 [6.947 ± 4.336] (≤ 10 cpd) 18577 ± 6539 [18.577 ±6.539] (≥ 20 cpd)	36.97 ± 54.20 [0.03697 ± 0.05420]	ng/mg creat [mg/g creat]; mean ± SD
Roethig (2009) [67]	13.3 ± 0.14	NM	mg/24h; weighted mean ± SE
Lindner (2011) [68]	10.62 ± 7.31	0.05 ± 0.34	mg/24h; mean ± SD
Shepperd (2013) [69]	12 ± 5.9 (0.2-23.6)	0.04 ± 0.04 (0.02-0.26)	mg/24h; LS mean ± SD (ranges)
Scherer (2014) [70]	15.9 ± 7.4	<0.01 (<0.01)	mg/24h; mean ± SD
Morin (2011) [71]	18.9 (17.4-20.5) (14 cigarette/day)	0.03 (0.01-0.03)	mg/24h; mean (95% CI)

Table 2 Representative urinary nicotine equivalents levels after smoking abstinence

Author (year)	Levels in smokers	Levels in ex-smokers or switchers	Units; variability
Roethig (2007) [72]	20.58 ± 6.79	2.21 ± 4.02 [a]	mg/24h; mean ± SD
Frost-Pineda (2008) [73]	16.1 ± 6.2	0.17 ± 0.07 [a]	mg/24h; mean ± SD
Tricker (2012) [74]	6.91 ± 3.71	0.62 ± 0.87 [b]	mg/24h; mean ± SD
Tricker (2012) [75]	14.32 ± 4.62	0.00 ± 0.01 [a]	mg/24h; mean ± SD
Tricker (2012) [76]	6.45 ± 3.00	0.06 ± 0.02 [a]	mg/24h; mean ± SD
Tricker (2012) [77]	9.29 ± 5.28	0.21 ± 0.60 [a]	mg/24h; mean ± SD
Theophilus (2015) [43]	21.5 ± 5.7	0.5 ± 0.3 [b]	mg/24h; mean ± SD

[a] After 8 days of smoking abstinence
[b] After 5 days of smoking abstinence

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List of abbreviations

3R4F	Kentucky 3R4F reference research cigarette [78]
AAPCC	American Association of Poison Control Centers
ACToR	Aggregated Computational Toxicology Resource
AD	Addictive
ADME	Absorption and Distribution, Metabolism, and Excretion
CA	Carcinogen
CAS number	Chemical Abstracts Service number
CI	Confidence interval
CNS	Central nervous system
CT	Cardiovascular toxicant
Creat	Creatinine
FDA	Food and Drug Administration
HC	Health Canada
HCI	Health Canada Intense regime [79]
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
ISO	International Organization for Standardization smoking regime [80]
IUPAC	International Union of Pure and Applied Chemistry
IV	Intravenous
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LS	Least squares
MSS	Mainstream smoke
MW	Molecular weight
NA	Not assessed
NEQ	Nicotine equivalents
NM	Not measured
NRT	Nicotine replacement therapy
PMI	Philip Morris International
RDT	Reproductive or developmental toxicant
RT	Respiratory toxicant
SE	Standard error
SD	Standard deviation
WHO	World Health Organization

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