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A LC-MS/MS Method for Concurrent Determination of Nicotine Metabolites and Role of CYP2A6 in Nicotine Metabolism in U937 Macrophages: Implications in Oxidative Stress in HIV + Smokers

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Abstract

Nicotine, the major constituent of tobacco, is predominantly metabolized by liver CYP2A6 into cotinine and many other compounds, including nicotine-derived nitrosamine ketone (NNK), which is known to cause oxidative stress. We have recently shown that CYP2A6 is highly expressed in U937 monocyte-derived macrophages. In this study we investigated the role of CYP2A6 in nicotine metabolism and oxidative stress in U937 macrophages. To study nicotine metabolism, we developed a highly sensitive LC-MS/MS method for simultaneous quantitative determination of nicotine, cotinine, and NNK. The LC-MS/MS analysis was carried out by multiple reaction monitoring mass transitions with m/z of 163.2/130.1, 177.4/98.3, and 208.4/122.1 for nicotine, cotinine, and NNK, respectively. The calibration curves were linear within 3.3–1028.1 ng/ml for nicotine and 0.3–652.6 ng/ml for cotinine and NNK. This novel method was then applied to quantify nicotine metabolites, cotinine and NNK, in nicotine-treated U937 macrophages. Cotinine and NNK initially formed at 30 min, followed by a peak at 2–3 h. The role of CYP2A6 in nicotine metabolism in U937 macrophages was further confirmed by using CYP2A6-selective inhibitor, tryptamine, which significantly decreased cotinine (70%) and completely inhibited NNK formations. Finally, we showed that nicotine-treated macrophages increase the formation of oxidant at 30–60 min, which is consistent with the initial formation of cotinine and NNK. In conclusion, we have developed a new LCMS/MS method for concurrent determination of nicotine metabolites and analyzed the role of CYP2A6 in nicotine metabolism and oxidative stress in U937 macrophages, which may have implications in viral replication among HIV+smokers.

Keywords

Nicotine; CYP2A6; Oxidative stress; Macrophages; HIV-1; LC-MS/MS

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Introduction

Nicotine, the major constituent of tobacco, is predominantly metabolized by liver cytochrome P450 2A6 (CYP2A6) to cotinine (75%) and many other metabolites (25%) (Benowitz et al. 2009). Some of these metabolites are further metabolized by CYP2A6 into several reactive procarcinogenic compounds, including nicotine-derived nitrosamine ketone (NNK) (Hecht et al. 2000; Kamataki et al. 2002). In addition, lung CYP2A13 is involved in the metabolism of nicotine and cotinine, as well as metabolic activation of NNK (Wong et al. 2005; Bao et al. 2005). CYP2A6-mediated nicotine metabolic pathway is known to cause liver damage and pancreatic cancer (Smith et al. 1995, 1997; Kadlubar et al. 2009; Benowitz 2009), however, CYP2A13-mediated nicotine metabolic pathway is known to cause lung cancer (Smith et al. 1997; Wong et al. 2005; Benowitz 2009). Although not well understood, CYP-mediated liver damage and lung/pancreatic cancers in smokers are likely through induced oxidative stress (Smith et al. 1995; Demizu et al. 2008; Benowitz 2009). Similarly, tobacco constituents, especially nicotine and NNK have been shown to cause oxidative stress in microglia (Ghosh et al. 2009), neurons (Bhagwat et al. 1998), and white blood cells (Chuang and Hu 2006). However, the mechanism by which tobacco/nicotine increases oxidative stress in these cells is not clear.

Monocytes/macrophages are one of the important cellular targets of HIV-1 replication and also function as a critical viral reservoir (Kedzierska and Crowe 2002; Montaner et al. 2006). Furthermore, infiltration of infected macrophages into the brain results in the spreading of the virus to resident glia leading to NeuroAIDS (Aquaro et al. 2005). In view of the existing information that nicotine can damage liver and cause cancer, it becomes imperative to determine the effect of nicotine in monocytes/macrophages that are known to cross blood brain barrier and play an important role in neuroAIDS. We have recently shown that CYP2A6 is expressed >20- to 1000-fold higher than several other CYP enzymes in U937 macrophages (Jin et al. 2011). However, the role of CYP2A6 in macrophages is not known. The advantage associated with the use of U937 cell line is that it is free from complications of CYP2A6 polymorphisms, which are known to be associated with altered nicotine pharmacokinetics (Mwenifumbo and Tyndale 2007).

The present study was designed to determine whether CYP2A6 is involved in nicotine metabolism and oxidative stress in U937 macrophages. Therefore, first we needed a sensitive analytical technique to estimate the intracellular concentrations of nicotine and its metabolites. Various analytical techniques, including radio immunoassays, enzyme linked immunosorbent assay, gas chromatography, and high performance liquid chromatography (HPLC), have been reported for the analysis of nicotine and its metabolites (Davis and Curvall 1999). Over the past decade(s) fast and sensitive liquid chromatography tandem triple quadrupole linear ion Qtrap mass spectrometry (LC-MS/MS) method has been developed and improvised for measurement of nicotine and several nicotine metabolites in urine for clinical studies (Byrd et al. 1992; Byrd and Ogden 2003; Byrd et al. 2005). The limits of quantification of nicotine and its metabolites in these methods were approximately 10–20 ng/ml, which are sufficient for determination of nicotine pharmacokinetics in clinical studies. More recently, LC-MS/MS method was developed for nicotine and its metabolites in human plasma with 2.5 ng/ml lower limit of quantitation (Miller et al. 2009). To best of

our knowledge, no LC-MS/MS method has been reported to measure the intracellular concentrations of nicotine and its metabolites in nicotine-treated macrophages at physiologically relevant concentrations. In this study we have developed a simple, highly sensitive, and cost-effective LC-MS/MS method for simultaneous measurement of nicotine, cotinine, and NNK, which allowed us to determine the role of CYP2A6 in nicotine metabolism and oxidative stress in U937 macrophages.

Materials and methods

Cell culture and nicotine treatment

The U937 monocytic cell line was obtained from ATCC (Manassas, VA). U937 monocyte cells were grown in RPMI 1640 media (Sigma Aldrich, St. Louis, MO) and differentiated into macrophages by phorbol 12-myristate 13-acetate as described before (Jin et al. 2011). Nicotine treatments (1 μ M) for the measurements of nicotine metabolites and oxidant contents were performed in triplicate in 12-well plates containing 1 ml media in each well, and each experiment was independently repeated 2–3 times. Nicotine concentration (1 μ M) was selected based on initial optimization using varying concentrations (0.25–10 μ M) and monitoring the concentrations of nicotine, cotinine, and NNK. For inhibition experiment, the cells were pre-treated with 50 μ M CYP2A6-selective inhibitor, tryptamine, for 1 h followed by treatment with 1 μ M nicotine for 30 min and 1 h. Nicotine, cotinine, and NNK from U937 cells were extracted according to the following sample preparation and extraction procedure, and their intracellular concentrations were measured using newly developed highly sensitive LC-MS/MS method as described below. A 180 μ g protein was extracted from each nicotine-treated cell for the measurements of nicotine, cotinine, and NNK.

Preparation of standard stocks and spike solutions

Nicotine, cotinine, and NNK (Sigma Aldrich) stock solutions were prepared in methanol. Calibration curve and quality control dilutions were spiked in a mobile phase to obtain calibration curve standards (3.3–1028 ng/ml for nicotine and 0.3–652.6 ng/ml for cotinine and NNK) and quality control standards (3.3–858.7 ng/ml for nicotine and 10.3–625.1 ng/ml for cotinine and NNK) as previously reported (Earla et al. 2010). Ritonavir (Sigma Aldrich) was used as an internal standard to ensure reproducibility and reliability of the method. All the solutions were stored at 4° C for further analysis. Similarly, calibration curve and quality control dilutions were spiked in U937 macrophage samples in PRMI 1640 media to obtain calibration curve and quality control standards for nicotine, cotinine, and NNK, as described (Earla et al. 2010). These samples were stored at –80° C until further use.

HPLC and mass spectrometry conditions

Chromatographic separation was carried out on UFLC Shimadzu prominence system consisting of LC-20 AD liquid chromatography low pressure gradient pump, SPD-M20A diode array detector, SIL-20AST auto sampler and DGU-20As degasser (Shimadzu USA manufacturing Inc., Torrance, CA) using a reverse phase Xterra MS C18 column (50 $\times$ 4.6 mm, i.d. 0.5 μ m particle size) (Waters Corporation, Milford, MA). Isocratic mobile phase composed of 75% acetonitrile and 0.05% formic acid was run at a flow rate of 0.3 ml/min. Sample injection volume used was 15 μ l and the total analytical run time was 4 min.

LC-MS/MS (API 3200 Q TRAP instrument, Applied Biosystems/MDS Sciex, Foster City, CA), is interfaced with electrospray turbo ionization source. LC-MS/MS optimization was carried out by multiple reactions monitoring (MRM) positive ion mode based on *mass to charge* (m/z) ratio with a protonated molecular ion $[M+H]^+$. Mass spectrometry was tuned/optimized using 200 ng/ml solution of nicotine, cotinine, NNK, and ritonavir for its ionization. Precursor ions were optimized in quadrupole 1 (Q1) based on m/z by modifying parameters such as declustering potential, ion spray voltage, and source gas 1. One of the most stable abundant product ions of each precursor ions of nicotine, cotinine, NNK, and ritonavir, generated in Q2 as collision cell, were optimized. Precursor ions were fragmented to product ions by using nitrogen gas, which served as collisionally activated dissociation (CAD) and collision energy for Q3. Precursor and product ions obtained by direct infusion mode were injected at a flow rate of 5 μ l/min with a built-in Harvard infusion syringe pump. The MRM chromatogram peak resolutions were optimized by regulating desired turbo ion source temperature, adjusting the composition and proportion of mobile phase mixtures, source gas 2, and dwell time.

Extraction of nicotine, cotinine, and NNK from U937 macrophages

Nicotine-treated U937 macrophages were lysed in 1 ml of 0.1 M Hepes buffer (pH 7.4), containing 5% trichloroacetic acid. The crude lysate samples (0.2 ml) were supplemented with 25 μ l of 10 μ g/ml freshly prepared ritonavir and vortex-mixed for 30 s followed by addition of 30 μ l of 30% v/v ammonia solution and again vortex-mixed for 1 min. Nicotine, cotinine, and NNK were extracted by adding 1 ml of ethyl acetate, vortex-mixing for 2 min, and finally centrifuging at 12,000 rpm for 45 min at 4° C. Then, 850 μ l of the upper layer was transferred to fresh tubes and evaporated in the genevac DD-4X evaporator (Genevac Inc., Gardiner, NY) at 37°C for 90 min. The residue was reconstituted with 200 μ l of mobile phase, and the reconstituted samples were transferred into a HPLC autosampler vial with silanized inserts. The resulting solutions were injected into LC-MS/MS and data were collected, analyzed, and validated.

Validation of selectivity, accuracy, precision, and recovery

The validation and acceptance criteria were performed according to “Guidance for Industry Bioanalytical Method Validation, FDA, 2001” guidelines and acceptance criteria (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf>). Briefly, selectivity was performed by spiking the limit of quantitation concentration to assess the capacity of analytical method by differentiating and quantifying the analytes in the presence of other components in the sample. The accuracy was determined by replicate analysis of samples containing known amount of the analyte. The acceptance criteria for accuracy was 100 $\pm$ 15%, whereas it was 100 $\pm$ 20% for the lower limit of quantification level. The precision of an analytical method is described by the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous solution. It was measured using six determinations at every concentration range. The acceptance criteria for precision was 100 $\pm$ 15%, however, it was 100 $\pm$ 20% for the lower limit of quantification level. The recovery was performed by comparing the analytical results for extracted samples at a minimum of three concentrations (low, medium, and high) with unextracted standards. The acceptance criteria for recovery

was 100%. In addition, the extent of recovery of the analyte and IS was consistent, precise, and reproducible.

Measurements of oxidant contents

Measurements of oxidant contents were performed essentially as described previously (Jin et al. 2011). Briefly, the production of oxidant contents was measured by flow cytometry using the fluorogenic dye dichlorofluorescein diacetate (DCFDA) (Invitrogen). U937 macrophages were treated with 0 and 1 μ M nicotine for 15 min to 2 h. The cells were harvested and incubated with DCFDA in a humidified incubator at 37°C for 30 min. DCF emission (corresponding to FITC) was immediately measured at 525 $\pm$ 20 nm by flow cytometry (BD Biosciences, San Jose, CA), and mean fluorescence intensity (MFI) presented by the software was analyzed.

Statistical analysis

Statistical analysis was performed to determine mean $\pm$ SD from 3 replicates, and a student *t*-test was applied to determine *p* values. A *p* value of 0.05 was considered significant.

Results and discussion

LC-MS/MS method development

We developed and validated a highly sensitive LC-MS/MS analytical method for simultaneous quantitative determination of nicotine and its metabolites, cotinine and NNK in U937 macrophages. The mass spectrometry parameters, such as curtain and CAD gases, gas 1, and gas 2 were optimized as 20, 45 and 50 psi, respectively, whereas ion spray voltage, ionization source temperature, and dwell time were optimized as 4800 V, 400°C, and 200 milliseconds, respectively. The declustering potential for nicotine, cotinine, and NNK were fixed at 80, 40, and 40 V, respectively, while collision energy for nicotine, cotinine, and NNK were adjusted at 26, 28, and 15 V, respectively. The optimized MS/MS mass spectra for nicotine, cotinine, NNK, and ritonavir are shown in Fig. 1 (A–D). Analysis was carried out by MRM positive mode using ion mass transitions of *m/z* 163.2/130.1 for nicotine, *m/z* 177.4/98.3 for cotinine, *m/z* 208.4/122.1 for NNK, and 721.6/296.4 for ritonavir (Fig. 1 and Table 1).

The LC-MS/MS MRM chromatograms of nicotine, cotinine, NNK, and ritonavir in U937 macrophages are shown in supplemental Fig. 1. The results showed that there was no endogenous or exogenous interfering peak in the blank at the retention time of nicotine, cotinine, NNK, and ritonavir. Similarly, there was a prominent baseline peak at the lower level of quantification of all the analytes, suggesting that the signal to noise ratio is greater than 4 for all the compounds. The LC-MS/MS results from nicotine, cotinine, and NNK showed that the method is capable of quantifying these analytes at very low concentration levels (3.3 ng/ml for nicotine and 0.3 ng/ml for cotinine and NNK). Finally, our data from supplemental figure shows that the peak area response is proportional to concentrations from lower limit to upper limit of quantification for all the analytes, suggesting that the results are reproducible and robust.

The accuracy, coefficient of variation, retention times, and correlation coefficient of precision and accuracy batch of nicotine, cotinine, and NNK are presented in Table 1. The calibration curve accuracies for nicotine, cotinine, and NNK were in the range of 84–117%, 86–118%, and 90–121%, respectively. The recoveries for nicotine, cotinine, and NNK were 89.5%, 91.3%, and 94.4%, respectively. The calibration curves were linear within the concentration range of 3.3–1028.1 ng/ml for nicotine ($r=0.996$) and 0.3–625.1 ng/ml for both cotinine ($r=0.9827$) and NNK ($r=0.9952$) (Table 1). The coefficient of variation for nicotine, cotinine, and NNK was within 6.1%, 17.9%, and 20.0%, respectively, whereas their accuracy at lower level of quantification was within 17.3%, and 18.2%, and 21.2%, respectively.

In this method, ritonavir was used as an internal standard because we have earlier developed the extraction technique for protease inhibitors, including ritonavir (Kumar et al., 2010). We further optimized the extraction method of ritonavir using nicotine extraction protocol, which showed more consistent result and better recovery than the protocol used earlier for protease inhibitors (Kumar et al., 2010). Thus, we used ritonavir in this study even though it is not chemically similar to nicotine-related compounds. It is not necessary to have chemical similarity to utilize it as internal standard.

We have demonstrated a simple, rapid, and cost-effective LC-MS/MS analytical technique for concurrent determination of nicotine, cotinine, and NNK in U937 macrophages. Our analytical method is rapid and cost-effective, because we achieved the chromatographic separation on HPLC reversed phase MS C18 column, with a shorter run time of 4 min, which has the ability to measure nicotine, cotinine, and NNK simultaneously. However, other investigators used Solid Phase Extraction (SPE) procedure. This method requires 48 or 96 well plate manifold instrument, many SPE- cartridges/columns, and inert nitrogen gas for sample elution (Bao et al. 2010), which is expensive and time-consuming. Similarly our extraction procedure is simple and cost-effective, because we used two-step liquid-liquid extraction procedure using ammonium hydroxide and ethyl acetate. Other researchers have used liquid-liquid extraction method by using methylene chloride to extract nicotine and cotinine from the alkalized plasma (Curvall et al. 1982; Davis 1986). This extraction procedure resulted into emulsion formation, which is not only time consuming, but also leads to poor extraction efficiency.

Our LCMS/MS method is extremely sensitive, which can detect nicotine, cotinine, and NNK at >10-fold lower concentrations than most analytical methods used for the detection of these analytes (Byrd and Ogden 2003; Byrd et al. 2005; Miller et al. 2009; Marclay and Saugy 2010). These LC-MS/MS methods were inadequate to determine the intracellular concentrations of nicotine and its metabolites in nicotine-treated macrophages, as well as in macrophages of non-HIV-infected/HIV-infected smokers. In addition, our method showed approximately 20% deviation from the nominal concentration for precision and accuracy batches. This LC-MS/MS method follows the most rigorous Food and Drug Administration (FDA) guidelines and Good Laboratory Practice acceptance criteria for linear calibration and quality control standards for the detection of nicotine, cotinine, and NNK (FDA 2001). Therefore, our method can be used for simultaneous estimation of intracellular

concentrations of nicotine, cotinine, and NNK in all kinds of cells, and therefore, is also applicable to preclinical and clinical sample analysis.

Metabolism of nicotine in U937 macrophages

We analyzed the metabolism of nicotine in U937 macrophages by measuring its major metabolite, cotinine, and an important procarcinogenic metabolite, NNK, using highly sensitive LC-MS/MS technique as described above. Figure 2a shows representative LC-MS/MS and MRM chromatogram profiles of nicotine, cotinine, and NNK. Cotinine and NNK were initially formed at 30 min, followed by a peak at 3 h and 2–3 h, respectively (Fig. 2b). The levels of cotinine and NNK declined after 3 h, however, minimal concentrations were present throughout 6 h observation period. On the other hand, nicotine could not be detected in the cells after 30 min (data not shown). The profiles of nicotine and cotinine are consistent with the report that nicotine has very short half-life (30 min) compared to that of cotinine (2–3 h) (Benowitz et al. 2009).

The concentrations of cotinine (0.03–0.1 μM) and NNK (0.016–0.038 μM) at different time points were 10% of total concentration of nicotine used for the treatment (1 μM). The remaining nicotine may be accounted for as other metabolites. The decline in cotinine and NNK concentrations after 3 h can be attributed to the fact that both cotinine and NNK have been shown to undergo further metabolism in to other procarcinogenic and carcinogenic compounds (Hecht et al. 2000; Benowitz et al. 2009).

Role of CYP2A6 in nicotine metabolism in U937 macrophages

Although the metabolism of nicotine and subsequent formations of cotinine and NNK in U937 macrophages are likely to be through CYP2A6 pathway, in part, these can also occur through other minor CYP enzymes, such as CYP2A13, CYP2B6, CYP2D6, CYP2C9, and/or CYP2E1 (Flammang et al. 1992). We have earlier shown that CYP2A13 is not expressed in U937 macrophages and CYP2B6, CYP2C9, CYP2D6, and CYP2E1 are expressed at much lower level than CYP2A6 in these cells (Jin et al. 2011), which strongly suggests that CYP2A6 is predominantly responsible for nicotine metabolism in U937 macrophages. To ensure that CYP2A6 is involved in nicotine-metabolism, we measured cotinine and NNK in the presence of CYP2A6-selective inhibitor, tryptamine in nicotine-treated cells (Zhang et al. 2001). Tryptamine is known to inhibit CYP2A6 ($K_i=1.7 \mu\text{M}$) with 6.5- to 213-fold greater potency than other CYPs, except for CYP1A2 ($K_i=1.7 \mu\text{M}$) (Zhang et al. 2001), which is not expressed in U937-macrophages (Jin et al., 2010). Tryptamine (50 μM) decreased the formation of cotinine by 70% and 35% at 30 min and 1 h, respectively (Fig. 3). On the other hand, tryptamine completely blocked the formation of NNK at 30 min and 1 h. A relatively less inhibitory effect by tryptamine at 1 h compared to 30 min could be due to decreased concentration of tryptamine in 1 h ($\text{Tryptamine}_{1/2}=1 \text{ h}$; Lemberger et al. 1971). A complete inhibition of NNK formation by tryptamine is in line with the fact that NNK is one of the terminal metabolites of nicotine/cotinine pathway, in which CYP2A6 is involved at multiple steps (Benowitz et al. 2009).

Formation of oxidant contents in nicotine-treated U937 macrophages

Tobacco/nicotine is known to cause oxidative stress in the liver and lungs (Benowitz 2009), as well as in microglia (Ghosh et al. 2009), neurons (Bhagwat et al. 1998), and white blood cells (Chuang and Hu 2006). Therefore, we sought to measure the formation of oxidant contents as a direct indicator of oxidative stress in nicotine-treated U937 macrophages. Figure 4a shows a representative flow cytometry profile of control and nicotine-treated cells at 1 h. The results showed an increase in fluorescence intensity indicating the formation of oxidant contents in U937 cells. The data analysis showed significant increase in mean fluorescence intensity (MFI) upon nicotine treatment at 30 min (15%) and 60 min (25%) (Fig. 4b). Similar increase in oxidant contents were found in other studies, including ours, in alcohol-treated cells (Jin et al., 2010). Furthermore, the formation of oxidant contents was consistent with the initial formation of cotinine and NNK (Fig. 2b vs. Fig. 4b). To rule out the possibility that nicotine, cotinine, and NNK do not oxidize DCFDA, we incubated nicotine, cotinine, and NNK, with DCFDA, which did not increase MFI. Our results are consistent with earlier observations that nicotine increases oxidative stress in macrophages (Mahapatra et al. 2009). Taken together, the results suggest that oxidant contents are formed as a result of nicotine metabolism in U937 macrophages. Nicotine metabolism-mediated oxidative stress can occur as a result of formation of reactive metabolites of nicotine metabolic pathway and/or production of oxidants (e.g. peroxides) through CYP-mediated reaction (Yildiz et al. 1998; Benowitz 2009; Benowitz et al. 2009).

Recently, it has been shown that mouse CYP2A5 (a counterpart of human CYP2A6) is induced by oxidative stress generated by CYP2E1-mediated alcohol metabolism in hepatocytes (Lu et al. 2011). In independent studies, oxidative stress-mediated induction of CYP2A5/2A6 has been shown to be through the induction of Nuclear factor-erythroid 2 p45-related factor 2 (Nrf-2) (Lämsä et al. 2010; Yokota et al. 2011). Consistent with this, we have also shown that CYP2A6 and Nrf-2 are simultaneously induced in chronic alcohol- and nicotine-treated U937 macrophages (unpublished observations). Taken together, we hypothesize that CYP2A6-mediated nicotine metabolism increases oxidative stress, which induces CYP2A6 and in turn enhances the metabolism of nicotine and oxidative stress through Nrf-2 pathway in macrophages.

Tobacco/nicotine has been shown to enhance HIV-1 replication in alveolar macrophages (Abbud et al. 1995) and microglia (Rock et al. 2008). However, the mechanism by which smoking or nicotine increases HIV-1 replication is unknown, except a report that suggests the role of iron and oxidative stress (Boelaert et al. 1996a). In independent studies, in-vitro HIV infection has been shown to be associated with iron-mediated oxidative stress, which is likely to contribute to viral cytopathogenicity (Boelaert et al. 1996b; Savarino et al. 1999). Furthermore, the interaction between iron and HIV may be reciprocal, since viruses with a life-cycle involving a DNA phase require chelatable iron for optimum replication (Savarino et al. 1999). Our findings along with the report from Boelaert et al. strongly suggests that CYP2A6-mediated nicotine metabolic pathway induces oxidative stress in U937 macrophages, which may be responsible for increased HIV-1 replication in smokers. This is consistent with another independent finding that tobacco use has been shown to increase the

risk factor of HIV infection, and nicotine has been shown to affect HIV-1 interaction with macrophages (Sopori and Kozak 1998).

HIV-1-infected individuals who smoke show decreased immune response, poorer responses to antiretroviral therapy (ART), and greater risk of virological rebound, compared to HIV-1-infected non-smokers (Feldman et al. 2006). It is therefore possible, at least in part, that CYP2A6-mediated oxidative stress followed by increased HIV-1 replication may be responsible for these effects. In the U.S., the prevalence of cigarette smoking in the HIV-1-infected population is three times higher than general population (Burkhalter et al. 2005; CDC 2005), which further increases the risk of HIV-1 infection, progression, and AIDS development and decreases the effects of ARTs. Therefore, studies to further examine the role of CYP2A6 pathway in oxidative stress and HIV-1 replication in HIV-1-infected smokers are highly desirable. Furthermore, it also offers a possible therapeutic target that will be expected to block nicotine-mediated oxidative stress, which may in-turn decrease HIV-1 replication and increase response to ARTs. This strategy is also being pursued in other scenario where CYP2A6 inhibitors are being explored as a means to treat nicotine dependence (Sellers et al. 2003) and to prevent cancer among smokers (von Weymarn et al. 2006).

Conclusions

In conclusions, we have developed a highly sensitive, cost-effective, and robust LC-MS/MS analytical technique for concurrent determination of nicotine, cotinine, and NNK in macrophages. In addition to measuring nicotine, cotinine, and NNK in U937 macrophages, this method may also be useful for simultaneous quantitation of intracellular concentrations of these analytes in preclinical and clinical samples. Using this method, we have demonstrated that CYP2A6 is involved in nicotine metabolism in U937 macrophages, leading to oxidative stress. These findings are very important in view of earlier reports that tobacco/nicotine is known to increase viral replication in HIV-1-infected patients (Abbud et al. 1995; Rock et al. 2008), perhaps through oxidative stress pathway (Boelaert et al. 1996a; 1996b; Savarino et al. 1999). Our future study using smokers/HIV-1-infected samples will further help examining the role of CYP2A6 in nicotine-mediated HIV-1 replication, which eventually may lead to a novel therapeutic intervention in HIV-1-infected smokers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Coupled mass spectra in positive scan mode for **a.** nicotine, **b.** cotinine, **c.** NNK, and **c.** ritonavir at 200 ng/ml in methanol. Structures, precursors, and its product ion fragmentation patterns for nicotine, cotinine, NNK, and ritonavir are shown. The intensity (cps, counts per second) is presented in Y-axis and m/z is presented in X-axis. The data is representative of more than ten independent determinations

Fig. 2.

Nicotine metabolism in U937 macrophages. The U937 macrophages were incubated with 1 μM nicotine from 30 min to 6 h and cotinine and NNK were determined using LC-MS/MS.

a. Representative MRM profiles of nicotine and its metabolites cotinine and NNK. **b.** Kinetic profiles of the formation of cotinine and NNK. The nicotine treatment times (h) are presented in X-axis, while concentration of cotinine and NNK are presented in Y-axis. The graphs were plotted as mean±SD from three replicates. The experiment was repeated two times

Fig. 3.

Effect of CYP2A6-selective inhibitor tryptamine on the formation of cotinine and NNK in nicotine-treated U937 macrophages. The percent of cotinine and NNK formations are presented in Y-axis. The X-axis represents the time points of nicotine treatment. The graphs were plotted as mean $\pm$ SD from four replicates. The experiment was repeated two times. The significant differences ($p < 0.05$) between control and tryptamine-treated cells are represented (*). The significance was determined using a t -test

Fig. 4.

Effect of nicotine on CYP2A6-mediated formation of oxidant contents in U937 macrophages. **a.** Representative figures of the formation of oxidant contents at 1 h nicotine treatment. The events (cell population) are presented in Y-axis and relative fluorescence intensity is presented in X-axis. **b.** Bar graphs of mean fluorescence intensity (MFI). The percent of MFI is presented in Y-axis. The formation of oxidant contents was measured using flow cytometer from control and nicotine-treated cells from 15 min—2 h. * Represents $p < 0.05$ compared to controls. The mean $\pm$ SD was calculated from four replicates and significance was determined using a t -test. The experiment was repeated three times

Table 1
Nicotine and its metabolites average calibration curve standards (CC, $n=4$), quantity control standards (QC, $n=6$), average retention times (for CC, $n=4$; QC, $n=6$), and correlation coefficient (r , $n=4$)

	Nominal (ng/ml)	Nicotine Calculated (ng/ml)	Accuracy (%)	Cotinine Calculated (ng/ml)	Accuracy (%)	NNK Calculated (ng/ml)	Accuracy (%)
CC-1	0.33			0.39±0.07	118.2	0.38±0.08	121.2
CC-2	3.3	2.8±0.1	84.8	2.8±0.10	84.8	3.3±0.81	100.9
CC-3	9.8	11.5±0.7	117.3	11.0±1.4	112.2	11.6±0.6	117.6
CC-4	28.9	33.2±1.4	114.9	32.2±2.8	111.4	33.2±1.4	115.0
CC-5	96.3	96.4±1.4	100.1	98.9±5.0	102.7	110.4±21.2	114.7
CC-6	240.7	213.5±7.1	88.7	217.0±12.0	90.2	217.0±12.0	90.2
CC-7	437.6	439.7±7.1	100.5	459.7±35.3	105.1	464.7±42.4	106.2
CC-8	652.6	581.2±99.0	89.1	676.7±34.1	103.7	676.7±34.1	103.7
CC-9	822.5	728.3±4.2	88.5				
CC-10	1028.1	1046±64	101.7				
QC-1	3.3			2.9±0.06	87.9	3.9±0.05	118.2
QC-2	10.3	12.2±2.6	118.4	11.7±1.9	113.6	12.2±2.6	118.4
QC-3	100.5	97.9±3.6	97.4	100.4±0.1	99.9	104.9±6.3	104.4
QC-5	251.2	234.9±23.1	93.5	240.9±14.6	95.9	235.9±21.7	93.9
QC-6	456.8	470.8±19.7	103.1	476.3±27.5	104.3	476.3±27.5	104.3
QC-7	625.1	646.3±4.9	103.4	641.3±12.0	102.6	656.3±9.2	105.0
QC-8	858.7	792.0±94.3	92.2				
Retention time (min)			1.58	1.77		2.75	
Correlation Coefficient (r)			0.99	0.9827		0.9952	
Linear equation (y)= $mx+c$			0.032x+25.954	0.0334x+24.588		0.0003x-12.85	
Precursor ion (Q1) : m/z [M+H] ⁺			163.2	177.4		208.4	
Product ion (Q3) : m/z [M+H] ⁺			130.1	98.3		122.1	
MRM transitions (Q1/Q3)+mode			163.2/130.1	177.4/98.3		208.4/122.1	

Standard error is represented as $\pm$ m and c stands for slope and intercept, respectively, for linear equation. Lower limit of quantitation for nicotine, cotinine, and NNK are 3.27 ng/ml, 0.30 ng/ml, and 0.30 ng/ml, respectively. Upper limit of quantitation for nicotine, cotinine, and NNK are 1028.1 ng/ml, 652.6 ng/ml, and 625.1 ng/ml, respectively. Coefficient of variation for nicotine, cotinine, and NNK are 6.1%, 17.9%, and 20%, respectively. The mean $\pm$ SD was calculated from three replicates and significance was determined using a t -test.