**INTRODUCTION**

Cigarette smoking is the leading cause of preventable death in the United States. The Centers for Disease Control and Prevention estimated that cigarette smoking produces 430,000 deaths each year in 2000-2004 and generated an estimated $157 billion in annual health-related economic losses. A recent national survey by the Substances Abuse and Mental Health Services Administration in 2007 estimated that 60 million people or 24% of the US adult population aged 12 or older had smoked cigarettes in the past month, highlighting the widespread use of nicotine.

Although the prototypical cigarette smoker is characterized as someone who consistently smokes at least 1 pack of cigarettes per day for a long period of time, a large number of current smokers neither smoke daily nor consume 1 pack of cigarettes on days when they do smoke. These “low-volume” irregular smokers pose an increased health risk as a consequence of the inhalation of tobacco smoke including increased rates of coronary heart disease, myocardial infarction and lung cancer compared to non-smokers. The transition from “low-volume” to nicotine dependence has been documented however little is known about the processes that underlie changes in smoking behavior from the first use of a cigarette, subsequent regular use and eventual addition.

The relationship between cigarette smoking, exposure to ETS and serious health conditions has resulted in the need for analytical methods for the determination of nicotine and metabolites in biological samples. This current research is focused on the investigation of biomarkers of nicotine use in the oral fluid of “low-volume” smokers.

**AIM**

There were several study objectives: (1) To develop and validate a sensitive and specific liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of nicotine-N-glucuronide (NIC GLUC), cotinine-N-glucuronide (COT GLUC), nicotine (NIC), cotinine (COT), N-nicotine (NNIC), cotinine-N-oxide (NCOT), anatabine (AB) and anatabine-N-oxide (ANAB) in human oral fluid collected as part of a clinical study investigating biomarkers of low-level smoking; and (2) To obtain preliminary data on the use of the COT microplate enzyme-linked immunosorbent assay (ELISA) as a screening tool in the analysis of oral fluid collected from low-level smokers.

**HUMAN SUBJECTS**

Clinical samples were collected as part of an Institutional Review Board approved study (IRB #21414, University of Utah). One milliliter of oral fluid was obtained from three non-smoker humans using the Quantisol™ collection device (Immunocap Corporation, CA) prior to the application of a 7.7 mg transdermal nicotine patch (Novartis, Basel, Switzerland) and then at 0.5 and 3.75 hours following patch removal. The patch was worn for four hours to mimic the plasma nicotine concentration observed after smoking 1 cigarette. Anatabine and anatabine-N-oxide were included in the method to monitor monitoring of unextracted tobacco use by participants during the study since these two alkaloids are derived from the leaves of the tobacco plant.

**SPE METHOD**

Oasis HLB® combined with Oasis MCX® SPE cartridges

**Conditioning**: (1) 2 mL of methanol (v/v) (2) 2 mL of 10 % aqueous trifluoroacetic acid (TFA)

**Wash**: None

**Elution**: 1 mL 5 % MeOH/50 mM HAc in MeOH/ACN (v/v)

**LC-MS/MS CONDITIONS**

20 μL of oral fluid Quantisol™ buffer homogenate (1:4, v/v) were added to the LC microplate ELISA wells in duplicate using a Minispot 75 automatic pipet system (Tecan Group Ltd, Männedorf, Switzerland). 100 μL of a LC-MS grade acetonitrile reagent was subsequently added and the plate left in the dark at room temperature for 30 minutes. After the incubation, the microplate Wells were washed with deionized water (15 x 300 μL) in order to remove any unbound sample or residual enzyme conjugate reagent that might have been present in the wells. The plate was then washed 10 times with 0.5 % acetic acid/then 10 times with 0.5 % acetic acid/then 10 times with 0.5 % acetic acid/then 10 times with 0.5 % acetic acid.

The ELISA dose response curve was generated by comparing the absorbance value for fortified analyte-free oral fluidQuantisol™ homogenate (B) with the analyte-free oral fluidQuantisol™ homogenate (A) over the range 0-500 ng/mL.COT. Intraday imprecision within the same batch in (B) was calculated for all analytes in the LC-MS/MS method at a concentration of 50 ng/mL, relative to the COT dose response curve. A cut-off concentration of 10 ng/mL COT was selected for the screening of clinical samples.

**LC-MS/MS METHOD VALIDATION RESULTS**

The ELISA dose response curve was linear, for COT, with an R² value of ≥ 0.99 over the range 5-100 ng/mL. (Figure 2). Intra-day and inter-day imprecision were < 3 % for a chosen assay cut-off concentration of 10 ng/mL COT. Analytes which were targeted in the LC-MS/MS method were tested for cross-reactivity with the COT microplate ELISA and did not cross-react, with the exception of 3-HC which cross-reacted by 80 % at a COT concentration of 50 ng/mL.

**CONCLUSION**

A sensitive and specific LC-MS/MS and ELISA method have been successfully applied for the quantification and detection of nicotine and nicotine metabolites in oral fluid samples collected after application of a transdermal nicotine patch which had been worn for four hours as part of a clinical study investigating nicotine biomarkers in low-smokers.