

2. Abstract and Specific Aims

This study will provide an accessible and affordable means for future monitoring of tobacco use in Alaska and to determine the exacerbating effects of tobacco use on Vitamin D deficiency. Currently, there is no low-cost method available in Alaska for detecting nicotine levels in humans and tobacco and cigarette use in the State is common and particularly high among Alaska Natives. In fact, according to the National Health Interview Survey, the rate of cigarette smoking among Alaska Natives is almost twice higher than in the general US population. Alaska Natives have high rates of tobacco-related deaths and lung cancer remains to be one of the leading causes of all cancer-related deaths especially in rural regions of Alaska. Residents of Northern climates have been found to have lower levels of Vitamin D than populations in southern or mid latitudes. Vitamin D deficiency has been linked to many forms of cancer, osteoporosis, diabetes, autoimmune diseases and heart disease. This second phase of this study will determine whether nicotine contributes to Vitamin D deficiency in Alaskans.

This study has two main aims: the first is to establish a mass spectrometry protocol for detecting nicotine and its metabolites such as cotinine in human blood and urine samples for future use in practices relevant to human health such as: monitoring smoking cessation efforts, detecting cigarette exposure in children and fetuses of smoking pregnant women, developing a tobacco control plan and also encouraging the smoking part of the population to reduce cigarette and tobacco use. The second aim is to determine whether nicotine and its metabolites contribute to Vitamin D deficiency in tobacco users.

3. Introduction

a) Statement of Research Questions:

- 1) Is it possible to develop a low cost mass spectrometry protocol for use in Alaska to detect the presence of nicotine and its metabolites in human blood and urine?
- 2) Do tobacco users have lower levels of Vitamin D than non-tobacco users?

b) Historical discussion of impacts

Lung cancer is a primary disease of cigarette smokers, with estimated smoking-attributable mortalities in excess of 80% among both men and women. Despite the well-known health risks from smoking and its consequences, people in Alaska and especially in western Alaska still use cigarettes as well as even more popular smokeless tobacco (ST), called Iqmik, which appears to be highly prevalent, particularly among women. (Renner et al. 2005). Iqmik consists of leaf tobacco mixed with ash derived from burning punk fungus, mistakenly considered to be safer to use than cigarettes during pregnancy by some regional residents which poses a certain risk for birth outcomes (Hurt et al. 2005). Currently, analyses of nicotine and cotinine levels in the blood and urine of Alaskans are performed at high cost by out-of-state labs. A locally available, low-cost protocol for detecting nicotine and its metabolites such as cotinine will assist efforts in Alaska aimed at reducing tobacco use and in detecting passive smoke exposure in non-users of tobacco products.

Residents of Northern climates have been found to have lower levels of Vitamin D than Southern or mid-latitude populations (Gilman et al. 2006, Huotari and Herzig 2008, Mohr et al. 2008). It has been well-established that Vitamin D is essential to calcium absorption in humans. Insufficient levels of Vitamin D may lead to bone thinning, rickets and osteoporosis among other ailments (Binkley et al. 2007, Huotari and Herzig 2008, National Institute of Health 2008). Vitamin D deficiency has also been linked to diabetes, several forms of cancer, and increased risk of cardiovascular disease (Brot et al. 1999; Binkley et al. 2007; Garland et al. 2007; Wang et al. 2008). Nicotine has been shown to inhibit Vitamin D metabolism (Brot et al. 1999). Given the high level of tobacco use in Alaska, nicotine may exacerbate an already serious health problem: Vitamin D deficiency. If a direct correlation can be shown between tobacco use and low levels of Vitamin D, then dietary Vitamin D supplementation may become an addition to tobacco use cessation programs or advised for those subject to passive tobacco smoke on a regular basis.

c) State of knowledge

In recent years, much has been written about Vitamin D deficiency in humans, its causes and effects. What had been a topic of little interest to the health community has become a subject of great interest in scientific writings and in the popular press. While articles have addressed Vitamin D deficiency in Northern climates (Gilman et al. 2006, Huotari and Herzig 2008, Mohr et al. 2008), no studies could be found that focused on the Alaska population. It is the intention of this study to serve as a first step to further work on Vitamin D deficiency in the Alaska populace.

4. Research Design

A research proposal will be prepared and submitted to the UAA Institutional Research Board (IRB). Included in this proposal will be an IRB approved participant consent form.

Participants: A total of 40 participants of age 19 years and above will be recruited through posted advertisements on the University of Alaska, Anchorage campus. The participants will be selected according to their claim of being a current smoker or never smoker. The subjects will be divided in two groups. The first group will consist of 20 current smokers and the second will consist of 20 never smokers. Efforts will be made to ensure sample groups reflect the student demographics on the UAA campus.

Sampling

Blood (10 ml) for the serum will be collected by venipuncture. Urine will be collected as a single-void specimen (30 ml). Both collections will be done in conjunction with health-care professionals through the UAA Medical Technology Laboratory.

Materials and Methods

1. Establishing protocol for detecting nicotine and its metabolites [similar to that described by Moyer et al. (2002)]

Reagents: Nicotine, nicotine-d3, cotinine, cotinine-d3, anabasine, nornicotine, formic acid ammonium salt, acetonitrile, ammonium hydroxide, methanol.

Other materials: Clean Screen extraction columns, PromChrom Technologies Polyhydroxyethyl A HPLC columns, urine specimen cups, BD vacutainer tubes, glass tubes, and bovine calf serum

Calibrators and controls: Stock reference solutions of nicotine, nicotine-d3, cotinine, cotinine-d3, anabasine, and nornicotine, will be prepared in methanol. These reagents will be prepared and stored at -20 °C in sealed plastic vials. A working internal standard will be prepared in type I water to contain 2 mg/L each of nicotine-d3 and cotinine-d3.

Working serum-based calibrators will be prepared to contain four increasing concentrations of nicotine and cotinine. Serum control pools will be prepared in bovine serum. Working urine-based calibrators will be prepared to contain four increasing concentrations of nicotine, cotinine, anabasine, and nornicotine. Urine control pools will be prepared to contain typical physiologic concentrations of these compounds in urine from a volunteer not exposed to tobacco or nicotine products.

Sample Preparation: Samples will be processed following the protocol of Moyer et al. (2002). Calibrator, control, or patient sample (1 mL), 0.1 mL of working internal standard, and 4 mL of 0.1 mol/L acetic acid will be mixed in a glass test tube for 2 s. Extraction columns will be prepared by washing with 4 mL of methanol followed by 4 mL of 0.1 mol/L acetic acid, taking care not to dry the columns. Samples will be poured into the columns and drawn slowly through the bed. The columns will be washed with 4 mL of 0.1 mol/L acetic acid followed by 4 mL of methanol. Analytes will be eluted into glass test tubes with 4 mL of freshly prepared 20 mL/L ammonium hydroxide in methanol. Samples will be evaporated to dryness at 30 °C with nitrogen. Samples will be reconstituted in 0.2 mL of acetonitrile for liquid chromatography and tandem mass spectroscopy analysis (LC-MS/MS).

Column Separation

Separation will be achieved with a Polyhydroxyethyl A HPLC column; 5- μ m bead size; 100-Å pore size appropriate to the new ASET Lab Agilent 1200 Series HPLC and 6410 Triple

Quadrupole LC-MS/MS. The mobile phase will consist of 200 mL of type 1 water, 1800 mL of acetonitrile, 1.3 g of ammonium formate, and 15 mL of formic acid (88%).

LC-MS/MS

Sample separation and analysis will be performed using the new ASET Lab Agilent LC-MS/MS. Detection of ions at the electron multiplier will be in two periods using the positive ion mode.

Quantification

The concentration of each analyte will be determined by calculating the ratio of each analyte ion-pair peak area relative to its respective internal standard peak area. Each analytical batch will be calibrated by performing analysis of the calibrators outlined in the **Calibrators and controls** section above; results from calibrator analysis will be used to create a calibration curve using simple linear regression analysis. The slopes and intercepts from the resulting calibration equations will be used to calculate control and specimen results.

Recovery, Limit of Quantification, and Linearity Studies

Sample recovery will be evaluated by adding each control analyte into human serum or urine at concentrations of 5, 50, 100 and 1000 µg/L. Three replicates of each will be processed as described above in **Sample preparation**.

The limit of quantification will be evaluated by adding the analytes of interest to human serum or urine collected from a non-tobacco user. The serum or urine will be tested before the addition, using the procedure outlined in this section to demonstrate that the ion-pair signal at the retention time unique to any of the analytes will be no different from background, indicating the specimens are tobacco free.

Linearity will be evaluated by analyzing tobacco-free human serum or urine to which control analytes have been added.

2. Measuring 25-Hydroxyvitamin D

This study will employ the testing protocol recommended by the kit supplier, Immunodiagnostic Systems PLC (2008), and summarized below.

Kit Materials

The 25-Hydroxyvitamin D EIA test includes lyophilized calibrators and controls (buffered human serum containing 25-hydroxyvitamin D and <1% sodium azide (0.09% reconstituted), and 25-D Biotin concentrate that will be reconstituted according to procedures set out in kit instructions. The kit also includes a wash solution (phosphate buffered saline), an enzyme conjugate (phosphate buffered saline containing avidin, peroxidase, protein, enzyme stabilizers and preservative), buffer (proprietary reagent for dissociating 25-hydroxyvitamin D from binding proteins), substrate (aqueous formulation of TMB and hydrogen peroxide) and stop solution (0.5M hydrochloric acid). Other materials in the kit include a microplate coated with 25-hydroxyvitamin D sheep polyclonal antibody. The microplate contains 12 x 8 strip wells. Adhesive plate sealers are also included in the kit.

Assay Procedure

Calibrator, control and sample (25 µL) will be diluted with 25-D Biotin Solution (1 ml). Each diluted calibrator, control and sample (200 µL) will be added to the appropriate wells of the microplate in duplicate. The microplate will be covered and incubated at 18-25°C for 2 hours. After incubation, all wells will be washed three times with wash solution, and enzyme conjugate (200 µL) will be added to all wells. The microplate will be covered and incubated at 18-25°C for 30 minutes. Following the incubation, the microplate wash step will be repeated. After washing,

TMB substrate (200 μ L) will be added to all wells and the plate incubated at 18-25°C for 30 minutes. The reaction is stop by adding stop solution (100 μ L) to all wells. The absorbance of each well will be measured at 450 nm (reference 650 nm) using a microplate reader within 30 minutes of adding the Stop Solution.

Calibration

25-OH D Calibrators are standardized using U.V. quantification.

Quality Control

Control samples at several analyte levels will be used to ensure validity of results. The controls will be tested as unknowns. Quality Control charts will be maintained to follow the assay performance.

Calculation of Results

The percent binding (B/Bo%) of each calibrator, control and unknown sample will be calculated as follows:

$$B/Bo\% = \frac{\text{(mean absorbance)}}{\text{(mean absorbance for '0' calibrator)}} \times 100$$

A calibration curve will be prepared using SoftMax Pro software by plotting B/Bo% on the ordinate against concentration of 25-hydroxyvitamin D on the abscissa. B/Bo% for each unknown sample will be calculated and concentrations will be reported in nmol/L (nM).

5. Anticipated Results

The anticipated results include the development of a local testing protocol to determine nicotine levels in humans and, using this method, that blood and urine samples from smokers will exhibit high levels of nicotine and cotinine and there will be no, or low, peaks in the samples of never smokers. The expected results for the 25-hydroxyvitamin D assay portion of the project are that most samples taken will demonstrate Vitamin D deficiency with smokers exhibiting the highest deficiencies in the study's population.

6. Project Budget

ITEM	QTY	UNIT PRICE	SHIPPING	Ext. Price
1) Reagents & Supplies				
Chemicals				\$1,042.20
Disposables				\$684.88
Vitamin D Test Kit	3	\$565	\$195	\$1,890.00
2) Gift Cards for Sample Groups	40	\$20		\$800.00
BUDGET TOTAL				\$4417.08

Budget Justification

1) The reagents and supplies are the minimum supplies necessary to establish the mass spectrometry protocol and to assay Vitamin D levels in the test population. The disposables will include some items not included in the Vitamin D test kit such as pipettes and glass test tubes.

2) The gift cards will be an inducement for participation in the study.

7. Project References

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8. Project Timeline

- a) January 31, 2009 Complete background research
- b) January 31, 2009 Order Equipment & materials
(first budget expenditure)
- c) February 28, 2009 Finalize Mass Spectrometry Protocol and complete selection of sample groups
- d) May 30, 2009 Complete sample collection & lab work
- e) October 31, 2009 Complete analysis **(final budget expenditure)**
- f) December 31, 2009 Final Report Deadline
- g) April, 2010 Undergraduate Research Symposium Presentation