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# The Addition of Cotinine to an Existing HPLC/MSMS Method to Detect Eleven Over The Counter Medications and Nicotine Exposure In Human Urine

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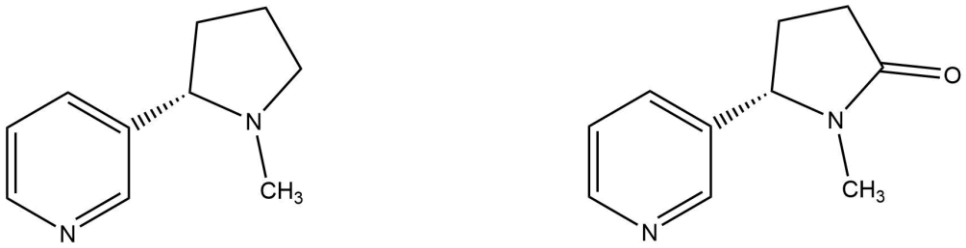
## Abstract

Cotinine, a metabolite of nicotine, serves as an effective biomarker for nicotine use and exposure at low levels (< 1 ppb). Nicotine exposure is ubiquitous and can occur through second and even third-hand sources. Several studies have investigated nicotine and its metabolites potential role on uridine 5'-diphospho-glucuronosyltransferase activity, also known as UGT's, but there has been no conclusive evidence that these compounds regulate UGT activity in humans. UGT's are a class of enzymes which catalyze the transfer of glucuronic acid to hydrophobic molecules. These enzymes are responsible for the excretion of several drugs, pollutants, endobiotic and xenobiotic compounds such as phthalate diesters. The purpose of this project is to develop an HPLC/MSMS method for cotinine using a previously existing HPLC/MSMS method. This method separates, detects and quantifies eleven over-the-counter medications in human urine samples. By adding cotinine to this existing method, human urine samples can be analyzed for the presence of compounds that could regulate UGT activity. In addition, this method allows scientists to distinguish nicotine users from non-users, as well as distinguishing first-hand and second-hand exposure. This method can analyze eleven common over-the-counter medications as well as cotinine in various matrices such as urine samples. This information allows scientists to look for potential correlations between OTC medication use, tobacco use or second-hand exposure and UGT activity.

## 1. Introduction

Nicotine exposure can occur through inhalation, ingestion, skin contact and through the mucous membranes. While smoking is the primary source for nicotine exposure in the general population, exposure can occur through first, second and third hand sources. Nicotine exposure is pervasive and can occur through exposure to second-hand smoke, residual nicotine content or consumption of several nicotine containing plants, including potatoes, tomatoes, cabbage and eggplant[1]. Mean air concentrations of nicotine in public spaces that permitted smoking range from 0.3 to 30 $\mu g/m^3$. Home air concentrations of nicotine where there is one or more smoker range from 2 to 14 $\mu g/m^{2,3}$.

Cigarettes contain approximately 1.5% nicotine by weight, producing about 1-2 mg of bioavailable nicotine per cigarette.[3] Other common routes of exposure include oral snuff, chewing tobacco and nicotine gum which deliver approximately 3.6, 4.5, and 2.0 mg of bioavailable nicotine respectively[4]. Cotinine has a much higher presence in the blood of smokers and nonsmokers than that of nicotine[4]. Cotinine and 3-hydroxycotinine are minor alkaloids found in tobacco plants and major metabolites of nicotine. Nicotine blood plasma levels range from 10-50 ng/mL in smokers, while cotinine plasma levels range from 250-300 ng/mL[4].




**Nicotine** **Cotinine**

Figure 1: Structures of Nicotine and Cotinine

Nicotine has a pharmacological half-life of 2-3 hours upon absorption. Cotinine, the primary metabolite of nicotine, has a much larger half-life of 15-19 hours.[4] Upon absorption, nicotine enters the bloodstream. Nicotine is then distributed to body tissues, primarily the liver, kidney, spleen and lungs. Nicotine also binds to brain tissues with a high affinity. The slow release of nicotine from these tissues result in a longer half-life of cotinine from administered nicotine in comparison to the half-life of cotinine from administered cotinine[4].

Cotinine's large pharmacological half-life coupled with its high blood plasma, urine and salivary levels make it an excellent biomarker for probing nicotine exposure. Cotinine can be detected in serum, urine, saliva, and hair samples. It is well known what concentrations of cotinine in various matrices distinguishes tobacco users from non-users, however, optimal cutoff points for urinary concentrations of cotinine have shown a lack of standardization. A review of these cutoff points and their derivations shows a urinary cutoff point for smoker status at 50-200 ng/mL[5]. Cotinine's use as a biomarker has several applications. It allows scientists to differentiate whether an individual is a smoker or a nonsmoker. Concentrations of cotinine can be used to help identify the route and medium of exposure. It also provides information on how much nicotine an individual has absorbed. Cotinine's use as a biomarker to measure nicotine exposure in human urine samples via HPLC/MSMS can give deeper insight on how lifestyle choices such as smoking or secondhand exposure can affect metabolic processes such as glucuronidation[6].

Glucuronidation is a two-phase metabolic process responsible for the metabolic conversion and clearance of several drugs and xenobiotics in humans including nicotine, phthalate diesters, and acetaminophen. Glucuronidation proceeds via uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes. These enzymes catalyze the transfer of glucuronic acid to small hydrophobic molecules, increasing their polarity. This increase in polarity results in clearance from the body, commonly in the form of urinary excretion. They are among the most important phase II metabolic enzymes[7].

Alterations in metabolic processes involved in drug clearance can have severe toxicological consequences[7]. Diester phthalates are common endocrine disruptors that are used in plastics such as polyvinyl chloride (PVC) and selective commercially available cosmetic products such as perfumes and body washes. Phthalates are metabolized in a two-phase process in the human body via UGT enzymes. Phase I biotransformation oxidizes phthalate diesters, transforming them in phthalate monoester. Phase II metabolism proceeds via UGT enzymes, which transfer a glucuronic acid onto the carboxyl group of the phthalate monoester. Phthalate monoesters are the bioactive form of phthalates in humans and have adverse effects on male reproductive development[8]. Alterations to glucuronic activity, such as inhibition of UGT enzymes, results in an increased half-life for phthalate monoester in the body. In 2001, it was discovered that several over-the-counter medications and natural organic compounds that are found in human foodstuffs inhibit UGT enzymes[7].

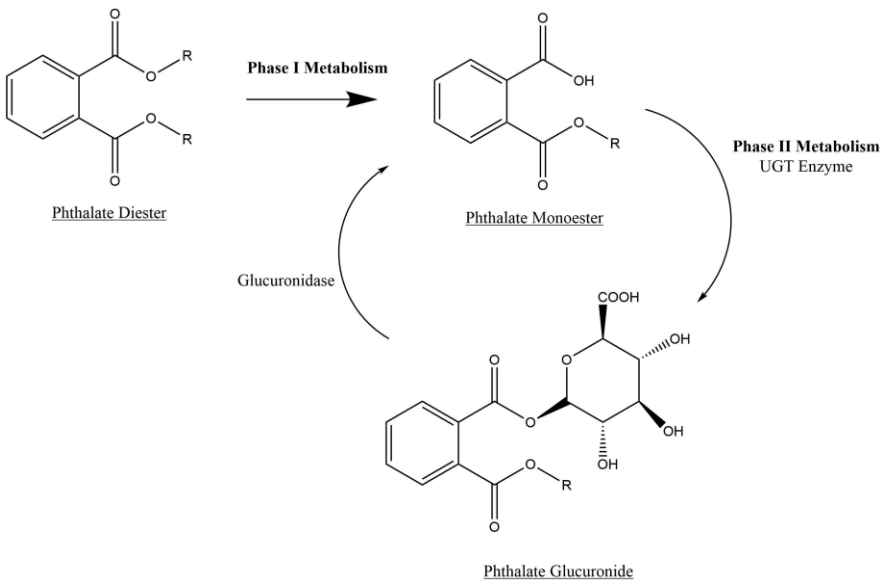



Figure 2: Metabolic Pathway of Diester Phthalates

In humans, 70-80% of absorbed nicotine is metabolized into cotinine, which occurs via CYP2A6 and cytoplasmic aldehyde oxidase. Unchanged cotinine accounts for just a small degree (10-15%) of nicotine metabolites in human urine, while the remainder is composed of primary metabolites including *trans*-3'-hydroxycotinine (33-40%), cotinine glucuronide (12-17%), and *trans*-3'-hydroxycotinine glucuronide (7-9%)[4]. Nicotine and cotinine metabolism vary amongst sex and ethnicities. Women have a higher metabolism of cotinine and nicotine than men, and women who take oral contraceptives metabolize these compounds quicker than women who do not take oral contraceptives[9]. Metabolism of cotinine proceeds more slowly in non-Hispanic black subjects than non-Hispanic white subjects[9].

Tobacco smoking has been implicated in affecting drug clearing enzymes Cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) enzymes. Mice exposed to cigarette smoke from ages 4 to 30 days showed induced several CYP enzymes in the lung and kidney, while induction glucuronidation activity was enhanced in the liver and lung. Polycyclic aromatic hydrocarbons are believed to be responsible for the induction of CYP enzymes. Nicotine is also a proven regulator of UDP-glucuronosyltransferase in humanized *UGT1* mice brains[10].

Prior research has been conducted in Dr. Brock's toxicology lab that relates to UGT inhibition and its effects phthalate metabolism in humans. Unpublished research performed by Dr. Brock's research lab investigated several OTC medications interactions with UGT isozymes using in *Vitro* enzyme assays. The ability to detect and quantify common over-the-counter medications in matrices like human urine allows for a more direct approach at monitoring UGT inhibition. Urine samples with known glucuronic activity can be analyzed for these compounds which will allow data to be extrapolated in regard to whether or not any one of these compounds could impact the glucuronic pathway. One point of interest is pregnant women, whose fetus in *Utero* could experience the adverse effects of an inhibited glucuronic pathway. The role of UGT enzymes in the clearance of pervasive xenobiotic and anti-androgenic compounds such as phthalates is well documented. Through inhibition of UGT enzymes, the resulting increase in biological half-life for phthalates results in prolonged fetal exposure to the anti-androgenic form of phthalates. A male fetus that has prolonged exposure to these monoester phthalates is at higher risk of possessing reproductive defects such as reduced anogenital distance, decreased testicular size and delayed testicular descent[11]. However, while it is well documented that tobacco use and secondhand exposure is harmful to a fetus in *Utero*, the use of common over-the-counter medications and their implications on glucuronic activity is unknown. Michael Way developed a method to separate, detect and quantify concentrations of 11 over-the-counter medications in human urine samples[12]. This method allows scientists to gain insight on the possible implications of OTC medication administration and their effects on glucuronidation.

This experiment adds cotinine to an existing HPLC/MSMS method that detects and quantifies eleven common over-the-counter medications. Use of this method serves several purposes, it can detect and quantify common over-the-counter medications in various matrices including human urine. Use of OTC medications is unregulated and their implications from a toxicological standpoint requires a method that can detect and quantify use as opposed to relying upon surveyed data. Through the addition of cotinine, insight on lifestyle choices can be understood. In particular, the ability to distinguish smokers from nonsmokers is useful. Gathering this data from urine samples can allow scientists

to extrapolate gathered data and gain a better understanding of these compounds' interactions with glucuronidation. The toxicological implications of this data provide insight into the possible inhibition of a major metabolic pathway involved in the biotransformation and clearance of drugs, pollutants and endogenous compounds[11].

## 2. Experimental

Seven cotinine standards with concentrations of 5, 15, 50, 100, 350, 500 and 750 ppb were made from a 212 ppm stock composed of cotinine and deionized water. These standards were made in 10 mL quantities and were stored at 0 ℃ until used. Storage of cotinine at room temperature was nonoptimal after an initial HPLC/MSMS analysis using 750 ppb cotinine that was stored at room temperature for several days resulted in poor detection. Therefore, each sample was thawed at room temperature before analysis. After the third thawing and refreezing cycle, standards demonstrated reduced detection via HPLC/MSMS. As a result, standards were disposed of after the third thawing.

High pressure liquid chromatography was performed using two Shimadzu LC20-AD HPLC modules. An ammonium acetate buffer was created by dissolving ammonium acetate in HPLC/MS grade water. The buffer's pH was then brought to 3.20 by adding glacial acetic acid slowly, stirring, and measuring intermittently using a pH electrode. A solvent composed of HPLC/MS grade methanol with 1.3 mM formic acid was also made. The ammonium acetate buffer was used in pump A while methanol was used in pump B. All standards were analyzed using a liquid chromatograph for 20 minutes with a flow rate of 0.5 mL/min. Initial conditions were 5% solvent B and rose to 85% at 14 minutes, after which the column was left to re-equilibrate to initial conditions for the remainder of the run.

A Shimadzu C18 3 µm 50 x 4.6 mm column was employed for separation of the standards. Samples were detected using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer. Positive ion MRM was employed to detect cotinine due to the acidic pH of the ammonium acetate buffer and its ability to protonate cotinine. Prior to optimization, the precursor ion was set to detect 177.1 m/z and the product ion at 79.6 m/z, with a CE of -35.0 and a Q1 and Q3 bias at -15.0 V. After optimization, the precursor ion was set at 177.5 m/z and the daughter ion at 80.0 m/z, with a CE of -20.0, a Q1 bias of –18.0 V and Q3 bias of -15.0 V. After optimization, standards were analyzed in increasing order of concentration in order to construct a standard curve. Areas were determined using the LabSolutions software's manual peak integration feature. These peak areas were then placed in an Excel spreadsheet in order to construct a standard curve.


Figure 3: ESI Fragmentation of Cotinine

## 3. Results and Discussion:

An existing HPLC/MSMS method was modified to detect cotinine standards as low as 5 ppb. Optimization through the LabSolutions software resulted the original Q1 bias from -15.0 V to -18.0 V. Quadrupole 1 serves as the mass selector for precursor ions immediately after the sample has passed through the ionization source. Quadrupole 3 selects the fragmented ions that have passed through Q2, the collision quadrupole. Q3's bias was unchanged post-optimization. A more negative quadrupole bias voltage increases the transmission of ions, resulting in the higher sensitivity seen between figure 4 and figure 5. In doing so however, mass resolving power can be lowered. The optimization of both the precursor and product ion masses in addition to lowering the collision energy resulted

in higher sensitivity throughout the method.

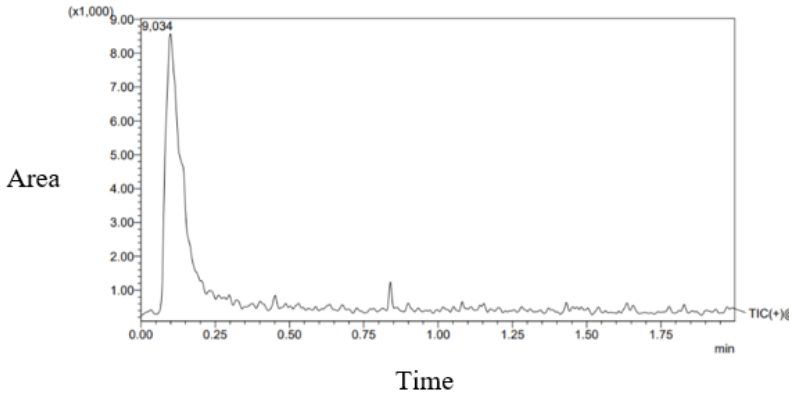



Figure 4: 100 ppb Cotinine Standard, Pre-Optimization

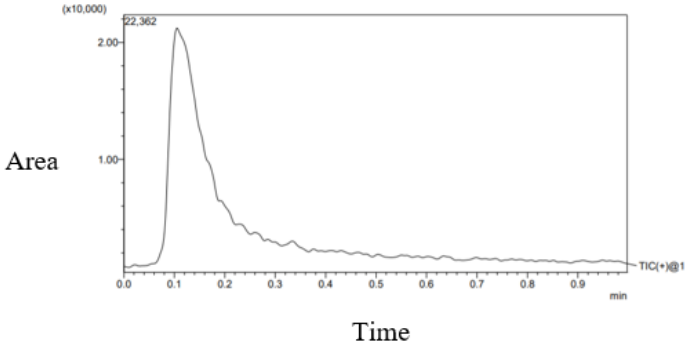



Figure 5: 100 ppb Cotinine Standard, Post-Optimization

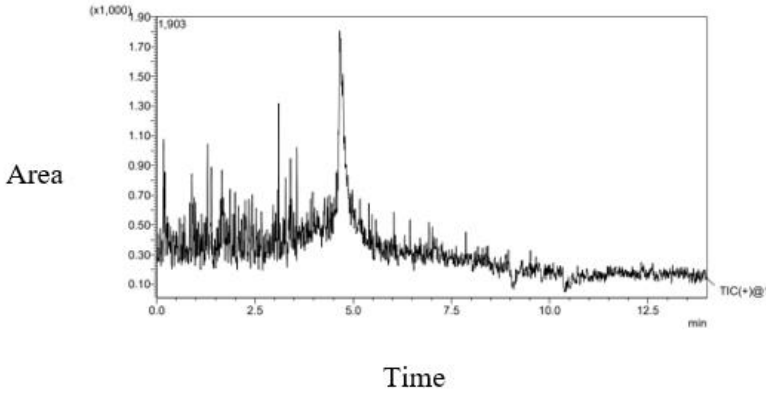



Figure 6: 5 ppb Cotinine Standard

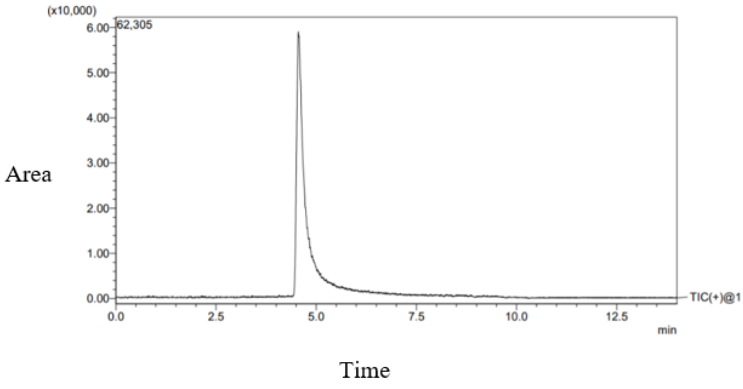



Figure 7: 750 ppb Cotinine Standard

Although the five ppb standard shown in figure 6 was detected with a substantial amount of noise, it is possible to quantify the amount of cotinine accurately. Further optimization using the Shimadzu software could reduce the noise at low levels. In addition, cotinine standards have shown that they degrade rapidly after thawing which could attribute to noise at lower levels. However, all standards above 5 ppb, such as the 750 ppb spectra shown in figure 7, did not show considerable amounts of noise.

This standard range encompasses the wide urinary cutoff range that assigns smoker status. In addition, this range goes as low as 5 ppb to account for second and even third hand exposure to nicotine. Although further optimization may be required at the lowest level for optimal quantification, detection is still possible and therefore allows scientists to determine if an individual has been exposed to very low levels of nicotine, far below the established cutoff points that designate smoker status. Standards showed tailing after elution, which could indicate exposed silica in the column. A column replacement could alleviate this problem as well as lowering operating at a lower pH to mitigate possible interactions with the analyte and silica.

A calibration curve, shown in figure 8, was constructed from the seven cotinine standards that will allow for quantification of unknown concentrations of cotinine in human urine samples. While the 5 ppb standard demonstrated considerable noise post optimization, detection and quantification is still possible through LabSolutions manual peak

integration software. For the purposes of this method, data gathered from higher standard concentrations resulted in low noise mass spectra after optimization. This allows for scientists to distinguish a smoker from a nonsmoker, as well as quantify cotinine in matrices such as urine. In the future, further optimization of cotinine detection can reduce the levels of noise at the lowest concentrations if necessary for analyzing exposure routes beyond that of direct tobacco use. However, this method can still detect cotinine and quantify those levels at concentrations as low as 5 ppb.

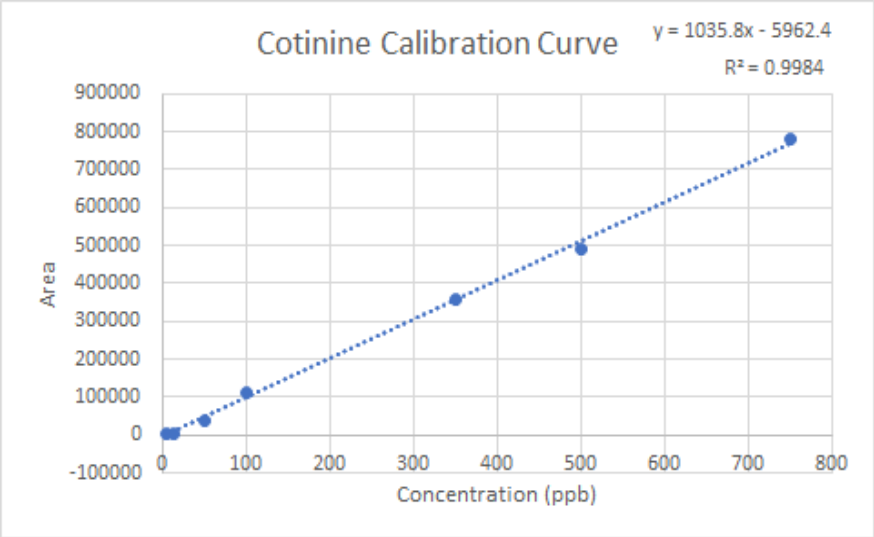



Figure 8. Cotinine Standard Curve

Cotinine proceeds to elute around 4.5 minutes. Cotinine's elution does not coincide with any of the eleven analytes that this method also detects and quantifies whose retention times are listed in figure 9. Therefore, adjustments to the liquid chromatography portion of this method were not necessary.

| Analyte | Retention Time Window (mins) |
|---|---|
| Theobromine | 0-5 |
| Acetaminophen | 0-5 |
| Cotinine | 0-5 |
| Caffeine | 5-7 |
| 3-hydroxy desloratadine | 10.5-12 |
| Loratadine | 11.25-14 |
| Desloratadine | 10.5-12 |
| Salicylic Acid | 6-8.5 |
| 2-hydroxy ibuprofen | 8-12 |
| Carboxy-ibuprofen | 9-12 |
| Naproxen | 11-14 |
| Ibuprofen | 11.25-14 |

Figure 9: Analytes in HPLC/MSMS Method and Retention Time Windows

## 4. Conclusion:

Cotinine, an important biomarker for nicotine exposure, was successfully added to an existing HPLC/MSMS method. This method can detect and quantify eleven over-the-counter medications and cotinine in matrices such as human urine. In addition to detection and quantification of cotinine, this method allows scientists to distinguish smokers from nonsmokers, allowing toxicological studies for example to not rely upon surveyed data which can be inaccurate or falsified by subjects. By analyzing human urine samples using this method, future work can be done to extrapolate information on a potential correlation between alterations in glucuronic activity and the role that several over-the-counter medications and lifestyle choices such as smoking may have on the regulation of UGT enzymes. The potential uses for this method in toxicology specifically allow for analysis of several OTC medications and investigating their potential role in regulation of a major metabolic pathway.

## 5. Acknowledgements

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## 6. References

1. Biomonitoring Summary. https://www.cdc.gov/biomonitoring/Cotinine_BiomonitoringSummary.html (accessed Nov 23, 2019).
2. NTP (National Toxicology Program). 2016. Report on Carcinogens, Fourteenth Edition.; Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service. https://ntp.niehs.nih.gov/go/roc14
3. Kozlowski, L. T.; Mehta, N. Y.; Sweeney, C. T.; Schwartz, S. S.; Vogler, G. P.; Jarvis, M. J.; West, R. J. Filter Ventilation and Nicotine Content of Tobacco in Cigarettes from Canada, the United Kingdom, and the United States. *Tobacco Control* 1998, *7* (4), 369–375.
4. Benowitz, N. L.; Hukkanen, J.; Jacob, P. Nicotine Chemistry, Metabolism, Kinetics and Biomarkers. *Handb Exp Pharmacol* 2009, *192*, 29–60.
5. Sungroul, Kim. Overview of Cotinine Cutoff Values for Smoking Status Classification. *Int J Environ Res Public Health* 2016, *13*, 1236
6. Benowitz NL, Perez-Stable EJ, Fong I, Modin G, Herrera B, Jacob P III. Ethnic differences in N-glucuronidation of nicotine and cotinine. J Pharmacol Exp Ther 1999;291(3):1196-1203.
7. Grancharov, K.; Naydenova, Z.; Lozeva, S.; Golovinsky, E. Natural and synthetic inhibitors of UDP-glucuronosyltransferase. *Pharmacol. Ther.* 2001, *89*, 171-186
8. Mylchreest, Eve; Wallace, Duncan G.; Cattley, Russell C.; Foster, Paul M. D. Dose dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicol Sci.* 2000, *55*, 143-151
9. Benowitz NL, Lessov-Schlaggar CN, Swan GE, Jacob P., 3rd Female sex and oral contraceptive use accelerate nicotine metabolism. Clin Pharmacol Ther. 2006;79(5):480–488.
10. Sakamoto, M.; Itoh, T.; Tukey, R. H.; Fujiwara, R. Nicotine Regulates the Expression of UDP-Glucuronosyltransferase (UGT) in Humanized UGT1 Mouse Brain. *Drug Metabolism and Pharmacokinetics* 2015, *30* (4), 269–275.
11. Swan S H; Liu F; Sathyanarayana S; Barrett E S; Janssen S; Nguyen R H N; Redmon J B. First trimester phthalate exposure and anogenital distance in newborns. *Hum. Reprod.* 2015, *30*, 963-972.
12. Way, Michael. Development of A Liquid Chromatography Tandem Mass Spectrometry Method for The Detection and Quantification of Eleven Common Over-The-Counter Medications in Human Urine. **2018.**