Detection of Drugs in Exhaled Breath Condensate

By

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Abstract:

When humans breathe, air enters the lungs leading to an interchange of gases between the blood circulation and the air in the lungs. Thus, it has been questioned whether it could be possible to detect chemicals/drugs present in a person's blood circulation from this interchange process. This has already been demonstrated for volatile substances such as alcohol and even some other less volatile substances (THC, opioid metabolites, etc. (6)(10)), but has not been confirmed for the listed substances (Table 1) yet. In this study, several common over-the counter (OTC) drugs were administered to a group of volunteer subjects and several exhaled breath fractions were analyzed for these drugs. Exhaled breath condensate (EBC) and exhaled breath aerosols (EBA), together with the saliva fraction were collected over a period of 3-time intervals to determine if the drugs (acetaminophen, naproxen, and ibuprofen) or their potential known metabolites can be detected. The sampling intervals were chosen for each drug based on the average expected detection window from their pharmacokinetic profile (i.e. Drugbank, HMDB, and etc.) in the blood stream following administration. Exhaled breath samples were collected by several different methods: 1) a cooled glass tube that retained condensate volatiles and semi-volatile compounds, and 2) filters, such as N95 mask and C18 filters, that capture aerosols and larger molecules. Processed samples were then analyzed by liquid chromatography mass spectrometry, Quadrupole-Time of Flight (LC-MS/MS, qTOF). The data obtained was analyzed to identify the drugs detected, their metabolites and determine their concentrations.

This study has been done using a selected group of common OTC drugs that would most likely be found in the breath of everyday individuals due to their widespread use. Individual volunteers used a custom-made breath condenser (K-tube) and commercial filters (C18 disks and N95 masks) to collect several breath samples following the OTC drug ingestion. These samples included

collections at an initial time 0 (T_0) before any dose intake along with two or three time-points at specific time intervals after a dose intake. While filters collected aerosol fractions (EBA) from the direct breath exhalations, the K-tube collected lighter breath fractions, EBC, together with an additional ethanolic rinse fraction of the glass tube to get less polar molecules adhered on the surface walls. All fractions, after a proper sample treatment, were then injected into an LCMS/MS system, where the triple quadrupole – Time of Flight (qTOF) analyzer detects presence of the analytes along with their metabolites through exact mass determination and fragmentation. When no targeted compounds were detected, we evaluated the potential impact of possible interference factors such as dose ingested, individual metabolism, sample collection/preparation technique or limited partitioning of the drugs from blood to breath. The final multi-drug experiment yielded a few promising results. Out of the six compounds tested, two (Phenylephrine HCL and Oxymetazoline HCL) were successfully detected based on mass and retention time. These results indicate a potential for more extensive tests in the future to focus in on the low detection range of these drugs.

1 Introduction

With the current opioid abuse crisis as well as increasing numbers of Driving Under the Influence of Drugs (DUID) cases occurring in America, there is great need for quick, roadside testing for drugs in a person's system to aid law enforcement in determining the correct course of action (1–3). The absorption, distribution, metabolism and elimination processes of drugs in the body is called pharmacokinetics (4)(5). Law enforcement and forensic analysts must also consider pharmacokinetics in the criminal drug cases, and not just the detection of the drugs themselves. The amount of time the drug has been present in their system will determine the resulting substances found. In fact, drugs can be completely or partially transformed in the body into their chemical metabolites. Some metabolites can remain longer in a person's system before being removed than others (6–9). Thus, someone found to be below the alcohol per se limit for driving can still be found to have committed a DUI if, based on elimination rates, a higher alcohol percentage can be determined to have been present in the past (10). In this way, the determination of the past presence of drugs can be made based on the presence of drug metabolites.

Based on this need and theory, the detection process for illicit chemicals would have to capture a variety of compounds with varying properties. While there are currently several different techniques for detecting drugs in a person's bodily fluids, a method compatible with the fast-paced and direct testing nature of law enforcement roadside stops remains elusive. Exhaled breath testing fits the current model for detecting alcohol associated with drunk driving as it provides a sensitive, non-invasive procedure that can easily be applied by law enforcement during roadside stops. Up to this point, however, the field of exhaled breath research remains relatively novel and is particularly lacking in publications related to detection of trace amounts of drug of abuse (6,11,12). Forensic and law enforcement practitioners are faced with little to no on-site methods to determine

specific drugs and dosages present in an individual after ingestion except for testing breath for the presence of alcohol (10). Novel devices and techniques have been used for cannabinoids, but no widely accepted standard has thus been set (13). No standardized technique has been developed thus far and results vary with how data correlates to current known, best practices. The only currently available procedure requires use of Drug Recognition Experts (DREs), who are highly trained and skilled individuals following a very specific set of procedures to evaluate subjects for impairment due to potential drug influence and determine probable cause for arrest. While current DREs are very good at their jobs, using evaluations based on observable signs and symptoms of drug influence, the final determination is somewhat subjective and based on the experience/skill of the officer. More and more, as oversight of law enforcement increases, there is an increased need for more scientific approaches that will be better accepted by the general public as well as provide more solid, repeatable evidence in court. This creates a need for highly specific and accurate tests that can be scientifically validated. There is also the added concern that DREs require a large amount of training, necessitating significant time and adding to budget constraint burdens for law enforcement agencies. Development of exhaled breath analysis allowing detection of drugs in conjunction with current alcohol breath analysis would provide an objective and far-ranging process for law enforcement to determine driving impairment and establish probable cause (4). This would streamline the process law enforcement has to go through, as well as reducing doubt associated with subjective determinations.

Technology for the collection and road-side detection of psychoactive drugs in exhaled breath is still in its early stages. One of the first steps in developing such technology is determining whether it really is possible to identify any or all ingested drugs in breath samples. Most drugs have vapor pressures much lower than alcohol (10,14); thus, the question remains as to what

concentration is required in circulation for the drug's volatility to allow detection in breath samples. It has previously been shown that ingested drugs can be found in oral fluids, thus, suggesting that those same drugs could be also be detected in the breath (1,14). In addition, based on the concept that breathing involves an air-blood interface, drugs could theoretically be released into lung air according to a specific ratio similar to the release of blood alcohol (10). This study is focused on the development and optimization of a new procedure for the detection of drugs and their metabolites in breath samples. The evaluation has been tested on a wide variety of drugs to provide insight into the detection differences for drugs which affect different systems of the body and including drugs that have administration routes that differ from each other. In order to evaluate possible differences in detection of various drug forms, a wide range of medicines with varying routes of administration were chosen. These routes included drugs delivered orally (liquid or pill), topically, or in an aerosol. Different techniques were tested to collect the breath from subjects after drug administration. Initial tests were studied using a relatively new technique based on a custom made K-tube (7,8,15), developed in Prof. Davis BioMEMS group, where the exhaled breath is frozen into a condensate in a glass tube inside an isolated container surrounded with dry ice for collection and testing. The experiment followed methodology from a preliminary study for breath sample collection followed by LC/MS drug analysis (9). In this study, samples were collected with the K-tube from patients with acute pain episodes and were under continuous opioid infusion treatment. Exhale Breath Condensate (EBC) was collected from each patient followed by an ethanol rinse fraction after EBC was removed from the device. This allowed the removal of molecules with higher polarity that could remain attached in the glass surface of the K-tube. LC/MS was also used to determine the sensitivity of our methods as it relates to drug dosage as well as the vapor pressure of each specific drug. In addition to the K-tube collection, the breath

collection was also tested by different commercial filters, such as N95 masks, surgical masks, and C18 filters. This was based on the fact that in addition to the EBC sampled with the K-tube, there was the potential loss of analytes through a heavier fraction of the breath exhalation containing aerosols, EBA, as well as any exhaled saliva or heavy particles that are breathed out during normal respiration (16,17). Specifically, the C18 filter was placed inside the K-tube apparatus before the cooling phase with the goal of potentially capturing these aerosols that were not able to condensate and pass straight through the glass-tube cooled section. In the same context, the filtering masks (N95 and surgical) were worn as a way of determining if the drug-related compounds were present in saliva or were being lost through other avenues. Although some literature describes the presence of drug-related compounds in breath, there is no clear collection technique to be able to detect as much drugs as possible. Moreover, the published literature is focused on drugs-of-abuse, such as opioids, cannabinoids and other stimulants (e.g. cocaine, amphetamine) (18,19).Through this research, this study aims to answer the questions: How well will different drugs partition from blood to breath and will common/typical doses be sufficient for detection? For that, this study was first focused on optimizing the best technique to collect breath samples containing these substances. Considering the limitation to access to individuals that consume drugs-of-abuse, all testing and optimization steps were developed using OTC non-prescribed drugs.

This research study will be performed in parallel with other technological projects ongoing at the BioMEMS lab in order to create a quick, portable analyzer of substances in air and breath. The results of this project will also be used in the developmental validation of any procedures successfully utilized in the study for detection of drugs in breath samples.

2 Materials and Methods

2.1 Chemicals and standards

All chemical reference substances were purchased from Cerilliant Co (via Sigma-Aldrich, Round Rock, TX, US). The stock and working solutions were prepared in methanol and stored at −20° C. The list of substances used in the study and mass spectrometric parameters are summarized in Table 1. Retention time (RT, min) and theoretical exact mass of the main precursor ion detected with MS ([M + H]+) parameters were used for confirmation of analyte detection. All chemicals including LC-MS grade acetonitrile (Fisher Scientific AB, Gothenburg, Sweden) and formic acid (VWR international) were of the highest analytical grade, and the water used in the study was filtered and deionized. Pure standards of each drug together with a commercial drug solution were used to optimize the instrumental method and protocols. These same standards were used to spike breath condensate samples by adding different drug concentrations to 1 mL of pooled clean EBC extracted from a group of control participants.

Table 1. List of substances used in the experiment and their corresponding LC-MS parameters. (Data from Drugbank.com)

Compound	Formula	RT (min)	Exact mass	Precursor [M+H+]
Naproxen	$C_{14}H_{14}O_3$	9.910	230.2592	231.1021
Acetaminophen	$C_8H_9NO_2$	2.687	151.1626	152.0716
Ibuprofen	$C_{13}H_{18}O_2$	11.477	206.2808	207.1385
Dextromethorphan HBR	$C_{18}H_{25}NO$ * HBR	8.763	271.404	272.2014
Etodolac	$C_{17}H_{21}NO_3$	11.070	287.3535	288.1599
Albuterol Sulfonate	$C_{13}H_{21}NO_3$	9.210 (Unknown)	239.3107	240.1599
Phenylephrine HCL	$C_9H_{13}NO_2$ * HCL	0.798	167.205	168.1024
Hydrocortisone	$C_{21}H_{30}O_5$	8.992	362.4599	363.2171
Oxymetazoline HCL	$C_{16}H_{24}N_2O$ * HCL	8.789	260.3746	261.1966

2.2 Sample collection

Two fractions were obtained using the K-tube device: EBC and ethanol rinse fraction. This collection was done using the K-tube device previously developed by the lab. Throughout testing, collections were also performed with commercial filters including C18 disks, N95 masks, and surgical masks.

2.2.1 K-tube collection

The K-tube consists of a glass tube surrounded by dry ice in an insulated refrigeration system that sets the internal temperature of the device to around −80° C, the warm exhaled breath will then condensate and freeze to the inside of the tube with the intent of trapping any analytes within it (Figure 1).

Figure 1. Description of the K-tube device (left) and the mouthpiece with the trap structure (right)

The device first filters the exhaled breath from a mouthpiece through a trap that separates the saliva and larger particles using the flow of air. The airflow then proceeds through the glass tube as listed. Participants were asked to perform normal tidal breathing (no nose clip) through

their mouth. The K-tube has a one-way valve that facilitates this type of tidal breathing, thus allowing as much of the air to stay in the system as possible. All study participants avoided food and drink (except for water) for 1-2 hours before the breath collection. Sample collection was done for 15 min for the first tests of the study before being eventually extended to 20 min for further testing. Between 3 to 4 separate collections were performed for each participant depending on the time of action of the drug ingested. After each breath collection, the EBC samples were scraped out of the glass tube into a glass vial and stored in a −80 °C freezer. The ethanol rinse fraction was then collected by flushing the glass tubes with approximately 3 mL of pure ethanol and rinsing for 1 min before being collected in glass vial and stored in a −80 °C freezer. This additional fraction was an attempt to capture any less volatile or nonpolar molecules that may have adsorbed or stuck onto the glass tube which were not removed with the EBC. In addition, this step was also applied due to low amounts of EBC being provided by some of the subjects and, thus, attempt to extract more information. Later in the testing, ethanol was also used to rinse out the saliva trap in order to test if any analytes had been lost in the saliva or with the larger particles that were filtered out.

2.2.2 C18 collection

During the optimization process, it was observed that some potential analytes were lost in the saliva trap or flowing straight through the K-tube device during the EBC collection. Some papers suggested that capturing drug-related compounds in filters or other solid barrier devices were possible (16,17). Thus, it was decided to add an additional filter into the trap between the mouthpiece and the K-tube (Figure 2), an Empore C18 adsorbent disk, similar to how a SPME fiber can be added to the K-tube for certain collections (data not published, (15)). The idea being that as the air flows by the adsorbent material, the larger molecules that cannot reach the glass tube will adsorb and stick onto the filter. When the C18 adsorbent filters were implemented, they were

incorporated into the K-tube trap mechanism so as not to interfere with the flow path, while still receiving full contact with the flow stream in order to increase the odds of adsorbing the compounds of interest.

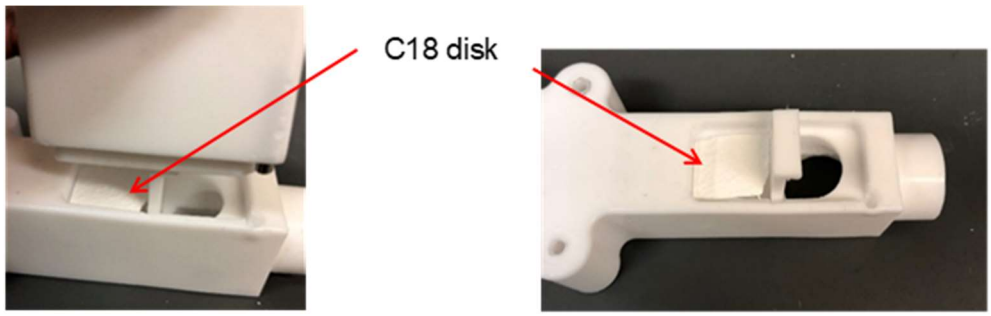

Figure 2. Position where the C18 disk was placed inside the K-tube device

2.2.3 Mask collection

Much in the same way as with the C18 filter, it was determined that drug-related compounds were being lost somewhere in the process of sample collection or preparation. As analytes had been previously found in certain filters from other studies, we decided to test breath collections using a mask filter. This system could show if the larger particles were just not traveling far enough for the other forms of collection to function. Both N95 and surgical masks were used in the experiment. N95 masks were selected to test as they were one of the most common masks used to filter most particles from saliva to larger fractions containing drug-related compounds. Surgical masks were also tested due to their wide availability (at the time when the study began) in addition to testing whether the material for collection really mattered. Both N95 and surgical mask were used for 20 min during the sample collection step. Masks were stored in sealed plastic bags and stored in a −20 °C freezer after the breath collections until they could be transferred to a −80 °C freezer.

2.3 Sample preparation

2.3.1 EBC and solvent rinse from K-tube

If possible, 1 mL of EBC sample was lyophilized at low temperature and pressure. For samples under 1 mL all the available volume was used for the lyophilization step. All the volume from the ethanol rinse fractions was dried with nitrogen at room temperature until completely dry. Both dried extracts were reconstituted with 50 µl of acetonitrile in water (95/5:H2O/ACN), vortexed, sonicated for 10 min at 4° C and centrifuged at 13,000 rpm for 10 min at 4° C. Supernatant was stored at −80° C until LC-MS/MS analysis. Samples were spiked with IS before lyophilization and drying, when available.

2.3.2 Filters: C18, N95 and surgical mask

A solid liquid extraction was performed for all the filters studied. C18 disks (0.5 x 0.5 inch) were broken into 2-3 pieces inside 20 mL glass vials. Both N95 and surgical masks had a 2 x 2 inch square cut off from the front filter where the majority of direct breathing took place. The squares were cut into small strips and placed inside 20 mL glass vials.

All the small pieces of filters were mixed with 300 uL of 2-isopropanol before being soaked in a 5 mL Methanol/Ethyl Acetate (20/80:MeOH/EtAc) solution. The solution was shaken at room temperature for an hour before being centrifuged at 3000 g for 15 min at 10° C. The supernatant was transferred to a separate vial, while another 5 mL of MeOH/EtAc was added to the remaining filter pieces and mixed, using the same process as before. Once completed, both 5 mL supernatants were combined with 10 uL of 10% formic acid (10/90:formic acid/diH2O). After mixing, the solution was evaporated with nitrogen until approximately 1 mL was left. This volume was then filtered through a 0.2 um PTFE syringe to remove any large obstructions/fibers from the sample.

The remaining solution was completely dried and then reconstituted in 100 uL of H2O/ACN (95/5:H2O/ACN) solution.

2.4 LC-MS/MS analysis

Analytes were separated with an InfinityLab Poroshell 120 EC-C18 column (3.0 mm × 50 mm, 2.7 μm) (Agilent Technologies, Palo Alto, CA, US) held at 35 °C. An autosampler at 5 °C maintained the samples stable until 20 μl of sample were injected onto the column. The mobile phase flow rate was 600 μl min−1 operating in a gradient mode. Mobile phase A consisting of water and mobile phase B consisting of acetonitrile, both with 0.1% formic acid. The gradient profile was as follows: starting with 3% B (hold time 3 min) and then began a linear change up to 10% B after 3 min and up to 50% B after another 4 min. The gradient continued up to 100% B after 3 more min and was held for 6 min. Sample analysis lasted 19 min and equilibration was performed at 3 min to stabilize the system. An electrospray ionization source, with an Agilent Jet Stream nebulizer was used. It was operated at 300 °C with ionization set at 2500 V and fragmentor voltage at 175 V in positive mode. Nitrogen gas was used for nebulization and desolvation. Nebulizer gas pressure, temperature and drying gas flow rate were set at 45 psi, 400 °C and 10 l min−1. Mass measurements were recalibrated using the reference masses m/z 121.0508 (purine) in positive ion mode. Identification of the targeted drugs was performed using ’All Ions’ mode MS/MS of preferred precursor ion list (Table 1) at collision energies of 0 and 20 V. Mass spectra was acquired at a scan rate of 3 spectra/s between 125 and 800 m/z for MS resolution and at 5 spectra/s between 50 and 700 m/z for MS/MS resolution (9)

2.5 Data analysis and statistics

LC-MS/MS data provided experimental retention times and mass spectra. The experimental masses found were compared to theoretical known information and quality control (QC) samples. One way to get the accuracy of the detected mass with a mass spectrometer is calculating the statistical error in ppm. This quality parameter describes the difference in mass allowed for a specific instrument. The error is calculated by [(difference between experimental and exact mass)/exact mass x 10^6] (ppm). qTOFs are usually in the range of error between 1 and 50 ppm. For an additional confirmation, the fragmentation patterns of the detected molecules were compared to drug databases that are publicly available (i.e. PubChem, HMDB, and Metlin).

The peaks from compounds of interest were integrated to assess the amount detected and to potentially demonstrate linkages between time of drug intake and dose. Descriptive and comparative statistics will be performed along with correlation analysis and significance testing with regards to study hypotheses.

3 Results

Although some studies have been done regarding detection of ingested drugs in breath, this will be the first study using the simple K-tube collection device to collect OTC drug samples (10,13). Based on the current information available, it was suspected that the non-volatile compounds would be found in low concentration in EBC. All study data for each drug tested was compared to control samples from subjects, who had no opioid or medication administrated for the previous 48h. This ensured that possible signals detected after intake of specific drugs were exclusively related to the drug itself and not from the subject. Blanks and QC were also collected and prepared

for each experiment in the study. Blanks included the pure solvent used in the final sample reconstitution, a 'blank-process' sample constituting an empty vial, and a 'blank-matrix' sample of a vial with a clean EBC or filter. Both 'blank-process' and 'blank matrix' went through the same sample preparation as the corresponding matrix. QCs were prepared by spiking clean EBC from control subjects or clean/ unused filters with commercial drug standards.

3.1 Breath collection optimization

3.1.1 Preliminary experiments with K-tube

The first experiments were done using the standard method that had been established for the K-tube to collect samples of basic NSAID over-the-counter (OTC) drugs previously selected to test. The first step was to confirm if any changes were required from the past studies and to test viability. These tests involved only the use of the K-tube to test acetaminophen (APAP), ibuprofen (IBUP), and naproxen (NAPR) with 3 subjects each. For each subject, EBC and ethanol rinse samples were collected before the intake (T0) and at two different times after the intake (T1 and T2 at 30 and 90 min at this point in the experiment). Although the tests showed some promising results by providing correct retention times for IBUP and NAPR, experimental masses detected were slightly different from the theoretical mass in the subject samples with an error of 320 ppm (being acceptable below 50 ppm). Moreover, some peaks appeared in all the collection times for some subjects, despite the fact the T0 should have no signal of any drug. APAP was detected in one of the subjects with reliability. This raised enough concern that the test was repeated with different subjects and attention focused on the variables present in the analysis/collection. It was decided that improvements needed to be made in the collection process to determine viability and get better signals. Past studies and literature reviews were reviewed to assess what was missing

from our analyses and other problems that had been encountered (3-6, 8-9, 11, 14-17). All the following experiments were useful to identify and isolate specific variables to optimize the entire methodology for determining exact drug action times, avenues other than EBC, collection times, collection methods, dosage, and etc.

3.1.2 Experimental optimization

(a) Optimization of collection times and modifications in K-tube:

First test was aimed to find the optimal sample collection technique and enhance the signal for the specific drugs. To ensure a significant signal and good timings for the sample collection, we adapted the collection times after intake to each specific drug as well as increasing the dosage to the maximum dose prescribed. To test the methodology, we focused on just one drug, acetaminophen (at 500 mg) due to its good results in the preliminary tests. The times of collection were changed from 30 to 70 min (T1) and from 90 to 110 min (T2) based on the action time in blood for APAP (approximately 1.5 hours). From there, we aimed to improve our collection techniques in order to achieve better results. Two additional fractions/samples were also collected for this set of experiments with the K-tube device: the saliva trap was rinsed with an organic solvent and a C18 disk was added before the condenser section. Finally, N95 mask collections were performed in conjunction with the K-tube collections. These modifications in the previous protocol provided a good test bed for us to assess the different variables present in the testing. A single subject was used for this part of the experiment.

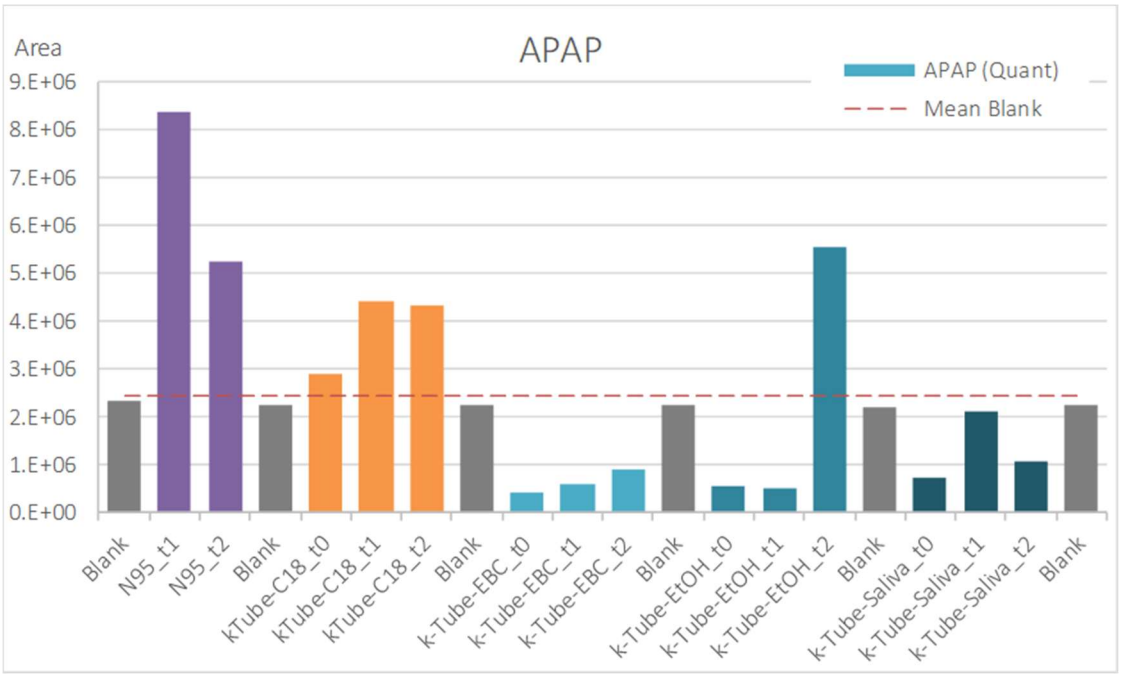

Figure 3. Acetaminophen (APAP) amounts detected in the different optimization experiments

The results, as shown in Figure 3, were mixed. There were some encouraging peaks with the C18, but for the most part, there was no correlation between analyte amount and time. The biggest concern was from the detection of APAP peaks in solvent blanks and in time zero fractions (T0). Fortunately, the solvent blanks (Figure X - gray) had the same abundance from all the injections, showing that there was no variability in the instrument. However, these signals seemed to indicate a possible source of contamination. The fact that peaks appeared in all injections for APAP and not for the IS (only spiked in samples and not blanks), showed that if there was contamination, it would come from in our sample preparation process. Standards were prepared and injected simultaneously to confirm the APAP signal found in the samples. From the results, we decided to repeat the experiment with a stricter control on contamination or other possible problems to account for signal in the blanks. N95 and C18 data seemed promising, however, K-tube fractions

such as EBC, ethanol, and saliva trap showed no relevant results and were discarded from following tests.

(b) Testing of several collection techniques

For the next phase of the experiment, various measures were taken to attempt to achieve positive results and decrease possible contamination. This phase included 5 sets of experiments to tackle different problems that were found from each previous experiment. Two major changes implemented: first, the collection time T1 was adjusted from 70 to 50 min, and all collections were performed for 20 mins to improve overall time coverage. The first set involved K-tube w/ C18 filter, N95 masks, and surgical masks. All collections were performed by 2 different subjects to reassess the original signals that had been detected previously. New standards and reagents were used as well as the addition of a 'blank-process' to study the effects of the preparation process.

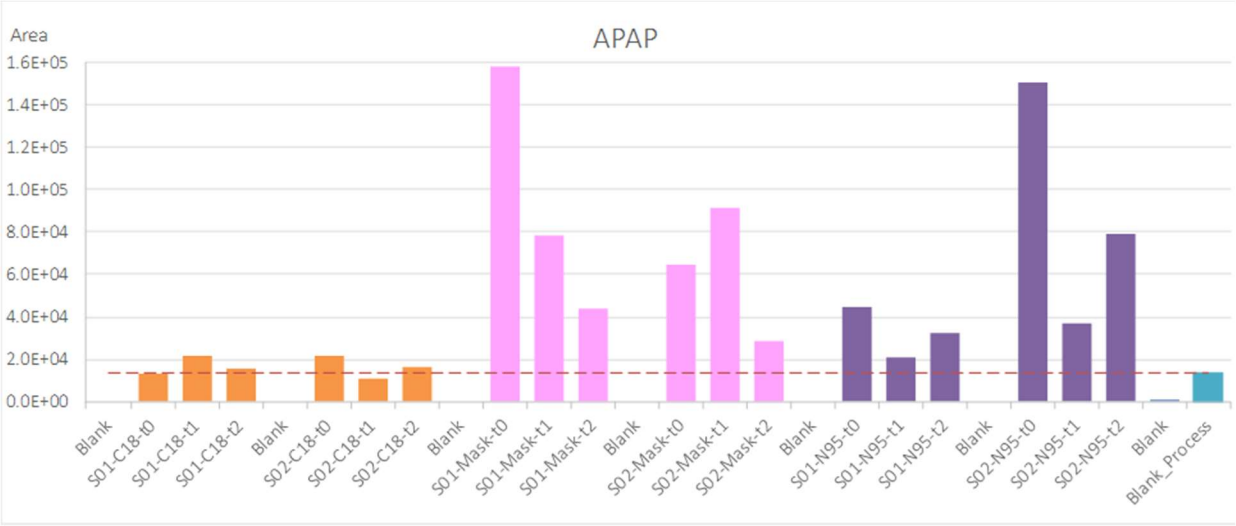

Figure 4. Acetaminophen (APAP) amounts detected with different collection techniques

Figure 4 shows the results obtained from this test. Although solvent blanks showed no contamination for APAP, there was a clear contamination in the 'blank-process'. This was a clear

sign that something was happening during the sample preparation. This is empathized when looking at the sample results, showing APAP signal in all T0 samples for the C18, N95, and surgical masks. When looking into the individual methods: mask filers showed higher signals than C18, however, the fact that T0 presented the highest amount of APAP in almost all the replicates makes it difficult to arrive at a clear conclusion from this experiment.

Interestingly, when looking at the IS signals, those are quite consistent in intensity throughout the samples, but when we decided to check the signal of a pure solution of IS (Figure 5). Considering the masses: 156 (APAP-d4, IS) and 152 (APAP) m/z, it was discovered that the pure standard on its own had both masses present. This raised alarms as this would have meant we were injecting impurities of APAP (152 m/z) into our samples. Due to this, we decided to not include internal standards in our testing as there was the possibility those peaks were a result of degradation of the IS from keeping it too long.

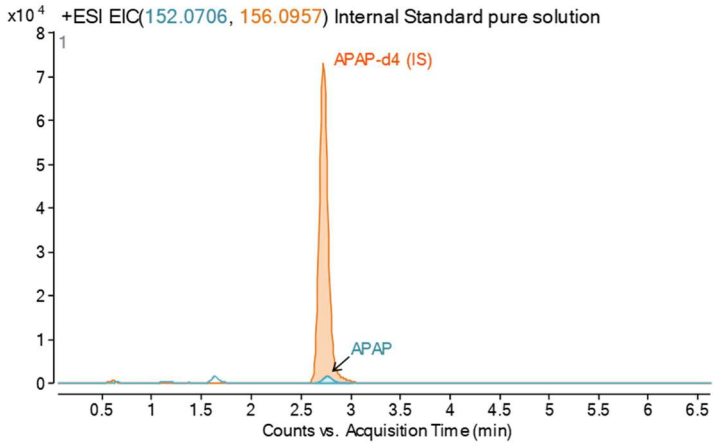

Figure 5. Extracted Ion Chromatograms (EICs) for APAP and APAP-d4.

(c) 'Process contamination' study with C18

The third set of experiments involved testing just the C18 disks without the IS being present. C18 disks have been used in several publications (with different modifications on the

collection devices) showing promising results for the drugs detection (14,20). We collected samples from 2 different subjects and run blanks. The results presented in Figure 6 showed signal of acetaminophen in all fractions of the samples including the blank process, blank-C18 and T0 from both subjects. This was concerning as we had been isolating procedures and ensuring quality control so as not to contaminate any of our samples. Thus, it was determined that there was the possibility that there was a source/sources of acetaminophen contamination through the sample processing material. All equipment and materials were thoroughly cleaned before the next set of experiments.

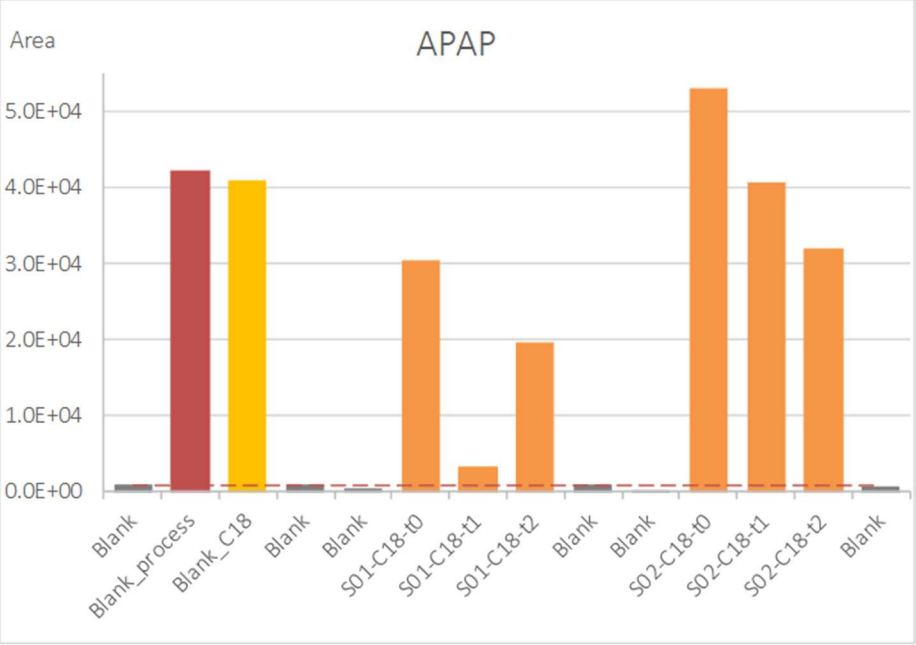

Figure 6. Acetaminophen (APAP) amounts detected in the study

To negate the possibility of acetaminophen contamination we used another OTC drug for the experiments. The same previous experiment with C18 disks was repeated with NAPR. In this case, a different collection step was done right after taking the drug (T1) in addition to the normal 50 min (T2) collection. In our best effort to obtain a signal, we introduced the drug into subjects

by swishing them around in their mouth before intake to ensure the best possible chance that the procedures could detect the presence of the drug. The subjects swished crushed-up naproxen pills dissolved in a small amount of water in their mouths and performed an additional collection just after intake (new T1) to achieve the same results.

The results are presented in Figure 7, where the signals for the standard and pill were added to confirm the signal of NAPR. In this case, the signals for the T0 were much lower than the rest of the collected times, but these were inconsistent between subjects. The peak evolution that was consistent with what we were looking for only presented in one of the subjects (S02). Although there were no signals of contamination in the sample preparation process (blank-process clean), a huge NAPR signal appeared in the blank of the filter C18 (new filter without contact with breath). The problem with the idea of contamination had not been solved. So, we tried to change the collection method to evaluate the process.

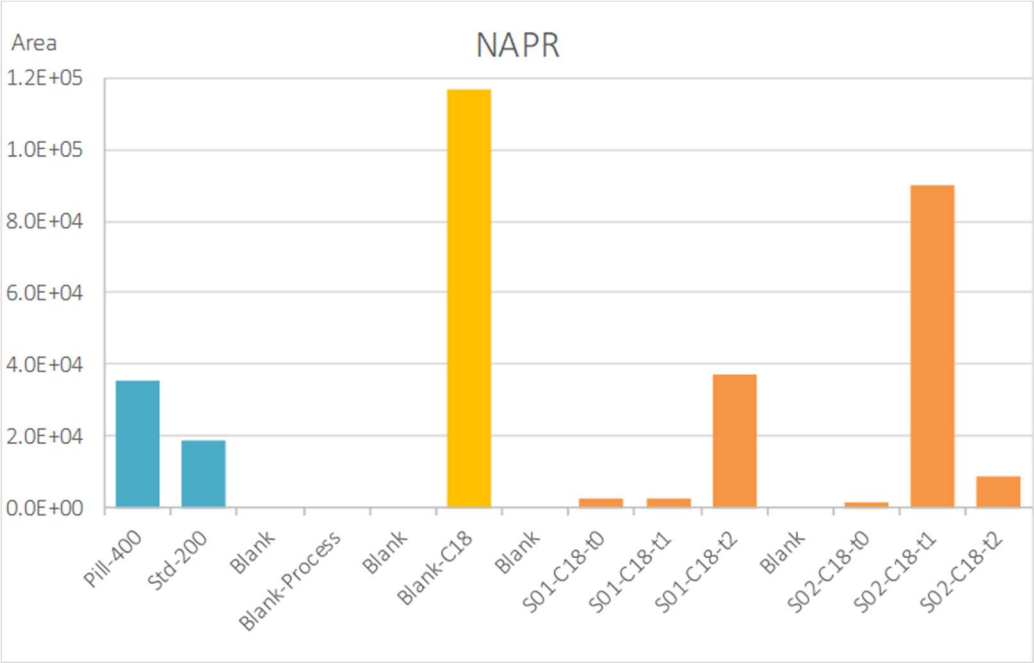

Figure 7. Naproxen (NAPR) amounts detected in the study

(d) Contamination study with N95

The next set of experiments was performed with the N95 masks to ensure that different collection methods had the same problems that we were discovering in our testing. Naproxen was taken in the same manner as the previous set, with the difference being collection with the N95 masks as opposed to the C18 filter. New, clean N95 masks were spiked with NAPR standard before sample preparation to test signal detection. Only two subjects performed the experiment. The results (Figure 8) showed promise for the NAPR spiked masks with clear, strong signals that correlated to spiked concentrations (SpkN95-50 and SpkN95-100, spiked with 50 and 100 uL at 200 ng/mL, respectively). However, the present problems from the previous experimentation remained with clear signals in the N95 blank and in the T0 fractions. At this point, we had settled on contamination being a significant problem that we could not eliminate from the variables we had isolated and attempted to correct. We shifted focus to the environment and equipment the drugs had been prepared in. While they had been cleaned and used properly up to this point, the problems had not been alleviated. The next steps were to isolate these variables as well as the human factor involved in testing.

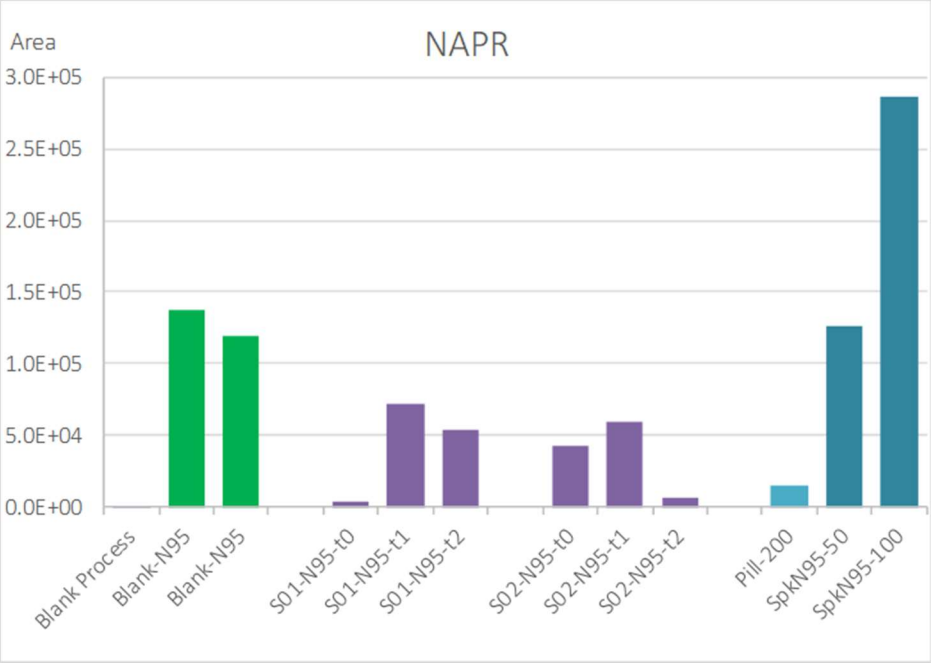

Figure 8. Naproxen (NAPR) amounts detected in the experiment

For that, several blank-N95 samples were collected to understand the source of contamination. We ordered completely new boxes of N95 masks from different sources and shipped them to different locations. Six subjects then proceeded to take the masks to their own locations and perform a 20 min collection without taking any drugs before sealing the masks on their own. Once collected, the masks were sent to a different person who had never been involved in testing to be prepared in a completely separate lab location. The samples were prepared following the exact procedure. This resulted in no signs of acetaminophen or naproxen contamination in any of the samples when tested on a LC-MS/MS (qTOF). This was significant as it pointed to the previous lab equipment, location that was used in all the preliminary experiments, or user error during preparation.

The next test addressed the possibility to contain contamination sources for lab equipment in the initial location. In this case, two clean N95 samples were collected and prepared in the second lab space. One of our primary sources of worry for contamination was the n2 dryer used in all the initial tests. This n2 dryer was more efficient than the dryer that was used in the second lab space. Thus, we used it in this experiment to test whether we could use this equipment as well as determine if it was a possible source of the contamination. Fortunately (and mysteriously), the results showed no signs of contamination for both of the drugs (NAPR and APAP). That allowed us to continue preparing the samples in the second lab and while knowing the original n2 dryer was safe to use for the sample preparation. However, there was no clear explanation for the initial contamination tests, except for the possibility of having the first batches of filters already contaminated.

(e) Verification study with N95

With no signs of contamination thus far in the isolated variables, we moved onto testing a final validation of the procedure for signal and potential cross contamination. Naproxen and acetaminophen were both used with N95 collections with 2 different subjects for each drug (APAP and NAPR). In this case, collections were taken before drug intake (T0) and two more at 50 (T1) and 110 (T2) mins after the OTC drug intake.

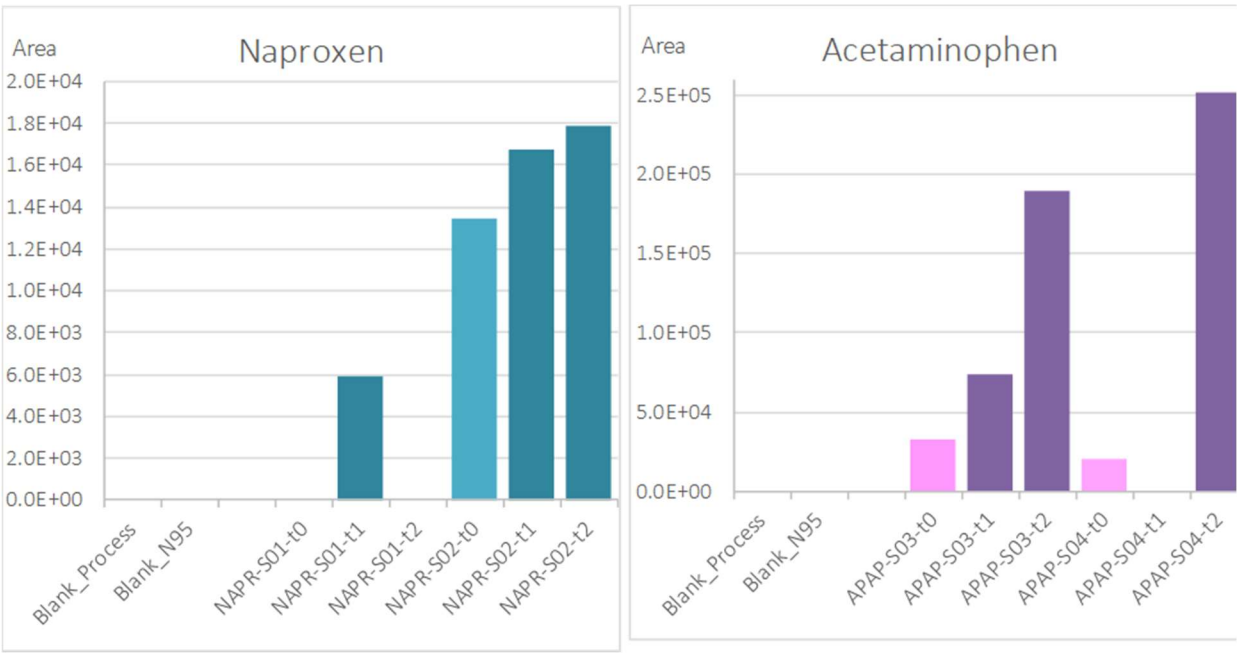

Figure 9. Naproxen (NAPR) and Acetaminophen (APAP) amounts detected in the experiment

The results presented in Figure 9 proved inconclusive for the replicate samples from the subjects. It has to be highlighted that no signs of the drugs were detected in both of the blanks, from the blank-process, and in the clean N95 mask itself. Naproxen was only detected at T1 for one of the subject's (S01) and at all times, including T0, for the other subject(S02). On a different note, Acetaminophen was detected in really small concentrations at T0, but in higher concentrations during collection times after ingestion.

To verify these promising and non-process contaminated results, the exact same experiment was repeated as previous with the additional rinse step of the crushed-up pill. This step was added to the intake of the pill to increase the chances of seeing signal. In this case, an initial collection (T0) before drug intake and 3 additional collections were taken at 0 (T1), 50 (T2) and 110 (T3) mins after the OTC drug intake. The same subjects participated in the repetition of the experiment (Figure 10).

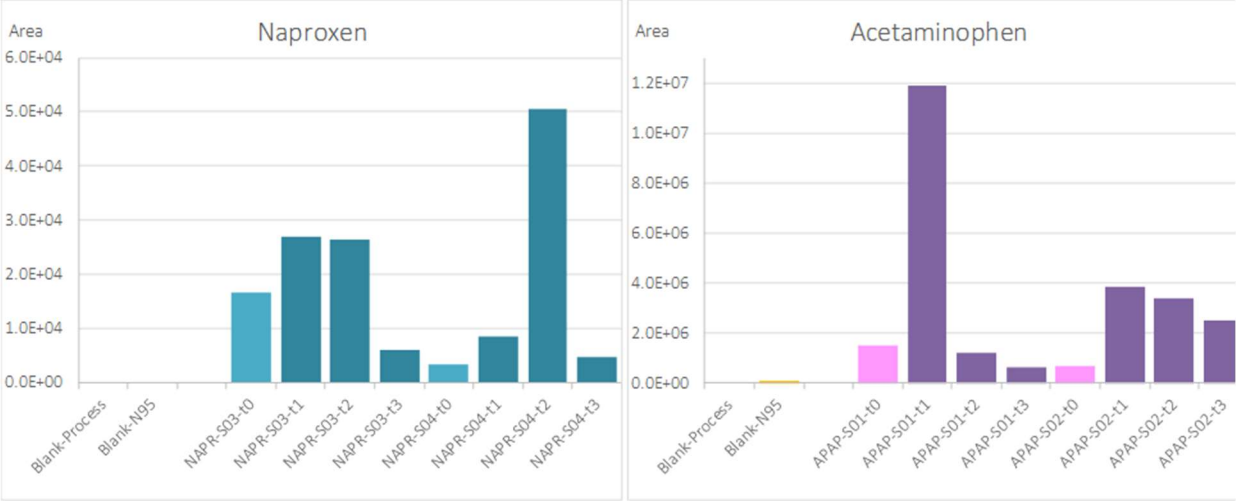

Figure 10. Naproxen (NAPR) and Acetaminophen (APAP) amounts detected in the confirmation experiment

3.1.3 Collection and analysis of real samples

This confirmation test allowed experimentation to continue with the primary goal of the study, which was to test various drugs and their viability in being detected using a novel breath test. The drugs originally chosen for this study were guaifenesin, dextromethorphan HCl, Cortaid (hydrocortisone topical), benzocaine, bismuth subsalicylate, diphenhydramine HCl, melatonin, etodolac, albuterol sulfonate, and oxymetazoline HCl. This spread of common drugs would encompass a wide range of drug types and drug route options that could provide a baseline study as to whether breath testing would be sufficient for certain analytes or not. They were sampled and prepared with the changes and lessons learned from the testing phase that was done.

Due to unforeseen circumstances from the Covid-19 pandemic, only samples for dextromethorphan HCL, phenylephrine HCl, etodolac, albuterol sulfonate, hydrocortisone, and oxymetazoline HCl were able to be collected. There was only one subject for each drug and masks

were not employed due to the sudden importance of masks for Covid-19. Collection was done with the K-tube for collecting EBC and EtOH, with two collections having a filter present in the saliva trap. The lessons and modifications that we were obtained from our optimization experiments were used for the collection of these final drugs. The collection times for T0, T1, and T2 were based on the action times of each individual drug in the body.

The blank process did not show any signs of the drugs, showing the lack of contamination in the system. However, there appeared to be some potential contamination in the H2O/ACN blank as peaks for etodolac and oxymetazoline HCL were present at the same retention times.

a. Dextromethorphan HCL:

This drug was not successfully detected in the subject (data not shown). The drug sample had clear peaks that were not present in any of the collections. Being that the drug is taken in liquid dose and with a very small dose of only 20 mg, it made sense that detection would be difficult.

b. Phenylephrine HCL:

This drug was successfully detected in the subject based on the single, clear strong peak at the exact same retention time as the standard (Figure 10). The mass present in the peak also correlated with that of the standard. This peak was present in the T1 collection. However, the T0 collection had the peak slightly shifted to earlier in the run but had the correct mass. In addition, there was also a peak at 4.249 mins that had the correct mass. While, this could be possible contamination, with the difference in retention time, it was determined that they could be ruled out as negative for the T0 collection.

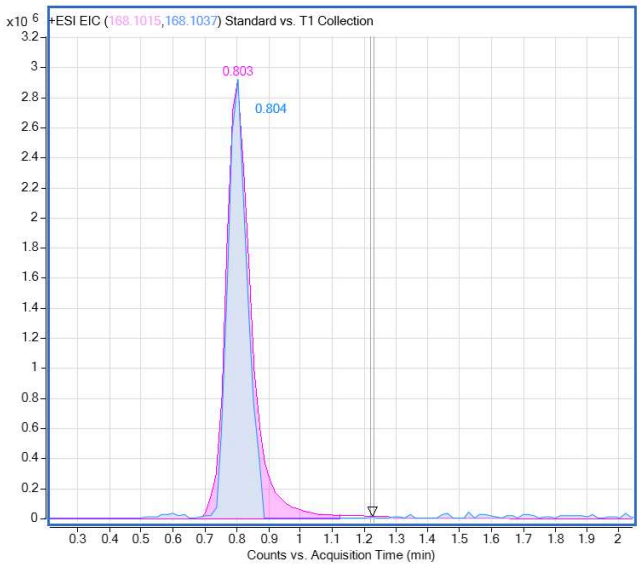

Figure 11: Extracted Ion Chromatograms (EICs) for Phenylephrine HCL in EBC (blue) and Phenylephrine HCL Pill Standard (pink).

c. Oxymetazoline HCL:

This drug was successfully detected in the subject based on the exact mass at the correct retention time as the standard. This was present in the T1 collection. The T0 collection had a peak at the correct retention time, but the mass was slightly off. This could indicate a contamination as the mass was close enough to be considered, but the drug would then be present in the wrong collection time. The T2 collection did not have a peak and the drug was not detected.

d. Etodolac:

This drug was not successfully detected in the subject (data not shown). There were no peaks present in any of the collections at the correct retention time. Fortunately, this does go to show that the detected contamination of etodolac in the H2O/ACN blanks did not contaminate these samples and thus likely not have contaminated the other samples.

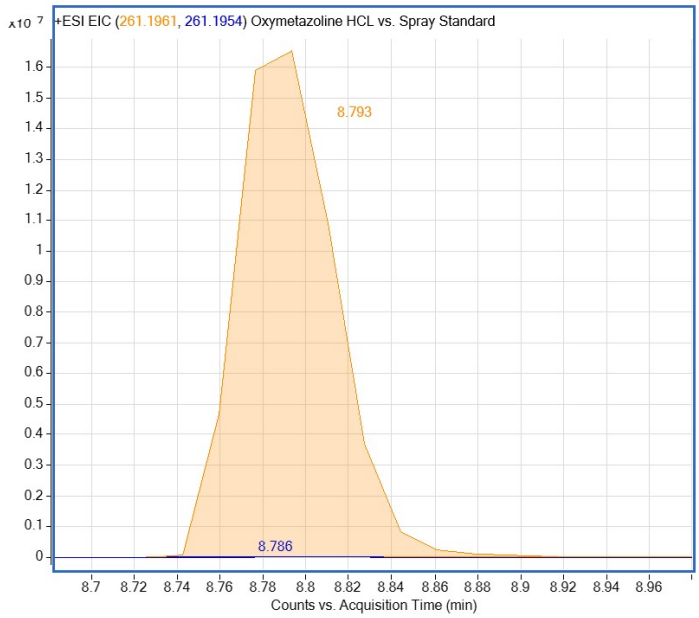

Figure 22: Extracted Ion Chromatograms (EICs) for Oxymetazoline HCL EBC (blue) and Oxymetazoline HCL Spray (orange)

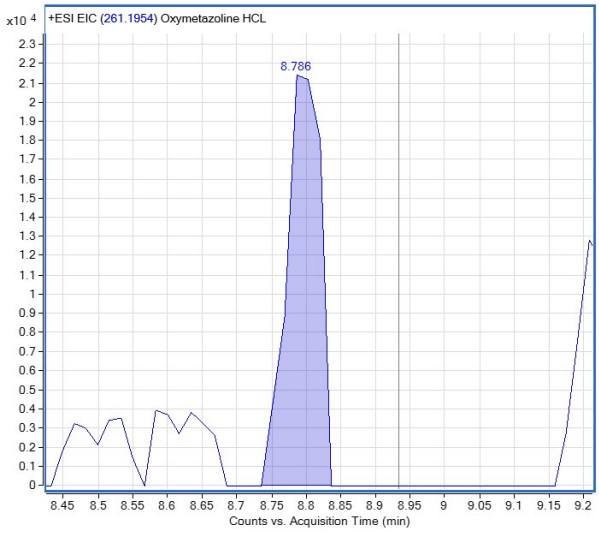

Figure 33: Extracted Ion Chromatograms (EICs) for Oxymetazoline HCL EBC to scale

e. Hydrocortisone:

This drug was not successfully detected in the subject (data not shown). There were no corresponding peaks at the correct retention time that had the mass we were looking for. The T0, T1, and T2 collections looked to mainly have noise and not any defined peaks.

f. Albuterol Sulfonate:

The drug could not be completely marked as having been detected. There was no standard that we were able to make for the drug as it is an aerosol. However, we were able to find databases that gave predicted masses. The T2 collection had a peak at 9.21 min that corresponded exactly with the predicted mass. This mass peak was not detected in T0 or T1 collections. This could show a good indication that the peak detected in T2 was a confirmation due to the drug needing time to manifest in the blood.

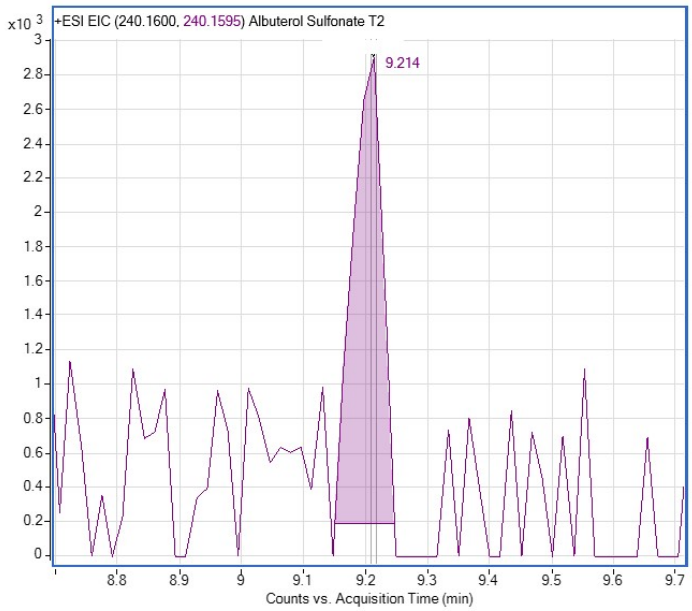

Figure 54: Extracted Ion Chromatograms (EICs) for Albuterol Sulfonate at T2

4 Conclusion

The determination of analyte detection for this experiment was based on retention time and theoretical exact mass of the main precursor ion. For example, Acetaminophen was determined to have an early retention time of approximately 2.7 minutes and a mass of 152.07 m/z. If this was not exact or close enough to the level of confirmation that we required, then mass of metabolites were quantified based on the parent molecules pharmacokinetics. The retention time and theoretical exact mass were tested with our drug standards that were run through the same method and a pattern of recognition established for their determination. Especially when an insufficient amount of sample is collected, there was a procedure to attempt to equalize the amounts acquired. The final test battery of drugs, while short and less than ideal due to circumstances, showed a strong potential for detecting a variety of drugs.

While this experiment did not yield the results that we were searching to discover, there shows enough promise to expect another simple and non-invasive proposed technique to detect and determine concentration of drugs in EBC. That technique would provide a good way to preliminarily assess drug concentrations in impaired driving subjects and determine what steps to take from that point on. With further validation and improved quality control, these methods could be further fleshed out for a larger scale. Unfortunately, parts of the experiment were also affected by the Covid-19 pandemic that led to less samples and less subjects than anticipated. However, the respiratory nature of the virus and the masks used created other research questions on EBC that helped show the efficacy of EBC testing. If effective, EBC testing will reduce the need for drawing blood from suspects and would likely reduce or at least simplify the means of obtaining probable cause for further analysis in DUI/DUID cases. In addition, accurate detection and quantification of drugs in breath could eventually allow for technology to simplify DUI/DUID detection

procedures down to a single breathalyzer test after impairment has been determined by a trained officer or DRE.

References:

1. Fierro I, Colás M, González-Luque JC, Álvarez FJ. Roadside opioid testing of drivers using oral fluid: the case of a country with a zero tolerance law, Spain. Substance Abuse Treatment, Prevention, and Policy 2017;12(1). https://doi.org/10.1186/s13011-017-0108-3.

2. Lipari RN. Key Substance Use and Mental Health Indicators in the United States: Results from the 2018 National Survey on Drug Use and Health. 2018;:82.

3. Vadivelu N, Kai AM, Kodumudi V, Sramcik J, Kaye AD. The Opioid Crisis: a Comprehensive Overview. Curr Pain Headache Rep 2018;22(3):16. https://doi.org/10.1007/s11916-018-0670-z.

4. Schepens E, Inman S, McCullough BJ, Hopley C. Rapid confirmation and quantitation of drugs-of-abuse in oral fluid using a low cost, small footprint mass spectrometer. Forensic Chemistry 2017;4:75–81. https://doi.org/10.1016/j.forc.2017.03.002.

5. Overview of Pharmacokinetics - Clinical Pharmacology. Merck Manuals Professional Edition. https://www.merckmanuals.com/professional/clinical-pharmacology/pharmacokinetics/overview-of-pharmacokinetics (accessed January 2, 2021).

6. Martin AN, Farquar GR, Jones AD, Frank M. Human breath analysis: methods for sample collection and reduction of localized background effects. Analytical and Bioanalytical Chemistry 2010;396(2):739–50. https://doi.org/10.1007/s00216-009-3217-7.

7. Aksenov AA, Zamuruyev KO, Pasamontes A, Brown JF, Schivo M, Foutouhi S, et al. Analytical methodologies for broad metabolite coverage of exhaled breath condensate. Journal of Chromatography B 2017;1061–1062:17–25. https://doi.org/10.1016/j.jchromb.2017.06.038.

8. Zamuruyev KO, Aksenov AA, Pasamontes A, Brown JF, Pettit DR, Foutouhi S, et al. Human breath metabolomics using an optimized non-invasive exhaled breath condensate sampler. Journal of Breath Research 2016;11(1):016001. https://doi.org/10.1088/1752-7163/11/1/016001.

9. Borras E, Cheng A, Wun T, Reese KL, Frank M, Schivo M, et al. Detecting opioid metabolites in exhaled breath condensate (EBC). Journal of Breath Research 2019;13(4):046014. https://doi.org/10.1088/1752-7163/ab35fd.

10. Jones A. Measuring Alcohol in Blood and Breath for Forensic Purposes - A Historical Review. 1996.

11. Lawal O, Ahmed WM, Nijsen TME, Goodacre R, Fowler SJ. Exhaled breath analysis: a review of 'breath-taking' methods for off-line analysis. Metabolomics 2017;13(10). https://doi.org/10.1007/s11306-017-1241-8.

12. Anderson K, Coats T, Monks P, White I, Pandya H, Beardsmore C, et al. A COMPARISON OF TIDAL AND INCENTIVE BREATH COLLECTION METHODS FOR THE DETERMINATION OF BREATH VOLATILES CONCENTRATION. Emerg Med J 2015;32(12):983. https://doi.org/10.1136/emermed-2015-205372.15.

13. Himes SK, Scheidweiler KB, Beck O, Gorelick DA, Desrosiers NA, Huestis MA. Cannabinoids in Exhaled Breath following Controlled Administration of Smoked Cannabis. Clinical Chemistry 2013;59(12):1780–9. https://doi.org/10.1373/clinchem.2013.207407.

14. Chen D, Bryden WA, McLoughlin M. A Novel System for The Comprehensive Collection of Nonvolatile Molecules from Human Exhaled Breath. :16.

15. Zamuruyev KO, Borras E, Pettit DR, Aksenov AA, Simmons JD, Weimer BC, et al. Effect of temperature control on the metabolite content in exhaled breath condensate. Analytica Chimica Acta 2018;1006:49–60. https://doi.org/10.1016/j.aca.2017.12.025.

16. Leung NHL, Chu DKW, Shiu EYC, Chan K-H, McDevitt JJ, Hau BJP, et al. Respiratory virus shedding in exhaled breath and efficacy of face masks. Nat Med 2020;26(5):676–80. https://doi.org/10.1038/s41591-020-0843-2.

17. Pleil JD, Wallace MAG, Madden MC. Exhaled breath aerosol (EBA): the simplest non-invasive medium for public health and occupational exposure biomonitoring. J Breath Res 2018;12(2):027110. https://doi.org/10.1088/1752-7163/aa9855.

18. Beck O, Stephanson N, Sandqvist S, Franck J. Detection of drugs of abuse in exhaled breath using a device for rapid collection: comparison with plasma, urine and self-reporting in 47 drug users. J Breath Res 2013;7(2):026006. https://doi.org/10.1088/1752-7155/7/2/026006.

19. Beck O, Leine K, Palmskog G, Franck J. Amphetamines Detected in Exhaled Breath from Drug Addicts: A New Possible Method for Drugs-of-Abuse Testing. Journal of Analytical Toxicology 2010;34(5):233–7. https://doi.org/10.1093/jat/34.5.233.

20. Beck O, Sandqvist S, Böttcher M, Eriksen P, Franck J, Palmskog G. Study on the Sampling of Methadone from Exhaled Breath. Journal of Analytical Toxicology 2011;35:7.