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Development Of A Liquid Chromatography Tandem Mass Spectrometry Method For The Detection And Quantification Of Eleven Common Over-The-Counter Medications In Human Urine

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Abstract

Common over-the-counter medications such as ibuprofen, loratadine, and naproxen inhibit glucuronosyl transferase, which are responsible for the metabolism and promoting excretion of phthalates, ubiquitous industrial chemicals and environmental pollutants. Existing studies on phthalate diesters and their monoester metabolite concentrations in humans have shown individual metabolism of phthalates varies both between and within individuals, but the etiology of this inter- and intra-individual variation remains unknown. Glucuronosyl transferase inhibition by over-the-counter medications may affect individual phthalate metabolism, and therefore may explain these patterns. In the following study, novel liquid chromatography tandem mass spectrometry and solid phase extraction methods were developed to quantify over-the-counter medication levels in human urine. This method development was completed in tandem with data analysis of human urine samples of known phthalate monoester levels, with the ultimate purpose of these studies being the assessment of over-the-counter medications in individual human metabolism of phthalates.

1. Introduction

Glucuronidation is a metabolic pathway activated by a superfamily of enzymes known as UDP-glucuronosyltransferases (UGTs). UGTs catalyze the conversion of hydrophobic substrates into more hydrophilic glucuronide conjugates to facilitate excretion through either urinary or biliary routes.[1] Glucuronidation, therefore, usually reduces toxicity because it can turn potentially toxic metabolites of xenobiotics into inactive conjugates.

Phthalate diesters are a class of ubiquitous industrial chemicals found in a wide variety of common products including plastics and plasticizers such as polyvinyl chloride (PVC), floor and wall coverings, solvents, personal care products such as cosmetics, and food packaging.[3,4,13] Phthalate exposure is widespread,[5] and in 2000 it was determined that phthalate exposure is higher and more common than suspected previously.[3] Upon entering the body, phthalate diesters are rapidly hydrolyzed into their respective monoester form. These phthalate monoesters then usually undergo Phase II biotransformation via UDP-glucuronosyltransferase enzymes to form glucuronide-conjugated monoesters in order to render them hydrophilic enough to facilitate renal excretion. Short chain and more hydrophilic phthalate monoesters, such as monoethyl phthalate (MEP) are often rapidly excreted in urine unconjugated ("free") without glucuronidation, whereas longer chain monoesters such as mono-(2-ethylhexyl) phthalate (MEHP) and its metabolites mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) are more likely to be excreted in their conjugated form.[5]

Exposure to phthalates has been connected with an array of negative health effects in rodents and humans. In laboratory animal studies, phthalate exposure has been linked to impaired development of the male reproductive system.[6] Likewise, in human subjects, a positive dose-response relationship between urinary levels of the MEP and DNA damage in sperm was found, indicating phthalates may have detrimental effects on the human reproductive

system as well.[11] Research suggests phthalate monoesters are the primary agent responsible for the toxicity associated with phthalate exposure.[6] Phthalate monoester exposure has been characterized by previous studies,[5,13] but few studies have examined the factors that affect phthalate monoester metabolism.

Glucuronidation of endogenous and exogenous compounds varies significantly both between individuals and within the same individual, but the etiology of that interindividual and intraindividual variation are not well characterized.[1,6,7,8] UGT deficiency, in association with other risk factors, is thought to be linked to an increased risk of developing cancers of the solid tissue, including colorectal, gastrointestinal, lung, and liver cancer.[7] A genetic deficiency in UGT expression also causes congenital diseases such as Gilbert's Syndrome and Crigler-Najjar syndrome.[2]

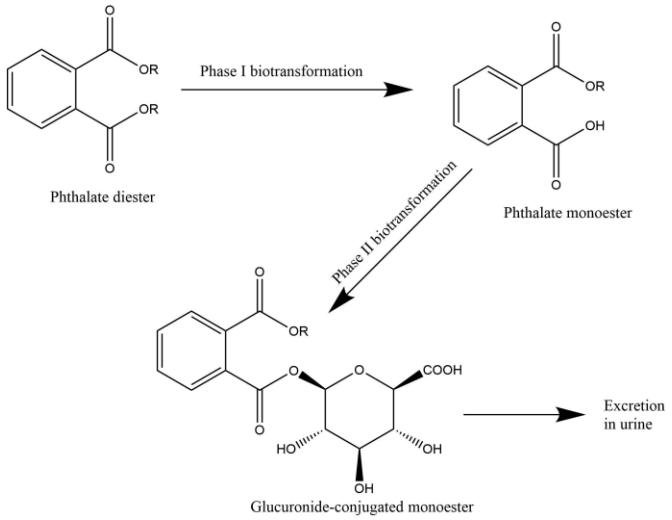

Figure 1. General structure of the metabolic pathway of a phthalate diester.

One possible explanation for differences in individual UGT activity is genetic variation. In patients with Gilbert's Syndrome, the presence of an additional TA nucleotide pair in a TATAA element in the promoter region for the bilirubin-specific UGT enzyme, bilirubin UDP-glucuronosyltransferase 1, has been shown to be associated with the disease.[2] While various other UGT enzymes are responsible for the glucuronidation of xenobiotics such as phthalates, evidence was found for a genetic basis for UGT deficiencies.[2] Males with the addition TA nucleotide pair did exhibit increased levels of bilirubin in serum, whereas females did not,[2] indicating a possibility that males are more susceptible to genetic UGT deficiency.

Furthermore, studies suggest that UGT activity is higher in the jejunum of the small intestine than elsewhere in the gastrointestinal tract, including the liver, and that polymorphic regulation of UGT mRNA effects UGT expression in the jejunum, but not in the liver.[1] Polymorphic regulation of UGT genes in the jejunum can lead to variation in catalytically active UGT enzymes between individuals, which could therefore explain interindividual differences. However, as was found with nicotine glucuronidation,[10] it is likely that genetics are not the sole determinant of interindividual variability.

Studies of demographic and lifestyle factors have been examined in relation to the metabolism of phthalates have been undertaken.[3,5,9] Broadly, characteristics and individual factors analyzed in recent literature include: age, sex, race/ethnicity, socioeconomic status, urban/rural residence, education, body mass index, cancer history, menopausal status (for women), smoking habits, alcohol use, caffeine consumption, prescription medication use, postmenopausal hormone use (in women), season, and time of sample collection.[3.5.9] These studies have identified a variety of associations, including the fact that unconjugated di-2-ethylhexyl phthalate (DEHP) metabolites occur in greater concentrations between 3-6 pm,[5] indicating that the time of day can play a role in individual glucuronidation capacity. Other findings include that women of childbearing age were determined to have higher concentrations of unconjugated monobutyl phthalate (MBP) in their urine,[3] and these findings are corroborated by the finding that women taking postmenopausal hormones were found to have lower levels of MEHP than premenopausal women.[9]

Table 1. Summary of drugs known to inhibit UGT activity. Adapted from Grancharov *et at*.[12]

Inhibitor	***Substrate***	***Apparent K_i (μM)***
Probenecid	AZT	530
Salicylic acid	4-MU	367
Naproxen	AZT	200
S-fenoprofen	4-MU	220
Naloxone	Morphine	630
Morphine	AZT	900
Diazepam	Morphine	230
Flunitrazepam	Morphine	130
Lorazepam	AZT	160
Oxazepam	4-MU	188
Temazepam	4-MU	217
Amitriptyline	Codeine	130
Novobiocin	Bilirubin	50
Chloramphenicol	Codeine	270

Over-the-counter drugs (OTCs) are an attractive avenue of investigation because several common medications, including many OTCs, are known to inhibit glucuronidation, such as the antibiotic novobiocin, antidepressants amitriptyline and imipramine, uricosuric agent probenecid, salicylic acid (a metabolite of aspirin) and salicylamide, and nonsteroidal anti-inflammatory drugs (NSAIDs) (S)-naproxen, ibuprofen, fenoprofen, and ketoprofen, as well as loratadine and its metabolites, desloratadine and 3-hydroxy desloratadine.[12] A brief summary of these data can be found in Table 1. The potential effects of OTC-based inhibition of UGT activity on an individual's glucuronidative profile will be examined in the following study. For the purposes of the following study, the OTCs and common drugs loratadine, desloratadine, 3-hydroxy desloratadine, ibuprofen, 2-hydroxyibuprofen, carboxy-ibuprofen, acetaminophen, salicylic acid, caffeine, theobromine, and naproxen have been identified for urinalysis via liquid chromatography and tandem mass spectrometry (LC/MS/MS).

Analysis of OTCs in urine or plasma is typically done using liquid chromatography-tandem mass spectrometry.[14-19] LC-MS/MS is useful in the analysis of urinary metabolites because it allows for the characterization of dissolved compounds based on mass, and since all of the identified OTCs have different masses, it can be useful in determining whether any or all of them are present in a given urine sample.

The goal of the following study, therefore, consisted of three phases. The first phase consisted in the analysis of a large dataset of human urine samples to identify individuals with chronically low or episodically low glucuronidative activity. The second phase consisted in developing an LC/MS/MS method for the identification and quantification of eleven common OTCs in human urine. The third phase will consist in examining the relationship between low UGT activity and the presence of certain OTCs. By examining whether these compounds are present in samples of urine known to contain unusually high levels of unconjugated phthalate monoester, as measured by higher levels of percent free MEHHP than the population average, we can begin to determine whether the inhibitory effects of these OTCs plays a role in an individual's varying glucuronidative profile. The first two of this study's three objectives are reported below.

2. Methods

2.1. Sample acquisition

The identification of over 50 urine samples with unusually high levels of unconjugated phthalate monoester prompted this investigation into potential causes of chronic or episodic glucuronosyltransferase inactivity. From a previous Environmental and Reproductive Health (EARTH) study,[5] a dataset was acquired containing data on the levels of various free and conjugated phthalate monoesters in a varying number of urine samples from 268 people, male and female, who attended a fertility clinic. The percent of free MEHHP to conjugated MEHHP was selected as a test for individual glucuronidative capacity due to the fact that MEHHP is a secondary metabolite of DEHP, a long-chain phthalate diester and therefore more likely to be excreted in the conjugated form,[5] thus serving as a more accurate measure of individual UGT activity. During this study, a population average percent free MEHHP of 14.36% was calculated from the entire 268 individual population, and the highest and lowest percent free MEHHP samples were selected from 15 randomly selected female individuals who had an above average percent free MEHHP (Table 2). The highest and lowest free MEHHP samples were selected from 10 randomly selected female individuals with a consistently average or below average percent free MEHHP to serve as a control. These data also demonstrate that intra-individual glucuronidation capacity can vary greatly, as all individuals identified for having above average free MEHHP levels were below average at certain testing times.[5] This indicates that UGT activity even in these individuals may fall along a baseline level, but certain episodic disruptions in activity can be triggered, and the hypothesis of this study is that OTCs may serve as that deactivator.

Table 2. Percent free MEHHP values for the 15 women whose samples were acquired for analysis for the presence of OTCs.

Subject	***Percent free MEHHP***
Population average	14.36%
F2013	39.28%
F2017	22.95%
F2031	16.67%
F2052	47.23%
F2054	27.28%
F2059	44.39%
F2060	20.81%
F2072	26.65%
F2077	27.93%
F2084	35.19%
F2088	46.42%
F2103	25.11%
F2107	37.81%
F2134	23.10%
F2145	22.51%

2.2. Sample preparation

A 1 M ammonium acetate buffer was prepared, and glacial acetic acid was added dropwise until a pH of 6.5 was reached. To 2 mL of this ammonium acetate buffer, 40 µL of β-glucuronidase enzyme was added and mixed. 5 µL of the resultant solution was added to each sample. A 200 ppm solution of 4-methylumbelliferone glucuronide (4-Me-Um) was prepared. For enzymatic deglucuronidation of samples, 2 mL of urine sample was combined with 1 mL of HPLC-grade water, 5 µL of 200 ppm 4-Me-Um glucuronide, 50 µL of internal spiking solution, and 5 µL of β-glucuronidase/ammonium acetate buffer. The resultant mixture was incubated in a water bath at 37 °C for 90 minutes.

2.3. Solid phase extraction

All analytes were extracted from urine using an Oasis HLB solid phase extraction (SPE) cartridge under a vacuum. Extraction cartridges were pretreated with 5 mL of methanol, followed by 5 mL of water, both at 1 mL/min (-0.3 bar). 2 mL of urine samples prepared as described above were then loaded onto the cartridge and pulled through slowly. The cartridge was then washed with 5 mL HPLC-grade water and air was pulled through the sorbent to remove excess water for 2 minutes. Analytes were then eluted using 4 mL of methanol and 4 mL of methanol:formic acid (97.5:2.5 v/v) and then air was pulled through for 30 seconds to capture all analytes. Samples were then placed in a TurboVap Evaporator under a gentle stream of nitrogen at 65 °C for 90 minutes. After evaporation was complete, samples were reconstituted in 200 µL of HPLC-grade water:methanol (95:5 v/v). This protocol yielded recovery rates between 55% and 99%.

2.4. Liquid chromatography mass spectrometry analysis

Liquid chromatography tandem mass spectrometry was performed using a Shimadzu triple quadrupole electrospray ionization (ESI) LC/MS/MS using a Shimadzu C18 3 µm 50 x 4.8 mm column. The solvent protocol used in the developed method was A: 5 mM ammonium acetate and formic acid in water, pH 3.20, and B: 1.33 mM formic acid in methanol. Samples were run on the liquid chromatograph for 20 minutes with a flow rate of 0.5 mL/min. Initial conditions were 5% solvent B which then climbed to 85% over the course of 14 minutes, after which time the column was re-equilibrated back to initial conditions for the remainder of the run.

In order to detect the 11 different analytes and their respective internal standards in urine, mass spectrometry was employed using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer.

3. Results

Table 3 contains the data gathered for each individual analyte and its respective internal standard during method development, including ESI mode, precursor and product m/z, collision energies, and quadrupole pre-biases. An example spectrum can be seen in Figure 3. Limits of detection were found for all eleven analytes, as seen in Table 4.

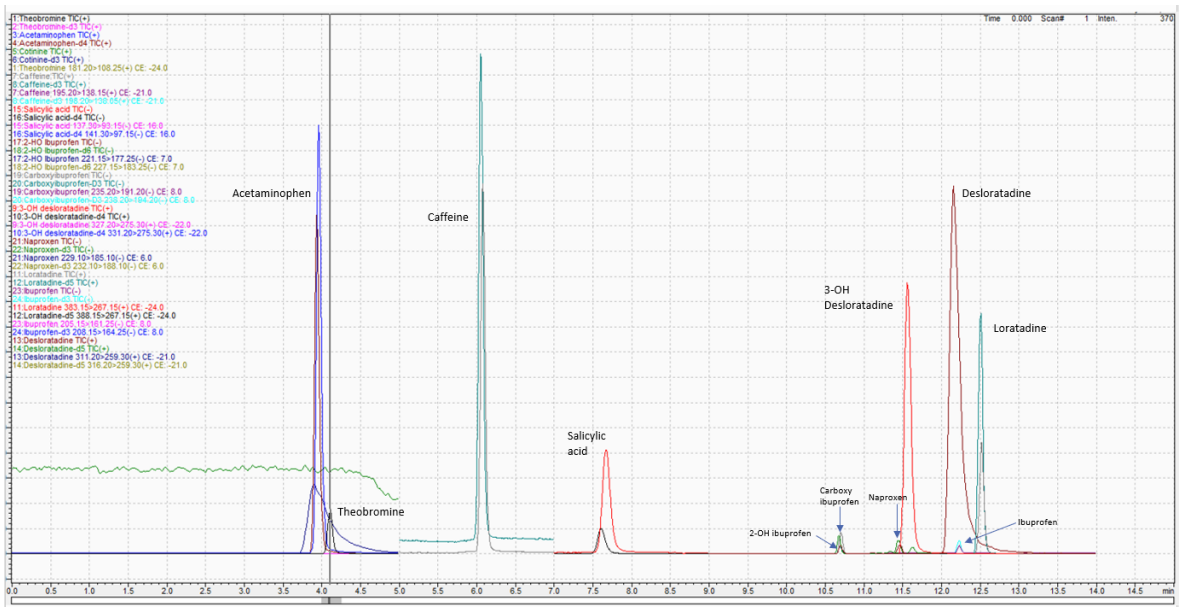

Figure 3. A complete chromatogram of the LC/MS/MS method developed during the course of this study.

Table 3. LC/MS/MS conditions for all tested OTCs. All compounds were tested in multiple reaction monitoring (MRM) mode.

Analyte	*Retention time window (min)*	*Mode*	*Precursor m/z*	*Product m/z*	*Q1 pre bias (V)*	*CE (V)*	*Q3 pre bias (V)*
Theobromine	0-5	+	181.20	108.25	-11.0	-24.0	-18.0
Theobromine-d3	0-5	+	184.20	70.05	-11.0	-24.0	-18.0
Acetaminophen	0-5	+	152.15	110.15	-15.0	-18.0	-20.0
Acetaminophen-d4	0-5	+	156.15	114.10	-15.0	-24.0	-18.0
Caffeine	5-7	+	195.20	138.15	-10.0	-21.0	-24.0
Caffeine-d3	5-7	+	198.20	138.05	-10.0	-21.0	-24.0
3-hydroxy desloratadine	10.5-12	+	327.20	275.30	-16.0	-22.0	-29.0
3-hydroxy desloratadine-d4	10.5-12	+	331.20	275.30	-16.0	-22.0	-29.0
Loratadine	11.25-14	+	383.15	267.15	-18.0	-24.0	-23.0
Loratadine-d5	11.25-14	+	388.15	267.15	-18.0	-24.0	-23.0
Desloratadine	10.5-12	+	311.20	259.30	-15.0	-21.0	-27.0
Desloratadine-d5	10.5-12	+	316.20	259.30	-15.0	-21.0	-27.0
Salicylic acid	6-8.5	-	137.30	93.15	11.0	16.0	18.0
Salicylic acid-d4	6-8.5	-	141.30	97.15	11.0	16.0	18.0
2-hydroxy ibuprofen	8-12	-	221.15	177.25	14.0	7.0	16.0
2-hydroxy ibuprofen-d6	8-12	-	227.15	183.25	14.0	7.0	16.0
Carboxy-ibuprofen	9-12	-	235.20	191.20	13.0	8.0	24.0
Carboxy-ibuprofen-d3	9-12	-	238.20	194.20	13.0	8.0	24.0
Naproxen	11-14	-	229.10	185.10	10.0	6.0	17.0
Naproxen-d3	11-14	-	232.10	188.10	10.0	6.0	17.0
Ibuprofen	11.25-14	-	205.15	161.25	13.0	8.0	24.0
Ibuprofen-d3	11.25-14	-	208.15	164.25	13.0	8.0	24.0

Table 4. Limits of detection for each of the 11 OTCs.

Analyte	**LOD (ppb)**
Acetaminophen	0.41
Caffeine	1.4
Theobromine	0.91
Loratadine	0.51
Desloratadine	1.9
3-hydroxy desloratadine	0.27
Salicylic acid	2.5
Ibuprofen	7.1
2-hydroxy Ibuprofen	21
Ibuprofen carboxylic acid	3.0
Naproxen	15

4. Discussion

A LC/MS/MS method was developed to identify and quantify eleven common OTCs in human urine. To optimize the chromatographic separation phase, a variety of different mobile phase systems were examined and adapted from the literature. A 5 mM ammonium acetate and formic acid solution at pH 3.20 and 1.33 mM formic acid in methanol was found to be the most optimal for allowing the separation of the broad list of OTCs of interest.

The mass spectrometry parameters listed in Table 3 were optimized via a series of automated precursor ion scans, followed by a product ion scan to search for the fragments that yielded the greatest sensitivity based on previously acquired precursor ions. Q1 pre-rod biases, collision energies, and Q3 pre-rod biases were optimized via an automated procedure in order to find the voltages that allowed for the greatest detection of analytes. Six of the eleven compounds analyzed showed greater sensitivity in the positive ionization mode, while the non-steroidal anti-inflammatory drugs ibuprofen, naproxen, and the metabolites of ibuprofen, as well as salicylic acid showed greater sensitivity in the negative ionization mode. For all eleven analytes, the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ ions were selected as precursor ions.

In the positive ionization mode, the fragmentation of theobromine m/z 181.20 resulted in a negative ion m/z 108.25, and for caffeine, similar fragmentation resulted in the transition from m/z 195.20→138.15. For acetaminophen, a transition of m/z 152.15→110.15 was observed. For loratadine, desloratadine, and 3-hydroxy desloratadine, transitions of m/z 383.15→267.15, m/z 311.20→259.30, and 327.20→275.30 were observed respectively.

In the negative ionization mode, fragmentation patterns typically corresponded to the loss of a carboxylic acid group m/z 44. This resulted in transitions m/z 137.30→93.15 for salicylic acid, m/z 229.10→185.10 for naproxen, m/z 205.15→161.25 for ibuprofen, m/z 235.20→191.20 for carboxy-ibuprofen, and m/z 221.15→177.25 for 2-hydroxy ibuprofen.

5. Conclusion

Both an SPE and LC/MS/MS method were developed to facilitate the quantification of eleven common OTCs in human urine. With these methods, future work continuing where this study left off will include the analysis of urine samples of known glucuronidation activity in order to determine whether OTCs play a role in inhibiting UGT activity in humans.

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