Application Note 00317

Simultaneous Detection of Steroid, Amine and Opiate Glucuronidation with a Single, Rapid LC/MS/MS Method

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Introduction

Glucuronidation is part of the Phase II or Conjugation metabolism within the body. It involves the transfer of glucuronic acid from uridine-diphosphoglucuronic acid to the xenobiotic being metabolized. Other examples of conjugation reactions in drug metabolism include sulfonation, amino acid addition, and glutathione detoxification.

Uridine-glucuronyl Transferases (UGTs), the enzyme family responsible for glucuronidation of xenobiotics, are named based on the divergent evolution of the UGT genes. Three families of UGTs have been identified in humans: UGT1, UGT2, and UGT8. Of these, UGT1 and 2 have been shown to be most relevant in the glucuronidation of xenobiotics. The enzymes are further divided into sub-families determined by alternative splicing of the first exon (UGT1 family) or the unique gene encoding them (UGT2 family). In humans, at least 12 different splicings have been identified for the UGT1 family. The UGT2 family appears to have 18 members, all of which are in the UGT2B subfamily. Only one 2A sub-family member is known to exist, 2A1, and very little is known about the substrates of 2C1 (1).

An understanding of UGTs, and their substrates, is becoming increasingly important from an epidemiological perspective, as well as a pharmacological one. For example, UGT1A1 polymorphisms have been implicated in increased risk of breast cancer in African American women (2). It has been found that steroid metabolism catalyzed by UGT1A1 is important for maintaining the intracellular steady-state levels of estrogens in certain tissue(s) (3). Furthermore, pharmaceutical companies are increasingly interested in the ADME/Tox profile(s) of the new chemical entities (NCEs) being produced. The kinetics of phase II metabolism may also be of interest, depending on the compound. However, UGTs demonstrate a very broad reactivity towards xenobiotics and, consequently, there is considerable overlap in their substrate specificities. Although individual UGTs cannot be classified as having only reactivity for a particular functional group – that is, no single UGT catalyzes **only** amino groups – UGTs can be differentiated based on their reactivity for an array of classical (or typical) compounds. We present here a method for the qualification and quantification of the glucuronidation of three prototypical compounds – steroids, amines, and opiates. This method is of interest from an enzyme kinetics perspective (with the use of microsomes or supersomes) and as a means to detect opiate metabolites in plasma for the possible detection of a disease state or the classification of ADME/Tox properties of an NCE.

Instrumentation

Varian 320-MS LC/MS/MS equipped with an ESI source

Two Varian ProStar™ 210 Solvent Delivery Systems

CTC Analytics HTS PAL AutoSampler

Materials and Reagents

All chemicals were reagent or HPLC grade from Sigma-Aldrich (St. Louis, MO) with the exception of the morphine and morphine-glucuronides (Cerilliant, Little Rock, TX) and Nicotine-N-ß-glucuronide (Toronto Research Chemicals, Inc., Toronto, ON).

Sample Preparation

All stock solutions were prepared in 50:50 Acetonitrile:Water at 1 mg/ml. All dilutions were prepared in 50% aqueous acetonitrile + 0.1% acetic acid.

Conditions

Mass Spectrometry Conditions

Ionization Mode ESI (positive and negative)

Collision Gas	2.0 mTorr Argon
API Drying Gas	40 psi at 330 ˚C (Ramping to 260 ˚C over 3 min after a 2 min delay)
API Nebulizing Gas	55 psi
SIM Width	0.7 amu
Needle	5000V
Capillary	Scanning (See Scan Parameters below)
Shield	600V
Detector	1700V

LC Conditions

Column	Varian Pursuit® C18 3 µm 100 x 2 mm (Varian Part #A3001100X020)
Solvent A	0.1% acetic acid in water
Solvent B	Acetonitrile
Injection Volume:	5 µL

LC Program

Time	%A	%B	Flow ml/min
0:00	95	5	0.2
0:30	95	5	
1:30	95	5	
2:00	57	43	
3:45	5	95	
4:30	5	95	
4:45	95	5	
5:00	95	5	
6:00	95	5	

MS/MS Scan Parameters

Polarity	Cap (V)	Name	Prec. Ion	Prod. Ion	CE (V)	Dwell (sec)
Pos	50	NicGluc	339.3	132	26.5	0.25
Pos	50	NicGluc	339.3	163	12.5	0.25
Neg	75	NapGluc	319	113	13	0.25
Neg	75	NapGluc	319	143	24	0.25
Pos	90	MorphGluc	462.2	286	28	0.25
Pos	90	MorphGluc	462.2	268	26	0.25
Neg	100	EstraGluc	447	113	23	0.25
Neg	100	EstraGluc	447	271	26.5	0.25

Discussion

Good separation of all glucuronides is obtained in less than five minutes using a Pursuit C18 (3 µ) column with 0.1% aqueous acetic acid (Solvent A) and acetonitrile (Solvent B) (see Figure 1). Calibration curves were prepared as serial dilutions from 1,000 to 2 ppb in10 steps (triplicate runs per sample). All calibration curves appear linear in this range (see examples in Figures 2A and 2B) with correlation coefficients for all the glucuronide metabolites as follows: 0.999 (Estradiol-Glucuronide), 0.992 (Nicotine-Glucuronide), 0.986 (Naphthol-Glucuronide) and 0.999 (Morphine-Glucuronide).

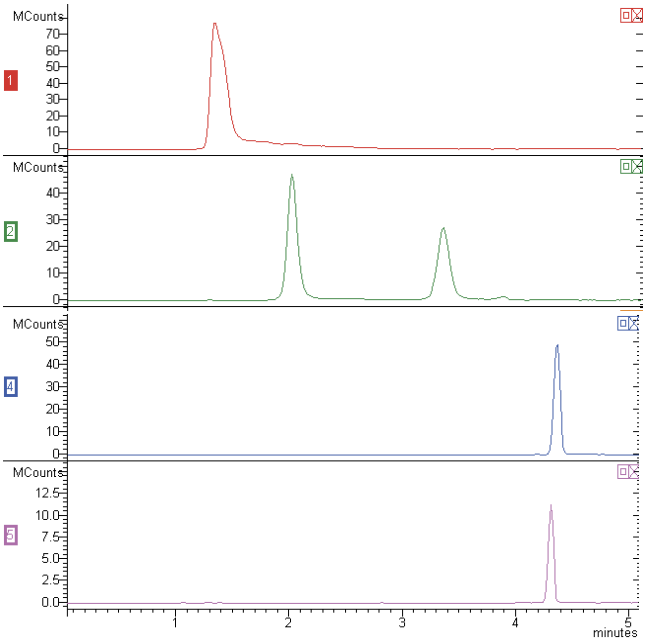

Figure 1 – Separation of all metabolites in a single 5 min run; from the top, Nicotine, Morphine, Estradiol, and Naphthol.

Sensitivity of the instrument is excellent, showing 244 fg detection (LOQ) for morphine-glucuronide in a clean matrix. As a "real world" test for this assay, two different matrices were tested to determine how the 320-MS could handle "dirty" matrices – enzyme kinetics assay mixture (i.e., high concentrations of salts and buffers) and a plasma-like matrix consisting of BSA in PBS (i.e., very high protein content and high salts).

An assay kinetics mixture was chosen to demonstrate the use of this procedure in a screening method to test an NCE for activity with a certain UGT (e.g., to test a new drug with UGT2B7 to determine its kinetics and if there is activity). To simulate a UGT kinetics mixture, a solution consisting of

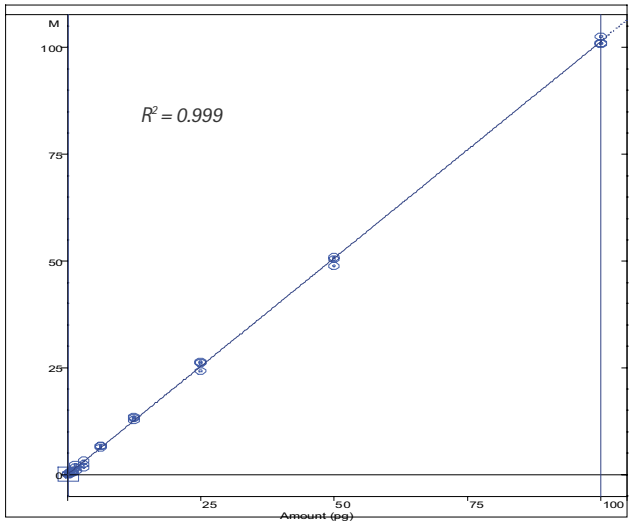

Figure 2A - Calibration curve of estradiol-glucuronide.

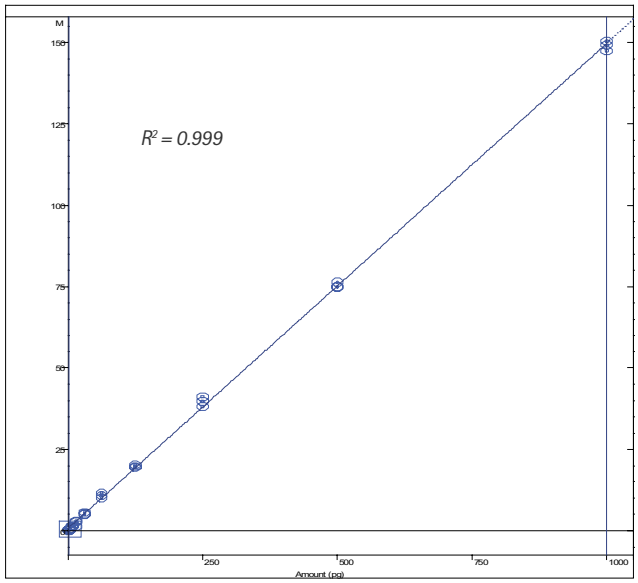

Figure 2B – Calibration curve of morphine-glucuronide.

50 mM Tris-HCl (pH = 7.5) with 8 mM MgCl2 was used to dilute morphine-6-glucuronide into various concentrations. Due to the fact that enzyme kinetics mixtures tend to be high in substrate concentration (the Km of morphine with UGT2B7 is approximately 350 µM in human liver microsomes (4)), a 1-to-100 dilution of this mixture is made with 50/50 water/acetonitrile so that the mass spectrometer is not overwhelmed. Figures 3A and B show the limits of quantitation (including spectra) of M6G vs. blank runs to be approximately 490 fg (on column).

Detection of morphine-6-glucuronide in a plasma-like matrix is another example for the use of this application. Drug metabolism studies are frequently undertaken in rats or dogs to determine the metabolic profiles of NCEs before clinical trials in humans. Determination of blood plasma levels of these drugs and their metabolites by LC/MS/MS is a common way of detecting clearance of the drug. As a surrogate to dog or rat plasma, bovine serum albumin (BSA) was used because of its availability and low cost. The matrix consists of 6 mM BSA in phosphate buffered saline (PBS).

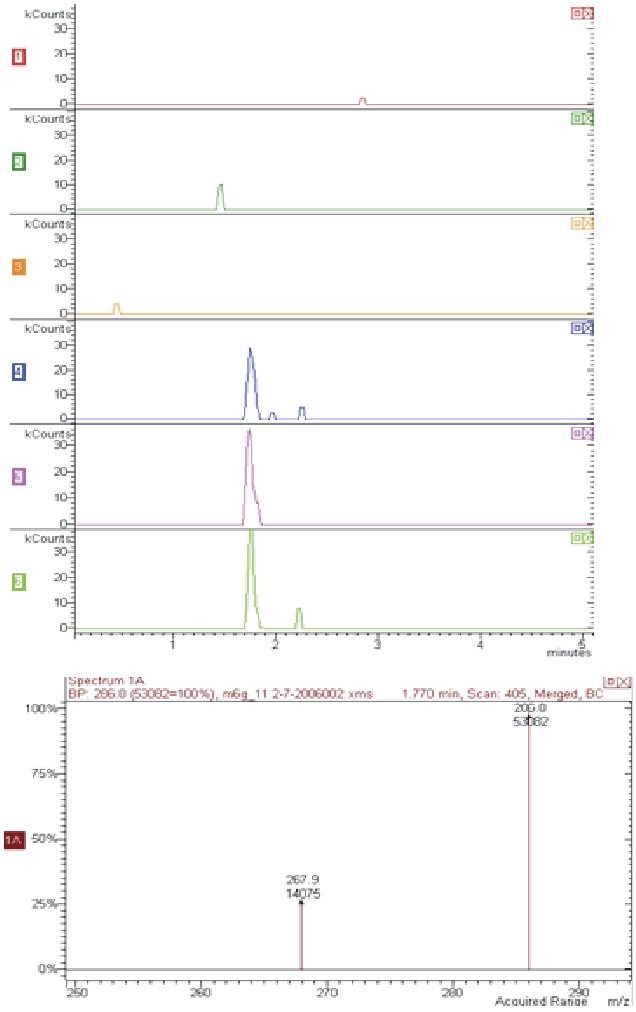

Figure 3A (Top) Morphine-glucuronide in an enzyme kinetics matrix (chromatogram 4-6) vs. blank injections (injections 1-3). Figure 3B (Bottom) Mass spectra of first injection of morphine-glucuronide.

Three concentrations spanning the range of typical blood plasma concentrations of M6G were created by adding 1, 2, or 3 µL of 100 ng/µL M6G into 500 µL of the plasma surrogate. 500 µL of a protein-crashing solution consisting of 50/50 methanol/acetonitrile with 0.5% acetic acid was added to the plasma surrogate mixture. Samples were placed in the refrigerator for 1 hr, centrifuged, and the supernatants collected. A 5 µL injection (in triplicate) of each yields approximately 50, 100, or 150 pg on column (see Figure 4). Typical blood concentration for a 100 mg CR dose of morphine is 37 +/- 15 ng/ml, equivalent to 110 pg, for the low end concentration, which is within the test sample limits (4).

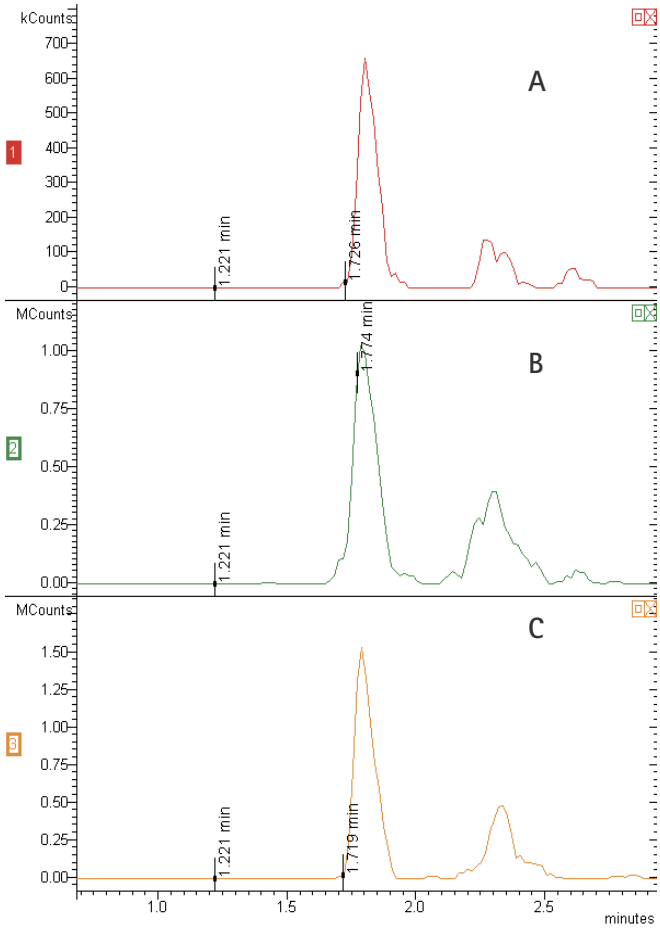

Figure 4 – Chromatograms of morphine-glucuronide at 50 (A), 100 (B), and 150 (C) pg (on column) in a plasma-like matrix.

Conclusion

This application note details the detection and quantitation of opioid-, amino-, and steroid- glucuronidation with a single, rapid, and sensitive LC/MS/MS method. Several different matrices were tested including an assay screening mixture and a plasma-like matrix consisting of BSA in PBS. It demonstrates femtogram sensitivity of morphine-glucuronide in a screening matrix as well as rapid and efficient detection of a drug metabolite in a plasma-like matrix. This application is useful for any pre-clinical pharmaceutical laboratory looking to characterize the UGT activity of an NCE or detect UGT metabolites in plasma-containing samples.

References

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3. Belanger, A., et al J. Steroid Biochem. Mol. Biol., **65** 301-310, 1998.
4. J Health Sciences **51(3)** 325-322 (2005).

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