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Liquid chromatography–tandem mass spectrometry method for measurement of nicotine *N*-glucuronide: A marker for human UGT2B10 inhibition

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ABSTRACT

Nicotine is considered to be a specific substrate for UGT2B10, an isoform of human uridine diphosphate glucuronosyltransferase (UGT). In the present study, a sensitive and selective liquid chromatography/tandem mass spectrometry (LC–MS–MS) method for quantification of nicotine *N*-glucuronide in pooled human liver microsomal incubates was developed and validated. Proteins in a 200 μL aliquot of incubation solution were precipitated by adding 40 μL 35% perchloric acid. The overall extraction efficiency was greater than 98%. Nicotine *N*-glucuronide and internal standard were recorded using selected reaction monitoring in positive ion electrospray with ion transitions of *m*/*z* 339–163 and *m*/*z* 342–166, respectively. The linear calibration curve was obtained over the concentration range of 10–1000 nM, with a lower limit of quantification of 10 nM. The intra-day and inter-day precision (% CV) and accuracy (% bias) of the method were within 15% at all quality control levels. Nicotine glucuronide in processed samples was stable for 24 h at room temperature and 48 h at 4 °C based on the stability experiments performed in this study. This established method was employed to evaluate the inhibitory effects of five target compounds including amitriptyline, hecogenin, imipramine, lamotrigine, and trifluoperazine on enzymatic activity of UGT2B10. IC_{50} values for inhibition of nicotine *N*-glucuronidation by amitriptyline, imipramine, lamotrigine, and trifluoperazine were calculated. Trifluoperazine was found to be a non-substrate inhibitor for human UGT2B10.

1. Introduction

Glucuronidation, catalyzed by the uridine diphosphate glucuronosyltransferases (UGTs), is an important metabolic pathway for the detoxification and elimination of many endobiotics (bilirubin, bile acids) and xenobiotics (zidovudine, acetaminophen) [1–3]. To date, at least 19 human UGTs have been identified and they are divided into two subfamilies in humans: UGT1 and UGT2 [4]. Liver is the major organ involved in glucuronidation, although some UGT isoforms are highly expressed in intestine and kidney [5,6]. UGTs participate in the transfer of glucuronic acid to a large number of structurally diverse substrates containing such acceptor groups as phenols, alcohols, amines, and carboxylic acids. Aliphatic or aromatic tertiary amine groups are quite common in many drugs and bioactive components, such as nicotine and tricyclic antidepressants and anticonvulsants. Human UGT1A4 has been believed to be the only enzyme involving N^+-glucuronidation of these amine-containing drugs until the recombinant human liver UGT2B10 expressed in insect cells was shown to catalyze the same metabolic reaction [7–9]. Studies using human liver microsomes and recombinant human UGT enzymes indicated that human UGT2B10 is another important UGT isoform contributing to the glucuronidation of some drugs containing aliphatic or aromatic tertiary amine functional groups and subsequently resulting in the formation of quaternary ammonium glucuronide metabolites [9–11]. However, only a limited number of isoform-selective UGT substrates and inhibitors have been identified due to the overlapping substrate specificity of human UGT isoforms [12,13], and no isoform-selective UGT inhibitory antibody is available to date [8]. Hecogenin and trifluoperazine were known substrates and selective inhibitors of UGT1A4 [8,14,15]. Whereas, it remains unclear whether hecogenin and trifluoperazine can inhibit the activity of UGT2B10.

Nicotine is one of the major bioactive constitutes in tobacco plants and detected in urine and plasma of smokers. Given that nicotine is a selective substrate of UGT2B10 at low substrate concentrations (approximately 0.1 mM) [10], monitoring nicotine *N*-glucuronide generated by human liver microsomes in the presence of compounds containing aliphatic or aromatic tertiary amine could be used to evaluate the potential effect of these drugs

Abbreviations: HLM, human liver microsomes; CID, collision induced dissociation; SRM, selected reaction monitoring; HPLC, high performance liquid chromatography.

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on nicotine *N*-glucuronidation catalyzed by human UGT2B10 and anticipate specific drug–drug interaction in clinical use.

High performance liquid chromatography coupled tandem mass spectrometry (HPLC–MS–MS) is an effective analytical approach to determine low concentrations of drugs or other ingested materials in biological matrices. Nicotine has two basic nitrogen moieties in pyrrolidine and pyridine rings with pK_a values at 8.1 and 3.0, respectively. In an aqueous solution, nicotine exists as free base, or in the mono- or diprotonated forms, depending on the pH of the solution [16]. *N*-Glucuronidation of nicotine occurs at the tertiary amine on its pyridine ring to generate a charged quaternary amine, which can greatly affect the retention and peak shape of nicotine glucuronide on an HPLC column. These problems can be resolved by HPLC column selection, selecting suitable composition of mobile phase, or controlling the pH value of mobile phase. Specifically, aqueous phosphate solution and heptane sulfonate sodium were used to adjust pH of mobile phase and optimize the HPLC retention time and separation of nicotine *N*-glucuronide [7,17]. However, the nonvolatile buffer used in these methods is not compatible with electrospray ionization (ESI) and ion-pairing agents significantly reduce the signal intensity in ESI mass spectrometry [18]. To determine *N*-glucuronidation of [5-^{3}H]nicotine generated by human liver microsomes and expressed UGTs, an HPLC analysis was developed using 0.025% trifluoroacetic acid (pH 2.5) as mobile phase [19]. Another LC–MS method has been reported to quantify nicotine glucuronide in recombinant human UGT2B10 with limit of quantification of 5 nM, in which the mobile phase consists of methanol and water with trifluoroacetic acid (pH 2.2) [10]. Whereas, the relatively long chromatographic cycle time (>10 min) limited the application of these methods in high-throughput analysis. Recently, Miller et al. proposed a LC–MS–MS method to determine eight nicotine metabolites including nicotine glucuronide in human plasma and urine with lower limit of quantitation (LLOQ) at 1 ng/mL and 2.5 ng/mL, respectively [20]. However, the reported sample preparation method required multiple steps of manipulation including acidification, solid-phase extraction, neutralization, evaporation, dryness, and reconstitution.

The present study describes the development and validation of a fast and selective electrospray LC–MS–MS method for the determination of nicotine glucuronide in human liver microsomes (HLM). A simple protein precipitation using perchloric acid was applied to extract nicotine glucuronide in HLM incubation solution. The stability of *N*-glucuronide in acidic condition was validated. The dynamic range is 10–1000 nM, and the LLOQ is 10 nM. This method was then successfully applied to the analysis of nicotine *N*-glucuronide in HLM in support of determination of the inhibitory effects of compounds on UGT2B10 catalyzed nicotine *N*-glucuronidation.

2. Materials and methods

2.1. Reagents and chemicals

HPLC-grade acetonitrile, methanol, deionized water, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Nicotine, amitriptyline, hecogenin, imipramine, lamotrigine, trifluoperazine, alamethicin, uridine 5′-diphosphoglucuronic acid (UDPGA), trifluoroacetic acid (TFA), and perchloric acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Nicotine *N*-glucuronide and nicotine *N*-glucuronide (Methyl-d_3) were purchased from Toronto Research Chemicals (North York, ON, Canada). Five lots of pooled human liver microsomes (20 mg/mL) were purchased from BD Gentest (Bedford, MA, USA), In Vitro Technologies (Chicago, IL, USA), and XenoTech (Lenexa, KS, USA). Recombinant human UGTs were generated in baculovirus-infected insect cells as described previously [9].

2.2. Preparation of standard solutions

Primary standard stock solutions of nicotine *N*-glucuronide and nicotine *N*-glucuronide (methyl-d_3) (internal standard) were prepared separately in methanol at 10 mM and were stored at −20 °C. The secondary stock solution of nicotine *N*-glucuronide at 80 μM was obtained by dilution with 20% methanol. The calibration standards were freshly prepared by spiking 35 μL secondary stock solution into 665 μL blank incubation solution containing human liver microsomes to get a standard solution at 4 μM, followed by a serial dilution to give final nicotine glucuronide concentrations of 10, 25, 50, 100, 250, 500, 800, and 1000 nM. Quality control (QC) samples were prepared in a manner similar to the calibration standards at four concentration levels (10, 25, 200 and 800 nM). A working solution of internal standard was prepared in water at a final concentration of 4 μM. A 40-μL aliquot of 35% perchloric acid was added to calibration standard or QC to precipitate the protein. Then 20 μL of internal standard working solution was added after the addition of perchloric acid. After vortex-mixed for 10 s and centrifuged at 10,000 × *g* for 10 min, a 5-μL aliquot of the supernatant was injected into the LC–MS–MS system for analysis.

2.3. Liver microsome incubation

The enzyme reactions were conducted under linear conditions respective of incubation time and protein concentration. In all cases, incubation mixtures containing 0.5 mg/mL microsomal protein, 50 μg/mg alamethicin, 50 mM potassium phosphate buffer (pH 7.4), and 5 mM $MgCl_2$ were kept on ice for 15 min. After 3 min pre-incubation, reactions were initiated by adding UDP-glucuronic acid (UDPGA) (2 mM final concentration) in a total volume of 200 μL. Reactions were allowed to proceed for 90 min at 37 °C, and terminated by the addition of 40 μL perchloric acid (35%) followed by the addition of 20 μL of nicotine glucuronide (methyl-d_3) (4 μM). After centrifugation at 10,000 × *g* for 10 min to remove the precipitated proteins, the supernatant was transferred to 96-well plate. The assays to study enzyme reaction kinetics were performed in duplicate at 10 concentrations of nicotine (0.05–5 mM) in recombinant UGT2B10 and 12 concentrations (0.015–5 mM) in pooled human liver microsomes.

The potential inhibitory effects of amitriptyline, hecogenin, imipramine, lamotrigine, and trifluoperazine on nicotine glucuronidation were evaluated in pooled human liver microsomes. Nicotine was co-incubated at 200 μM in duplicate with pooled human liver microsomes in the presence of 8 concentrations of amitriptyline, hecogenin, imipramine, lamotrigine, or trifluoperazine (0.05–100 μM). The formation of nicotine glucuronide was compared to that in their absence (vehicle control).

2.4. LC–MS–MS

The Acquity ultra performance liquid chromatography system (Waters Corporation, Milford, USA) interfaced to an API 4000 Qtrap mass spectrometer (Applied Biosystems, Foster City, USA). Chromatographic separation was carried out using a Discovery HS F5 analytical LC column (2.1 mm × 50 mm, 3.0 μm) (Sigma–Aldrich, St. Louis, USA). A mobile phase consisting of water containing 0.06% TFA (pH 2.0) (A) and methanol containing 0.06% TFA (B) was used for the LC-separation. The elution gradient program was as follows: held 10% B over first 0.5 min, increased B from 10% to 80% within 1.5 min followed by 80% B for 1 min. Column was equilibrated with initial condition of gradient for 2 min. The flow rate was 0.35 mL/min. The injection volume was 5 μL. The column and the autosampler were maintained at room temperature (~25 °C). Nicotine glucuronide and the internal standard eluted at 0.9 min.

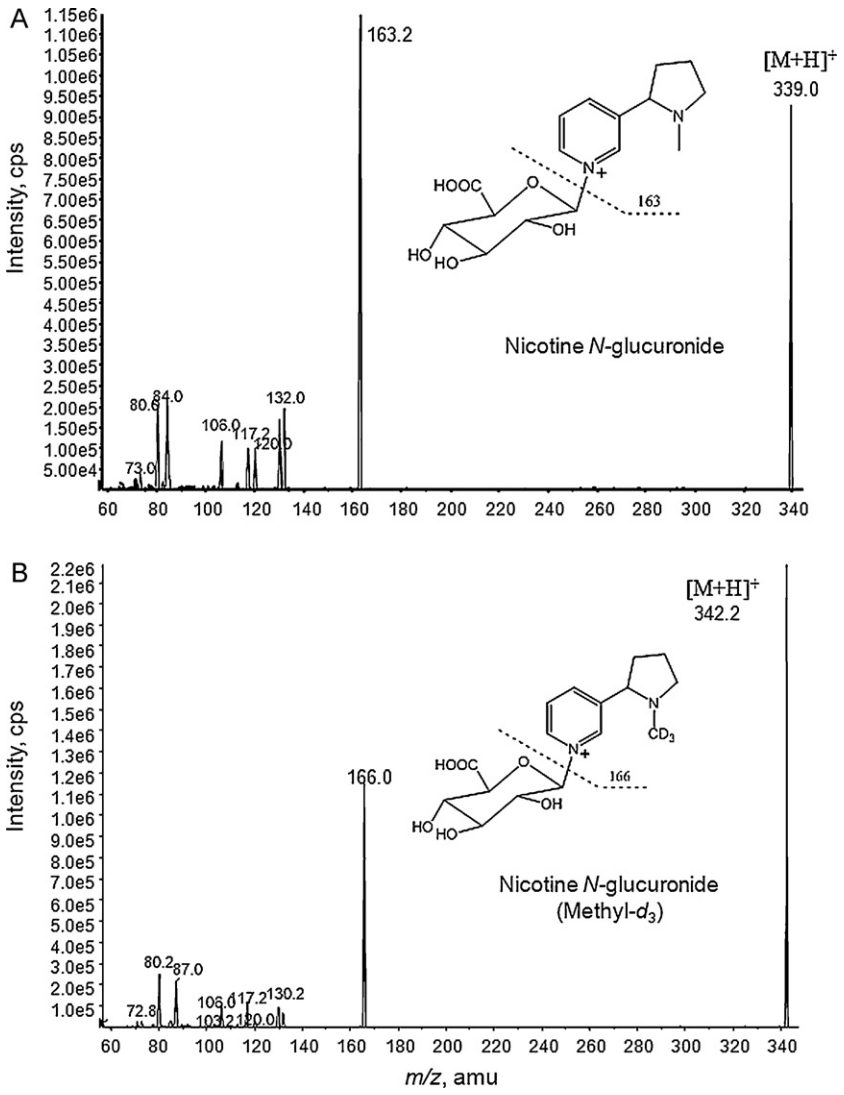

Fig. 1. Positive ion electrospray product ion mass spectra of (A) nicotine glucuronide and (B) nicotine glucuronide (methyl-d_3).

Positive ion electrospray tandem mass spectrometric analysis was carried out at unit resolution with collision-induced dissociation and selective reaction monitoring. The source temperature was 450 °C, the electrospray voltage was 5000 V, and the declustering potential was 56 V for both nicotine glucuronide and the internal standard. Nitrogen was used as the collision gas at energies of 23 V for nicotine glucuronide and the internal standard. The ion transitions of m/z 339–163 for nicotine glucuronide and m/z 342–166 for the internal standard were monitored with a dwell time of 150 ms per transition. Data were acquired and analyzed using Applied Biosystems Analyst 1.4.2 software.

2.5. Method validation

The selectivity of the method was investigated by screening at least five lots of drug-free pooled human liver microsomes in the incubation solution for the exclusion of any endogenous co-eluting interference at the retention times of nicotine glucuronide and internal standard.

Three validation batches were used to assess the precision and accuracy by evaluating intra-day and inter-day accuracy and precision of four quality control samples concentrations. Each batch was processed on a separate day and had two sets of calibration standards and six replicates of QC samples at 10, 25, 200, and 800 nM. QC samples and other test samples were interspersed between two calibration curve samples. A blank sample was always placed right after the upper limit of quantification (ULOQ) standard to evaluate the carry-over of the LC–MS–MS. The lower limit of quantification was defined as the lowest concentration of nicotine glucuronide in calibration standards at which the concentration can still be reliably quantified (within 20% CV and RE, and it typically requires a signal-to-noise ratio of larger than 10:1). Accuracy was defined as the percent bias of the measured concentration relative to the nominal concentration of each quality control sample. Precision was defined as percent coefficient of variation obtained from replicates ($n = 6$) of the QC samples. Both the precision and accuracy were less than or equal to 15%.

The recovery of nicotine glucuronide from incubation solutions using protein precipitation was evaluated using quality control samples. The measured concentrations of normal QC samples were compared to blank microsomal extracts post-spiked with the standard solution at the same concentration. Matrix interference caused by the suppression or enhancement of ionization during LC–MS–MS was evaluated by comparing the LC–MS–MS response of nicotine glucuronide that was spiked in extracted blank samples,

with that obtained from QC neat solutions at the same concentrations (10 nM, 25 nM, 200 nM and 800 nM).

The stability of un-extracted QC samples was evaluated by keeping six aliquots of each of QCs (10 nM, 25 nM, 200 nM and 800 nM) at ambient temperature (~25 °C) for 4 h in order to establish the short-term stability of nicotine *N*-glucuronide in microsomal incubation solution. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples. The short-term post-preparative stability was included in one of three batches of extracted samples that were stored at 4 °C for 48 h or in the auto-sampler for approximately 24 h before injection onto the LC–MS–MS system to determine the storage stability and re-injection reproducibility of the processed samples. The results were compared with those of freshly prepared QC samples. Nicotine glucuronide was considered to be stable when the measured concentrations in the test samples ranged from 85% to 115% of the initial concentrations.

3. Results and discussion

3.1. Selectivity

Positive ion electrospray MS/MS product-ion spectra of nicotine glucuronide and nicotine glucuronide (methyl-d_3) are shown in Fig. 1. Protonated nicotine glucuronide was detected at *m*/*z* 339, and collision-induced dissociation of this precursor ion produced product ions of *m*/*z* 163, 132, and 106, corresponding to $[M+H-176]^+$, $[M+H-176-CH_3NH_2]^+$, $[M+H-176-C_3H_7N]^+$ (Fig. 1A). As the ion of *m*/*z* 163 was the base peak of the product tandem mass spectrum of the protonated molecule of *m*/*z* 339, the mass transition of *m*/*z* 339–163 was selected for monitoring and quantification of nicotine glucuronide. In addition, the ion transition of *m*/*z* 342–166 was selected to measure the internal standard nicotine glucuronide (methyl-d_3) (Fig. 1B). The selected reaction chromatograms of nicotine glucuronide and internal standard at extracted blank matrix are shown in Fig. 2. No interfering peaks from endogenous compounds were detected at the retention times of nicotine glucuronide or the internal standard during the analysis of blank microsomal extracts.

Initial LC–MS–MS methods were developed using various HPLC columns (XTerra RP18 column from Waters and Acclaim mixed-mode HILIC column from Dionex) with mobile phase having various pH values (pH 2.0–9.0). However, peak splitting was observed for the nicotine glucuronide. The peak shape of analyte was improved when a Discovery HS F5 column was used with mobile phase containing 0.06% TFA (pH ~2.0). Furthermore, the retention time of nicotine *N*-glucuronide on column was reduced to less than 1 min.

3.2. Matrix effects

Matrix effects are common during electrospray mass spectrometry when co-eluting matrix components cause the analyte signal to become either attenuated or, less frequently, enhanced. To determine whether matrix interference affected the analysis of nicotine glucuronide, an extracted blank microsomal sample was injected into the LC–MS–MS system while nicotine glucuronide was introduced continuously post-column. No significant matrix

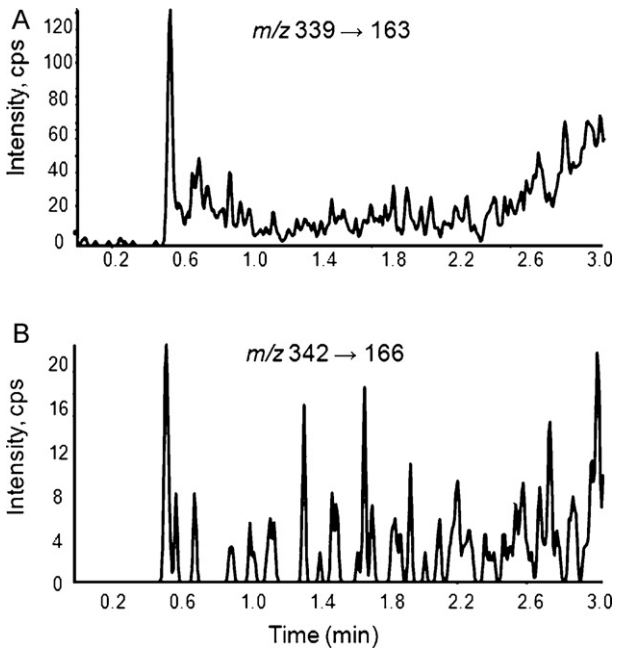

Fig. 2. Representative LC–MS–MS chromatograms of matrix blank samples. (A) Nicotine glucuronide (*m*/*z* 339–163) and (B) nicotine glucuronide (methyl-d_3) (*m*/*z* 342–166).

interference was observed between 0.9 min and 1.0 min corresponding to elution time of standard (data not shown). The absolute matrix effect was evaluated by comparing LC–MS response for standard spiked into microsomal extracts with that in neat solution. The mean value of matrix effect ranged from −2.3 to 2.4%, indicating a minimal matrix effect under current ionization condition (Table 1).

3.3. Linearity and sensitivity

The calibration curve for nicotine-glucuronide in incubates was linear over the concentration range of 10–1000 nM using a $1/X^2$ weighting factor. On three different days, the calibration curves for nicotine glucuronide were represented as the regression equations $y = 0.0192x - 0.0132$, $y = 0.0218x - 0.0189$, and $y = 0.0211x - 0.0418$, respectively, all with correlation coefficients (r^2) greater than 0.996. The limit of detection (LOD) of nicotine-glucuronide in incubation matrices was 0.5 nM (0.17 ng/mL, 5 μL injection on-column), based on a signal-to-noise (S/N) ratio equal to 3:1 (Fig. 3A and B). Using the lowest concentration of the calibration curve as the lower limited of quantification (LLOQ) according to FDA guidance, the LLOQ of this method is 10 nM (3.3 ng/mL) (Fig. 3C and D).

3.4. Precision and accuracy

The precision of the assay was defined as the coefficient of variation (CV) calculated from six measurements of QCs at four different concentrations. The accuracy of the assay was defined as the percent bias of the mean of the measurements ($n = 6$) of

Table 1
Results of matrix effect for human liver microsomes.

	Matrix	Absolute matrix effect (%) QCs ($n = 6$)			
		10 nM	25 nM	200 nM	1800 nM
Nicotine glucuronide	HLM	−2.3	2.4	2.2	−1.5

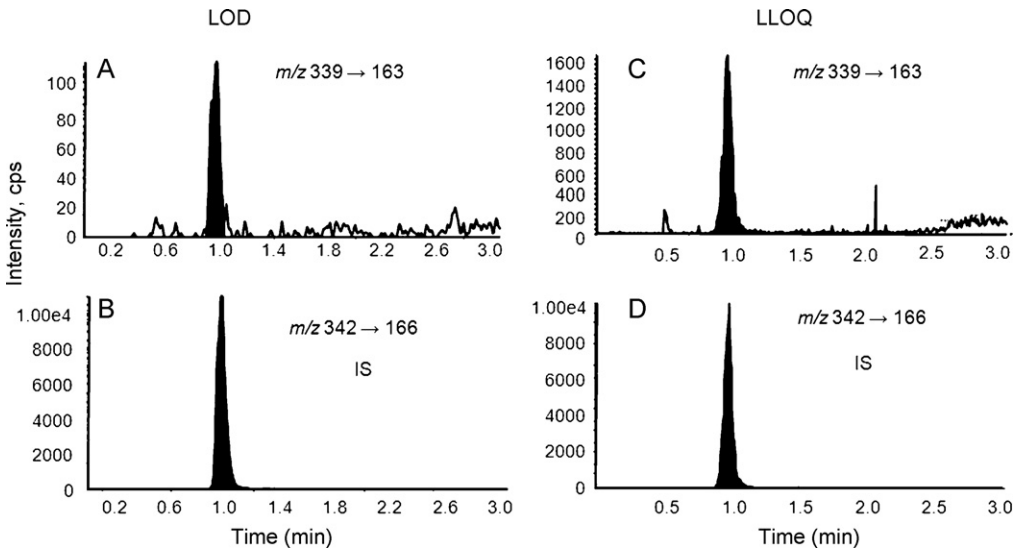

Fig. 3. Representative LC–MS–MS chromatograms of LOD and LLOQ samples. (A) Extracted samples spike with nicotine glucuronide at 0.5 nM (LOD) and (B) internal standard nicotine glucuronide (methyl-d_3); (C) extracted samples spike with nicotine glucuronide at 10 nM (LLOQ) and (D) internal standard.

Table 2
Intra-assay and inter-assay accuracy and precision.

		QCs			
		10 nM	25 nM	200 nM	800 nM
Day 1	*n*	6	6	6	6
	Mean	9.0	25.7	200	796
	CV %	3.7	3.3	4.3	4.2
	Bias %	−10.5	2.7	0.4	−0.4
Day 2	*n*	6	6	6	6
	Mean	9.3	26.4	216	864
	CV %	8.5	6.7	3.8	4.2
	Bias %	−6.9	5.4	9.1	8.1
Day 3	*n*	6	6	6	6
	Mean	8.9	24.3	212	794
	CV %	8.1	2.3	3.6	3.9
	Bias %	−11.4	−2.8	6.4	−0.7
Inter-day	*n*	18	18	18	18
	Mean	9.0	25.4	210	818
	CV %	7.9	5.5	5.1	5.6
	Bias %	−9.6	1.8	5.3	2.3

four QCs compared to the true value. The resulting precision (CV) and accuracy are presented in Table 2. The intra-day precision (CV) and accuracy ranges were 2.3–8.5% and −11.4 to 9.1%, respectively. The inter-day precision values were in the range of 5.1–7.9% for all the QC samples, and accuracy was between −9.6 and 5.3%. All intra-day and inter-day precision and accuracy values met the required ±15%, indicating that the assay is accurate, precise and reproducible for the quantification of nicotine-glucuronide over the concentration range of 10–1000 nM in incubation matrices.

Table 3
Storage stability and re-injection reproducibility of processed samples.

	QCs (n = 6)			
	10 nM	25 nM	200 nM	800 nM
(1) Short term (un-extracted samples in room temperature for 4 h)				
Mean	9.3	24.3	207	823
CV %	8.5	11.4	4.3	6.7
Bias %	−6.6	−2.7	3.4	2.9
(2) 4 °C for 48 h				
Mean	10	24.6	224	851
CV %	10	13.5	5.5	10.8
Bias %	0.4	−1.5	12.2	6.4
(3) Re-injection reproducibility after 24 h				
Mean	10.1	25.9	205	780
CV %	7.6	9.7	3.8	2.0
Bias %	0.8	3.7	2.9	−2.4

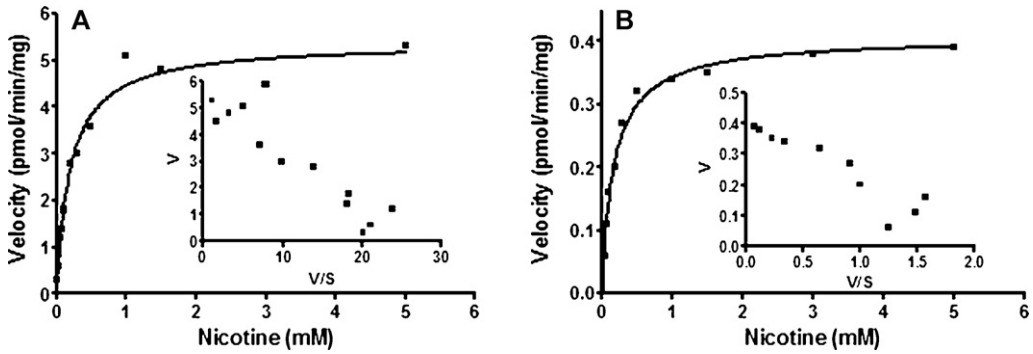

Fig. 4. Kinetic analysis of nicotine glucuronidation by human liver microsomes and recombinant UGT2B10. (A) Nicotine glucuronidation catalyzed by human liver microsomes. The substrates were incubated at 12 concentrations in the range of 0.015–5 mM. (B) Nicotine glucuronidation catalyzed by recombinant UGT2B10. The substrates were incubated at 10 concentrations in the range of 0.05–5 mM. Eadie–Hofstee plot for nicotine glucuronidation is also presented.

Table 4
Kinetic parameters of nicotine glucuronidation in human liver microsomes and recombinant UGT2B10. Data are presented as mean ± standard error of parameter fit.

	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m (μl/min/mg)
HLM	200.8 ± 27.6	5.3 ± 0.2	0.026
UGT2B10	179.8 ± 19.1	0.41 ± 0.01	0.002

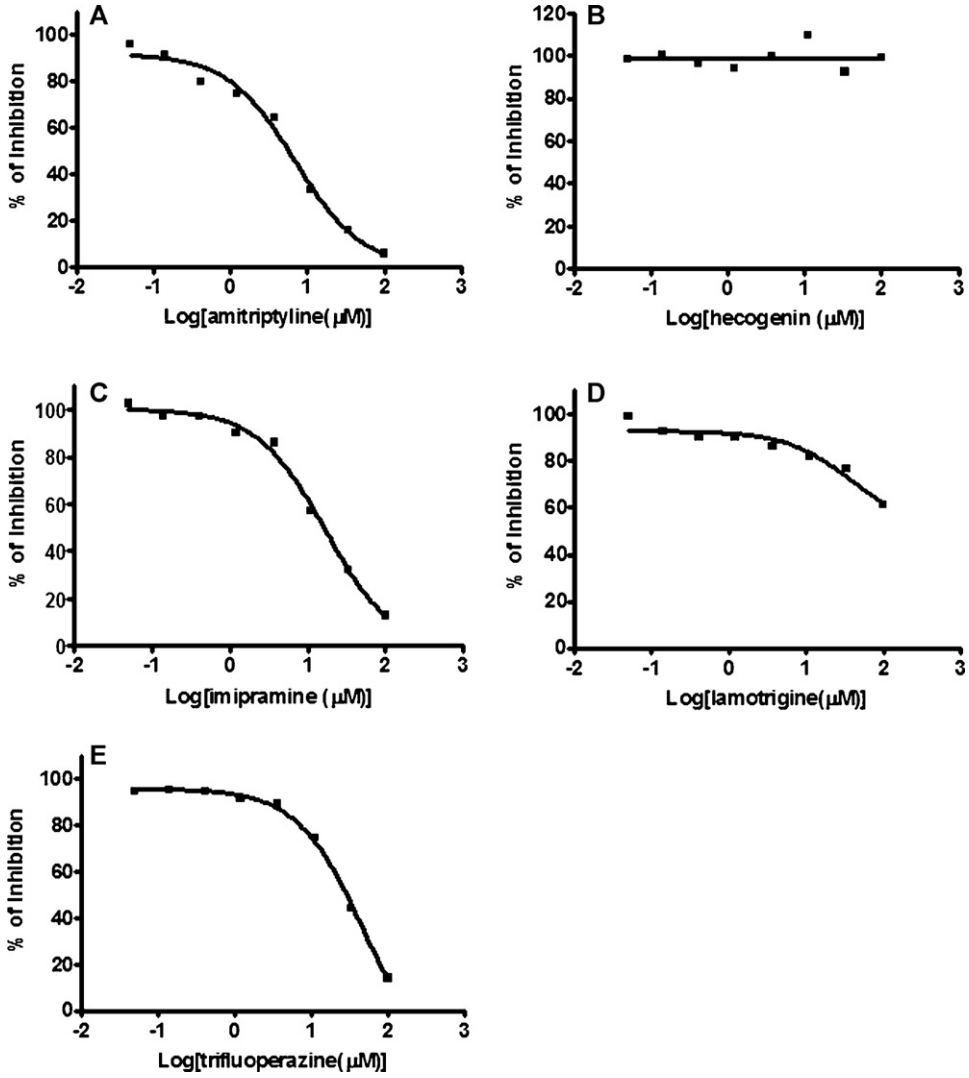

Fig. 5. Inhibitory effects of amitriptyline, hecogenin, imipramine, lamotrigine, trifluoperazine (0.05–100 μM) on nicotine glucuronidation in HLM.

Table 5
Average IC_{50} values for inhibition of human UGT2B10 by amitriptyline, hecogenin, imipramine, lamotrigine, and trifluoperazine.

	Amitriptyline (μM)	Hecogenin (μM)	Imipramine (μM)	Lamotrigine (μM)	Trifluoperazine (μM)
IC_{50}	6.8	NA[a]	16.5	40.5	49.1

[a] Not available.

3.5. Recovery

The extraction efficiency was assessed by comparing concentrations of nicotine glucuronide in the extracted QC samples with those obtained from the extracted blank microsomal fractions post-spiked with corresponding neat standard solutions. Treatment of microsomal fractions with 40 μL of 35% perchloric acid served the dual purpose of precipitating proteins and adjusting the pH value of incubates. The recoveries of nicotine-glucuronide from microsomal fraction at initial concentrations of 10, 25, 200 or 800 nM were 99.4%, 94.4%, 98.7% or 101.8%, respectively, using the protein precipitation procedure. This protein precipitation procedure not only allows the high efficiency of extraction, but also improved the peak shape of nicotine glucuronide during LC–MS–MS analysis through adjustment of the pH value of extracted incubates.

3.6. Stability

In general, *N*-glucuronides are susceptible to acid hydrolysis. Therefore, it was critical to evaluate the stability of nicotine glucuronide in processed samples by comparing the concentration remaining after storage under different conditions to the concentration of nicotine glucuronide in freshly prepared samples. These results are shown in Table 3. Nicotine glucuronide was stable in processed microsomal fraction for 24 h at room temperature in HPLC auto-sampler, and for 48 h at 4 °C based on the stability experiments performed in this study. In addition, the short-term stability of un-extracted analyte in microsomal incubation mixtures was evaluated at room temperature for up to 4 h, based on the expected maximum period of time that samples would be maintained at this temperature during assay. The results of the experiments showed that no significant degradation occurred during assays (Table 3).

3.7. Inhibition of nicotine N-glucuronidation catalyzed by UGT2B10

The method described above has been successfully employed to investigate the kinetics of the formation of nicotine glucuronide catalyzed in pooled human liver microsomes and by recombinant human UGT2B10. The results are shown in Fig. 4 and Table 4. The kinetics parameters were calculated using a nonlinear regression analysis fit of the data to the Michaelis–Menten equation (Fig. 4). Eadie–Hofstee plot of nicotine *N*-glucuronidation by human liver microsomes and UGT 2B10 were monophasic (Fig. 4), indicating that one enzyme and one binding site was involved. These results suggested that UGT2B10 is the only enzyme contributing to nicotine glucuronidation at current low substrate concentration. The apparent K_m values (the apparent affinity constants) for the metabolism of nicotine to form *N*-glucuronide by HLM and UGT2B10 were 201 and 180 μM, respectively.

To further evaluate the potential inhibitory effects of amitriptyline, hecogenin, imipramine, lamotrigine, and trifluoperazine on nicotine glucuronidation, nicotine was incubated with HLM in the presence of each compound at various concentrations. As shown in Fig. 5, amitriptyline and imipramine have been demonstrated as UGT2B10 substrates with higher affinity to UGT2B10 than UGT1A4 [9]. Here, the average estimated IC_{50} values for the inhibition of UGT2B10-catalyzed nicotine glucuronidation by amitriptyline and imipramine were 6.8 μM and 16.5 μM, respectively (Table 5). The approximately 60% residual activity after inhibition with 100 μM lamotrigine indicated a minimal inhibitory effect on UGT2B10 catalyzed nicotine glucuronidation. Hecogenin has been reported as a selective inhibitor of UGT1A4 [8]. In consistent with this finding, no inhibitory effect of hecogenin on nicotine glucuronidation was observed across the concentration range used, indicating that hecogenin is not an inhibitor of UGT2B10. This study provides additional evidence for the selective inhibition of hecogenin against UGT1A4. Inconsistent with the previous report that trifluoperazine is a selective substrate and inhibitor probe for UGT1A4 [8], 100 μM trifluoperazine inhibited 86% of nicotine glucuronidation, suggesting that trifluoperazine is also an inhibitor of UGT2B10. In addition, trifluoperazine *N*-glucuronide was not observed during incubation with recombinant UGT2B10 (data not shown), indicating that trifluoperazine is a non-substrate inhibitor for human UGT2B10.

4. Conclusions

In the present study, a simple and rapid LC–MS–MS method was successfully developed for the quantitative analysis of nicotine glucuronide in human liver microsomal incubates. This assay requires less than 3 min per sample for LC–MS–MS analysis. The extraction procedure not only precipitated proteins in samples, but also optimized the chromatographic peak shape of analyte. Under our experimental conditions, nicotine glucuronide was stable in the processed samples. This method was further employed to evaluate potential inhibitory effects on human UGT2B10 in human liver microsomes using nicotine as probe. Trifluoperazine was found to be non-substrate inhibitor of UGT2B10. Certain UGT2B10 substrates, such as amitriptyline and imipramine, exhibited potent inhibitory effect on human UGT2B10. Therefore this method could be implemented in drug metabolism studies using nicotine as UGT2B10 probe.

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