Application Note: 359

Determination of Direct Glucuronidation of NCEs in Liver Microsomes using SIM, Data Dependent Full-Scan MS/MS and CNL Scanning

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Key Words

- Finnigan™ TSQ Quantum Discovery™
- Drug Discovery
- Metabolism
- UGTs

Introduction

Glucuronidation represents an important conjugation reaction during the metabolism of xenobiotics and is catalyzed by various uridine diphosphate glucuronosyltransferases (UGTs). It is considered to be an important detoxification pathway. UGTs are membrane-bound enzymes localized primarily in the endoplasmic reticulum (ER) which facilitate the transfer of the glucuronic acid-moiety from UDP-glucuronic acid to a large number of structurally diverse drugs and xenobiotics. The hydrophilic glucuronide formed is removed from the body via excretion in bile or urine (Tukey, 2000)[1]. *In vitro* tools for predicting *in vivo* clearance of drugs, which are predominantly glucuronidated, are rarely determined at early drug discovery stages. In this work, we describe methods to study the glucuronidation rate of New Chemical Entities (NCEs) using liver microsomes.

Goal

The goal of this study was to identify NCEs that are prone to phase II glucuronidation. This report demonstrates the use of the Finnigan TSQ Quantum Discovery in carrying out *in vitro* glucuronidation studies of NCEs in the liver microsomes.

Experimental Conditions

Chemicals and Reagents

Uridine 5'-diphosphoglucuronic acid (UDPGA), alamethicin, saccharic acid-1,4-lactone, were purchased from Sigma-Aldrich Co. (St. Louis, MO). Dimethylsulfoxide and magnesium chloride were obtained from Merck (Darmstadt, Germany). Reference drugs Diclofenac were received from Novartis, Oxazepam from Ayerst and Haloperidol from Janssen Pharmaceutica. Acetonitrile and methyl alcohol were purchased from Acros Organics (Geel, Belgium), while ammonium acetate 10 mM in water/ACN solution 95:5% v/v from Biosolve (Valkenswaard, The Netherlands). All other chemicals were of analytical grade quality and solvents were high-performance liquid chromatography (HPLC) grade.

Microsomal Preparations

Liver microsomes were prepared according to the method described by Gorrod et al (1975).[2] Protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad, Belgium) based on Bradford,[3] using BSA (fraction V) as standard. Microsomes were aliquoted and stored at –80 °C until use.

Microsomal UGT Enzyme Assay

The UGT assays were performed as described previously by Fisher et al (2000).[4] Aliquots of microsomal suspensions, containing 1 mg/mL of protein, were pipetted into 5-mL glass tubes which were immersed in ice. Alamethicin (50 µg/mL) was added and the mixture was placed on ice for 15 min. After addition of saccharic acid-1,4-lactone (5 mM), test-compound (5 µM) and $MgCl_2$ (3 mM), the mixture was pre-incubated (5 min at 37 °C). The incubations (0, 5, 10, 20, 30 and 60 min, 37 °C) were initiated by adding 50 µL of a solution of UDPGA (2 mM). Blanks (0 and 60 min) containing boiled microsomes were incubated under the same conditions. The reaction was stopped with two volumes of DMSO. Samples were subsequently centrifuged at 3000 g for 10 min (RT). All experiments were performed in triplicate.

Instrumentation Methods

HPLC Conditions

Column: BDS Hypersil™ C18
(50 × 4.6 mm, 5 micron, Thermo)
Flow: 1.2 mL/min
Mobile phases: Solvent A: 10 mM ammonium acetate in H_2O/Acetonitrile (95:5)
Solvent B: Acetonitrile (100%).
Injection volume: 100 µL
Gradient run time: 15 min

Time (min)	%A	%B
0	100	0
0.5	100	0
10	5	95
12	5	95
15	95	5

MS Conditions

Mass Spectrometer: Finnigan TSQ Quantum Discovery (Thermo, San Jose CA). All analyses were carried out in positive electrospray mode.
ESI conditions: Spray voltage 3800
Sheath gas pressure: 30
Aux gas flow: 10
Capillary temperature: 370 °C
Selected Ion Monitoring (SIM): Scan time: 0.5 sec
Scan width: 1 Da
Unit resolution: (0.7 FWHM)

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Scan Conditions

Data Dependent™ scans: 320–800 Da in 1 s.

Data Dependent scan (MS/MS) of most intense ions above threshold of 10^5 counts.

Collision energy of 35 V and collision gas (Ar) pressure of 1.5 mTorr was used.

Constant neutral loss (CNL) scan for loss of 176 Da with scan time 1 s.

For the determination of compound metabolic stability the samples were quantified in Selected Ion Monitoring mode (SIM). The metabolic turnover and *in vitro* glucuronidation rates in terms of half-life were calculated. CNL scans for the loss of 176 Dalton were performed in order to identify glucuronidated species.

Calculations of Half-life and Clearance

Metabolic half-life determination

The metabolic *in vitro* half-life was calculated using the slope of the log-linear regression from the concentration remaining parent compound versus time relationship (k), $t_{1/2} = - \ln(2)/ k$.* (*slope of linear part of the log-linear curve, Figure 5.)

Prediction of hepatic clearance

Intrinsic clearance at the enzyme site (CL'_{int}, mL per min per mg protein) was calculated using: $CL'_{int} = 0.693/t_{1/2} \times fu$. Where fu is the unbound fraction. As fu is not known for the tested compounds, the calculations are performed with fu = 1. The *in vitro* protein concentration is 1 mg/mL.

For humans the intrinsic clearance is calculated as,

$$\mathbf{CL_{int} = CL'_{int} \times (45\ mg\ microsomes/g\ liver) \times (25\ g\ liver/kg\ b.w.)}$$

Human hepatic clearance (CL_H) is defined as,

$$\mathbf{CL_H = (Q_H \times CL_{int} \times fu)/(Q_H + [fu \times CL_{int}])}$$

Scaling factors are: 45 mg of microsomal protein per g liver, liver weight 1800 g, body weight 70 kg, liver blood flow of 1450 mL/min (Q_H = 21 mL/min/kg b.w.) Mouse and rat clearances were calculated according to literature data[8]

Results and Discussion

In vitro systems, including microsomes and hepatocytes, have been routinely used in early drug discovery metabolism studies to obtain an estimate of metabolic stability, usually expressed as intrinsic clearance (CL_{int}). Several methods have been used to predict hepatic *in vivo* clearance from *in vitro* tissue preparations. The prediction of *in vivo* hepatic metabolic clearance of drugs primarily undergoing phase II metabolism has been estimated by an *in vitro* glucuronidation assay.

In this approach the assay was set up with two application scenarios in mind. The first application aims to identify issues pertaining to chemical classes at Hit to Lead stages: rapid identification of "scaffolds" prone to direct phase II glucuronidation. For that purpose, compounds are incubated with liver microsomes at 5 µM for 60 min. CNL scan detection set for 176 Dalton is used to identify the compounds prone to direct glucuronidation. Haloperidol,[5] Oxazepam[6] and Diclofenac[7] were chosen to exemplify (Figures 1-3) the assay in the HtL stage for aliphatic hydroxyl and carboxylic acid functional group containing compounds.

The second application was aimed at acquiring more detailed information on *in vitro* metabolic half-lifes and predictions of hepatic clearances to be made of individual NCEs due to phase II at the lead optimization (LO) stages. In the LO phase, this enables Medicinal Chemists to trans-

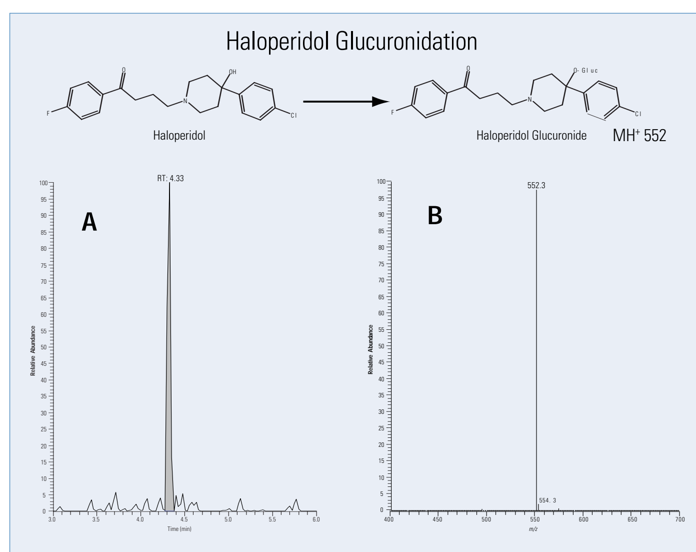

Figure 1: (A) CNL chromatogram of 176 Da for Haloperidol incubated with HLM+UDPGA (B) The CNL mass spectrum of the peak at 4.33 min in the chromatogram

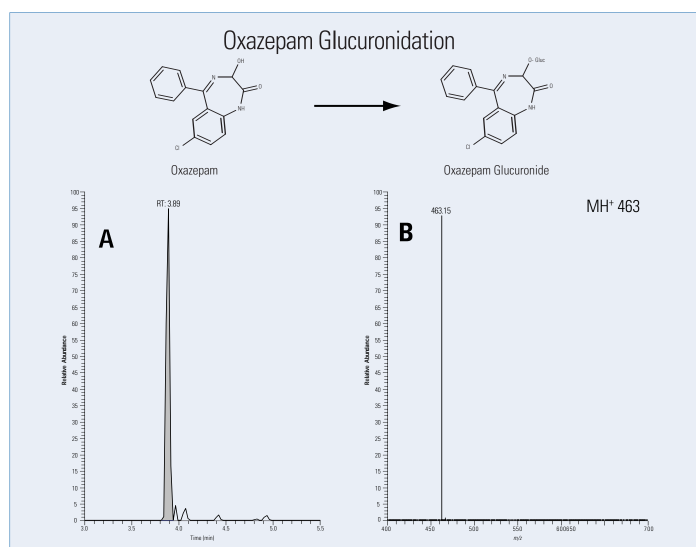

Figure 2: (A) CNL chromatogram of 176 Da for Oxazepam incubated with HLM+UDPGA (B) The CNL mass spectrum of the peak at 3.89 min in the chromatogram

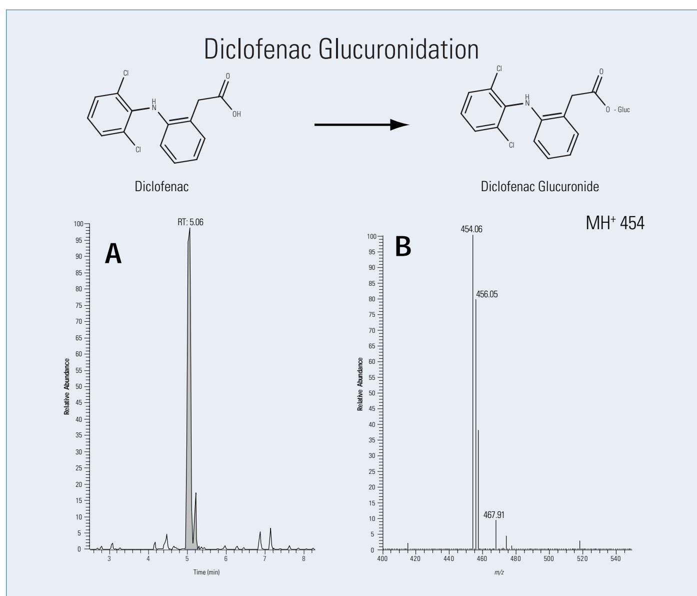

Figure 3: (A) CNL chromatogram of 176 Da for Diclofenac incubated with HLM+UDPGA
(B) The CNL mass spectrum of the peak at 5.06 min in the chromatogram

late this into the physiological relevant hepatic clearance data to understand the ***in vitro*** PK structure-property relationship (SPR) and to bridge into the physiology. For this purpose, the compounds are incubated at 5 µM. A time curve is taken to calculate an ***in vitro*** $t_{1/2}$ and to predict hepatic clearance values. Figure 5 illustrates the phase II mediated turnover in human, rat and mouse liver microsomes using JNJ compound number 1 (structure not disclosed). Derived from that, an ***in vitro*** half-life is calculated and the hepatic clearance is predicted. Table 1 shows the predicted hepatic clearances for JNJ compound 1 and a series of four JNJ compounds ranging from low to high phase II hepatic clearances. Quantification was carried out by SIM.

In addition, CNL and SIM/MS^2 dependent scans are performed to verify the phase II metabolite formation as exemplified for diclofenac (Figure 4).

Diclofenac Glucuronidation

Figure 4: (A) Extracted ion chromatogram of the MH+ ion of Diclofenac in the SIM mode; (B) Mass spectrum of the peak at 5.06 min from chromatogram A;
(C) CNL chromatogram of Diclofenac incubated with HLM+UDPGA; (D) CNL mass spectrum of the peak at 5.06 min from chromatogram C;
(E) XIC of the MH+ ion of Diclofenac; (F) MS/MS spectrum of the peak at 5.07 min from chromatogram E;
(G) XIC of the MH+ ion of Diclofenac glucuronide; (H) MS/MS spectrum of peak at 5.10 min from chromatogram G

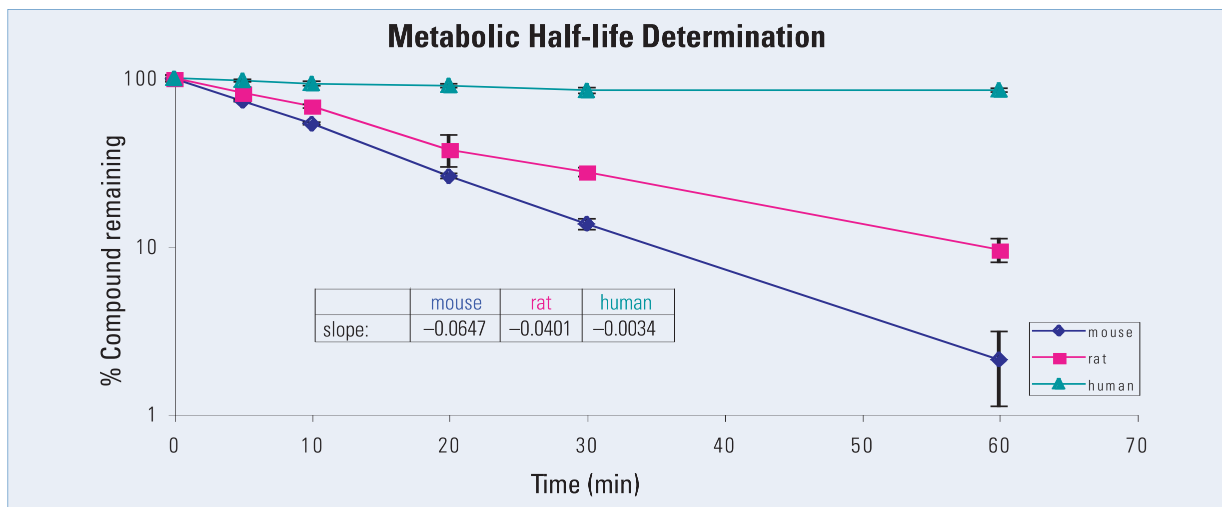

Figure 5: Metabolic phase II turnover of JNJ compound 1 using mouse, rat, and human microsomes

	mouse		rat		human	
	Clh [l/h.kg]	t1/2 [min]	Clh [l/h.kg]	t1/2 [min]	Clh [l/h.kg]	t1/2 [min]
JNJ Comp 1	4.27	8	2.58	7	1.06	21
JNJ Comp 2	3.96	11	2.58	7	< 0.49	> 60
JNJ Comp 3	2.12	75	< 0.97	> 60	< 0.49	> 60
JNJ Comp 4	< 1.82	> 60	< 0.97	> 60	< 0.49	> 60
JNJ Comp 5	< 1.82	> 60	< 0.97	> 60	< 0.49	> 60

Table 1: Calculated in vitro half-live values ($t_{1/2}$, given in minutes) and predicted hepatic clearances (Clh, given in Liter per hour and kg body weight) for a series of JNJ compounds

Conclusion

The *in vitro* glucuronidation assay described is a valuable tool for rapid identification of scaffolds that are directly prone to glucuronidation as well as for the estimation of the hepatic clearance mediated by UGTs.

The utility of the TSQ Quantum Discovery, as an analytical device for quantification of compounds in phase II metabolic stability studies and for the identification of the phase II metabolites formed, has been illustrated. The measurement of the *in vitro* metabolic phase II turnover in terms of half-life was performed using SIM. The identification of the phase II metabolites was facilitated by applying CNL scanning. However, using information of the Data Dependent full-scan MS/MS in addition to the CNL scanning provides a confirmation of the proposed metabolite information.

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