

# Measurement of Metabolic Stability Using SIM and Identification of Metabolites by Data Dependent Full-Scan MS/MS and CNL Scanning

Peter B Ebmer, Ethirajulu Kantharaj, Katie De Wagter, Anne Van Vlaslaer, Claire Mackie and Ron AHJ Gilissen; Department of ADME/Tox, In Vitro Pharmacokinetics Group, Johnson & Johnson Pharmaceutical Research & Development, 2340 Beerse, Belgium  
Dipankar Ghosh and Karel Lazou; Thermo Fisher Scientific, San Jose, CA, USA

## Key Words

- TSQ Quantum Discovery™
- Constant Neutral Loss
- Data Dependent™
- Drug Discovery
- SIM Quantitation

## Introduction

*In vitro* pharmacokinetic data, such as metabolic stability and the identification of formed metabolites have become important tools in early drug discovery.<sup>[1, 2]</sup> This information can help steer a medicinal chemist to improve on a compound's metabolic stability and also allow predictions to be made for *in vivo* clearances.

The routes by which drugs may be metabolized or biotransformed, include oxidation, reduction, hydrolysis and conjugation reactions. It is important for medicinal chemists to understand these pathways, as the rate of metabolism of a drug can impact the ultimate pharmacological, pharmacokinetic and/or toxicological activities.

The most commonly applied model in the pharmaceutical industry to determine metabolic stability utilizes NADPH supplemented liver microsomes from various species. However, only certain biotransformations, mainly mediated by the cytochrome P450 (CYP450) enzymes, are characterized by this method. It is well known that certain compounds are not prone to CYP450 mediated metabolism, but to direct conjugation with a sugar, sulphate or other hydrophilicity enhancing moieties.

Hepatocytes offer an excellent tool to elucidate metabolic pathways, as they comprise an intact cell system with both CYP450 (Phase I) and conjugation (Phase II) enzymes and their required cofactors. In Johnson & Johnson Pharmaceutical Research and Development, rat hepatocytes are applied in early drug discovery for studying both compound metabolic stability and metabolite formation. The analytical procedure utilizes LC-MS/MS for the study of compound turnover (*in vitro* half-life and subsequent calculation of physiological relevant hepatic clearance data) and the identification of Phase I and II metabolites.

## Goal

This report demonstrates the role of the Thermo Scientific TSQ Quantum Discovery in carrying out *in vitro* studies of compound metabolic stability and identification of Phase I and II metabolites. For the determination of compound metabolic stability, samples were quantified using SIM and from the metabolic turnover an *in vitro* half-life was calculated. Full-scan MS/MS Data Dependent scans were carried out for the identification of Phase I metabolites. For phase II metabolites, Constant Neutral Loss (CNL) scans followed by Data Dependent triggered MS/MS analysis were carried out for identification and structural elucidation.

## Experimental

### Rat hepatocyte metabolism

Test compounds (exemplified here with verapamil) were incubated in rat hepatocyte suspension cultures using 24-well high recovery plates, purchased from BD-Gentest (Woburn, MA, USA). Per well, 1 million viable cells were used in a 0.5 ml volume incubation medium.

For *in vitro* half-life calculations, incubates contained 5  $\mu$ M verapamil. Time points were 0, 15, 30, 60 and 120 minutes and assayed in triplicate. The reactions were stopped with two volumes of DMSO.

For metabolite identification, incubations were carried out using 50  $\mu$ M verapamil. The reaction was stopped with two volumes of DMSO after 60 minutes.

For sample preparation, the incubates were centrifuged for 10 minutes at 4°C in glass vials (3000  $\times$ g) and the supernatants were transferred to 96 well plates for analysis by LC-MS/MS.

## HPLC

Column: Thermo Scientific Hypersil™ BDS C18, 50×4.6 mm, 5 μ

Flow: 1.2 mL/min (from which 0.1 μl was split off and infused into the source)

Mobile phase A: 10 mM ammonium acetate (95%), acetonitrile (5%) pH 7.5

Mobile phase B: Acetonitrile (100%)

Injection volume: 50 μl

Gradient I: Quantitation using SIM, starting with 100% A to 5% A at 2.5 min, hold for 2 min, and return to 100% A in 0.5 min. Total time 6 minutes.

Gradient II: Qualitative metabolite ID, starting with 100% A, hold for 2 min, to 5% A in 4 min, hold for 5 min, return to 95% A in 1 min. Total time 12 minutes.

## Mass Spectrometry

TSQ Quantum Discovery from Thermo Fisher Scientific, San Jose, CA, USA. All analyses were carried out in positive electrospray mode.

ESI conditions: flow inlet 0.1 mL, spray voltage 3800, sheath gas pressure 30 λ, auxiliary gas pressure 10, capillary temperature 370°C.

Single Ion Monitoring (SIM): scan time 0.5 sec, scan width 1 Da, unit resolution (0.7 FWHM)

Full-scan MS/MS data dependent scans: 320–800 Da in 1 second. Data dependent scan (MS/MS) of most intense ions above threshold of 105 cps. Collision energy of 35 V and collision gas (Ar) pressure of 1.5 mTorr was used.

Constant Neutral Loss (CNL) scans: for losses of  $m/z$  176 (glucuronic acid adducts),  $m/z$  80 (sulphate) and  $m/z$  129 (glutathione adducts). Scan time 1 second. Collision energy 35 V, collision gas (Ar) 1.5 mTorr.

### Determination of *in vitro* metabolic turnover and calculation of metabolic half-life

The percentage compound remaining at a certain time, point (x), was determined by comparing the peak areas (measured in SIM) of the parent compound at  $T_x$  with that at  $T_0$  minutes and was calculated as follows:

$$\begin{aligned} \% \text{ remaining} &= [\text{Area at } T_x / \text{Average Areas } T_0] \times 100\% \\ \% \text{ metabolized} &= [100 - \% \text{ remaining}] \end{aligned}$$

(The averaged areas of the  $T_0$  minute triplicates are used for calculation)

Metabolic *in vitro* half-lives ( $t_{1/2}$ ) were calculated using the slope of the log-linear regression from the concentration remaining parent compound versus time relationship (k) as reported by Obach et al<sup>[3]</sup>.

$$t_{1/2} = -\ln 2 / k.$$

## Results

### Determination of the Verapamil *in vitro* half-life.

The determination of the metabolic *in vitro* half-life of Verapamil from the log-linear curve of the Verapamil turn-over is graphically outlined in Figure 1. The calculated *in vitro*  $t_{1/2}$  was 20 min.

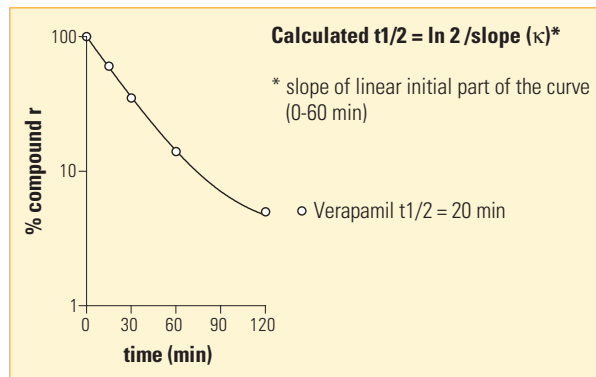


Figure 1: Log-linear curve of Verapamil turn-over with rat hepatocytes

### Phase I Metabolite identification

Several metabolites were detected in full-scan mode, however we exemplify with a major M-14 and a minor M-28 metabolite. The extracted ion chromatogram of verapamil and the corresponding M-14 and M-28 metabolites are illustrated in Figures 2a, 2b and 2c, respectively. Verapamil is observed to elute at 7.57 minutes, with the major M-14 metabolite at 7.15 minutes, and the minor M-28 at 6.76 minutes. The corresponding full-scan mass spectra of these three components are illustrated in Figures 2d, 2e and 2f. As is evident from these full-scan mass spectra, it is possible to determine the molecular weight but not a great deal of structural information. However, the Data Dependent full-scan MS/MS spectra shown in Figures 3a and 3b are more information rich, allowing a prediction of the structure for the M-14 metabolite.

### Phase II Metabolite identification

Phase II reactions in drug metabolism are classified as conjugation reactions and give products that in most cases account for the majority of the inactive, excreted products of a drug. Examples include glucuronidation, glycosidation and sulphation.

Figures 4a and 4b show the full-scan extracted ion chromatograms of the M-14 metabolite glucuronic acid conjugate (at 5.49 minutes) and the M-28 metabolite glucuronic acid conjugate (at 5.25 minutes), with their respective mass spectra in Figures 4c and 4d, respectively.

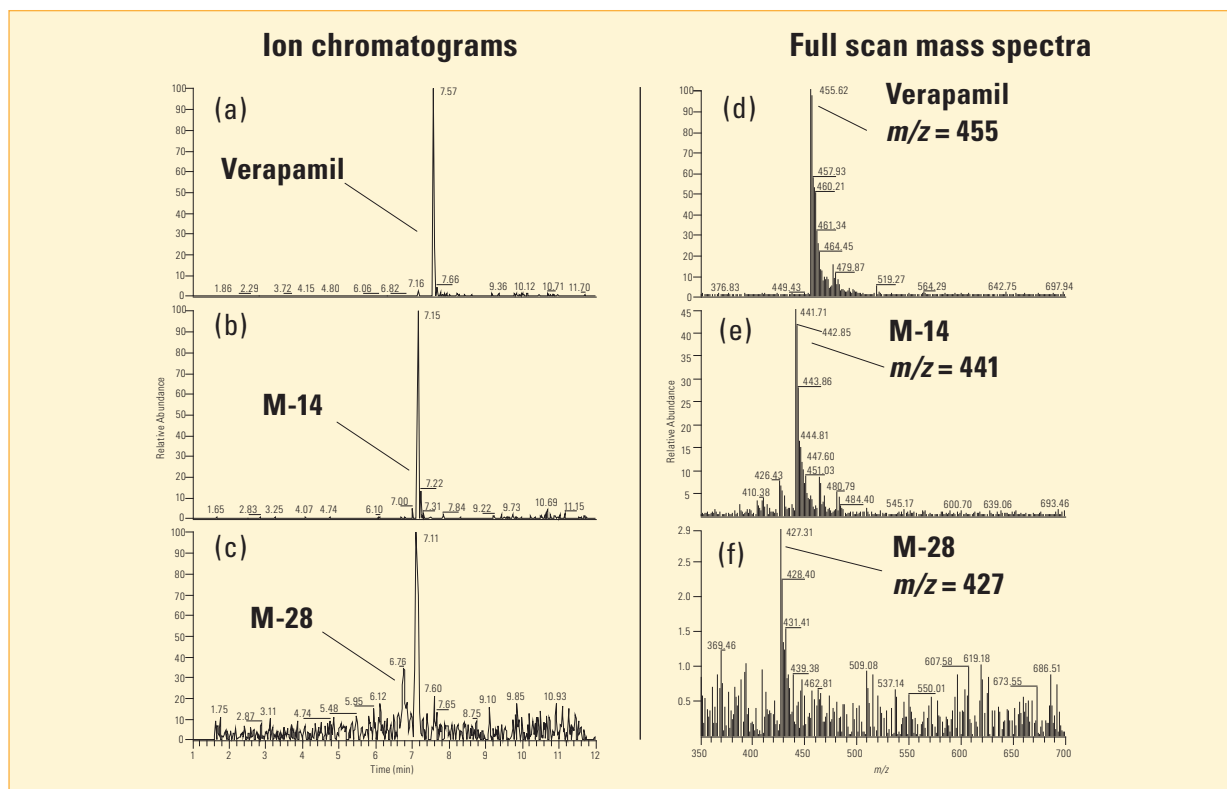


Figure 2: Extracted ion chromatograms (XIC's) of a) Verapamil, b) a M-14 metabolite, c) a M-28 metabolite and the corresponding full-scan spectra of d) verapamil, e) the M-14 metabolite and f) the M-28 metabolite, respectively, formed with rat hepatocytes

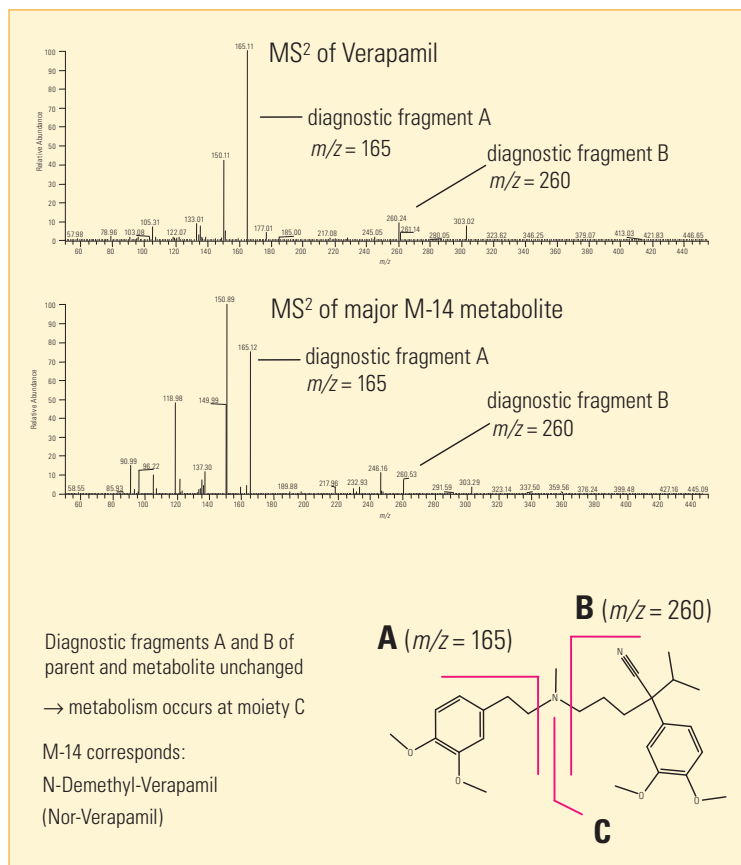


Figure 3: MS/MS spectra of Verapamil (a) and a major M-14 metabolite (b) obtained by Data Dependent full-scan MS/MS for proposal of phase I metabolite structure

In comparison with Figure 4, the CNL chromatograms (Figures 5a, and 5b) for the same M-14 and M-28 metabolite conjugates are more specific (due to the characteristic loss of 176 mass units), and provide much cleaner mass spectral data (Figures 5c and 5d). *This clearly demonstrates the advantages of using CNL scanning when looking for specific Phase II metabolites such as glucuronide conjugates.*

The MS/MS spectra of these two particular metabolic conjugates are shown in Figure 6 for structure elucidation. In Figure 6a, the presence of the diagnostic fragments at  $m/z$  165 and  $m/z$  260 (which are characteristic of nor-verapamil, see fragmentation schematics in Figure 3), indicate nor-verapamil glucuronide as the major Phase II metabolite. Added confirmation comes from the  $m/z$  441, which can be assigned to the free M-14 metabolite fragment. Following this rationale, it is suggested that the minor Phase II metabolite eluting at 5.25 minutes is a bi-demethyl-verapamil glucuronide.

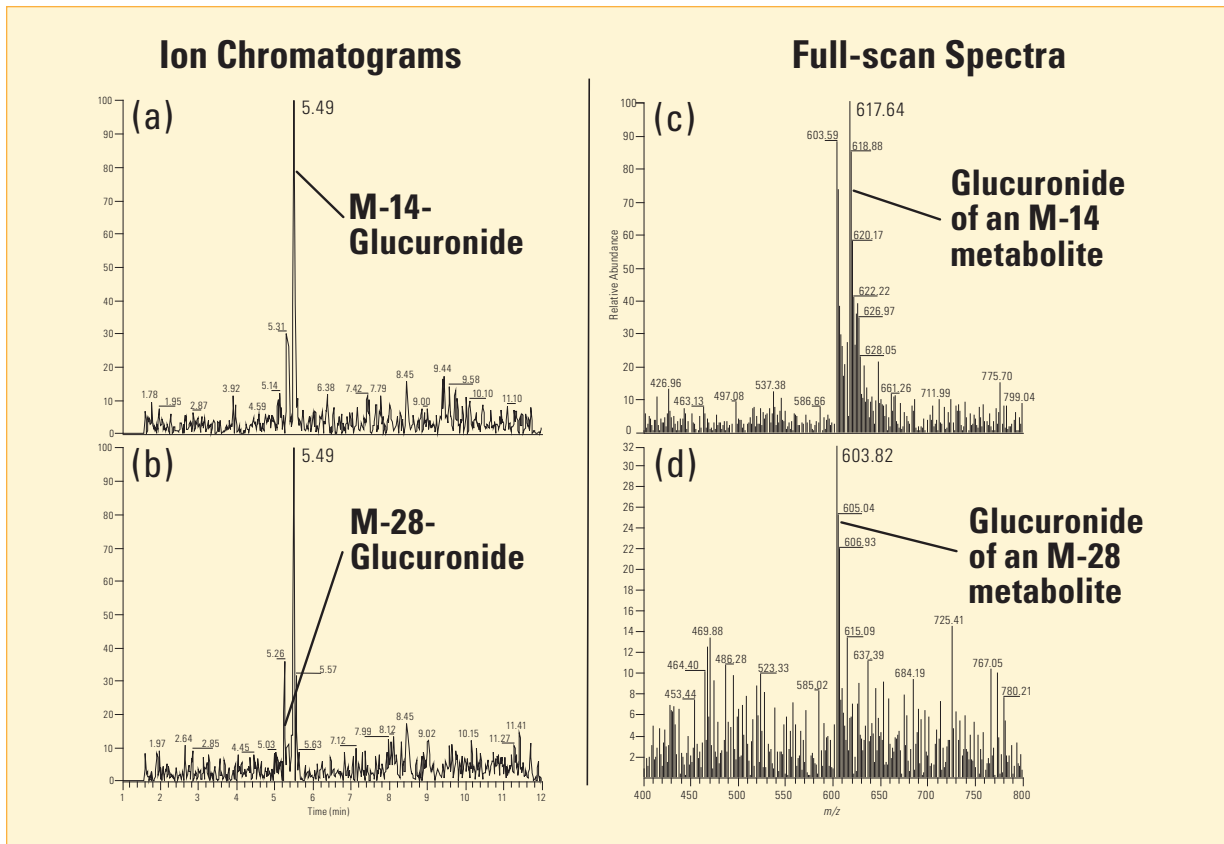


Figure 4: Confirmation of glucuronide formation in full-scan mode: Full-scan XIC of a) the M-14 metabolite glucuronic acid conjugate and b) the M-28 metabolite glucuronic acid conjugate and corresponding full-scan spectra c) of the M-14 metabolite glucuronic acid conjugate and d) of the M-28 metabolite glucuronic acid conjugate

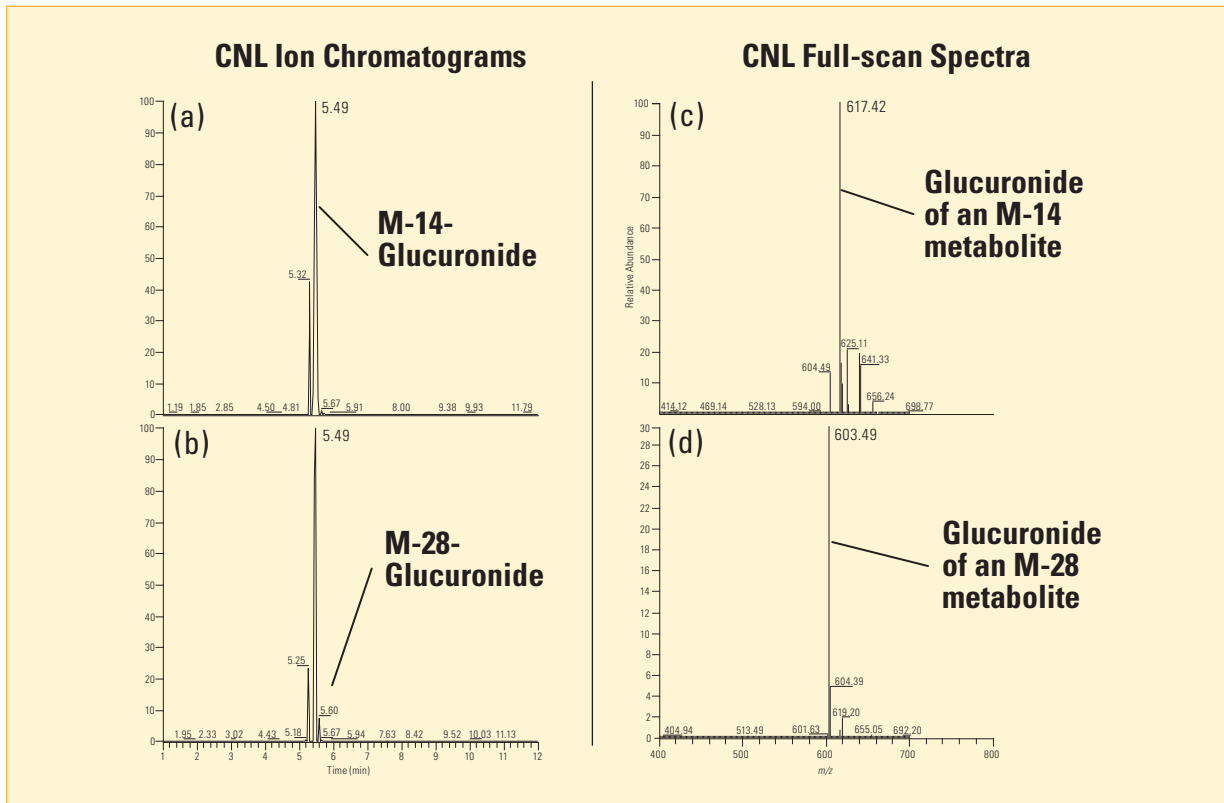


Figure 5: CNL XIC's of the a) M-14 metabolite glucuronic acid conjugate and b) M-28 metabolite glucuronic acid conjugate and CNL spectra c) M-14 metabolite glucuronic acid conjugate and d) M-28 metabolite glucuronic acid conjugate

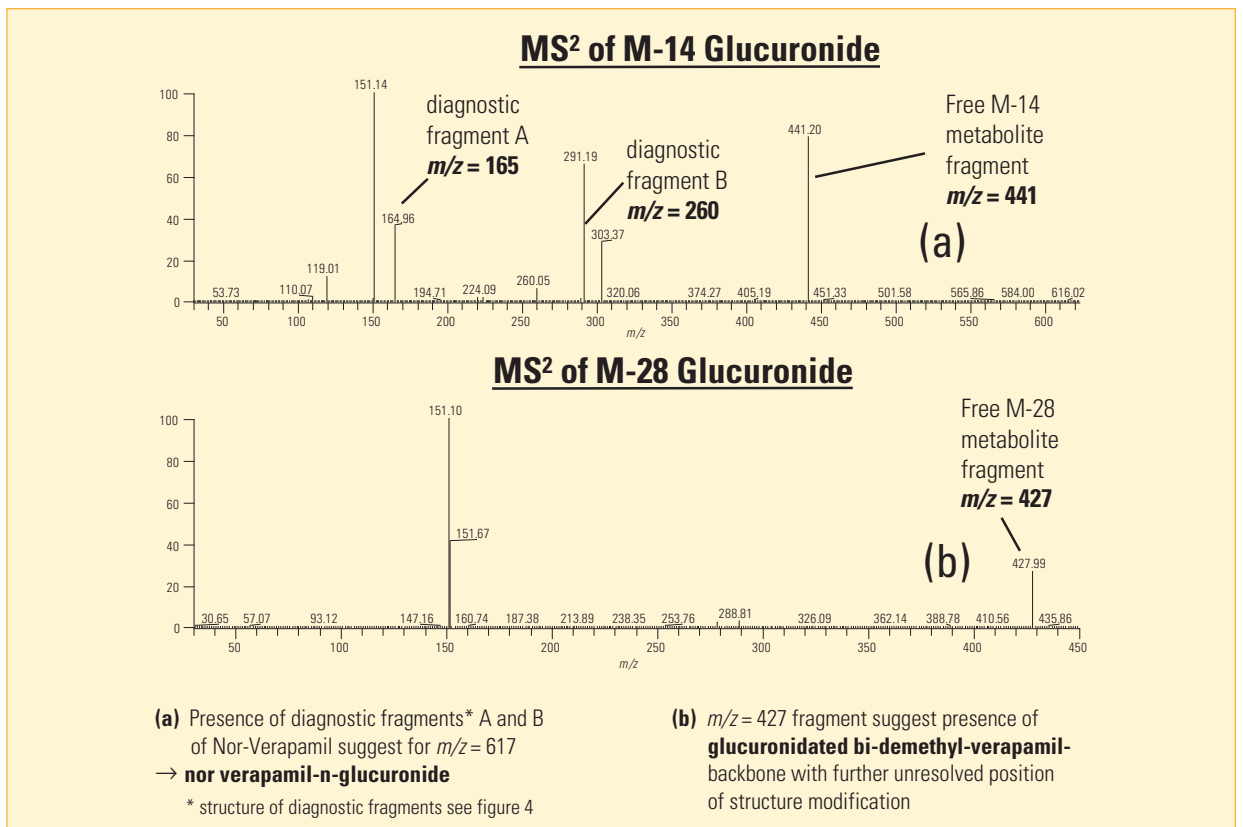


Figure 6: MS/MS spectra of phase II metabolites for structure elucidation a) M-14 metabolite glucuronic acid conjugate and b) M-28 metabolite glucuronic acid conjugate

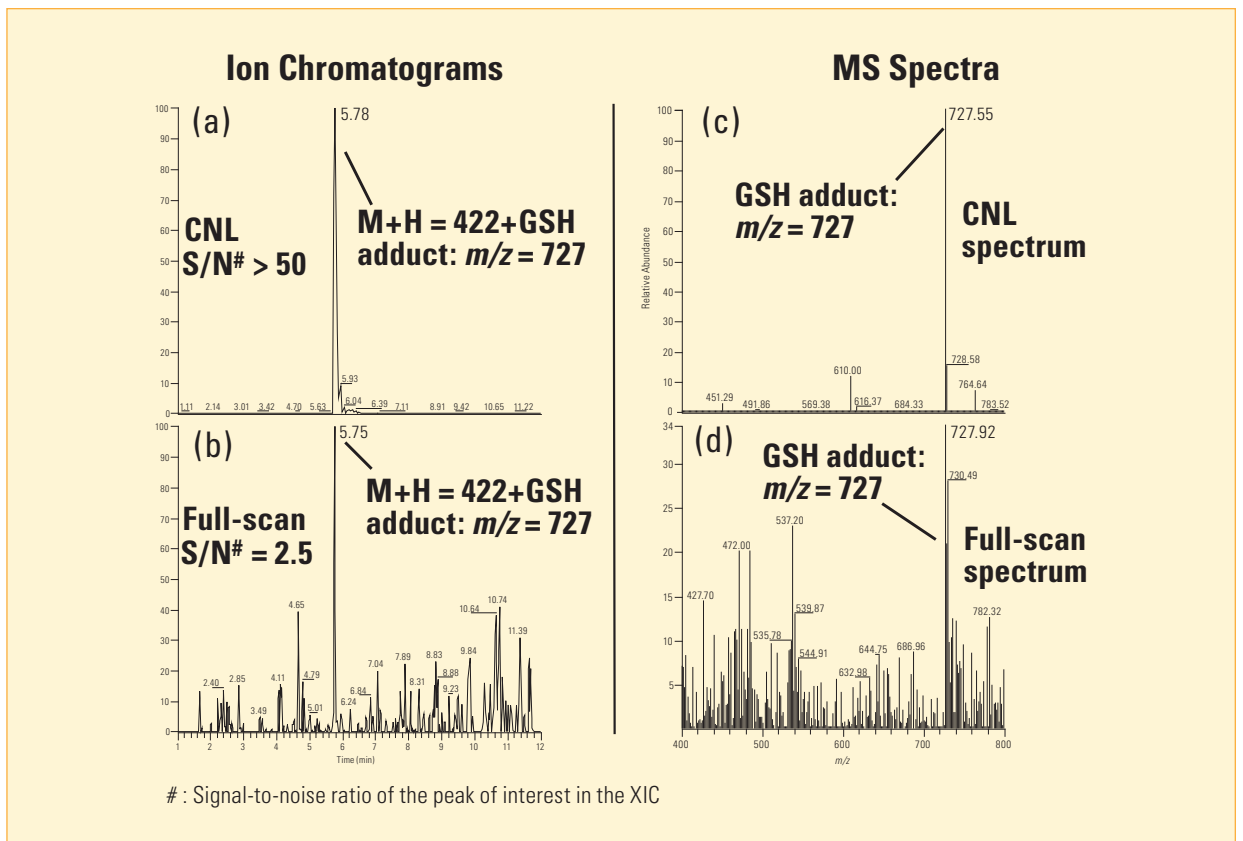


Figure 7: Detection of a glutathione adduct using CNL (129) scan and confirmation with MS full-scan mode: XIC of the GSH adduct a) in CNL mode and b) in full-scan mode and the corresponding mass spectra c) from CNL mode and d) measured in full-scan

A further example of the advantage of CNL scanning over full-scan mode is illustrated by the detection of another Phase II metabolite in Figure 7. This shows the glutathione conjugate of a proprietary drug discovery compound (mwt 421 amu, structure not disclosed). Figure 7a shows the ion chromatogram obtained using CNL and Figure 7b shows the same chromatogram but obtained in full-scan mode. The respective mass spectra, in Figures 7c and 7d, respectively, again clearly highlights the benefit of CNL data for structural elucidation: while the signal-to-noise ratio (S/N) was 2.5 in full-scan, in CNL the S/N was greater than 50.

## Conclusions

The utility of the TSQ Quantum Discovery in early drug discovery for the measurement of a compound's metabolic stability with rat hepatocytes and for the identification of its phase I and II metabolites has been illustrated.

The measurement of the in vitro metabolic turn-over in terms of an in vitro half-life was performed using selective ion monitoring.

The identification of phase I metabolites was facilitated by applying Data Dependent full-scan MS/MS. Phase II metabolites were identified using Constant Neutral Loss scanning especially for glucuronides and glutathione conjugates. A significantly better signal-to-noise ratio in CNL as compared to full-scan could be demonstrated. However, using information of the Data Dependent full-scan MS/MS also for phase II metabolite identification in addition to the CNL scanning can give even more structural information on the metabolite and provides an additional confirmation of the proposed metabolite formation.

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