

Identification of Metabolites Using Data Dependent Constant Neutral Loss Scans on the Finnigan LTQ Linear Ion Trap Mass Spectrometer

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Overview

Purpose: To illustrate the application of automated Data Dependent™ constant neutral loss (DDCNL) scan function on the Finnigan™ LTQ™ for identification of metabolites.

Methods: Liquid chromatography with on-line electrospray ionization mass spectrometry was used for the analysis of drug samples following incubation with rat hepatocytes.

Results: The ultra-high sensitivity of the Finnigan LTQ, together with DDCNL scanning was used to trigger MS³ fragmentation based on a neutral loss. This resulted in unambiguous determination of Phase I and Phase II metabolites in a single chromatographic run.

Introduction

Determination of the metabolic fate of drugs is an essential and important part of the drug development process. *In vitro* biotransformation studies are commonly performed in the early drug discovery stage to determine the metabolic pathways and to generate Phase I and Phase II metabolites for their further characterization. In Phase I reactions, the parent compound undergoes either hydrolysis, oxidation and/or reduction. Phase II reactions involve glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. Each of these transformations typically shows a characteristic mass shift with respect to the molecular ion of the parent compound.

The wide variety of potential metabolites and resulting complex mixtures make their identification a challenging and labor-intensive task. The Data Dependent constant neutral loss (DDCNL) scan function that is available on all Finnigan Ion Trap Mass spectrometers can significantly expedite identification of these metabolites.

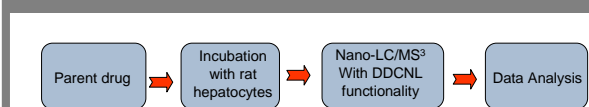
Methods

Sample preparation: Verapamil (5 µM) was incubated with rat hepatocytes (0.25 million cells/mL) in a 12 well plate for 3 hrs at 37 °C. The incubation was quenched by addition of equal volume (1 mL) of acetonitrile and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to vials for analysis.

LC conditions: Finnigan Surveyor™ MS pump (Thermo Electron) with a 2.1 x 50 mm BetaBasic™ C18 column. A gradient from 5-90% B in 10 min was employed. Solvents A and B were water and acetonitrile, respectively, each containing 0.1% formic acid.

MS conditions: The incubated drug sample was analyzed on the Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron), using Data Dependent scans. Spray Voltage 4.5 kV, Heated capillary temperature :350 °C, Normalized Collision Energy™: 30 %, Isolation width: 2.0 amu.

FIGURE 1. Workflow diagram for analysis of drug metabolites using DDCNL (Data Dependent Constant Neutral Loss) scan functionality



Results

Neutral loss scans were used to search for metabolites based on MS/MS structural features shared by the metabolite and the unmodified drug, i.e. a common mass difference between the protonated molecular ion of the parent and the MS/MS fragment ions. The flow chart of scan events for this Data Dependent Neutral Loss experiment is shown in Figure 2. An MS survey scan was first performed, followed by 5 MS/MS scan events. When the MS/MS scan event detected a neutral loss and the intensity of the parent ion was among the top 5 (or top 3, depending on the user's setting), it triggered the MS³ scan event. When a neutral loss is not detected, another MS scan was repeated.

FIGURE 2. Automated metabolite identification using Data Dependent MS³ on the Finnigan LTQ

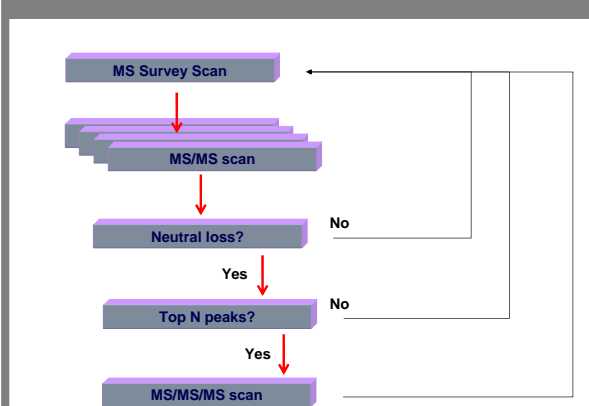


FIGURE 3. Data Dependent acquisition parameters for analysis of metabolites

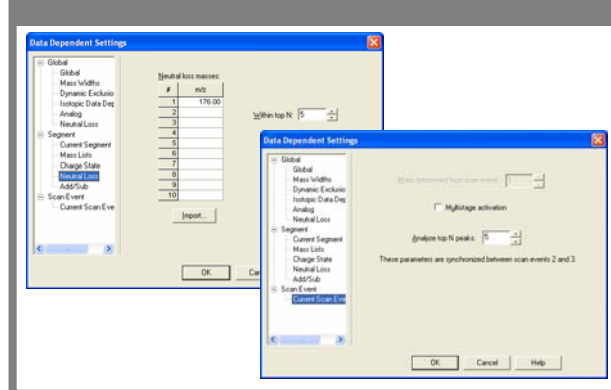


FIGURE 4. MS/MS spectra for Verapamil and a list of possible neutral loss masses

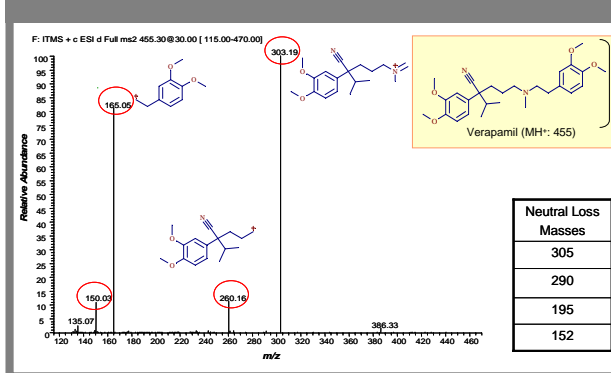


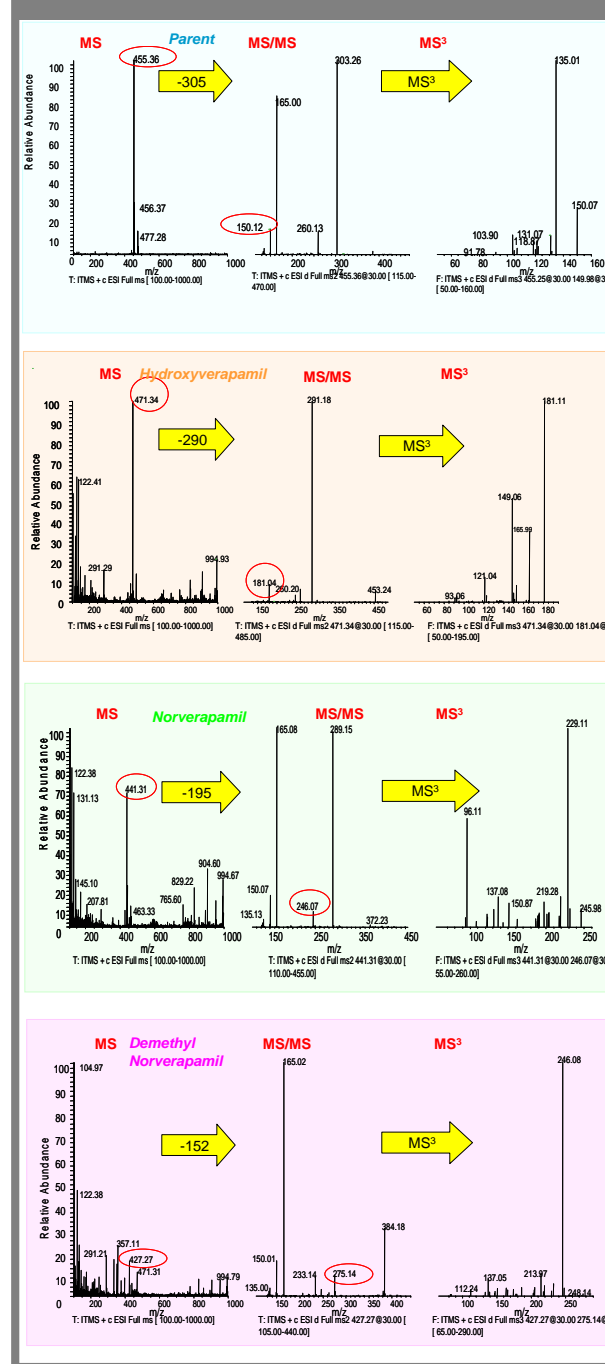
FIGURE 5. Metabolites identified as a result of DDCNL scanning for the defined neutral losses

Neutral loss mass	m/z	455 (parent)	441	427	471
305					
290					
195					
152					
All of the above					

The set-up for performing neutral loss analysis is shown in Figure 3. The user can define either single or multiple neutral loss masses. The "Within top N" dialog box directs the software to look for the neutral loss among top N ions in the MS/MS spectra. The "Analyze top N peaks" dialog box enables the acquisition of MS/MS spectra on the top N peaks in the MS scan. The neutral loss masses were determined by looking at the fragment ions in the MS/MS spectra of the parent drug. Figure 4 shows the MS/MS spectra of the parent drug. Verapamil incubated with rat hepatocytes was analyzed for identification of possible Phase I metabolites using these neutral loss masses.

Figure 5 shows the results obtained from analysis of an incubated Verapamil sample at different neutral loss mass settings. For a neutral loss mass of 305, DDCNL scan function triggered an MS³ scan on only the parent drug whereas for a neutral loss mass of 290 an MS³ spectra was obtained for m/z 455 (parent drug), 441 and 471. Similarly, the neutral loss mass of 152 indicated the presence of putative metabolites at 441 and 427. The DDCNL functionality can be used to monitor more than one neutral loss mass in the same experiment in which case all the putative metabolites will be identified in a single run. This is indicated by the last row in the table. The MS/MS and MS³ spectra for Verapamil and its metabolites are illustrated in Figure 6.

FIGURE 6. MS, MS/MS and MS³ spectra for Verapamil and its metabolites identified using DDCNL scanning

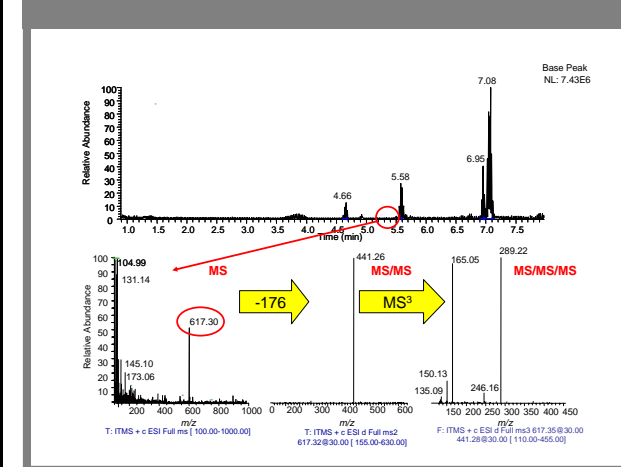


The neutral loss masses for Phase II biotransformations were determined from the mass shifts as listed in Figure 7. For example, the DDCNL scanning with a neutral loss mass of 176 amu was used to screen for Phase II metabolites of verapamil that had undergone glucuronidation reaction. Figure 8 shows identification of Nor-Verapamil-N-Glucuronide using DDCNL scan with a neutral loss of 176. The triggering of the MS³ scan points to the position of elution of this low level metabolite, whereas the MS/MS and MS³ spectra were used for structure elucidation.

FIGURE 7. List of Phase II Biotransformations

Biotransformation	Change in mass
Methylation	M+14
Acetylation	M+42
Sulfation	M+80, M+96
Glucuronidation	M+176, M+192
Taurine Conjugation	M+107
Cys Conjugation	M+121, M+119
GSH Conjugation	M+307, M+305

FIGURE 8. Identification of Nor-Verapamil-N-Glucuronide using DDCNL scanning



Conclusions

Data Dependent constant neutral loss (DDCNL) scanning was used for the identification of metabolites and their structural elucidation. Multiple neutral loss masses were defined in a given experiment resulting in identification of several putative metabolites in a single chromatographic run. The triggering of the MS³ scan event with a user-defined neutral loss points to the ions that exhibit a common structural motif (e.g. neutral loss of 176 points to metabolites that have lost glucuronic acid). The benefit of DDCNL is that by performing MS³ scans only on those compounds that show the desired neutral loss, the mass spectrometer can rapidly scan for low intensity ions thereby increasing the selectivity for identification of metabolic modifications. It also simplifies the analysis of complex mixtures in biological matrices, and enables characterization of low abundance species. The ultra-fast cycle time of the Finnigan LTQ combined with unmatched high MS³ spectral quality are key to the success of DDCNL scanning.

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