

# Identification of Metabolites using LC-MS<sup>n</sup>

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## **Introduction**

Many xenobiotics are extensively metabolized by humans and animals in order to improve their hydrophilic character and therefore to improve their clearance e.g. in urine or bile [1].

Chemical modifications provided by different enzymes can be classified in phase I and phase II metabolism. Phase I metabolism improves the hydrophilic properties of a compound e.g. by introducing hydrophilic moieties (hydroxylation) or cleavage of lipophilic moieties (dealkylation). Moreover, it introduces sites for conjugation reactions into the molecule. Phase II metabolism leads to a further improvement of the hydrophilic character of a compound or phase I metabolite by conjugation with different substrates such as glucuronic acid, sulfate, amino acids, or glutathione.

In order to detect a compound via its metabolites in body fluids -one of the major tasks in clinical and forensic toxicology as well as doping control [2]- the metabolic pathways of the compound must be studied. Detection of metabolites increases thereby the reliability of compound identification, allows confirmation of the body passage, and finally, minimizes the risk of false negative and positive screening results. Therefore, several metabolism studies are performed using different hyphenated mass spectrometric techniques including low and high resolution mass spectrometry providing high identification power [3].

This article will give a short overview on fast identification of metabolites using liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques. More detailed information on this topic can be found elsewhere and are strongly recommended [3-11].

## **General aspects**

In order to detect a metabolite, various information obtained during a LC-MS/MS run must be considered. This includes, beside chromatographic behaviour, the specific MS/MS fingerprint spectrum obtained by collisions-induced dissociation (CID). Therefore, the knowledge of typical

fragmentation reactions for certain chemical moieties and metabolism steps leading to typical “ $m/z$  shifts” of the corresponding fragments is essential to successfully identify a metabolite. High resolution mass spectrometry (HRMS) can be very useful in distinguishing the empirical formula either of a MS/MS precursor or of a corresponding fragment formed by CID as described elsewhere [8, 11].

## Typical fragmentation reactions

In contrast to electron impact (EI) fragmentation [12] CID fragmentation is so far not fully understood. Nevertheless, systematic investigations have shown more or less specific neutral losses occurring in CID for certain functional groups or chemical moieties as described by Levsen et al. [13, 14]. For example, a water loss (-18 da) can be commonly observed for alcohol moieties, but it is less common for phenols. A neutral loss of ammonia (-17 da) can be observed for primary amines e.g. formed by dealkylation reactions. As mentioned above, this information can be very useful to “elucidate” the structure or specific structure elements of a metabolite. Table 1, modified according to reference [14], exemplarily summarizes some neutral losses for specific moieties.

## Phase I metabolism

As mentioned above, phase I metabolic reactions enhance the hydrophilic properties of compounds leading -with some exceptions, e.g. N-oxides, carboxylic acids- to lower relative retention times of the corresponding phase I metabolite using standard reverse phase liquid chromatography (RP-LC). A quick screen for possible metabolite targets can be easily performed by investigation of the corresponding  $m/z$  shifts of the MS/MS precursors caused by metabolic changes to the molecule. As depicted in Figure 1 (A-C), a nominal  $m/z$  shift (NMZS) of +16 da to the corresponding MS/MS precursor of trimipramine (B) is observed for the arylhydroxy- (A) and the N-oxide- metabolite (C) of trimipramine. Table 2 (top), modified according to reference [8], summarizes typical  $m/z$  shifts observed for the corresponding MS/MS precursor for phase I metabolites. In order to confirm the presence of a metabolite spectrum, interpretation of the spectrum of the parent compound and the corresponding putative metabolite candidates must be performed and will be discussed exemplarily for trimipramine and its hydroxyl metabolites depicted in Figure 1. In detail: hydroxylation of the ring system will lead to a corresponding

NMZS of +16 da for fragment  $m/z = 250$  to  $m/z = 266$  (D). As a water loss ( $\Delta -18$  da), typical for aliphatic alcohols (see above), can not be observed, aromatic hydroxylation to a phenol must be assumed for the trimipramine metabolite depicted in Figure 1 (A). Comparison of the MS<sup>3</sup> spectrum of the shifted fragment  $m/z = 266$  (D) showed a similar fragmentation pattern as fragment  $m/z = 250$  (E) observed for trimipramine. Nevertheless, the position of the aromatic hydroxylation can not be determined exactly by MS techniques (even HRMS), highlighting one of their limitations for structure elucidation [13]. The presence of fragment  $m/z = 100$  - representing the alkyl side chain - for the trimipramine metabolite depicted (A) and the parent compound (B) further confirms that metabolism had taken place in the ring system.

In contrast, a corresponding NMZS of +16 da of the side chain fragment can be observed for the putative trimipramine metabolite depicted in Figure 1 (C). The formation of a *N*-oxide must be assumed as the corresponding side chain fragment is shifted by +16 da but no typical water loss is observed (see above). Additionally ions formed by the fragment  $m/z = 250$  da can be used for confirmation of the “trimipramine structure” (D+E) as described by Wissenbach et al. [15]. As mentioned above, chromatographic behaviour must also be considered for metabolite identification. Lower relative retention to trimipramine can be observed for trimipramine-M (HO-aryl-), while trimipramine-M (*N*-oxide-) shows higher relative retention (for RP-LC) [8]. While derivatization can provide useful structural information [16], it is not limited to GC-MS analysis as described extensively by Liu et al. [6].

## Phase II metabolism

Due to their higher hydrophilicity, lower relative retention times can be observed for the phase II metabolites of the corresponding phase I metabolites (or parent compound) with some exceptions as described more in detail by Holcapek et al. [8]. As described above, typical mass shifts of the MS/MS precursor can be observed for phase II metabolites and are summarized in Table 2 (bottom), modified according to reference [8]. As the conjugate moiety usually represents the most labile chemical bond of the metabolite molecule, typical neutral losses can be observed by CID fragmentation as the most prevalent fragmentation pathway of a phase II metabolite as depicted in Figure 2. Therefore screening for these neutral losses can be a useful strategy for efficient detection of phase II metabolites. Confirmation of the phase II metabolite can be performed e.g. by multistage MS techniques as described by Wissenbach et al. [15].

Nevertheless, the position of conjugation can often not be distinguished directly from MS/MS data even when using HRMS [6].

## Summary

Various information obtained by LC-MS/MS analysis must be considered to successfully detect a metabolite: mass shifts for corresponding MS/MS precursors, chromatographic behavior, mass shifts for corresponding MS/MS fragments and group-specific neutral losses provided by CID. HRMS can be used to additionally confirm the empirical formula of a metabolite or its fragments. Nevertheless, even when using HRMS the position of e.g. an aromatic hydroxylation or conjugation sites can not easily be determined by LC-MS/MS.

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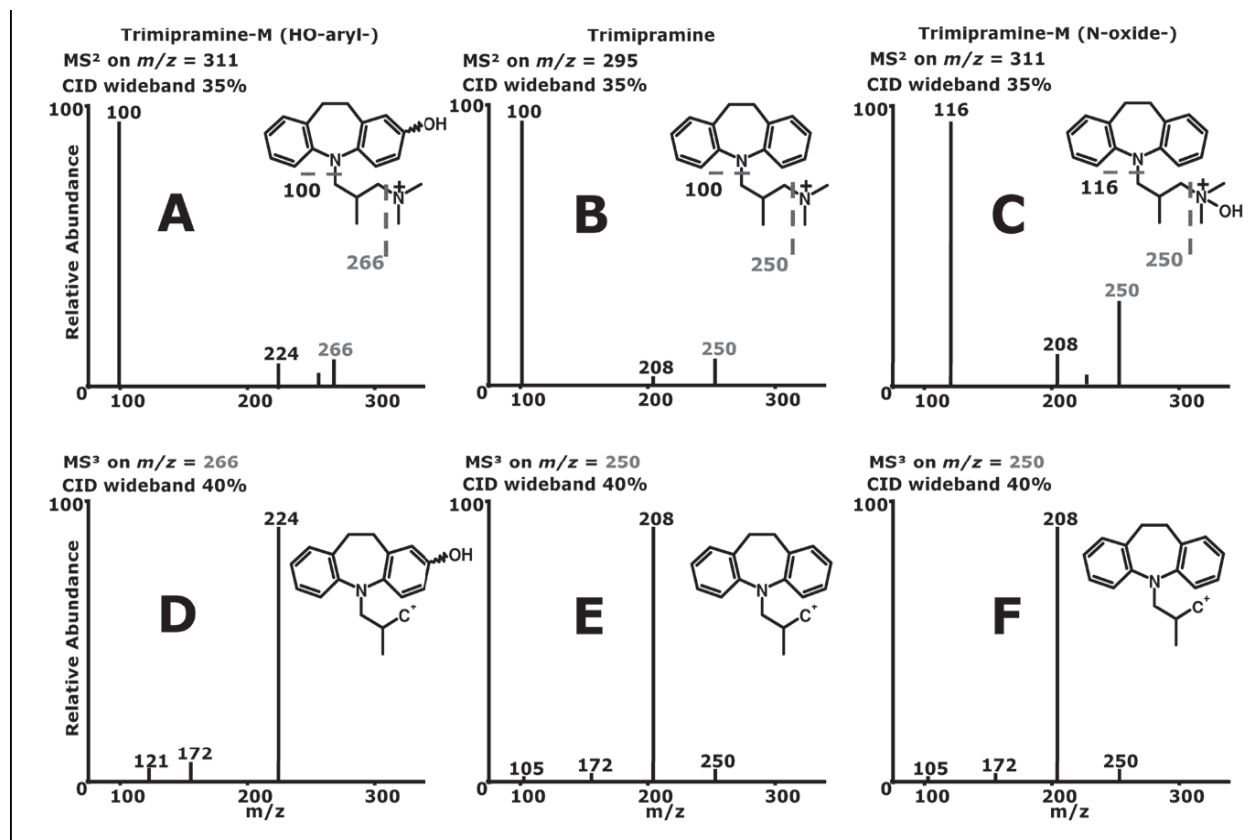
**Table 1:** Examples of neutral losses occurring during collision-induced dissociation of specific functional groups or chemical moieties (modified according to reference [14]).

Nominal mass shifts ( $\Delta$ da)	Neutral loss	Functional group or chemical moiety
-17	NH <sub>3</sub>	primary alkyl amines
-18	H <sub>2</sub> O	alcohols, carboxylic acids
-20	HF	fluorides
-28	CO	aldehydes, ketones, carboxylic acids
-42	CH <sub>2</sub> -C=O	N-acetyl-derivatives
-44	CO <sub>2</sub>	carboxylic acids
-46	HCOOH	carboxylic acids
-80	SO <sub>3</sub>	sulfonic acids
-176	anhydroglucuronic acid	glucuronides
-194	glucuronic acid	glucuronides (benzylic)

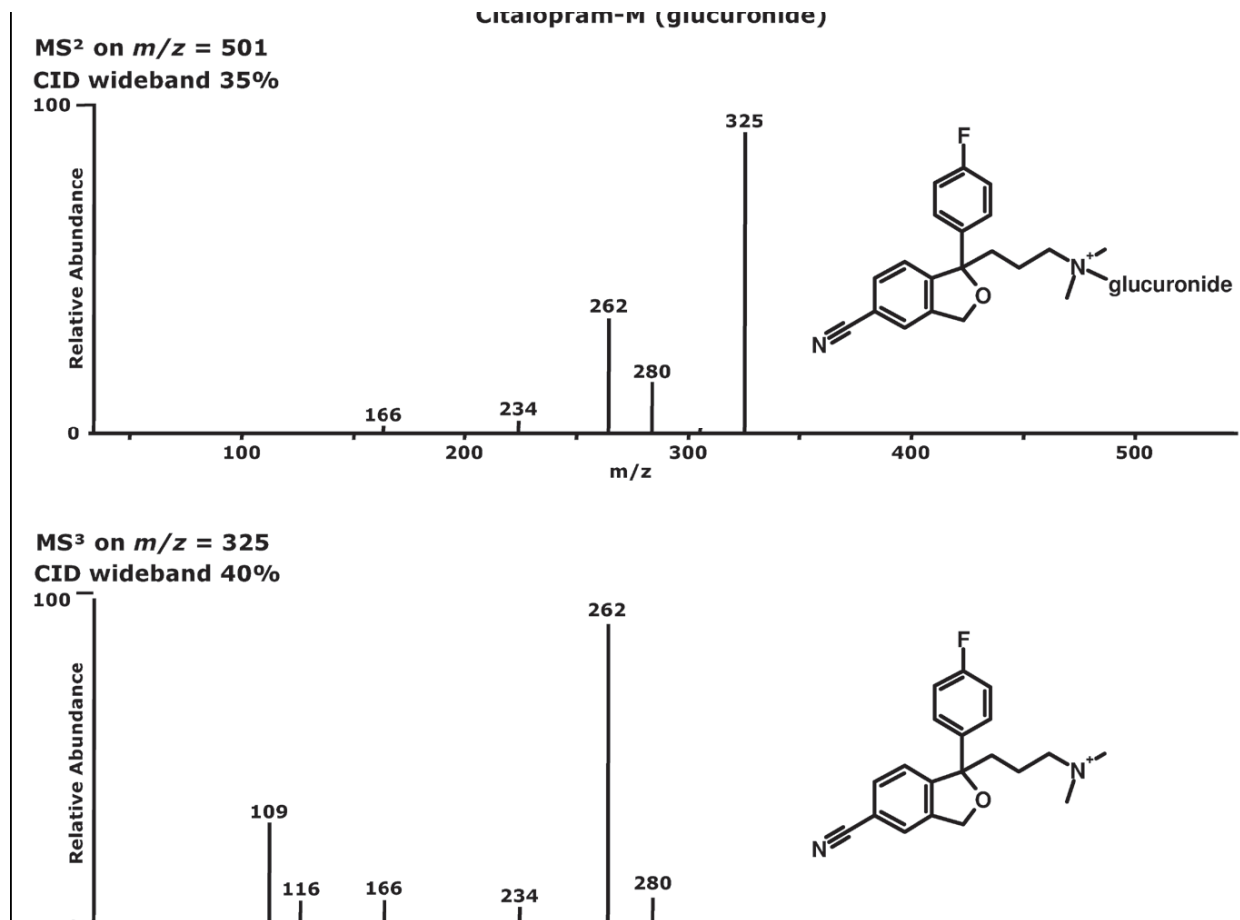
**Table 2:** Examples of nominal mass shifts for specific metabolic reactions phase I (top) and phase II (bottom) (modified according reference [8]).

<b>Phase I Metabolism</b>	
<b>Nominal mass shifts(<math>\Delta da</math>)</b>	<b>Metabolic reaction</b>
-62	debromation (oxidative)
-28	deethylation
-18	dechlorination (oxidative)
-14	demethylation
-2	first/second alcohols to aldehyd/ketone
2	ketone to alcohol
16	hydroxalytio
	alkene to epoxide
32	hydroxalytion (2X)
48	hydroxalytion (3X)
<b>Phase II Metabolism</b>	
<b>Nominal mass shifts(<math>\Delta da</math>)</b>	<b>Metabolic reaction</b>
14	methylation
42	acetylation
57	conjugation (glycine)
80	conjugation(sulfate)
161	conjugation (N-acetylcysteine)
162	conjugation (glycosides)
176	conjugation (glucuronidation)
305	conjugation (S-GSH)

**Figure 1:** MS<sup>2</sup> (A-C) and MS<sup>3</sup> (D-F) spectra of trimipramine-M (HO-aryl-) (A), trimipramine (B) and trimipramine-M (N-oxide-) (C) according to reference [15].



**Figure 2:** MS<sup>2</sup> (top) and MS<sup>3</sup> (down) spectra of citalopam-M (glucuronide) according to reference [15].



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