

Structural Determination of By-Products Obtained in Acetylation of Cysteine and Cysteine-Conjugated Metabolites by LTQ-Orbitrap XL – Leading to the Establishment of an Alternative Esterification and Amidation Method

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Introduction

Chemical derivatization in combination with LC/MS has been widely used in drug metabolism to increase analyte stability, separate analytes from the matrix, resolve isomers/enantiomers, and/or enhance their LC/MS mass sensitivity. During the course of identification of a cysteine-conjugated metabolite of XBL00001 (M1) from goat liver, acetylation of the metabolite was conducted to remove the matrix and retain it on a reversed-phase HPLC column to facilitate LC/MS/MS analysis. In addition to the regular di- and tri-acetylated products, two major unusual acetylated by-products were obtained, each having a molecular weight of 14 Da higher than respective di- or tri-acetylated products. The objectives of this study were to 1) identify the acetylation by-products by LC/MS/MS including fragmentation and accurate mass determination using an LTQ Orbitrap XL; 2) propose a possible mechanism of by-product formation; and 3) apply this esterification and amidation method in drug metabolite identification.

Experimental

Liquid chromatography:

Pump: Shimadzu LC-20AT Pumps
Mobile Phases: A: 10 mM ammonium acetate in water, pH 5.6
B: MeOH

Mass Spectrometry:

MS Systems: Thermo LTQ Orbitrap XL
Ion spray (IS): 4.5 kV
Capillary temp: 350 °C
Sheath gas: 60
Auxiliary gas: 40
Sweep gas: 10

Acetylation of M1 and L-Cysteine:

The acetylation of M1 and L-cysteine with acetic anhydride was performed in a pyridine solution. The reaction mixture was evaporated to dryness under a nitrogen stream and the residues were dissolved in methanol for LC/MS/MS analysis.

Results and Discussion

1 Methylation during Acetylation

Acetylation of a metabolite (designated M1) with this method yielded two major derivatives (Figure 1). The first at R_t ca. 26 min showed an MH^+ at m/z 315.11188, corresponding to a molecular formula of $C_{12}H_{19}O_4N_2S$ (-0.87 ppm), consistent with a di-acetylated M1 plus 14 Da. Likewise, the second at R_t ca. 43 min showed an MH^+ m/z 357.12224, consistent with a tri-acetylated M1 plus 14 Da (-1.33 ppm). Both di- and tri-acetylated M1 had one additional CH_2 unit (14 Da) over the respective di- and tri-acetylated M1, based on the LC-FTMS data. MS/MS analysis of the di-acetylated by-product yielded a product ion at m/z 283 (Figure 2), resulting from loss of $HOCH_2$ (32 Da), indicating the presence of a methyl ester moiety. The acetylation of L-cysteine with the same procedure yielded a similar di-acetylated by-product, producing an MH^+ at m/z 220.06378, corresponding to a molecular formula of $C_8H_{14}O_4NS$ (-0.11 ppm).

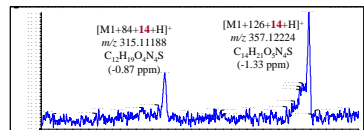


Figure 1. HPLC/RAM Chromatogram of Acetylated M1.

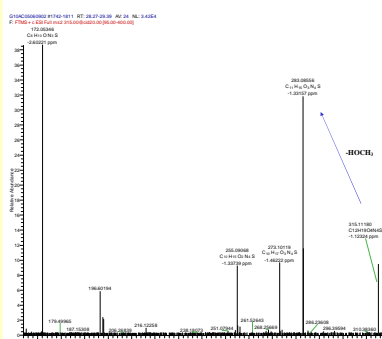


Figure 2. (+)-CID FT-MS/MS of di-acetylated M1 by-product

2 Confirmation of the Reaction:

A cysteine acetylation reaction mixture was aliquoted into three vials containing acetonitrile, methanol, or ethanol, followed by LC/MS/MS analyses. The methylated (m/z 220, Figure 3B) and ethylated (m/z 234, Figure 3C) products of acetylated cysteine were formed in the methanol and ethanol solutions, respectively, however, only regular acetylation products with m/z 206 were formed in the acetonitrile solution (Figure 3A).

3 Reaction with an Amine

Succinic acid was dissolved in pyridine, followed by the addition of acetic anhydride, and then 2-dimethylaminoethylamine. An amide derivative was obtained.

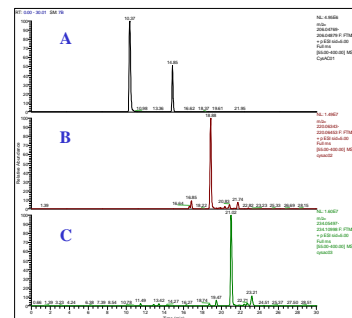
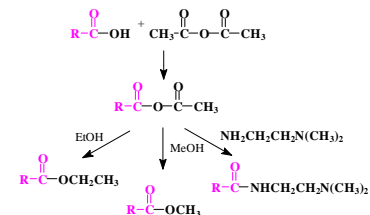


Figure 3. Extracted ion chromatograms of di-acetylated cysteine (m/z 206) (Panel A), methylated di-acetylated cysteine (m/z 220) (Panel B), and ethylated di-acetylated cysteine (m/z 234) (Panel C).

4 Mechanism of the Reaction:

We propose that the carboxyl moiety replaces one acetyl moiety of acetic anhydride to form a mixture acid anhydride, which then reacts with alcohols or amines to form respective esters or amides.



5 Application in Drug Metabolite Identification

- Methylation in drug metabolism studies is conventionally obtained by a reaction with diazomethane. Diazomethane is toxic and explosive, and needs to be freshly prepared. The reaction proposed herein is much simpler and less time-consuming.
- Methylation is commonly used to confirm the presence of a carboxylic acid drug metabolite, but a methyl ester could also be a real drug metabolite. Ethylation or amidation reaction may also be very useful in the quantitative bioanalysis field to enhance the HPLC chromatography and mass sensitivity of plasma or other matrix extracts.
- For weak ionizing compounds, formation of an amide derivative will dramatically enhance the sensitivity (10-100x) during LC/MS analysis in the positive ion mode.

Conclusion

An alternative esterification and amidation method was established, and has demonstrated to be very useful in drug metabolite identification.