K57 Incomplete Recovery of Codeine in Urine Using Common Enzymatic Hydrolysis Procedures

Sumandeep Rana, MS, Tara Brunson, BS, and Wayne B. Ross, MCLS, Redwood Toxicology Laboratory, 3650 Westwind Blvd, Santa Rosa, CA 95403*

The goal of this presentation is to inform attendees about the effectiveness of various commonly used hydrolysis techniques and conditions for the hydrolysis of opiates, stressing the incomplete hydrolysis of codeine following common enzymatic hydrolysis procedures.

This presentation will impact the forensic science community by demonstrating that choosing the right combination of hydrolyzing agent and hydrolysis conditions is critical to accurate results and leads to significant improvement in recovery of opiates from urine samples.

Methods: Opiates included in this method are: morphine, hydromorphone, codeine, and hydrocodone. Deuterated analogues of all four analytes are used as the internal standards.

Extraction: Authentic urine samples were hydrolyzed using β-glucuronidase from *Escherichia coli and Helix-Pomatia* for 3 hr and 16 hr each. Samples were spiked with internal standard, centrifuged and supernatant was diluted with mobile phase before injecting on the column. In separate experiments, the amount of enzyme added was doubled to evaluate optimal concentration of the enzyme for efficient hydrolysis. In addition, one set of samples was hydrolyzed using acid hydrolysis with 0.1N HCl and the results were used as the reference (100% recovery) to evaluate recovery from different enzymatic procedures.

Analysis: Liquid Chromatography with Tandem Mass Spectrometry (LC/MS/MS) analysis was performed in Electronic Spray Ionization (ESI) mode by Multiple Reaction Monitoring (MRM) using a 3200 triple quadrupole mass spectrometer connected to a Shimadzu prominence HPLC system. Separation was achieved on an ultra II biphenyl 5µ column (50 X 2.1mm). Mobile phases were 0.1% formic acid and 0.2% ammonium formate in de-ionized water (A) and in acetonitrile with 2% water (B). All analytes were eluted within four minutes. Two ion transitions for each analyte; morphine (286/152, 286/128), hydromorphone (286/185, 286/157), codeine (300/152, 300/115), hydrocodone (300/199, 300/128), and one ion transition for each internal standard; morphine-D6 (292/152), hydromorphone-D3 (289/185), codeine-D6 (306/165), and hydrocodone-D6 (306/202) were monitored.

Results: The procedure was applied to 50 authentic urine specimens previously tested positive for two or more analytes using acid hydrolysis and GC/MS. Results showed that efficient hydrolysis is essential to the optimum recovery of all analytes. β-glucuronidase from both *H. Pomatia* and *E. Coli* were not able to cleave codeine glucuronides efficiently and recovered only 25% and 50% of the free drug after 3 hr and 16 hr hydrolysis time, respectively. On the contrary, 100% recovery was achieved for hydrocodone after 3 hr with both *H. Pomatia* and *E.Coli*. Average morphine recovery was 84% with *H. Pomatia* at 3 hr and 100% after 16 hr of incubation. *E. Coli* recovered 77% and 89% morphine at 3 hr and 16 hr, respectively. Average hydromorphone recovery with *H. Pomatia* was 80% after 3 hr and 95% after 16 hr. *E. Coli* recovered only 41% hydromorphone at the end of 3 hr and 58% after 16 hr of incubation. Doubling the amount of enzyme did not improve the recovery for any of the opiates.

Conclusion: Acid hydrolysis for opiates has been commonly used with GC/MS analysis in the past. With the advancement of instrumentation, LC/MS/MS is gaining popularity in the clinical and forensic labs and enzymatic hydrolysis is the preferred method for releasing the free drugs. Post-enzymatic hydrolysis specimens can simply be diluted and injected on the column, eliminating the need for time-consuming extractions. It is essential, however, to optimize the hydrolysis conditions for the opiate glucuronides specific to each source of β-glucuronidase. Codeine glucuronide is the most difficult to cleave and only 50% of the drug was recovered in free form after 16 hr of hydrolysis with β-glucuronidase from *H. Pomatia.* In general, the enzyme from *H. Pomatia* performed better than the one obtained from *E. Coli*, under the conditions tested. Although *H. Pomatia* was able to release 100% of the free drug form morphine, hydrocodone, and hydromorphone conjugates in the urine samples at the end of 16 hr (3 hr in case of hydrocodone), it was found to be ineffective in cleaving codeine glucuronide. Further investigation is necessary to find the optimal conditions for enzymatic hydrolysis of codeine. The labs must carefully evaluate the hydrolysis efficiency of various enzymes for opiates and specifically for codeine.

Opiates, Hydrolysis, LC/MS/MS

 ** Presenting Author*