Development and validation of an opioid LC-MS/MS assay: evaluation of different β-glucuronidase enzymes and protein precipitation plates

He S. Yang, Alan H.B. Wu, Kara L. Lynch.

Department of Laboratory Medicine, University of California San Francisco and San Francisco General Hospital, San Francisco, CA 94110.

Background: Opioid confirmatory testing using mass spectrometry is more sensitive and specific for the detection of several opioids and their metabolites compared to immunoassay screening. Thus, it plays an important role in managing chronic pain patients on opioid therapy. In our laboratory, the current gas chromatography mass spectrometry (GC-MS) assay for opioid confirmation employs acid hydrolysis and derivatization. It detects codeine, morphine, hydrocodone, hydromorphone, oxycodone and oxymorphone. The whole procedure is quite laborious and time-consuming. Therefore, this work aimed to develop and validate a qualitative liquid chromatography tandem mass spectrometry (LC-MS/MS) method along with enzyme hydrolysis for the identification of thirteen opioid compounds. Analytes include morphine, codeine, hydromorphone, hydrocodone, oxymorphone, oxycodone, 6-MAM, methadone, EDDP, fentanyl, norfentanyl, buprenorphine, norbuprenorphine. In addition, we report a comprehensive evaluation of the performance of different β-glucuronidase enzymes and protein precipitation plates that are currently on the market.

Methods: Seven opioid glucuronides ordered from Cerilliant Corporation, including morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide, codeine-6-β-D-glucuronide, oxymorphone-3-β-D-glucuronide, hydromorphone-3-β-D-glucuronide, buprenorphine-glucuronide, norbuprenorphine-glucuronide, were spiked into certified drug free urine at a concentration of 1 ug/ml. Enzyme hydrolysis was carried out with three β-glucuronidase enzymes including IMCSzme™ (DPX Labs), Red Abalone β-glucuronidase (Kura Biotec), and E. Coli Recombinant β-glucuronidase (Kura Biotec). Hydrolysis efficiency at different time points was evaluated for each enzyme at its optimal temperature and buffer condition. In addition, the Supelco protein precipitation filter plate, Phenomenex Impact™ PLD+ plate, and Biotage Isolute PLD+ plate were evaluated for optimal compound recovery and minimal matrix effect.

Urine samples spiked with a mixture of thirteen opioid compounds and deuterated internal control (fentanyl-D5) were mixed with hydrolysis enzyme and its buffer, and incubated at the optimal temperature at different time points. After hydrolysis, the samples were transferred to a protein precipitation plate and mixed with 3X volume of ACN for sample clean-up and removal of the enzyme. The flow through was diluted 1:5 in mobile phase A (10mM ammonium formate). LC-MS/MS was performed using an Agilent HPLC with an ABSciex 5500 LC-MS/MS in positive ESI mode. Compounds were identified by a combination of retention time, two MRM transitions and an ion ratio. Separations were performed using a Kinetex 2.6 um Phenyl-Hexyl 100Å, 50X4.6mm LC column, with a 7 min gradient from 20% to 100% organic (Mobile phase A: 10 mM ammonium formate; Mobile phase B : methanol and 0.1% formic acid)

Validation of the final method included determining the lower limit of detection (LOD), linearity, precision, matrix effect, carry over, and recovery for each analyte. Thirty-five patient samples were analyzed by both GC-MS and LC-MS/MS methods. Some samples contained extremely high levels of opioids, such as morphine, oxycodone. These samples were tested straight and at various dilutions to determine the optimal dilution scheme, based on the immunoassay value, to minimize any potential carryover.

Results: Enzyme comparison test revealed that IMCSzyme and Red Abalone β-glucuronidase gave comparable hydrolysis efficiency which range from 54-76% for different opioid glucuronides at 1 hour; whereas hydrolysis efficiency of the E.Coli Recombinant β-glucuronidase was lower (41-65%). We chose IMCSzyme for this assay because of its hydrolysis efficiency and cost effectiveness. Supleco protein precipitation plate gave a higher and more consistent efficiency of recovery (88-103%) compared to the Phenomenex Impact™ plate and Biotage Isolute PLD+ plate, the latter two were around 50-90%. The calibration curves for each analyte exhibited consistent linearity and reproducibility in the range of 25-2000 ng/ml (2.5 – 200 ng/ml for fentanyl, norfentanyl, 6-MAM). Precision was determined by using two QC samples at 80 and 120 ng/ml (N = 5). Within-run coefficients of variation (CV) ranged from 1.7% to 9.6% for all analytes. Between-run CV ranged from 4.2% to 13.3%. Matrix effects were determined by using five donor urine matrices. No significant ion suppression was detected for any of the 13 analytes, with a few showing ion enhancement (83.2-135.4%). Recovery ranged from 88.1 to 103.1%, with an average of 98.6%. Comparison of 35 patient samples between LC-

MS/MS and GC/MS showed 82.5% concordance. LC-MS/MS assay was more sensitive for hydrocodone and hydromorphone compared to the GC-MS compounds due to interference or co-eluting peaks. More patient samples are undergoing the comparison analysis.

Conclusions: We have developed a sensitive and specific LC-MS/MS assay for opioid confirmations. The combination of IMCSzme β-glucuronidase and the supleco protein precipitation plate provided an efficient and cost-effective hydrolysis and sample clean-up. The procedure of LC-MS/MS sample preparation provides an approximately 60 % reduction in time and 75% reduction in cost compared to the GC-MS method.