A Rapid and Accurate LC-MS/MS Method for the Analysis of Nicotine, Nicotine Metabolites, and Minor Tobacco Alkaloid in Urine

Shun-Hsin Liang, Frances Carroll, Sharon Lupo, Ty Kahler, Paul Connolly, Rick Lake, Rob Freeman, Carrie Sprout

Restek Corporation, 110 Benner Circle, Bellefonte, PA 16823

Nicotine is the major tobacco alkaloid which underlies addiction of tobacco users. In humans, more than 70% of nicotine is transformed to cotinine which subsequently converted to trans-3'-hydroxycotinine, the main nicotine metabolite detected in urine. Nornicotine and norcotinine are the minor metabolites (0.5–2%) resulted from demethylation of nicotine and cotinine, respectively. The urinary measurement of nicotine metabolites has several aspects including monitoring public tobacco exposure, evaluation of nicotine replacement therapy, drug therapy assessment, forensic toxicology analysis, and life or health insurance application. In addition, nicotine metabolites can be used as the biomarkers for the pharmacogenomics evaluation and disease profiling. The minor tobacco alkaloid such as anabasine can be used as a unique marker for tobacco use and can only be detected in urine.

A variety of chromatographic methods have been developed for nicotine metabolite analysis. Most of the modern methods adapt the usage of high pH chromatography with relatively high concentration of additive reagents, which may not be applicable to all LC-MS instrumentation. The intent of this study was to develop a method for the analysis of nicotine related compounds in urine using "friendly" LC-MS/MS solutions and the highly efficient and selective Raptor™ Biphenyl column. The clinical applicability of the method was demonstrated by accurate and reproducible analysis of fortified analytes in the urine of non-tobacco user.

The fortified standard and QC samples were prepared with a liquid-liquid extraction procedure. An aliquot of 250 µL urine was mixed with 50 µL of internal standard solution (250 ng/mL in methanol) and 50 µL of 5N NaOH in a 4 mL glass vial. Extraction was performed by adding 1.5 mL of 50/50 methylene chloride/diethyl ether and stirred for 1.5 minutes. After centrifugation at 4000 rpm for 5 minutes, 1 mL of organic phase was transfer to a 1.5 mL HPLC vial and mixed

with 10 µL of 0.25N HCl before evaporating to dryness at 35°C under a gentle stream of nitrogen. The dried extract was reconstituted with 200 µL of water and injected into the Raptor™ Biphenyl column (100x2.1mm, 5µm) for the analysis with the Waters ACQUITY UPLC® I-Class System coupled with a Waters Xevo® TQ-S mass spectrometer using electrospray ionization in positive ion mode. Simultaneous analysis of all 6 analytes (nicotine, cotinine, trans-3'-hydroxycotinine, nornicotine, norcotinine, and anabasine) was performed with a fast 3 minute gradient and a total of 5 minute run time for each injection.

The calibration standards were prepared in blank urine with a range from 2 – 5000 ng/mL. The linearity range was from 2-1000 ng/mL for nicotine, nornicotine, norcotinine, and anabasine; 5-5000 ng/mL for cotinine; 10-5000 ng/mL for trans-3'-hydroxycotinine. The r^2 was ≥ 0.995 and the % deviation was within 15% of the nominal concentration (≤ 20% for the lowest concentrated standard). Per the noise-to-signal value at 10, the LLOQ is 0.4 ng/mL for all analytes.

Three levels of QC samples (7.5, 75, and 750ng/mL for nicotine, nornicotine, norcotinine, and anabasine; 75, 750, and 10,000 ng/mL for cotinine and trans-3'-hydroxycotinine) were prepared in urine for the test of accuracy and precision with established calibration standard curves. The 10,000 ng/mL QC sample for cotinine and trans-3'-hydroxycotinine was diluted 5-fold in water for the analysis. Analyses were performed on 3 different days. The method accuracy was demonstrated from the %recovery of within 10% of the nominal concentration for all QC levels. The %RSD was from 0.6-8.2% and 1.4-9.9% for intra-day and inter-day, respectively, indicating a good method precision.

Anabasine can only be detected in the urine of current tobacco users but not the urine of non-smoker or nicotine patch/gum users. With urinary analysis of anabasine from a light smoker, a moderate smoker, and a non-smoking nicotine patch user, it was demonstrated that the established method could clearly distinguish different tobacco users with specific and sensitive detection of anabasine.

The Raptor™ Biphenyl column was demonstrated to be excellent for simultaneous analysis of nicotine, two major metabolites (cotinine and trans-3'-hydroxycotinine), two minor metabolites (nornicotine and norcotinine), and a minor tobacco alkaloid, anabasine, in human urine. The

accurate and reproducible analysis can be achieved in less than 5 minutes of chromatographic run time and is thus applicable to low-cost and high through-put analysis of nicotine related compounds.