Analytical Method Validation of Metformin and Estrone-3-Sulfate for *In Vitro* Uptake Assays

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Abstract

Metformin and estrone-3-sulfate are well-known probe substrates for OCT2 and BCRP/OAT3 transporters, respectively, and are utilized in experiments to predict clinical drug-drug interactions. This study was performed to develop and validate analytical methods using UV-HPLC and LC-MS/MS to quantify, with high accuracy and precision, metformin and estrone-3-sulfate, respectively. Both assays were optimized and validated for precision, accuracy, 37°C stability, freeze-thaw stability, benchtop stability, selectivity, and injection carry-over according to FDA's 2018 bioanalytical method validation guidance. Linearity range of the analytical methods for metformin and estrone-3-sulfate were constructed according to the preliminary kinetic experiments in human embryonic kidney (HEK293) cells transfected with each uptake transporter. A UV-HPLC method for metformin was developed using a pentafluorophenyl C_{18} column. Metformin was detected with a high sensitivity at 280 nm. The lower and upper limit of quantitation were 800 nM and 11 µM, respectively. The results showed that the method was accurate (102.5 ± 3.5%; 103 ± 10%) and precise (<1%; <2%) for surrogate (10% acetonitrile in water, v/v) and cell-lysate matrices, respectively. A sensitive method for estrone-3-sulfate was developed using triple quadrupole LC-MS/MS with negative electrospray ionization operating in multiple reaction monitoring mode with an ion transition *m/z* 349.2 > 269.25 and 354.2 > 274.25 for estrone-3-sulfate and its internal standard, respectively. Linearity was established over a concentrate range of 3-150 nM using a BEH C_{18}-Acquity UPLC column which provides excellent chromatographic separation with no interference from the cell-lysate matrix. The method was accurate (104.8 ± 1.7%; 106.5 ± 1%) and precise (<7%; <5%) for surrogate (same as above) and cell-lysate matrices, respectively. Surrogate matrix and cell-lysate matrix were diluted with an equal volume of methanol before injection. The analytical methods meet all predefined performance acceptance criteria for specificity, sensitivity, and all stability experiments. The validated methods will be utilized to optimize *in vitro* uptake assays in transfected cells and to screen different transporter inhibitors for drug interaction predictions.

Introduction

The kidneys play an critical role in the kinetics of xenobiotics through a variety of organic cation/anion transporters that present the possibility of drug-drug interactions (DDIs). Metformin and estrone-3-sulfate (E3S) serve as known substrates for renal (OCT2) and (OAT3) transporters, respectively. Several analytical methods have been developed to study metformin and E3S. However, there were challenges to detect the lower limit of quantification that are necessary to evaluate drugs via *in vitro* transporter experiments. The present study aims to develop and validate sensitive analytical methods to quantify, with high accuracy and precision, metformin and E3S using HPLC and LC/MS, respectively. These assays will be utilized to optimize *in vitro* uptake assays using transfected cell lines to predict clinical DDIs.

Materials and Methods

The HPLC and LC-MS/MS methods were optimized and validated as per FDA's 2018 bioanalytical method validation guidance. The parameters validated are specificity, precision, accuracy, 37°C stability, freeze-thaw stability, benchtop stability, selectivity, and injection carry-over

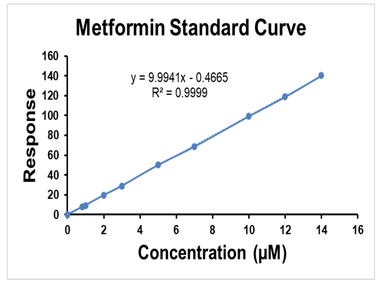
Analytical method parameters

	Metformin	E3S
Instrument	HPLC 1200 series of Agilent Technologies with a diode array detector (DAD)	Waters Acquity UHPLC coupled with a Xevo® TQ-XS mass spectrometer
Column	Hypersil GOLD PFP 150x4.6 mm, 3 µm	ACQUITY UPLC BEH C18 Column 100x2.1 mm, 1.7 µm
Mobile phase A	5 mM ammonium acetate in water (pH=6.5)	0.02% NH4OH in water (pH=8.9)
Mobile phase B	Acetonitrile	Acetonitrile
Separation mode	Isocratic elution	Gradient elution
Gradient	A:B (90:10 v/v)	0-0.5 min 5% B; 0.5-3 min 5-40% B; 3-4 min 80% B; 4-6 min 5% B
Surrogate matrix	10% Acetonitrile in water	Water
Flow rate	1.2 mL/min	0.4 mL/min
Injection volume	30 µL	3 µL
Run time	5 min	6 min

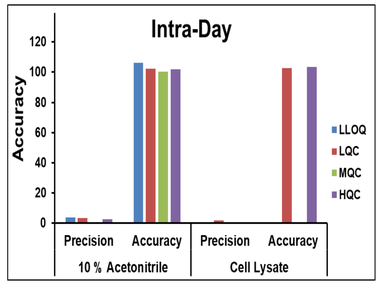
Analytical assay

	Metformin	E3S
Linearity range	0.8-14 µM	3-150 nM
Limit of Quantification	2.4 µM	9 nM
Retention time (min)	3.15	2.65
Correlation coefficient (R^2)	0.999	0.998

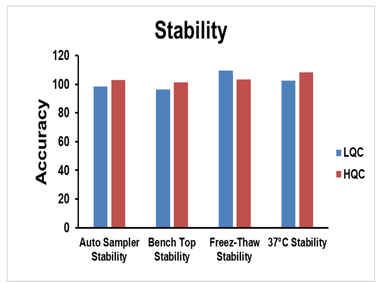
Metformin Results



The metformin standard curve was linear and accurate over a concentration range of 0.8-14 µM.

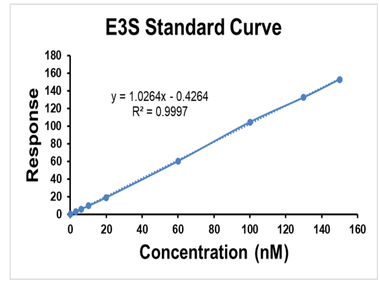


Samples were evaluated for intra- and inter-day accuracy and precision in 10% acetonitrile (surrogate matrix) and cell lysate. All samples showed high precision and repeatability of the analytical method.

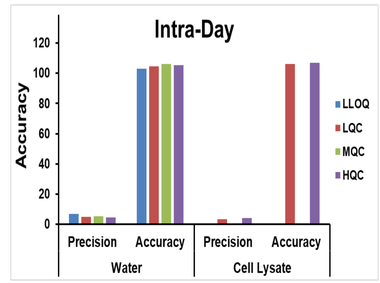


Metformin was stable in surrogate matrix and cell lysate under auto-sampler conditions (6°C) up to 24 h, on benchtop at RT up to 4 h, for three freeze-thaw cycles at -20°C/RT, and at 37°C for 30 min.

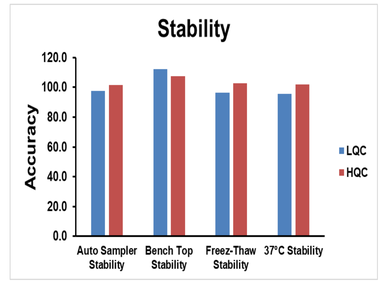
E3S Results



The E3S method was found to be linear in the concentration range of of 3-150 nM with correlation coefficient of 0.999.



QC samples were evaluated for intra- and inter-day accuracy and precision for the surrogate matrix and cell lysate. No significant changes detected upon testing all samples.



E3S was stable in surrogate matrix and cell lysate under auto-sampler conditions (6°C) up to 24 h, on benchtop at RT up to 4 h, for three freeze-thaw cycles at -20°C/RT, and at 37°C for 30 min.

Conclusion

The validated analytical methods enable accurate and sensitive quantification of metformin and E3S from cell uptake assays. Both compounds were stable under various conditions in both the surrogate matrix and cell lysate. These analytical assays will be used to optimize *in vitro* uptake assays in transporter-transfected cells to predict clinical DDIs.

Disclaimer

The ideas, findings, and conclusions in this presentation have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

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