Introduction

Fumaric acid is an unsaturated organic acid, extensively used as an excipient in pharmaceutical products. Fumarate is the ionized dicarboxylic form of fumaric acid at physiological pH and a key intermediate in the tricarboxylic acid (TCA) cycle. Fumarate was recently identified as a metabolic biomarker for diabetic kidney disease.

The endogenous concentration of fumarate was regulated by way of TCA cycles and other metabolic pathways and it is not known if exogenously administered fumaric acid may perturb the endogenous concentration of fumarate. A LC-MS/MS method was required to measure both endogenous and exogenous fumarate.

Stability discrepancy was discovered between endogenous fumarate and exogenously added fumaric acid in rat plasma during method development. Stability profiles were established for both endogenous and exogenous fumarate.

Furthermore, the conversion product of fumarate was identified by monitoring the intermediates from TCA cycle and the possible cause of stability discrepancy was also investigated.

Methods and Experimental

Sample Preparation

A surrogate matrix approach was used for the analysis of fumarate. The standard curve were prepared in 4% BSA in PBS with a range of 40 to 10,000 nM. QC's were prepared in native rat plasma except the LLOQ QC. Samples were extracted using protein precipitation by adding 400 µL of methanol to a aliquot of 50 µL sample. Stability evaluation of endogenous fumarate was conducted in both fresh and aged plasma.

LC-MS/MS

Sample extract was analyzed on an AB Sciex API 4000 mass spectrometer equipped with a Shimadzu LC-20 system. Chromatographic separation was achieved on a C18 column. Sample extract was analyzed on an AB Sciex API 4000 mass spectrometer equipped with a Shimadzu LC-20 system. Chromatographic separation was achieved on a C18 column using mobile phases of 0.1% FA in water and methanol at 0.4 mL/min for 6.5 min. The LC gradient ramped from 0 to 40% methanol in 2 min, followed by two forward flush cycles with 95% methanol. The API4000 was operated in negative electrospray ionization mode and validated for the determination and stability investigation of endogenous and exogenous fumarate.

Results

A high-throughput LC-MS/MS method using surrogate matrix approach was developed and validated for the determination and stability investigation of endogenous and exogenous fumarate.

The endogenous fumarate showed stability in rat plasma for at least 6 hours at room temperature, while exogenously added fumaric acid encountered 80% loss within 2 hours and reached a plateau after 6 hours. Fumaric acid was fortified at various concentrations, but none of the concentrations showed any impact on the stability profile of endogenous fumarate.

Intermediates from TCA cycle, malate, succinate, oxaloacetate and pyruvate were monitored and malate was the only conversion product that was detected. It confirmed the fumarase activity in rat plasma and suggests that other enzymes from the cycle may not be active or present in rat plasma.

Product/Reactant (malate/fumarate) ratio of endogenous fumarate showed 1.5 to 3-fold value of the fortified 13C4-fumaric acid. It suggests that the endogenous fumarase may be subjected to a highly regulated environment which may persist even when plasma was isolated in vitro. On the contrary, the added fumarate went through a fast reaction and reached a plateau, a direct result of the fumarase activity in rat plasma. The results demonstrate that the added fumarate may not fully represent the endogenous fumarate in matrix.

No isotope effect was observed while using labeled fumaric acid for stability evaluation.

Conclusions

Our results demonstrate for the first time that endogenous and exogenous fumarate showed two distinct stability profiles, indicating that exogenously added fumarate did not fully represent the analytical performance of endogenous fumarate.

Our findings confirm that there could be stability discrepancy between endogenous and exogenous small-molecule biomarker, and also demonstrate the complexity of stability evaluation for biomarkers.

We propose that a prudent investigation should be undertaken on the stability of both exogenously added and endogenous compound during biomarker and metabolomics analysis.

References

Massey V, The Biochemist J 1963, 8(1), 172-177.

Yao Shi1, Susanna Tse2, Brian Rago2, Udeni Yapa3, Fumin Li3 and Douglas M. Fast3
1Covance Laboratories Inc., Madison, WI; 2Pfizer Inc., Groton, CT; 3Former employees of Covance Laboratories Inc.