**LC-MS/MS Method for Quantitation of Physiological Levels of Glucagon in Human Plasma**

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**Purpose**

Historically glucagon plasma concentrations have been quantified using immunological methods to achieve the sensitivity required to measure endogenous concentrations. Glucagon and several other peptides such as oxyntomodulin, GLP-1, and GLP-2, are the result of post-translational processing of proglucagon. Significant sequence overlap and similarity in amino acid sequences may be detrimental to the selectivity of immunological methods, resulting in poor reproducibility, particularly when comparing concentrations over time within the same laboratory or between laboratories in the treatment of glucagon-related disorders. A sensitive LC-MS/MS method was developed for measurement of full-length glucagon at endogenous concentrations. The method addresses key parameters of selectivity, matrix effect, and stability of glucagon in human plasma.

**Methods**

Prescreened charcoal-stripped (2X) human K2EDTA plasma (acidified) was used as blank control matrix for preparation of calibration standards. Human plasma (0.250 mL sample volume) spiked with stable isotope labeled glucagon as an internal standard (IS) was extracted using an Oasis® MAX micro-elution solid phase extraction. The analyte and IS in the eluents were chromatographically separated from matrix components on an ACQUITY UPLC® BEH C18 (100 x 2.1 mm, 1.7 μm) analytical column. Positive ions were generated by electrospray ionization (ESI) and detected with an AB Sciex Triple Quad™ API 6500 tandem mass-spectrometer. Data were acquired in multiple reaction-monitoring (MRM) mode using transitions 697.4 → 813.3 and 698.6 → 814.7 m/z, for glucagon and the IS, respectively.

**Results**

A glucagon transition at 697.4 → 693.8 m/z has been employed in several previous glucagon methods; however, while having high absolute response, this transition was not sufficiently selective for an LLOQ (lower limit of quantitation) of 10 pg/mL. The transition at 697.4 → 813.3 m/z provided sufficient sensitivity using 0.250 mL of plasma when coupled with significantly improved chromatographic selectivity. Calibration standards (10.0-1000 pg/mL) prepared in blank control matrix showed parallelism with matrix effect test samples prepared in multiple lots of human plasma with varying endogenous levels of glucagon. Acidification of human plasma was critical for elimination of a strong (up to 55%) negative bias observed for quantitation of some lots of human plasma spiked with known glucagon concentrations. Evaluation of glucagon stability in the presence of a number of commercially available inhibitors (and inhibitor cocktails) with and without acidification demonstrated that acidification of plasma after collection was necessary to protect glucagon against degradation during storage or freeze/thaw cycles. The acidification conditions were optimized to suppress “glucagon-degrading” activity without denaturation or aggregation of plasma proteins that would subsequently cause SPE micro-elution plate clogging during extraction. All matrix effect test samples prepared in human plasma from 17 individual donors with endogenous concentrations from 10 to 98 pg/mL passed acceptance criteria, with the majority (12 of 17) of low matrix effect samples (spiked with glucagon at 0.5x - 2x endogenous concentration) being within 97-108% of the expected values. Both low quality control (QC) samples (pooled human plasma with low endogenous concentration < 25 pg/mL) and high QCs (spiked with glucagon at 750 pg/mL) were stable after 6 freeze/thaw cycles in an ice water bath (at least 5 hours each thaw cycle).

**Conclusion**

A sensitive and selective LC-MS/MS method for measurement of endogenous glucagon in human plasma. The method could potentially be introduced as standardized glucagon assay that would improve comparison of glucagon concentration data over time within laboratories as well as between laboratories.