INTRODUCTION:
• Glucagon is a polypeptide hormone that increases blood glucose concentration and has been used as an emergency treatment of hypoglycemia.
• Historically, methods for glucagon analysis to support studies, were based on immunochromatographic approaches intended for diagnostic purposes. These production ranges of these methods were inappropriate and inefficient even now, and the methods often lack selectivity and typically display strong matrix effect.
• An LC-MS/MS approach offers high selectivity of the analysis by minimizing interference from endogenous and exogenous compounds. An automated solid-phase procedure has been developed coupled with LC-MS/MS detection enabling sensitive glucagon analysis using only 0.250 mL of plasma sample. Due to inefficient isotopic purity of isotopically labeled glucagon, an analog with a deletion in the amino acid sequence of glucagon was used as an internal standard.

SAMPLE PREPARATION:
• Aliquot of human plasma (0.250 mL) was spiked with internal standard and diluted with 1 mM glycine buffer pH 8.2.
• Analytical sample solutions were filtered using a 0.22 μm filter and were analyzed within 2 hours of preparation.
• Each sample was analyzed in a separate lot.

INSTRUMENTATION:
• An Agilent Technologies, ZORBAX Rapid Resolution 300SB-C18, 50 x 2.1 mm, 3.5 μm analytical column was used for LC-MS/MS.
• Mobile phase containing 30% ACN with formic acid was used for separation.
• Liquid chromatography/tandem mass spectrometry was used to detect multiply charged positive ions in the multiple-reaction-monitoring mode.

RESULTS:
• The validated analytical range was from 100 to 10,000 pg/mL, with dilution integrity determined up to 35,000 pg/mL.
• The signal-to-noise at the lower limit of quantitation (LLOQ, 100 pg/mL) was typically 15 with only about 20% of the sample volume injected (Figure 1).
• The inter-batch precision (% CV) of quality control samples at 300, 1000, and 7500 pg/mL was 3.0 to 6.0%, respectively.
• Assay linearity was determined for standards spiked into six separate lots of blank human plasma (EDTA). No significant matrix effect was observed in all lots spiked at the LLOQ and in 5 of 6 lots at high QC concentration. Precision (% CV) of the LLOQ and high QC concentration spiked samples quantitation in multiple lots were 2.4 and 3.1%, respectively (Table 1). The average extraction recovery of glucagon was about 60% (Table 3).
• Demonstrated lot-dependent glucagon degradation in human plasma and established composition of an inhibitory cocktail to improve substantially stability of clinical samples.
• Demonstrated post-preparative stability in injection solvent (quantification against freshly extracted standards) for 120 hours at 0°C and processed sample integrity in injection solvent (re-injection stability) was established for 120 hours at 0°C.
• Demonstrated accurate and precise glucagon quantitation in turbid and hemolyzed samples (Tables 4 and 5).

CONCLUSIONS:
• A sensitive, accurate and reproducible method for glucagon was developed and validated with improved selectivity as compared currently available immunochemical methods.
• Developing a diagnostic antibody to enhance significantly glucagon stability in plasma samples.