Human insulin is a peptide hormone which has a crucial role in regulating blood glucose. Monitoring insulin levels in patients can give diagnostic data regarding insulin resistance and pancreatic function. Insulin control is especially important in treating and diagnosing diabetes and medical conditions. Aptamers are short, single DNA or RNA strands that bind with high selectivity and specificity to molecular targets such as peptides and proteins. Isotope dilution is established as a gold standard for quantitation of biomarkers. Presented here is a high-performance liquid chromatography/mass spectrometry method enhanced by isotope dilution and aptamer enrichment for quantitation of human insulin. A QTRAP 6500+ (Sciex) coupled to a Shimadzu HPLC system was used. For selected ion monitoring the +5 and +6 ions were chosen. Calibration curves were generated from serial dilution of native and isotope labeled insulin. In summary, we have developed a novel method for quantitating human insulin using LC-MS.