Development of a high throughput UHPLC-MS/MS (SRM) method for the quantitation of endogenous glucagon from human plasma

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Abstract

Background: Published LC-MS/MS methods are not sensitive enough to quantify endogenous levels of glucagon. **Results:** A UHPLC-MS/MS (SRM) method for the quantitation of endogenous levels glucagon was successfully developed and qualified. A novel 2D extraction procedure was used to reduce matrix suppression, background noise and interferences. Glucagon levels in samples from healthy volunteers were found to agree with RIA derived literature values. Bland-Altman analysis showed a concentration-dependent positive bias of the LC/MS-MS assay versus an RIA. Both assays produced similar pharmacokinetic profiles, both of which were feasible considering the nature of the study. **Conclusions:** Our method is the first peer reviewed LC-MS/MS method for the quantitation of endogenous levels of glucagon, and offers a viable alternative to RIA based approaches.

Introduction

Glucagon is a 29 amino acid peptide which is one of multiple hormones that modulates glucose production or utilisation to regulate blood glucose levels. It is also a biomarker for pathologies such as diabetes, pancreatic cancer or certain neuroendocrine tumours [1]. It is known to be degraded by peptidases such as dipeptidyl peptidase IV [2][3] and consequently blood samples are typically collected in tubes containing protease inhibitors.

Endogenous glucagon levels in healthy patients are reported between 25-80 pg/mL, which may be raised by about 10 pg/mL in pancreatic cancer patients, and can reach up to 160

32 pg/mL in diabetic patients [1]. Following treatments using glucagon infusion levels can reach
33 ~906 pg/mL [4]. Glucagon concentrations are routinely measured using radioimmunoassay
34 (RIA) based approaches, however these assays can be time consuming to perform (up to 3
35 days) and the kits hasuppleve limited lifetimes (e.g. 2 months). In addition they can suffer
36 from poor precision and accuracy, as there is potential for cross reactivity with similar
37 compounds or inactive degradation fragments leading to inaccurate quantitation [5][6][7]. For
38 example, whilst a comparison between two glucagon immunoassays resulted in a high
39 correlation (R=0.97), the concentrations between individual samples differed by 2-4 fold [8].
40 The radioactive nature of RIAs also necessitates additional health and safety precautions
41 during set-up, and specialised disposal of radioisotopes.

42 A LC-MS/MS assay would have the potential to circumvent such problems [9], and may offer
43 additional benefits such as a reduced sample volume and a higher throughout. However,
44 published LC-MS/MS methods [10][11] are not sensitive enough to detect endogenous
45 glucagon levels. As described in a recent review paper [12] the lowest reported LLOQ in the
46 peer reviewed literature is 250 pg/mL [11], although assays of 100 pg/mL [13] and 10 pg/mL
47 [14] have been described at recent conferences.

48 Furthermore, as glucagon is produced endogenously, this presents additional experimental
49 challenges as an authentic analyte free matrix cannot be obtained to construct calibration
50 standards. Either a standard addition, surrogate analyte, or a surrogate matrix approach
51 must therefore be used [15][16].

52 In the standard addition based approach, analyte is spiked on top of the authentic matrix to
53 create a calibration line, which is extrapolated to measure concentrations below the matrix's
54 endogenous value. However the USA FDA Guidance for Bioanalytical Method Validation
55 [17] actively discourages the extrapolation of calibration curves beyond their range. The
56 surrogate analyte based approach uses an analogue to the analyte in place of the analyte
57 itself in calibration samples. As this will have a Selected Reaction Monitoring (SRM)
58 transition unique from the authentic analyte these can be prepared in authentic biological
59 matrix [15] . However, this approach requires the relationship between the authentic and
60 surrogate analyte to be thoroughly investigated, the approach is not commonly used, and is
61 not considered in the FDA [17] or EMA guidelines [18]. Alternatively, in the surrogate matrix
62 approach, calibration lines are constructed by spiking analyte into a surrogate matrix. QCs
63 can be prepared in actual sample matrix, and the accuracy calculated to demonstrate the
64 absence of a matrix effect. Surrogate matrices may be the authentic matrix stripped of
65 analyte (e.g. by charcoal [16] or immuno-afffinity methods [19]) or an alternative matrix (e.g.
66 protein buffers, dialysed serum [20]). Although not ideal, the EMA Guideline on bioanalytical

67 method validation [18] concedes that such an approach may be necessary for endogenous
68 analyte quantitation, and therefore this is the approach we adopted.

69

70 This article outlines the first peer reviewed high throughput UHPLC-MS/MS (SRM) based
71 approach capable of quantifying endogenous levels of glucagon from human plasma. The
72 high throughput nature of the assay is due to its ability to relatively quickly analyse large
73 numbers of samples. This is enabled by an extraction procedure that is relatively quick,
74 simple, and cheap in comparison to many immunochemistry based approaches [21], and
75 which can analyse large number of samples (~60) within an analytical batch. In addition,
76 UHPLC is used to minimise sample run times [22].A calibration range of 25–1000 pg/mL is
77 qualified, making the assay suitable for measuring both endogenous levels of glucagon and
78 elevated levels following treatments. Consequently the assay can be used for both
79 biomarker (PD, Pharacodymaic) and Pharmacokinetic (PK) analysis. However, the
80 calibration range could be easily truncated if only endogenous level analysis (PD) is
81 required. In addition we present the first comparison of glucagon concentrations determined
82 by an LC-MS/MS assay and a traditional RIA method using a large number of clinical
83 samples derived from a physiological study of glucagon's actions in the body (n=88).

84 The assay's performance has been evaluated using experiments described in the latest
85 EMA [18] and FDA [17] guidance and in accordance to the principles of GCP [23].

86 **Key Terms**

87 *Radioimmunoassay (RIA)* - A highly sensitive technique used to measure concentrations of
88 antigens (e.g. peptides) by use of antibodies. Pre-bound radioactively labelled antigens are
89 displaced by non-radioactive antigens from a sample. Monitoring the change in radioactivity
90 allows quantitation.

91 *UHPLC-MS/MS (SRM)* – An analytical methodology that combines the use of ultra-high
92 performance liquid chromatographic (UHPLC) separations with sensitive mass spectrometer
93 selected reaction monitoring (SRM). Traditionally used for small molecule quantitation, but
94 increasingly used for the quantitation of biological molecules (e.g. peptides).

95 **Experimental**

96 *Chemicals and materials*

97 Certified human glucagon (HSQGTFTSDYSKYLDSRRAQDFVQWLMNT) was obtained from
98 EDQM (Strasbourg, France) and the analog internal standard (IS) (des-thr^{7}-glucagon)
99 (HSQGTFSDYS KYLDSRRAQDFVQWLMNT) from Bachem (Bubendorf, Switzerland). This
100 internal standard has given suitable performance in LC-MS/MS glucagon assays [13] [14],
101 and it avoids the expense of synthesising a heavy labelled internal standard. Water was
102 produced by a Triple Red water purifier (Buckinghamshire, U.K.). BD glass collection tubes
103 (5 mL) containing K3 EDTA anticoagulant and 250 Kallikrein Inhibitor Units (KIU) of Aprotinin
104 were obtained from BD (Oxford, UK). Following collection, tubes were placed on ice, then
105 centrifuged at 2300 x g for 10 minutes to obtain plasma, which was stored at -80°C when not
106 in use. All chemicals and solvents were HPLC or analytical reagent grade and purchased
107 from commercial vendors.

108 *Instrumentation: LC-MS/MS*

109 The LC-MS/MS system consisted of a Waters Acquity UPLC system (Waters Corporation,
110 Massachusetts, USA) coupled to an AB SCIEX 5500 QTRAP (Applied Biosystems / MDS
111 SCIEX, Ontario, Canada) with an electrospray ion source. Data acquisition and processing
112 were performed using Analyst 1.5.2 (Applied Biosystems/ MDS SCIEX). The majority of the
113 chromatograms were integrated using fully automated settings. A minority had their
114 integration settings (peak selection, peak splitting factor, noise percentage) altered to ensure
115 appropriate and consistent integration. No samples were integrated using manual integration
116 mode.

117 Glucagon was separated on a Waters UPLC BEH C18 1.7 µm (2.1 x 100 mm) column
118 maintained at 60 °C. The mobile phase consisted of (A) 0.2% formic acid (FA) in acetonitrile
119 (MeCN) and (B) 0.2% FA (aq). The gradient for separation was 22–32% A over 2 minutes.
120 The column was then cleaned with 95% A for approximately 1 minute then 22% A for
121 approximately 4 minutes. The flow rate was 0.8 mL/min and the total run time 7.1 minutes.

122 The mass spectrometer was operated in positive ion mode with an electrospray voltage of
123 5500 V, an entrance potential of 10 V, and a declustering potential of 70 V. The source
124 temperature was 600°C, the curtain gas 40 Psi, and the desolvation gases, GS1 and GS2,
125 were set at 60 psi and 40 psi respectively. Quantitation was performed using the selected
126 reaction monitoring (SRM) transitions 697.5→693.8 and 677.2→673.8 for glucagon and the
127 internal standard respectively. The N_2 collision gas was set to medium and both transitions

used collision energies of 15 V and collision exit cell potentials of 13 V. The Q1 and Q3 quadruples were both operated at unit resolution.

Preparation of stock, standards and QC MED and HIGH plasma samples

1 mg/mL stock solutions of glucagon and glucagon internal standard were prepared in borosilicate vials using surrogate matrix [Methanol (MeOH): H_2O: Formic acid (FA): Bovine serum albumin (BSA), (20:80:0.1:0.1, v/v/v/w)]. Glucagon working solutions were prepared by dilution with this solvent to create nine calibration standard spiking solutions (125, 225, 375, 500, 1000, 2000, 3000, 4500, 5000 pg/mL), and four quality control spiking solutions (125, 250, 10000, 75000 pg/mL). Additional calibration standard and QC spiking solutions at 75 and 50 pg/mL were also prepared for the assessment of assay performance at the 10 and 15 pg/mL levels. Internal standard working solution (ISWS) was similarly prepared at 20 ng/mL. The stock and working solutions were prepared to a volume of 10 mL and were stored at -20 °C when not in use. QC MED and QC HIGH plasma samples were prepared by diluting the appropriate spiking solution 100 fold with plasma to create samples at 100 and 750 pg/mL respectively. These were either used immediately, or stored at -80 °C prior to use.

Extraction method development & surrogate matrix quantitation

Additional details of the extraction method development experiments described are provided in the supplementary information. In summary:

Protein precipitation optimisation The following precipitation solvents were investigated; Acetonitrile (MeCN), MeCN:H_2O (50:50,v/v), and MeCN:H_2O (75:25, v/v). Each solvent was investigated with and without 0.1% formic acid. In addition MeCN: H_2O: NH_3 (75:25:0.1, v/v/v) was investigated.

Solid phase extraction optimisation Extraction efficiencies of the MAX, MCX, and WCX phases from a 96 well Oasis sorbent selection plate (10 mg) (Waters Corporation) and from a size exclusion hydrophobic (SEH) Bond Elut Plexa 96 round-well (30 mg) plate (Agilent Technologies, California, USA) were evaluated. The Oasis extraction used generic conditions for peptide analysis based on those provided by the manufacturer, whilst we used our in house generic conditions for the Plexa evaluation.

Surrogate matrix quantitation- The calibration standard spiking solutions described above were diluted 5 fold with surrogate matrix. 400 µL aliquots were then extracted according to the procedure below. The matrices investigated were H_2O, MeOH: H_2O:FA:BSA (20:80:0.1:0.1, v/v/v/w), 6% BSA (aq) and 6% rat plasma (aq).

Extraction method for validation

Plasma sample (aprotinin stabilised, K3 EDTA) (400 µL) was placed into a 5 mL polypropylene tube and 20 µL of ISWS was added to all non-blank samples. The samples were briefly vortex mixed, precipitated using 3.2 mL of MeCN:H_2O:NH_3 (72:25:0.1,v/v/v), vortex mixed again, and then centrifuged for 10 minutes at 2300 x g. The supernatant was transferred to a new tube and evaporated to dryness overnight under vacuum. Samples were reconstituted in 800 µL 2% NH_3 (aq) and then vortex mixed. A Bond Elut Plexa 96 round-well solid phase extraction (SPE) plate (30 mg) was conditioned using 1 mL MeOH, then equilibrated with 1 mL H_20. The samples were loaded, washed with 1 mL 5% MeOH (aq), eluted with 2 x 225 µL MeCN:H_2O:FA (75:25:0.1, v/v/v), and then evaporated under nitrogen at 40°C, before being reconstituted in 200 µL 0.2% FA (aq).

Calibration standards, QC LLOQs and QC LOWs were then prepared freshly for each batch by spiking 80 µL of the appropriate spiking solution into the plate, along with 20 µL of ISWS and 100 µL surrogate matrix. Taking into account the 2-fold concentration experienced by plasma samples (400 µL of plasma sample is reconstituted into 200 µL of solvent) this gives final calibration levels of 25, 45, 75, 100, 200, 400, 600, 900, and 1000 pg/mL, and final QC levels of 25 and 50 pg/mL. The plate was centrifuged for 10 minutes at 2300 x g, and 50 µL of sample injected on to the LC-MS/MS system for analysis.

Validation Experiments

The validation experiments chosen were based on those described in the latest EMA guidance [18]. Calibration standards were analysed in duplicate with each batch. Data was imported into Watson LIMS 7.2 (Thermo Fisher Scientific Inc, Massachusetts, USA) and linear regression with $1/x^2$ weighting was applied to the peak area ratios-concentration plot for the construction of calibration lines. The precision and accuracy of the method was determined by analysis of replicate (n=6) QC samples at four different concentrations (25, 50, 100, and 750 pg/mL), and was assessed within a batch (intra-batch, n = 6 replicates) and between batches (inter-batch, 3 batches). The ability to dilute was assessed by diluting an over range dilution sample (7500 pg/mL) 10-fold with blank plasma. Carryover effects were evaluated by injection of blank samples immediately after injection of the highest point in the calibration range.

Selectivity was assessed by qualitatively examining chromatograms from six independent control matrix samples for the presence of potentially interfering peaks. It was not feasible to monitor multiple charge states or SRM transitions to further ensure selectivity as only the selected transition demonstrated sufficient sensitivity at the endogenous concentration .The

195 modification of analyte and internal standard responses to the presence of matrix was also
196 determined in such samples. These were extracted and post spiked at either the medium or
197 high level, and compared to the mean response from samples in surrogate matrix (minimum
198 n=6). The effect of haemolysed (3%) plasma and hyperlipidaemic plasma (~4 mmol/L of
199 triglycerides) upon on quantitation was investigated by preparing QCs in these matrices at
200 the medium and high level (n=6 replicates). Recovery of the analyte was evaluated by
201 comparing the analytical results for extracted analyte samples at the medium and high level
202 with unextracted analyte samples that represent 100% recovery.
203
204 The stability of the glucagon in aprotinin stabilised human plasma was evaluated at the
205 medium and high concentrations in replicate (n=6). Stability was assessed after
206 6 hr 20 min on ice (4 °C), after storage for 11 and 75 days at -20°C, and for 7, 11, 51, and 64
207 days at -80°C. Similarly stability was assessed after 4 freeze-thaw cycles from -20 °C to 4 °C
208 and also 4 freeze cycles from -80 °C to 4 °C. Stability was similarly assessed in whole
209 blood following storage on ice for 1 hour. The ability to re-inject sample extracts at medium
210 and high concentrations was assessed after storage at +4°C for 6 days. The stability of the
211 stock solution was assessed after storage at -20°C for 66 days and that of LLOQ and ULOQ
212 working solutions after 163 days at -20°C.
213
214 All results are quoted from batches where the standards and QCs passed our prospectively
215 defined acceptance criteria, which were based on the EMA and FDA guidelines. These
216 required that at least 75% of standards in each batch had back calculated accuracy within
217 15% (20% at the LLOQ) of the nominal concentration, with standards outside these criteria
218 excluded from the regression. QCs in precision and accuracy batches needed to have mean
219 intra-batch accuracy within 20% of the nominal concentration, and intra-batch precision that
220 did not exceed 20%. In other batches at least 2/3 of the individual QCs had accuracy within
221 20% of the nominal concentration, with at least one QC passing criteria at each level.
222 Although the guidelines suggest a 15% criteria (20% at the LLOQ) should be applied to QC
223 performance, they state it can be widened prospectively in special cases. We felt it was
224 justified to raise the QC acceptance criteria to 20% (CV and RE) due to the surrogate matrix
225 nature of the assay. The 20% (RE) acceptance criteria was also applied to plasma, blood
226 and extract stability experiments, as well as to the assessment of the matrix effect in
227 different individuals (matrix factor ratio) and of the effect of haemolysed or hyperlipidaemic
228 plasma.
229

230 *Collection of samples from volunteers to assess endogenous glucagon concentrations*

231 Plasma was collected from 12 healthy males and 12 healthy females using glass collection
232 tubes containing K3 EDTA and aprotinin, as described above. Glucagon levels were
233 determined using the qualified LC-MS/MS method. Plasma was collected at the start of the
234 working day and volunteers were not asked to change their usual eating regime.

235

236 *Collection of physiological study samples*

237 Physiological study samples (n=117) were collected by Imperial College London. The
238 samples originated from 7 different individuals who were each infused with a glucagon
239 solution at either 16 or 20 pmol/kg/min for 12 hours subcutaneously. Blood samples at
240 various time points were collected in 5 mL lithium heparin collection tubes containing 1000
241 KIU of Aprotinin, spun down in a cold centrifuge within 5 to 10 mins of collection, and then
242 stored at -20 °C.

243 *Analysis of physiological study samples*

244 A selection of the physiological study samples (n=100) were analysed by LGC using the LC-
245 MS/MS method described above. Additional QCs prepared in aprotinin stabilised plasma
246 with lithium heparin anticoagulant were analysed to ensure assay performance in the sample
247 matrix. 38 of the study samples were analysed over the calibration range 25–1000 pg/mL,
248 whilst the remainder were analysed over the calibration range 10–1000 pg/mL. For these
249 samples additional calibration points and QCs were included at the 10 and 15 pg/mL levels
250 to evaluate assay performance. Samples (n=105) were also analysed by Imperial College
251 using their established radioimmunoassay method over the calibration range 5 -1000 pg/mL,
252 which is directed against the C-terminal region of glucagon [24][25]. Samples were analysed
253 upon their first freeze-thaw.

254

255 **Results and discussion**

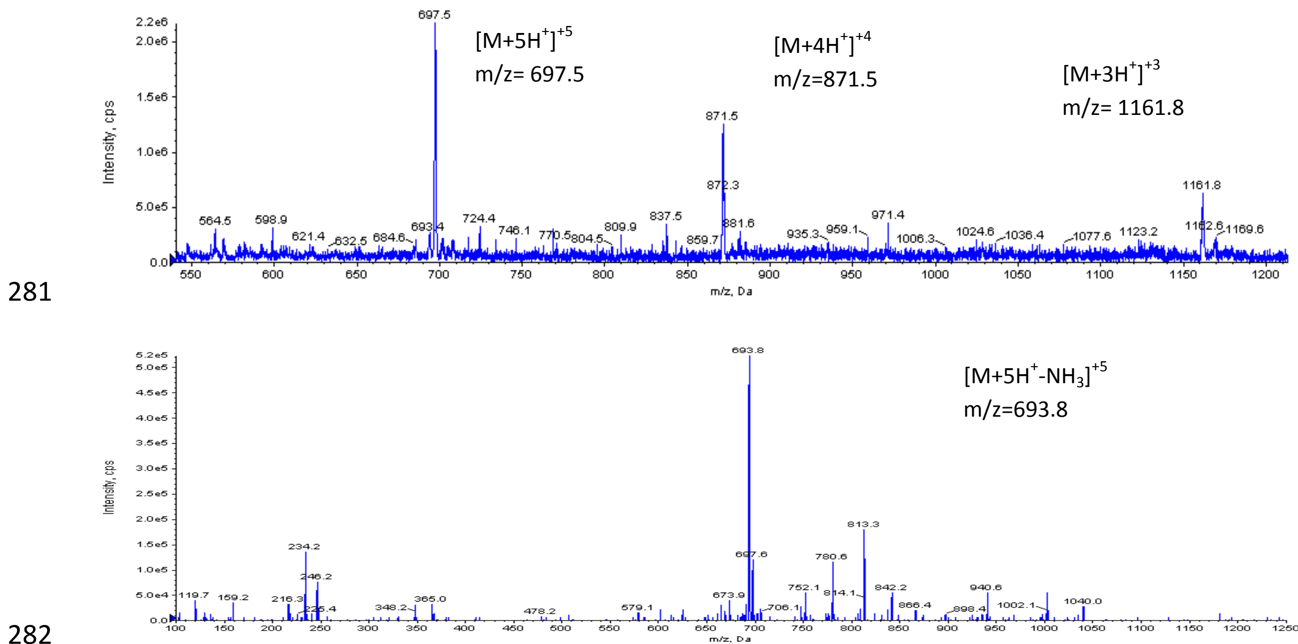
256 *Method development*

257 Analysis of endogenous levels of glucagon by LC-MS/MS poses a significant technical
258 challenge. Not only are the low endogenous concentrations difficult to measure, an
259 endogenous analyte quantitation strategy must be used, and stability issues must be
260 addressed.

261 Extensive assay optimisation was therefore performed to obtain the low 25 pg/mL LLOQ. A
262 QTRAP mass spectrometer was used in SRM mode, and parameters were optimised.
263 UHPLC was chosen for chromatographic separation because it results in greater efficiencies
264 [26] and/or shorter runtimes [27] than the HPLC commonly used for such separations. The
265 greater efficiency can lead to lower matrix effects due to improved separation from matrix
266 suppressants [28] and to higher sensitivities due to sharper peak shapes [22].

267 The $[M+5H^{+}]^{5+}$ ion was found to give the highest intensity during MS method development
268 (Figure 1a), although other studies have found the $[M+4H^{+}]^{+4}$ to be optimal [11][10]. MS2
269 experiments showed that showed that the ionic species generated by ESI of glucagon were
270 able to absorb substantial collision energy without undergoing major fragmentations, as
271 demonstrated previously [10] (Figure 1b). As also reported [13][12] an SRM transition
272 corresponding to the loss of ammonia ($[M+5H^{+}]^{+5}/[M+5H^{+}-NH_3]^{+5}$ was found to be optimal.
273 Although this is not a particularly specific transition, the intensity was significantly greater
274 than other transitions and was therefore chosen; selectivity was fully investigated during the
275 validation. Resolution settings for Q1 and Q3 were optimal at unit-unit, rather than high-high
276 as reported by others [11]. The optimal ion pairs of the transitions were 697.5/693.8, which
277 corresponds to a 18.5 Da loss. The small difference between our optimal pair, and that
278 previously reported (697.6/694.2) [13][12] is attributed to the resolution limitations of the
279 mass spectrometer used [29], as is the difference between the theoretical mass loss of
280 ammonia (17 Da) and that observed (18.5 Da).

281
282



283 Figure 1a (top)-Glucagon full scan MS spectrum A mass window of 400 -1250 m/z was isolated.
284 b)(bottom) MS spectrum of production ion scans (Parent= 697.5, CE= 25 V)

285

286 A relatively large 400 µL plasma volume was chosen for extraction, to enable concentration
287 of extracts to achieve higher sensitivities. The volume does, however, compare well to the
288 2 x 200 µL typically required for RIA methods. Initially, protein precipitation based extraction
289 techniques were investigated, as they are quick and cheap, and are amenable to automation
290 and high throughput analysis. Additionally, pure acetonitrile precipitation has been previously
291 selected for glucagon extractions [10] [11]. We have previously demonstrated that diluting
292 acetonitrile with various proportions of water can lead to more specific extractions [30], as
293 can the addition of acids or bases to due to the differences between the isoelectric points
294 (pI) of the proteins or peptides of interest and the background proteins [31]. Precipitation
295 solvents containing various proportions of acetonitrile, water, acid and base were
296 investigated, with MeCN:H_2O:NH_3 (75:25:0.1,v/v/v) giving the best response. However, in all
297 cases background noise and interferences were relatively high, as was matrix suppression.

298 It was therefore decided to investigate solid phase extraction (SPE) based approaches, as
299 these should lead to cleaner samples with reduced background noise and interferences.
300 These studies are described in the supplementary information.

301 Combining protein precipitation with size exclusion hydrophobic (SEH) SPE was found to
302 reduce the on column matrix effects, whilst providing adequate recovery. To our knowledge
303 this is the first time protein precipitation has been combined with SEH SPE for quantitative
304 peptide analysis, although protein precipitation has been combined with other SPE phases
305 for this purpose[32]. Due to the satisfactory performance of this extraction methodology,
306 alternatives such as immunoaffinity enrichment were not investigated [33].

307

308 Various UHPLC gradients were investigated to further reduce matrix build-up on the column
309 and it was found that a 4 minute flush at the starting conditions gave the best performance.
310 This gradient combined with the 2D extraction methodology significantly increased the
311 robustness of the assay.

312 Glucagon is known to be degraded by the blood enzymes and consequently sample
313 stabilisation is required [2] . The enzyme inhibitor aprotinin was used to reduce degradation
314 and samples were extracted on ice. As there have been reports of enzyme inhibitors
315 interfering with peptide quantitation [34] assay performance was closely monitored during
316 the validation for any such issues.

317

318 *Surrogate matrix quantitation*

319 Several mixtures were screened for their suitability as surrogate matrices. A dilute buffer
320 matrix was evaluated, as such matrices have been shown to be suitable for some assays.
321 [35] [19]. A buffer solution containing a relatively high percentage of BSA was also evaluated
322 to minimise any non-specific analyte binding that may occur. In addition a diluted rat plasma
323 matrix was chosen to investigate whether biological matricies improved assay performance.

324 The dilute buffer matrix, Water and MeOH: H_2O: FA: BSA (20:80:0.1:0.1, v/v/v/w), resulted
325 in low signals following extraction, which is attributed to non-specific binding of glucagon to
326 plastic consumables used during the extraction procedure, as has been described previously
327 [10]. The 6% BSA (aq) matrix, selected to minimise non-specific binding in solvent led to a
328 very high background noise, whilst the 6% rat plasma (aq) led to poor calibration line
329 accuracy against prepared concentrations. It was therefore decided to use MeOH: H_2O: FA:
330 BSA (20:80:0.1:0.1, v/v/v/w) as the surrogate matrix, but not to extract samples prepared in
331 this, in order to prevent large losses by nonspecific binding. Whilst plasma samples require
332 extraction, their high protein content prevents binding and the use of an internal standard
333 was expected to take into account recovery differences between the surrogate matrix
334 calibrants (which will necessarily have recovery of 100% for the analyte and IS) and the
335 extracted plasma samples. The internal standard was also expected to take in to account the
336 differences in matrix effect between the two matrices, as well as any small losses that
337 occurred due to non-specific binding that occurred in the injection plate. Whilst the buffer
338 solution selected as the surrogate matrix is of quite a different nature to the plasma samples,
339 assays for small [35] and large molecules [19] have been successfully validated using such
340 an approach, and the validation experiments described later in this manuscript fully assess
341 the assay's performance. It was decided to proceed with this approach rather than
342 investigate alternative matrices such as charcoal stripped plasma. It has been suggested
343 that when a surrogate matrix approach is used that aliquots of the authentic matrix
344 containing the endogenous analyte should be used as QC MED samples and QC HIGH
345 samples should be prepared by spiking analyte in addition to this endogenous level [35].QC
346 LOW samples are then made by diluting authentic matrix with surrogate matrix, and
347 QC LLOQ samples prepared in pure surrogate matrix. Unfortunately this strategy cannot be
348 used for glucagon quantitation due to its relatively low endogenous levels (~LLOQ to ~3x
349 LLOQ). It was therefore decided to construct QC LOW using surrogate matrix, and QC MED
350 and QC HIGH samples were prepared by spiking analyte on top of the endogenous level in
351 authentic matrix. Due to the low endogenous levels it was decided to limit the LOW level to 2
352 x LLOQ (rather than the 3x LLOQ typically used [18].

353 Human plasma (K3 EDTA) from a commercial supplier was analysed using the assay to
354 determine its suitability as an authentic matrix. As shown in Supplemental Figure 4 such
355 plasma has a significantly raised background compared to plasma collected from volunteers
356 in house. This may be a result of the lack of stabiliser upon collection, the age of the plasma
357 and/or storage conditions. The raised background makes it unsuitable for the construction of
358 QC samples, and therefore it was decided to use plasma collected in house as the integrity
359 of these samples could be ensured. Similarly, sample collection and storage regimes for
360 any clinical samples should be carefully controlled to ensure their integrity.

361 *Validation*

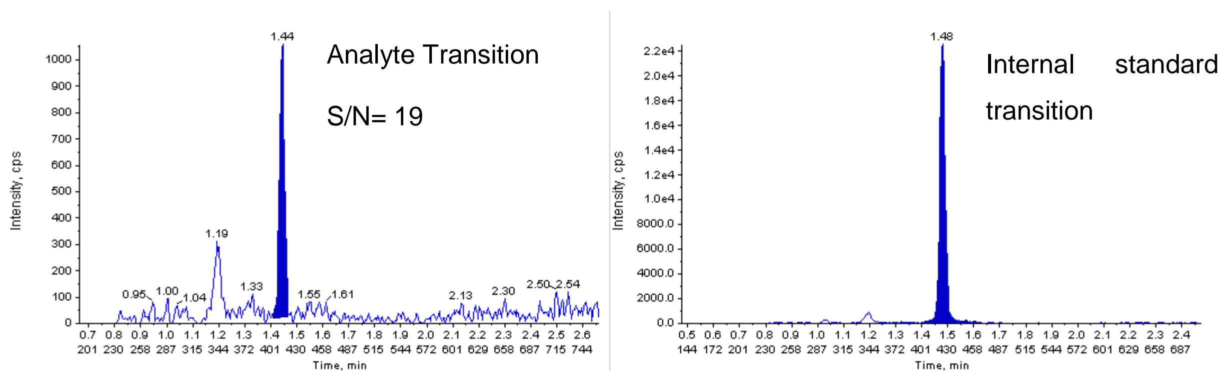
362 The precision and accuracy of the method was determined by analysis of replicate (n=6) QC
363 samples at four different concentrations (25, 50, 100 and 750 pg/mL). Precision and
364 accuracy was assessed within a batch (intra-batch, n = 6 replicates) and between batches
365 (inter-batch, 3 batches). The intra- and inter-assay precision did not exceed 20%, nor did the
366 intra- and inter-assay accuracy demonstrating the method was performing robustly
367 (Supplemental Table 1). No carryover after high calibration standards was observed and no
368 potentially interfering peaks were observed during the selectivity assessment. The 10-fold
369 dilution of an over range QC sample (7500 pg/mL) with control plasma was used to
370 demonstrate the absence of dilution effects (Supplemental Table 2).
371

372
373 The analogue Internal standard (IS) compensated for differences in suppression observed
374 by the analyte in different matrices, with mean matrix factor (MF) ratios being 1.08 and 1.05
375 at the medium and high level; a perfect correction would have a ratio of 1 (Supplemental
376 Table 3).

377 Recovery was assessed across three different batches with a minimum of 3 replicates at
378 each level. In order to investigate whether the nature of the matrix affected recovery it was
379 assessed from; samples where the analyte was spiked into control matrix then immediately
380 extracted, samples where the analyte was spiked into 3 freshly acquired matrix pools then
381 immediately extracted, and finally from samples where the analyte was spiked into matrix
382 then stored for a week at -80 °C before extraction (Supplemental Table 4). No significant
383 difference between these experiments was observed, which gave an average analyte
384 recovery of 51.2%

385

386 Acceptable sensitivity is usually demonstrated by assessing whether the analyte response at
387 the LLOQ level is at least 5 times [18] the average response due to background noise
388 (Figure 2), which was the case for all accepted batches. It is then assumed that an unknown
389 sample at the LLOQ concentration would also have a similarly acceptable response.
390 However, this will not necessarily be the case for surrogate matrix assays, due to differences
391 in the recovery and matrix factor between the surrogate and authentic matrices. By taking
392 into account the mean analyte recovery (51.2%) and mean matrix factor (0.746) for our
393 assay, it was calculated that signal-to-noise (S/N) at the LLOQ should be at least 13.1 to
394 ensure that S/N for an authentic sample at the LLOQ level ≥5 (assuming an unchanged
395 background level). This criterion was not formally part of our validation, but it was met by all
396 accepted batches.
397

398 

399 Figure 2- Representative LLOQ for glucagon in plasma (25 pg/mL) surrogate matrix chromatogram
400 demonstrating a signal-to-noise of ≥ 13.1

401 Although we used Aprotinin, a degree of glucagon instability within human plasma was
402 apparent and most experiments gave results outside the acceptance criteria of ±20% of the
403 nominal concentration (Table 1). Even if 0 hr concentrations were used, to take into account
404 any assay bias or preparation differences, many results remain outside ±20% of this
405 concentration. Glucagon plasma samples were found to be within 23.7% of their nominal
406 concentrations following storage at the extraction temperature (+4°C) for 6 hours 20
407 minutes, and within 21.4% of their 0 hr concentration following storage for 75 days at -20°C,
408 or within 20.2% following storage for 51 days at -80°C. Greater instability was observed
409 following multiple freeze-thaw cycles, and these should therefore be minimised during
410 analysis. The accuracy of the method is therefore limited by the sub-optimal sample
411 stabilisation procedure. The effect of such pre-analytical parameters has been described by
412 others [36] , and future assay development should include an evaluation of these. For
413 example, stability would likely be improved if specific DPP-IV inhibitors were used [37],
414 rather than the broad serine protease inhibitor Aprotinin.
415

416 As stability in Human K3 EDTA plasma with Aprotinin stabilisation did not pass our
417 acceptance criteria, the method is described as qualified, rather than validated. However, the
418 instability was moderate, and the data generated is likely to "fit for purpose" for many
419 applications.
420

421 **Key Terms**

422 *Validated assay* –An assay where experiments based on those described in the USA FDA
423 Guidance for Industry: Bioanalytical Method Validation (2001) and those described in the
424 EMA Guideline on Bioanalytical Method Validation (2012) meet their prospectively defined
425 acceptance criteria.

426 *Qualified assay* – An assay where not all of the validation experiments described in the
427 guidance have been assessed or have passed their prospectively defined acceptance
428 criteria. However the assay may still be considered "fit-for-purpose".

429 *Fit- for-purpose assay*- An assay where its performance characteristics have been assessed
430 and are reliable for the intended application. For example, a biomarker assay which is used
431 to assess a sole pharmacodynamic end point requires better performance characteristics
432 than an assay used as part of a panel of measurements.

433

434 Table 1- Glucagon stability data; Freezer and, extraction temperature stability of glucagon in plasma

Nominal Concentration		Stability of Glucagon in Aprotinin stabilised human plasma (K3 EDTA)								
		+4 °C	- 20 °C			-80 °C				
		6 hr 20 min	4 F/T	11days	75days	4 F/T	7days	11days	51Days	64days
MED (100 pg/mL)	Mean Measured Conc. (pg/mL)	76.9	54.8	83.6	81.8	75.0	89	-	81.4	71.4
	SD	4.23	6.48	6.75	5.35	5.23	5.16	-	8.97	4.16
	%CV	5.5	11.8	8.1	6.5	7.0	5.8	-	11	5.8
	% Stability (c.f. nominal)	76.9	54.8	83.6	81.8	75.0	89.0	-	81.4	71.4
	% Stability (c.f. 0hr)	-	51.6	85.5	83.7	70.6	91.0	-	81.7	71.7
HIGH (750 pg/mL)	Mean Measured Conc. (pg/mL)	572	332	581	526	464	530	615	533	445
	SD	9.50	25.3	21.9	52.8	57.7	11.9	32.7	46	30.6
	%CV	1.7	7.6	3.8	10	12.4	2.2	5.3	8.6	6.9
	% Stability (c.f. nominal)	76.3	44.3	77.5	70.1	61.9	70.7	82.0	71.1	59.3
	% Stability (c.f. 0hr)	85.6	41.5	86.8	78.6	58.0	79.2	91.9	79.8	66.7

435

436 SD Standard deviation CV Coefficient of variation - No data available

437 % Stability (c.f. nominal) = 100 * mean measured concentration / nominal concentration

438 % Stability (c.f. 0 hr) = 100 * mean measured concentration / mean measured 0hr concentration

439 Statistics are of n=6 replicates, expect for 64 days (-80°C), which have n=4 and n=5 replicates at the MED and HIGH level respectively.

440 The ability to re-inject extracts was demonstrated after storage at +4°C for 6 days
441 (Supplemental Table 5). The stability of stock and working solutions of glucagon, which were
442 stored at -20 °C when not in use, was demonstrated for 67 and 163 days respectively
443 (Supplemental Table 6).

444 The stability of glucagon in Aprotinin stabilised whole blood following storage on ice for 1
445 hour was found to be within acceptance criteria (Supplemental Table 7).
446
447 Haemolysed samples (plasma spiked with 3% whole blood) contained a large neighbouring
448 peak, and did not pass acceptance criteria, demonstrating haemolysed samples cannot be
449 accurately quantified using this method (Supplemental Figure 5). The presence of
450 hyperlipidaemic plasma did not significantly affect the quantitation of glucagon
451 (Supplemental Table 8).
452
453

454 *Using the qualified LC-MS/MS method to assess endogenous glucagon concentrations from*
455 *volunteers*

456 Plasma was collected from 12 healthy males and 12 healthy females and glucagon levels
457 determined using the qualified LC-MS/MS method. As shown in Table 2 levels agreed well
458 with the 25-80 pg/mL range determined by RIA [1]. Chromatograms from samples which
459 gave glucagon concentrations above the LLOQ showed good signal to noise ratios (Figure
460 3). Some samples which gave glucagon concentrations below the LLOQ showed
461 integratable peaks (Figure 3) and their approximate concentrations were determined by
462 extrapolation (Table 2)

463 Table 2- Glucagon concentrations from healthy volunteers.

Male Volunteer ID	Measured glucagon concentration (pg/mL)	Female Volunteer ID	Measured glucagon concentration (pg/mL)
M1	34.2	**F1**	BLQ (10.4)
M2	27.4	**F2**	BLQ (16.5)
M3	BLQ (16.0)	**F3**	BLQ (12.1)
M4	31.2	**F4**	41.6
M5	50.2	**F5**	BLQ (17.7)
M6	63.0	**F6**	44.4
M7	BLQ (21.3)	**F7**	29.6
M8	53.7	**F8**	59.5
M9	40.4	**F9**	31.7
M10	39.4	**F10**	BLQ
M11	BLQ (20.0)	**F11**	BLQ
M12	153	**F12**	BLQ

464 BLQ – Below limit of quantitation (25 pg/mL). Extrapolated values are in parenthesis. No integratable peaks were observed for
465 F10, F11, F12. No haemolysis was observed in the samples.

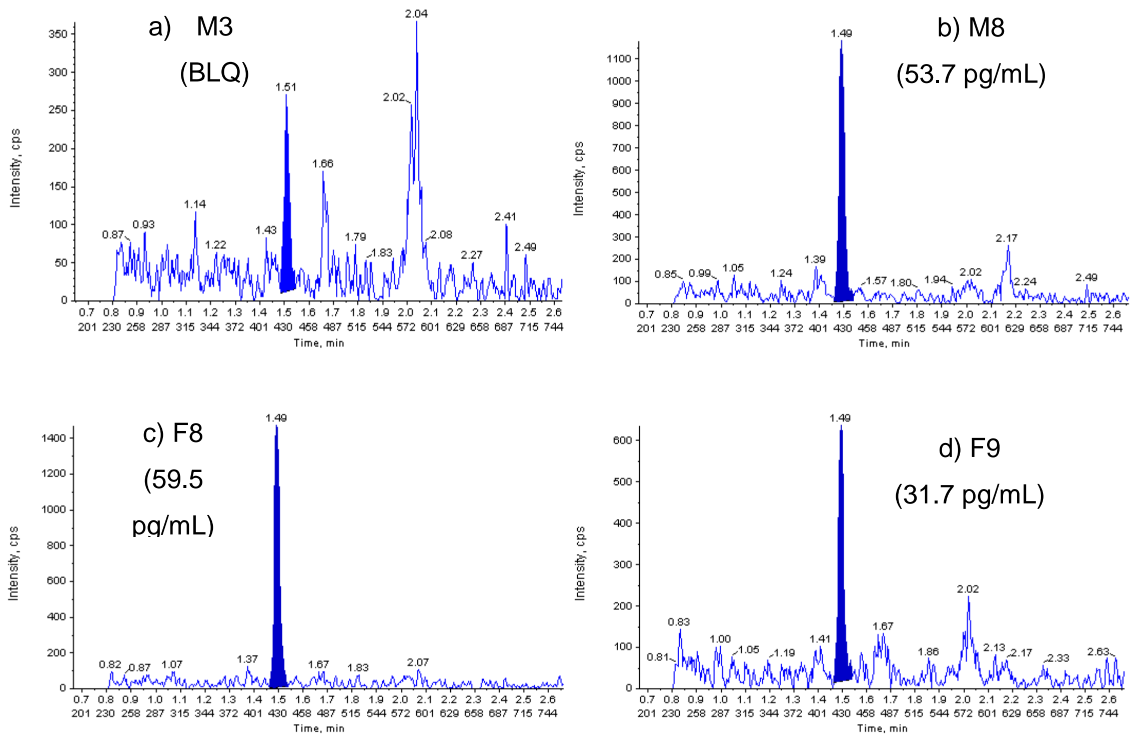


Figure 3 Chromatograms showing endogenous levels of glucagon in plasma samples from healthy volunteers.M3 (a),M8 (b), F8 (c), and F9 (d)

The majority of samples (58%) gave glucagon concentrations above the 25 pg/mL qualified LLOQ, demonstrating the assay's utility for endogenous level analysis. However as glucagon concentrations in some individual plasmas were very close to, or below, this level, for subsequent analysis we decided to include additional standards and QCs at the 10 and 15 pg/mL concentrations. These allowed assessment of whether a lower LLOQ could be achieved on a batch to batch basis. The acceptable LLOQ was experimentally determined by ensuring that its performance was within acceptance criteria (signal to noise >5, and CV and RE (<20%).

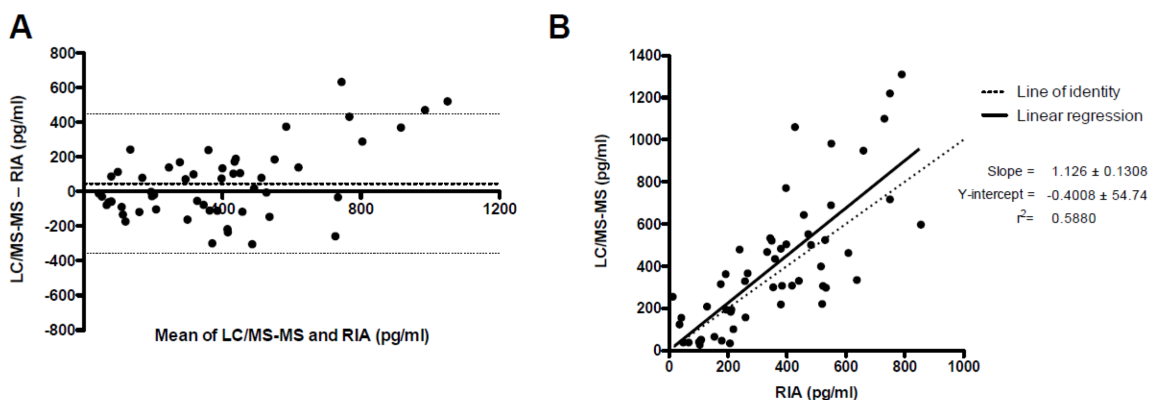
To assess whether quantitation was reproducible at the endogenous level, samples containing endogenous glucagon were pooled together, and analysed multiple times in 3 different batches (n=6 replicates in each batch) using the approach above. An overall mean of 26.5 pg/mL was observed with an overall CV of 19.8%, demonstrating reproducible quantification at the endogenous level (Supplemental Table 9). QCs (n=6 replicates) consistently performed within 20% (RE and CV) at the 15 pg/mL level in each of the 3 batches, and were within 20% (RE and CV) at the 10 pg/mL level in 2 out of the 3 batches (Supplemental Table 10). This allowed the LLOQ to be reduced from the 25 pg/mL level in the qualified assay, to increase the proportional of quantifiable concentrations.

488 *LC-MS/MS vs. RIA assays for physiological study samples*

489 Plasma samples (n= 117) were collected from a physiological study involving the infusion of
490 glucagon. 100 of these samples were analysed using our LC-MS/MS assay and 105
491 samples using the established RIA assay. Both assays contained QC samples, which
492 performed within their established acceptance criteria.

493 Bland-Altman analysis of the 88 common samples shows that the mean bias of the LC/MS-
494 MS assay versus the RIA is +45.06 pg/ml with 95% bias confidence intervals of -358.5 to
495 448.6 pg/ml. Inspection of the plot (Figure 4 a) shows that there is a concentration-
496 dependent positive bias, particularly at values above 600 pg/ml, which is also evident in the
497 scatter plot (Figure 4 b) This would be expected if the RIA assay was suffering from the
498 hook effect at higher concentrations, which has been reported for other biomarkers such as
499 calcitonin [38].

500



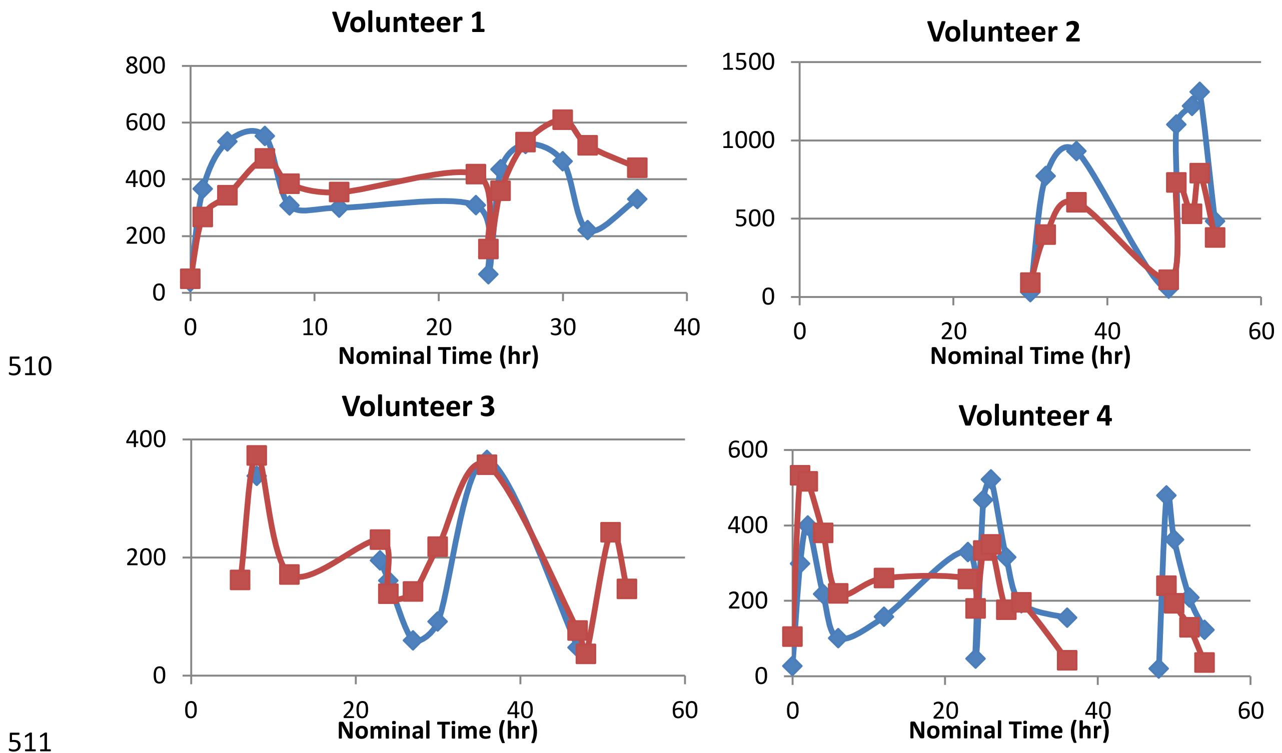
501

502

503 Figure 4a – Bland-Altman plot and b) scatter plot comparing performance of LC-MS/MS and RIA
504 methods for glucagon.

505

506 RIA and LC-MS/MS assays produced pharmacokinetic (PK) profiles of similar shapes, which
507 fitted with expectations from the nature of the study (Figure 5). It is therefore not possible to
508 determine which assay gives the “right” answer, and the approaches should be regarded as
509 complementary.



510

511

512 Figure 5- A selection of PK profiles from RIA assay concentrations (red squares) and LC-MS/MS
513 method concentrations (blue diamonds).Y axis units are pg/mL. See supplemental information Figure
514 6 for the complete set of 9 profiles

515

516 **Conclusion**

517 The developed procedure is the first peer reviewed LC-MS/MS method capable of
518 quantifying endogenous levels of glucagon in human plasma. Glucagon levels from healthy
519 volunteers agreed well with the range expected from RIA assays. Our method avoids the
520 radioactivity (and precautions this requires) associated with RIA assays, has a shorter
521 extraction time and good precision and accuracy.

522 The 25 pg/mL LLOQ in our qualified assay is a considerable improvement over the lowest
523 LC-MS/MS LLOQ previously reported (250 pg/mL) in the peer reviewed literature [11]. A 10
524 pg/mL LLOQ has been reported in a conference presentation [14], using a highly sensitive
525 QTRAP mass spectrometer. We were on occasion able to see such levels using our
526 instrument, although we performed the qualification using a 25 pg/mL LLOQ to improve
527 assay robustness. Transferring this assay on to a more modern instrument may enable the
528 LLOQ of 10 pg/mL to be achieved routinely. Our 2D extraction procedure was key to
529 achieving such sensitivity, by reducing matrix suppression, background noise, and
530 interferences. To our knowledge this is the first time protein precipitation and size exclusion
531 SPE have been combined for such a purpose for high throughput peptide analysis. Our

532 surrogate matrix approach, using a mixture of non-extracted surrogate matrix STDs and QCs
533 and extracted authentic matrix QCs, is also a novel strategy for endogenous peptide
534 analysis.

535 Bland-Altman analysis shows a mean positive bias of the LC/MS-MS method versus the RIA
536 that appears to be a concentration-dependent, as would be expected if the RIA was suffering
537 from the hook effect at higher concentrations. The PK profiles from both assays were similar
538 shapes, and both profiles fitted with the nature of the physiological study suggesting the
539 methods are complementary.

540 The assay‘s performance has been qualified using experiments described in the latest EMA
541 [18] and FDA [17] guidance and in accordance to the principles of GCP [23].
542
543

544 **Executive Summary**

545 **Introduction**

546 • Published LC-MS/MS methods are not sensitive enough to quantify endogenous
547 levels of glucagon.
548 • Endogenous compounds, such as glucagon, can be quantified using either a
549 standard addition, surrogate analyte, or a surrogate matrix approach.
550 • We favoured the surrogate matrix approach as it avoids extrapolation and is
551 described in the EMA Guideline on bioanalytical method validation.

552 **Results and Discussion**

553 *Method development*

554 • Extensive optimisation has generated the most sensitive LC-MS/MS method for
555 glucagon quantitation in the peer reviewed literature.
556 • A novel 2D extraction technique, combining protein precipitation with size exclusion
557 hydrophobic (SEH) SPE, was key to achieving such sensitivity, by reducing matrix
558 suppression, background noise, and interferences.
559 • Quantitation used a mixture of non-extracted surrogate matrix STDs and QCs and
560 extracted authentic matrix QCs. Such approach is a novel strategy for endogenous
561 peptide analysis.
562

563 *Validation*

564 • Validation experiments performed were based on those described in the latest EMA
565 and FDA guidelines.
566 • Most experiments, including the precision and accuracy of the method, were within
567 the prospectively defined acceptance criteria.
568 • However, a degree of plasma sample instability was apparent, and it fell outside of
569 our prospectively defined acceptance criteria.
570 • The assay is therefore described as qualified, over the range 25 – 1000 pg/mL,
571 rather than validated. The assay will however be fit-for-purpose for many
572 applications.

573

574

575 *Using the qualified LC-MS/MS method to assess endogenous glucagon concentrations from*
576 *volunteers*

577 - Glucagon levels in healthy volunteers measured by LC-MS/MS showed good
578 agreement with literature values determined by RIA.
579 - Assessment of assay performance at the 10 and 15 pg/mL levels allowed the assay
580 LLOQ to be lowered from 25 pg/mL on a batch to batch basis.
581 - Reproducible quantitation at the endogenous glucagon level was demonstrated.

582

583 *LC-MS/MS vs. RIA assays for physiological study samples*

584 - Bland-Altman analysis shows a concentration-dependent positive bias of the LC/MS-
585 MS assay versus an RIA, with a mean bias of +45.06 pg/mL
586 - Both assays produced similar PK profiles, both of which were feasible considering
587 the nature of the study, and the methods should be regarded as complementary.

588

589

590

591 **Future Perspectives**

592 We believe that experimentally demanding or troublesome immunoassays, such as the
593 glucagon RIA assay, will increasingly become replaced with LC-MS/MS based
594 methodologies to circumvent issues with cross reactivity, increase sample throughout and
595 avoid the use of radioactivity. To achieve the low LLOQs often required we also believe that
596 approaches such as 2D extraction will become more commonly used. For regulated
597 bioanalytical studies of endogenous compounds, strategies such as surrogate matrix
598 quantitation, which avoids the need to extrapolate the calibration curve, will become the
599 favoured approach.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval (West London Research Ethics Committee: 11/LO/1782) and have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations.

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