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Utilizing LC-MS/MS to Provide Adaptable Clinical Bioanalytical Support for an Extended Half-Life Bioactive Peptide Fused to an Albumin-Binding Domain Antibody

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24 ## Abstract

25 Bioactive peptides are often unstable in the body leading to short half lives and requiring
26 frequent dosing intervals. Linking these peptides to moieties such as albumin, fatty acids and
27 polyethylene glycol has been shown to extend the half-lives of various therapeutics allowing less
28 frequent dosing regimens. In this study the GLP-1 receptor agonist, therapeutic under
29 investigation (GSK2374697), was a bioactive peptide (exendin-4) that was fused to an albumin-
30 binding domain antibody (AlbudAb™) to increase the half-life of the therapeutic. However,
31 developing selective quantitative methods for these molecules to provide a complete
32 understanding of the pharmacokinetic (PK) properties using immunoassay, has proved to be
33 challenging. Methods utilizing LC-MS/MS for the determination of GSK2374697 in human
34 plasma were based on the selection and quantification of two surrogate peptides after enzymatic
35 digestion using either Lys-C or trypsin. These methods were validated and used for the analysis
36 of clinical samples from a first time in human (FTIH) study. Method validation data for both
37 surrogate peptides indicate that the methods are rugged, accurate, precise and well suited for
38 support of regulated clinical studies. The pharmacokinetic results obtained from the two
39 surrogate peptides indicate that the peptide derived from the bioactive portion of the molecule
40 has a much shorter terminal half-life than the peptide derived from the AlbudAb portion of the
41 molecule. Development of assays for these multiple molecular fragments allowed for the
42 accurate quantification and integrity of the molecule from different binding regions illustrating
43 different AUCs and half lives.

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47 # Introduction

48 The presence of biotherapeutics in the pipelines of pharmaceutical companies has
49 increased dramatically over the last 10 years [1]. Recently the FDA has approved a number of
50 peptides as therapies for multiple indications including diabetes mellitus type 2 (exenatide,
51 liraglutide, lixisenatide), osteoporosis (teriparatide), congestive heart failure (nesiritide) and
52 hormone-responsive cancer (triptorelin). However, the limited oral bioavailability and short
53 half-lives typically associated with peptide therapeutics has lead to the need to administer these
54 drugs by subcutaneous or intravenous administration at frequent dosing intervals. To overcome
55 these challenges, researchers have used various strategies including modification of the peptides
56 native sequence to resist catabolism or by chemical modification (ie. pegylation) to increase the
57 half-life of these molecules.

58 One class of next generation molecules being investigated to increase the half-life of
59 peptides, small proteins and small molecule therapeutics are engineered protein scaffolds such as
60 domain antibodies (dAbs) that have high affinity for human serum albumin (AlbudAbs) [2].
61 These AlbudAbs are approximately 110 amino acids in length and have been found to be
62 extremely stable and well expressed in culture. This small size would normally lead to a short
63 half life due to rapid renal clearance but the ability of these molecules to bind to serum albumin
64 increases the half-life to that approximating serum albumin itself [3]. In addition, these
65 molecules can be genetically or chemically fused to various peptides and proteins to increase
66 half-life, solubility, or impart bispecific functionality to a molecule, imparting unique therapeutic
67 pharmacokinetic characteristics [2-4].

68 Along with the cost of discovering and developing a new drug of between $1.2 and $1.7
69 billion dollars [5], drug development processes requires pharmacokinetic (PK) analysis to be

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70 performed as part of safety and efficacy studies in both nonclinical species and human subjects.
71 Currently, immunoassay is considered the bioanalytical 'gold standard' for the detection and
72 quantification of biopharmaceuticals for the support of PK and toxicokinetic (TK) exposure
73 studies. However, due to the large amount of interfering endogenous immunoglobulins present,
74 dAbs are an analytical challenge to selectively detect using immunoassay. In addition, the
75 bispecific nature of the AlbudAb-fusion therapeutics makes it particularly challenging to fully
76 understand the integrity of the molecule using immunoassay alone. Recently, mass spectral
77 (MS) assays, coupled with liquid chromatography (LC), have been shown to allow development
78 of a robust, sensitive and selective method for a domain antibody therapeutic and expedited
79 method development time compared to traditional immunoassay methodologies [6, 7].
80
81 This manuscript describes the method development and validation of LC-MS/MS methods for
82 the determination of GSK2374697 (GLP-1 receptor agonist, peptide therapeutic genetically
83 fused to an AlbudAb) in human plasma to support a clinical study where GSK2374697 was
84 dosed to healthy volunteers. The developed methods allowed quantification of a Lys-C derived
85 twelve amino acid peptide form the N-terminus of the molecule or a tryptically derived peptide
86 from the complimentary determining region (CDR) of the molecule. This strategy allowed
87 quantification of both active drug (from N-terminus) and drug-related material (from CDR)
88 giving information on the integrity of the molecule that would not have been possible using a
89 single immunoassay approach. This clinical study was approved by the GSK institutional
90 review board and informed consent was obtained from all participants.
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Results and Discussion

Assay Design and Surrogate Peptide Selection

GSK2374697 consists of a bioactive GLP-1 receptor agonist peptide genetically linked to an AlbudAb moiety with a total molecular weight of ~17kDa (Figure 1). Ideally, a bioanalytical method that measures the intact molecule is preferred as this eliminates concerns about catabolized or transformed forms of the molecule. However, for larger molecules (>10 kDa), it becomes increasingly difficult to use this strategy due to difficulties with sample preparation and reduction in sensitivity [4]. Thus, current practice relies on enzymatic digestion of the intact molecule to produce peptide fragments (surrogate peptides) that then are quantified and used to represent a portion of or the entire molecule. Proper surrogate peptide selection is extremely important when designing the assay as modifications or truncations to that particular peptide may cause inaccurate representation of the molecule. In this case, the surrogate peptides selected were the Lys-C derived twelve amino acid peptide from the N-terminus of the molecule as the N-terminus of the peptide is necessary for bioactivity of the molecule, and the Trypsin derived six amino acid peptide from one of AlbudAbs three complimentary determining region (CDR) which is responsible for the molecules albumin binding specificity and contains drug-related material.

Internal Standard Selection

Even with extensive method optimization, the ability to accurately account for differences due to enzymatic digestion, sample extraction, LC injection volume, and variability needs to be addressed with internal standard selection for mass spectrometer methods. In the last several years there have been a variety of internal standard molecules used to develop quantitative assays

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utilizing an enzymatic digestion step for large molecule proteins including: analog proteins [4], stable isotopically lableled (SIL) peptides, SIL peptides with a N- and/or C-terminal extendion (extended sequence SIL peptides) or fully labeled peptides [8]. Just as with small molecule IS selection, a closely matched internal standard will correct for assay variability [9]. In addition, a fully labeled molecule allows selection of any surrogate peptide without the need for synthesis of a new labeled peptide. Unlike monoclonal antibodies, domain antibodies can be efficiently expressed in *E. coli* [10]. This allowed the use of ISOGRO® 15N to make fully-uniformly labeled ($^{13}C^{15}N$ or ^{15}N) internal standard molecule. Initially, GSK2374697 was grown in medium with either $^{13}C^{15}N$ containing 99 atom percent ^{13}C and 98 atom percent ^{15}N or ^{15}N containing 98 atom percent ^{15}N. As the selected surrogate peptide contained 54 carbon atoms and 15 nitrogen atoms the $^{13}C^{15}N$ labeled peptide would increase in mass by 69 and the ^{15}N labeled peptide would increase in mass by 15.

The $^{13}C^{15}N$ or ^{15}N labeled GSK2374697 molecule was digested with Lys-C and injected onto the LC-MS/MS system monitoring for both the unlabeled peptide and the labeled version of the peptide. In both cases there was no unlabeled peptide detected, indicating at least partial incorporation of either ^{13}C and/or ^{15}N. However, a ten-fold difference in signal was noted between the $^{13}C^{15}N$ and the ^{15}N labeled peptide. Likely, this was due to incomplete incorporation of the label into the protein. With this information the ^{15}N labeled peptide was selected as the internal standard for the assay.

Assay Development and Validation

Following method development and assay validation, Assay A (50 to 10000 ng/mL) was used to quantify initial plasma samples following administration of GSK2374697 in a FTIH clinical

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3 139 study. After several runs it was observed that there was a large variation in IS response when
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6 140 comparing the standards and QCs to the samples with a greater than 10-fold difference in IS
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8 141 response in certain samples was observed. In addition, following clinical review of the first few
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11 142 subjects at the lowest dose, it was deemed necessary to lower the analytical range of the assay.
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13 143 To improve the IS response and develop a more sensitive method, various modifications to the
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15 144 method were made including increasing the sample volume from 50 to 200 µL as well as
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18 145 including a denaturation step by incubating the sample with 6M guanidine and heating to 65°C.
19
20 146 As a result, the IS response consistency was vastly improved with no bias associated with
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22 147 standards, QC or study samples (Figure 2). Assay B then was fully validated (sample
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24
25 148 chromatograms in Figure 3, and validation statistics in Table 2) and used to quantify over 3000
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27 149 samples from 10 dosing regimens in support of the FTIH study.
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32 151 ***Pharmacokinetic Analysis***
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34 152 The PK parameters obtained for the half-life of GSK2374697 in human were not as expected
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37 153 based upon data obtained from prior TK studies in preclinical species where the N-terminal
38
39 154 peptide was quantified using LC-MS/MS. A bioanalytical investigation using the FTIH study
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42 155 samples was undertaken to determine if the stability of the molecule attributed to the observed
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44 156 reduction in half-life. The specific peptide fragment being monitored in the original analysis
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46 157 (Assays A and B) correlated to the active portion of the molecule (N-terminus) conjugated to the
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48
49 158 AlbudAb. As a result, an additional assay (Assay C) was rapidly developed to monitor for a
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51 159 specific peptide from the complimentary determining region (CDR) of the dAb portion of the
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54 160 molecule. As shown in Figure 4, the results from the reanalysis of the dAb portion of the
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56 161 molecule resulted in a much longer half-life compared to the active peptide. These results
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162 suggest that the active portion of the molecule is being catabolized *in-vivo* with the AlbudAb
163 portion of the drug at least partially intact. For additional information on the PK results and
164 discussion regarding the clinical study please see reference 11.

165

166 Experimental

167 *Materials and Methods*

168 The therapeutic AlbudAb molecule, GSK2374697, was developed and manufactured by
169 GlaxoSmithKline (GSK) and supplied at 10 mg/mL in sodium acetate buffer and stored at 4 °C.
170 A ^{15}N and a $^{15}N/^{13}C$ uniformly labeled version of GSK2374697 was prepared using ISOGRO®
171 complex growth media (Sigma) by GlaxoSmithKline and used as the internal standard (IS) for
172 method development, validation and sample analysis. The IS was supplied at 1 mg/mL in buffer
173 and stored at -20ºC. Control whole human blood was collected from in-house GSK volunteers
174 while control human plasma was purchased from Bioreclaimation. All study participants were
175 presumed healthy and provided written informed consent forms. Chemicals such as sodium
176 bicarbonate, sodium hydroxide, methanol, formic acid, isopropanol, guanidine HCL, and acetic
177 acid were purchased from Fisher Scientific. Endoproteinase Lys-C was purchased from Roche
178 Diagnostics. Trypsin gold was purchased from Promega. Strata XC-L 30 mg solid phase
179 extraction (SPE) plates were purchased from Phenomenex.

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181 *Assay Details*

182 Over the course of method development and sample analysis for this clinical study, three
183 separate assays were used. The specific assay details can be found below and are summarized in
184 Table 1.

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185 *Assay A Sample Preparation:*

186 The initial validated assay had an analytical range from 50 to 10000 ng/mL (Assay A). A 50 μL
187 aliquot of plasma (standard, quality control, blank, or subject sample) was placed into a 1.4 mL
188 polypropylene tube (Micronic, Aston PA), followed by 50 μL of IS. After mixing, a 75 μL
189 aliquot of Lys-C solution (1 μg/mL in 100 mM sodium bicarbonate, pH 8.5) was added to all
190 tubes for sample protein digestion. Tubes were capped, vortexed and allowed to mix gently for
191 approximately 24 hours under ambient conditions. The following day, the Strata XL-C SPE
192 plates were conditioned with methanol followed by 2% (v/v) formic acid in water. After SPE
193 conditioning, formic acid was added to all samples to halt the digestion, followed by loading of
194 the samples onto the SPE plate. The SPE plate was washed with 2% formic acid followed by
195 methanol. After drying with vacuum, the samples were eluted using 5% ammonium hydroxide
196 in methanol. The samples then were dried under nitrogen and reconstituted in a mixture of 80/20
197 0.1% formic acid/acetonitrile (v/v). LC-MS/MS conditions stated below were used to quantify
198 the Lys-C derived N-terminal peptide.

199

200 *Assay B Sample Preparation:*

201 To improve the sensitivity of the assay and limit internal standard variability, Assay B was
202 developed. Most extraction and digestion steps remained identical to Assay A with the
203 exception of increasing the plasma volume to 200 μL and including a denaturation step by
204 adding 0.2 mL of 6M guanidine hydrochloride and heating at 65°C for 30 minutes, to denature
205 the proteins and increase digestion efficiency, prior to the addition of the Lys-C solution. LC-
206 MS/MS conditions stated below were used to quantify the Lys-C derived n-terminal peptide.

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Assay C Sample Preparation:

A third method (Assay C) was developed by modifying Assay B to enable different peptide fragments to be quantified. To achieve this, Lys-C was replaced with trypsin gold. Digestion with trypsin was used to obtain the specific peptide of interest from the CDR of the AlbudAb. An evaluation of the assay precision and accuracy was performed. LC-MS/MS conditions stated below were used to quantify the trypsin derived peptide.

LC Configuration

Similar LC equipment was used for the three assays detailed above. The UHPLC system was an Acquity system from Waters, consisting of a pump, autosampler, sample organizer and column compartment. To minimize or eliminate any carryover, the autosampler washes consisted of 40/40/20 acetonitrile/isopropanol/0.1% formic acid in water (v/v/v) and 0.1% formic acid in water. The analytical column was a Waters Acquity 135 Å BEH C18, 1.7 µm (2.1 x 50 mm) held at 65ºC. The mobile phase consisted of linear, gradient conditions of 0.025% formic acid (mobile phase A) and 50/50 isopropanol/acetontrile (mobile phase B) with a flow rate of 700 µL/min. As the assay range was lowered, the LC conditions had to be modified slightly to separate various endogenous interferences. An LC-diverter valve was also incorporated with the assays that included guanidine (Assay B and C). Without the use of the divert valve, IS response dropped rapidly and significantly following the initiation of the run. With the 4-minute run time, flow was diverted to waste for the first minute, followed by flow to the MS from 1 – 2.5 minutes, followed by flow to waste. A make-up pump was incorporated that pumped 50/50 acetonitrile/0.1% formic acid into the MS when the LC line consisting of the analytical column was diverted to waste.

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232 ***Mass Spectrometry and Quantification***

233 An API5500 mass spectrometer (Applied Biosystems) with a TurboIonSpray®
234 Electrospray interface running Analyst software version 1.5 was used for method development
235 and validation. The following source conditions were used: ion spray voltage 5500 V,
236 nebulizing gas 70 psi, turbo gas 70 psi, curtain gas 40, collision gas 10, declustering potential 95,
237 collision energy 36, and temperature 750ºC. For Assay A and B, MRM transitions were
238 monitored for the Lys-C derived doubly charged native and IS peptide, respectively: 640 double
239 charged parent ion to 932 single charged daughter ion, and 647 double charged parent ion to 943
240 single charged daughter ion (daughter ions correspond to the b^9 product ion fragment). The
241 dwell time was 150 ms and Q1 and Q3 were operated in low resolution. For Assay C, MRM
242 transitions were monitored for the tryptically derived doubly charged native and IS peptide,
243 respectively: 416 double charged parent ion to 606 single charged daughter ion, and 421 double
244 charged parent ion to 612 single charged daughter ion. The dwell time was 50 ms and Q1 and Q3
245 were operated in low resolution. The precursor ions were selected for monitoring based on the *in*
246 *silico* analysis of GSK2374697 using either the endoproteinase enzyme Lys-C or trypsin and the
247 daughter ions were selected to ensure high selectivity.

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249 Linear responses in the analyte/internal standard peak area ratio(s) were observed over
250 the range 50 – 10000 (Assay A) and 10 to 2500 ng/mL (Assay B and C). The correlation
251 coefficients obtained using $1/x^2$ weighted linear regression were better than 0.9991, 0.9968 or
252 0.9963, for Assay A, B, and C respectively. UHPLC MS/MS data were acquired and processed
253 (integrated) using the proprietary software application Analyst (Version 1.5, Applied

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254 Biosystems/MDS Sciex). Concentrations of GSK2374697 in QC samples were determined
255 from the appropriate calibration line, and used to calculate the bias and precision of the method
256 within the Study Management System, SMS2000 (Version 2.3, GlaxoSmithKline).
257
258 ***Method Validation***
259 Both Assay A and B were validated according to the FDA procedures outlined in the
260 Guidance for Industry, Bioanalytical Method Validation, May 2001 [12] and departmental
261 Standard Operating Procedures. Assay A was used for the initial assessment (10% of study
262 samples) of compound concentrations in the clinical samples. However it was soon discovered
263 that the assay range was not sensitive enough to quantify at all desired timepoints. The more
264 sensitive, Assay B, was then used for the analysis of the remaining FTIH study samples, along
265 with re-analysis of BLQ (below limit of quantification) samples that utilized Assay A. The
266 method validation discussion going forward will focus on Assay B. The three validation batches
267 comprised an eight-point calibration curve extracted in duplicate with GSK2374697 calibrants at
268 10, 25, 50, 100, 250, 500, 1000 and 2500 ng/mL; various blanks with and without the addition of
269 IS; and six replicates of the following QC levels: 10, 30, 200, 2000 and 2500 ng/mL. The
270 precision and accuracy limits were ±15% (±20% at the lower limit of quantification; LLQ), and
271 the statistical analysis of the validation data is presented in Table 2. The selectivity of the
272 method was established by the analysis of blank and double blank samples of control human
273 plasma from six individual volunteers. UHPLC MS/MS chromatograms of the blanks and
274 validation samples were visually examined and compared for chromatographic integrity and
275 potential interferences. Representative chromatograms of a double blank sample, validation
276 sample at the LLQ (10 ng/mL) and internal standard are shown in Figure 3. No unacceptable

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3 277 interferences at the retention times of GSK2374697 and its internal standard were observed.
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6 278 Finally, the validation included stability assessment of GSK2374697 in stock solution, human
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8 279 plasma (room temperature, long term frozen stability and freeze thaw), human whole blood, and
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11 280 after processing. In addition, as mandated by various regulatory authorities, the method was
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13 281 investigated for incurred sample reproducibility (ISR). In this case, approximately 10% of the
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15 282 total study samples assayed with both Assay A and B were selected for ISR. The evaluation of
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18 283 bioanalytical methods through the reanalysis of incurred samples can be taken as one additional
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20 284 measure of assay reproducibility. ISR results were within the acceptable limits set forth by
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23 285 regulatory agencies (95% of the selected results were within 20% of the original), indicating
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25 286 assay reproducibility, stability and ruggedness. A further breakdown of the ISR results indicates
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27 287 that 85% of the 48 samples chosen from Assay A, and 98% of the 191 samples chosen from
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30 288 Assay B were within the acceptance limits, indicating the improved robustness of Assay B.
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32 289 Method qualification of Assay C included a single run consisting of duplicate calibration
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35 290 curves, and 6 replicates of quality control samples at 10, 30, 200, 2000, and 2500 ng/mL. For
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37 291 Assay C, precision and accuracy limits were extended to ±20%. Selectivity assessment in
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39 292 plasma from six different volunteers was also investigated with no noted interferences.
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47 295 **Conclusion**
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49 296 We have illustrated the performance of a novel analytical method for the determination of
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52 297 GSK2374697 (range 10 – 2500 ng/mL) in human plasma using UHPLC MS/MS. GSK2374697
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54 298 was extracted from 200 µL of human plasma, after the addition of an isotopically labeled internal
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57 299 standard by protein digestion followed by solid phase extraction (SPE). Assay throughput,
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robustness, stability and other performance characteristics were found to be acceptable for clinical sample analysis. The incorporation of the LC-MS/MS based assay provided information on the AlbudAb from the n-terminus and CDR peptide region in a single assay. In addition, we have highlighted one of the key strengths of LC-MS/MS, which is the ability to develop assays in real time providing adaptability in comparison to immunoassay methods.

Contributors

Each author made significant contributions to the scholarly content of this manuscript in the following domains:

CLB: concept & design of the analyses, data analysis and interpretation, manuscript preparation and revisions in terms of important intellectual content

JK: concept & design of the analyses, data analysis, acquisition and interpretation, manuscript preparation and revisions in terms of important intellectual content

TM: data analysis, acquisition and interpretation, manuscript preparation

BO: data analysis; manuscript preparation and revisions in terms of important intellectual content.

MS: data analysis, acquisition and interpretation, manuscript preparation

All authors were critically involved in revising the manuscript and all reviewed the final manuscript and gave approval for submission.

Conflicts of interest

CLP, JK, TM, BO, MS are employed by and shareholders of GlaxoSmithKline.

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326

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397 **Table 1 – Assay Comparison**
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	Assay		
	A	B	C
Assay Range (ng/mL)	50 to 10000	10 to 2500	10 to 2500
Aliquot Volume	50	200	25
LC Run Time (min)	3	4	3.5
Fully Validated*	YES	YES	NO
Denature Reagent	NONE	Guanidine	Guanidine
Digestion Reagent	Lys-C	Lys-C	Trypsin Gold
Divert Valve	NO	YES	YES
Monitored Fragment	N-terminus	N-terminus	CDR region

399 *According to FDA guidance

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429 **Table 2 – Quality Control Statistics from Validation for Assay B**

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Concentration (ng/mL)	10	30	200	2000	2500
RUN 1, n=6					
Mean	9.7	31.1	192	1837.2	2252.2
Precision (%CV)	13.2	3.0	1.9	2.1	2.5
Bias %	-2.8	3.7	-4.0	-8.1	-9.9
RUN 2, n=6					
Mean	9.6	32.7	204.7	1957.2	2428.1
Precision (%CV)	7.6	7.1	1.6	1.3	2.4
Bias %	-3.7	8.9	2.4	-2.1	-2.9
RUN 3, n=6					
Mean	8.4	30.8	200.5	1920.8	2412
Precision (%CV)	11.2	11.0	1.5	1.9	1.4
Bias %	-15.6	2.6	0.3	-4.0	-3.5
Overall Totals, n=18					
Mean	9.3	31.5	199.1	1905	2364.1
Precision (%CV)	12.1	7.7	3.2	3.2	4.0
Bias(%)	-7.4	5.1	-0.5	-4.7	-5.4
Between-run precision (%)	6.3	0.7	3.2	3.1	4.0

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452 Figure 1 – Graphical representation of GSK2374697 and the Albudab complex

453 Figure 2 – Internal standard variation with and without guanadine in the digestion procedure

454 Figure 3 – Example chromatograms of blank (top), LLQ of 10 ng/mL (middle) and Internal

455 Standard (bottom)

456 Figure 4 – Concentration and half-life comparison for dAb and n-terminus cleavage and

457 monitoring

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475 **Figure 1 – Graphical representation of GSK2374697 and the Albudab**
476 **complex**
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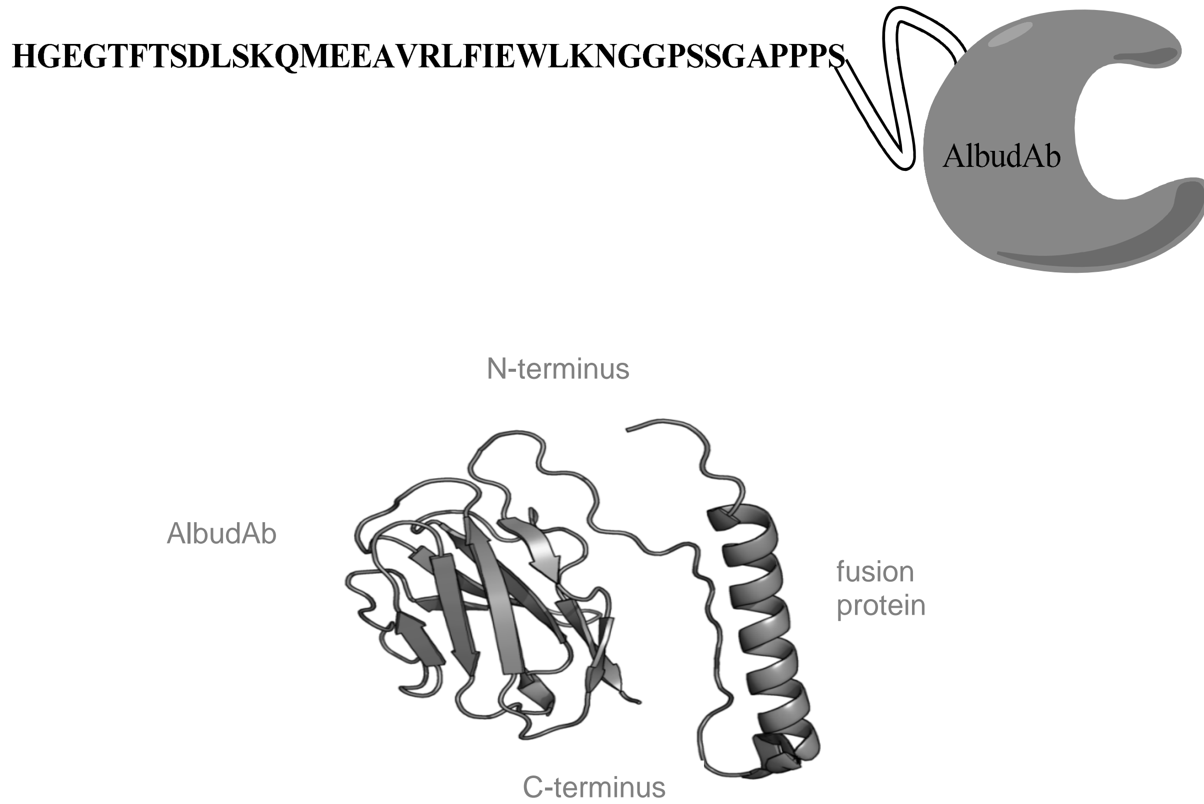

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4 494 **Figure 2 – Internal standard variation with and without guanadine in the**
5 495 **digestion procedure**
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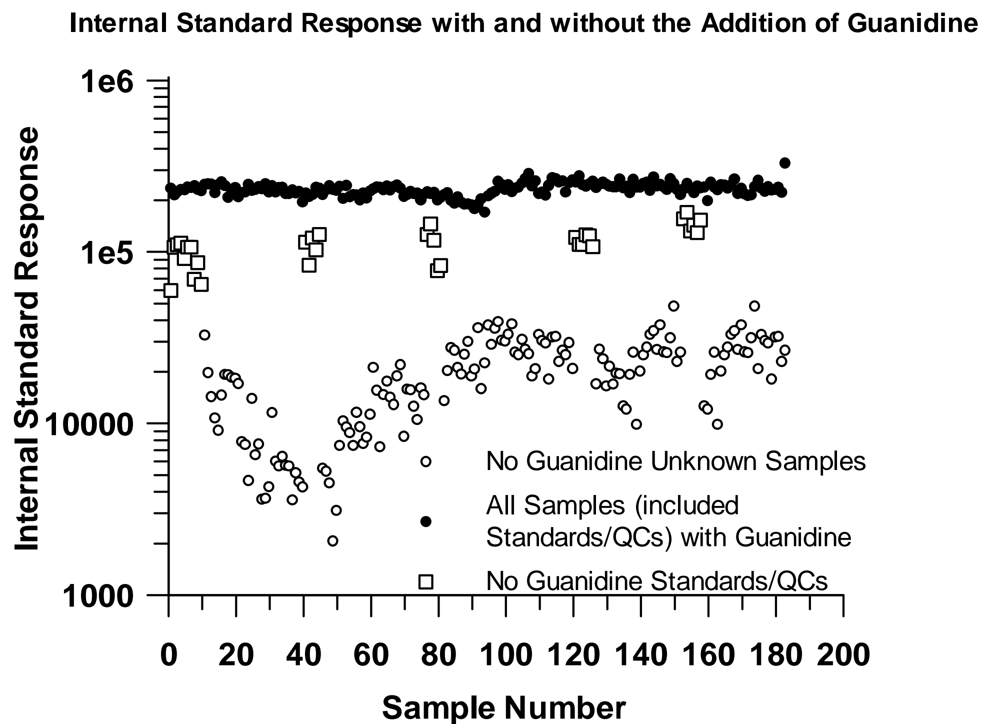

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4 517 **Figure 3 – Example chromatograms of blank (top), LLQ of 10 ng/mL (middle)**
5 518 **and Internal Standard (bottom)**
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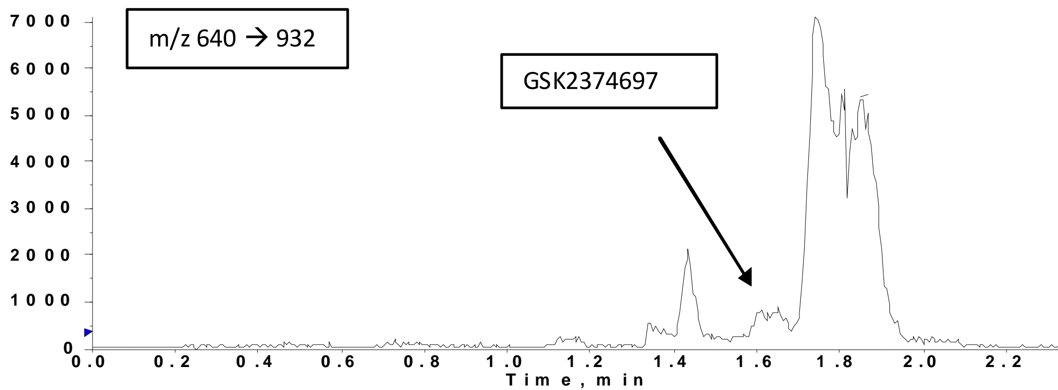

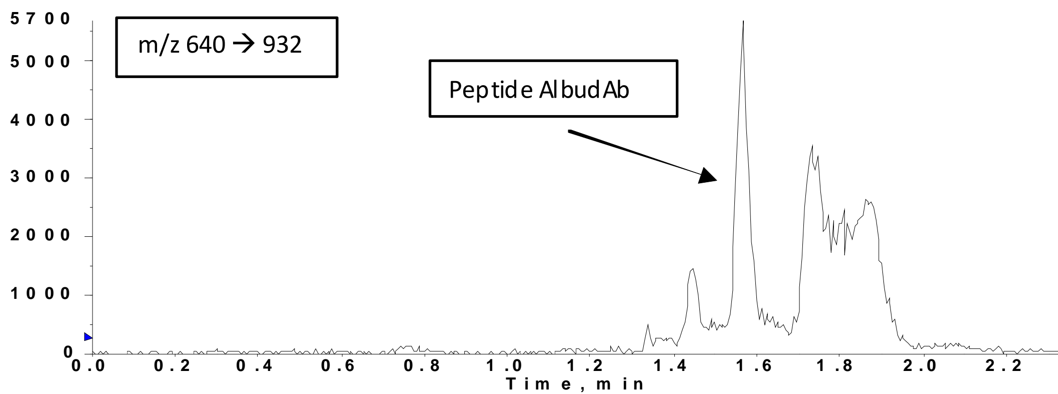

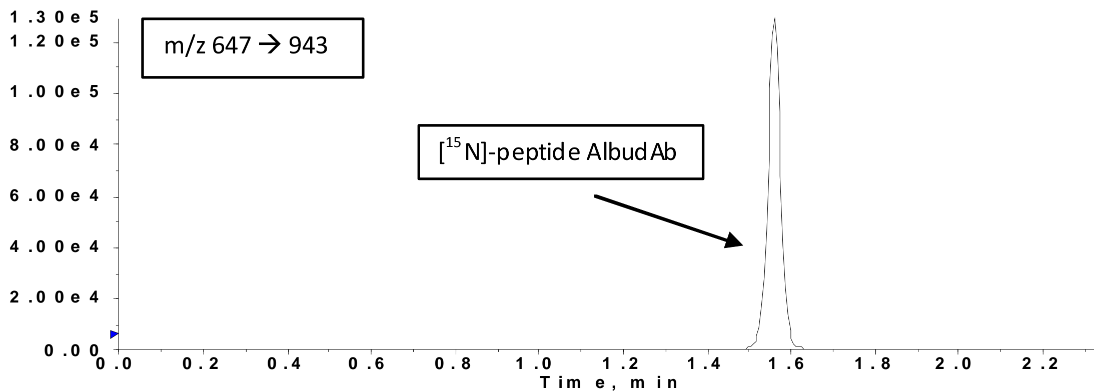

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523 **Figure 4 – Concentration and half-life comparison for dAb and n-terminus**
524 **cleavage and monitoring**
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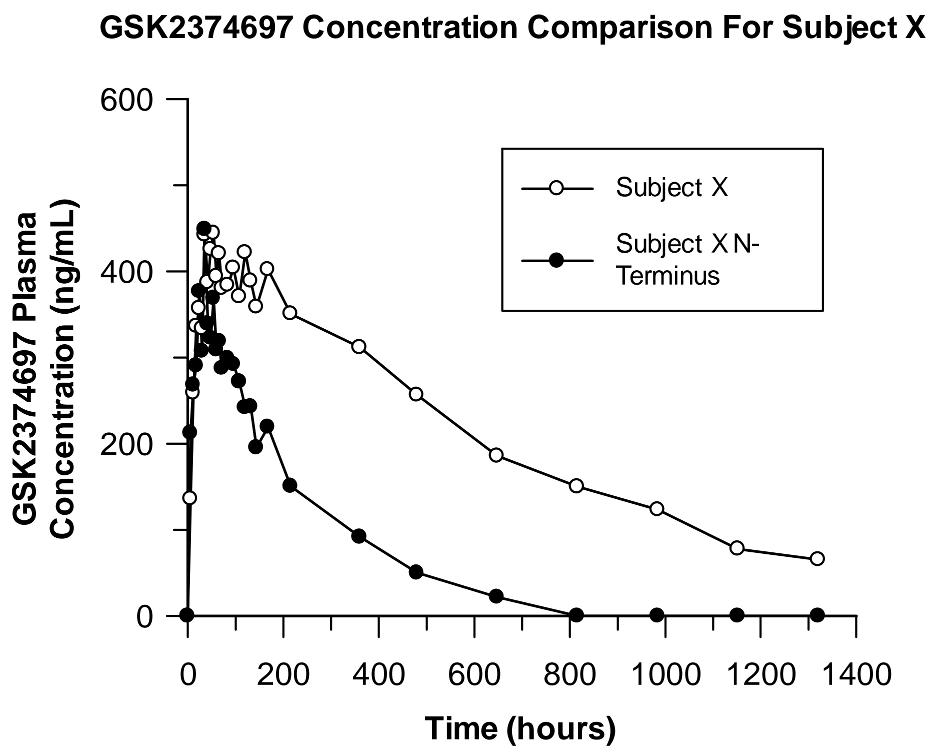

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