The preanalytical stability of glucagon as measured by liquid chromatography tandem mass spectrometry and two commercially available immunoassays

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Full title: The pre-analytical stability of glucagon as measured by LC-MS/MS and two commercially available immunoassays.

Jaimini Cegla¹, Ben J Jones¹, James Howard²³, Richard Kay⁴, Colin S Creaser³, Stephen R Bloom¹, Tricia M Tan¹

¹Division of Diabetes, Endocrinology and Metabolism, Imperial College London, UK
²LGC Limited, Cambridgeshire, CB7 5WW, UK
³Centre for Analytical Science, Department of Chemistry, Loughborough University, UK
⁴Institute of Metabolic Science, University of Cambridge, UK

Corresponding Author:

Professor Tricia Tan
Division of Diabetes, Endocrinology and Metabolism,
6th Floor Commonwealth Building,
Hammersmith Hospital, Imperial College London,
Du Cane Road, London W12 0NN, U.K.
Tel 020 8383 3242 Fax 020 8383 8320,
Email: t.tan@imperial.ac.uk

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Guarantor: Dr Jaimini Cegla

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Abstract

**Background:** One of the main challenges in the measurement of glucagon is the premise that it is unstable in human plasma. Traditionally, protease inhibitors have been used to prevent its degradation, however, their use is controversial. Here we investigated the optimal method of sample collection for glucagon, with measurement by liquid chromatography tandem mass spectrometry (LC-MS/MS) and two commercially available immunoassays.

**Methods:** Blood from healthy fasting volunteers (n=10) was processed under a variety of pre-analytical condition including collection in EDTA vs lithium heparin tubes and the addition of aprotinin and/or a DPPIV inhibitor. Additionally, the effect of freeze thaw was assessed. Plasma glucagon levels were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) and two commercially available immunoassays (HTRF® sandwich immunoassay, Cisbio and Milliplex MAP Human Metabolic Hormone Panel, Merck Millipore).

**Results:** A systematic bias of Milliplex > LC-MS/MS > HTRF was noted and plasma glucagon levels were significantly different between methods (Milliplex vs. LC-MS/MS P<0.01; Milliplex vs. HTRF P<0.0001; LC-MS/MS vs. HTRF P<0.001). The addition of aprotinin, DPPIV inhibitor or a combination of aprotinin and DPPIV inhibitor had no effect on plasma glucagon levels when compared to ‘non-stabilised’ samples or each other. Whether samples were taken in EDTA tubes or lithium heparin tubes made no difference to plasma glucagon levels. These findings were consistent for all three methods. Plasma glucagon levels were not significantly different after 2 freeze-thaw cycles (performed on samples in EDTA tubes containing aprotinin and DPPIV inhibitor).

**Conclusions:** This study demonstrates that glucagon is stable in both EDTA and lithium heparin tubes when stored at -80°C. Furthermore, the addition of aprotinin and DPPIV inhibitors is unnecessary.
Introduction

Glucagon has an important physiological role in energy homeostasis as well as cardiovascular regulation. Furthermore, it is a biomarker for diseases such as diabetes and neuroendocrine tumours. One of the main challenges in measurement of glucagon is the premise that it is unstable in human plasma. Studies indicate that the circulating serine protease dipeptidyl-peptidase IV (DPPIV) is the primary enzyme for glucagon degradation in blood. In order to counteract degradation, inhibitors such as the serine protease inhibitor aprotinin (1) or the proprietary P800 inhibitor cocktail are often added to samples to stabilise them (2). However, their use is controversial as other studies have found these to be ineffective in preventing degradation (3, 4). Here we investigated the optimal method of sample collection for glucagon, with measurement by liquid chromatography tandem mass spectrometry (LC-MS/MS). Additionally, measurement was also made by two commercially available immunoassays in order to ascertain whether peptide degradation could cause a positive bias as immunoassays could potentially cross-react with inactive fragments.

Methods:

A 20ml blood sample was collected from 10 fasting healthy volunteers (West London National Research Ethics Committee, ref. 11/LO/1782). Immediately after collection, 2 ml samples were decanted into various collection tube types: 1) K3 EDTA anticoagulant (ref 368860 – Becton Dickinson Vacutainer System, NJ, USA) with the addition of aprotinin (Bayer, Newbury, UK; final concentration 250 kallikrein inhibitor units/ml) and a DPPIV inhibitor (Ile-Pro-Ile, Sigma-Aldrich, Dorset, UK; final concentration 10 μg/ml); 2) K3 EDTA anticoagulant with the addition of aprotinin; 3) K3 EDTA anticoagulant with the addition of a DPPIV inhibitor; 4) K3 EDTA anticoagulant alone; 5) lithium heparin (ref 367883 – Becton Dickinson Vacutainer System, NJ, USA) with the addition of aprotinin and a DPPIV inhibitor; 6) lithium heparin with the addition of aprotinin; 7) lithium heparin with the addition of a DPPIV inhibitor and 8) lithium heparin alone. Following collection, tubes were placed on ice and centrifuged at 2300 x g for 10 minutes to obtain plasma, which was stored at -80°C for up to 9 months. Samples were analysed on ice within 4.25 hours of defrosting. Two further aliquots, collected in K3 EDTA tubes with aprotinin and DPPIV inhibitor, underwent 1 and 2 further freeze/thaw cycles, respectively.
Glucagon levels were measured by LC-MS/MS based on a method previously described (5). This consisted of a Waters Acquity ultra-high performance liquid chromatography UPLC system (Waters Corporation, MA, USA) coupled to an AB SCIEX 5500 QTRAP (Applied Biosystems/MDS SCIEX, Ontario, Canada) with an electrospray ion source (intra-assay CVs were ≤11.9% at 4, 7 and 13 pmol/l). Secondly, glucagon was assayed by a Homogenous Time Resolved Fluorescence (HTRF®) sandwich immunoassay (Cisbio, Codolet, France).

Thirdly, we used the Milliplex MAP Human Metabolic Hormone Panel which involves a sandwich immunoassay with fluorescence detection (Merck Millipore, Darmstadt, Germany). For both immunoassays, intra-assay CVs were <10% across the working range. Differences between tube types and methods were analyzed by two-way analysis of variance with correction for repeated measurements. P<0.05 was considered statistically significant.

Results:
The results of plasma glucagon levels taken from healthy fasting volunteers, processed under a variety of pre-analytical conditions, are shown in Table 1 and Figure 1. A systematic bias of Milliplex > LC-MS/MS > HTRF was noted and plasma glucagon levels were significantly different between methods (Milliplex vs. LC-MS/MS P<0.01; Milliplex vs. HTRF P<0.0001; LC-MS/MS vs. HTRF P<0.001).

Importantly, glucagon levels were not statistically different comparing the various tube types. The addition of aprotinin, DPPIV inhibitor or a combination of aprotinin and DPPIV inhibitor had no effect on plasma glucagon levels when compared to ‘non-stabilised’ samples or each other. Whether samples were taken in EDTA tubes or lithium heparin tubes made no difference to plasma glucagon levels. These findings were consistent for all three methods. The LC-MS/MS data suggested a trend towards lower levels following freeze-thaw cycles, but no significant difference was observed using the immunoassays.

Discussion:
This paper describes, for the first time, the pre-analytical stability of glucagon, as measured by LC/MS-MS and two different immunoassays. Consistent with Emmen (3), we find that aprotinin has no effect on endogenous glucagon stability. Consistent with Albrechtsen (4), we find that glucagon stability is unaffected by DPPIV inhibition and freeze-thaw when measured using immunoassay. Albrechtsen et al did not measure endogenous glucagon,
rather, samples were spiked in vitro, so the work described here measuring endogenous levels of glucagon provides robust confirmation of Albrechtsen’s findings. An explanation for the discrepant findings in historical studies of the effect of stabilisers on glucagon stability may be related to the varying specificity of the antibodies used in the glucagon immunoassays resulting in cross-reactivity with glucagon-like peptides, where DPPIV inhibition is more important.

The LC-MS/MS data suggested a trend towards lower levels following freeze-thaw cycles, as reported previously (5), but no significant difference was observed using the immunoassays. This finding for LC-MS/MS is consistent with previous data where after 4 freeze-thaw cycles, glucagon was approximately 40-50% compared to a non-freeze-thaw sample when measured by LC-MS/MS (5). It is possible that freeze-thaw increases glucagon aggregation, leading to glucagon precipitating out during LC-MS/MS extraction and thus resulting in artefactually lower levels. This requires further investigation.

A marked systematic bias is noted between the three assays. Bak et al previously compared 7 commercially available immunoassays for glucagon measurement and found striking differences in recovery between the assays (6). These differences may be explained by poor standardisation of reference materials, varying levels of antibody cross-reactivity, different matrix effects or the presence of factors in the plasma, causing non-specific interference. LC-MS/MS in theory circumvents some of these issues, particularly the potential for cross-reactivity with similar compounds or inactive degradation fragments leading to inaccurate quantitation.

As we were primarily interested in physiological values of glucagon in clinical use, this study examined fasting glucagon levels in normal human volunteers. Further study may include basal and stimulated glucagon levels (e.g. post-arginine) or hyperglucagonaemic patients with type 2 diabetes or glucagonoma.

In conclusion, glucagon is stable in both EDTA and lithium heparin tubes when centrifuged immediately and stored at -80°C. Importantly, the addition of aprotinin and DPPIV inhibitors is unnecessary. We have confirmed this by three separate analytical methods.
References


Table 1: Glucagon levels as measured by liquid chromatography tandem mass spectrometry (LC-MS/MS), Milliplex immunoassay (Milliplex) and Cisbio HTRF immunoassay (HTRF) in various tube types (EDTA = K3 EDTA anticoagulant; LH = lithium heparin tube; A = aprotinin added; D = DPPIV inhibitor added (mean plasma glucagon level (pmol/l) ± SEM, n=10).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Tube type</th>
<th>Additive</th>
<th>Freeze thaw</th>
<th>LC-MS/MS (pmol/l) ± SEM</th>
<th>Milliplex (pmol/l) ± SEM</th>
<th>HTRF (pmol/l) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EDTA</td>
<td>AD</td>
<td>no</td>
<td>5.8 (± 0.6)</td>
<td>6.9 (± 1.2)</td>
<td>2.8 (± 0.8)</td>
</tr>
<tr>
<td>2</td>
<td>EDTA</td>
<td>A</td>
<td>no</td>
<td>5.6 (± 1.6)</td>
<td>6.6 (± 1.4)</td>
<td>3.0 (± 1.1)</td>
</tr>
<tr>
<td>3</td>
<td>EDTA</td>
<td>D</td>
<td>no</td>
<td>6.1 (± 1.7)</td>
<td>7.8 (± 1.4)</td>
<td>3.8 (± 1.1)</td>
</tr>
<tr>
<td>4</td>
<td>EDTA</td>
<td>-</td>
<td>no</td>
<td>5.4 (± 1.3)</td>
<td>6.6 (± 1.4)</td>
<td>3.3 (± 1.0)</td>
</tr>
<tr>
<td>5</td>
<td>LH</td>
<td>AD</td>
<td>no</td>
<td>6.7 (± 1.9)</td>
<td>9.2 (± 2.1)</td>
<td>2.3 (± 0.6)</td>
</tr>
<tr>
<td>6</td>
<td>LH</td>
<td>A</td>
<td>no</td>
<td>5.3 (± 1.4)</td>
<td>8.1 (± 1.8)</td>
<td>1.7 (± 0.5)</td>
</tr>
<tr>
<td>7</td>
<td>LH</td>
<td>D</td>
<td>no</td>
<td>5.8 (± 1.8)</td>
<td>9.1 (± 2.2)</td>
<td>2.8 (± 0.9)</td>
</tr>
<tr>
<td>8</td>
<td>LH</td>
<td>-</td>
<td>no</td>
<td>5.8 (± 1.8)</td>
<td>8.7 (± 1.9)</td>
<td>2.4 (± 0.8)</td>
</tr>
<tr>
<td>9</td>
<td>EDTA</td>
<td>AD</td>
<td>1 cycle</td>
<td>3.9 (± 1.2)</td>
<td>7.1 (± 1.2)</td>
<td>4.0 (± 1.2)</td>
</tr>
<tr>
<td>10</td>
<td>EDTA</td>
<td>AD</td>
<td>2 cycles</td>
<td>3.3 (± 1.0)</td>
<td>7.3 (± 1.6)</td>
<td>3.9 (± 1.2)</td>
</tr>
</tbody>
</table>
Figure 1: Glucagon levels as measured by Milliplex immunoassay (Milliplex), Cisbio HTRF immunoassay (HTRF) and liquid chromatography tandem mass spectrometry (LC-MS/MS) in various tube types (EDTA = K3 EDTA anticoagulant; LH = lithium heparin tube; A = aprotinin added; D = DPPIV inhibitor added; FT1 = EDTA AD + one freeze thaw cycle; FT2 = EDTA AD + two freeze thaw cycles) (n=10).