Domestic Animal Endocrinology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.journals.elsevier.com/domestic-animal-endocrinology

Short Communication: Identification of equine corticotropin-like intermediate lobe peptide (CLIP) binding to an adrenocortipcotrophic hormone (ACTH) assay capture antibody

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ARTICLE INFO

Article history:
Received 2 December 2022
Received in revised form 7 January 2023
Accepted 10 January 2023
Available online xxx

Keywords:
ACTH
CLIP
Pituitary *pars-intermedia* dysfunction
Equine

ABSTRACT

A chemiluminescent immunoassay is commonly employed to measure adrenocorticotrophic hormone (ACTH) concentrations to assist pituitary *pars intermedia* dysfunction diagnosis. In a previous study, seasonally dependent assay cross-reactivity to endogenous equine corticotropin-like intermediate lobe peptide (CLIP, ACTH 18–39) was suspected. The present study aimed to demonstrate binding of endogenous equine CLIP to the capture antibody of the ACTH chemiluminescent immunoassay. Liquid chromatography – mass spectrometry (LCMS) methods were optimised to identify selected ions from synthetic human ACTH, α-melanocyte stimulating hormone (α-MSH, ACTH 1–17) and CLIP. Synthetic ACTH and CLIP bound to the capture antibody of the chemiluminescent ACTH assay, but α-MSH did not. Equine endogenous CLIP was detected by LCMS in pony plasma taken in the autumn and could be eluted from the capture antibody of the ACTH chemiluminescent immunoassay. Further research is required to enable quantification of CLIP. Equine CLIP may alter measured ACTH concentrations *in vivo*.

1. Introduction

Equine pituitary *pars intermedia* dysfunction (PPID) is a common neurodegenerative disease often diagnosed using adrenocorticotrophic hormone (ACTH) concentrations measured by a chemiluminescent sandwich immunoassay (Immulite/ Immulite 1000 ACTH, Siemens). The assay's monoclonal capture antibody is raised to human corticotrophin-like intermediate lobe peptide (CLIP), a-C-terminal fragment of ACTH (ACTH 18-39) and is immobilized onto a plastic bead contained within a reaction cup; the detection (polyclonal) antibody is raised to ACTH 1-24 (personal communication, Siemens UK). In a previous study, this ACTH assay showed suspected seasonally-dependent cross-reactivity when analyzing equine samples [1]. When the results of 2 ACTH assays were compared, samples collected in the spring showed a small bias between the 2 assays, whereas samples collected in the autumn showed a marked bias with wide limits of agreement [1]. The package insert for the ACTH chemiluminescent assay reports 13% to 15% cross-reactivity to CLIP, although the species and matrix are not specified. In experimental work, the addition of human synthetic CLIP to equine plasma revealed 9% to 21% cross-reactivity [1]. Equine endogenous CLIP is therefore a candidate *in vivo* cross-reactant, although it is unknown whether endogenous CLIP is present at detectable concentrations in apparently normal equids nor whether endogenous CLIP shows

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0739-7240/$ – see front matter © 2023 Published by Elsevier Inc.
https://doi.org/10.1016/j.domaniend.2023.106785

Table 1
Optimised LCMS conditions for detection of ACTH, α-MSH and CLIP.

Column Type	Aeris XB-C8
Column Dimensions	100 × 2.1 mm
Gradient	5- 21% B, 3.5 mins
Mobile phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile:Methanol:IPA (1:1:1)
Column oven temperature	85°C
Autosampler Temperature	15°C
Nebulising Gas flow	3L/min
DL temperature	250°C
Heat Block temperature	400°C
Drying gas flow	15 L/min
Total Run time	15 min

similar cross-reactivity to synthetic CLIP. The predicted sequence of equine ACTH [2] is identical to human ACTH [3], however partial sequence transcriptome analysis suggests sequence differences [4]. Endogenous human ACTH includes 3 post-translationally modified residues [3]. Endogenous equine ACTH (and CLIP) may therefore interact differently with assay antibodies compared with synthetic peptides based on human sequences.

The present study aimed to determine whether synthetic human CLIP and endogenous equine CLIP bind to the capture antibody of the chemiluminescent ACTH assay.

2. Materials and methods

Ethical approval was obtained from the Royal Veterinary College Ethics and Welfare Committee. The study was conducted under a UK Home Office license (PPL 70/8195) as the study used surplus plasma from 12, non-laminitis British native ponies enrolled in another study.

Liquid chromatography-mass spectrometry (LCMS) (LCMS8040, Shimazdu) was used for analysis. Methods were optimized for detection of synthetic human ACTH, α-MSH and CLIP (A0423, M4135 and A0673 Sigma- Aldrich) in distilled water at supra-physiological concentrations (60ng/ml). The most abundant detectable ions were identified for subsequent selective ion monitoring (SIM). Interactions between the synthetic peptide solutions and the assay capture antibody were determined by analysis of solutions of each peptide prior to and following incubation with the antibody bound beads and following washing and elution of the peptide from the beads. (Table 1)

The elution process was optimized as follows: 750µl of synthetic peptide solution (60ng/ml) was mixed with 250µl of Reagent 1 (a buffer) from the chemiluminescent ACTH assay reagent kit and added to 5 antibody bound beads in a syringe barrel. The syringe was mixed for 30 min at 250 rpm on an orbital shaker. The solution was removed under vacuum. The 5 beads were washed twice with 2ml water for 30 min each. Finally, 500µl of an elution solution (see below) was added and the syringe and mixed for a further 30 minutes at 250rpm on an orbital shaker. The resulting solution was transferred to a tube by a vacuum manifold, dried under nitrogen and reconstituted in 100µl of distilled water prior to analysis by LCMS. For initial trials 0.1M glycine: HCl was used for elution however this damaged the chromatography column and subsequent analysis used a solution of 1% formic acid in water: acetonitrile (1:1). Conditions for elution are summarized in Table 2.

Table 2
Mass to charge ratio (m/z), detection time for each ion (dwell time) and optimized quadrupole energy (Q3 bias) for the most abundant detectable ions from synthetic peptides based on human sequences used for subsequent analysis.

Peptide	m/z	Dwell time (msec)	Q3 bias (V)
ACTH	909.1	100	-26
	757.5	100	-38
	649.7	100	-32
CLIP	822.6	10	
	1233.3	10	
α-MSH	555.7	10	
	833	10	

To investigate the binding of endogenous CLIP, EDTA plasma from samples taken from 12 ponies in the autumn was used to create 2 pools with high and low measured ACTH concentrations using the chemiluminescent assay. Pooled plasma samples were incubated with the antibody-bound beads. Elution and LCMS detection of equine endogenous ACTH and CLIP was compared with results obtained from charcoal stripped equine plasma [5] spiked with synthetic ACTH and CLIP.

3. Results

The most readily detectable ions from initial analysis of synthetic ACTH, α-MSH and CLIP are shown in Table 2. The solutions of synthetic α-MSH, ACTH and CLIP in distilled water overloaded the binding capacity of the antibody-bound beads. After incubation with the bead, most of the peptide remained in solution but there was a reduction in the peaks for CLIP and ACTH but not α-MSH, indicating binding of ACTH and CLIP but not α-MSH.

The high and low pools of equine plasma yielded ACTH results of 232pg/ml and 25.7pg/ml respectively when analyzed using the chemiluminescent immunoassay according to the manufacturer's instructions. LCMS SIM analysis identified endogenous ACTH and CLIP in eluted solutions created from both pools, with higher concentrations of both analyzes in the high pool (Figs. 1 and 2). The twin-peaked appearance of the chromatograms may indicate different conformational forms of the peptides. Unfortunately, these analyzes could not be quantified accurately because the mass range of the LCMS analyzer is 1-2000Da, such that the molecular ions of ACTH and CLIP were too large to be detected, limiting sensitivity.

4. Discussion

To cause the previously reported cross-reactivity [1], synthetic CLIP must bind both the assay capture and detection antibodies of the ACTH chemiluminescent immunoassay. The present data confirm that synthetic CLIP binds to the capture antibody. In addition, one or more CLIP-like endogenous peptides are present in plasma collected from ponies in the autumn and bind to the capture an-

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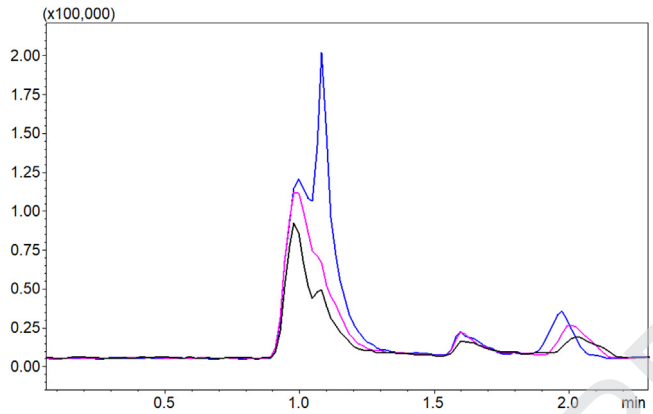

Fig. 1. Chromatogram of selected equine ACTH ions following LCMS analysis of pony plasma spiked with: synthetic ACTH (black line) and low (magenta line) and high (blue line) pooled pony plasma samples following elution from the chemiluminescent (CL) assay capture antibody. The y axis indicates signal intensity (counts), the x axis time (min). The low and high pools yielded apparent ACTH concentrations of 25.2pg/ml and 232pg/ml respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

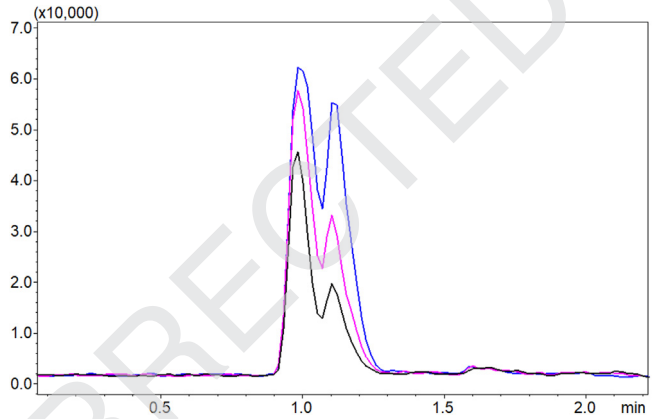

Fig. 2. Chromatogram of selected equine CLIP ions following LCMS analysis of pony plasma spiked with: synthetic CLIP (black line) and low (magenta line) and high (dark blue line) pooled pony plasma following elution from the chemiluminescent (CL) assay capture antibody. The y axis indicates signal intensity (counts), the x axis time (min). The low and high pools yielded apparent ACTH concentrations of 25.2pg/ml and 232pg/ml respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tibody. Given that CLIP is the antibody target (Siemens UK, personal communication) this binding was anticipated but has not been demonstrated previously for endogenous equine CLIP in equine plasma. Whilst the exact mechanism of cross-reactivity was not determined, it is speculated that this results from the overlap between the antibody targets; the 6 N-terminal residues of CLIP (ACTH 18–24) may be an epitope for the polyclonal detection antibody raised to ACTH 1 to 24 (Siemens, personal communication). It is presumed therefore that the epitope for the capture antibody lies in the 24 to 39 residue region.

Previous studies have also indicated the presence of an endogenous cross-reactant in pony plasma in basal plasma samples collected in the autumn [1] and following *pars intermedia* stimulation with exogenous thyrotropin-releasing hormone (TRH) [6] that is present at sufficiently high concentrations to affect the results of diagnostic tests. The present study supports the suggestion that endogenous CLIP is that cross-reactant and may alter measured ACTH concentrations *in vivo,* however binding by endogenous CLIP to the detection antibody has not been conclusively demonstrated.

The potential for equine CLIP to increase measured ACTH concentrations raises several questions including the potential for other CLIP-like peptides to exert similar effects. A seasonal pattern of measured equine ACTH concentrations occurs as a physiological process and, in an exaggerated manner, in equids with PPID [7]. The extent to which the seasonal autumnal increase and the pathological increases measured in PPID are caused by CLIP rather than ACTH requires investigation. It is also unclear whether CLIP has direct metabolic effects in the horse. In rats, CLIP exerts orexigenic effects during fasting [8] andthe CLIP metabolite β-Cell tropin is a potent insulin secretagogue [9] with a more potent secretogenic effect on fatty rather than lean rat pancreas [10]. Although speculative, the clinical implications would be important if CLIP or β-Cell tropin were to exert similar effects in the horse.

5. Conclusions

Circulating equine endogenous CLIP can be detected by LCMS in plasma from healthy ponies sampled in the autumn and binds to the capture antibody of a commonly

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used chemiluminescent immunoassay. This finding provides further support for the hypothesis that endogenous CLIP affects measured ACTH concentrations *in vivo* which may have implications for our understanding of the diagnosis and pathophysiology of PPID.

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Uncited References

[11]

Acknowledgments

The study received funding from: Petplan Charitable Trust and MARS Petcare UK. E.J. Knowles' PhD was also supported by The Mellon Trust. There are no conflicts of interest for any of the authors.

The authors thank Michelle Moreton Clack for laboratory assistance and visiting students for assistance with sample collection.

Q7 Q8

CRediT authorship contribution statement

E.J. Knowles: Conceptualization, Funding acquisition, Writing – original draft, Investigation. **C. Hyde:** Methodology, Investigation, Data curation, Visualization, Writing – review & editing. **P.A. Harris:** Supervision, Writing – review & editing, Funding acquisition. **J. Elliott:** Supervision, Writing – review & editing, Funding acquisition. **N.J. Menzies-Gow:** Funding acquisition, Project administration, Supervision, Writing – review & editing.

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