Towards an untargeted mass spectrometric approach for improved screening in equine anti-doping

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Abstract

The emergence of novel doping agents is a continuous issue for analysts who aim to maintain the integrity of horseracing together with the wellbeing and safety of the animals and riders involved. Untargeted mass spectrometric analysis presents a potential improvement for anti-doping as it enables the detection of compounds being indirectly affected by an administered drug. In this study, liquid chromatography-high resolution mass spectrometry was used to investigate a 12-horse administration study of the synthetic opioid, butorphanol. A mass spectrometric workflow capable of detecting metabolic differences for an extended period of time was successfully developed. This proof-of-concept study demonstrates the potential of untargeted workflows to provide a list of biomarkers of exposure and effect that are indicative of drug administration which may be implemented into routine testing for improved doping control.

Keywords: Drug profiling; mass spectrometry; butorphanol, doping control

1. Introduction

Biomarkers are compounds indicating a change in the physiological state of an individual, including exposure to, and effect of a doping agent[1-3]. Indirect detection aims to identify biomarkers of effect[4]. Common metabolomic or untargeted detection workflows follow a chronological order of sample collection and preparation, analytical analysis/data collection, and statistical analysis[5]. MS is the preferred technique for analysis of small molecules or compounds at low levels[5]. Statistical analysis generally involves the use of deconvolution software to process the data and transform it into a suitable format for the analyst to process[5]. The use of indirect detection as a screening tool has been proposed as a preliminary step to identify abnormal samples requiring more conventional targeted analysis[4]. Untargeted detection methods are a developing field in the racing industry aiming to identify biomarkers that experience significant change within a system in response to doping[6]. Within the racing

industry, the detection of any substance which may be performance altering is crucial[7,8], therefore, there is an effort to establish new, more appropriate detection methods.

Butorphanol is a synthetic opioid which has narcotic analgesic effects in humans as well as producing minor stimulating effects in horses [9-15]. Butorphanol presents with many side effects including nausea, sedation, raised pulmonary vascular pressure and central nervous system (CNS) excitation which leads to increased locomotor activity[10,16,17]; which creates an unfair advantage in horse racing. It is crucial that these doping agents are controlled for both horse welfare and the integrity of the sport [9,18,19]. To date, *in vivo* equine studies of synthetic opioids, such as butorphanol, have not investigated large-scale metabolic changes.

The aim of this study was to compare conventional target-based data analysis methods with an untargeted mass spectrometric approach using liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis in conjunction with data mining and processing software tools. The potential of an untargeted mass spectrometric approach for non-targeted screening was investigated through monitoring the effects of butorphanol administration.

2. Methods

2.1 Chemicals and Reagents

Acetonitrile (ACN), ammonium acetate (NH_4OAc), ammonium hydroxide (NH_4OH), ethyl acetate (EtOAc), hydrochloric acid (HCl), methanol (MeOH), trichloroacetic acid (TCA) and petroleum spirits (hexanes) of LC-MS grade were purchased from Merck (Darmstadt, Germany). Ultrapure grade water was (18.2 MΩ.cm) obtained from a ThermoFisher Scientific Barnstead Smart2Pure system (ThermoScientific; Langenselbold, Hungary). Two sources of butorphanol tartrate were purchased, from different companies, to complete the study with one for calibration (Chiron AS Stiklestadvn; Trondheim, Norway) and the other for quality control (QC) (National Measurement Institute; North Ryde, NSW, Australia).

2.2 Animal administration study

The study involved 12 geldings, either Thoroughbred or Standardbred, with body weight 500 ± 41.8 kg. Butorphanol was intravenously (IV) administered in a single dose of 20 mg/horse. Blood samples were taken via a jugular catheter in the opposite vein to the butorphanol administration using Lithium Heparin Vacutainer collection tubes (BD, Oakville, ON,

Canada). Samples were taken at 0 (pre-administration), 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120 min, and 2.5, 3, 3.5, 4, 5, 6, 7, 8, 12, 24, 36, 48, 72, and 96 hours post-administration. Blood samples were immediately centrifuged at 1500 x *g* for 10 minutes to obtain plasma and stored at -20 °C until analysis. Animal ethics approval (08/031) was obtained for this study from the Charles Sturt University Ethics Committee.

2.3 Sample preparation

Plasma samples (2 mL) were fortified with d_6-butorphanol (500 ng/mL, 50 µL) prior to the addition of trichloroacetic acid (10% v/v, 200 µL). The samples were diluted with approximately 6 mL of water and the pH was adjusted, using dilute HCl (3% v/v and 10% v/v) and dilute NH_4OH (3% v/v), to between 3 and 3.5. The tubes were then centrifuged at 1500 x *g* for 10 minutes to separate the supernatant from the protein precipitate.

Solid phase extraction, using a UCT positive pressure manifold and UCT XTRACKT Gravity Flow DAU cartridges (Part code: XRDAH203. UCT; Bristol, PA, USA), was performed. Cartridges were conditioned with 3 mL of MeOH, followed by 3 mL water. Samples were loaded using the supernatant prepared above, washed with 3 mL of acetic acid and dried. To simulate the routine method used by the Australian Racing Forensic Laboratory (ARFL) the acid-neutral fraction was eluted with EtOAc:hexane (3:2) before the cartridges were then washed with 3 mL of MeOH and dried. The polar basic fraction used in this study was eluted with a solution of 0.5% MeOH/3% ammonium solution in EtOAc before the addition of a drop of methanolic HCl from a Pasteur pipette and dried under a stream of nitrogen at 60 °C. The dried fraction was then reconstituted in one drop of MeOH from a Pasteur pipette and 100 µL of 10 mM ammonium acetate buffer solution (pH 3.9). The samples were transferred to the corresponding labelled liquid chromatography (LC) vials and stored at 4 °C until analysis.

2.4 LC-HRMS analysis

Liquid Chromatography-High Resolution Accurate Mass Spectrometry (LC-HRMS) analysis was undertaken using an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to a 6545 quadrupole-time-of-flight (QTOF) mass spectrometer. The LC system was equipped with a Phenomenex (Torrance CA, USA) Gemini C18 column (50 mm x 2 mm, 5 µm). Mobile phase A consisted of aqueous NH_4OAc (10 mM, pH 9.0), and mobile phase B 0.1% acetic acid in ACN. The gradient used was: 0-2 min A-B (99:1 v/v), 2-8.5 min

A-B (99:1 v/v) to A-B (20:80 v/v), 8.5-11.5 min A-B (99:1 v/v) with a 2.7 min re-equilibration time. The flow rate was constant at 0.5 mL/min and injection volume was 1 µL.

Mass spectrometry (MS) data was collected in all ions positive ionisation mode in the range of 35 to 1000 *m/z* with a scan rate of 10 spectra/sec. Collision energies of 10, 20 and 40 eV were selected with a resolution of 17,500 (FWHM). The gas temperature and flow rate were 320 °C and 8 L/min, respectively. The nebuliser was set to 50 psi. The sheath gas temperature and flow were 350 °C and 11 L/min, respectively. The VCap was set to 3500 V and the nozzle voltage was 1000 V. The fragmentor, skimmer and octopoleRFPeak were 100, 65 and 750, respectively. Agilent MassHunter Quantitative software (B 08.00) was used to quantify samples.

2.5 Validation of butorphanol quantification

The selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effects and stability for the quantification of butorphanol in equine plasma were assessed according to the procedures provided in the Supporting Information. Validation criteria followed for this method was specific to the field of toxicology and has been detailed by *Peters et al*[20].

2.6 Profinder

Agilent Profinder software (V10.0 SP1, Build 10.0.10142.1) was used to analyse the mass spectral data and identify entities (e.g. metabolites, adducts, artefacts) within the data. A recursive molecular feature extraction (rMFE) was performed using the parameters provided in the Supporting Information (Table S2). The rMFE algorithm works by plotting the data in a 3D space in terms of retention time, *m/z*, and abundance. The rMFE algorithm was set to re-evaluate the data after obtaining a target list thus reducing the number of false entities. A score was generated which was based on the isotopic abundance of the feature, the retention time variance, charge state and the overall quality of the peak in relation to surrounding noise. Interpretations of the data were made based on status (Table 1); this was defined as ‘Pre’ for samples collected prior to butorphanol administration, and ‘Post’ for samples after butorphanol was administered. Detailed explanation of Profinder and the entity identification process are outlined in the Supporting Information.

2.7 Mass Profiler Professional

Agilent Mass Profiler Professional (MPP) (V 15.0) was used for differential analysis of multiple sample sets through implementation of statistical tools ultimately aiding the identification of compounds of interest[21]. The pre-processing of the data in MPP was a 3-step process (Figure 1). Detailed explanation of the MPP analysis steps is provided in the Supporting Information. The MPP workflow was a sequential workflow with each step furthering the information gathered from the last.

3. Results and Discussion

3.1 Quantification of butorphanol

The method was validated with respect to the quantification of butorphanol. The calibration was linear up to 100 ng/mL (R^2=0.9938) with an estimated LLOQ and LOD of 0.30 ng/mL and 0.1 ng/mL, respectively. Residual analysis demonstrated no observed bias in the calibration. Quantitative accuracy and precision were deemed to be acceptable based on relative error and relative standard deviation (%RSD) being ± 20% RE and less than 20%RSD at LLOQ and ±15% RE and 15%RSD for other assessments, respectively. Recovery and matrix effects were 108% and 131%, respectively. Butorphanol demonstrated stability at 4 °C and -20 °C over a 4-week period, consistent with a previous study by *Paine et al*[22]. LC retention time of butorphanol was 5.0 ± 0.1 min and QTOF-MS error within 1.8 ppm. Detailed results for the method validation are presented in the Supporting Information.

Butorphanol was quantified for samples from 3 horses out of the 12 involved in the study. The quantified levels presented in Figure 2 for all three horses were comparable to a previous study by *Knych et al*[18]. Maximum levels (n=3) were observed at 5 min post-administration followed by a rapid elimination to LLOQ at 3-hours to 7-hours and LOD at 5-hours to 48-hours. This limited timeframe presents a challenge for race day screening in a routine testing laboratory; therefore, complementary data analysis methods were investigated for improved retrospective detection of butorphanol administration.

3.2 Profinder

Profinder extracted 864 entities from the LC-QTOF-MS acquired data. Quality control checks were performed to ensure sufficient recursive molecular feature extraction (rMFE) was

achieved as shown in Supporting Information under the titles of 'Profinder: Pre-processing of data for quality assurance' (Figure S5 and S6) and 'PCA with QC Samples' (Figure S7).

3.3 Mass Profiler Professional

3.3.1 Statistical analysis

The volcano plot reduced the number of entities from 864 to 116, an 86.6% reduction, by applying filters of 2.0 and 0.05 for the fold-change and p-value, respectively. While this may appear to be a large decrease in the amount of data, it remains considerable for effective review by an analyst. The 25 up- and 91 down-regulated entities can be visualised in the volcano plot (Figure 3).

Five clusters (Figure 4) can be reviewed by PCA in a 3-D space which show sufficient separation for the continuation of the statistical workflow. A table detailing the correlation of the sample, cluster and interpretation is included in the Supporting Information (Table S5).

This was compared to the quantification values of butorphanol in plasma (Figure 2). Samples collected pre-administration clustered closely together which provided additional quality assurance that the statistical analysis was suitable. Samples at 0 and 15 minutes would be expected to be separated in the spatial arrangement due to the large difference in their butorphanol concentrations. Butorphanol and its metabolites were treated as part of the endogenous entities to test the validity of the data treatment for the detection of exogenous compounds.

Samples within a cluster have similar entity profiles to one another. The cluster furthest from the pre-administration sample cluster (Cluster 1) had an entity profile most different to the pre-administration sample. The *k*-means clustering result is shown in a heat map (Figure 5) which illustrates the different clusters and their relation to one another with up- and down-regulation of the entities thus furthering the information in Figure 4 by including the entities and their up- or down-regulation profile over time. The majority of entities fall into Cluster 1 visualised at the top of Figure 5. This includes samples collected prior to administration and samples collected up to 0.08 hours (5 min) after butorphanol was administered. Butorphanol has a large distribution value of 1.4 L/kg, however, metabolic changes due to the drug would likely take longer to be noticed[18,23]. Therefore, having these sampling times cluster together was

reasonable as they would not be expected to be significantly different in terms of their entity profile.

The majority of Cluster 2 involved samples taken 12-hours post-administration. A major difference between this and Cluster 1 was the larger number of down-regulated entities. This may indicate negative-feedback mechanisms attempting to achieve equilibrium, thus providing the opportunity for at least a 12-hour detection period following butorphanol administration.

Clusters 3 and 5 were part of the samples collected 3-hours after administration that shows considerable up-regulation at the same time that butorphanol has decreased to levels approximating 1 ng/mL or lower. Cluster 4 was a broad collection of samples from 15 min, 3-hours and 12-hours post-administration highlighting inter-individual metabolism but which all show significant down-regulation.

If the detection of butorphanol was performed by conventional data analysis methods then the presence would only be detectable for 2.5-4-hours with a LOD of 0.1 ng/mL. The *k*-means clustering highlights the benefits of using mass spectrometric data analysis approach as the administration of a doping agent could potentially be detected for a longer period of time than conventional target-based data analysis methods. When applied to routine batch analysis, this would reduce the amount of time required for data checking as this workflow is looking to detect outliers and abnormalities within a sample.

Clusters 1 and 5 were the most distinct from one another, as seen in the heatmap (Figure 5). Therefore, a 2-D scores plot (Figure S8) was created to visualise the separation which was based on the spatial arrangement of entities. The 2-D scores plot was created in relation to Figure 4. Thus, components 1 and 3 were used to create the scores plot where the most separation between the clusters could be visualised. The 2-D PCA loadings plot (Figure S9) visually displays the entities affecting the separation of the samples in Clusters 1 and 5. The loadings plot uses the same components as Figure S8, therefore, a comparison between the cluster separation and entity separation can be made through visual assessment of the two figures. No overlap can be seen in the 2-D scores plot emphasising how distinct the two clusters were from each other.

The list of up- and down-regulated entities was reviewed for those with the largest fold change values; 2.21 to 34342.54 for up-regulated entities and 2.03 to 64.34 for down- regulated entities. A small number of the up-regulated entities were suspected to be metabolites of the administered butorphanol. To this end, it was prudent to consider the presence of hydroxybutorphanol, norbutorphanol and glucuronide metabolites of butorphanol and hydroxybutorphanol[24]. To confirm the statistical analysis was adequate for extracting entities relating to the drug administration, putative identification of these entities was attempted; the information is presented in the Supporting Information under the titles of 'Equine hydroxybutorphanol' (Figure S10) and 'RR profile of the suspected butorphanol metabolites' (Figure S11-S15 and Table S6).

The same mass spectrometric workflow was completed without the suspected butorphanol metabolites and compared to the original analysis. Figures and further information on this analysis were provided in the Supporting Information under the title 'Reprocessed data without suspected butorphanol metabolites' (Figure S16-S20). There were 5 of the same up-regulated entities and 23 of the same down-regulated entities. The list of down-regulated entities in both analyses was larger in both analyses, thus there was a larger amount of matching down-regulated entities. This comparison provided evidence to support the separation between pre- and post-administration samples as being due to multiple entities and was not solely reliant on the suspected butorphanol metabolites, even though they displayed the largest fold change. Down-regulated entities are of interest as they had the opposite effect to what butorphanol was having on the system. Preliminary efforts to identify the entities used the entity nominal mass to search the Human Metabolome Database (HMDB) and ChemSpider for possible compounds. Compounds were filtered based on their ionisation ability based on this method using positive ionisation mode and a polar basic extraction. A detailed description is provided in the Supporting Information.

3.4 Future work

While the aim for untargeted detection of the doping agent, butorphanol, was achieved to identify butorphanol metabolites, more work needs to be completed on verifying these suspected metabolites. Currently, this work would be useful as a screening tool, however, it would still require confirmatory analysis to definitively state a finding of butorphanol administration. The use of Molecular Structure Correlator is proposed as the continuation of

the workflow as this enables visual comparison of fragmentation by *in silico* dissociation mechanisms. Collection of MS/MS data would aid the comparison of entities to library searching which would be the final identification step of the workflow. Once this entity identification workflow has been established further expansion to identify and validate other potential biomarkers from the down-regulated entity list could be made possible. The long-term goal for this work would be the creation of a class prediction model, based on these up- and down-regulated biomarkers to complement current routine detection efforts.

4. Conclusion

An untargeted mass spectrometric data analysis approach provides a complementary approach to current methods of identifying doping agents within the racing industry. The analysis of a butorphanol administration study involving 12-horses was completed using LC-QTOF-MS and statistical analysis workflow. Conventional detection methods could detect the presence of butorphanol for 2.5 to 4-hours post-administration. This untargeted workflow has the potential to identify butorphanol doping within samples through entities which are not related to the doping agent itself. Changes within the horse's system were observed for up to 12-hours post-administration, which extended the detection period by at least 8-hours compared to conventional targeted screening. For equine anti-doping, the retrospectivity of this workflow would ideally be implemented as a routine approach to detect outliers in batch analyses which would then require further investigation.

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Conflict of interest: Profinder and Mass Profiler Professional software packages were provided to the ARFL by Agilent Technologies in a collaborative arrangement.

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Table 1: Data file labels for the sampling times used for Profinder analysis*

Sampling Time	Sample label	Interpretation
0 min	P0	Pre
5 min	P1	Post
15 min	P3	Post
3 h	P15	Post
12 h	P22	Post

*all labels also include the horse and number prior to the sampling time label. For example, horse 1 sampled at 0 min is written as H1P0.

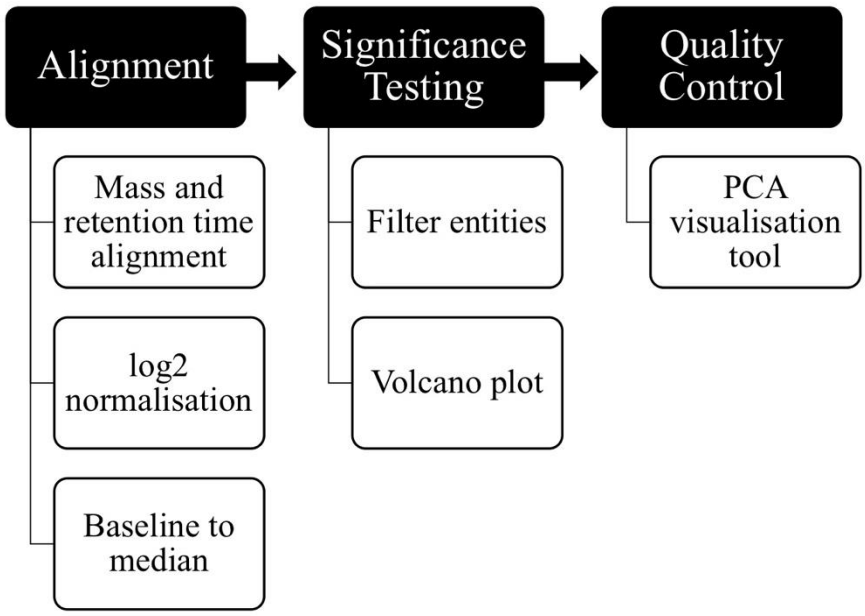

Figure 1: The 3-step data pre-processing workflow for Mass Profiler Professional used in this study

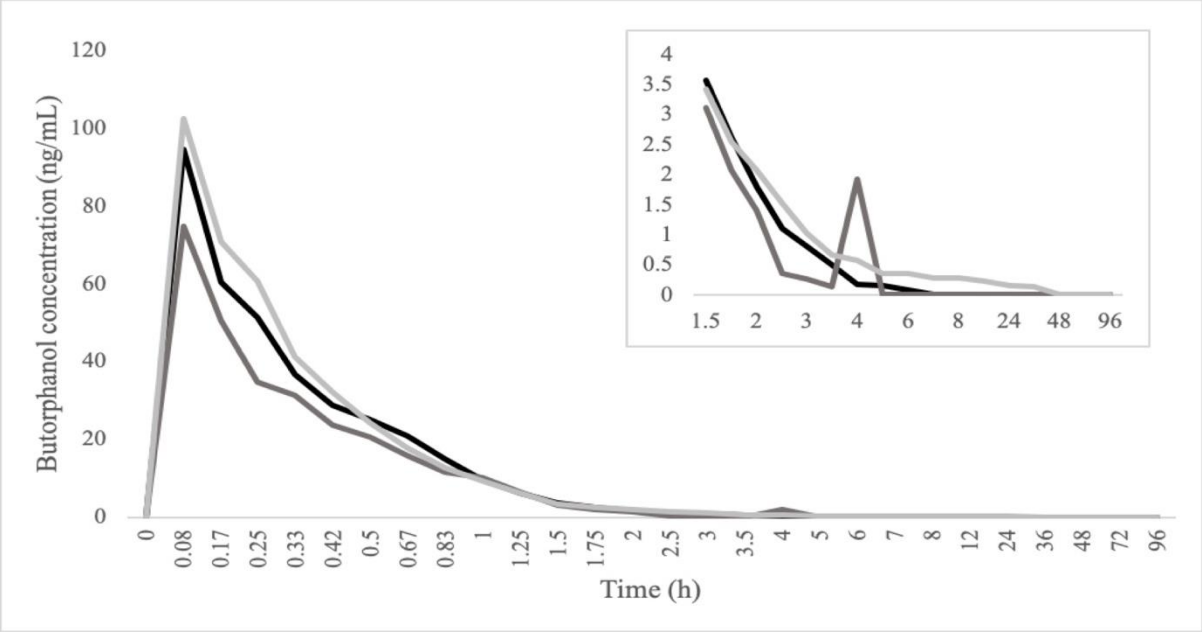

Figure 2: Plasma elimination profiles for butorphanol in 3 horses

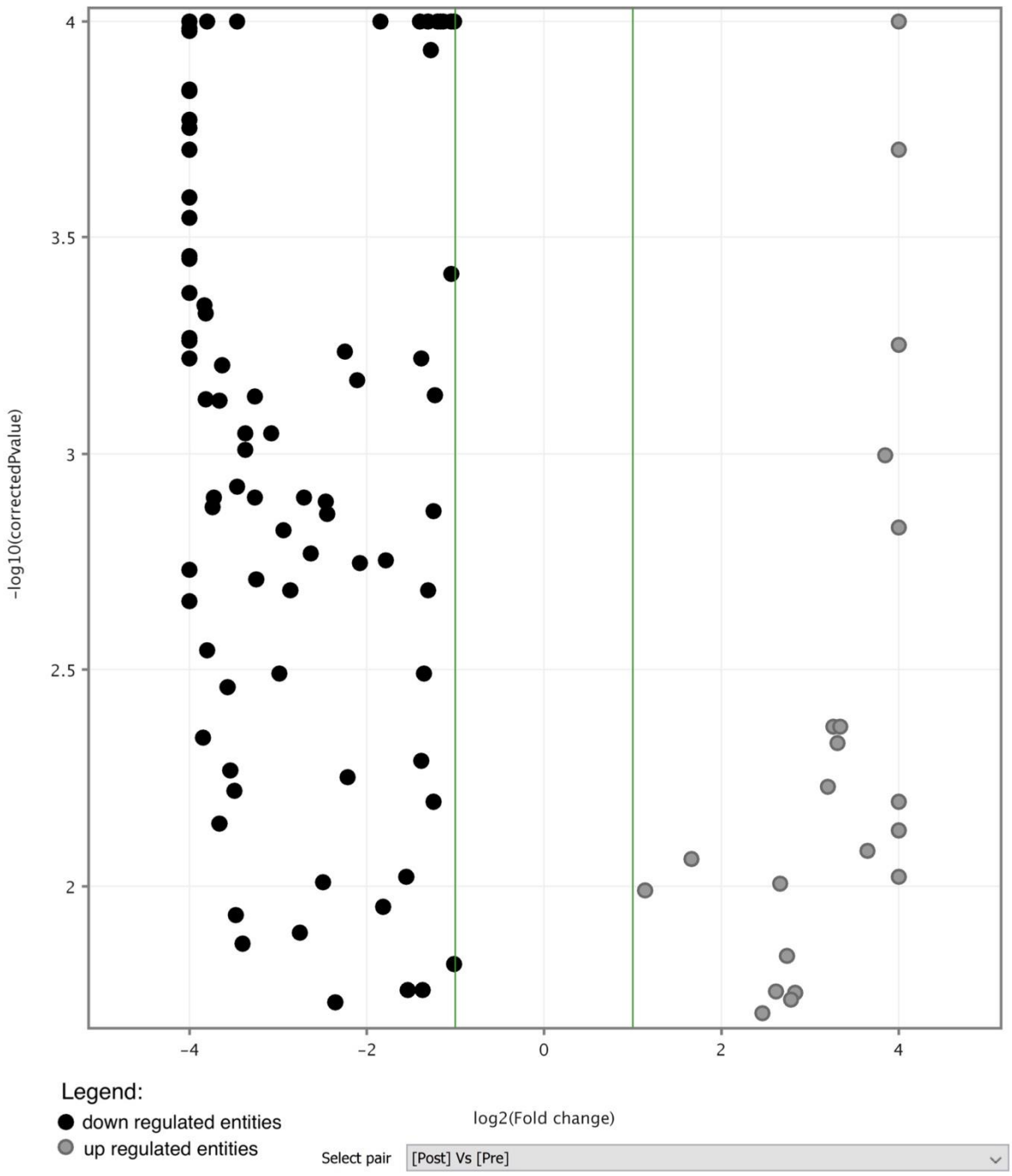

Figure 3: Volcano plot of reduced entities down-regulated (left) and up-regulated (right)

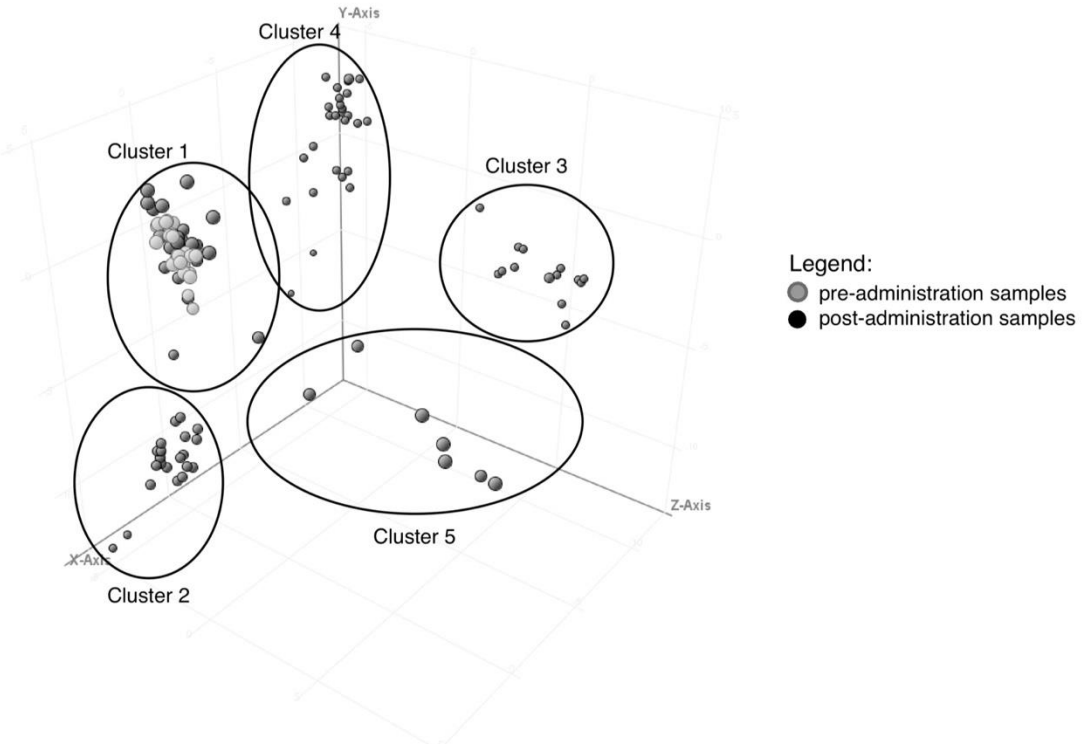

Figure 4: Principal Component Analysis visualisation with annotated clusters for 12 horses administered with butorphanol. Components of x=1, y=2, and z=3. A detailed version of each cluster and the related timepoints can be found in Table S5 in the Supplementary

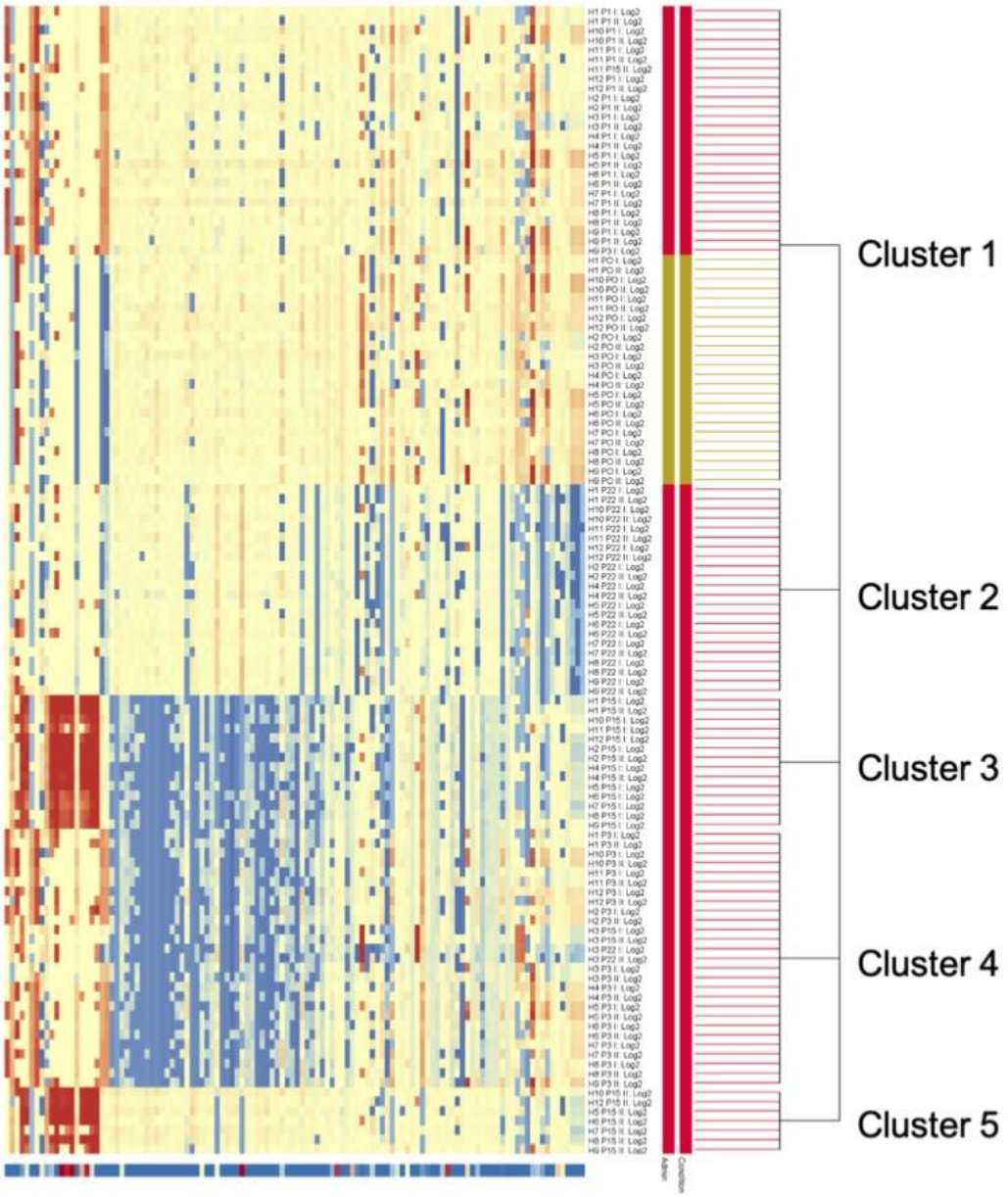

Figure 5: Heat map of up-(red) and down-regulated (blue) entities clustered following k-means clustering