RESEARCH ABSTRACT

ANIMAL WELFARE

Title: A study to develop and validate assays to measure and compare four circulating neuropeptides as objective pain biomarkers in piglets – **NPB #13-198**

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Scientific Abstract:

The primary objective of this project was to analytically and clinically validate a novel liquid chromatography-mass spectrometry (LC-MS) method for determining parent and metabolite substance P (SP), beta-endorphin, calcitonin-gene related peptide (CGRP) and neuropeptide Y (NPY) concentrations in swine plasma. Current enzyme tests have not been appropriately validated in pigs and yield inconsistent and non-reproducible results in pain-free individuals. This has prevented establishment of a reference range for circulating neuropeptides in pain-free subjects thus hampering efforts to use these measures as objective pain assessment tools. Furthermore, ELISA and radioimmunoassay (RIA) tests also lack specificity, with significant cross-reactivity reported with neuropeptides and their metabolites.

This research project resulted in development of a sensitive and specific analytical methodology using LC-MS to measure Substance P, beta-endorphin, and several Substance P metabolites in spiked piglet plasma at the low parts-per-trillion level. The developed methodology was then applied to un-spiked piglet plasma samples from several sources. The majority of the tested samples had little if any Substance P or its metabolites. A few samples gave detectable amounts of Substance P in concentrations below 5 pg/mL (parts-per-trillion). In contrast, many of the samples demonstrated immunoreactivity in ELISA tests in the 100 pg/mL concentration range. The stark difference between the results measured by the highly specific and sensitive LC-MS and methodologies based on immunoreactivity cast serious doubt on the ability of the ELISA tests to specifically and accurately measure Substance P and other neuropeptides. It is likely that the ELISA tests are detecting interference from other biological molecules in the piglet samples. Although the LC-MS method developed in the project meets the criteria for analytical reproducibility when "spikes" of a known concentration of piglet Substance P are analyzed, the fact that no significant amounts of Substance P were detected (despite repeated efforts) in piglet plasma suggests that this assay will not be useful for assessment of pain in piglets.

The second objective was to compare the results of commercial ELISA kits and Radioimmunoassay (RIA) kits for determining Substance P, Neuropeptide Y, CGRP and beta-endorphin concentrations in porcine plasma with the LC-MS results to determine the utility of using these assays in swine pain assessment. Multiple commercially available ELISA test kits were analyzed. The selected porcine Substance P ELISA kit gave the best results with the sample concentration being ~6.6ug/mL; however, this is likely not low enough for use in pigs. Two pig calcitonin gene related peptide ELISA kits were tested. One kit had severe problems with apparent matrix interference and we concluded additional research is necessary to refine the extraction technique. The second kit could possibly be used with a low (1:2) dilution of the pig plasma; however, further method validation is needed. Two porcine neuropeptide Y (NPY) ELISA kits were evaluated and both were determined to be acceptable. It was concluded that the selected porcine beta-endorphin ELISA kit needed further validation before use on piglet serum.

The third objective was to determine the *in-vitro* stability of Substance P, Neuropeptide Y, CGRP and beta-endorphin after collection to determine the optimum handling and storage conditions. The fourth objective was to determine Substance P, Neuropeptide Y, CGRP and beta-endorphin concentrations in samples collected from piglets before and after castration for acute and chronic pain. Unfortunately, because the "gold standard" LC-MS method to determine Substance P, Neuropeptide Y, Calcitonin gene-related peptide (CGRP) and Beta-endorphin concentrations in porcine plasma was not able to detect these substances in un-spiked pig plasma, objectives 3 and 4 were not met.