RESEARCH REPORT
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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Industry Summary:

Without a current gold standard method to objectively recognize and quantify pain perception in animals, addressing questions about animal welfare is extremely challenging. Furthermore, the lack of scientific evidence makes it very difficult for stakeholders to respond to societal concerns with science-based recommendations for reducing pain caused during castration, tail docking, teeth clipping, animal identification and other procedures. The main goal of this project was to develop and validate a new "gold standard" test for measuring circulating physiological biomarkers of pain in piglets and to compare the results with existing commercial immunoassays.

The primary objective of this proposal 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. Long known major obstacles to investigating the role of these neuropeptides as a pain biomarker are (1) the inaccuracy of existing immunoassays for measuring plasma concentrations and (2) the correlation of circulating neuropeptide biomarkers with acute and chronic pain following a routine husbandry procedure such as castration. Current enzyme-linked immunosorbent assays (ELISA's) 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. Therefore, many of the physiological effects of neuropeptide metabolites have been mistakenly attributed to the parent peptide hindering our understanding of the specific role of the peptides in pain perception.

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.

Key Words: Pigs, Pain, Measurement, Biomarkers, Neuropeptides

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.

Introduction:

A "gold standard" method to objectively detect and quantify pain in animals is needed to address animal welfare concerns with common techniques such as castration, tail docking, and teeth clipping and other procedures utilized in pork production. The main goal of this project was to develop and validate a better test for measuring circulating physiological biomarkers of pain in piglets. This would allow producers to make better-informed decisions on management of pain in pigs and in doing so satisfy consumer and public concerns.

Objectives:

1. To develop and validate a "gold standard" Liquid Chromatography-Mass-Spectrometry (LC-MS) method to determine Substance P, Neuropeptide Y, Calcitonin gene-related peptide (CGRP) and beta-endorphin concentrations in piglet plasma.
2. To compare the results of commercial enzyme-linked immunosorbent assay (ELISA) kits and Radioimmunoassay (RIA) kits for determining Substance P, Neuropeptide Y, CGRP and Beta-endorphin concentrations in porcine plasma with the "gold standard" LC-MS results to determine the utility of using these assays in swine pain experiments.
3. 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.
4. 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.

Materials and Methods:

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is a highly sensitive and specific analytical technique that is more accurate, robust, and reproducible than current enzyme immunoassay techniques. Our laboratory developed a sensitive, specific, accurate, and robust quantitative LC/MS/MS method for the analysis of Substance P and its metabolites in plasma. This method was extended to include beta-endorphin, neuropeptide Y, calcitonin gene-related peptide and their metabolites. A structurally related peptide, substance P (Tyr8), was used as the

internal standard for all analytes. The analytes and internal standards were isolated from plasma using protein precipitation and/or solid phase extraction (SPE) cartridges, such as Waters HLB or similar. Extracts were subjected to digestion using trypsin or other protease enzymes to generate smaller peptide fragments. Extracts were evaporated to dryness and re-constituted in starting mobile phase. LC/MS/MS was carried out using an Agilent 1260 Infinity HPLC system coupled with an AB Sciex QTrap 4500 triple quadrupole/ion trap mass spectrometer. Chromatographic separation of all analytes and internal standards was achieved using a Waters XBridge Shield RP18 with elution using an acetonitrile/water gradient with acetic acid as a mobile phase modifier. The LC/MS/MS method for the quantitation of substance P, beta-endorphin, neuropeptide Y, and calcitonin gene-related peptide in porcine plasma was validated according to the FDA guidance for regulated bioanalysis.

Development of an analytical methodology for neuropeptides utilizing LC-MS required overcoming obstacles not usually encountered in LC-MS analysis of small molecules or antibiotics. Among these are peptide sorption to surfaces and ion suppression by matrix components in the MS instrument. The first obstacle was overcome by using polypropylene containers for all analytical operations. The second obstacle, that of ion suppression, was more difficult and required a multi-faceted approach involving elimination of matrix components in the sample preparation process and separation of analytes from matrix components in the liquid chromatography part of the LC-MS methodology.

Several different sample preparation strategies were explored. Ultrafiltration to remove larger sized proteins from plasma provided a largely matrix-free filtrate but exhibited very low recovery of Substance P. Direct application of diluted plasma to solid phase extraction (SPE) cartridges resulted in excessive matrix components in the extract and unacceptable ion suppression. Precipitation of plasma proteins with acetonitrile removed the bulk of the plasma proteins but the resulting extract was still too crude for analysis by LC-MS. Only the combination of a precipitation step with acetonitrile followed by clean up by SPE provided an extract amenable to LC-MS analysis. LC-MS analysis of these relatively clean samples still suffered from the effects of ion suppression by matrix components (other peptides/small proteins). To overcome this obstacle, two LC columns were coupled together and the LC conditions modified to separate the peptides of interest from matrix components. This strategy was successful in being able to get specific and sensitive detection of Substance P, beta-endorphin, and several Substance P metabolites in piglet plasma. The downside of this strategy was that the sample analysis time for each sample increased from 15 to 32 minutes. In addition, sensitive detection of several Substance P metabolites as well as Neuropeptide Y and Calcitonin gene relate peptide (CGRP) still suffered from the effects of ion suppression.

Conditions for the clean up of precipitated piglet plasma by SPE were examined in depth. The purpose of this in depth examination was to maximize the recovery of Substance P/metabolites while minimizing the recovery of interfering matrix components. The SPE cartridges were of a weak cation exchange type. They work by retaining basic peptides while acidic and neutral compounds are unretained. Recovery of the basic peptides from the SPE cartridges required a very acidic solvent to wash the peptides from the resin. Combinations of acetonitrile/water/formic acid/acetic acid were investigated. Solutions containing 20% formic acid resulted in rinsing off an undesirable amount of extraneous peptides while a solution with 10% formic acid did not recover enough Substance P/beta-Endorphin. Acetic acid by itself was not very effective in rinsing off the desirable peptides even at a concentration of 30%. A combination of formic acid and acetic acid each at a concentration of 15% proved to be a

workable solution. A solvent containing 10% formic acid and 20% acetic acid was also effective. These rinses with high acid content were somewhat difficult to dry down prior to LC-MS analysis. Although the recovery of desirable peptides was acceptable, there were concerns about stability of Substance P etc. in these solutions of 30% organic acid content at the dry down temperature of 50°C. Because of these concerns, rinse solutions utilizing the more volatile trifluoroacetic acid (TFA) were investigated. TFA is widely used in the peptide/protein field. A solution of only 2% TFA was more than equivalent to solutions containing 30% of the other acids and was much easier to evaporate to dryness. Unfortunately, it also washed off a much larger amount of the matrix peptides/proteins that led to unacceptable ion suppression during LC-MS analysis. Another widely used tool in the peptide/protein field consists of SPE with reversed phase resins. These type cartridges were also investigated as another way to avoid the high acid content rinses from the weak cation SPE method. Processing plasma samples with these SPE cartridges also resulted in an unacceptable level of ion suppression.

The recovery of Neuropeptide Y and CGRP from the weak cation SPE cartridges was somewhat limited with the mixed formic acid/acetic acid rinses. A more aggressive solvent containing 20-25% formic acid and no acetic acid was needed for acceptable recovery of these peptides. Unfortunately, a greater amount of extraneous peptides were also washed off with theses rinses giving rise to ion suppression issues.

Results:

Objective 1: To develop and validate a "gold standard" Liquid Chromatography-Mass-Spectrometry (LC-MS) method to determine Substance P, Neuropeptide Y, Calcitonin gene-related peptide (CGRP) and Beta-endorphin concentrations in porcine plasma.

Analytical Methodology:

After finding little to no Substance P in a sizeable number of incurred piglet plasma samples a small stability study was conducted. Pooled piglet plasma samples were prepared containing added Substance P and beta-endorphin. One pool of piglet plasma was kept in ice while a second pool was kept at room temperature. Samples from each pool were taken at 0, 0.25, 0.5, 1, 2, 4 & 6 hours after addition of Substance P/beta-endorphin and the samples immediately protein precipitated and processed. Samples out to 2 hours at room temperature and 4 hours on ice were indistinguishable from the 0 hour pooled sample. The absence of Substance P from incurred samples thus was not due to instability upon bringing samples to room temperature. All of the incurred samples contained a protease inhibitor and had been stored at -80°C. It is unlikely that Substance P had degraded at these temperatures without at the same time showing evidence of metabolite formation.

Similarly, further analysis to identify Substance P in piglet samples via ELISA and RIA was performed to identify further candidate samples to screen via LC-MS. Samples of a higher concentration determined on RIA were compared to the same sample via ELISA. There was not a reproducible similarity in concentrations between samples.

Summary:

The analytical methodology was developed to measure Substance P in piglet plasma to a limit of detection of about 1-2 pg/mL. This is an increase in detection limit of about 25-fold compared to a previous study in bovine plasma. Although the LC-MS method meets the criteria

for analytical reproducibility when "spikes" of a known concentration of piglet Substance P are analyzed, when un-spiked samples are analyzed no significant amounts of Substance P are measured. This is in contrast to the values in the same samples via immunoassay suggesting that these assays are detecting interference from other biological molecules in the piglet samples and cast doubt on the ability of the ELISA tests to specifically and accurately measure Substance P and other neuropeptides.

Objective 2: To compare the results of commercial Enzyme-Linked immunosorbent assay (ELISA) kits and Radioimmunoassay (RIA) kits for determining Substance P, Neuropeptide Y, CGRP and beta-endorphin concentrations in porcine plasma with the "gold standard" LC-MS results to determine the utility of using these assays in swine pain experiments.

Material and Methods:
Commercially available ELISA kits were researched for Substance P (Porcine Substance P(SP) ELISA Kit; Catalog #: MBS296080, My BioSource, Inc; San Diego), Neuropeptide Y (Porcine Neuropeptide Y (NPY) ELISA kit; Catalog #: MBS734700; My BioSource, Inc; San Diego, CA and Pig Neuropeptide Y (NPY) ELISA kit; Catalog # CSB-EL016034PI; Cosmo Bio USA Inc; Carsbad, CA), CGRP (Pig calcitonin gene related peptide ELISA kit; Catalog #: MBS269711; My BioSource, Inc; San Diego, CA 92195-3308 and Pig calcitonin gene related peptide ELISA kit; Catalog # CSB-E16438p; Cosmo Bio USA Inc; Carsbad, CA) and Beta-endorphin (Porcine Beta-Endorphin (β-EP) ELISA kit; Catalog #: MBS260544; My BioSource, Inc; San Diego, CA).

Substance-P
Porcine Substance P (SP) ELISA Kit Lot#20140514 exp. Date: Nov 14, 2014
Catalog #: MBS296080
My BioSource, Inc.
P.O. Box 153308
San Diego, CA 92195-3308
1-888-627-0165
www.mybiosource.com

This assay uses enzyme-linked immunosorbent assay based on biotin double antibody sandwich technology to assay porcine Substance-P. The kit is validated for serum, blood plasma, saliva, urine and other related tissue liquid.

Assay range: 2ug/mL – 60ug/mL
Sensitivity: 1.2ug/mL

Blank Piglet plasma was set up in the following dilutions and run, in triplicate against a spike curve in buffer. The spike curve was made up from kit standard and run according to kit instructions and levels.
Dilutions:
Undiluted
1:2 with EIA buffer 200ul blank plasma: 200ul buffer

1:20 with EIA buffer 25ul blank plasma: 475ul buffer
1:100 with EIA buffer(1:5 of 1:20) 100ul of 1:20 : 400ul buffer
1:200 with EIA buffer(1:2 of 1:100) 200ul of 1:100 : 200 ul buffer

Spike levels:
0ugmL, 2ug/mL, 4ug/mL, 8ug/mL, 16ug/mL, 32ug/mL, 64ug/mL.

Kit Results
The undiluted sample gives the best results with the sample concentration being ~6.6ug/mL, falling in the middle of the curve. This assay range is probably not low enough for use in pigs.

Calcitonin gene related peptide (CGRP)
Pig calcitonin gene related peptide ELISA kit Lot#36252922 exp. Date: April 2015
Catalog #: MBS269711
My BioSource, Inc.
P.O. Box 153308
San Diego, CA 92195-3308
1-888-627-0165
www.mybiosource.com

This kit employs Double Antibody Sandwich Technique. The principle of Double Antibody Sandwich is based on characteristics of the tested antigen with more than two valances, which can identify coated antibody and detection antibody at the same time. This kit is validated for detection of Porcine CGRP in serum, plasma or cell culture supernatant.

Assay range: 31.2pg/mL – 2000pg/mL
Sensitivity: 12pg/mL

Blank piglet plasma was set up in the following dilutions and run, in triplicate, against a spike curve in buffer. The spike curve was made up from kit standard and run according to kit instructions and levels.
Dilutions:
Undiluted
1:2 with EIA buffer 250ul blank plasma : 250ul of buffer
1:20 with EIA buffer 35ul blank plasma : 665ul of buffer
1:100 with EIA buffer(1:5 of 1:20) 140ul of 1:20 : 560ul of buffer
1:200 with EIA buffer(1:2 of 1:100) 250ul of 1:100 : 250ul of buffer

Spike levels:
31.2pg/mL, 62.5pg/mL, 125pg/mL, 250pg/mL, 500pg/mL, 1000pg/mL

Kit results:
The kit “negative” sample had OD readings of 0.383 and 0.363. The 1:200 diluted sample had reading of 0.446, 0.342 and 0.338. However, when calculation adjustments were made for the dilution the 1:20 sample had a concentration of 5455pg/mL. It was recommended that further dilutions be looked at such as 1:2, 1:4, 1:8 and 1:16.

Dilutions:
Undiluted
1:2 with EIA buffer 400ul blank plasma : 400ul buffer
1:4 with EIA buffer 400ul of 1:2 : 400ul buffer
1:8 with EIA buffer 400ul of 1:4 : 400ul buffer
1:16 with EIA buffer 200ul of 1:8 : 200ul buffer

Same spike levels were run as before.
All sample dilution ODs are similar. Undiluted = 0.554, 1:2 = 0.488, 1:4 = 0.389, 1:8 = 0.355, 1:16 = 0.367. Kit blank OD = 0.079 and kit negative OD = 0.208.

Conclusion: There is matrix interference and an extraction may be necessary. Additional research is necessary to refine the extraction technique.

Pig calcitonin gene related peptide ELISA kit Lot#B05170247 exp. Date: 20Jan 2015
Catalog # CSB-E16438p
Cosmo Bio USA Inc.
2792 Loker Ave. West Suite 101
Carsbad, CA 92010
P: 760-431-4600
www.cosmobiousa.com

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for CGRP has been pre-coated onto a microplate. Standards and samples are pipette into the wells and any CGRP present is bound by the immobilized antibody. After removing any unbound substances, biotin-conjugated antibody specific for CGRP is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of CGRP bound in the initial step. The color development is stopped and the intensity of the color is measured.

This kit is validated for pig serum, plasma and tissue homogenates.
Assay range: 31.2pg/mL – 2000pg/mL
Sensitivity: Typically <7.8pg/mL.

Blank pig plasma was diluted as follows and run in triplicate against a spike curve in buffer.
Undiluted
1:2 with kit buffer
1:5 with kit buffer
1:10 with kit buffer
1:20 with kit buffer

Spike levels run, in buffer, according to kit instructions; 31.25pg/mL, 62.5pg/mL, 125pg/mL, 250pg/mL, 500pg/mL, 1000pg/mL and 2000pg/mL.

Kit results:
The "blank" kit diluent run had OD readings of 0.086 and 0.088. The 1:10 dilution had similar readings. The 1:2 dilution had OD readings similar to the 31.25 spike, with a reading of 30pg/mL, when corrected, 60pg/mL.

Conclusion: Could possibly use a 1:2 dilution of the plasma. Further method validation is needed before testing of incurred samples can commence.

Neuropeptide Y (NPY)

Porcine Neuropeptide Y (NPY) ELISA kit Lot#20140512 exp. Date: Nov 12, 2014
Catalog #: MBS734700
My BioSource, Inc.
P.O. Box 153308
San Diego, CA 92195-3308
1-888-627-0165
www.mybiosource.com

This ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-NPY antibody and an NPY-HRP conjugate. The assay sample and buffer are incubated together with NPY-HRP conjugate in a pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450nm in a microplate reader. The intensity of the color is inversely proportional to the NPY concentration since NPY from samples and NPY-HRP conjugate compete for the anti-NPY antibody-binding site. Since the number of sites is limited, as more sites are occupied by NPY from the sample, fewer sites are left to bind NPY-HRP conjugate. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The NPY concentration in each sample is interpolated from this standard curve.

This kit is valid for pig serum, plasma and tissue homogenates.
Assay range: 31.2pg/mL – 2000pg/mL
Sensitivity: 1.0pg/mL.

Blank pig plasma was diluted as follows and run in triplicate against a spike curve in buffer.
Undiluted
1:2 with kit buffer
1:5 with kit buffer
1:20 with kit buffer
1:100 with kit buffer
1:200 with kit buffer

Spike levels run, in buffer, according to kit instructions; 100pg/mL, 250pg/mL, 500pg/mL, 1000pg/mL and 2500pg/mL.

Kit results:

Sample	Dilution	Rep	OD result	Ave OD	Dilution correction	%Difference from previous dilution
Blank piglet plasma	Undiluted	1	0.454	0.459		
		2	0.465			
		3	0.459			
Blank piglet plasma	1:2	1	0.489	0.496	0.992	1.16
		2	0.483			
		3	0.516			
Blank piglet plasma	1:20	1	0.692	0.668	13.36	12.47
		2	0.687			
		3	0.764			
Blank piglet plasma	1:100	1	0.764	0.699	69.9	4.23
		2	0.725			
		3	0.609			
Blank piglet plasma	1:200	1	0.616	0.641	125.2	0.83
		2	0.654			
		3	0.653			

Conclusion: For accuracy, the sample should be diluted to or below the middle of the analytical range of the assay. This would be the 1:100 dilution. This kit is ready to progress to the next stage of method validation, namely the testing of incurred samples.

	Conc.			Back Calc. Conc.			
Samplepg/mL	OD	Ave. OD		pg/mL		SD	CV
Std A	0.0	1.438	1.473	1.934		0.05	3.4
		1.509					
Std B	100	0.959	0.941	105.136		0.025	2.7
		0.923					
Std C	250	0.753	0.717	235.037		0.051	7.1
		0.681					
Std D	500	0.541	0.519	484.591		0.031	6.0
		0.497					
Std E	1000	0.342	0.322	1200.685		0.028	8.7
		0.303					
Std F	2500	0.226	0.227	2215.895		0.002	0.7
		0.228					

Accuracy and Spike recovery still need to be performed on this kit.

Pig Neuropeptide Y (NPY) ELISA kit Lot#V25170248 exp. Date: 19 Jan 2015

Catalog # CSB-EL016034PI
Cosmo Bio USA Inc.
2792 Loker Ave. West Suite 101
Carsbad, CA 92010
P: 760-431-4600
www.cosmobiousa.com

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for NPY has been pre-coated onto a microplate. Standards and samples are pipette into the wells and any NPY present is bound by the immobilized antibody. After removing any unbound substances, biotin-conjugated antibody specific for NPY is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of NPY bound in the initial step. The color development is stopped and the intensity of the color is measured.

This kit is valid for pig serum, plasma and tissue homogenates.
Assay range: 31.25pg/mL – 2000pg/mL.
Sensitivity: 7.81pg/mL

Blank pig plasma was diluted as follows and run in triplicate against a spike curve in buffer.
Undiluted
1:2 with kit buffer (sample diluents)
A blank sample was diluted 1:2 with sample diluent. 400uL was taken from that diluted sample and 100uL of a freshly made NPY standard, 500pg/mL was added. Concentration to be 125pg/mL. Another blank sample was diluted 1:2 with sample diluents. 400uL was taken from that diluted sample and 100uL of a freshly made NPY standard, 50pg/mL was added. Concentration to be 12.5pg/mL. Freshly made NPY standards at 500pg/mL and 50pg/mL were also run, undiluted. The freshly made NPY standard was made by taking 200ug of NET peptide from a standard manufactured by Phoenix Pharmaceuticals, Inc. Lot#430393, and adding 2mL of 1% acetic acid/de-ionized water. Concentration of 100ug/mL.

Kit results:
All dilutions along with the 1:2 blank sample with standard added look similar. The 500pg/mL fresh standard is reading about 9.791pg/mL. The 50pg/mL standard is reading similar to the blank buffer.

Conclusion: This kit is ready to progress to the next stage of method validation, namely the testing of incurred samples.

Porcine Beta-Endorphin
Porcine Beta-Endorphin (β-EP) ELISA kit Lot#38266973 exp. Date: April 2015
Catalog #: MBS260544
My BioSource, Inc.
P.O. Box 153308
San Diego, CA 92195-3308

1-888-627-0165
www.mybiosource.com

This kit employs Double Antibody Sandwich Technique. The principle of Double Antibody Sandwich is based on characteristics of the tested antigen with more than two valances that can identify coated antibody and detection antibody at the same time.

This kit is valid for the following matrices: Porcine serum, plasma or cell culture supernatant.
Assay range: 15.6pg/mL – 1000pg/mL.
Sensitivity: 5pg/mL

Blank pig plasma was diluted as follows and run in triplicate against a spike curve in buffer.

Undiluted	
1:2 with kit sample diluent	250:250
1:20 with kit sample diluent	35:665
1:100 (1:5 of 1:20)	140uL of 1:5 to 560uL of kit sample diluent
1:20 (1:2 of 1:100)	250uL of 1:2 to 250uL of kit sample diluents

Spike levels run in buffer, according to kit instructions: 1000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, 31.2pg/mL, 15.6pg/mL.

Kit results:
The undiluted blank porcine plasma gave a reading of approximately 10pg/mL. The 1:2 gave a reading of 5pg/mL. The undiluted sample looks good.

Ran a spiked sample curve undiluted. Took out the upper 2 spike levels and added a lower spike by diluting the 15.6pg/mL low spike 1:2 to level of 7.8pg/mL. Ran a lower spike at 3.9pg/mL.

Results of spike curve in plasma:
All spike levels run in plasma with the kit standard looked the same. OD Readings were 4.000 – 3.592 with no differentiation between spiked levels.

Spike levels run in buffer looked good. Could not differentiate between the 3.9 and the 7.8pg/mL spikes. Would take the curve down to 7.8pg/mL.

Conclusion: Additional validation is needed before we can progress to the next stage of method validation, namely the testing of incurred samples.

Summary for Objective 2: Multiple commercially available ELISA test kits were analyzed. However, without the ability of the LC-MS method to detect Substance P, Neuropeptide Y, CGRP and beta-endorphin in un-spiked pig plasma, we are unable to complete this objective.

Objective 3: 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.

Note: Without the ability of the "gold standard" Liquid Chromatography-Mass-Spectrometry

(LC-MS) method to detect Substance P, Neuropeptide Y, Calcitonin gene-related peptide (CGRP) and Beta-endorphin concentrations in un-spiked porcine plasma this objective is unable to proceed as scheduled.

Objective 4: 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.

Note: Without the ability of the "gold standard" Liquid Chromatography-Mass-Spectrometry (LC-MS) method to detect Substance P, Neuropeptide Y, Calcitonin gene-related peptide (CGRP) and Beta-endorphin concentrations in un-spiked porcine plasma this objective is unable to proceed as scheduled.

Discussion:

Pain experienced during routine animal husbandry procedures is an area of growing consumer interest and concern. One example of the impact this has had on livestock production practices is a recent European directive mandating the use of prolonged analgesia during surgical castration in pigs. Given that future access to international markets will require compliance with emerging animal welfare regulations, it is critical that U.S. livestock producers develop practical and cost-effective strategies to provide analgesia at the time of processing. Unlike in Europe, there are currently no analgesic compounds specifically approved for the alleviation of pain in food animals in the United States. In accordance with United States Food and Drug Administration Guidance Document 123, validated methods of pain assessment must be used to prove that a pharmaceutical is efficacious before it can be labeled as an analgesic. This point supports basic biological research on pain markers in pigs conducted in a controlled environment. Furthermore, the lack of scientific evidence makes it very difficult for stakeholders to respond to societal concerns with science-based recommendations for reducing pain caused during castration, tail docking, teeth clipping and identification.

concentrations in pig plasma is a big goal that would potentially allow for more detailed analysis of painful procedures in pigs. This research project was successful 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. Unfortunately, when 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. The marked difference between the results measured by the highly specific and sensitive LC-MS and methodologies based on commercially available ELISA kits 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.