Effects of sample handling methods on substance P concentrations and immunoreactivity in bovine blood samples

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Objective—To determine the effects of protease inhibitors and holding times and temperatures before processing on the stability of substance P in bovine blood samples.

Samples—Blood samples obtained from a healthy 6-month-old calf.

Procedures—Blood samples were dispensed into tubes containing exogenous substance P and 1 of 6 degradative enzyme inhibitor treatments: heparin, EDTA, EDTA with 1 of 2 concentrations of aprotinin, or EDTA with 1 of 2 concentrations of a commercially available protease inhibitor cocktail. Plasma was harvested immediately following collection or after 1, 3, 6, 12, or 24 hours of holding at ambient (20.3° to 25.4°C) or ice bath temperatures. Total substance P immunoreactivity was determined with an ELISA; concentrations of the substance P parent molecule, a metabolite composed of the 9 terminal amino acids, and a metabolite composed of the 5 terminal amino acids were determined with liquid chromatography–tandem mass spectrometry.

Results—Regarding blood samples processed immediately, no significant differences in substance P concentrations or immunoreactivity were detected among enzyme inhibitor treatments. In blood samples processed at 1 hour of holding, substance P parent molecule concentration was significantly lower for ambient temperature versus ice bath temperature holding conditions; aprotinin was the most effective inhibitor of substance P degradation at the ice bath temperature. The ELISA substance P immunoreactivity was typically lower for blood samples with heparin versus samples with other inhibitors processed at 1 hour of holding in either temperature condition.

Conclusions and Clinical Relevance—Results suggested that blood samples should be chilled and plasma harvested within 1 hour after collection to prevent substance P degradation. (*Am J Vet Res* 2014;75:109–116)

Because of societal concerns for the welfare of farm animals, various organizations have implemented or are considering the implementation of regulations concerning painful husbandry procedures such as dehorning and castration.[1] To provide proper scientific evidence for regulatory decisions and for the development of pain-mitigating options, there is a need for studies in which pain-relieving techniques for food animal species are investigated. A major impediment to determination of conclusions from such studies is the lack of an objective measure of pain in animals. Circulating cortisol concentration has been used as a response variable to estimate the severity of noxious stimulation attributable to procedures in various studies, but that method has limitations.[2] Substance P concentration has

Abbreviations

KIU	Kallikrein inhibitor units
LC-MS-MS	Liquid chromatography–tandem mass spectrometry
$SP_{1–11}$	Parent substance P molecule composed of 11 amino acids
$SP_{3–11}$	Metabolite of substance P composed of the last 9 C-terminal amino acids
$SP_{7–11}$	Metabolite of substance P composed of the last 5 C-terminal amino acids

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been evaluated as a response variable that may be more specific for the measurement of pain in animals.[3–10]

Substance P is a biologically active peptide that has a role in neural transmission of nociceptive signals from peripheral sites to the CNS.[11] Substance P is released by peptidergic peripheral sensory nerve fibers at central synaptic junctions in the dorsal horn of the spinal cord and at peripheral nerve terminals, where the peptide has a role as a signaling molecule in the transmission of pain impulses and the induction of inflammation and central sensitization.[12]

Substance P is a member of the tachykinin family and is synthesized in cell bodies from transcripts of the gene preprotachykinin-A. The substance P parent molecule is a peptide composed of 11 amino acids with an amidated carboxyl terminus (arginine-proline-lysine-proline-glutamine-glutamine-phenylalanine-phenylalanine-glysine-leucine-ethionine-NH_2). Enzymes that cleave $SP_{1–11}$ include members of the serine and metalloprotease families: angiotensin I–converting enzyme, aminopeptidase, neutral endopeptidase, dipeptidyl peptidase IV, and postproline cleaving enzyme.[13,14] Enzymes of these classes require inhibitors with specific affinity for their catalytic sites; therefore, protection of $SP_{1–11}$ from enzymatic degradation may require at least 2 inhibitor types. Various degradation products are derived from $SP_{1–11}$ and are named in accordance with the amino acids they contain. For example, cleavage of $SP_{1–11}$ between the number 2 and number 3 amino acids (proline and lysine) results in the formation of 2 fragments: an arginine-proline fragment and $SP_{3–11}$. Fragments containing the 5 C-terminal amino acids (fragments $SP_{7–11}$ through $SP_{1–11}$) have similar biological effects, although the potency of such effects decreases with fewer amino acids.[15] Fragments composed of the hydrophobic C-terminal and those composed of the hydrophilic N-terminal have opposite biological effects. Results of another study[16] suggest that the antinociceptive effects of morphine are enhanced when combined with the substance P fragment composed of the first 7 amino acids. Knowledge of the fragmentation profile of substance P may therefore increase understanding of pain processing and perception in animals.

Concentrations of substance P can be measured by means of ELISA or LC-MS-MS. Whereas concentrations of the substance P parent molecule are specifically measured with LC-MS-MS, ELISA measures substance P immunoreactivity that includes $SP_{1–11}$, C-terminal fragments, and other related immunoreactive peptides.

In studies involving livestock, sample handling conditions are not always ideal. Barns often are distant from laboratory facilities, and refrigeration or ice may not be available for chilling samples. Furthermore, samples may be kept in batches for transport to a laboratory where processing is delayed.

In support of research that may use substance P concentration or immunoreactivity as a response variable, the purpose of the study reported here was to determine the effects of various sample handling techniques on substance *P* values in blood samples when analyzed by means of LC-MS-MS or ELISA. An objective was to compare the effects of various handling procedures on concentrations of $SP_{1–11}$, $SP_{3–11}$, and $SP_{7–11}$ as measured with LC-MS-MS and on total immunoreactivity of substance P (ie, immunoreactivity of $SP_{1–11}$, metabolites of substance P, and other related immunoreactive molecules) as measured with ELISA. Another objective was to determine the effects of delayed blood sample processing (up to 24 hours) and the temperature (ambient or ice bath) at which a sample was kept during that period. In addition, 2 concentrations of the enzyme inhibitor aprotinin in EDTA, 2 concentrations of a commercially marketed complete protease inhibitor cocktailin EDTA, EDTA alone, and heparin were compared with respect to protection of $SP_{1–11}$ and total immunoreactivity of substance P from degradation.

Materials and Methods

Samples—Blood samples collected from a healthy 6-month-old Holstein calf were used in the study. All experimental procedures in this study were approved by the Kansas State University Institutional Animal Care and Use Committee under the supervision of the university veterinarian.

Collection tube preparation—Enzyme inhibitor treatments were prepared and added to tubes approximately 8 hours prior to blood sample collection. Aprotinin powder was dissolved and serially diluted with deionized water to create 2 stock solutions that would provide final concentrations of 50 and 500 KIU/mL when diluted in the collected blood samples as recommended by manufacturers of the ELISA kit[a] used for determination of total substance P immunoreactivity in this study. A commercially available protease inhibitor cocktail tablet[b] was dissolved and serially diluted in deionized water in accordance with the manufacturer's recommendations to provide solutions of single strength and double strength when diluted in blood samples. To prepare tubes containing aprotinin or complete protease inhibitor cocktail, 100 µL of a concentrated stock solution was added to an uncapped 4-mL blood collection tube containing K_3EDTA. All tubes were recapped and refrigerated (4°C) overnight.

Approximately 1 hour prior to blood sample collection, a spiking solution of $SP_{1–11}$ was prepared by adding 1 mL of 0.1% formic acid to a vial containing 425,000 ng of $SP_{1–11}$. From this solution, serial dilutions were made with 0.1% formic acid to obtain a solution containing $SP_{1–11}$ at a concentration of 50 ng/mL. Fifty microliters of the $SP_{1–11}$ solution was added to each of the prepared tubes containing enzyme inhibitors, lithium heparin, or K_3EDTA alone to provide a final concentration of 625 pg/mL when mixed with 4 mL of whole blood.

The blood sample collection tubes were arranged in 3 replicate arrays for each blood sample holding period (0, 1, 3, 6, 12, and 24 hours of holding before processing). The arrays to be used for holding periods of 1, 3, 6, 12, and 24 hours each contained 2 sets (1 for each temperature treatment [ambient and ice bath temperatures]) of the following 6 enzyme inhibitor treatments spiked with 625 pg/mL of $SP_{1–11}$: heparin (15 U/mL; USP standard), EDTA (1.8 mg/mL), aprotinin (50 or 500 KIU/mL) combined with EDTA (1.8 mg/mL), or protease inhibitor cocktail (single strength or double

strength) combined with EDTA (1.8 mg/mL). Arrays to be used for immediate processing (0 hours of holding) contained only 1 set of the 6 inhibitor treatments because there was no temperature treatment. In addition to the tubes with enzyme inhibitor treatments, each array also contained 1 tube containing double-strength protease inhibitor cocktail that was not spiked with $SP_{1–11}$; contents of this tube were to be processed immediately to detect endogenous concentrations of substance P. For samples in the tube containing double-strength protease inhibitor cocktail that was not spiked with $SP_{1–11}$, it was assumed a priori that this inhibitor cocktail would provide the fullest extent of protection against the various types of enzymes that degrade substance P. All tubes were then kept in a cooler filled with crushed ice for transport to the barn facility for blood sample collection.

Blood sample collection—Whole blood (total volume, 864 mL) was obtained from the calf. Approximately 18 hours prior to blood sample collection, the calf was restrained in a chute and an indwelling jugular venous catheter was placed with local anesthesia. To maintain patency overnight, the catheter was flushed once with 3 mL of a 3 U/mL heparin solution (heparin sodium[c] diluted with saline [0.9% NaCl] solution[c]; USP standard).

On the morning of blood sample collection, the calf was restrained in a chute while the tubes in 3 arrays were uncapped in preparation for filling of the first blood sample collection series. The heparin solution was cleared from the catheter by attaching a syringe and, in 3 repetitions, drawing 5 mL of blood into the syringe, then depressing the plunger to return the blood into the jugular vein of the calf. A collection series consisted of 3 syringes of blood and was performed as follows. A 60-mL syringe was attached to the uncapped catheter and filled during 30 to 60 seconds, after which 4 mL of blood was rapidly transferred to each of the 13 tubes in the first array. As soon as a tube was filled, it was recapped and rapidly inverted 10 times to ensure thorough mixing of the contents. During this time, another 60-mL syringe was attached to the catheter and the next blood sample collection was performed. After the filling of tubes in each array was completed (time, approx 1 minute), the tubes were immediately placed in their holding environment and the start time and temperature were recorded. In this manner, the process was repeated to quickly obtain 3 complete replicate treatment arrays for each holding period. To facilitate the processing of samples, the order of collection for each holding period was as follows: 12-, 6-, 3-, 24-, 0-, and then 1-hour periods. Collection and processing of the blood samples for the 3 arrays in each holding period was completed in a mean ± SD time of 5 ± 2 minutes. Approximately 5 minutes was needed between collection of blood samples for each array to prepare for the next blood sample collection series, resulting in a mean ± SD time of 11 ± 2 minutes between times that blood sample collection was started for each collection series.

Blood samples for ambient temperature holding were placed in a test tube rack, whereas samples for chilled holding were immediately plunged into a bath of crushed ice in an insulated portable cooler. For chilled blood samples, care was taken to ensure that only the cap and a small amount of each tube remained above the ice surface. Ambient temperature was recorded with a digital thermometer placed on the benchtop near the samples. Temperature in the cooler at the ice-air interface was recorded with a similar thermometer that was protected in a plastic bag. Ambient temperatures ranged from 20.3° to 21.2°C during the 1-hour sample collection period at the barn, then ranged from 23.9° to 25.4°C during the remaining 24-hour holding period in the laboratory. The temperature of the ice bath–air interface in the cooler ranged from 4.5° to 7.3°C during the 1-hour period during blood sample collection and ranged from 0.1° to 2.2°C during the remaining holding period in the laboratory; however, the blood samples in those tubes were kept below the ice surface, where the presumed temperature at the ice-water interface was 0°C.

Blood samples were either processed within 5 minutes after collection (0 hours) or kept for 1, 3, 6, 12, or 24 hours prior to processing. During processing, blood samples were centrifuged in a refrigerated unit (4°C) at 10,000 × *g* for 15 minutes. Tubes were then removed from the centrifuge and kept in an ice bath while plasma was harvested and divided among paired cryovials (2 aliquots); plasma samples were kept at –70°C until analysis. The 0-hour blood samples spiked with $SP_{1–11}$ and the samples in tubes with double-strength protease inhibitor cocktail that were not spiked with $SP_{1–11}$ were processed in the barn with the same refrigerated centrifuge that was used to process the other blood samples. For holding periods processed in the barn, cryovials with harvested plasma were immediately placed in a cooler on ice packs (which had been kept overnight at –70°C); these plasma samples were then transported to the laboratory, where they were observed to have frozen during the < 10-minute transit. These plasma samples were kept at –70°C until analysis. Blood samples for all other holding periods were processed in the laboratory, with care taken to ensure that they were handled in a manner similar to samples that were processed in the barn.

LC-MS-MS analysis of $SP_{1–11}$ and metabolite concentrations—Only samples for 0-, 1-, 3-, and 6-hour holding periods were analyzed by means of LC-MS-MS. Analysis of 1 aliquot of the plasma samples was performed within 6 weeks after collection and storage at –70°C.

An LC-MS-MS method was used for the analysis of concentrations of $SP_{1–11}$, $SP_{3–11}$, and $SP_{7–11}$ in plasma samples. A structurally related peptide, [Tyr8]-substance P, was used as the internal standard for all analytes. The analytes and internal standard were isolated from 0.5 mL of each aliquot of plasma samples by means of solid-phase extraction with hydrophilic, lipophilic balanced cartridges. Extracts were evaporated until dry and reconstituted in the starting mobile phase. Electrospray ionization and tandem mass spectrometry were performed with a high-performance liquid chromatography system coupled with a triple quadrupole mass spectrometer.[d] Chromatographic separation of all analytes and internal standard was achieved with an analytic column[e] and a gradient elution from 100% of

one mobile phase (0.2% acetic acid in water) to 80% of another mobile phase (0.2% acetic acid in acetonitrile) and re-equilibration during a runtime of 15.0 minutes. This method was accurate and precise for a linear dynamic range from the lower limit of quantitation (50 pg/mL) to 1,000 pg/mL (data not shown).

ELISA analysis of total immunoreactivity of substance P—Approximately 18 months after blood sample collection and plasma sample storage at –70°C, an aliquot of plasma samples for all holding periods from 0 to 24 hours were analyzed for determination of total immunoreactivity of substance P with a validated ELISA method.[3] Briefly, analytes were extracted from plasma by acidification with acetic acid and fractionating with reverse-phase, solid-phase extraction columns. Peptides were eluted from the column with an organic aqueous solvent mixture and concentrated by drying in gaseous nitrogen. The dried extract was reconstituted and analyzed in duplicate in accordance with the manufacturer's instructions for the substance P ELISA kit.[a] Assay performance was monitored with 5 replicates of bovine plasma samples containing 0, 200, or 800 pg/mL of substance P purified standard. The method was linear for the 5 replicates of each concentration ($R^2 > 0.99$), and the coefficient of variation for each concentration within an assay run was < 15%. The coefficient of variation for the 5 ELISA runs in the study was 42% for 0 pg/mL samples, 20% for 200 pg/mL samples, and 35% for 800 pg/mL samples. This ELISA[a] had the following cross-reactivities between the assay and metabolites: $Sp_{1–11}$ (100%), SP_{3-11} (87.9%), the fragment composed of the 8 terminal amino acids (11.7%), and $SP_{7–11}$ (5.9%).

Statistical analysis—Because the 3 syringes of blood obtained for each collection series were not independent and because holding period was confounded with collection series, we refrained from investigating rates of decay and from making comparisons across holding periods. Instead, we confined our analysis to comparisons of the 3 replicates of time and temperature combinations within each holding period. Furthermore, because the analyses of samples by means of LC-MS-MS and ELISA were not performed after the same freezer storage time and because the ELISA method cross-reacted with substance P metabolites, we also refrained from statistical comparison of total immunoreactivity of substance P (determined with ELISA) with a summary measure of $SP_{1–11}$, $SP_{3–11}$, and $SP_{7–11}$ concentrations (determined with LC-MS-MS).

The response variables of $SP_{1–11}$, $SP_{3–11}$, and $SP_{7–11}$ concentrations and total immunoreactivity of substance P were transformed (natural log) and analyzed with ANOVA models. For graphic presentation, the means and 95% confidence intervals were transformed back to the original units. Fixed effects included temperature (ice bath holding conditions vs ambient temperature holding conditions) and enzyme inhibitor (aprotinin [50 or 500 KIU/mL] in EDTA, protease inhibitor cocktail [single or double strength] in EDTA, EDTA alone, and heparin). For analyses of total immunoreactivity of substance P, the ELISA run number was used as a random effect. Differences among treatment groups were assessed on the basis of results of Tukey pairwise *t* tests by time. Data analyses were performed with statistical software.[f] Values of $P \leq 0.05$ were considered significant.

Results

Analysis of blood samples that were not spiked with $SP_{1–11}$—For blood samples with double-strength protease inhibitor cocktail that were not spiked with $SP_{1–11}$ and were processed immediately following collection, results of LC-MS-MS analysis indicated no detectable concentrations of endogenous $SP_{1–11}$, $SP_{3–11}$, or $SP_{7–11}$. In such samples analyzed by means of ELISA, the

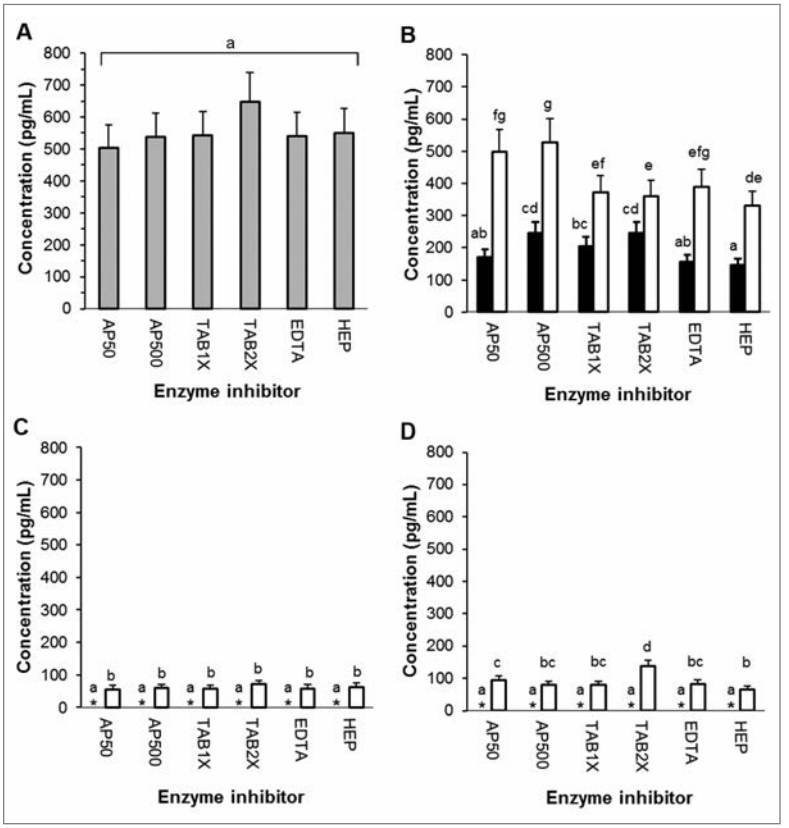

Figure 1—Back-transformed least squares median concentration estimates and upper 95% confidence intervals of LC-MS-MS assay total concentrations of $SP_{1–11}$ in blood samples collected from a 6-month-old calf, spiked with 625 pg of $SP_{1–11}$/mL, mixed with various enzyme inhibitors, and processed immediately (gray bars; A) or after 1 (B), 3 (C), or 6 (D) hours of holding at ambient (20.3° to 25.4°C; black bars) or ice bath (white bars) temperatures. The enzyme inhibitors included heparin (15 U/mL; HEP), EDTA (1.8 mg/mL), aprotinin (50 KIU/mL [AP50] or 500 KIU/mL [AP500]) combined with EDTA (1.8 mg/mL), or a protease inhibitor cocktail (tablet dissolved and diluted in deionized water to provide concentrations of single strength [TAB1X] or double strength [TAB2X]) combined with EDTA (1.8 mg/mL). Processing included centrifugation and harvesting of plasma. *Sample had no detectable analyte or a peak concentration less than the lower limit of quantitation of the assay. [a–g]Within each holding period, values without a common letter are significantly ($P < 0.05$) different.

amount of immunoreactivity was similar to that for reference samples containing 0 pg of substance P standard/mL.

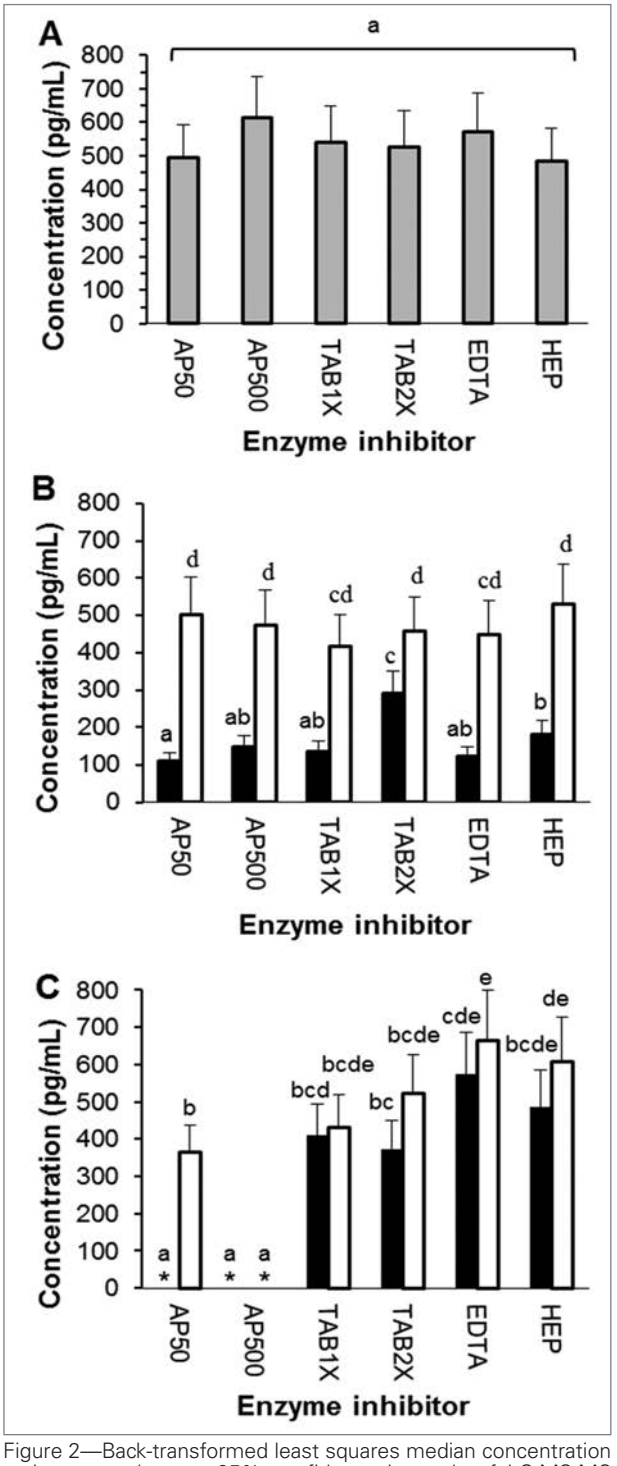

Figure 2—Back-transformed least squares median concentration estimates and upper 95% confidence intervals of LC-MS-MS assay total concentrations of $SP_{3–11}$ in blood samples collected from a 6-month-old calf, spiked with 625 pg of $SP_{1–11}$/mL, mixed with various enzyme inhibitors, and processed immediately (gray bars; A) or after 1 (B) or 3 (C) hours of holding at ambient (20.3° to 25.4°C; black bars) or ice bath (white bars) temperatures. Data for the 6-hour holding time are not shown because no analyte peaks were detected in those samples. [a–e]Within each holding period, values without a common letter are significantly ($P < 0.05$) different. *See* Figure 1 for remainder of key.

LC-MS-MS analysis of blood samples spiked with $SP_{1–11}$—Results of LC-MS-MS analysis regarding ex vivo degradation of $SP_{1–11}$, $SP_{3–11}$, and $SP_{7–11}$ indicated the main effects of holding temperature and enzyme inhibitor were significant ($P < 0.001$). Although there was significant ($P < 0.001$) interaction between holding temperature and enzyme inhibitor for $SP_{3–11}$ and $SP_{7–11}$, interaction between those variables was not significant ($P = 0.06$) for $SP_{1–11}$. Concentrations of $SP_{1–11}$ (**Figure 1**), $SP_{3–11}$ (**Figure 2**), and $SP_{7–11}$ (**Figure 3**) were summarized. For blood samples processed immediately following collection (0 hours of holding), there were no significant ($P > 0.12$) differences in concentrations of $SP_{1–11}$, $SP_{3–11}$, or $SP_{7–11}$ among the enzyme inhibitor treatments.

Within 1-, 3-, or 6-hour holding periods, $SP_{1–11}$ concentrations in blood samples kept in an ice bath were significantly ($P < 0.005$) greater than concentrations in blood samples kept at ambient temperature. For blood samples kept in an ice bath for 1 hour prior to processing, the only significant differences in $SP_{1–11}$ concentrations were between samples with either concentration of aprotinin (50 or 500 KIU/mL) and samples with other enzyme inhibitors. The $SP_{1–11}$ concentration in blood samples with 500 KIU of aprotinin/mL was significantly ($P < 0.018$) greater than the concentrations in samples with heparin or single- or double-strength protease inhibitor cocktail. The $SP_{1–11}$ concentrations in blood samples with 50 KIU of aprotinin/mL were significantly ($P < 0.04$) greater than they were in samples with heparin or double-strength protease inhibitor cocktail. For blood samples kept in an ice bath for 1 hour prior to processing, there were no significant ($P > 0.99$) differences in $SP_{1–11}$ concentration between samples with either concentration of aprotinin (50 or 500 KIU/mL) or between samples with either concentration of protease inhibitor cocktail (single or double strength).

ELISA analysis of blood samples spiked with $SP_{1–11}$—Results of ELISAs regarding total immunoreactivity of substance P for all holding periods (0 to 24 hours) were summarized (**Figure 4**). The interaction between holding temperature and enzyme inhibitor was significant ($P < 0.001$).

For blood samples that were processed immediately after collection, there were no significant ($P > 0.93$) differences in total immunoreactivity of substance P among the enzyme inhibitor treatments. Within the 1- and 3-hour holding periods for ice bath and ambient temperature holding conditions, there were no significant ($P > 0.92$) differences in total immunoreactivity of substance P among samples treated with either concentration of aprotinin, either concentration of protease inhibitor cocktail, and EDTA alone. Within the 1-hour holding period, the total immunoreactivity of substance P in blood samples treated with heparin was significantly ($P < 0.001$) lower than that in samples treated with any other enzyme inhibitor for ambient temperature holding conditions and significantly ($P < 0.04$) lower than that in samples treated with 50 KIU of aprotinin/mL or double-strength protease inhibitor cocktail for ice bath holding conditions.

Although not statistically compared, total immunoreactivity of substance P as measured with

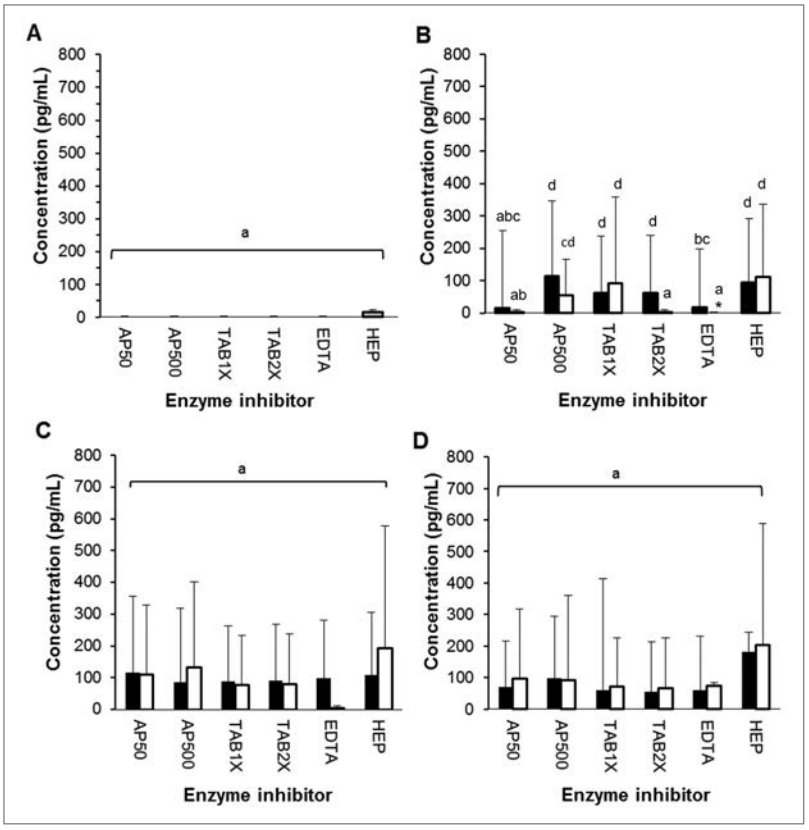

Figure 3—Back-transformed least squares median concentration estimates and upper 95% confidence intervals of LC-MS-MS assay total concentrations of SP_{7-11} in blood samples collected from a 6-month-old calf, spiked with 625 pg of SP_{1-11}/mL, mixed with various enzyme inhibitors, and processed immediately (gray bars; A) or after 1 (B), 3 (C), or 6 (D) hours of holding at ambient (20.3° to 25.4°C; black bars) or ice bath (white bars) temperatures. [a–d]Within each holding period, values without a common letter are significantly ($P < 0.05$) different. *See* Figure 1 for remainder of key.

ELISA seemed to increase in blood samples kept > 3 hours prior to processing. The immunoreactivity in blood samples kept 6 and 24 hours prior to processing was similar to that in samples that were immediately processed (0 hours of holding). Within each enzyme inhibitor group in ice bath holding conditions, the total immunoreactivity of substance P as measured with ELISA seemed to be lower at 12 hours than it was at 6 and 24 hours of holding before processing.

Discussion

In the present study, we investigated the effects of various enzyme inhibitor types and holding times and temperatures on substance P concentrations as determined by means of LC-MS-MS and ELISA. Because there is variation among animals regarding stress responses and chemical, enzymatic, and cellular components of blood and because it was not possible to collect and pool whole blood samples from several animals, only 1 animal was used in the study. This was meant to control for those potential sources of variability, which might have obscured differences among groups. The animal used in this study was a healthy randomly selected calf and was considered to be representative of other calves; therefore, the use of blood samples from only 1 calf was not thought to have affected inferences made on the basis of the results of the study. Furthermore, because blood components may vary over time within an individual and because analyses for each holding period were performed with blood samples obtained at a distinct time, we refrained from making inferences for results between holding periods (because differences might have been caused by intra-individual variation).

As suggested by results that indicated approximately equal concentrations of SP_{1-11} and SP_{3-11} in the 0-hour blood samples analyzed by means of LC-MS-MS, there seemed to be rapid early cleavage of SP_{1-11} to SP_{3-11}, which was found for all enzyme treatments. Immediately after blood samples were added to the test tubes, there was a short period during the tube inversion process before the inhibitors were homogenously mixed with blood samples; during this time, enzymes in blood samples may have been able to act freely on SP_{1-11}. This source of substance P degradation might have been minimized by spiking the tubes with exogenous substance P after, rather than before, the addition of whole blood; however, the intent of the study was to evaluate substance P degradation attributable to handling procedures, as would occur when SP_{1-11} is present in circulating blood. Given that inhibitor molecules may be dissociated from the protease enzymes, a portion of the enzymatic cleavage of SP_{1-11} to SP_{3-11} could also have occurred during the 15-minute centrifugation, the 5- to 10-minute period of plasma sample harvest, the storage period at –70°C, and the thaw process prior to analysis.

When blood samples were kept at ambient temperature, enzymatic processes seemed to proceed more rapidly than when they were kept in an ice bath. Enzymes are typically more active at physiologic temperatures than they are at colder temperatures, so these results were not unexpected. In samples kept for 1 hour prior to processing, concentrations of SP_{1-11} as determined by means of LC-MS-MS were approximately 50% lower in samples kept at ambient temperatures than they were in samples kept in an ice bath. Even when blood samples were in an ice bath, SP_{1-11} concentrations were near the lower limit of quantitation when kept for 3 hours prior to processing. Therefore, results of LC-MS-MS analysis of SP_{1-11} concentrations suggested that blood samples should either be processed immediately or kept in an ice bath and processed within 1 hour after collection. When blood samples were processed immediately, the type of enzyme inhibitor did not significantly affect concentrations of SP_{1-11} or SP_{3-11}. When blood samples were kept for 1 hour in an ice bath, aprotinin seemed to be the best enzyme inhibitor for protection of SP_{1-11} from enzymatic degradation, and no significant differences were detected between the 50 or 500 KIU/mL concentrations of that inhibitor. Because the composition of the complete protease inhibi-

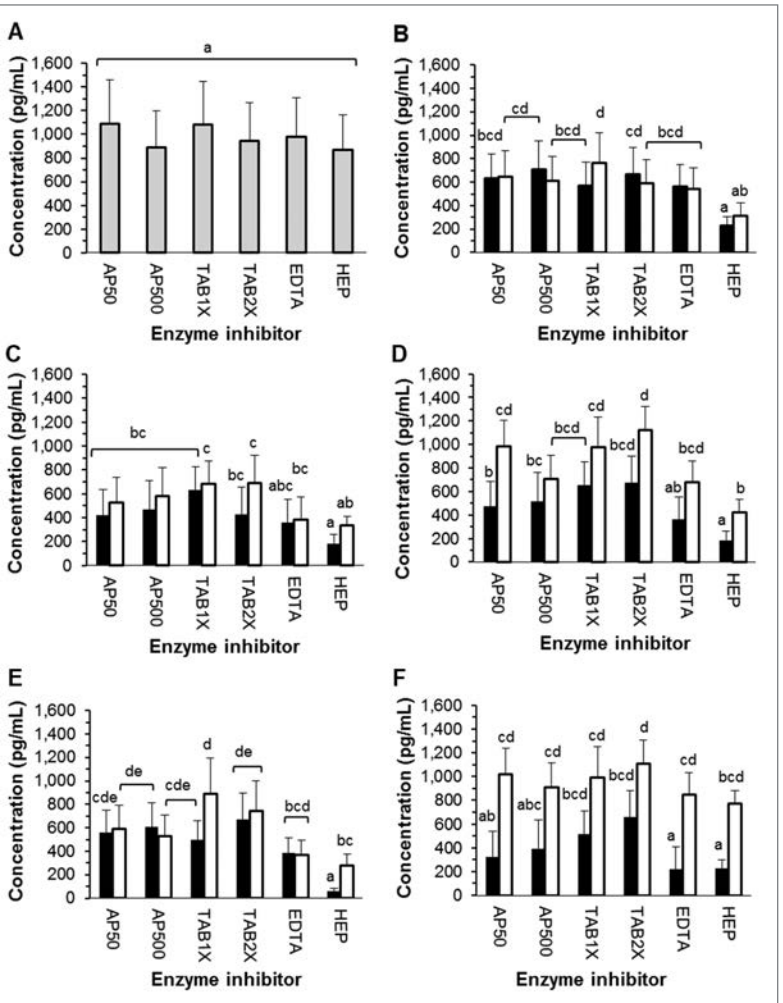

Figure 4—Back-transformed least squares median concentration estimates and upper 95% confidence intervals of ELISA total immunoreactivity of substance P (ie, immunoreactivity of SP_{1-11}, metabolites of substance P, and other related immunoreactive molecules) in blood samples collected from a 6-month-old calf, spiked with 625 pg of SP_{1-11}/mL, mixed with various enzyme inhibitors, and processed immediately (gray bars; A) or after 1 (B), 3 (C), 6 (D), 12 (E), or 24 (F) hours of holding at ambient (20.3° to 25.4°C; black bars) or ice bath (white bars) temperatures. [a–f]Within each holding period, values without a common letter are significantly ($P < 0.05$) different. *See* Figure 1 for remainder of key.

tor cocktail tablet is proprietary information, it was not known whether aprotinin was included in that formulation; however, for blood samples kept in an ice bath for 1 hour prior to processing, neither the single- nor double-strength concentrations of the cocktail seemed to provide more protection of SP_{1-11} than EDTA alone.

Similar to LC-MS-MS analysis results for SP_{1-11} and SP_{3-11} concentrations, results of ELISAs for blood samples processed immediately indicated the type of enzyme inhibitor did not significantly affect total immunoreactivity of substance P. Unlike the results determined with LC-MS-MS, there were no significant effects of temperature for blood samples kept 1 or 3 hours before processing as determined with ELISA. This was likely because the ELISA measured SP_{1-11} and substance P degradation products that had a carboxyl terminus group.

This study may not have had a high enough power to detect some differences between treatments as determined with the ELISA, given the interassay coefficient of variation of 20% to 35% of that assay in a substance P concentration range of 200 to 800 pg/mL. Although the ELISA results seemed to be unaffected by holding temperatures for blood samples, especially for those processed within 1 hour after collection, it is important to recognize that the assay has 100% cross-reactivity only for SP_{1-11}; therefore, the goal of sample handling should be to preserve SP_{1-11} so that accurate results are obtained. For studies in which a difference in substance P concentrations between groups is the alternate hypothesis, true differences between groups might not be detected if SP_{1-11} is unnecessarily allowed to degrade to SP_{3-11} and if other metabolites for which the percentages of cross-reactivity for the ELISA are not as high. Pronounced intra- and interindividual variability of plasma substance P immunoreactivity has been detected for control and castrated calves in another study.[3] Therefore, it is important to control for as much procedure-related variability and to measure the highest concentrations of the substance P parent molecule in blood samples as possible so that true differences between treatment groups are not missed because of artificially low substance P immunoreactivity and high errors in values.

For both SP_{1-11} concentrations and total immunoreactivity of substance P, we could not determine whether the differences between the 1- and 0-hour holding period blood samples were significant, although such differences were typically large. This suggested that a strict processing order should be adhered to; the processing order should follow the blood sample collection order, without large discrepancies in the amount of time between collection and processing. Otherwise, substantial differences in results could occur because of sample handling. Alternately, variability in results among samples may have been high enough to prevent identification of significant differences attributable to treatments.

The finding of low concentrations of SP_{1-11} typically detected in blood samples after a 3-hour holding period supported the recommendation that samples obtained for analysis of substance P should be processed within 1 hour following collection. For holding times > 3 hours between collection and processing, a factor other than enzymatic degradation seemed to affect substance P concentrations in whole blood samples; the ELISA total immunoreactivity of substance P seemed to increase after more than 3 hours. Notably, for blood samples kept in an ice bath for 24 hours, the ELISA total immunoreactivity of substance P was similar to that for samples processed immediately following collection, regardless of the type of enzyme inhibitor treat-

ment. The ELISA total immunoreactivity of substance P in samples kept 6 and 12 hours before processing were typically similar to those for samples kept 0 and 24 hours before processing, depending on the enzyme inhibitor. Similarly, concentrations of $SP_{1–11}$ in blood samples kept in an ice bath seemed to increase from 3 to 6 hours after collection.

The finding of high immunoreactivity of substance P in blood samples kept for > 3 hours prior to processing suggested the possibility that substance P (or other molecules with cross-reactivity to the ELISA) was being produced by cells in the whole blood samples after collection. Production of substance P in blood samples in vitro would not account for the ELISA results that indicate total immunoreactivity of substance P at 12 hours of holding was typically lower than it was at 6 hours of holding prior to processing. This finding might be explained by the order in which the blood samples for each holding period were collected. The blood samples in the 12-hour holding period group were obtained during the first collection time. Although the calf was well acclimated to the facility and to most of the study personnel, there was more activity than usual on the day of blood sample collection and the animal was unfamiliar with the person collecting the samples. Therefore, it was likely that the procedure caused stress in the calf. Results of an in vitro study[17] of bone marrow aspirates of clinically normal humans indicate that the addition of ACTH (100 ng/mL) to bone marrow stromal cells induces peak concentrations of substance P at 48 hours after stimulation. In the present study, it was possible that the samples collected first (and kept 12 hours prior to processing) contained lower concentrations of ACTH than those obtained during subsequent collections. That possibility might explain the apparent lower ELISA total immunoreactivity of substance P detected for blood samples kept 12 hours prior to processing versus that for samples kept 6 or 24 hours before processing; determination of ACTH concentrations in blood samples would have been necessary to confirm that theory.

Results of this study suggested that various biological processes may affect the concentration of substance P following blood sample collection. Thus, to accurately determine substance P concentrations, blood samples should be processed as soon as possible and handled in accordance with a protocol that ensures samples are immediately chilled to the temperature of an ice bath. Furthermore, results suggested it is important that plasma is harvested from blood samples in the same order in which samples were collected, with similar times between collection and harvesting of plasma for all groups. Otherwise, substantial differences among samples regarding substance P concentrations may develop or differences may be obscured because of sample handling techniques. This procedure is recommended for samples analyzed by means of LC-MS-MS or ELISA. Although results of the ELISA in this study seemed to be minimally affected by the holding temperature during the first hour after blood sample collection, the goal of sample handling should be to preserve $SP_{1–11}$, for which that test had the highest percentage of cross-reactivity.

Results of this study suggested that use of a standard blood collection tube containing heparin or EDTA seemed to be as effective for preserving substance P as the combination of EDTA with aprotinin or an enzyme inhibitor cocktail, as long as blood samples were chilled and processed within 5 minutes after collection. These findings were similar for blood samples analyzed by means of ELISA and those analyzed by means of LC-MS-MS.

a. Assay Designs, Enzo Life Sciences Inc, Farmingdale, NY.
b. Complete protease inhibitor tablet, Santa Cruz Biotechnology Corp, Santa Cruz, Calif.
c. Baxter Healthcare Corp, Deerfield, Ill.
d. API 4000, AB SCIEX, Framingham, Mass.
e. XBridge Shield RP18, Waters Corp, Milford, Mass.
f. SAS, version 9.2, SAS Institute Inc, Cary, NC.

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