Novel Method for Determination of Substance P Levels in Unextracted Human Plasma by Using Acidification

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Substance P (SP) is a member of the tachykinin family and has an important role in immune responses. SP is detectable in plasma in a free and bound state. Simple modification of a commercially available SP enzyme-linked immunosorbent assay allows the dissociation and capture of plasma SP without solid-phase extraction.

The undecapeptide substance P (SP) is a member of the tachykinin family. Its role as a neurotransmitter (21, 22, 23) includes regulation of immune responses (19). Elevated levels of serum or plasma SP have been associated with disorders such as inflammatory bowel disease (16), sickle cell crisis (20), depression and anxiety (2, 11, 14, 19, 26), rheumatologic diseases (1, 17), infectious diseases (28), including human immunodeficiency virus (7, 8, 13), and cancer (24, 27). SP exerts its influence on immune responses through the induction of regulatory cytokines (3, 18).

Considerable variability has been reported for SP levels in similar biological fluids from healthy subjects and patients with diverse disease processes (5, 7, 8, 9, 11, 12). Corbally et al. (6) reported that endogenous human plasma-derived SP was reversibly and nonspecifically bound to both high-molecular-mass proteins (>400,000 Da) and intermediate-molecular-mass proteins (58,000 Da). SP was readily dissociated from proteins by repeat gel filtration. These observations suggested that SP binding to plasma proteins was mediated by weak hydrogen bonding and led to the development of methods for the extraction of SP from a variety of biological fluids (10, 25). Such extractions should be considered carefully since most are designed for the enrichment of low-molecular-mass peptides that can potentially exclude peptides nonspecifically bound to high-molecular-mass plasma or serum proteins. Such procedures may lead to underestimates of the total quantity of SP (4, 6).

This study investigated the influence of hydrogen ion concentrations on nonspecific SP-plasma protein dissociations that allow for the optimal binding of dissociated SP by capturing antibody.

**Human plasma.** Whole-blood-derived EDTA anticoagulated plasmas obtained from 28 healthy adult control subjects were stored at −70°C until evaluated for SP levels.

**SP EIA.** A commercially available SP antigen competition enzyme immunoassay (EIA; Cayman Chemical Company, Ann Arbor, MI) was modified to investigate the influence of hydrogen ion concentration on the dissociation and subsequent capture of SP in pooled normal adult human plasma. The SP EIA uses microtiter wells coated with monoclonal mouse anti-rabbit immunoglobulin G, an SP-specific polyclonal rabbit capture antibody, and an acetylcholinesterase-conjugated SP tracer. The substrate for acetylcholinesterase consists of acetylthiocholine and 5,5-dithio-bis-(2-nitrobenzoic acid). The colored end product is 5-thio-2-nitrobenzoic acid with absorbance read at 412 nm. A phosphate-citrate (PC) buffer was selected as the SP dissociation buffer with the evaluation of buffers having pH values of 7.3 to 2.2. Pooled normal adult human plasma was first diluted 1:8 in Cayman buffer (pH 7.3) followed by a 1:2 dilution in the various PC buffers yielding pH values of 7.3 to 3.6 for the treated plasma. SP standards used for construction of the standard curve were initially prepared in Cayman buffer (pH 7.3), yielding final SP concentrations of 3.9 to 125 pg/ml. After identification of the optimal-pH PC buffer that yielded maximum recovery of plasma-derived SP, SP standards were first diluted in Cayman buffer yielding SP concentrations of 250 to 7.8 pg/ml. This was followed by a 1:2 dilution in the optimal-pH PC buffer to yield SP concentrations of 125 to 3.9 pg/ml. After acidification, 100 μl of the treated plasma described above and SP standards were added in quadruplicate to the appropriate microtiter wells. Then, 50 μl of SP-specific polyclonal rabbit capture antibody and 50 μl of the acetylcholinesterase-conjugated SP tracer were added to the mixture. The capture antibody and SP tracer were prepared with Cayman buffer (pH 7.3) according to the manufacturer’s instructions. After an overnight incubation at 4°C, the plates were washed with Cayman buffer followed by the addition of 200 μl of reconstituted enzyme substrate. The plates were allowed to develop until the absorbance readings at 412 nm for Bb (maximum binding) were between 0.3 and 1.0 absorbance unit, using a PowerWave XS plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Absorbance data were downloaded and analyzed using Bio-Tek KC4 v.3.4 software.

**Influence of pH on recovery of human plasma-derived SP.** Figure 1 illustrates the effect of pretreatment of human plasma with PC buffers of decreasing pH on the quantitation of SP in plasma. Maximal SP recovery occurred using a PC buffer (pH 2.6) that yielded a plasma pH of 4.3 and a final pH of 6.5. The...
human plasma pool yielded an SP value of 18 pg/ml when evaluated under a physiologic pH of 7.3 and a value of 61 pg/ml upon exposure to pH 2.6 conditions. This indicated that approximately 70% of the plasma SP is present in a reversibly bound state.

**Influence of pH on SP standard curve.** In order to exclude the possibility that the observed increase in plasma SP levels after exposure to PC buffers of decreasing pH may be attributable to artifacts introduced by the altered pH of the modified assay, SP standards (3.9 to 125 pg/ml) were prepared in parallel using both the manufacturer’s recommended Cayman buffer (pH 7.3) and the modified procedure that employed the PC buffer (pH 2.6). Figure 2 illustrates a regression analysis of SP standards assayed by both methods, yielding a correlation coefficient of 0.9865. These data indicate that the observed increase in plasma SP levels using the modified assay was not attributable to alteration of the standard curve.

**Correlation of individual plasma SP levels derived from SP standard curves prepared in Cayman buffer (pH 7.3) or PC buffer (pH 2.6).** Although the modified procedure had little effect on the quantitative measurement of SP standards, individual plasma samples (n = 28) were evaluated using SP standard curves prepared as described in the legend to Fig. 2. As shown in Fig. 3, linear regression analysis revealed excellent correlations of SP values derived from either standard curve, yielding an $R^2$ of 0.978.

**Difficulty in the accurate measurement of SP in plasma is attributed, in part, to reversible nonspecific binding of SP to plasma proteins (4, 6).** This study was undertaken to develop a gentle nonchromatographic, liquid-phase procedure that permits the dissociation of SP from plasma proteins through limited hydrogen ion pressure, allowing for the capture of SP by a specific antibody of higher avidity after a return to more physiologic conditions. Exposure of human plasma to PC buffers of decreasing pH led to an enhanced release of protein-bound SP with optimal dissociation occurring at a plasma pH of 4.3 produced by a 1:2 dilution of plasma (diluted 1:8 in Cayman buffer [pH 7.3]) with PC buffer (pH 2.6). A final pH of 6.5 was achieved after the addition of the capture antibody and SP tracer, both of which were prepared in Cayman buffer (pH 7.3). These data indicate that a significant proportion of SP in human plasma exists in a bound state.

Possible alteration of the SP standards and/or other reactive components in the assay as a result of the introduction of the PC buffer (pH 2.6) was investigated by the parallel evaluation of the SP standard curve, formulated as recommended in Cayman buffer (pH 7.3), and the low-pH-modified procedure. Linear regression analysis of standard SP values obtained with both buffer systems yielded an $R^2$ of 0.9865. These data indicate insignificant differences between the two buffer systems regarding assay performance. An evaluation of human plasma (n = 28) using SP standard curves formulated by both methods.
also revealed insignificant differences in SP values upon linear regression analysis (R² = 0.978).

This study describes a simple liquid-phase method for the recovery of a significant proportion of SP bound to human plasma proteins. The modified EIA described here should allow for more accurate estimates of total SP present in human plasma.

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REFERENCES


