Substance P (SP), a bioactive undecapeptide, was discovered in 1930 (7) and is found at varying concentrations in the central nervous system (11). Observations by Lembeck that there is a much higher concentration of SP in the dorsal than in the ventral roots of the spinal cord led to the speculation that SP was associated with sensory neuron transmission (18). Chang and Leeman (5) reported the chemical structure of SP, which they determined to be an undecapeptide (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-amide), leading to its synthesis and availability for experimentation. Recently, immunooassay techniques have made it possible to study the distribution of SP in different tissues (6). SP is widely distributed in the central and peripheral nervous systems and has been implicated in immune (14) and hematopoietic (20, 21) modulation. We have recently demonstrated that human immune cells such as monocytes and macrophages express SP at both mRNA and protein levels (10). SP may have a major role in the pathogenesis of several inflammatory diseases such as rheumatoid arthritis, bullous pemphigus, asthma, inflammatory bowel disease (Crohn’s disease), and ulcerative colitis as well as in the transmission of pain (4, 16).

An area of intense investigation within the field of psycho-neuroimmunology centers on the elucidation of the roles played by certain neuropeptides in the modulation of immunological responses in various disease states. SP, one of three well-characterized neuropeptides of the tachykinin peptide family, has been extensively studied in terms of its role in physiological processes such as vasodilation, smooth-muscle contraction, and nerve conduction (3, 12, 27). More recently, SP has been shown to play an important role in the induction of cytokines such as interleukin β (IL-1β), IL-6, and tumor necrosis factor alpha, which are central to the initiation of inflammatory responses leading to modulation of both specific and nonspecific immune responses (13, 24). With the observation of SP receptors on both T and B lymphocytes (26), several studies have strengthened the hypothesis that SP plays a pivotal role in the trafficking of lymphocytes (15) leading to enhanced humoral (25) as well as cellular humoral responses (16, 17). Elevated plasma SP and immunoglobulin A levels were noted in human immunodeficiency virus type 1 (HIV-1)-infected infants compared to their uninfected cohorts (2, 23). Macrophages play a central role in both inflammatory responses and antigen presentation. The finding of SP augmentation of lipopolysaccharide (LPS)-induced cytokine production (IL-1, IL-6, and tumor necrosis factor alpha) by macrophages lends additional evidence for the role of SP in the modulation of host immune function (4).

The measurement of SP levels in various body fluids may serve as an important surrogate marker in monitoring host immune responses in a variety of disease states. Therefore, development of techniques to accurately measure SP is of obvious importance. Historically, attempts to measure neuropeptides in various body fluids, particularly serum or plasma, have been difficult due to the nonspecific binding of neuropeptides to other plasma or serum proteins (1, 22). Thus, various liquid organic extraction methods have been described for the partial purification of neuropeptides including SP with varying levels of success (9, 22). Further, until recently, labor-intensive radioimmunoassays (RIA) were the only methods available for the quantitation of SP after partial purification (9). The RIA technique, however, is less than satisfactory in terms of safety, ease of handling, and sensitivity.

In an effort to improve upon existing procedures for both the...
preparation and quantitation of SP in serum or plasma, the present paper describes the development of a simple chromatographic procedure for the partial purification of SP and describes the evaluation of a sensitive enzyme immunoassay (EIA) for the determination of SP level in serum and plasma samples from patients with several clinical disorders.

MATERIALS AND METHODS

Patient populations. The 305 samples were from adults undergoing a diagnostic procedure three times, twice in the hospital and once at home (n = 23 [69 samples]), children hospitalized with sickle-cell disease (n = 51), women with human immunodeficiency virus (HIV) infection (n = 11), patients infected with HIV (n = 43), patients with chronic inflammatory demyelinating polyneuropathy (n = 3), patients with multiple sclerosis (n = 23), healthy adult volunteers (n = 19), umbilical cord blood from HIV-positive mothers (n = 4), cultured monocytes from umbilical cord blood treated with cytokines (n = 77), and a cultured monocyte cell line from umbilical cord blood (n = 5). The choice of these diverse groups was based on sample availability as well as the desire to test the procedures on a wide range of patients.

Specimen collection. Blood was collected in serum separation tubes and centrifuged at 1,500 × g for 20 min. The blood was processed in the laboratory approximately 2 h after the specimen was drawn. The serum was collected, aprotinin (5 U/ml), a protease inhibitor (Sigma Chemical Co., St. Louis, Mo.), was added to each specimen, and the specimen was stored at −70°C until analysis was performed. The mean storage time prior to analysis was 8 days. The samples were dried under nitrogen gas and reconstituted with deionized water to 1/3 of the initial volume, and the assay was performed with the EIA as described below.

Extraction of SP from plasma and sera. The method used for the extraction of SP from acidic plasma or serum samples is a modification of a previously described procedure in which plasma samples were extracted with acetone followed by ether. The extracts were then air dried and reconstituted with assay buffer (9). Rissler (22) reviews the problems associated with liquid-liquid extraction of peptides such as SP from body fluids and advocates solid-phase extraction buffer (9). Rissler (22) reviews the problems associated with liquid-liquid extraction of peptides such as SP from body fluids and advocates solid-phase extraction of samples on small disposable cartridges such as the ones used in this study.

Briefly, 1.0 ml C18 reverse-phase columns (Bond Elute; Varont, Harbor City, Calif.) were activated by first rinsing with 5 ml of high-performance liquid chromatography grade methanol followed by a final rinse with 5 ml of distilled water. Plasma samples (0.5 ml) were diluted 1:4 with 4% (vol/vol) acetic acid. Initially, acidified plasma samples were spiked with known amounts of radiolabeled SP to evaluate SP recovery from the C18 reverse-phase columns. Further, known amounts of purified SP were processed through the column to evaluate the influence of preparation on immunoreactivity of the eluted peptide. The acidified samples were then added to the activated reverse-phase columns and allowed to pass through the columns by gravity. The columns were then washed five times with 2 ml of 4% acetic acid to remove all unbound material. Three buffer systems were evaluated for efficiency of elution of SP bound to C18 reverse-phase columns. The buffer systems tried were (i) 90% (vol/vol) ethanol, 10% (vol/vol) water, 0.4% (vol/vol) acetic acid; (ii) 80% (vol/vol) acetonitrile, 1% (vol/vol) trifluoroacetic acid (prepared in distilled water); and (iii) 60% (vol/vol) ethanol, 40% (vol/vol) acetic acid, 1% (vol/vol) trifluoroacetic acid (prepared in distilled water). The buffer systems were applied to the SP column, and the bound SP was then eluted with the buffer as described above.

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RESULTS

Recovery of SP from C18 reverse-phase columns. In order to determine the efficiency and efficacy of using the chromatographic extraction procedure on clinical specimens, 24 specimens were processed in duplicate both with and without the C18 reverse-phase columns. The paired specimens were plated and EIA readings were obtained at the same times. Values were obtained for these duplicate specimens from B/Bo plots and were compared statistically. The mean value for SP obtained for the duplicates without the column extraction was 6.87 with a standard deviation (SD) of 6.49. By comparison, the mean value of SP obtained from the duplicates with the column was 18.63 with an SD of 8.91 (Fig. 1). The paired coefficient of correlation between values obtained with and without the column was 0.98 (P < 0.001). The changes in the SP concentrations for the individual specimens based on the extraction procedure are graphically presented in Fig. 2. In addition to eliminating the interference with EIA measurement due to the nonspecific binding of SP to plasma or serum proteins, the purification procedure provides an additional advantage. Since the mean values for the specimens subjected to the chromatographic procedure were consistently three times the values for specimens not treated, and the interassay coefficient of correlation was very high, the purification procedure represents a worthwhile step in measuring SP levels since it also serves to place the values closer to the center of the EIA measurement scale.

Determination of immunoreactivity of eluted SP. In order to evaluate the influence of sample preparation on the immunoreactivity of SP, known amounts of synthetic SP (0.5 ml of 125 pg/ml) provided with the EIA kits were subjected to the same preparative procedure as that performed on unknown plasma and serum samples and then quantified by EIA. The results of eight individual experiments yielded a mean value of 124.5 pg/ml with an SD of 21.4 pg/ml and a coefficient of variance of 9.4%. The 95% confidence limits (mean ± 2 SD) were 81.7 to 167.4 pg/ml. We therefore used these limits for acceptance or rejection of individual evaluations of unknown samples. Figure 3 illustrates the mean B/Bo values ± SD for each SP standard used to produce reference standard curves for eight individual EIA experiments and represents the typical calibration curve for this EIA of SP. A linear displacement of acetylcholinesterase-linked SP by synthetic SP standard concentrations was obtained, when plotted as a semilogarithmic function from 7.8 to 1,000 pg of SP per ml. The coefficient of variation for each curve was greater than 0.98, while the coefficient of variation for the composite curve was 0.97. These data demonstrate an acceptable level of interassay precision for the EIA formatted assay, particularly when the coefficient of variance (9.4%) obtained with the multiple evaluations of the accuracy control mentioned above is taken into consideration.

Evaluation of SP level in normal control specimens. The limit of detection of SP in plasma and serum samples by the
EIA evaluated in the present study was stated by the manufacturer to be 17.5 pg/ml, which places the limit of detection near the level of normal ranges reported elsewhere for control subjects (21.94 ± 18 pg/ml) (9). We, therefore, elected to reconstitute eluted and dried samples in a volume (0.15 ml) which represented a 3.33× concentrate of the original sample volume (0.5 ml), which was applied to the C<sub>18</sub> reverse-phase columns. This was found to yield SP values near the middle of the three-decalog standard curve or about 100 pg/ml. The evaluation of 19 control sera yielded an uncorrected mean value of 126 pg of SP per ml (range, 17 to 382 pg/ml), with corrected values having a mean of 38 pg of SP per ml (range,
5 to 115 pg/ml), which is well within the quantitation range of the assay. The correction used was for the 3.33x concentration effect of the purification procedure. These conditions allow for the evaluation of pathological samples which might have both higher and lower levels of SP compared to a normal cohort.

**Evaluation of SP levels among patient populations.** Among all 306 specimens analyzed, the mean value for SP was 32 pg/ml, with corrected values ranging from a low of 2 pg/ml to a high of 242 pg/ml (SD, 39 pg/ml). The mean values for each group of subjects are presented in Table 1. Statistical analysis of the mean SP values using analysis of variance revealed significant differences among the groups ($F(9,312) = 3.7; P < 0.01$). The post hoc Tukey-B test revealed that the SP values obtained from the cord blood from mothers infected with HIV-1 were significantly higher than those in the diagnostic-procedure group, the children hospitalized with sickle-cell disease, the women with HPV, patients infected with HIV-1, and patients with multiple sclerosis. The concentrations of SP released by monocyte cell cultures in serum-free medium were similar to those obtained from the plasma or serum samples presented in Table 1 (18 ± 31.5 pg/ml for monocytes treated with cytokines [n = 77] and 35 ± 52.1 pg/ml for untreated monocytes [n = 5]; the value for lymphocytes reported in reference 10 is 32 pg/ml [n = 3]).

**DISCUSSION**

This study illustrates the usefulness of the procedures in obtaining valid measures of SP from a variety of clinical settings. The mean values we obtained compare favorably with those obtained by means of RIA from plasma samples from healthy blood bank donors (20 to 151 pg/ml) reported by Powell et al. (19). Similar results were also reported by Fernandez-Rodriguez et al. (9), who used an RIA technique to measure SP and found mean values ranging from 65 to 128 pg/ml in a comparison between normal control volunteers and patients with cirrhosis. In the experiments designed to study the relationship between SP and anxiety we found that they are significantly correlated and that EIA is sufficiently sensitive to detect changes within individual subjects over time (8). Interestingly, umbilical cord plasma from HIV-positive mothers had significantly higher SP levels than the other groups studied. Although we had only four samples of cord blood, these data support the results obtained by Azzari and coworkers (2, 23),

![FIG. 3. SP standard curve of mean absorbance for the eight calibration concentrations used (±1 SD).](image)

| TABLE 1. Comparison of corrected SP concentrations among groups |
|---------------------------------------------------------------|
| **Group (n)** | **Mean SP concn (pg/ml)** | **SD** |
| Healthy adult volunteers (19) | 38 | 35.7 |
| Diagnostic procedure patients (23) | 35* | 27.2 |
| Children with sickle-cell disease (51) | 45* | 51.1 |
| Women with HPV (11) | 14* | 8.4 |
| Patients with multiple sclerosis (23) | 33* | 23.8 |
| Patients with CIDP (3) | 40 | 19.2 |
| Patients with HIV (43) | 35* | 45.2 |
| Umbilical cord blood from HIV* mothers (4) | 98* | 68.5 |

*a Adjusted for the extraction concentration effect. *, $P \leq 0.05$ (post hoc Tukey-B analysis of variance).

*b Performed at three different time points.

* CIDP, chronic inflammatory demyelinating polyneuropathy.
who found that the SP levels of HIV-seropositive children born to HIV-seropositive mothers were significantly higher than the SP levels of HIV-seronegative children born to HIV-seropositive mothers. Elevated SP levels in the HIV-seropositive children might be associated with the imbalance between T-helper lymphocyte subsets and the cytokines they secrete.

The variability of SP concentration within certain groups such as children with sickle-cell disease (SD = 51.1) and umbilical cord blood from HIV-positive mothers (SD = 68.5) might be due to the heterogeneous factors within the groups. The children with sickle-cell disease may have had different levels of pain or have been at different stages of the disease, resulting in the high variability exhibited by the group. Similarly, the umbilical cord blood from HIV-positive mothers might or might not have been infected with HIV, possibly causing the high variability demonstrated. Thus, the variability of SP values seen within the diagnostic groups might be a further demonstration of the usefulness of the techniques described in this paper.

We have demonstrated that the described procedures for purifying and quantifying SP are reliable and useful. The SP measures obtained from diverse sources were comparable to the published results obtained by other researchers using different techniques. The use of the chromatographic procedure to partially purify SP in plasma and serum samples can be omitted when cell culture medium is serum free.

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