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## Note

# Comparison of two methods for the determination of substance P immunoreactivity in human cerebrospinal fluid: sulphopropyl-Sephadex ion-exchange chromatography and/or reversed-phase high-performance liquid chromatography combined with radioimmunoassay

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The undecapeptide, substance P, is a putative neurotransmitter, which is found in the peripheral and central nervous system. The role of substance P in the pathophysiology of extrapyramidal movement disorders has been investigated in detail in immunohistochemical [1,2], electrophysiological [3] and pharmacological [4,5] studies. It has been suggested that substance P is of significance in determining the intensity of psychoses [6].

Various groups have carried out determinations of substance P and its metabolites in cerebrospinal fluid (CSF) in an attempt to throw light on the cerebral metabolism of substance P in patients. Up till now, such measurements have been carried out with various radioimmunoassay (RIA) techniques either without previous fractionation of the CSF [7–13] or after a purification step on Sep-Pak cartridges [14]. RIA of peptides is complicated by the absence of a "specific" antiserum and/or additional cross-reactivity with various structurally related peptides, precursor fragments or degradation products, and can give rise to a misleading interpretation of the data.

The object of this study was therefore the development of a fractionation method for recording substance P as selectively as possible. We have determined substance P immunoreactivity (SPIR) in human CSF using two highly sensitive RIAs combined with sulphopropyl-Sephadex C-25 ion-exchange chromato-

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graphy or, in another experiment, a high-performance liquid chromatographic (HPLC) method. Because of limited availability of the substance P antiserum used in the sulphopropyl method, a new antiserum was raised for the HPLC experiments.

## EXPERIMENTAL

### *Reagents and chemicals*

All chemicals used were of analytical grade. Sodium phosphate, chloramin T, sodium sulphite, acetonitrile and trifluoroacetic acid (TFA) were from Merck (Darmstadt, F.R.G.), charcoal, Triton X100 and gelatin were from Serva (Heidelberg, F.R.G.) and thiomerosal from Sigma (Taufkirchen, F.R.G.). Sephadex C-25 was from Pharmacia (Uppsala, Sweden) and substance P from Serva. The peptides for determination of cross-reactivities were products of Serva and of Bachem (Bubendorf, Switzerland). $Na^{125}I$-$Tyr^8$-substance P was from New England Nuclear (Dreieich, F.R.G.).

### *Sulphopropyl-Sephadex C-25 ion-exchange method*

*Cerebrospinal fluid.* Seven patients with various neurological disorders [multiple sclerosis in quiescent phase (three), pseudobulbar paralysis (one), tonic fit (one), abducens-nerve palsy (one), viral meningitis (one); mean age 44.8 years, range 23–72 years] were subjected to lumbar puncture in the course of diagnostic examinations. Samples (2.5–6.5 ml) were collected in polypropylene tubes [15] and frozen immediately in dry ice–methanol at −70°C. The samples were stored at −20°C for not more than four weeks before analysis. The CSF samples were thawed at room temperature and heated by microwave irradiation (up to 90°C for 5 s) to prevent proteolysis of peptides. The samples were then adjusted to the pH and ionic strength of the equilibration buffer (pyridinium formate, 30 m*M*, pH 3.0) and loaded on the sulphopropyl-Sephadex column.

*Sulphopropyl-Sephadex C-25 chromatography.* For each sample, a silanized glass column (20 cm × 1.5 cm I.D.) was packed to a depth of 16 cm with Sephadex C-25 ion-exchange gel, which had been equilibrated at least four times with buffer (30 m*M*, pH 3.0) to stabilize the chromatographic bed until the pH and ionic strength of the eluate were identical with those of the buffer. The CSF sample was washed on to the column with 100 ml of the same buffer. Stepwise elution was performed by washing the column with 100 ml each of a series of pyridinium formate buffers of increasing pH and ionic strength (60 m*M*, pH 3.3; 80 m*M*, pH 3.5; 0.32 *M*, pH 4.6; 1.0 *M*, pH 4.6; 1.6 *M*, pH 4.6). SPIR was eluted with the 1.6 *M*, pH 4.6 buffer. The eluate was collected in polypropylene tubes, frozen at once at −70°C in dry ice–methanol and then lyophilized (Leybold Heraeus, Lyovac GT 2). The residue was redissolved in 300 μl of RIA buffer, and the SPIR level was determined by RIA. All assays were carried out in duplicate. The recovery of the method, determined by adding 300 fmol of synthetic substance P to pooled CSF, was 51% (mean value).

*Radioimmunoassay [16].* The solvent used for tracer, antiserum and lyophilized samples was phosphate-buffered saline (PBS, 50 m*M* sodium phosphate, 145 m*M* sodium chloride, 0.02% sodium azide, pH 7.4), which contained 1% gel-

atin. None of the buffer compounds interfered with the RIA. In an ice-bath, 100 $\mu$l of a 1:100 dilution of substance P antiserum was added to 300 $\mu$l of standard or sample in RIA buffer and incubated at 4°C for 4 h. The guinea pig antiserum had been prepared by a method described elsewhere [17]. No cross-reactivity was observed with bombesin, met-enkephalin, leu-enkephalin, $\beta_h$-endorphin, somatostatin, adrenocorticotropin (ACTH), dynorphin A or neurotensin. The cross-reactivities of eledoisin and physalaemin were not examined because these peptides have not been found in vertebrates to date. After addition of 100 $\mu$l of $^{125}$I Tyr$^8$-substance P (2400 cpm) the mixtures were incubated at 4°C for a further 20 h. Neither the substitution of substance P with tyrosine in position 8 nor the iodination impaired the immunoreactivity compared with the native peptide. Free and bound $^{125}$I-substance P were separated by adding 250 $\mu$l of a 2.5% dextran-coated charcoal slurry in PBS to each sample, followed by incubation at 4°C for 15 min and centrifugation for 10 min (refrigerated centrifuge Christ Junior II KS, Heraeus Christ). The supernatant was decanted and counted for 5 min in a $\gamma$-counter (Packard Model 3380). The inter-assay coefficient was 12%; the RIA had a detection limit of 15 fmol per tube.

*Reversed-phase HPLC method*

Seven patients with various neurological disorders [slipped disk (four), amyotrophic lateral sclerosis (two), multiple fractures (one); mean age 44.4 years, range 20–61 years] were subjected to lumbar puncture. Samples were allocated to the two chromatographic techniques at random, so we cannot comment on the differences of the SPIR content in CSF between these neurological disorders. Samples (3.0–6.5 ml) were collected and stored as for ion-exchange chromatography. For further processing the samples were lyophilized (Leybold Heraeus, Lyovac GT 2), taken up in 650 $\mu$l of eluent A and centrifuged at 4°C for 20 min (1000 *g*). Then 600 $\mu$l of the supernatant were pipetted off, and the residue was resuspended in 650 $\mu$l of eluent A and centrifuged at 4°C for 20 min (1000 *g*). Again 600 $\mu$l of the supernatant were pipetted off and added to the first supernatant. The samples were then lyophilized and taken up in 250 $\mu$l of eluent A, and 200 $\mu$l of this solution were applied to the HPLC column.

*HPLC fractionation.* The HPLC system used (Waters Assoc., Eschborn, F.R.G.) consisted of two pumps (Model 510), a gradient programmer (Model 680), an injection system (Model U6K) and a fraction collector (LKB, F.R.G.). A reversed-phase column packed with $\mu$Bondapak $C_{18}$ (150 mm × 3.9 mm I.D., 10 $\mu$m particle size) and a Radial-Pak guard column also containing $\mu$Bondapak $C_{18}$ were used. The aqueous eluent (A) was 0.1% TFA in water (deionized water, double-distilled from a quartz still and collected in glass containers). The organic eluent (B) was 80% acetonitrile in water, containing 0.1% TFA, 365 pmol/l arginine and 350 pmol/l lysine. Eluent A was degassed by vacuum filtration, eluent B by sonication. The gradient was from 22% B, first concave (programmer curve 8), then linear to 87% B in 30 min; the flow-rate was 1.5 ml/min. Fractions (1.5 ml) were collected in polypropylene tubes that contained 50 $\mu$l of RIA buffer. The fractions were frozen at once and then lyophilized. The lyophilisate was taken up

in 300 $\mu$l of RIA buffer, and the SPIR level was determined by RIA. All assays were carried out in duplicate.

*Radioimmunoassay.* The solvent for tracer, antiserum, standard and lyophilized samples was a sodium phosphate buffer (50 m*M* sodium phosphate, 150 m*M* calcium chloride, 0.01% thiomerosal, 0.1% gelatin, 0.01% Triton X100 and 1.0% bovine serum albumin). None of the buffer compounds interfered with the RIA. A 100-$\mu$l volume of a 1:10 000 dilution of antiserum was added to 300 $\mu$l of standard and sample solutions in RIA buffer, and the mixture was incubated at 4°C for 4 h. The substance P antiserum was prepared by a method described elsewhere [18]. The antiserum cross-reacted with eledoisin (2.7%), physalaemin (0.01%) and substance P 4-11 (0.005%). No cross-reactivity were observed with substance K, neuromedin B, $\beta_h$-endorphin, bradykinin, substance P 1-4, bombesin, met-enkephalin, leu-enkephalin, $\alpha$-neoendorphin and dynorphin A. [$^{125}$I]-Tyr$^8$-substance P was prepared by the chloramin T method [19]. The iodinated peptide was separated by BioGel P4 gel chromatography. After addition of 100 $\mu$l of $^{125}$I-Tyr$^8$-substance P (10 000 cpm) the mixtures were incubated at 4°C for a further 24 h. Then 500 $\mu$l of a 2% active charcoal suspension (as RIA buffer but with 0.5% BSA) were added to each sample. The mixtures were incubated at 4°C for 15 min and then centrifuged for 10 min. The supernatant was decanted and counted for 5 min in a $\gamma$-counter (Packard Model PGD, Prias$^{TM}$). The sensitivity of the RIA was 5 fmol per tube, the inter-assay coefficient 7.2%. Calibration curves were prepared with standards processed under the same lyophilization conditions as the samples.

## RESULTS

SPIR was purified from human CSF by the sulphopropyl-Sephadex $C_{25}$ ion-exchange chromatographic method described, and determined with a highly sen-

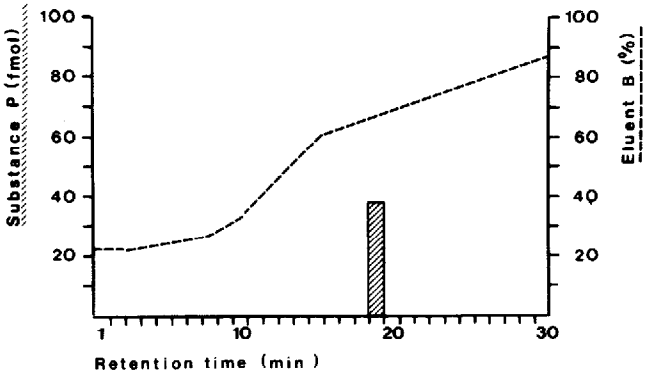


Fig. 1. Retention time for native SPIR (38 fmol in 3.2 ml of CSF). CSF was applied to reversed-phase HPLC column $\mu$Bondapak $C_{18}$ (150 mm×3.9 mm I.D.). Aqueous eluent (A), 0.1% TFA; organic eluent (B), 80% acetonitrile, 0.1% TFA, 365 pmol/l arginine, 350 pmol/l lysine. The gradient profile is shown by the dashed line. The left-hand axis refers to the amount of SPIR determined by RIA after purification by the HPLC method described.

sitive RIA. The recovery of this method, determined by the addition of 300 fmol of synthetic substance P to pooled CSF, was 51% (mean value). The concentration of SPIR in lumbar CSF of patients with various neurological disorders was $20.3 \pm 4.6$ fmol/ml (mean $\pm$ S.D.) with a range of 7.8–40.0 fmol/ml.

In another experiment the neuropeptide was separated from human CSF by reversed-phase HPLC, and the SPIR was determined by a different RIA. The recovery of this method was dependent on the concentration assayed: 52% for 15 fmol, 62% for 30 fmol and 101% for 90 fmol of synthetic substance P added to pooled CSF. With the TFA–acetonitrile gradient described the retention time for synthetic or native substance P was 18.5 min (see Fig. 1). With this method we determined a SPIR concentration of $12 \pm 0.9$ fmol/ml (mean $\pm$ S.D., $n=7$). The range of the individuals was between 9.3 and 16.4 fmol/ml.

## DISCUSSION

The careful assay of neurotransmitters in CSF using various immunoassay techniques [7–14] permits deductions as to their cerebral metabolism during the course of a disease.

Direct assay of SPIR in CSF without an adequate purification step presupposes that the RIA is in no way affected by the presence of unwanted substances. However, although an effect of organic salts as well as cross-reactions of the antiserum with structurally related substances, precursor fragments or degradation products must be expected in assays of biological material, extensive separation of the substance to be assayed in the RIA from its biological matrix is essential.

This point of view provides a ready partial explanation for the differences in the published mean levels of SPIR in human CSF. These range from $1.00 \pm 0.13$ pg/ml [8] to $74 \pm 21$ fmol/ml [13]. A recent report from our laboratory described a column chromatographic method for the separation of neuropeptides from human CSF [20]: sulphopropyl-Sephadex ion-exchange chromatography was applied to the isolation of SPIR from human CSF. Bergström et al. [21] have also shown that this method is suitable for the purification of SPIR in the picomolar range from the majority of cross-reacting components, such as the C-terminal fragments that arise in substance P metabolism [22]. We determined a recovery for 300 fmol of 51%.

Various groups [23–25] have described reversed-phase HPLC methods for the purification from biological material of neuropeptides in the nanomolar or picomolar range. The concentration of SPIR in human CSF, however, lies in the femtomolar range. With the frequently described simple TFA–acetonitrile gradient [25], we were unable to determine SPIR in this concentration range with acceptable reproducibility. Only after the addition of the amino acids arginine (365 pmol/l) and lysine (350 pmol/l) to eluent B we were able to establish a reproducible recovery rate (101% for 90 fmol of synthetic substance P in pooled CSF [26]). We account for the effect of the added amino acids as follows: in the reversed-phase column, the terminal silanol groups of the silica gel are not totally substituted with $C_{18}$ alkyl chains, and so the residual free silanol groups of the stationary phase can interact with the peptide to be fractionated and can prevent

reproducible elution. The recovery of SPIR which is dependent on the concentration of amino acids appear not to saturate all the silanol binding sites, high concentrations seem to affect the chromatographic interaction of the peptide with the stationary phase. So addition of 7.4 nmol/l arginine and 6.9 nmol/l lysine to eluent B resulted in a 63.7% recovery (90 fmol of synthetic substance P in pooled CSF). Cross-reactivity of arginine and lysine could be excluded.

The advantages of the sulphopropyl-Sephadex ion-exchange chromatography and the HPLC methods combined with a RIA over the previously described assays for SPIR are a result of the specific purification of the SPIR from CSF in good yield.

A disadvantage of the sulphopropyl-Sephadex ion-exchange method compared with HPLC is that for each sample a separate column must be used. In addition, the CSF preparative procedures for the HPLC method are simpler; the procedure has fewer steps, which decreases the chance of errors and improves the reproducibility of the results. The higher recovery achieved with this method makes it possible to work with a lower volume (ca. 2 ml) of sample. Not least, the HPLC method is less time-consuming, which favours its clinical applicability.

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