*Journal of Chromatography*, 222 (1981) 381—387
*Biomedical Applications*
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 767

# CHARACTERIZATION OF NEUROPEPTIDES BY REVERSED-PHASE, ION-PAIR LIQUID CHROMATOGRAPHY WITH POST-COLUMN DETECTION BY RADIOIMMUNOASSAY

## APPLICATION TO THYROTROPIN-RELEASING HORMONE, SUBSTANCE P, AND VASOPRESSIN

ELIOT SPINDEL, DOUGLAS PETTIBONE*, LAUREL FISHER, JOHN FERNSTROM and RICHARD WURTMAN*

*Laboratory of Neuroendocrine Regulation and Laboratory of Brain and Metabolism, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139 (U.S.A.)*

(Received August 29th, 1980)

## SUMMARY

Neuropeptide contents of rat brain samples were determined by radioimmunoassay (RIA) after fractionation of tissue extracts by high-performance liquid chromatography (HPLC). Solvent systems were composed of acetic acid, acetonitrile and short-chain (5—8 carbons) alkylsulfonic acids. Separate solvent systems were developed for thyrotropin-releasing hormone, substance P, arginine vasopressin and biologic analogs, and the enkephalins. All separation systems tested gave 80—90% recovery of picogram quantities of peptides. When lyophilized, the HPLC solvents did not interfere significantly with the RIAs, allowing quantitation of tissue concentrations of isolated neuropeptides using the lyophilized eluent from the HPLC. The combination of liquid chromatography with RIA should allow for very accurate identification and quantification of peptides in biologic samples containing large numbers of potentially cross-reacting species of molecules.

## INTRODUCTION

Although interest in the identification and functions of neuropeptides continues to increase, methods for their quantitation in tissue extracts remain inadequate. Neuropeptides are usually measured by radioimmunoassay (RIA); the accuracy of such measurements depends on the specificity and sensitivity of the RIA [1]. The problem of specificity (i.e., does the RIA antiserum cross-

*Present address: Department of Physiology, Uniformed Services Medical School, Bethesda, MD 20014, U.S.A.

react with other than the intended substance in the tissue extract) is often more acute than that of sensitivity because of the presence in tissues of many structurally similar peptides, in widely differing amounts. One strategy for increasing the likelihood that an RIA is actually measuring the intended compound, and not a cross-reacting substance, is to isolate that compound chromatographically prior to its estimation.

Recently, reversed-phase liquid chromatography has become popular as a technique for separating neuropeptides [2, 3] and as a purification step prior to RIA. However, while some peptides are readily isolated by conventional reversed-phase techniques, a number of biologically important peptides are not. For example, substance P (SP) tails when chromatographed reversed-phase, giving poor recovery of picogram quantities [4], and thyrotropin-releasing hormone (TRH) elutes too rapidly for adequate resolution from the solvent front [5]. Such problems in general are amenable to ion-pair chromatography [6—8]. We have explored the possibility as to whether the reversed-phase chromatography of SP and TRH, and of the nonapeptide hormones oxytocin (OXT), arginine vasopressin (AVP), and arginine vasotocin (AVT), can be improved by use of ion-pairing agents. Reversed-phase, ion-pair separations were achieved by complexing positively charged residues of the peptide with the negative charge of an alkylsulfonic acid. The correct choice of the alkylsulfonic acid and of the percentage of organic solvent employed allowed excellent separation and recovery of each of the peptides studied.

## EXPERIMENTAL

### *Materials*

Chromatography was performed on a Micromeritics 7000B high-performance liquid chromatograph (Norcross, GA, U.S.A.) or on a Bioanalytic LC-304 high-performance liquid chromatograph (West Lafayette, IN, U.S.A.) equipped with a column heater. Waters Assoc. (Milford, MA, U.S.A.) $C_{18}$ $\mu$Bondapak reversed-phase columns (30 cm × 3.9 mm I.D., particle size 10 $\mu$m) were used throughout. All chromatography was performed at 60°C with a flow-rate of 2 ml/min. All solvent systems used were combinations of acetonitrile and 0.1% 1-pentane-, 1-heptane- or 1-hexanesulfonic acid in 0.02 *N* acetic acid.

Reagents used were acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), acetic acid (Pierce, Rockford, IL, U.S.A.), 1-pentane-, 1-hexane-, and 1-heptanesulfonic acids (Eastman-Kodak, Rochester, NY, U.S.A.), and bovine serum albumin (BSA; ICN Pharmaceuticals, Cleveland, OH, U.S.A.). Distilled water was further purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Arginine vasopressin (AVP) was purchased from Ferring (Malmö, Sweden); arginine vasotocin (AVT) and MK-771* were gifts of Merck, Sharp and Dohme (Rahway, NJ, U.S.A.); oxytocin (OXT) was purchased from Bachem (Torrance, CA, U.S.A.). TRH was a gift of Abbott Labs. (North Chicago, IL, U.S.A.), 11-methionine sulfoxide-substance P (SP sulfoxide) was a gift of Dr. J.L.M. Syrier (Technische Hogeschool, Delft, The Netherlands). All other peptides were purchased from Peninsula Labs. (San Carlos, CA, U.S.A.).

*L-N-(2-oxopiperidin-6-yl-carbonyl)-1-thiazolidine-4-carboxamide.

*Detection of peptides*

*UV absorption.* Large quantities (500—3000 ng) of peptides were monitored by their absorption at 206 or 210 nm with either an LKB Uvicord detector or a Gilford 2400 spectrophotometer equipped with a flow-cell.

*RIA.* Small quantities (10—500 pg) of peptides were detected by RIA of the HPLC eluent. Fractions (0.5—2.0 ml) were collected with an LKB RediRac fraction collector into 12 × 75 mm culture tubes containing 100 μl 1.0% BSA in water. The tubes were placed directly into a Savant Instruments (Hicksville, NY, U.S.A.) Speed-Vac Concentrator for rapid, high recovery lyophilization. Fractions were reconstituted in RIA buffer and assayed for neuropeptides. Equivalent volumes of lyophilized HPLC solvent were also added to all RIA standard curves to prevent solvent artifacts.

*TRH.* Rat hypothalami were homogenized in 500 μl 2 *N* acetic acid, centrifuged, and the supernatants lyophilized. For injection into the chromatograph, the samples were resuspended in 400 μl HPLC solvent containing 0.1% BSA, centrifuged to remove particulate contamination, and 100 μl injected. The TRH RIA was modified after the method of Bassiri and Utiger [9] as described previously [10]. Maximum sensitivity was 2 pg TRH.

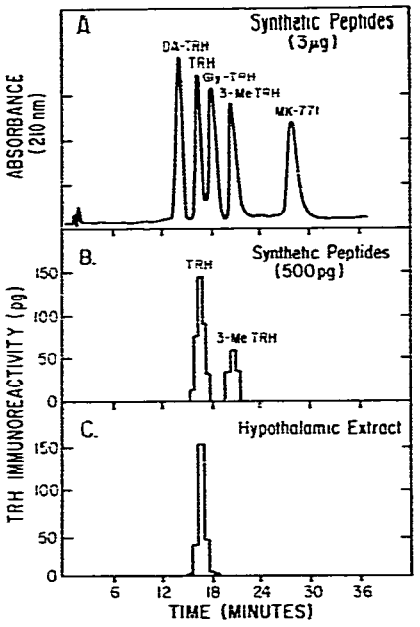
*AVP, AVT, OXT.* Posterior pituitary lobes were homogenized in 500 μl 10% trichloroacetic acid (TCA), centrifuged, and the pellets washed with 500 μl 0.25% acetic acid. The TCA supernatant and the acetic acid wash were combined, extracted with diethyl ether, and lyophilized. For injection into the chromatograph, the samples were resuspended as above. The RIAs for AVT, AVP and OXT were performed as before [11], except that the final dilution of the AVT antiserum was increased to 1:400,000 to give a maximum sensitivity of 0.6 pg AVT per tube.

*SP.* Rat striata were homogenized in 400 μl 2*N* acetic acid, centrifuged, and the supernatants lyophilized. For injection into the chromatograph, the samples were diluted 1:20 and resuspended as described above. The SP RIA was based on the method of Mroz and Leeman [12] as described previously [13]. Maximum sensitivity was 5 pg SP per tube.

## RESULTS

Optimal separation of TRH from its analogs was achieved with 2.75% acetonitrile plus 0.1% hexanesulfonic acid in 0.02 *N* acetic acid (Fig. 1A). Picogram amounts of TRH (pyroglu-his-pro$NH_2$) and 3-MeTRH (pyroglu-3-methyl-his-pro$NH_2$) were readily separated and recovered from the column for quantitation by RIA (Fig. 1B). Recovery of TRH from tissue extraction, HPLC, and lyophilization was greater than 80%. TRH immunoreactivity in rat hypothalamus was easily measured and corresponded chromatographically only to synthetic TRH (Fig. 1C).

The separation of TRH from its analogs varied with the ion-pairing agent used (Fig. 2). Optimization of separation with 1-heptane-, 1-hexane-, or 1-pentanesulfonic acid required decreasing amounts of acetonitrile. Only with 1-hexanesulfonic acid could all analogs be separated (Fig. 2B). With 1-heptanesulfonic acid, no conditions could be found to separate TRH from Gly-TRH (pyroglu-his-pro-gly$NH_2$). With 1-pentanesulfonic acid, Gly-TRH could be



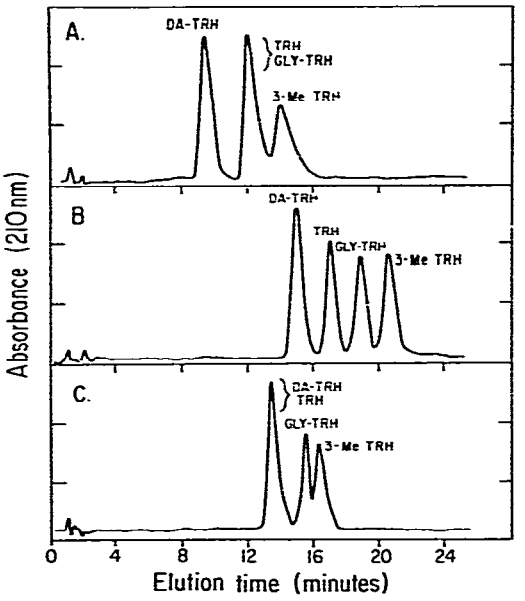



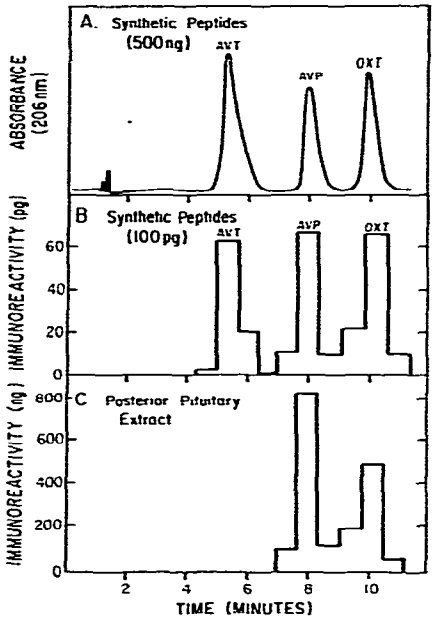

Fig. 1. Separation of TRH and TRH analogs by reversed-phase HPLC. The HPLC solvent was 2.75% acetonitrile plus 0.1% 1-hexanesulfonic acid in 0.02 *N* acetic acid. Chromatograms of (A) standards (3 μg of each peptide); (B) 500 pg TRH and 3-MeTRH; fractions were assayed by RIA; (C) an extract of rat hypothalamus; fractions were assayed by RIA. Peaks: THR = pyroglu-his-proNH$_2$; DA-TRH = pyroglu-his-pro; Gly-TRH = pyroglu-his-proglyNH$_2$; 3-MeTRH = pyroglu-3-methyl-his-proNH$_2$.

Fig. 2. Effect of different ion-pairing reagents on the separation of TRH analogs. (A) 0.1% 1-heptanesulfonic acid plus 6.5% acetonitrile; (B) 0.1% 1-hexanesulfonic acid plus 2.75% acetonitrile; (C) 0.1% 1-pentanesulfonic acid plus 2.0% acetonitrile. Peaks as in Fig. 1.

separated from TRH only by overlapping TRH with deamido-TRH (pyroglu-his-pro). However, when the objective was separation of TRH from deamido-TRH (an important TRH metabolite [14], a solvent with 0.1% 1-heptanesulfonic acid plus 8.5% acetonitrile was most effective, although TRH was no longer clearly separated from 3-MeTRH.

Optimal separation of AVT, AVP, and OXT was achieved with 20% acetonitrile plus 0.1% 1-hexanesulfonic acid in 0.02 *N* acetic acid. AVT eluted in 5.5 min, AVP in 8.1 min and OXT in 10.1 min (Fig. 3A). One hundred picograms each of AVT, AVP, and OXT were readily separated and recovered from the chromatograph for quantitation by RIA (Fig. 3B). Recovery of AVT and AVP was 76—78%; recovery of OXT was 85%. Chromatography of one rat posterior pituitary showed no AVT, 1.1 μg AVP, and 0.76 μg OXT.

Optimal separation of SP from its analogs was achieved with 35% acetonitrile plus 0.1% 1-pentanesulfonic acid (Fig. 4A). Recovery of picogram quantities of SP was greater than 80% (Fig. 4B). The SP immunoreactivity in 1/20 of a single rat striatum was readily quantitated and shown to be almost exclusively authentic SP. A small proportion of SP immunoreactivity was found to correspond to the oxidation product, SP sulfoxide.



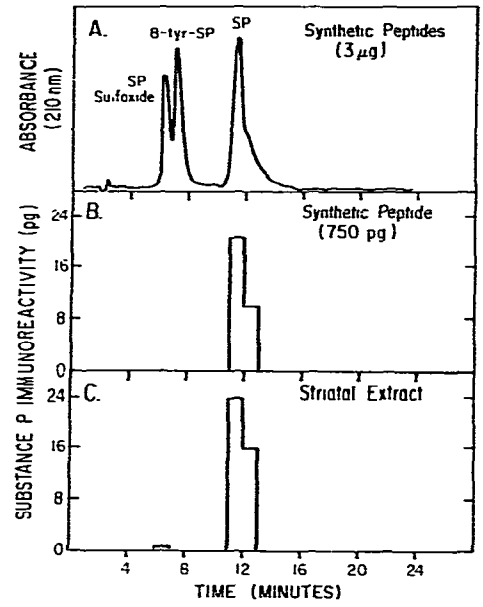




**Fig. 3. Separation of AVT, AVP and OXT. The HPLC solvent was 20% acetonitrile plus 0.1% 1-hexanesulfonic acid in 0.02 *N* acetic acid. Chromatograms of (A) standards (500 ng of each peptide); (B) 500 pg of each peptide; fractions were assayed by RIA; (C) an extract of one rat posterior pituitary; fractions were assayed by RIA.**

**Fig. 4. Separation of substance P and analogs. The HPLC solvent was 35% acetonitrile plus 0.1% 1-pentanesulfonic acid in 0.02 *N* acetic acid. Chromatograms of (A) standards (3 μg of each peptide); (B) 750 pg substance P; fractions diluted 1:20 before RIA; (C) an extract of 1/20 of rat striatum; fractions were assayed by RIA. Peaks: SP sulfoxide = 11-methionine sulfoxide-substance P; 8-tyr-SP = 8-tyrosine substance P; SP = substance P.**

The enkephalins could also be separated with a variation of this solvent system. With 0.1% 1-pentanesulfonic acid plus 20% acetonitrile in 0.02 *N* acetic acid, methionine enkephalin eluted in 4.8 min and leucine enkephalin in 9.0 min (chromatogram not shown).

## DISCUSSION

Reversed-phase, ion-pair HPLC is a versatile procedure for the high-resolution separation of neuropeptides. By varying the ion-pairing reagent and the percentage of acetonitrile, we have devised separation systems for TRH, AVP, SP, and the enkephalins. This general procedure is applicable to neuropeptides of varying size and relative positive charge.

With 1-hexanesulfonic acid as the ion-pairing reagent, TRH was readily separated from its analogs. This separation was better than that previously achieved with heptanesulfonic acid [5]. 1-Heptanesulfonic acid, however, remains the ion-pairing agent of choice when the objective is separation of TRH from deamido-TRH. The high resolution and high recovery of this technique have allowed us to demonstrate that TRH immunoreactivity in rat brain,

pancreas, and spinal cord does represent the authentic peptide [10].

With 1-hexanesulfonic acid as the ion-pairing reagent, rapid separation of AVT, AVP, and OXT was possible. The high recovery and good separation from the solvent front allowed easy quantitation of the relative amount of each peptide in the rat's posterior pituitary. Using this method, we have also found only very tiny amounts of AVT in the rat's pineal [15], where tissue concentrations of all three peptides are too low for fluorimetric detection [16]. The sequential combination of a non-ion-pairing HPLC isolation of AVP [2, 17] with an ion-pairing separation may provide a rapid isolation procedure for studying AVP biosynthesis.

Substance P could be chromatographed with high recovery by use of ion-pairing. Without ion-pair formation, recovery of picogram quantities of SP from HPLC has been poor, although Ben-Ari et al. [18] have used non-ion-pairing HPLC methods to identify SP in rat brain. Substance P has three positive charges, and requires a short-chain (5 carbon) ion-pairing reagent and a high percentage (35%) of acetonitrile.

The chromatographic methods described here are very flexible, and have been easily adapted to four different classes of neuropeptides. With high resolution, high recovery of applied peptide, and the lack of significant interference from the lyophilized solvent in RIAs, these chromatographic procedures may be ideal for characterizing other neuropeptide immunoreactivities in tissue extracts. Some cautions in the use of ion-pair chromatography are that (1) the ion-pair reagent remains in the solvent residue after lyophilization and might influence some RIAs (although apparently not those for the peptides considered here), and (2) minor variations in the composition of the HPLC solvent can cause marked variation in peptide retention times.

ACKNOWLEDGEMENTS

These studies were supported in part by grants from the National Institutes of Health (HD 11722) and The Center for Brain Sciences and Metabolism Charitable Trust. We thank Dr. S.E. Leeman for providing the anti-substance P serum used in these studies. Dr. Spindel and Dr. Pettibone were recipients of fellowships from the Whittaker Foundation. Dr. Fisher was the recipient of a predoctoral fellowship from NIMH.

REFERENCES

1 E. Straus and R.S. Yallow, in H. Gainer (Editor), Peptides in Neurobiology, Plenum Press, New York, 1977, p. 39.
2 R. Burgus and J. Rivier, in A. Loffet (Editor), Peptides, 1976, Éditions de l'Université de Bruxelles, Belgium, 1976, p. 85.
3 R. Burgus and J. Rivier, in M. Goodman and J. Meienhofer (Editors), Peptides -- Proceedings of the Fifth American Peptide Symposium, Wiley, New York, 1977, p. 52.
4 D.J. Pettibone and R.J. Wurtman, unpublished results.
5 E. Spindel and R.J. Wurtman, J. Chromatogr., 175 (1979) 198.
6 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, Science, 200 (1978) 1168.
7 W.S. Hancock, C.A. Bishop, L.J. Meyer, D.R.K. Harding and M.T.W. Hearn, J. Chromatogr., 161 (1978) 291.

8 J. Rivier, J. Liquid Chromatogr., 1 (1978) 343.
9 R.M. Bassiri and R.D. Utiger, Endocrinology, 90 (1972) 722.
10 E.R. Spindel and R.J. Wurtman, Brain Res., 201 (1980) 279.
11 J.D. Fernstrom, L.A. Fisher, B.M. Cusack and M.A. Gillis, Endocrinology, 106 (1980) 243.
12 E.A. Mroz and S.E. Leeman, in B.M. Jaffe and H.R. Behrman (Editors), Methods of Hormone Radioimmunoassay, Academic Press, New York, 1978, p. 121.
13 D.J. Pettibone and R.J. Wurtman, Brain Res., 186 (1980) 409.
14 H. Knisatschek and K. Bauer, J. Biol. Chem., 254 (1979) 10936.
15 L.A. Fisher, E.R. Spindel and J.D. Fernstrom, 62nd Annual Meeting of the Endocrine Society, Washington, DC, June 18—20, 1980, Abstr. No. 113.
16 K.A. Gruber, S. Stein, L. Brink, A. Radhakrishnan and S. Udenfriend, Proc. Nat. Acad. Sci. U.S., 73 (1976) 1314.
17 D.D. Blevins, M.F. Burke and V.J. Hruby, Anal. Chem., 52 (1980) 420.
18 Y. Ben-Ari, P. Pradelio, C. Gros and F. Dray, Brain Res., 173 (1979) 360.