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Short communication

Solid-phase extraction of arginine vasotocin and isotocin in fish samples and subsequent gradient reversed-phase high-performance liquid chromatographic separation

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Abstract

Gradient high-performance liquid chromatography (HPLC) preceded by a solid-phase extraction (SPE) step is described for determining arginine vasotocin and isotocin, the neurohypophysial nonapeptides, in fish plasma samples. The combination of these two methods significantly improves the separation and increases the sensitivity of the assay. The proposed assay may be a useful alternative for analysis of similar nonapeptides in plasma without the use of radioisotopes, while taking into consideration a difference in detection sensitivity.

1. Introduction

Arginine vasotocin (AVT) and isotocin (IT) are two nonapeptides produced in hypothalamic neurosecretory neurons in response to osmotic challenges in teleost fish (Fig. 1). The neurohypophysial secretion of these hormones is sensitive to exposure to different environmental salinities as these fish migrate between freshwater and seawater [1–4]. AVT, the non-mammalian counterpart of the well-known vasopressin, plays a role in osmoregulation, cardiovascular activity, endocrine secretion, and reproductive processes and appears to act as a neurotransmitter and/or neuromodulator in the central nervous system in fish [5–10]. It has been intensively studied recently with regard to its physiological actions, also in other vertebrates [11–25].

This paper describes a sensitive and rapid method for the determination of low levels of AVT and IT in plasma samples, employing high-performance liquid chromatography (HPLC) with UV detection preceded by a solid-phase extraction (SPE). The aim of this study was to combine the two methods in order to improve the separation and quantitative measurement without the use of radioisotopes.

ARGININE VASOTOCIN

Cys - Tyr - Ile - Gln - Asn - Cys - Pro - Arg - Gly - NH_2

ISOTOCIN

Cys - Tyr - Ile - Ser - Asn - Cys - Pro - Ile - Gly - NH_2

Fig. 1. Amino acid sequences of the natural neurohypophysial hormones in teleost fish.

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2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile, water, methanol and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Deventer, Netherlands). Glacial acetic acid and hydrochloric acid were supplied by E. Merck (Darmstadt, Germany). Synthetic AVT and IT and ethanol were obtained from Sigma (St. Louis, MO, USA).

Standard solutions of synthetic AVT (1 mg/ml) and IT (1 mg/ml) were prepared by dissolving each compound in HPLC-grade water immediately before use. Working standards were prepared by diluting a portion of these standard solutions with HPLC-grade water to give final concentrations ranging from 10 to 1000 ng/ml.

2.2. Animals and sampling

Rainbow trout (*Salmo gairdneri*) was obtained from a hatchery (Institute of Inland Fisheries in Rutki, Poland). Animals were kept in tanks on a commercial trout diet. All blood samples, taken at the time of sacrifice, were collected into centrifuge tubes, centrifuged at 1000 *g* for 5 min to obtain plasma and stored in dry ice.

2.3. Sample pretreatment with SPE

The Baker SPE 12G Column Processor (J.T. Baker, Phillipsburg, NJ, USA), a specially designed vacuum manifold capable of processing simultaneously up to 12 SPE columns, was used. AVT and IT were extracted from plasma by reversed-phase chromatography using a C_{18} Bakerbond SPE cartridge of 1 or 3 ml, respectively (pore size 60 Å, particle diameter 40 μm). The columns were conditioned according to the manufacturer's instructions with two portions of methanol (each of 1 or 3 ml, according to column volume) followed by two portions of HPLC-grade water. Plasma was acidified (100 μl 1 *M* HCl/ml plasma) according to the method presented previously by LaRochelle et al. [27]. The acidified sample was aspirated through the appropriate column (1 or 3 ml), washed with a portion of HPLC-grade water followed by a portion of glacial acetic acid–HPLC-grade water (4:96), each of 1 or 3 ml, according to column volume. The sample was eluted with two portions of 6 *M* HCl–absolute ethanol (1:2000). The eluate was collected, dried under air and stored at −20°C prior to analysis. Before assay, each sample was reconstituted with acidified ethanol to 100 μl and mixed well. The 10-μl samples were injected into HPLC for analysis.

2.4. High-performance liquid chromatography

HPLC was performed with a Beckman modular system (Beckman Instruments, San Ramon, CA, USA), consisting of two Model 110B solvent delivery modules, a system organiser with a Model 210A sample injector valve, and a column heater connected in series with a Model 166 programmable UV detector. Data were digitised by a Beckman 406 analog interface and processed by a Beckman analytical series System Gold data acquisition software on an IBM AT compatible computer.

Chromatographic separations were carried out on an Ultrasphere ODS column (250 × 4.6 mm I.D., 5 μm particle diameter, 80 Å pore size) connected to a precolumn (45 × 4.6 mm I.D.) filled with the same material and both obtained from Beckman Instruments. The column temperature was maintained at 22°C at a flow-rate of 1.0 ml/min, and the eluate was monitored at 215 nm. A gradient elution system was prepared. Solvent A was 0.1% TFA in water and solvent B 0.1% TFA in acetonitrile–water (3:1). A linear gradient from 20 to 40% eluent B in 20 min was used. All mobile phases were saturated with helium to remove the air bubbles and dissolved air.

3. Results and discussion

The preliminary extraction efficiency (percentage recovery) was obtained by comparing standard solutions analysed by HPLC before and after having been subjected to SPE. Recovery was 96% for AVT and IT. In order to establish

Table 1
AVT recovery test

AVT added (ng/ml)	AVT obtained (ng/ml)	Recovery (%)
500	479.5 ± 8.9	95.8
100	98.3 ± 9.0	98.3
50	48.3 ± 6.2	96.6
10	9.5 ± 0.4	94.5

Known amounts of AVT were added to 1 ml "zero plasma" extract containing no AVT. Values following purification represent mean ± S.D. ($n = 3$).

the real recovery of AVT and IT, the "zero plasma" extract was used (i.e. the plasma extract obtained from a former SPE procedure using a C_{18} SPE cartridge). The known amounts of synthetic AVT and IT added to 1-ml aliquots of "zero plasma" extract were mixed well for 20 min, subjected to SPE and analysed as described above. The recovery for various amounts of AVT and IT from plasma ranges from 94 to 98% and from 93 to 94%, respectively (Tables 1 and 2). These results are comparable with the data for synthetic vasopressin and oxytocin published elsewhere [26–28]. The recovery was found to be optimal when the sample was applied to the cartridge and then eluted at very low flow-rates (less than 1 ml/min). This result is in accordance with the flow-rates studied previously by Higa and Desiderio [29]. The chromatogram of "zero plasma" extract shows no peaks corresponding to endogenous AVT or IT. Separations of standards are presented in Figs. 2 and 3.

Fig. 2 shows the chromatogram resulting when

Table 2
IT recovery test

IT added (ng/ml)	IT obtained (ng/ml)	Recovery (%)
500	471.8 ± 10.0	94.4
100	93.5 ± 8.4	93.5
50	47.1 ± 6.2	94.2
10	9.3 ± 0.7	93.1

Known amounts of IT were added to 1 ml "zero plasma" extract containing no IT. Values following purification represent mean ± S.D. ($n = 3$).

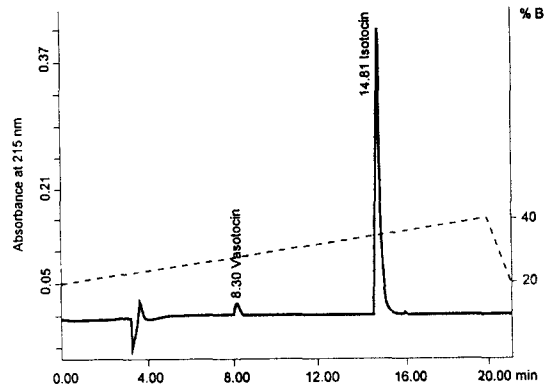

Fig. 2. Chromatogram of a sample obtained by spiking a 1-ml "zero plasma" extract with 10 ng of AVT and 250 ng of IT. Chromatographic conditions: injection volume 10 μl; column Ultrasphere ODS 5 μm (45 × 4.6 mm I.D. and 250 × 4.6 mm I.D.); elution: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile–water (3:1), linear gradient 20–40% eluent B in 20 min; flow-rate 1.0 ml/min; detection UV, 215 nm; temperature 22°C.

10 ng of AVT and 250 ng of IT were added to a "zero plasma" extract after SPE followed by HPLC separation. The retention time of synthetic AVT and IT was 8.30 and 14.81 min, respectively. Fig. 3 represents the chromatogram of a "zero plasma" extract containing 250 ng AVT and 25 ng IT. The retention times of synthetic AVT and IT were 8.23 and 14.88 min, respectively. Fig. 4 shows a representative chromatogram from a typical fish plasma sample after SPE. The retention times of endogenous AVT

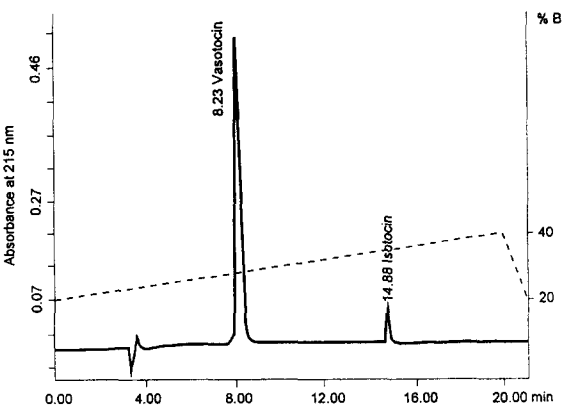

Fig. 3. Chromatogram of a sample obtained by spiking a 1-ml "zero plasma" extract with 250 ng of AVT and 25 ng of IT. Conditions as in Fig. 2.

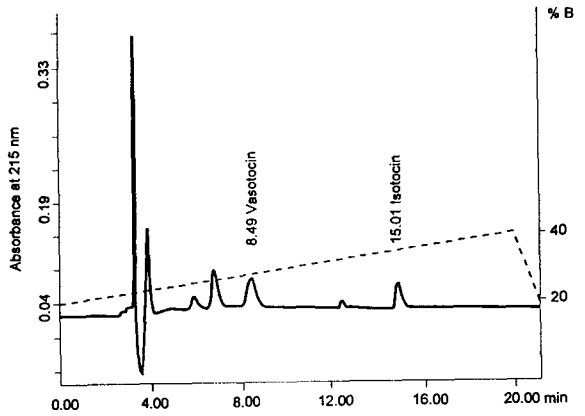

Fig. 4. Chromatogram for a typical fish plasma sample prepared by solid-phase extraction. Conditions as in Fig. 2.

and IT were 8.49 and 15.01 min, respectively. The endogenous AVT and IT were identified by their retention times compared with those of standards. Quantitative determination of these peptides in plasma was performed on the basis of a standard curve as described above, and linearity of signal responses was observed in the range of 10–500 ng/ml (coefficient of determination $r^2 = 0.989$). In this study, the detection limit for these peptides was 10 ng/ml (injected volume 10 μl).

The most difficult part of many chromatographic analyses of biological samples is the elimination of interfering matrix components. If the analytes are only present at trace levels, the selective removal of the large excess of matrix interferences without impairment of the recovery of the desired peptides is a striking challenge for the analyst. In addition, concentrating the analyte, increasing the concentration per unit volume of solvent, is often required when the sample solution is too dilute for direct injection. This alternative separation technique employs "trapping" of the analytes of interest by SPE, and thereby the desired components are extensively concentrated. They are then eluted from the cartridge with a small amount of solvent of strong elution potency and do not require a further extensive and tedious sample concentration procedure, as for example, would be the case in liquid–liquid extraction. As a consequence, SPE proved to be superior to the more or less traditional and widely used extraction methods [26–32] and thus allows a reliable and highly sensitive quantitation of small amounts of the peptides.

In this study, AVT and IT were well separated from each other and from matrix components in a repetitive manner. The described methodology was applied to the measurement of the concentration of endogenous peptides in fish plasma samples. The proposed method may therefore be a useful alternative to previously reported procedures for neurohypophysial peptides [26–28, 33–38]. Compared with radioimmunoassay, the principal disadvantage of the system is that a large volume of sample (10 ml) is required, when low concentrations are being measured. The principal advantage is that the system is a comparatively simple non-biological assay for AVT and IT.

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