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# Determination of arginine–vasotocin and isotocin in fish plasma with solid-phase extraction and fluorescence derivatization followed by high-performance liquid chromatography

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**Abstract**

A new HPLC assay for plasma arginine–vasotocin (AVT) and isotocin (IT) determination based on fluorescence detection preceded by combination of solid-phase extraction (SPE) and fluorescence derivatization is presented. Plasma samples retained on solid support were purified and then derivatized by the fluorescent compound 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The peptide derivatives were eluted from cartridges, pre-concentrated and analyzed by HPLC system with fluorescent detection. The separation was carried out on a reversed-phase column with solvent gradient system. The assay was linear in the range 15–220 pmol ml$^{-1}$ for AVT ($r^2 = 0.998$) and 10–220 pmol ml$^{-1}$ for IT ($r^2 = 0.996$). The detection limits for AVT and IT were 0.8 and 0.5 pmol ml$^{-1}$ (3:1, signal-to-noise), respectively. The recoveries of derivatized hormones were in the range 89–93%. Both of the inter- and intra-day assay precision were below 5.5 and 9% for AVT and IT, respectively. The assay should be also applicable to plasma and tissue samples from other animals with only minor modification.


*Keywords:* Derivatization, LC; Arginine–vasotocin; Isotocin

## 1. Introduction

Arginine–vasotocin (AVT) and isotocin (IT) are nonapeptides produced in the hypothalamic neurosecretory neurones of teleost fish. Both hormones are stored and released in neurohypophysis. AVT is present in all vertebrate classes being considered as the ancestral peptide in the neurohypophysial family. IT, however, is restricted to teleost fish only. Vasotocin and isotocin are analogues to well-known mammalian neurohypophysial hormone vasopressin and oxytocin, respectively. The physiological significance of AVT is connected with its well-known osmoregulation and cardiovascular activity in fish [1]. Moreover, AVT acts as neurotransmitter and/or neuromodulator in the central nervous system being responsible for reproductive behavior in many vertebrates [2–5].

There are two satisfactory approaches to measurement of fish neurohypophysial nonapeptides in plasma: RIA [6,7] and HPLC methods [8–11]. In general, HPLC assay is less-time consuming and does not require radioisotopes and antibodies. Moreover, the HPLC assay may be more specific due to potential cross-reactivity of antibodies in RIAs. However, the principal advantage of proposed method over RIA is that the determination of both, AVT and IT, in one plasma sample has been achieved.

To date, only a few HPLC methods for AVT determination have been reported: HPLC with UV [8], MS [9] and fluorescence detection [10,11]. However, simultaneous determination of AVT and IT is presented only in one case [8]. Low sensitivity of UV detection is a drawback of the method, thus the pre-concentration of large volume of fish plasma is required. HPLC assay with fluorescence detection, which is more sensitive and selective than UV and less expensive

*Abbreviations:* AVT, arginine–vasotocin; FD, fluorescent detection; IT, isotocin; ESI-MS, electrospray ionization mass spectrometry; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; RIA, radioimmunoassay; UV, ultra violet

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than MS, seems to be a satisfactory alternative. A main disadvantage of recommended fluorescence detection is that neurohypophysial hormones do not possess natural fluorescence and a label has to be incorporated into molecules. A pre-column derivatization is one of the methods widely used. Two assays for determination of neurohypophysial peptides with a pre-column derivatization have been reported, where vasopressin and oxytocin were transformed into fluorescent compounds with flluorescamine [11] and vasotocin with the addition of monobromobimane (mBBr) [10].

In the case of biological samples, which contain large number of individual compounds and very low concentrations of compounds of interest (e.g. hormones), direct pre-column fluorescent derivatization may lead to a detection problem. It can be overcome by purification the samples (e.g. by SPE) before derivatization or performing both procedures simultaneously on solid support. There are two attitudes towards the second method: derivatizing reagent (solid-phase reagent) is immobilized on a polymeric solid support and analyte is passed through the column [12,13] or analyte is adsorbed on solid carrier and derivatization is carried out by passing derivatizing reagent through the cartridge [14–17].

In this paper, a new HPLC assay with fluorescence detection for plasma AVT and IT has been presented. The combination of solid-phase extraction (SPE) and fluorescence derivatization significantly increases the sensitivity of the assay.

## 2. Experimental

### 2.1. Animals and sampling

Blood samples for AVT and IT were collected from fish (rainbow trout and flounder) at the time of sacrifice, centrifuged at $1000 \times g$ for 10 min to obtain plasma and stored in $-70\,^{\circ}C$ prior to analysis.

### 2.2. Chemicals and reagents

NBD-F was purchased from Sigma–Aldrich (Steinheim, Switzerland). Trifluoroacetic acid (TFA) and HPLC-grade acetonitrile were supplied by J.T. Baker (Deventer, Netherlands). Synthetic AVT and IT was obtained from BACHEM (Bubendorf, Switzerland).

All other chemicals of HPLC or analytical reagent grade were purchased from commercial sources.

Mili-Q pure water was used throughout the work.

The synthetic AVT and IT were dissolved in HPLC grade water ($1\,mg\,ml^{-1}$, 0.952 and 1.035 μM, respectively) and stored at $-20\,^{\circ}C$. Standard solutions were obtained by diluting stock solutions with HPLC grade water. Calibration curves were constructed using seven calibration standards. NBD-F solution was prepared in acetonitrile ($2\,mg\,ml^{-1}$, 10.92 μM) and kept at $-20\,^{\circ}C$.

Borate buffer (0.1 M, pH 9.5) was prepared from borax.

### 2.3. Apparatus and chromatographic conditions

SPE procedures were carried out on BAKERBOND spe™ Octadecyl cartridges (100 mg, 1 ml) connected to the Baker SPE 12G column Processor (J.T. Baker, Phillipsburg, NJ, USA).

HPLC analyses were performed with the Beckman Instruments (San Ramon, CA, USA) consisting of two solvent delivery modules (Model 110B), a system organizer with a Model 7725i sample injector valve, a column heater connected in a series with a spectrofluorometric detector RF-551 (Shimadzu, Columbia, MD) and a programmable UV detector (Model 166). Data were digitized by a Beckman analytical series System Gold data acquisition software.

Chromatographic separations were accomplished on an Ultrasphere ODS column (250 mm × 4.6 mm i.d., 5 μm particles diameter) preceded by a precolumn (45 mm × 4.6 mm i.d.) filled with the same material and both obtained from Beckman Instruments. The column temperature was $25\,^{\circ}C$ and flow rate $1\,ml\,min^{-1}$. Fluorescence detection was carried out at 530 nm with excitation at 470 nm. The eluate was monitored with UV at 215 nm.

All mobile phases were saturated with helium to remove the air bubbles and dissolved air.

Gradient elution system was employed for separation of derivatized hormones. Solvent A was 0.1% TFA in water and solvent B, 0.1% TFA in acetonitrile–water (3:1). A linear gradient was 48–80% of eluent B in 20 min.

Identification of peaks was made by comparison with retention times of standard peptides. Selected peaks were subjected to MS analysis.

### 2.4. Derivatization on solid support

Plasma sample (1 ml) or standard solution of AVT and IT (20 μl diluted to 1 ml with water) were acidified with 1 M HCl (100 μl) and transferred to the SPE column, which was previously conditioned with methanol (1 ml) and water (1 ml). Then water (1 ml), 4% acetic acid (1 ml) and again water (1 ml) were passed successively through the cartridge to wash out impurities. Solution of NBD-F in acetonitrile (50 μl) and borate buffer (450 μl, pH 9.5) were injected onto the column and passed through it slowly under gravity. The cartridge was covered with aluminum foil and kept at room temperature ($20\,^{\circ}C$) for 20 min. Then water (1 ml) and 30% methanol (1 ml) were successively passed through the column. The derivatized peptides were eluted with mixture of ethanol: 6 N hydrochloric acid (1000:1; 2 ml). The elutes were evaporated to dryness under a gentle stream of helium. The samples were dissolved in mobile phase (100 μl) and injected (20 μl) into the HPLC system.

## 3. Results and discussion

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was chosen for derivatization, as it reacts easily with amino group and gives highly fluorescent derivatives in short time [18,19].

Our preliminary experiments of derivatization in solution showed that this process is highly pH, temperature and time dependent. These parameters were tested using standard solutions of peptides to optimize reaction conditions on solid support.

Plasma nonapeptides, AVT and IT, were introduced onto SPE column in acidized medium [8]. Most of interfering plasma compounds from cartridges were eluted with 4% acetic acid. Additional washing (water) was recommended to remove traces of acid before derivatization step which required basic pH. As it was shown in Fig. 1, in lower pH peptides flowed out partially from solid carrier during passing the buffer through the cartridge. Thus, to minimize loss of peptides, derivatization step was performed at pH 9.5, and

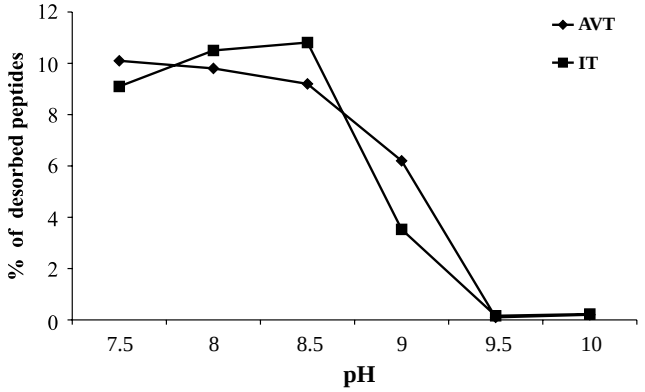


Fig. 1. Effect of different pH values of borate buffer (0.1 M) on the peptides desorption from solid support. The amounts of AVT and IT adsorbed on SPE cartridges were 120 nmol peptide/cartridge.

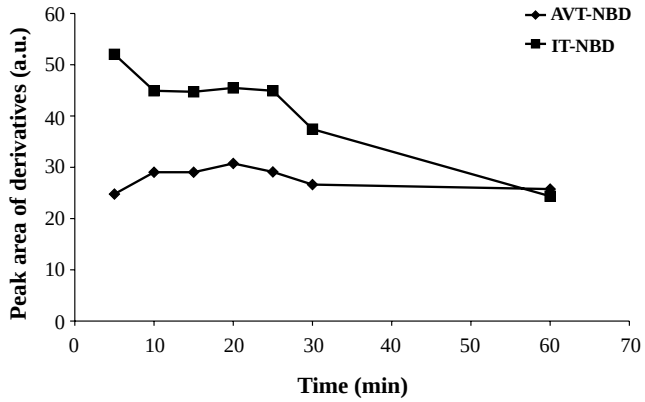


Fig. 2. Effect of reaction time on the formation of the reaction product of AVT and IT with NBD-F at 20 °C and pH 9.5 (a.u.: arbitrary units).

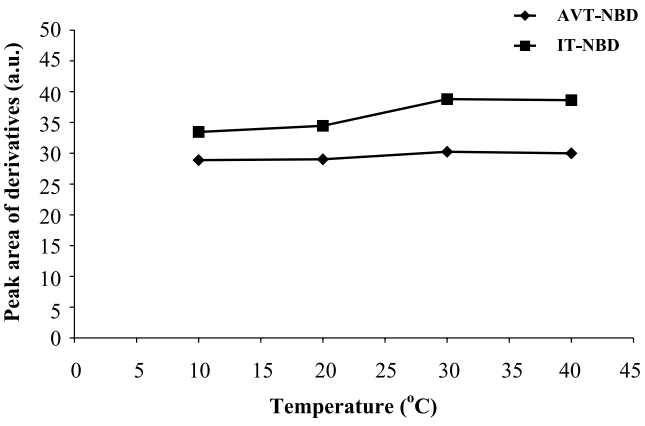


Fig. 3. Effect of temperature on the reaction of AVT and IT with NBD-F at pH 9.5 after 20 min on $C_{18}$ SPE cartridges (a.u.: arbitrary units).

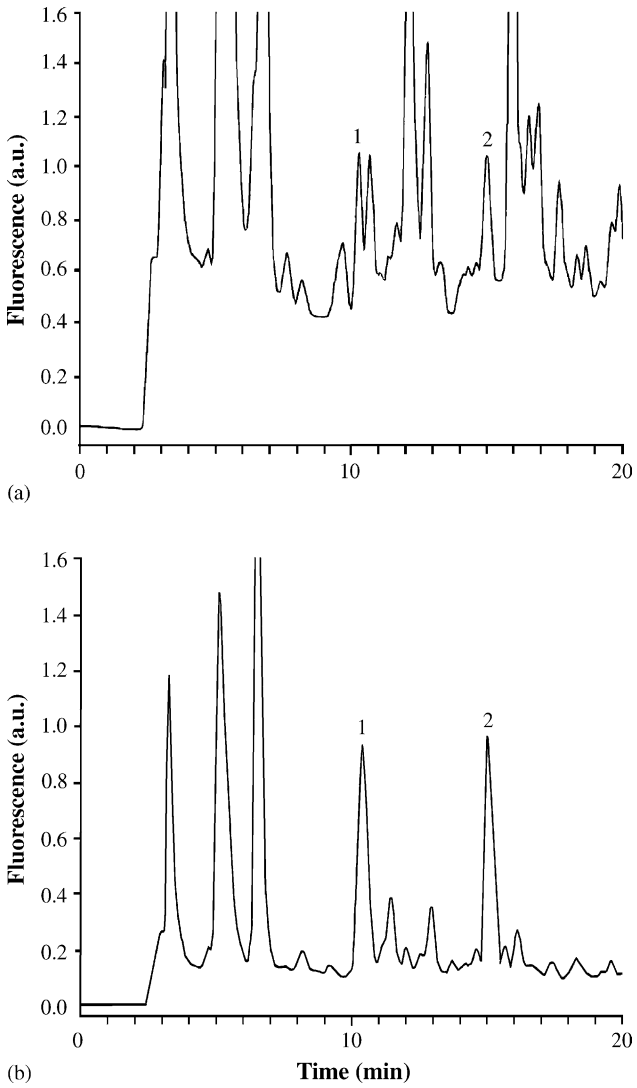


Fig. 4. Chromatogram of derivatized fish plasma sample on solid support: (a) without additional washing-up step, (b) with additional washing-up step (water followed 30% methanol)—the concentration of plasma AVT and IT: 1.3 and 2.8 pmol $ml^{-1}$, respectively. Chromatographic conditions: injection volume 20 μl; column: Ultrasphere ODS (250 mm × 4.6 mm, 5 μm); elution: A was 0.1% TFA in water and solvent B, 0.1% TFA in acetonitrile–water (3:1), linear gradient 48–80% eluent B in 20 min at flow rate 1 ml $min^{-1}$; fluorescence detection: $\lambda_{ex} = 470$, $\lambda_{em} = 530$. Peaks: (1) AVT-2NBD, (2) IT-3NBD (a.u.: arbitrary units).

Table 1
ESI-MS analysis of specified peaks

| Peptide derivative | Signal (*m*/*z*) | Retention time of corresponding peak (min) |
|---|---|---|
| **AVT-2NBD**<br>**NBD–NH–Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Arg–Gly–$NH_2$**<br>(S–S; O–NBD) | 1376.4<br>688.2 | 10.69 |
| **IT-3NBD**<br>**NBD–NH–Cys–Tyr–Ile–Ser–Asn–Cys–Pro–Ile–Gly–$NH_2$**<br>(S–S; O–NBD; O–NBD) | 1455.2 | 14.90 |

was preceded by combined buffer and NBD-F subjection onto cartridges.

The effect of time on derivatization was tested at pH 9.5 and at 20 °C (Fig. 2). It was established that shorter time of reaction resulted in higher fluorescent signal for IT derivative. Inversely, the most intense respond signal for vasotocin was observed after 20 min of reaction course. As a compromise between sensitivity for both hormones, 20 min of derivatization was selected.

Formation of derivatized peptides at different temperature ranged between 10 and 40 °C was studied at pH 9.5. As it was presented in Fig. 3, temperature did not influence on the reaction rate meaningfully and therefore an ambient temperature (20 °C) was selected for further studies.

The typical chromatogram showing separation of derivatized plasma peptides was shown in Fig. 4a and b.

An excess of derivatizing reagents and most of by-products of derivatization were removed by additional washing-up step (water followed by 30% methanol). This process unables to eliminate most of interfering compounds (Fig. 4b). A chromatogram corresponding to plasma derivatized on SPE cartridges without additional washing-up step was shown in Fig. 4a. The concentrations of arginine–vasotocin and isotocin in plasma samples were determined to be 1.3 and 2.8 pmol $ml^{-1}$, respectively.

Molecules, AVT and IT, posses multiple nucleophilic sites which theoretically can react with NBD-F. It has been reported that strongly hydrophilic phenol groups react with NBD-F similarly as amine site [20], so that it was necessary to provide an evidence that only one product was formed in this reaction. Selected peaks were analyzed by ESI-MS techniques and corresponding signals (*m*/*z*) were shown in Table 1.

All available functional groups were fully derivatized resulting in di-substituted vasotocin and three-substituted isotocin. Derivatized peptides when kept at +4 °C were stable for a few days.

The calibration, using standard peptides derivatized with NBD-F on solid support, was linear in the range 15–220 pmol $ml^{-1}$ ($r^2$ = 0.998) for AVT-NBD and 10–220 pmol $ml^{-1}$ for IT-NBD ($r^2$ = 0.996). The detection limit for AVT and IT was 0.8 and 0.5 pmol $ml^{-1}$, respectively (3:1 signal-to-noise).

Intra-day variations were determined calculating the recoveries and repeatability of the assay, using plasma samples spiked with standard peptides. Plasma samples were pooled and standards of AVT and IT at three different concentrations were added. The sample was derivatized on solid support five times within one day and the recovery and repeatability was determined. The precision of inter-day assays were determined by derivatizing (five times) the same sample on each day (3 days).

The recoveries of derivatized hormones were in the range 89–93%. The repeatability, expressed as relative standard deviation (R.S.D.), was 2–4.5 and 5.3–8.2% for vasotocin and isotocin, respectively. The inter-day precision (reproducibility) was in the range 2.5–5.5% for AVT and 5.5–8.5% for IT.

The higher R.S.D. values for isotocin derivative seem to be as a result of a chemical structure of the peptide. A hormone molecule posses three functional groups (more than AVT) which can be derivatized. Adsorbtion on solid support may affect the accessibility of functional sites. It may be reflected in poorer repeatability of IT.

Taking together, the method is adequate for determination of neurohypophysial peptides in fish plasma samples. The assay should be also applicable to other animals neurohypophysial nonapeptides, including humans vasopressin and oxytocin, with only minor modification.

## 4. Conclusions

A new sensitive method for determination of neurohypophysial peptides in plasma with HPLC and fluorescence detection was established. Sample purification and derivatization were integrated into the same process. Optimization of the reaction conditions made the detection limit of hormones as low as 0.5 pmol $ml^{-1}$.

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