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Development and validation of a capillary electrophoresis tandem mass spectrometry analytical method for the determination of Leu-Val-Val- and Val-Val-hemorphin-7 peptides in cerebrospinal fluid

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ABSTRACT

A CE-tandem MS method was optimised and validated for selective and specific determination of LVV- and VV-hemorphin-7 peptides in cerebrospinal fluid. These two small peptides originate from haemoglobin beta chains. They possess relevant biological activity and recently a potential biomarker role in posterior cranial fossa paediatric brain tumour disease was evidenced. The separation was optimised using formic acid as background electrolyte and a water/methanol mixture, containing 0.1% (v/v) formic acid, as sheath liquid. The two peptides, differing in only one amino acid of the sequence at the N-terminal side were baseline separated in less than 15 min. The method allowed a very reduced and rapid sample pretreatment and was successfully applied to hemorphins determination in patient samples without matrix interferences. The method successfully passed bioanalytical validation showing linearity, accuracy and precision data on cerebrospinal fluid matrix within the acceptable values. The analysis of cerebrospinal fluid of patients affected by different posterior cranial fossa tumour forms confirmed our previous findings showing the absence of hemorphins in the pre-surgical cerebrospinal fluid and their presence in the post-ones and controls. The present method saves costs and time due to capillary electrophoresis miniaturisation and to the absence of chromatographic column and gradient elution and allows numerous injections per sample starting from few microlitres of cerebrospinal fluid.

1. Introduction

LVV- and VV-hemorphin-7 (LVV- and VV-h7) are members of the hemorphins family, a group of non-classical opioid peptides with affinity towards μ- and σ-opioid receptors [1,2].

Based on the N-terminal sequence, the following hemorphins sub-families can be distinguished: LVV-, VV-, V-hemorphins and hemorphins [2]. The sequence YPW is essential for the opioid activity in the binding capacity to the opiate receptor. LVV- and VV-hemorphin-7 are deca- and nona-peptides, respectively, with sequence corresponding to fragment 32–41 and 33–41, respectively, of the haemoglobin beta chain (Table 1). In fact, the hemorphins are endogenous bioactive peptides derived from haemoglobin through the action of tissue specific proteolytic enzymes [2–4]. LVV- and VV-h7 were usually found in the central nervous system (CNS) tissues [1,4,5]. They are probably generated by cathepsin D [6,7]. Although, it was initially supposed that the source of hemorphins was erythrocyte haemoglobin (Hb), a key paper demonstrated that the brain Hb derived fragmentome origins only minimally from erythrocyte haemoglobin [8]. Rather, alpha and beta globin chains expressed in neurons and glial cells of rat and human could be the main source of brain hemorphins [9,10].

In addition to opioid like action, other relevant biological activities of hemorphins have been discovered: the capacity of lowering blood pressure, as inhibitors of angiotensin converting enzyme (ACE), their connection to beta-endorphin release and the low affinity towards orphan bombesin receptor subtype 3, involved in the modulation of intracellular calcium and protein phosphorylation [1,11]. Particularly, the LVV-h7 was supposed to be the endogenous ligand of the At4 receptor, the same receptor of angiotensin IV that is widely distributed in brain and peripheral tissues [12].

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Table 1
Characteristics of analysed peptides.

Amino acid sequence	Protein reference	M_r
LVVYPWTQRF	Hb (human) subunit β fragment 32–41	1308.4
VVYPWTQRF	Hb (human) subunit β fragment 33–41	1194.3

Recently, the important role of hemorphins in the organism resistance to homeostatic disturbance was also reported [13].

Due to the interesting biological activity of hemorphins, several papers studied their presence and variation in tissue and biological fluids in relation to different diseases. LVV-h7 was found in ventricular cerebrospinal fluid of patients with intracerebral bleeding but not in spinal samples of healthy subjects [14]. The ligand capacity of hemorphins to At4 receptor and the inhibition of ACE stimulated the investigation of their possible role in neurodegenerative diseases [15] and the study of their possible administration as therapeutic drug [16]. A general increase of all hemorphins, including LVV-hemorphin-7, was recognised in brain tissues from Alzheimer's disease patients, probably evidencing a vascular abnormality by amyloid angiopathy of the disease [15]. In another study the central administration of LVV-h7 improved learning and memory in normal rodents and was able to reverse memory deficits in animal models with amnesia [16].

LVV- and VV-hemorphin-7 were identified in both adrenal tissues of patient affected by pheochromocytoma tumour and healthy subjects [17]. LVV-h7 was found in an isolated BAL sample of lung cancer patient [18]. Reduced levels of hemorphins-7 were present in serum of breast cancer patients with respect to controls, suggesting their role as disease biomarker [19].

In vitro studies on tumour cell cultures evidenced a cytotoxic and antiproliferative activity of different hemorphin groups [20]. Particularly, the LVV-type resulted more antiproliferative than cytotoxic, whereas the VV-type and the valorphin, corresponding to the sequence VVYPWTQ, exhibited the reverse effects. The presence of Leu at the N-terminal produces the loss of cytotoxicity for hemorphin whereas in VV-hemorphins the presence of Gln is essential for cytotoxic effects.

Recent proteomic studies of our group evidenced a potential biomarker role of LVV- and VV-hemorphins-7 in paediatric posterior cranial fossa brain tumour [21]. The two peptides were present only in the post-surgery cerebrospinal fluid after total tumour removal and in the control samples of age in the range of patients and are good prognostic candidates. In fact a partial tumour resection or the presence of metastasis in the patient at the diagnosis did not restore the hemorphins content in post-surgery cerebrospinal fluid.

These results stimulated the development of a validated analytical method allowing fast screening of LVV- and VV-h7 in multiple samples characterised by precision, accuracy and fast analysis time for deeper investigations and possible future diagnostic applications.

The first analytical method for the determination of LVV-h7 in ventricular cerebrospinal fluid of patients with intracerebral bleeding was carried out by size exclusion chromatography and ESI mass spectrometry [14]. In recent years, together with HPLC analytical technique and mass spectrometry or UV detection, hemorphins determinations in biological matrix were also performed using capillary electrophoresis (CE) technique [21–23] with UV and/or off-line MS detection. In the view of administration of hemorphins as drug, a CZE-UV method was developed for the evaluation of LVV-hemorphin-7 catabolism induced by aminopeptidase M or ACE enzymes cleavage in spiked human plasma [21] and in vitro [23,24]. Off-line MALDI-TOF was used for metabolites characterisation. No papers report in literature the development of a CE analytical method in coupling with on-line ESI mass spectrometry for hemorphins quantitation.

CE and CE-ESI-MS techniques were successfully applied to proteins and peptides analysis, as documented in recent review papers [25–27]. The direct coupling of CE with mass spectrometry provides immediate specificity to CE analytes quantification well matching the analytical power of the technique with the selectivity and structure characterisation of tandem mass spectrometry detection.

The purpose of our work was the optimisation and validation of a new analytical method by capillary electrophoresis in coupling with on-line ESI-mass spectrometry for the dosage of LVV- and VV-h7 in cerebrospinal fluid using the corresponding deuterated peptides as internal standard.

The method was optimised by studying the most critical parameters influencing both the CE separation and the peptides mass spectrometry ionisation, and validated by testing linearity, precision, accuracy and recovery.

2. Materials and methods

2.1. Chemicals

Methanol and acetonitrile LC–MS, trifluoroacetic acid (TFA) (HPLC) and formic acid (98%) were from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Ultra pure water was obtained from P.Nix Power System Apparatus, Human, Seoul, Korea. Sodium hydroxide pellets, pro analysis, were from Merck, Darmstadt, Germany.

LVV-, VV-hemorphin-7 and the corresponding deuterated (d8) peptides were synthesised in laboratory following the procedure described in Section 2.3. The LVV- and VV-hemorphins stock solutions 2×10^{-3} M were prepared by dissolving the synthesised peptides hydrolysate with pure methanol and stored at −20 °C. Further dilutions were made in 0.1% (v/v) TFA aqueous solution and stored until use at −20 °C.

2.2. Apparatus

Capillary electrophoresis automated apparatus was from Agilent Technologies (Waldbronn, Germany) equipped with diode array UV detector and external nitrogen pressure. The CE apparatus was coupled to the Esquire 3000 plus mass spectrometer (Bruker Daltonics, Bremen, Germany) via a coaxial sheath liquid electrospray ionisation (ESI) interface (Agilent Technologies, Waldbronn, Germany). The sheath liquid was delivered by an external syringe pump (Cole Palmer, Vernon Hills, IL, USA) at a constant flow rate of 180 and 240 μL/h for CE-MS and flow injection analysis (FIA), respectively. Nebulising and drying gas (nitrogen) were set at 41368.5 Pa and 4.0 L/min, respectively. Dry gas temperature was 250 °C. Mass spectrometry capillary voltage was 4500 V. Separations were performed in 50 μm I.D., 375 μm O.D. fused silica uncoated capillaries (Composite Metal Services, Hallow, Worcs., UK) of total length of 87 cm. Effective length was 21.5 cm for UV detection and 87 cm for MS detection.

Tandem mass spectrometry (MS^2) detection of the analytes was performed in product ion scan mode by activating the Multiple Reaction Monitoring (MRM) windows using an isolation width of ±4.0 m/z and fragmentation amplitude of 1.0 V and 2.0 V for the hemorphins and d8-hemorphins, respectively, in positive ionisation mode and normal resolution scan. The acquisition of the MS^2 extracted ion current (EIC) signals was made in 400–1400 m/z mass scan range using a maximum accumulation time of 200 ms and a set target value of 50,000 and by activating the ion charge control (ICC) function. The sheath liquid consisted of water/methanol