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Preparation and evaluation of an immunoaffinity sorbent with Fab′ antibody fragments for the analysis of opioid peptides by on-line immunoaffinity solid-phase extraction capillary electrophoresis–mass spectrometry

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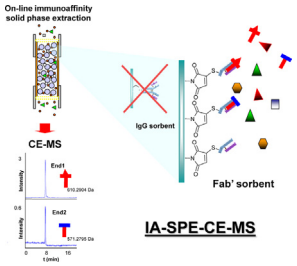
HIGHLIGHTS

- We prepared an IA sorbent with antibody fragments for the analysis of opioid peptides by IA-SPE-CE–MS.
- We followed a site-specific antibody immobilization approach.
- The main features of the IA sorbent were studied.
- Endomorphins 1 and 2 were analyzed in standards and plasma samples to achieve the best LODs.

GRAPHICAL ABSTRACT



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ABSTRACT

An immunoaffinity (IA) sorbent with antibody fragments was prepared for the analysis of opioid peptides by on-line immunoaffinity solid-phase extraction capillary electrophoresis–mass spectrometry (IA-SPE-CE–MS). The antibody fragmentation was evaluated by MALDI-TOF-MS. Fab′ fragments obtained from a polyclonal IgG antibody against Endomorphins 1 and 2 (End1 and End2) were covalently attached to succinimidyl silica particles to prepare the IA sorbent. An IA-SPE-CE–MS methodology was established analyzing standard solutions of End1 and End2 and acceptable repeatability, linearity ranges and LODs (0.5 and 5 ng mL^{-1}, respectively) were obtained. The LOD of End1 was slightly better than that previously obtained using an IA sorbent with intact antibodies (1 ng mL^{-1}). In human plasma samples, End1 and End2 could be detected at 1 and 50 ng mL^{-1}, respectively, which meant an improvement of 100 and 2-fold with regard to the LODs using an IA sorbent with intact antibodies (100 ng mL^{-1}).

1. Introduction

During decades, several innovations have been reckoned in order to improve CE selectivity, sensitivity and applicability [1–9], which have given rise to various techniques, such us CE–MS and immunoaffinity capillary electrophoresis (IACE). The benefits of CE–MS for the analysis of peptides and proteins are world-renowned [1–9]. IACE combines the resolving power of the electrophoretic separation with the sensitivity and specifity of immunoassays so that it can be extremely useful for enrichment and clean-up of low abundant components from complex matrices (e.g. peptides in biological fluids) [10–14]. Among the numerous IACE formats, which mainly differ in the set-up to establish the antigen–antibody interaction before the electrophoretic separation [10–14], it is worth highlighting the relevance of on-line immunoaffinity solid-phase extraction capillary electrophoresis (IA-SPE-CE) [12–14]. IA-SPE-CE is a variant of on-line solid-phase extraction capillary electrophoresis (SPE-CE) based on the

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immobilization of antibodies or antibody fragments onto the inner wall or an appropriate solid support as a small binding area integrated usually near the inlet of the separation capillary or the channels of a microchip [12–14]. One of the typical configurations consists in the insertion of an extraction microcartridge or analyte concentrator, which contains the IA sorbent that retains the antigenic analyte, enabling the introduction of large volumes of sample. The retained analyte is later eluted in a small volume of an appropriate solution, resulting in sample clean-up and concentration enhancement [13–18].

Unlike the typical chromatographic sorbents used in SPE [18–20], IA sorbents are highly appreciated because of their prominent selectivity and extraction efficiency when the immunoreactivity of the antibody and the active surface area on the IA sorbent are optimum. However, IA sorbents with the most appropriate features for IA-SPE-CE are not commercially available [11,12,21–28]. Between the wide variety of preparation methods that have been described, the covalent immobilization methods are the most popular to attach intact antibodies or their fragments onto different surfaces [22–28]. In general, first it is necessary to activate the solid support, the antibody or the fragments obtained after digestion [29–31], in order to generate the desired reactive groups so that the immobilization takes place through a specific region. Orientation of the antibodies or antibody fragments on the solid support is of great concern because the performance of the IA sorbent depends on the availability of the binding sites for antigen–antibody interaction [12,26,27]. In addition, the analysis conditions have to be carefully controlled because extreme ionic strengths, temperatures and acidic or alkaline pH values could affect the antigen–antibody interaction or could cause antibody denaturation [12,28]. The coupled antibody is usually an immunoglobulin G (IgG) because in general presents higher affinity for the antigen than other immunoglobulin classes [31]. The use of IgG fragments to prepare IA sorbents for IA-SPE-CE has been described by several authors [12,28]. However, there is no scientific evidence that points out that the use of antibody fragments over intact antibodies, and vice versa, provides improved results in IA-SPE-CE [25].

Laser-induced fluorescence detection has been commonly applied in IA-SPE-CE for the analysis of low molecular mass immunoreactive compounds in biological fluids and tissues [32–36]. In contrast, on-line MS detection has been explored little due to the difficulties of making it compatible with the requirements of IA-SPE-CE [16,37,38]. IA-SPE-CE–MS requires the use of volatile solutions in order to prevent salt build-up in the mass spectrometer. Furthermore, the use of extreme rinsing solutions and acidic BGE could cause the denaturation of the antibodies and antibody fragments of the IA sorbents, as well as analyte elution [12,28]. In a previous work, we demonstrated that the LODs for the CE–MS analysis of standard solutions of End1 and End2, which are opioid peptides related to nociception [39–41], could be improved 100-fold, until detecting 1 ng mL^{-1} by IA-SPE-CE–MS using an IA sorbent containing an intact IgG covalently attached to a solid support through the carbohydrates of the Fc region. However, in human plasma samples both peptides could be only detected down to 100 ng mL^{-1} [38]. As an alternative, the present article describes the preparation of an IA sorbent following the maleimide method (Fig. 1), based on the covalent immobilization of Fab′ fragments from the same IgG to succinimidyl silica particles [16,23,24,32–36]. The maleimide method was originally described to couple proteins and peptides to several materials such as quartz disks, polymers and sepharose [24] and it has been used to prepare IA sorbents in the last decade [16,23,24,32–36]. Following this immobilization method, the antigen binding sites of the Fab′ fragments are also supposed to be appropriately oriented at the opposite end of their attachment onto the solid support, facilitating the capture of the target analytes. The potential of the IA sorbent for the analysis by IA-SPE-CE–MS of End1 and End2 at the extremely low concentrations that are usually found in body fluids has been evaluated with standards and plasma samples [39–41].

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used in the preparation of buffers and solutions were analytical reagent grade. Acetonitrile, methanol, propan-2-ol, boric acid (99.8%), formic acid (HFor, 98–100%), acetic acid glacial (HAc), trifluoroacetic acid (TFA, 99%), ammonia (25%), sodium hydroxide (98–100%), sodium borate (98%), ammonium acetate (99%) and potassium dihydrogen phosphate anhydride (99.5%) were purchased from Merck (Darmstadt, Germany). Acetic anhydride (99.0%), sinapinic acid (99.0%), ethylendiaminetetraacetic acid disodium salt 2-hydrate (EDTA, 99–101%), sodium azide (99.5%), diethyl ether (99.7%), pyridine (99.8%), 4-(dimethylamino)pyridine (DMAP, 99%), 2-mercaptoethylamine·HCl (2-MEA, 98%) and polyethylene glycol (8000 Da, 50%) were provided by Sigma (St. Louis, MO, USA). ImmunoPure Fab′ micro preparation kit (Fab′ kit), dithiothreitol (DTT) and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) were supplied by Fisher Scientific (Madrid, Spain). Water with specific electric conductivity lower than 0.05 μS cm^{-1} was obtained using a Milli-Q water purification system (Millipore, Molsheim, France).

End1 (Tyr-Pro-Trp-Phe-NH_2, 610.2904 Da), End2 (Tyr-Pro-Phe-Phe-NH_2, 571.2795) and the polyclonal IgG fraction from rabbit serum (95%) were purchased from Sigma (St. Louis, MO, USA). The rabbit polyclonal IgG against End1 and End2 (immunogen affinity purified) was provided by Gene Tex (Irvine, CA, USA). Sep-Pak Classic NH_2 cartridges (55–105 μm particle diameters, 125 Å (1 Å = 0.1 nm) pore size, 970 μmol of NH_2 per gram of particle) were provided by Waters (Milford, MA, USA).

2.2. Standard solutions, electrolytes, sheath liquid

An aqueous stock standard solution (2500 mg L^{-1}) of each peptide was prepared and stored in a freezer at −20 °C when not in use. Diluted standard peptide mixtures of End1 and End2 were used for the analysis, as well as to spike human plasma samples. The 100 mM phosphate buffer solution (PBS) for the generation of IgG fragments was part of the Fab′ kit. The 50 mM borate buffer solution for the preparation of the IA sorbent was adjusted to pH 7.6 with boric acid. A phosphate buffer solution for the storage of the IA sorbent was prepared from 100 mM of potassium dihydrogen phosphate and pH was adjusted to pH 7.4 with NaOH (1 M). The background electrolyte (BGE) for CE–MS and IA-SPE-CE–MS was a solution of 20 mM of ammonium acetate adjusted to pH 7.00 with ammonia. The buffer and BGE concentrations are referred to the final solution and the pH value was adjusted before making up solution to the final volume and then measured again. The BGE was passed through a 0.45 μm nylon filter (MSI, Westboro, MA, USA) before the analysis. The sheath liquid solution consisted of a hydroorganic mixture of 60:40 (v/v) propan-2-ol:water, with 0.05% (v/v) of HFor and was degassed for 15 min by sonication before use. All solutions were stored at 4 °C when not in use.

2.3. Human plasma and sample preparation

Human plasma samples from healthy blood donors were kindly supplied by the *Banc de Sang i Teixits* (Hospital de la Vall d′Hebron, Barcelona). Venous blood was collected in standard clinical vials and placed on ice. Plasma was separated from the blood cells, pooled, placed into polyethylene tubes and frozen at −20 °C.