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Analysis of biological samples by capillary electrophoresis with laser induced fluorescence detection

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ABSTRACT

In this paper an overview is provided on practical difficulties as well as applications of capillary electrophoresis coupled to laser induced fluorescence detection methods in the field of analysis of biological samples. Various methodological approaches elaborated for determination of small molecules, peptides and proteins are outlined. Besides giving an overview on detection based on native fluorescence, immune and enzyme assays, the main focus is the problematics of sample derivatization and achievable detection sensitivities in the analysis of real biological samples. The characteristics and applicability of the most commonly used labeling reagents are discussed in details.

1. Introduction

Analysis of biological samples has its own difficulties, because of the usually limited amount of sample specimens, the low analyte concentration, the complex sample matrix, etc. While capillary electrophoresis requires small sample volumes, and has high efficiency to separate considerable number of sample components, it may suffer from the poor concentration sensitivity of the most widely used UV absorbance detection and the deteriorating effect of the sample matrix on separation efficiency. There are strategies developed to overcome or alleviate these difficulties, *e.g.* using a more sensitive detection mode or application of some sample clean-up methods or on-capillary concentration. Compared to UV absorbance, laser induced fluorescence detection offers better sensitivity, besides it is regarded to be more selective (for review see [1–5]). Although there are various applications of CE-LIF in the analysis of biological samples demonstrating these advantages, this method also has its own limitations and cannot be applied easily for all kinds of analytes and biological samples.

In this review it is intended to outline the use of CE-LIF in the analysis of endogenous small molecules, drugs, peptides and proteins in biological samples, giving examples of excellent applications, but mainly focusing on some of the difficulties. Analysis of nucleic acids and carbohydrates is not included into the present discussion.

2. Determination of analytes having intrinsic fluorescence

The advantages of CE-LIF can mainly be achieved when analytes with native fluorescence are to be determined, although these applications comprise a smaller proportion of CE-LIF determinations. Commonly three excitation wavelengths are used for detection of sample components having intrinsic fluorescence. These are 257/266/275/284 nm range, 325 nm and 488 nm wavelength of the common laser sources. The excitation wavelength is a fundamental determinant of the achievable detection sensitivity. At higher wavelength fewer compounds can be detected, thus the selectivity increases and sensitivity improves. Besides interfering peaks from the biological sample matrix, the quantum yield and the proper fit of the wavelength of excitation and the laser source are the main determinants of the detection limits.

UV lasers: solid state diode lasers, frequency doubled Argon-ion lasers and Krypton-ion laser are available as excitation sources at 266 nm, 257/275 nm and 284 nm, respectively. They have been used for the determination of biologically active amines and some drugs, as well. In low UV range relatively high number of analytes can be excited, however the noise from the sample matrix, and light

Abbreviations: CBQCA, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; CFSE, carboxyfluorescein succinimidyl ester; DTAF, 5-(4,6-dichrolotriazinyl) aminofluorescein; ERK, extracellular signal-regulated protein kinase; FITC, fluorescein isothiocyanate; FQ, 3-(2-furoyl)quinoline-2-carboxaldehyde; GABA, γ-amino-butyric acid; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NDA, naphthalene dicarboxaldehyde; OPA, *ortho*-phtalaldehyde; SAMF, 6-oxy-(*N*-succininmidyl acetate)-9-(2′-methoxycarbonyl) fluorescein; SIFA, *N*-hydroxysuccinimidyl fluorescein-O-acetate.

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scattering can limit the detection performance. In biological samples interference derives from the native fluorescence of proteins, peptides, nucleotides and other aromatic compounds resulting in rather complex electropherograms while impairing sensitivity analyzing real samples.

Serotonin, catecholamines and their metabolites could be measured in various biological samples with 10–100 nM detection limits [6,7]. On-capillary preconcentration methods [6,8] or selective sample extraction [9] was used to further improve detection sensitivity to the subnanomolar concentration range needed to analyze urine samples. Based on the native fluorescence in deep UV range propranolol and methylenedioxymethamphetamine (Ecstasy) have been determined in plasma and urine samples, respectively. However, the reported detection sensitivity was in the 10^{-7} M range, which is only slightly better than that of conventional UV detection [10,11].

HeCd laser source also can be used in the UV range at 325 nm wavelength. This higher wavelength is accompanied by a better selectivity due to less interfering compounds in the biological matrices resulting in improved sensitivity.

Impressive detection limit of 10^{-10} M concentration range has been reported for pteridines, probable cancer biomarkers; although aqueous standard solutions were used for the calibration and no data were provided on analyte concentration in the urine samples analyzed [12]. Several drugs, like fluoroquinolones [13,14], triamteren [15], phenprocoumon [16], tramadol [17], carvedilol [18], zaleplon [19] zopiclone [20] and salicylates [11,21] also exhibit native fluorescence fitting the lower wavelength of HeCd laser. In real biological samples, the improvement in detection sensitivity was 10–100 times compared to UV detection.

The application of UV laser induced native fluorescence detection is not really widespread so far, because the commercially available CE instruments are not equipped with these types of expensive laser sources.

The most widely used is the argon-ion laser having a 488 nm excitation wavelength that perfectly fits the detection of flavins and anthracyclines. Because of the low interference at the visible wavelength, 10^{-10} to 10^{-9} M detection limits were routinely achieved for flavin vitamers in plasma [22] and various tissues [23]. Terabe and co-workers combined LIF detection and dynamic pH junction-sweeping preconcentration method to further decrease the limit of detection by two orders of magnitude [24,25].

In case of anthracycline anticancer drugs, like doxorubicin, daunorubicin and idarubicin 10^{-9} to 10^{-8} M detection limit in human serum samples was achieved [26]. In a recent work liposome enclosed doxorubicin was separated from the free drug to assay the stability of liposomal preparation during various conditions, including in plasma samples. Due to quenching effect of the liposomes the fluorescence was more than five times less intense resulting in similarly poorer detection limit [27].

3. Detection of analytes without fluorofore

As considerably less compounds possess native fluorescence compared to those having UV absorbance, this detection mode usually requires sample derivatization, or indirect detection can be performed. This latter approach is hardly applicable in case of biological samples as it does not provide the needed selectivity since all the sample components are detected. Its sensitivity is much less compared to the direct methods, the detection limits are similar to that of the direct UV detection and are in the micromolar concentration range [28,29]. As the direct methods are considerably more sensitive, sample derivatization with a fluorofore or a fluorogenic tag is used in majority of cases, although this is not without difficulties, as is discussed in the next paragraphs.

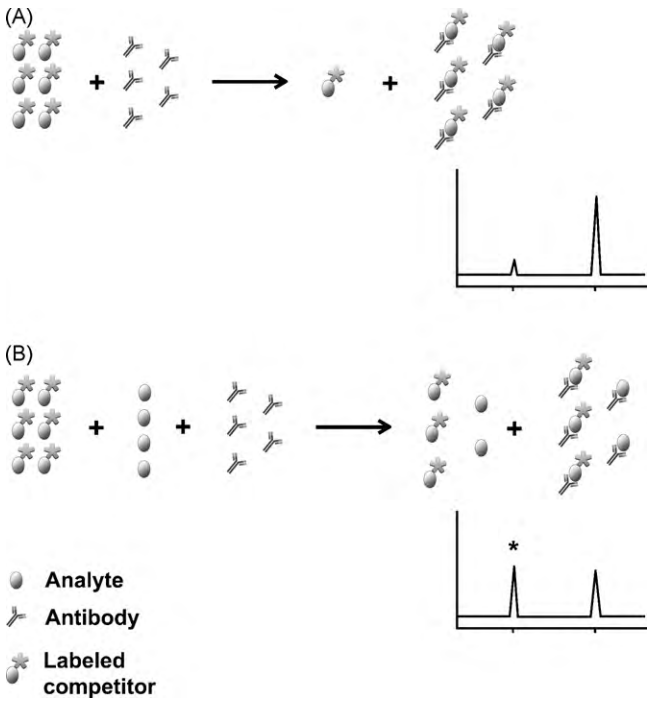

Fig. 1. Scheme of competitive immunoassay in the absence (A) and presence (B) of anlyte. The labeled competitor and the analyte are reacted with limited amount of antibody followed by the separation of the formed immunecomplex from the excess antigen. Asterisk indicates the peak corresponding to excess competitor. The increase of this peak correlates with analyte concentration.

Beside covalent derivatization, interaction with a fluorofore probe is another possibility to use for determination of the analytes, like dynamic labeling of proteins [4,30] or DNA [31–34] and immune and enzyme assays [35]. The advantage of this latter approach is that a preformed labeled probe is used, which is either commercially available or can be prepared at high concentration followed by purification. At high concentration the labeling reaction is more reliable and as the high excess of the labeling reagent and side products can be removed, there are no interfering peaks deriving from the derivatization reaction. Using prelabeled probes the electropherograms are less complex, resulting in easier separation and peak identification as well as higher sensitivity due to better selectivity. The same applies for monitoring enzyme reactions, when the separation of the two fluorescent sample components, the substrate and the product is usually easy to perform. The listed advantages allow achieving detection sensitivity in the subnanomolar concentration range.

3.1. Competitive immunoassays

Some of the immunoassays based on the competition between the analyte and its fluorescently labeled derivative for a limited amount of antibody. In this case the labeled antigen should be separated from its complex formed with the antibody. The amount of the competing analyte is proportional to the increase of peak corresponding to the labeled antigen (Fig. 1). Using this competitive immunoassay arrangement, proteins and peptides have been determined in biological samples. Method has been developed to assess prion protein in blood as a potential clinical diagnostic tool for spongiform encephalopathy [36]. Recombinant hirudin has been assessed in plasma samples with 20 nM detection limit [37]. Vasopressin has been measured in cerebrospinal fluid down to nanomolar concentrations using FITC-labeled vasopressin as fluorescent probe [38]. Methionine-enkephalin has been determined in the plasma and increased level has been found in cancer