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Rapid analysis of trace levels of flavins by pressurized capillary electrochromatography-laser induced fluorescence detection with sulfonated N-octadecyl methacrylate monolith

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ABSTRACT

In this paper, pressurized capillary electrochromatography (pCEC) with laser induced fluorescence detection (LIF) was demonstrated as a viable approach for the separation and determination of trace flavins in human plasma, where flavins tend to be degraded *ex vivo*. Using a sulfonated N-octadecyl methacrylate monolithic column in isocratic pCEC separation, symmetrical peak shapes and rapid separation could be obtained in a weakly acidic mobile phase. Baseline separation of riboflavin, flavin mononucleotide and flavin adenine dinucleotide could be achieved within 4.5 min in a mobile phase containing 60% (v/v) acetonitrile and 40% (v/v) of 20 mmol L^{-1} phosphate buffer (pH 4.0), with −22 kV of applied voltage and 290 psi of supplementary pressure and 0.02 mL min^{-1} of flow rate. Based on a 473 nm laser diode double pumped solid state source, flavins could be determined by LIF with the detection limit (LOD) as low as 0.5 nmol L^{-1} (S/N = 3). The concentration ranges were 0.005–2 μmol L^{-1} for RF and FMN, and 0.02–40 μmol L^{-1} for FAD. Owing to the weakly acidic condition selected in this experiment, the high fluorescence quantum yields and good stability of flavins contributed to a preferable analysis. Combined with a simple clean-up procedure, this method has been proved to be effective for the rapid and selective analysis of trace levels of flavins in human plasma without sample preconcentration.

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1. Introduction

Capillary electrochromatography (CEC) has gained increasing attention and rapid development in recent years. The interest in CEC could be explained by the fact that it combines both the high efficiency of capillary electrophoresis (CE) and the high selectivity of high performance liquid chromatography (HPLC). CEC has been demonstrated to be very promising for application-driven studies in complex matrix [1,2]. However, the extensive applications of pure packed CEC were always hindered by the poor reproducibility derived from bubble formation and column drying-out [3], which usually occurred at the retaining frit (interface of the packed and unpacked sections) of packed column as a result of the change in electroosmotic flow (EOF). Recent development in monolithic column [4], and pressurization technique of CEC by applying pressure at one end or two ends of capillary, provide alterative effective solutions for these problems. Pressurized capillary electrochromatography (pCEC), which uses a HPLC pump to superimpose a hydraulic pressure at the inlet end of the capillary column onto an electrically generated flow [5], is the most feasible way that could be conducted more rapidly, easily and reproducibly than "pure" CEC at little expense in column efficiency, as well as the use of buffers with higher ionic strengths and higher voltage were allowed.

Laser induced fluorescence (LIF) detection is to date one of the most sensitive detection methods that is well suitable for microseparation. The combination of CEC with LIF can offer high efficiency and sensitivity along with the benefits of small sample requirements and have attracted many attentions in the detection of trace levels of drugs analytes [6,7] since Yan et al. first introduced in 1995 [8]. Recently, Kato et al. applied a photopolymerized sol–gel monolithic column to determine micromolar level of amino acids in rat cerebrospinal fluid [9]. Due to the low content (usually at micromolar–nanomolar levels) in complex matrix and relative short half-life of analytes, the application of pCEC-LIF to the analysis of biological constituents in biofluids is still rare currently.

Riboflavin (RF) and its two biologically active metabolites, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)

Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; RF, riboflavin; pCEC, pressurized capillary electrochromatography; LD-DPSS, laser diode double-pumped solid-state.

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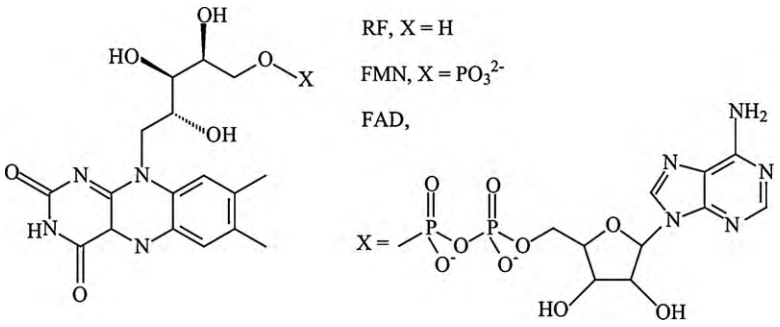

Fig. 1. Structures of Riboflavin (RF), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).

(as shown in Fig. 1), serve as essential redox-active coenzymes associated with numerous flavoenzymes that catalyze a variety of biochemical reactions, and present in most of biological organism [10]. As an important clinical nutritional index, quantification of flavins in plasma is essential for studying vitamins metabolism and evaluation of riboflavin deficiency [11]. However, flavins are instable *ex vivo* associated to chemical degradation by exposition to light, alkaline or extremely acidic media, and atmospheric oxidation [12]. They also tend to degrade in plasma samples within 10 min via rapidly enzymatic hydrolysis [13]. For the purpose of clinical assessment or biochemical studies, a rapid and sensitive method for simultaneous monitoring of flavins in biological fluids is of great demand.

So far, a series of analytical methods have been developed to determine flavins simultaneously in biological or food samples, including HPLC [14–16], capillary liquid chromatography [17], CE [12,18–21], and microchip capillary electrophoresis [22]. Gradient elution mode, high water content and relative long pretreatment and analysis time were usually needed in HPLC analysis of flavins with high hydrophilicity. Furthermore, asymmetrical and too wide peaks were always inevitable in commercial packed reversal phase HPLC column, due to the irreversibly adsorption of flavins with the silanol groups of stationary phase [15]. Although flavins have high fluorescence yields and stability in weakly acidic conditions [19], alkaline buffers were actually adopted in preconcentration–CE–LIF [18,20,21] for achieving rapid analysis and better resolution. In that case, additional peaks due to alkaline hydrolysis or photodegradation will hamper the quantitative analysis [22]. In neutral or weakly alkaline conditions, surfactants or cyclodextrins were needed in the buffers to separate FMN and FAD, which have similar charge–mass ratio.

Most of previous works with LIF detection [18–20] usually employed the sensitive but expensive and bulky gas laser as an excitation source. Recently, a less cost and more compact blue light-emitting-diodes (LEDs) as a light source was also introduced to the LIF analysis of RF [21], however, relative low detection efficiency and high-background signals limited its further bioanalysis application. The laser diode double-pumped solid-state (LD-DPSS), which combines the advantages of gas lasers with LEDs to a large extent [23], provides an ideal excitation source for LIF detection for endogenous flavins.

In this work, an isocratic elution pCEC coupled with LD-DPSS LIF detection method has been developed for the rapid separation and sensitive determination of flavins in human plasma. The sulfonated N-octadecyl monolith prepared in our lab [24] could generate strong EOF even at weakly acidic condition, which is quite meaningful for rapid monitoring of flavins with low degradation in clinical analysis. As a result, short analysis time and high responses of flavins could be achieved at weakly acidic condition. Several electrochromatography parameters were evaluated and optimized. With a minimal sample preparation, fast and sensitive pCEC-LIF analysis of flavins in human plasma sample was realized.

2. Experimental

2.1. Instrumentation

All experiments were performed on a modified TriSep-2010GV CEC system (Unimicro Technologies, Pleasanton, CA, USA) (see Fig. 2), which consisted of a solvent delivery gradient module, a micro fluid manipulation module (including a 0.8 μL six-port injection valve, a four-port split valve, and a back-pressure regulator), a high voltage power supply (0 ± 30 kV), a data acquisition module, and a LD-DPSS LIF detector (10 mW, λ_{ex} = 473 nm; λ_{em} = 530 nm) (Dalian Institute of Chemical Physics, Chinese Academy of Sciences). Samples are injected to the injection valve and introduced in the sample loop, and then be carried to the four-port split valve by the mobile phase flow. Under constant pressure controlled by a back-pressure regulator (Upchurch Scientific), the total flow from the four-port valve was split into two paths, one entered the capillary column, and the other was flow into waste solvent bottle. In that case, the flow rate in the capillary column was constant, while the waste flow rate varied correspondingly with the pump flow rate, therefore the splitting ratio can be adjusted. A negative voltage was applied to the outlet of column, and the inlet of column was connected to the four-port split valve and grounded.

2.2. Reagents and materials

Flavin adenine dinucleotide (FAD, 95%) and flavin mononucleotide (FMN, 95%) were purchased from Sigma–Aldrich (St. Louis, MO, USA); riboflavin (RF, 98%) was from Alfa (Ward Hill, MA, USA). Acetonitrile (ACN) and methanol (Chemical Reagent Corporation, Shanghai, China) were of HPLC grade. Sodium phosphate monobasic, sodium phosphate dibasic dodecahydrate, sodium hydroxide, and phosphoric acid (Chemical reagent Plant, Shanghai, China) were of analytical grade. Octadecyl methacrylate (OMA), 3-sulfopropyl methacrylate potassium salt (SPMA), ethylene dimethacrylate (EDMA), 2,2′-azobisisobutyronitrile (AIBN) and 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS) were purchased from Aldrich (Milwaukee, WI, USA). All the other chemicals were of analytical grade. All sample and buffer solutions were prepared with ultrapure water supplied by a Milli-Q purification system (Millipore, Milford, MA, USA). Fused-silica capillaries were purchased from the Yongnian Optic Fiber Plant (Hebei, China).

FMN and FAD stock solutions were prepared as 5.0×10^{-3} mol L^{-1} in methanol-water (50:50, v/v) respectively. RF stock solutions were prepared as 5.0×10^{-3} mol L^{-1} in Milli-Q water. The stock solutions were stored in darkness at −20 °C prior to use. These stock solutions were further diluted by mobile phase daily and used as standard solutions prior to pCEC injection. Peaks

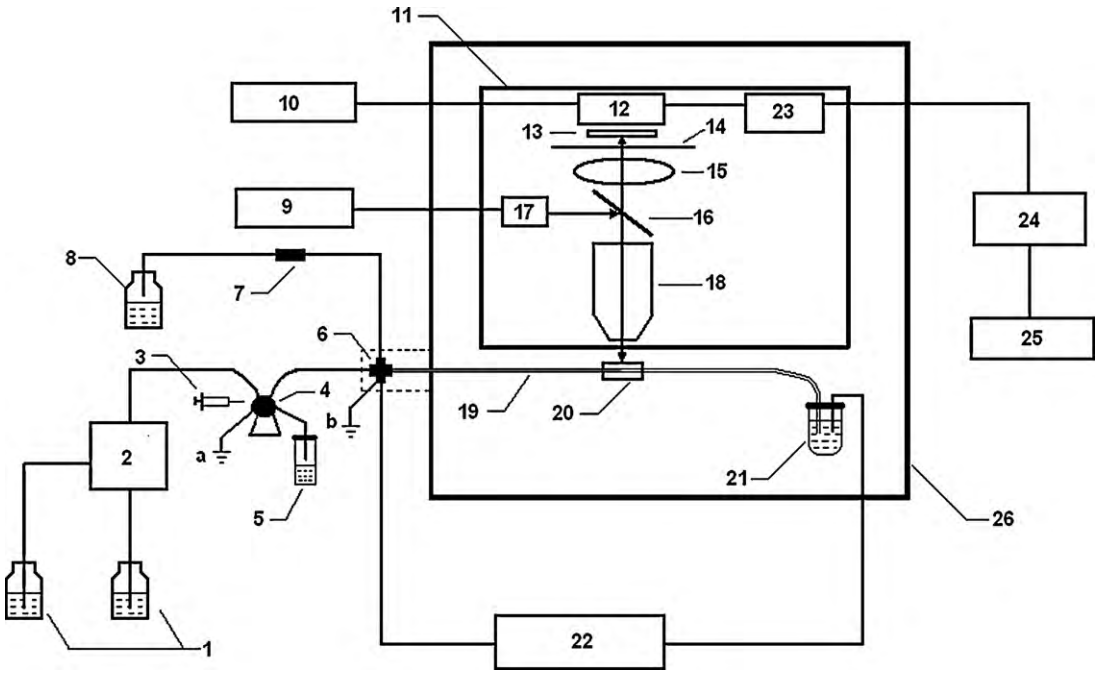

Fig. 2. The apparatus installation of pCEC–LIF: (1) mobile phase, (2) binary HPLC pump, (3) sample injector, (4) six-port valve, (5) waste reservior, (6) four-port split valve, (7) back-pressure regulator, (8) waste reservoir, (9) LD-DPSSL power supply, (10) high voltage supply, (11) laser induced fluorescence detector, (12) PMT, photomultiplier tube, (13) filter, (14) interference filter, (15) collimating lens, (16) dichroic mirror, (17) diode double pumped solid state laser driver, (18) microscope objective, (19) separation capillary electrochromatography column, (20) capillary flow cell, (21) buffer vials, (22) high voltage power supply, (23) integrator, (24) chromatogram acquisition unit, (25) computer, (26) black plexiglass box, a, b are grounded.

were identified by spiking the sample solution with standard solutions of each flavin.

2.3. Preparation of the monolithic column

The sulfonated N-octadecyl methacrylate monolithic column (100 μm i.d. × 365 μm o.d., total lengths 60 cm, effective lengths 28.5 cm) was prepared according to our previous report [24]. Prior to use, the inner wall of a capillary was treated with γ-MAPS. The solution for polymerization was consisted of the monomers OMA (17.82%, w/w), EDMA (12%, w/w) and SPMA (0.18%, w/w), the porogens cyclohexanol (49%, w/w) and 1,4-butanediol (21%, w/w), and the initiator AIBN (1.0 wt% with respect to the monomers). The mixture was sonicated for 20 min, and purged with nitrogen for 5 min to obtain a homogeneous solution, and then was injected into the pretreated capillary for suitable length. The capillary was plugged at both ends and submerged in a 60 °C water bath for 20 h. The resultant monolithic capillary column was washed with methanol using an HPLC pump to remove porogens and unreacted monomers, and then was ready for use.

2.4. Samples preparation

Human blood samples were collected from healthy volunteer and added into tubes containing heparin and EDTA immediately, then kept at −20 °C and protected from light. The plasma was then prepared by centrifugation at 1500 × *g* within 15 min, and stored frozen in the dark at −20 °C, and used within 1 week.

The extraction procedure of flavins from plasma was modified from the method described by López-Anaya and Mayersohn [14]. In a 5-mL test tube that was protected from light, 1 mL of plasma was mixed with 1 mL of acetonitrile to precipitate proteins. The tube was vortex-mixed for 1 min and centrifuged for 10 min at 12,000 rpm to separate proteins. The supernatant was transferred to another 10 mL test tube that was protected from light, and then mixed for 3 min with 6 mL chloroform to extract added acetonitrile. After filtration through a Millipore filter (0.45 μm pore size), the aqueous phase was diluted with mobile phase and kept at 4 °C prior to pCEC injection within 1 h. All samples and standard solutions were protected against light during the whole procedure.

2.5. Electrochromatography procedures

The phosphate buffer was prepared from a 0.2 mol L^{-1} solution of phosphate salt, then adjusting to various pH values by mixing 0.2 mol L^{-1} sodium hydroxide and/or 0.2 mol L^{-1} phosphoric acid, and then diluted to final concentration with water. Mobile phases were prepared by mixing appropriate volumes of acetronitrile, buffer solution, and degassed in an ultrasonic bath for 20 min before use. All the solutions were filtered through 0.22 μm filter. An isocratic elution pCEC mode was adopted in the experiment. Before pCEC experiments, the monolithic column was conditioned on the instrument with the mobile phase for 1 h, and the applied voltage applied on the outlet vial was firstly ramped from 0 to −20 kV and then operated at −20 kV. The column was equilibrated for about 30 min after the mobile phase was changed and the temperature of the column was kept at room temperature.

3. Results and discussion

Flavins are one class of water-soluble biomolecules with high hydrophilicity. The charged phosphate group of FMN and adenine diphosphate group of FAD always made the rapid separation of flavins by RP-HPLC or CE to be a troublesome task. In this study, pCEC–LIF using a sulfonated N-octadecyl capillary monolithic column was performed to separate three flavins in neutral or weakly acidic conditions, where flavins have high fluorescence quantum yields and good stability. A number of pCEC parameters were optimized to decrease the analysis time and increase the resolution.

3.1. Effect of the content of organic modifier

The organic modifier content in the mobile phase is a fundamental factor that influences the selectivity and resolution of the compounds mixture. Experiments showed that the volume percentage of acetonitrile affects not only the velocity of EOF but also the chromatographic interaction in CEC. Higher amounts of acetonitrile content induced an increase in electroosmotic mobility [24] and a lower retention of the analytes, indicating a weak interaction of the compounds with the stationary phase. Thus, a slightly decrease of the elution time and the resolution can be observed when the acetonitrile content increased from 50 to 70%. RF could be easily separated with FMN and FAD ($Rs \geq 2.0$) in this case. Satisfactory resolution ($Rs \geq 1.5$) of FMN and FAD could be achieved when the acetonitrile content set in the range of 50–65%. A mobile phase with ACN content of 60% provided the best compromise concerning resolution and analysis time.

3.2. Effect of buffer pH

The fluorescence intensity and the stability of flavins are strongly pH-dependent [19]. The fluorescence intensity of FMN and RF showed a maximum value between pH 3.7 and 7.5, whereas for FAD the fluorescence intensity was maximal at pH between 2.7 and 3.1. Under alkaline conditions, flavins exhibit lower fluorescence quantum yields and are easily degraded, especially for RF. For this reason, the influence of buffer pH on the retention of three flavins was studied at pH between 2.5 and 7.0, using the mobile phase containing 5 mM phosphate buffer and 60% (v/v) of acetonitrile.

Owning to the unique chemical structure of flavins, the change of buffer pH of the mobile phase has a great influence on the existence form of analytes. RF shows neutral at all examined pH values (pK_a 10.2). FMN is the phosphorylated product of RF. It is negatively charged at examined pH because of the additional phosphate group (pK_{a1} 1.3, pK_{a2} 6.5). As the condensation product of RF and adenosine diphosphate, FAD carries both negatively charged pyrophosphate group (both pK_a values of the pyrophosphate are less than 2.0 [25]) and positively charged adenine group with a pK_a value of ca. 4.5–5.0 [26].

Therefore buffer pH plays an important role in affecting the selectivity and resolution of flavins. As shown in Fig. 3, the retention time of neutral RF had no obvious change when pH value was raised from 3.0 to 7.0. The separation of FMN and FAD was improved initially as the buffer pH increased from 3.0 to 5.0, owing to the combined action of EOF and the increasing electrophoretic mobility (with a opposite direction to EOF) of more deprotonated solutes. The resolution between FMN and FAD was reduced when the pH further increased to 7.0. A complete loss of resolution was observed at pH 7.0, the reason is that FMN could gradually be more negatively charged in this circumstance (pK_{a2} of phosphate group in FMN is 6.5), and the electrophoretic mobility was increased correspondingly. FMN will migrate against EOF and elute later, while the opposite is expected in case of repulsion of negatively charged solutes with sulfonate groups on the surface of the monolith. Thus these two phenomena are not responsible for the same effect on the elution behavior and make the separation difficult at this pH. In addition, there were no significant changes of the elution behavior between pH 4.0 and 6.0. A good compromise between resolution, analysis time and fluorescence intensity was obtained with buffer pH 4.0 (Fig. 3).

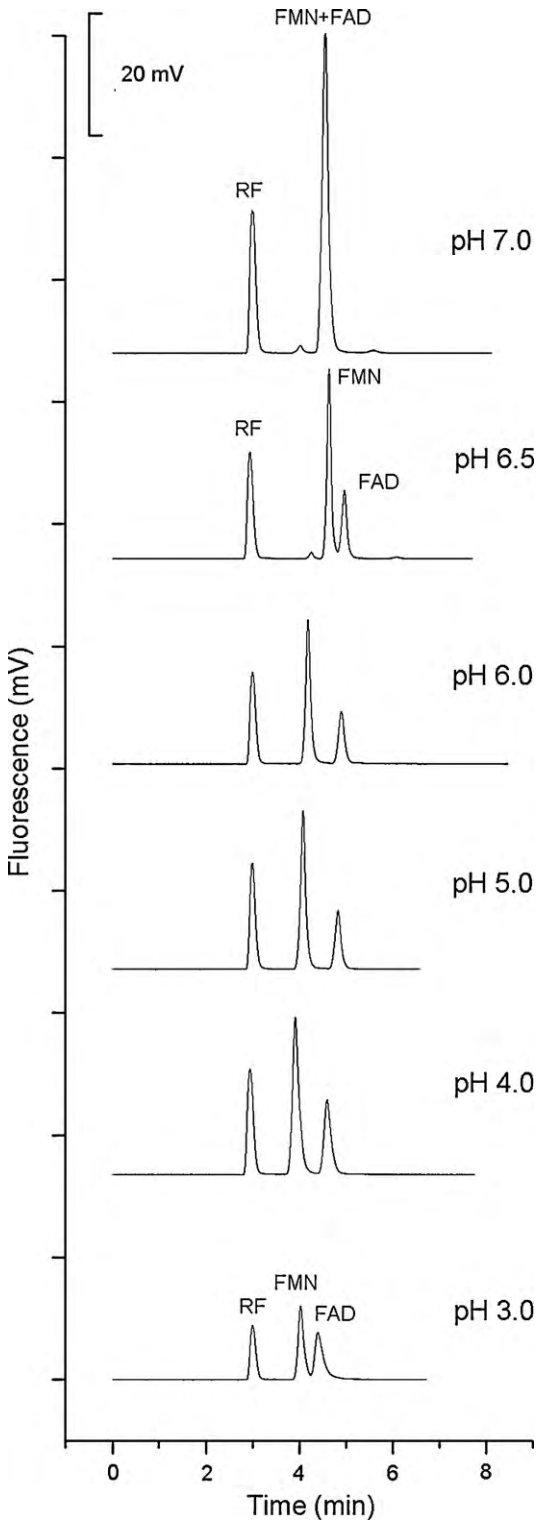

Fig. 3. Influence of pH of the running buffer. Experimental conditions: capillary column: 28.5 cm effective length, 60 cm total length × 100 μm, i.d.; mobile phase, 60% (v/v) acetonitrile, 40% (v/v) of 10 mmol L^{-1} phosphate buffer, applied voltage −20 kV, supplementary pressure 250 psi, flow rate 0.02 mL min^{-1}; LD-DPSS LIF detection (λ_{ex} = 473 nm; λ_{em} = 530 nm). Concentrations of RF: 5×10^{-7} mol L^{-1}, FMN: 6×10^{-7} mol L^{-1}, and concentration of FAD: 2×10^{-6} mol L^{-1}.

3.3. Effect of buffer concentration

The influence of ionic strength in the mobile phase on the separation of flavins was studied by changing the phosphate buffer concentration from 5 to 40 mmol L^{-1}, keeping the buffer pH at

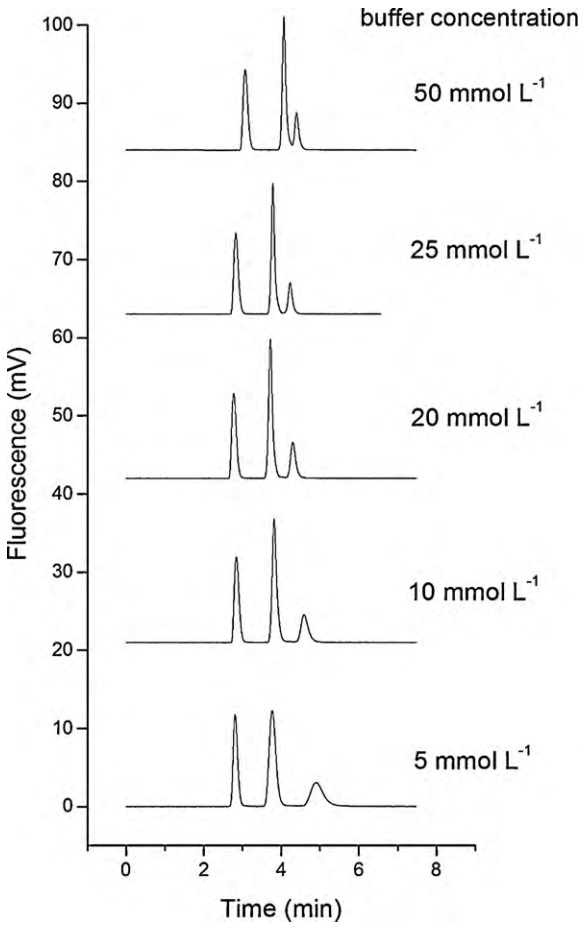

Fig. 4. Influence of buffer concentration. Experimental conditions: mobile phase, 60% (v/v) acetonitrile, 40% (v/v) of phosphate buffer (pH 4.0), other conditions as in Fig. 3. Concentrations of RF: 5×10^{-7} mol L^{-1}, FMN: 6×10^{-7} mol L^{-1}, and concentration of FAD: 1×10^{-6} mol L^{-1}.

4.0 and the ACN content at 60% (v/v). Generally, the increase of buffer concentration will reduce the EOF of the monolithic column. The retention time of neutral RF and negatively charged FMN increased slightly when buffer concentration increased from 20 to 50 mmol L^{-1} (Fig. 4).

In Fig. 4, a reduction of retention time and improvement of peak shape of FAD could be observed when the buffer concentration increased. FAD is an amphoteric compound, carrying both negatively charged pyrophosphate group and positively charged adenine group with a pK_a value of ca. 4.5–5.0 [26]. The increased phosphate buffer concentration in the mobile phase may contribute to the reduction of the electrostatic interactions between adenine group of FAD and sulfonate groups on the surface of stationary phase. Higher buffer concentrations could speed up the ion exchange interaction and finally reduce the retention and improve the peak shape [24]. But excessive phosphate buffer concentrations were not applicable, due to the unsatisfactory resolution of FMN and FAD, and the high-background noise that always led to the decrease of S/N. As a result, 20 mmol L^{-1} PBS buffer was selected as buffer solution in mobile phase.

3.4. Effect of the applied voltage

The applied voltage applied on the outlet vial was varied from 0 to −25 kV. In CEC, the increase of the electric field can enhance the EOF and alters the electrophoretic migration of the charged substances. As expected, the retention time of the flavins decreased

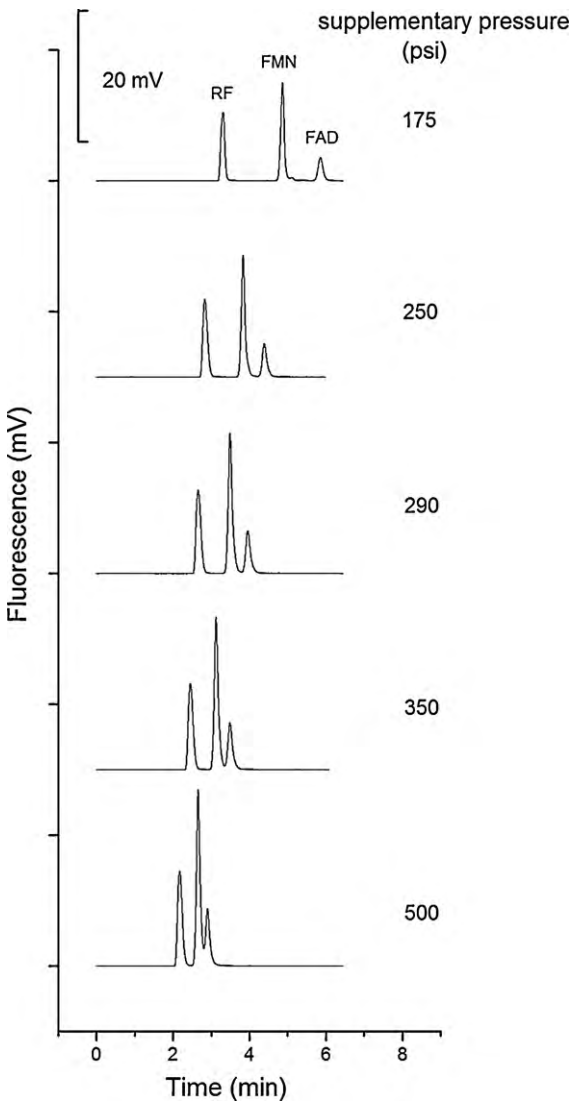

Fig. 5. Influence of supplementary pressure. Experimental conditions: mobile phase, 60% (v/v) acetonitrile, 40% (v/v) of 20 mmol L^{-1} phosphate buffer (pH 4.0), applied voltage −22 kV; other conditions as in Fig. 3. Concentrations of RF: 5×10^{-7} mol L^{-1}, FMN: 6×10^{-7} mol L^{-1}, and concentration of FAD: 1×10^{-6} mol L^{-1}.

with the increase of applied voltage. The resolution also increased obviously in this case, which may be attributed to the migration mobility changes of FMN and FAD with total negative charge at pH 4.0. All the three analytes could not be resolved when the applied voltage was 0 kV (i.e. capillary liquid chromatography). However, excessively high value of applied voltage also leads to Joule heating, results in the decrease of S/N. Considering the migration time, resolution, and Joule heating, −22 kV was selected as the optimum voltage.

3.5. Effect of supplementary pressure

In pCEC, supplementary pressure was applied to assure the reliability and reproducibility of the electrochromatographic system, and to speed the analysis [1]. As shown in Fig. 5, with the pressure increased from 175 to 500 psi, a decrease in retention time of all analytes and a loss of resolution for flavins were observed. At 290 psi, the analytes could be baseline separated within 4.5 min.

The optimal electrochromatographic separation was achieved with fulfillment of requirements as short retention time, absolute resolution, and symmetrical peak shapes (shown in Fig. 6A). The

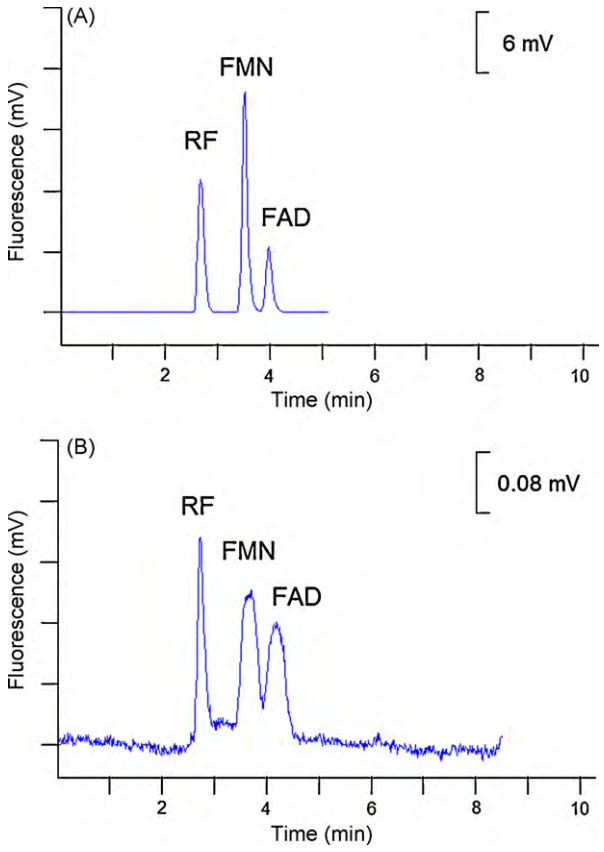

Fig. 6. (A) Typical pCEC chromatogram of RF, FMN, and FAD under the optimum conditions. Experimental conditions: Capillary column: 28.5 cm effective length, 60 cm total length × 100 μm, i.d.; experimental conditions: mobile phase, 60% (v/v) acetonitrile, 40% (v/v) of 20 mmol L^{-1} phosphate buffer (pH 4.0), applied voltage −22 kV, supplementary pressure 290 psi, flow rate 0.02 mL min^{-1}. LD-DPSS LIF detection (λ_{ex} = 473 nm; λ_{em} = 530 nm). Concentrations of RF: 5×10^{-7} mol L^{-1}, FMN: 6×10^{-7} mol L^{-1}, and concentration of FAD: 1×10^{-6} mol L^{-1}. (B) pCEC chromatogram of human plasma sample diluted 2.5-fold with mobile phase. All experimental conditions as same as in A.

multiple effects including chromatographic retention, electrostatic interaction and electrophoresis may simultaneous contribute to the separation process of flavins. Under weakly acidic condition (pH 4.0), satisfactory separation of flavins was obtained within 4.5 min. The retention times of RF, FMN and FAD were 2.66, 3.51 and 3.97 min, respectively. The resolution between FMN and FAD was 2.12, while the resolution between RF and FMN was 3.91. Number of theoretical plates per meter of flavins were 8700 (RF), 19,700 (FMN) and 20,100 (FAD) at the optimal conditions.

3.6. The linear range, detection limit and repeatability

The linearity of the method was determined by constructing a calibration curve with different concentrations of three flavins under the optimized conditions. All measurements were carried out for three replicate measurements and the results were shown in Table 1. To test the validity of the linear regression model, we performed a statistical lack-of-fit test on the data. The linear model adequately describes the calibration curve, as evidenced by the lack of departures from linearity observed in the quantitations (lack-of-fit: $F = 3.3224$, $P = 0.08976$ for RF; $F = 3.5684$, $P = 0.09557$ for FMN; and $F = 2.2218$, $P = 0.1534$ for FAD).

Owing to the high fluorescence yields and stability at weakly acidic conditions, the LOD (defined as S/N = 3) of flavins ranged from 0.5 to 3.5 nmol L^{-1}, and the LOQ (defined as S/N = 10) of flavins ranged from 5.0 to 20 nmol L^{-1}, with relative standard deviations (RSD) < 5%. Because the concentration levels of flavins in plasma were often at nanomolar level (from several to several hundreds of nanomolar), the favourable linear ranges of flavins in proposed method could satisfy the demands of clinical analysis. The sensitivity of this method was much higher than many previously reported HPLC methods for flavin analysis with offline sample extraction [25,26], and it also exceeded or reached the same level of some relevant CE-LIF methods with traditional gas lasers LIF detection [18,19], and far more sensitive than the detection with LEDs [21]. The stable DPSS-LIF light source (473 nm) with suitable output power (10 mW) was testified to improve the detection capability and ensure the lower detection limit of LIF. Owning the advantages of small size, low cost, long life, and reasonable light intensity, the all solid-state LD-DPSS LIF detection can act as a good alternative for capillary-based microanalysis of flavins. The short- and long-term repeatability of the retention time and peak area for flavins were presented in Table 2. The intra- and inter-day repeatability of retention times were lower than 2.7% and the value for peak area were lower than 4.0%.

Table 2
Retention time and peak area repeatabilities[a,b].

Analyte	Intra-day RSD (%) (n=7)		Inter-day RSD (%) (n=7)	
	Retention time	Peak area	Retention time	Peak area
RF	0.48	1.2	2.1	2.8
FMN	0.54	0.63	2.5	3.4
FAD	0.57	0.94	2.7	4.0

[a] Conditions identical to Fig. 3.
[b] Concentrations of RF: 5×10^{-7} mol L^{-1}; FMN: 6×10^{-7} mol L^{-1}, and concentration of FAD: 1×10^{-6} mol L^{-1}.

3.7. Sample analysis

In spite of the complexity of the plasma sample matrix, the proposed pCEC-LIF method allowed for a simple sample pretreatment at neutral conditions with the smallest requirement of human plasma sample and solvent, and therefore provided a short analysis time (<20 min) for the flavins. The relative short analysis time, the neutral or weak acidic condition used in the samples preparation and separation, made this approach be effective in minimizing the degradation of flavins and improving the sensitivity of fluorescence detection.

Fig. 6B presents pCEC chromatogram of human plasma extracts diluted 2.5-fold with mobile phase and analyzed by the proposed pCEC-LIF method. Few degradation peaks could be observed from the electrochromatograms. The bad peak shapes for FMN and FAD were possibly originated from the matrix effects of plasma sam-

Table 1
Analytical parameters of RF, FMN, and FAD.

Analyte	Calibration equation $Y = AX + B$	r^2	Linear range (μmol L^{-1})	LOD (nmol L^{-1})	LOQ (nmol L^{-1})
RF	$Y = 2.076 \times 10^5 X + 338.4$	0.9999	0.005-2	0.5	5.0
FMN	$Y = 2.679 \times 10^5 X + 312.9$	0.9998	0.005-2	0.7	5.0
FAD	$Y = 6.020 \times 10^4 X + 624.6$	0.9999	0.020-40	3.5	20.0

Y, peak area; X, analyte concentration in μmol L^{-1}; r^2, determination coefficient; LOD, the detection limit (signal-to-noise ratio = 3); LOQ, the quantitation limit (signal-to-noise ratio = 10).

Table 3
Endogenous contents and recoveries of flavins in human plasma sample with the proposed method ($n = 5$).

Analyte	Original amount[a] (nmol L^{-1})	Added amount (nmol L^{-1})	Found amount (nmol L^{-1})	Recovery (%)[b]	RSD (%)[c]
RF	29.7 ± 1.2	10	40.2	104.9	1.9
		400.0	437.1	101.9	5.1
FMN	15.0 ± 0.9	15.0	29.4	96.1	4.0
		400.0	446.9	108.0	5.2
FAD	52.6 ± 2.7	40.0	87.1	86.1	4.7
		1000.0	1155.4	110.3	3.1

[a] Expressed as mean ± standard deviation.
[b] Recovery (%) = (Found amount − original amount)/added amount × 100.
[c] RSD (%) = (SD/mean) × 100%.

ples. The measured contents of endogenous RF, FMN and FAD in human plasma sample from healthy volunteer were listed in Table 3. These contents correspond well to those previous clinical reports for flavins analysis in plasma by CE [18], and liquid chromatography–tandem mass spectrometry [16].

Recovery experiments were performed to verify the reliability of the proposed method. Human plasma samples from healthy volunteer were spiked with two levels of standard solution of flavins, then were extracted and analyzed as described in Section 2. The spiked concentrations were chosen at lower and higher concentrations of the endogenous flavins. The total amount of each analyte was calculated from the corresponding calibration curve with standard solutions. The recoveries were counted by the formula: Recovery (%) = (found amount − original amount)/spiked amount × 100%, and RSD (%) = (SD/mean) × 100%. The recoveries of the investigated analytes were in the range from 86.1 to 110.3% with RSDs lower than 5.2%. The results were given in Table 3. Considering the results, the method was of an acceptable precision.

4. Conclusion

An isocratic elution pCEC method coupled with LD-DPSS LIF detection, using a sulfonated N-octadecyl methacrylate monolithic column, allowed for a quite fast, sensitive and simply analysis of flavins in human plasma with the smaller requirements of sample and solvent. Under the optimum conditions, all the flavins could be completely separated within 4.5 min in a weakly acidic mobile phase, with low degradation and high fluorescence efficiencies. The detection limit (LOD) of flavins as low as 0.5 nmol L^{-1} was obtained. The potential for clinical analysis of relevant flavins in human plasma was demonstrated, without the need for off-line or on-line sample enrichment. This method is well suited to satisfy the demands for rapid, selective and sensitive detection of trace level of flavins in biological fluids, and it may become a promising tool to assess flavins status in clinical analysis.

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