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Pulsed multi-wavelength excitation using fiber-in-capillary light emitting diode induced fluorescence detection in capillary electrophoresis

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ABSTRACT

A novel instrument was developed using a multi-wavelength pulsed LED array with in-column optic-fiber induced fluorescence detection by capillary electrophoresis. The light from 2 different wavelength LEDs (450 nm and 480 nm) was pulsed for short intervals at high intensity. The beam from each LED was collimated and reshaped with the gradient index (GRIN) lens group to achieve a highly effective coupling between LED light source and an optical fiber. The optical fiber was placed inside the capillary for in-capillary LED-induced fluorescence detection. The advantages of this system were validated by the simultaneous determination of vitamin B2 and fluorescein. Detection limits for vitamin B2 and fluorescein were estimated to be 5 nM and 0.29 nM ($S/N = 3$), respectively. The relative standard deviations (RSDs, $n = 6$) of the both compounds for migration time and peak area were better than 0.83%, 2.20% and 1.21%, 2.75%, respectively. The method was applied to the determination of vitamin B2 in commercial tablets and fluorescein in fluorescein sodium injection and the recoveries obtained were in the range of 96.6–102.0% and 99.9–102.8%, respectively. It was also applied to human serum, where the recoveries were found to be in the range of 94.4–97.0% and 92.6–96.4%, respectively. The system has been successfully applied in separation and determination of the both biological samples with acceptable analytical performance.

1. Introduction

Since its first introduction in the 1980s, capillary electrophoresis (CE) has emerged as a popular analytical technique often seen as an alternative to liquid chromatography. However one of its main limitations is its sensitivity. This can be addressed by the use of one of the various approaches to on-line concentration, or through improved hardware to facilitate more sensitive detection. In the latter area, laser induced fluorescence (LIF) detection has been regarded for many years as one of the most sensitive forms of detection. While only a few analytes exhibit native fluorescence, there is a range of simple chemistry that can be used to add a fluorophore to facilitate sensitive detection [1,2]. As samples become more complex, there is a growing desire to be able to monitor and detect multiple fluorophores to increase throughput and maximize the information obtained from the sample. In recent years, a number of multi-wavelength fluorescence detectors for simultaneous monitoring of various analytes have been presented. Karlinsey and Landers used excitation at a single wavelength with an aucusto-optical tunable filter (AOTF) as a variable emission filter allowing multi-wavelength detection with a single PMT [3]. This is a simple, flexible and powerful way to achieve multi-wavelength detection, but it is expensive, and the optical transmission of the AOTF is not high. The more common method for multi-wavelength detection involves the use of a CCD camera in which different wavelength can be spectrally resolved. Takahashi et al. used two lasers (Ar+ 488 nm, and YAG 532 nm) as excitation light sources and the fluorescence image is split into four different color images with a polyhedral image-splitting prism coupled with optical filters [4], but the performance of the cooled CCD camera was lower than a PMT and they have a poor linear range, thus reducing their performance. Hsiung et al. used multiple excitation/emission fibers integrated into a microchip, with the disadvantage being that each emission fiber required its own unique PMT [5]. The UV–vis–NIR spectrometer in the dark-field optical system was expensive and it is difficult to obtain low detection limits and so it was not easy to popularize [6]. The approaches described above can all significantly improve the CE throughput compared with conventional single-wavelength fluorescence detection system for CE, but require complex optics or are expensive.

In this work, we build upon our previous work on coupling multiple light emitting diodes (LEDs) into a single optical fiber

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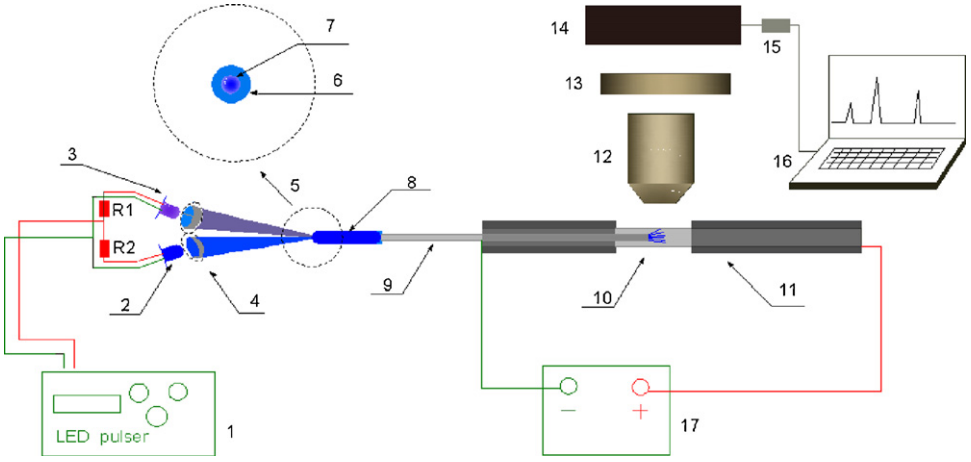

Fig. 1. Schematic of the CE instrument with pulsed LEDIF detection. (1) LED pulser; (2) LED: 480 mm; (3) LED: 450 mm; (4) focusing lens (Dia:10 mm; focus: 1.5 mm); (5) sectional view of end-face focused on the GRIN lens; (6) sectional view of GRIN lens (Dia: 1.8 mm); (7) focused spot (Dia: 1.4 mm); (8) GRIN lens (Dia: 1.8 mm); (9) optical fiber; (10) detection window; (11) capillary; (12) 60× microscope objective; (13) cut-off filter; (14) PMT; (15) data collector; (16) computer; (17) high-voltage power supply.

for in-capillary LED induced fluorescence (LEDIF) by pulsing the LEDs. The light from two LEDs was focused, reshaped and collimated by optical lens and GRIN lens combination, and then it was efficiently coupled to a single-mode optical fiber for fluorescence detection with a single long-pass filter and a single PMT [7]. LEDs are attractive light sources and their advantages have been discussed extensively in the literature [8–11]. Here, we pulse the LEDs at a higher current than it can be used under continuous operation which directly translates to more intense light being emitted from the LED, although for a shorter period of time. This strategy has recently been reported by Ren et al. with an array of 8 blue LEDs pulsed in sequence and a single detector to perform an 8-channel CAE of FITC labeled arginine. They demonstrated superior light intensity output from LEDs pulsed at currents of 200 mA at frequencies of 80 Hz than that was achieved with continuous operation at 20 mA. Detection limits between pulsed and continuous operation LEDs were not directly compared, but the light intensity was 10 times greater at 200 mA, compensating for the loss of light when each LED is turned off. There was also a noticeably less photobleaching with the pulsed LEDs, which should translate to improved performance. It has been reported in the literature that pulsed operation of the LED significantly increased the sensitivity, and greatly reduced the power consumption and photobleaching effect [12]. Moreover, high power LEDs are frequently being operated in a pulsed manner [13]. The performance of this system developed is demonstrated by the simultaneous separation and detection of the two biological important analytes (vitamin B2 and fluorescein).

2. Experimental

2.1. Multi-wavelength lamp-house system with pulsed LED array by coupling GRIN lens to SMF

The lamp-house system has been previously described [14] and a schematic is shown in Fig. 1. Briefly, the light from two individual 5 mm LEDs was focused onto the end-face of a GRIN lens using an appropriate optical lens. The function of the GRIN lens is to reshape the beam, correct dispersion and to collimate the light so that it can be efficiently coupled into the optical fiber.

2.2. Equipment fabrication

A high-voltage power supply (0–30 kV, Tianjin Dongwen High-voltage power Plant, Tianjin, China) was used to drive the electrophoretic separation and an uncoated fused-silica capillary (Hebei Yongnian Optical Fiber, China) was used for the sample separation. GRIN lens (Dia: 1.8 mm, NA ≥ 0.6) with anti-reflection coating and appropriate transmitting wavelength from 400 nm to 600 nm, were ordered from Shenzhen Sunboon technology Co. Ltd. The emission wavelength of both the LEDs (Riyueming Optic and Electronics Ltd., Shenzhen, China; applied voltage, +3.5 V; intensity, +3 mW; spectral half width, +25 nm) used in this work was measured to be 450 nm and 480 nm, respectively with a fluorescence spectrophotometer (FITACHI F-4500). The LEDs and filter were selected according to the excitation and emission wavelength of both the samples, vitamin B2 and fluorescein, respectively. As both the emission wavelengths are 530 nm, a 515 nm long pass filter can be used instead of a multiple filters. The LEDs were pulsed using an in-house constructed LED pulser designed to allow the use of up to 5 individual LEDs, although only two were used in this work. Each LED was pulsed on for 9 ms followed by a short dark pulse of 1 ms.

The multi-wavelength pulsed LED array excitation source was focused on an end-face of GRIN lens by two lens (Dia: 10 mm, focus length 13 mm, design wavelength ≥400 nm) before coupling GRIN lens to an optical fiber. The other end of the optical fiber was inserted into the end of the separation capillary to introduce the multi-wavelength pulsed LED exciting light to the sample detection window. Fluorescence light emitting from the detection window of the capillary was then collected by a 60× microscope objective with a spatial filter (Olympus, Japan) and passed through a yellow color filter (cut-on wavelength at 515 nm). The fluorescence signal was subsequently detected by a PMT (R105UH, Beijing Hamamatsu Photon Techniques Inc., Beijing, China) at a rate of 20 Hz. The output signal was recorded and processed with a computer using in-house written software.

2.3. Chemicals and reagents

Vitamin B2 (VB2, 98% purity), commonly called riboflavin, was obtained from Shanghai Biochemical Reagents Company (Shanghai, China). VB2 tablets were the product of Chengdu Jinhua pharmaceutical Co., Ltd. (Chengdu, China). Fluorescein was obtained from the Tianjin No. 3 Chemical Reagent factory (Tianjin, China). Fluorescein sodium injection solution (National Medicine Permit No. H44023410) was supplied by Guangzhou Baiyunshan Pharmaceutical Co., Ltd. Blank serum was provided by Chengdu No.1. People Hospital from healthy volunteers. Sodium dodecyl sulfate (SDS), sodium tetraborate, NaOH and other chemical reagents used

in this work were of analytical grade or better unless otherwise noted.

2.4. Sample preparation

Standard stock solutions of VB2 were prepared in Milli-Q water. The standard stock solution of the fluorescein was dissolved in 1 mM NaOH. Both VB2 and fluorescein stock solutions were stored in the dark and in a refrigerator at 4 °C. All stock solutions were diluted with Milli-Q water to the desired concentration and filtered through a 0.45 μm cellulose membrane filter before use. Milli-Q water (Millipore, Bedford, MA, USA) was used throughout the work.

Ten VB2 tablets (5 mg/tablet) were ground into a fine powder and the equivalent of one tablet was accurately weighed and extracted with 100 mL of 0.10 M acetic acid [15]. Then it was diluted to approximately 6.92×10^{-5} M VB2 and sonicated for 15 min with vortex mixing at 5 min intervals. The solution was filtered through an ordinary filter paper, washed with Milli-Q water several times and the filtrate plus washings were diluted to a 250.0 mL volumetric flask. The sample solutions were diluted with Milli-Q water to obtain solutions where the expected concentration of VB2 was within the calibration range before assay. Fluorescein sodium injection (China National Medicine Permit No. H44023410, 0.6 g/3 mL) was directly diluted to fall within the calibration range.

Before assay, 1.0 mL of serum sample (Chengdu No.1. People Hospital) was diluted with 1.0 mL of acetonitrile (≥99.9%, liquid chromatography grade, Shanghai Luzhong Trade Co. Ltd.) and shaken vigorously for 15 min to deposit proteins, then left on ice for 1 h. After centrifuging at 10,000 × *g* for 15 min with centrifuge (Anke TGL – 16C. Shanghai, China), the deproteinized serum was used as analyte in the experiment. 10 μL of VB2 tablets solution (VB2 concentration calculated, 6.92×10^{-6} M), 10 μL of fluorescein sodium injection solution (2.25×10^{-5} M) were mixed in a 0.5-mL microcentrifuge tube. Then the volume of the borate buffer was added to the tube according to the concentration of the both samples diluted within the calibration range when the application was processing. The tube was tightly capped, shaken and mixed adequately before injection.

2.5. CE procedure

At the beginning of each day, the capillary was pretreated with 0.1 M NaOH for 15 min, deionized water for 3 min and the running buffer for 4 min. New capillaries were rinsed sequentially with 1.0 M NaOH for 20 min, 0.1 M NaOH for 10 min, deionized water for 5 min, and finally running buffer for 10 min prior to the first use. Between two sequential injections, the capillary was rinsed with 0.1 M NaOH, water and running buffer for 3 min each. Running buffer was treated with ultrasonic degassing before experiment.

Sample injection was performed by siphon at a height of 20 cm. After the introduction of the sample solution, the voltage (10–26 kV) was applied across the capillary in a number of steps. All separations were performed on a 50.0 cm × 75 μm i.d. (365 μm o.d. and 48.0 cm effective length) uncoated fused-silica capillary with 10 mM borate buffer, pH 9.1.

3. Results and discussion

3.1. Optimization of separation conditions

In order to obtain an optimal electropherogram, the pH 8.5–9.5, concentration of borate buffer (8–14 mM), injection time (5–10 s) and applied voltage (12–22 kV) were varied. It was found that there was no significant difference in the migration times of VB2 and fluorescein except for the effect of applied voltage. The detection sensitivity also did not vary largely with the pH of the running buffer solution and concentration of the running buffer solution. Although an optimal pH value of 9.5 is reported in the literature for the separation of VB2 [16], a pH of 9.1 was determined to be better when considering the separation of both fluorescein and VB2. An applied voltage of 18 kV was chosen as the optimum when the separation efficiency, migration time and peak symmetry were all considered. The peak widths and peak shapes of VB2 and fluorescein were examined by changing injection time from 5 to 10 s at a height of 20 cm, and then 8 s was chosen as the optimum injection time. It should be noted that the right amount of SDS should be added to buffer solution, which benefits for siphonic mode to overcome the surface tension of the solution. Through above experiments, the optimum conditions were therefore 10 mM borate buffer at pH 9.1, applied voltage 18 kV, hydrodynamic mode injection for 8 s with the sample vials raised to a height of 200 mm and 10 mM SDS.

3.2. Optical principle of multi-wavelength excitation light source

In order to obtain the best excitation efficiency for fluorescence detection, the emission wavelength of the LED used has to be matched to the excitation wavelength of VB2 and fluorescein. The selection principle of LED and filter was described in the literature [17] where in order to maximize spectral overlap, the LED was selected such that its output wavelength was slightly lower than the excitation maxima of the fluorophore. Fig. 2(A) shows the excitation and emission spectra of VB2 as well as the emission spectrum of the two LEDs. VB2 is highly fluorescent with an excitation maximum at 455 nm. The proximal and optimal wavelength LED is 450 nm as seen from the emission spectra c in Fig. 2 (A). This LED therefore should be dominant in excitation of VB2. However, the 480 nm LED (spectra d in Fig. 2(A)) can also partially excite VB2. Similarly, Fig. 2(B) shows the excitation and emission spectra of fluorescein, as well as the output of the two LEDs. In this case, the 480 nm LED is responsible for most of the fluorescence, with a minor contribution from the 450 nm LED. As will be discussed below, the use of both of these LEDs is important to be able to detect fluorescein and VB2 in a single separation way.

3.3. Separation and detection of VB2 and fluorescein by multi-wavelength pulsed LEDs

To examine the individual contribution of both the LEDs to the overall detection response, the separation of VB2 and fluorescein was performed with the individual LEDs and then both of the LEDs operated simultaneously. The electropherogram using only the 450 nm LED is shown in Fig. 3(A) with the separation using only the 480 nm LED shown in Fig. 3(B). It can be seen that there is a slight response for fluorescein when the 450 nm LED and a slight response for VB2 with the 480 nm LED, as anticipated from the spectral data in Fig. 2. Fig. 3(C) shows the electropherogram obtained when both of the LEDs are used and it can be seen that the detection performance with both LEDs is superior for both VB2 and fluorescein because of the contribution of both LEDs to the output. This can be easily seen from comparison of the sensitivity for the 3 systems, which are given in Table 1. When using only the 450 nm LED, the LOQ for fluorescein was 1.43 μM, 0.87 nM with the 480 nm LED and 0.80 nM when both LEDs were used. A similar result was obtained for VB2, with a LOQ of 37.4 nM with the 450 nm LED, 1.7 μM with the 480 nm LED and 12 nM when both LEDs were used. Significantly, this demonstrates that the sensitivity of the multi-wavelength pulsed LED system is much higher than that of the conventional single wavelength CE system. One additional advantage is the ability to separate both medications in a single separation; in a conventional single-wavelength CE system, it would require two separate separations.

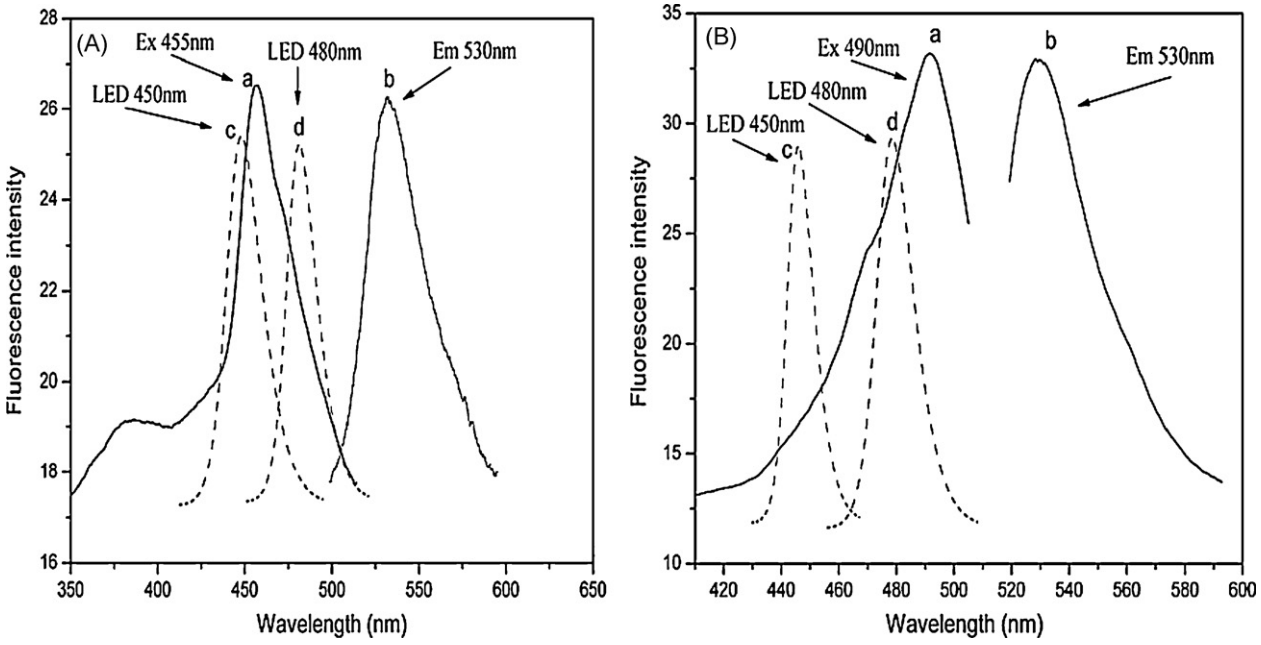

Fig. 2. Excitation (a) and emission (b) spectra of VB2 (A) and fluorescein (B). The dashed line (c and d) shows the emission spectrum of multi-wavelength pulsed LEDs (450 nm and 480 nm).

3.4. System characteristics

Validation of the multi-wavelength CE system was carried out by evaluating the detection limits, linearity and the reproducibility of migration time and peak area in Table 1. The limits of detection and quantification were calculated by measuring the noise at a signal-to-noise ratio of 3 for limit of detection (LOD) and 10 for the limit of quantification (LOQ). The linear ranges were 3.60×10^{-8}–2.53×10^{-6} M for VB2 and 1.01×10^{-9}–2.38×10^{-6} M for fluorescein. The correlation coefficients were better than 0.9993. The detection limits ($S/N = 3$) for VB2 and fluorescein were 5.0 nM and 0.29 nM, respectively. Although the LOD was higher than that of some literatures [16,18,19] by LIF, these LODs are excellent when compared to other methods in the literature. Specifically, de Jong and Lucy [20] using spectral filtering of light-emitting diodes, reported LODs of 50 nM for VB2 and 3 nM for fluorescein, Yang et al. reported 0.92 μM [21] with a blue concave LED as excitation source, de Jong and Lucy also reported 3 pM LODs using a high power blue Luxeon LED [22] and Chang et al. reported 3–7 ppb LODs for riboflavin using a UV-LED [23].

The reproducibility of the method was investigated by injecting VB2 (1.33×10^{-6} M) and fluorescein (2.25×10^{-7} M) standard solution 11 times and by measuring the repeatability of the peak migration time, the peak heights and the corrected peak area and were carried out in the optimum experimental conditions as described previously. The intraday precision was calculated as a percentage of relative standard deviation (%RSD) of migration times and corrected peak areas. The interday precision was evaluated over 3 days by performing six successive injections each day. As can be seen from Table 2, the repeatability %RSD calculated from the peak height and the peak area suggests that the proposed CE method presents acceptable reproducibility.

The accuracy of the method was performed by adding known amounts of VB2 and fluorescein to a commercial VB2 tablet and fluorescein sodium injection solution. Both analytes were determined by the mean concentrations measured and concentrations added according to label claim in the pharmaceutical preparations. The results obtained for intra and inter day accuracy are shown in Table 2.

3.5. Application

In order to demonstrate the applicability of our method, two types of samples were considered: pharmaceutical formulations and serum samples. In the pharmaceutical formulations, VB2 in a VB2 tablet and fluorescein in a fluorescein sodium injection solution were diluted as described in Section 2 and then mixed to facilitate simultaneous determination using the multi-wavelength CE system. Fig. 4(A) shows the results of the determination of VB2 in commercial tablets and fluorescein in fluorescein sodium injection, which was compared satisfactorily with those obtained by pharmacopoeia (PR China, 2005), moreover, the UV methods [24] described in China pharmacopoeia is tedious and time consuming. Each sample was analyzed with six independent determinations and each

Table 1
The method validation of the multi-wavelength CE system with pulsed LED.

Excitation wavelength	Vitamin B2					Fluorescein				
	Linearity range (nM)	Slope	R^2 [a]	LOD[b] (nM)	LOQ[c] (nM)	Linearity range (nM)	Slope	R^2 [a]	LOD[b] (nM)	LOQ[c] (nM)
450 nm	113.8–2513	0.2230	0.9998	15.7	37.4	–	–	–	–	1430
480 nm	–	–	–	–	1700	2.7–2310	0.1431	0.9996	0.32	0.87
450 nm and 480 nm	36.0–2527	0.3791	0.9993	5.0	12.0	2.2–2320	0.1583	0.9995	0.29	0.8

[a] Correlation coefficient.
[b] Detection limit ($S/N = 3$).
[c] LOQ were recorded as the concentration producing a signal to noise ratio of 10 ($S/N = 10$).

Table 2
Precision of the multi-wavelength CE system with pulsed LED and recoveries of VB2 and fluorescein from commercial tablet and fluorescein sodium injection samples ($n = 6$).

	Intra-day			Inter-day		
	Found ± SD (ng mL^{-1})	RSD[a] (%)	Recovery (%)	Found ± SD (ng mL^{-1})	RSD[a] (%)	Recovery (%)
Vitamin B2						
10	10.44 ± 0.30	2.87	104.4	10.20 ± 0.25	2.45	102.0
20	19.50 ± 0.63	3.23	97.5	19.31 ± 0.50	2.59	96.6
200	200.62 ± 3.68	1.83	100.31	199.45 ± 4.07	2.04	99.7
Fluorescein						
4	3.94 ± 0.05	1.27	98.5	4.11 ± 0.13	3.16	102.8
20	19.04 ± 0.71	3.73	95.2	20.17 ± 0.69	3.42	100.85
100	102.59 ± 3.74	3.65	102.59	99.86 ± 3.21	3.21	99.86

	Migration time (RSD, %)	Corrected area (RSD, %)	Migration time (RSD, %)	Corrected area (RSD, %)
Vitamin B2	0.83	2.20	0.80	0.91
Fluorescein	1.21	2.75	0.76	2.10

[a] Three replicates were performed.

Table 3
The determination data of VB2 and fluorescein in human serum ($n = 6$).

Human serum samples	Initial	Added	Found: mean ± SD	Recovery (%)	RSD (%)
Vitamin B2 concentration ($\times 10^{-7}$ M)					
Serum 1	6.42 ± 0.11	10	15.86 ± 0.45	94.40	2.73
Serum 2	7.18 ± 0.23	10	16.88 ± 0.33	97.00	1.82
Serum 3	5.92 ± 0.21	10	15.45 ± 0.37	95.31	2.43
Fluorescein concentration ($\times 10^{-8}$ M)					
Serum 1	7.06 ± 0.22	10	16.32 ± 0.62	92.62	3.71
Serum 2	8.38 ± 0.18	10	18.02 ± 0.48	96.41	2.53
Serum 3	6.97 ± 0.20	10	16.30 ± 0.53	93.29	3.21

series were injected three times. The recoveries test results of the both medicaments are shown in Table 2. The recoveries obtained are always higher than about 91% and lower than 104%, while RSD is always lower than 4%.

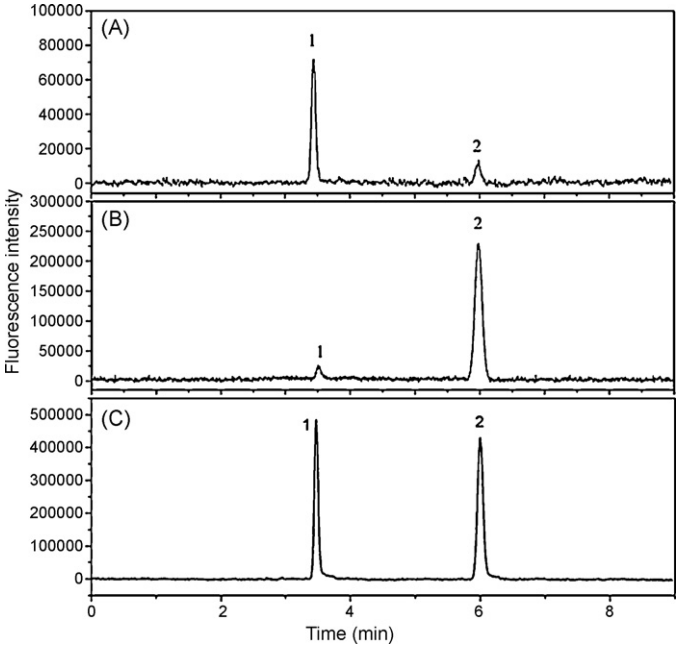

Fig. 3. (A) shows the separation of VB2 (3.46×10^{-7} M, peak 1) and fluorescein (2.25×10^{-6} M, peak 2) with a pulsed LED only excitation wavelength, 450 nm. (B) The separation of VB2 (1.33×10^{-6} M, peak 1) and fluorescein (1.21×10^{-7} M, peak 2) with a pulsed LED only excitation wavelength, 480 nm; electropherogram (C) is standard electropherograms of VB2 (1.33×10^{-6} M, peak 1) and fluorescein (2.25×10^{-7} M, peak 2) with pulsed LEDs excitation wavelength, 455 nm and 480 nm. CE conditions: run buffer, 10 mM borate at pH 9.2; SDS concentration, 20 nM; capillary dimensions, 50 cm × 75 μm id (365 μm od) with 48-cm effective length; siphonal mode, 8 s at a height of 20 cm; electrophoresis voltage, 18 kV.

The dual-wavelength CE system was also used to determine VB2 and fluorescein in spiked serum. A blank electropherogram of deproteinized human serum is shown in Fig. 4(B), with peaks 3, 4, 5 and 6 all being unidentified. These peaks mainly arise from contributions of riboflavin, bilirubin and its derivatives, β-carotene, zinc protoporphyrin, protoporphyrin IX etc. in human serum. It has been reported that riboflavin can be detected in human serum when excited at 450 nm [25], although it is found at levels slightly below our LOD. Thus it was not observed in this work. It is easily possible to

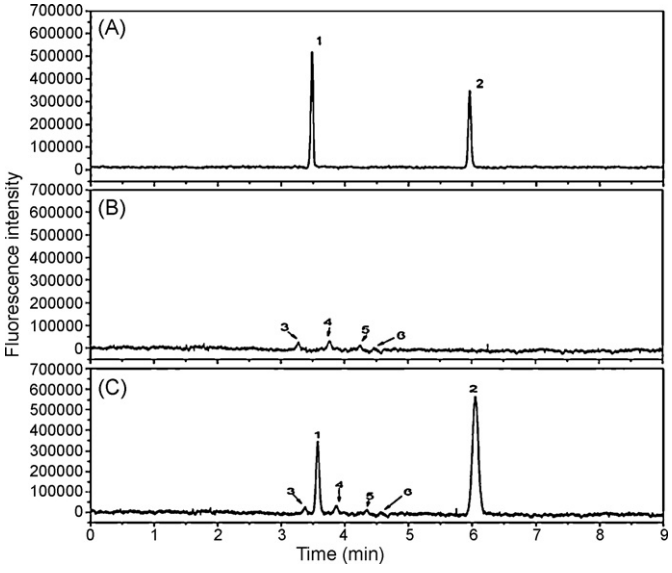

Fig. 4. (A) Electropherogram of VB2 tablet (peak 1, the relevance of the concentration of VB2 is 1.42×10^{-6} M) and the fluorescein sodium injection sample solution (peak 2, 1.81×10^{-7} M). Typical electropherogram (B) is of human plasma sample solution, the peak 3, 4, 5 and 6 are all unknown components in human serum. (C) Blank serum spiked with VB2 (peak 1, 9.6×10^{-7}M) and fluorescein (peak 2, 2.88×10^{-7} M). The conditions were the same as Fig. 3.

directly quantify VB2 in serum from supplements. Fig. 4(C) shows the same blank serum samples spiked with 9.6×10^{-7} M VB2 (peak 1) and 2.88×10^{-7} M fluorescein (peak 2). Recovery values for VB2 and fluorescein spiked into serum are shown in Table 3, which are all within 91–104%. Significantly, the endogenous components in serum did not interfere with the detection of VB2 and fluorescein indicating that this may be just the reason that the Eye Hospital carries out the diagnosis of human corneas with fluorescein sodium injection.

4. Conclusion

In this paper, a simple CE apparatus was successfully developed using multi-wavelength pulsed LED in-column fiber induced fluorescence detection with a capillary and a PMT. The LED light was efficiently focused onto an optical fiber using a GRIN lens combination. The use of 2 LEDs with different wavelength allowed the simultaneous detection of both VB2 and fluorescein in a single CE separation, and its applicability to quantify both of these in pharmaceutical formulations and spiked into human serum was demonstrated.

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