Asian Journal of Chemistry; Vol. 27, No. 9 (2015), 3143-3148

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.15412

Field-Amplified on-line Sample Stacking for Separation and Determination of Adefovir and Tenofovir Using Capillary Electrophoresis

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Received: 2 March 2014; *Accepted*: 9 April 2015; *Published online*: 26 May 2015; AJC-17212

An on-line sample stacking method known as field amplified sample stacking, using hydroxypropylmethyl cellulose as electro-osmotic flow suppressant, was developed for separation and identification of adefovir and tenofovir for the first time. A water plug (hydrodynamic injection for 15 s, 2.0 psi) was added to the system prior to the loading of sample (electrokinetic injection at cathode end (14.6 kV, 20 s)) in the capillary (80 cm × 75 μm). The separation was performed using phosphate solution containing 0.3 % hydroxypropylmethyl cellulose and measured at 18 kV and 214 nm. Comparing with the conventional capillary electrophoresis, the signal enhancement factor was found to be 146 for adefovir (LOD = 2.67 ng mL^{-1}) and 137 for tenofovir (LOD = 3.22 ng mL^{-1}) (S/N = 3). The proposed method has potential application in pharmacokinetics and can reach detection limits comparable to mass spectrometry, a more complicated and costly procedures.

Keywords: Adefovir, Tenofovir, Capillary electrophoresis, Field-amplified sample stacking, On-line sample enrichment.

INTRODUCTION

Chronic infection with the hepatitis B virus (HBV) is a major public health problem and causes 1.2 million deaths per year worldwide[1]. Tenofovir (9-[(R)-2-(phosphonomethoxy)propyl] adenine) and adefovir [Fig. 1(A, B)] are new nucleotide antiretroviral drugs used in the treatment of human immunodeciency virus type 1 (HIV-1) infection and hepatitis B virus infection[2-4]. To improve its low bioavailability, a prodrug of tenofovir *i.e.*, tenofovir disoproxil fumarate (TDF) is used instead. Tenofovir disoproxil fumarate also has been shown to inhibit replication of wild-type hepatitis B virus and lamivudine-resistant hepatitis B virus mutants *in vitro* while also inhibiting lamivudine-resistant hepatitis B virus in patients and hepatitis B virus in patients with human immunodeciency virus[5-9]. These inhibition processes involve the conversion of tenofovir disoproxil fumarate to tenofovir through human *in vitro* metabolization. Due to their increasing importance and disease fighting potential, a significant amount of researches have been directed towards studying these two drugs. Tenofovir treatment can be given either intravenously or orally. Recent studies have determined the level of tenofovir (in serum) for both treatment variations. In a group of patients given the intravenous treatment, the mean values for the maximum concentration of drug in serum (C_{max}) were 8.49 ± 5.33, 8.55 ± 4.74 and 47.5 ± 37.6 μg mL^{-1} for the 2.5, 7.5 and 12.5 mg kg^{-1} dose groups, respectively. For those who received oral treatment, the mean values for C_{max} were 0.171 ± 0.0687, 0.377 ± 0.217 and 0.524 ± 0.229 μg mL^{-1} for the 5.0, 15.0 and 25.0 mg kg^{-1} dose groups, respectively[10]. Moreover, the maximum concentration for adefovir after oral administration of 20 mg of adefovir dipivoxil capsules (single dose) in human plasma[11] is 37.40 ± 6.24 μg L^{-1}.

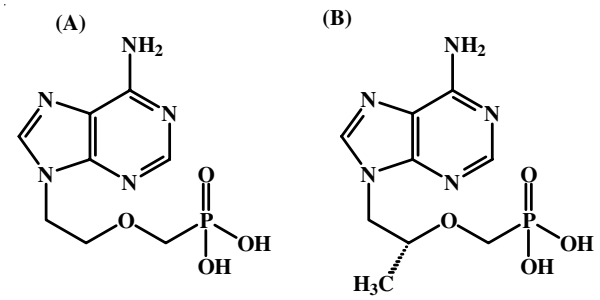

Fig. 1. Chemical structures of adefovir (A) and tenofovir (B)

Several methods for the determination of the two drugs in biological matrixes have been published, such as, LC/MS/MS[11-13] (the lower limit of quantification (LLOQ) is 10 ng/mL, 5 ng/mL, 0.5 ng/mL, respectively), HPLC-UV[14] (LLOQ

= 100 ng/mL), fluorescent derivatization-HPLC[15] (LLOQ = 5 ng/mL) and SPME-HPLC[16] (LLOQ = 10 ng/mL). Since adefovir and tenofovir have no fluorescent functional groups, fluorescence analysis requires an additional set of procedures, resulting in a laborious and time consuming process overall. Procedures such as MS also have a disadvantage due to the high instrumentation cost, limiting its usage in more practical situations. Hence, an accurate, rapid and novel method should be developed for the analysis of these two compounds. Capillary electrophoresis (CE) is a widely used technique in separation science on account of its high separation efficiency, superior separation speed, small sample requirement and low cost. Consequently, it has become one of the most outstanding separation techniques in the analysis of an increasingly large number of charged species, neutral compounds and macromolecules[17-19].

Capillary electrophoresis with UV detection (CE-UV) has been used to separate and analyze adefovir and tenofovir, but the internal diameter of the capillary is only 20-100 μm, which shortens the detection light path and limits detection sensitivity[20]. Moreover, using the most common on-column UV detection method yields high detection limits (10^{-6} to 10^{-5} mol L^{-1}) which can't meet the need for many real biological samples analyses. In order to improve the sensitivity, we have proposed a practical way to increase sensitivity through analyte on-line enrichment during separation. Several methods have been used to increase on-capillary concentration for improvements in sensitivity, namely field-amplified methods (FASS)[21], on-line isotachophoresis[22-24] and large volume sample stacking[25,26]. Field-amplified method is based on principle of conductivity difference between the sample solution and background electrolyte and are most often used for stacking due to their simplicity and practicality. With FASS, in the presence of a low conductivity solvent plug, concentration increase of 1000-fold is achievable[27].

Our work utilizes an on-line sample stacking method - field amplified sample stacking with hydroxypropylmethyl cellulose (HPMC) that is similar to a dynamic coating reagent - hydroxypropyl cellulose (HPC) used in capillary electrophoresis[28] as an electroosmotic flow suppressant to separate and identify adefovir and tenofovir. The proposed method has been used for successful determination of the above-mentioned analytes in bovine serum samples. The established method has the potential use in the pharmacokinetic studies which play an important role in the evaluation and development of a prodrug.

EXPERIMENTAL

The experiments were performed using a lab-constructed capillary electrophoresis instrument with a CL101A high voltage supply and a CL1020 UV detector from Cailu Scientific Instrument Company (Beijing, China). The data was collected using HW-2000 Chromatogram Software from Qianpu Software Company (Shanghai). The separation was performed in an 80 cm (the detection window is 48 cm to the cathode end and 32 cm to the anode end) × 75 μm i.d. fused-silica capillary (Yongnian-Ruifeng Photoconductive Fiber Factory, Hebei, China). The new capillary was washed using syringe for 10 min with each of the following solution: 1.0 M hydrochloric acid, 1.0 M sodium hydroxide and water, respectively. Between each run, the separation capillary was rinsed with 1.0 M sodium hydroxide, water and background electrolyte, respectively. The detection wavelength was set at 214 nm. Other instruments utilized in this experiment were the HC-3018 high speed centrifuge (Anhui USTC Zonkia Scientific Instruments Co., Ltd.) and CL-200 magnetic stirrer (Gongyi-Yuhua Instruments Co., Ltd.).

Standards of adefovir (ADV) and tenofovir (TNV) were purchased from Beijing Mediking Biopharm Co., Ltd.. Sodium dihydrogen phosphate (NaH_2PO_4, GR) was provided by Tianjin Chemical Reagents Development Centre. Hydrochloric acid and sodium hydroxide were purchased from Kelong Chemical Reagent Company (Chengdu, China) and were all analytical reagent grade. Hydroxypropylmethyl-cellulose (HPMC) was purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol came from Tianjin Chemical Reagents Development Center. Bovine serum was purchased from Luoshen Biotechnology Company (Shanghai, China). The water used for solution preparation was purified by Ultra-pure Water Purification System (Shanghai UPCo., Ltd., China). Stock solutions of 400 μg mL^{-1} adefovir, 400 μg mL^{-1} tenofovir, 200 mM NaH_2PO_4 and 2.4 % HPMC were prepared in ultra-pure water. Working solutions were obtained by diluting and mixing corresponding stock solutions to the desired concentrations with 1 mM NaOH (to maximum make the analytes into negative ion mode, adefovir: $pKa_1 = 2.0$, $pKa_2 = 6.8$). By preparing samples in a low-conductivity solution, 1 mM NaOH in this experiment and injecting the sample solution electro-osmotically into the column surrounded by high concentration buffer, one can achieve a field enhancement at the injection point[29]. In the experiment, we found that analytes in the dilute NaOH solution will achieve stronger signal response by the FASS mode compared with that in the deionized water for more ionic analytes, see the relavant article by Song *et al.*[30]. The solutions of standards were kept at 4 °C in a refrigerator prior to use.

Injection procedure: In non-stacking mode, the mixture containing adefovir and tenofovir was hydrodynamically injected into the cathode end of the capillary for 10 s (2.0 psi) using gravity generated with 14 cm height difference between anode and cathode. For FASS-CE, the water plug was first loaded using the sample injection procedures in non-stacking mode into the cathode end of the capillary. Thereafter, the cathode end of the capillary was put into sample vials with 1.5 mL solution and then a high voltage was applied for sample injection.

Sample pretreatment: 2 mL bovine serum was added into an Eppendorf tube followed by the addition of 6 mL methanol to precipitate protein. After vortex-mixing for 3 min, the samples were centrifuged at 12000 rpm (13363 × g) for 10 min. Supernatant solution was transferred into another vial and evaporated to dryness in nitrogen environment at 60 °C. The residue was redissolved in 2 mL 1 mM sodium hydroxide solution and then sonicated. The mixture was then filtered through a 0.22 μm membrane. Finally, the serum sample was diluted to 50 and 100 fold, respectively, with 1 mM sodium hydroxide solution. Varying amounts of adefovir and tenofovir

standard solution was added in the diluted serum sample to obtain the desired concentrations.

RESULTS AND DISCUSSION

FASS-CE model: To increase the detection sensitivity, the FASS technique was applied in present experiment. With FASS, charged analytes can be effectively stacked prior to the capillary electrophoresis separation. These targeted analytes are subjected to field-amplied enrichment and are subsequently stacked at the interface of the solvent plug (commonly water) and the background electrolyte[31]. The FASS method depends on conductivity difference between sample region and buffer region. When a high voltage is exerted to the two ends of the capillary, analyte ions prepared in a more diluted solution will experience higher electric field strength and, in turn, move faster than the ions inside the background electrolyte. The sample ions slow down when they pass the boundary between the sample and the background electrolyte compartments and stack into a zone much narrower than the original sample plug, analogous to the idea of a "traffic jam". In order to gain better enrichment, a solvent plug (commonly water) is usually introduced before the sample injection. Fig. 2 is a schematic illustration of the proposed FASS procedures, consisting of four main steps. (A) The buffer with high conductivity is filled into the capillary; then (B) low conductivity media (water in this work) is hydrodynamically loaded into the cathode end of the capillary by lifting the corresponding capillary end to a certain height; (C) the cathode end of the capillary is put into sample solutions and a high voltage is applied during the injection of anions. In the final step, (D) the two ends of the capillary are put into the separation buffer vials. With the application of a high voltage, the anions are separated in the capillary.

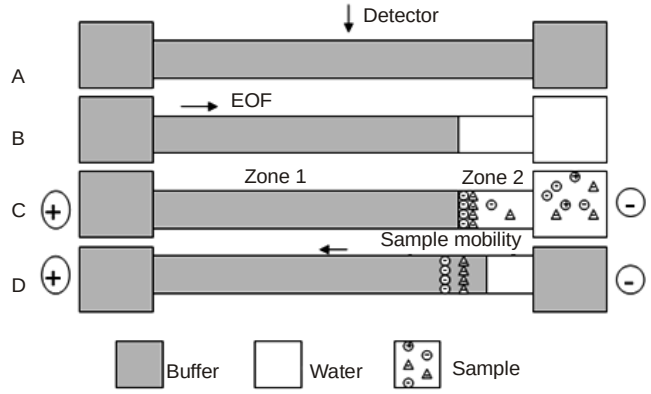

Fig. 2. Schematic illustration of FASS method. (A) Filling of the capillary with high concentration buffer; (B) loading of a water plug into cathode column end by gravity; (C) electrokinetic injection of anions at cathode end; and (D) separation and detection of stacked ions by a common detector placed in the center of the capillary

Migration time: The migration time of each compound was initially determined based on varied concentrations of the mixture of analytes hydrodynamically injected by gravity. The experiment reveals that the migration time of adefovir was less than that of tenofovir, with a constant time difference of 0.6 min. The reason for such an observation can be explained by the Stokes equation:

$$m = Q/6\pi r\eta$$

(m: the electrophoretic mobility of charged particles; r: the radius of particles; η: the viscosity of buffer solution; Q: the electric charges of particles).

From the equation, we can see the value of m has a negative relationship with the r of analytes. The molecular size of tenofovir is slightly greater than that of adefovir. Hence, according to the equation listed above, the electrophoretic mobility of adefovir should be larger than that of tenofovir under the same electric field, which means that adefovir would approach the detector earlier.

Optimization of FASS-CE

Effect of hydroxypropylmethyl cellulose: Hydroxypropyl cellulose has been previously used to suppress electro-osmotic flow[32]. In our experiment, we validated that the HPMC does the same function as an electro-osmotic flow suppressant to allow the analytes to reach the detection window. There is no observation of signals of both compounds after 30 min electrophoretic run without the addition of HPMC to the phosphate buffer. The mechanism of hydroxypropyl cellulose as electro-osmotic flow suppressant can be found in previously published work[28]. Hydroxypropylmethyl cellulose and hydroxypropyl cellulose are both polymer molecules. The monomers of these polymers are homologue because the structures of their monomer only have a -CH_2 difference. The mechanism of their influence on electroosmotic flow is almost the same. We selected the concentration of HPMC as 0.3 % according to a published paper[32].

Effect of buffer conductivity: In FASS, the stacking efficiency has strong dependency on the conductivity of background buffer, making buffer concentration a significant influence factor. For the purpose of electro-osmotic flow suppression, acidic running buffer was adopted to favour the formation of HPMC dynamic coating on capillary surface. So we choose phosphate solution as the buffer for HPMC will not have the function of suppressing the electroosmotic flow under the alkaline condition. The electropherogram in Fig. 3

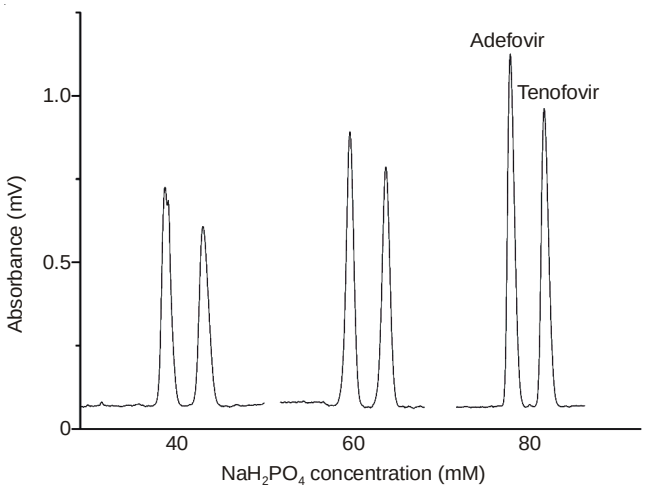

Fig. 3. Dependence of FASS performance on buffer conductivity. Separation buffer: 40-80 mM NaH_2PO_4 with 0.3 % HPMC; water-plug injection: time 10 s at cathode end by gravity with 14 cm height difference; sample electrokinetic injection: 20 s with 10.6 kV; separation voltage: 18.0 kV. Sample solution: 500 ng mL^{-1} adefovir and tenofovir

illustrates the stacking performance of the two compounds (500 ng mL^{-1} adefovir and tenofovir) dissolved in 1 mM NaOH with 40, 60, 80 mM NaH_2PO_4 (0.3 % HPMC, pH = 6.0) as running buffer, respectively. The water plug injection time for the cathode end was 10 s by gravity and the sample was injected at 10.6 kV for 20 s. According to FASS theory, higher buffer concentration has higher conductivity and therefore it will improve detection sensitivity. However, on the other hand, the buffer concentration can't be too high since it produces a higher current as well, resulting in elevated Joule-heat. Beyond a certain point, Joule-heat is detrimental to capillary electrophoresis performance and it will widen the peaks of electropherograms. In the case of NaH_2PO_4 concentration at 100 mM, the current reached 115 μA (the maximum current value demanded in our instrument is 120 μA), which is too high for successful separation for peak band broadening. Thus, 80 mM NaH_2PO_4 (0.3 % HPMC) was selected for the subsequent experiment.

Effect of water plug: Field amplification phenomenon can occur as long as the conductivity of the sample solution is lower than that of the running buffer. However, the sample ions electrokinetically injected will focus on the injection point (capillary tip) and cause the conductivity of sample band becoming higher, which will lead to the degradation of the field enhancement. Thus pre-injection of a short water plug prior to sample injection has been proposed for FASS to provide remarkable electric field enhancement and higher stacking efficiency. During electroinjection, the charged solutes migrate rapidly through the water zone. When the charged solutes reach the interface between the water zone and the running solution, their electromigrational transport decreases because the electric field within the water plug is much higher than that within the buffer. Consequently, many of the charged analytes are effectively concentrated before their electrophoretic separation[30]. Fig. 4 shows that the peak intensities of these two compounds are significantly increased by a 5-s gravity loading of water into the capillary ends before sample injection compared with no water injection. In addition, the presence of water plug can make the stacking system more stable as identified from the calculated RSDs. The effect of injection time of water plug on peak intensities was further investigated in the range of 5-20 s and 20 s as injection time was found to provide the highest signals for both adefovir and tenofovir (Fig. 4). The longer the water plug is and the higher intensity of the signal will be when the water plug injection time is less than 20 s, which means that the water plug should be long enough for proper field amplification. In case the water plug is too short, somewhat similar to that without water plug in FASS, rapid accumulation of sample ions will cause dramatic increase of conductivity at the capillary tip, consequently lowering stacking efficiency. Based on our experimental testing and results, the RSDs of adefovir and tenofovir signal response were 2.80 % and 5.72 % with 15-s as injection time. However, when the injection time increased to 20 s, the RSDs reached to 7.44 % (adefovir) and 5.97 % (tenofovir) respectively. In order to have a good repeatability, an injection time of 15-s was selected for the stacking system since the water injection time exceeding 15-s would make the stacking system less unstable.

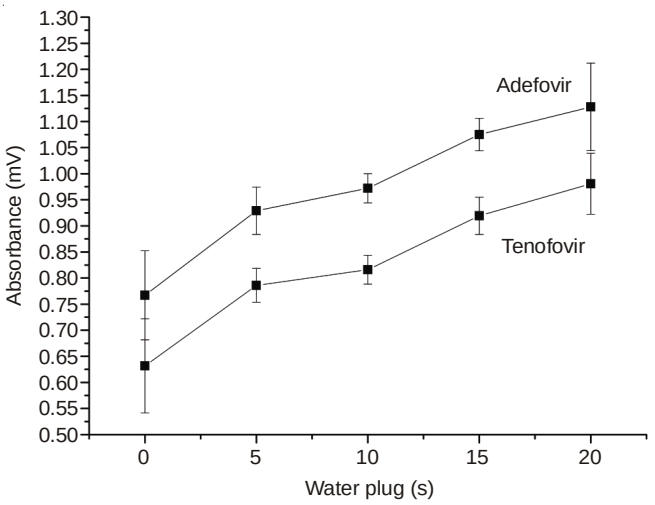

Fig. 4. Dependence of FASS performance on water plug length. Water-plug injection time: 0-20 s at cathode end by gravity with 14 cm height difference; separation buffer: 80 mM NaH_2PO_4 with 0.3 % HPMC; sample electrokinetic injection: 20 s with 10.6 kV; separation voltage: 18.0 kV. Sample solution: 500 ng mL^{-1} adefovir and tenofovir; mean values ± S.D., n = 3

Effects of sample injection voltage and injection time: The effect of sample injection voltage on peak intensities was also investigated in the range of 6.6-16.6 kV and the highest peak height was obtained at 14.6 kV. The sample injection time was further studied and explored at this injection voltage. The data in Fig. 5 indicates that increasing injection time in the range of 5-20 s will increase the signal significantly due to more analyte particles introduced. However, no further improvement in sensitivity was observed after 20 s through further prolonging injection time; instead, the stacking performance of both adefovir and tenofovir began to be worse somehow and the peaks became wider. At the interface of low and high conductivity media, the analytes still have electrophoretic mobility, so that the water plug can only trap the stacked ions for a certain time. If the injection time is too long, some of the

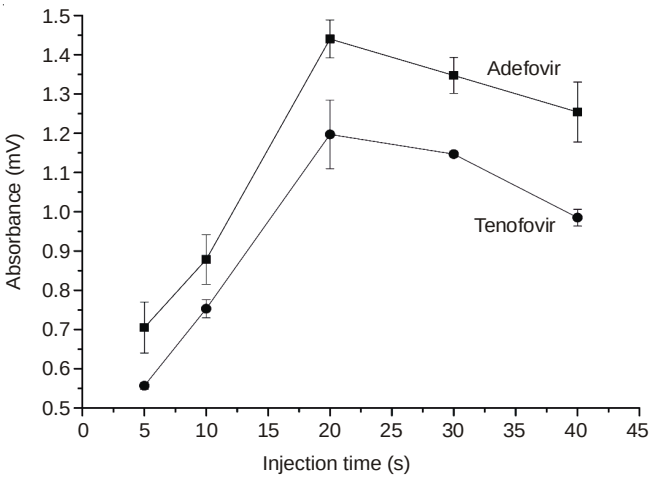

Fig. 5. Dependence of FASS performance on injection time. Sample electrokinetic injection: 5-40 s with 14.6 kV; 80 mM NaH_2PO_4 with 0.3 % HPMC; water-plug injection: time 15 s at cathode end by gravity with 14 cm height difference; separation voltage: 18.0 kV. Sample solution: 500 ng mL^{-1} adefovir and tenofovir; mean values ± S.D., n = 3

TABLE-1
LINEARITY, LOD, REGRESSION COEFFICIENT AND SENSITIVITY ENHANCEMENT BY FASS

Compound	Concentration (ng mL^{-1})	LOD (ng mL^{-1})		Enrichment factor[a]	Linear equation	R
		Non-stacking	FASS method			
Adefovir	10-500	390	2.67	146	y = 2887.8x - 10.517	0.99814
Tenofovir	10-500	440	3.22	137	y = 2391.2x - 4.377	0.99816

[a]Enrichment factor was simply calculated by (LOD of non-stacking)/(LOD of FASS method).

already stacked analytes would leave the boundary and enter the buffer zone from the water plug before separation, resulting in de-stacking and band broadening for the analytes.

Repeatability, linearity and detection enhancement: In order to evaluate the repeatability of the proposed approach, a mixed solution of 500 ng mL^{-1} adefovir and 500 ng mL^{-1} tenofovir was analyzed under the optimized FASS conditions. Although the injection of water plug and FASS sampling were all performed manually with renewal sample solution for each run, repeatable peak heights were obtained. Intra-day RSDs of the peak heights of analytes for 5 repeated injections were found to be 2.84 % (adefovir) and 2.79 % (tenofovir), respectively. Inter-day repeatability was assessed by analyzing the same concentration of sample solution above within 3 consecutive days; the RSDs of peak heights for the two compounds were 1.38 % (adefovir) and 3.26 % (tenofovir), respectively. The linearity of the method was tested using five different concentrations of standard mixture of adefovir and tenofovir, as listed in Table-1. The calibration curves show good linear relationship between peak amplitudes and the concentrations of tested compounds.

Typical electropherograms of the adefovir and tenofovir under non-stacking and FASS conditions are shown in Fig. 6(A) 10-s hydrodynamic injection by gravity for both adefovir and tenofovir was applied with 14 cm height difference (2.0 psi) using a mixture of 5 μg mL^{-1} adefovir and tenofovir as an example [Fig. 6(A)]. The FASS electropherogram was obtained by the electrokinetic injection under optimized conditions [Fig. 6(B)]. The sample solution used in Fig. 6(B) is 10-time dilution of that in Fig. 6(A), but much higher detector responses for the analytes were obtained under FASS condition, suggesting that significant improvement in sensitivity was achieved by the FASS procedures. From the limits of detection (LOD, S/N = 3, Table-1), it can be seen that over 150-time enhancement in sensitivity was achieved for both two compounds under FASS condition, compared with hydrodynamic injection by gravity.

Real sample analysis: Original serum sample was used as the blank matrix. The FASS method relies on the difference between the concentrations of the sample region and the buffer region. Therefore, the concentration of some existing ions in the sample solution will directly inuence the signal enhancement performance[33].

When the serum sample was not diluted, the signal enhancement was not significant because of the matrix effects; therefore, we diluted the serum to some extent based according to the treated sample procedures in a published article[33]. The original serum didn't contain the two compounds [Fig. 7(A)], so we need to spike some amount of standard solution of these two analytes into the diluted serum. From the experimental results,

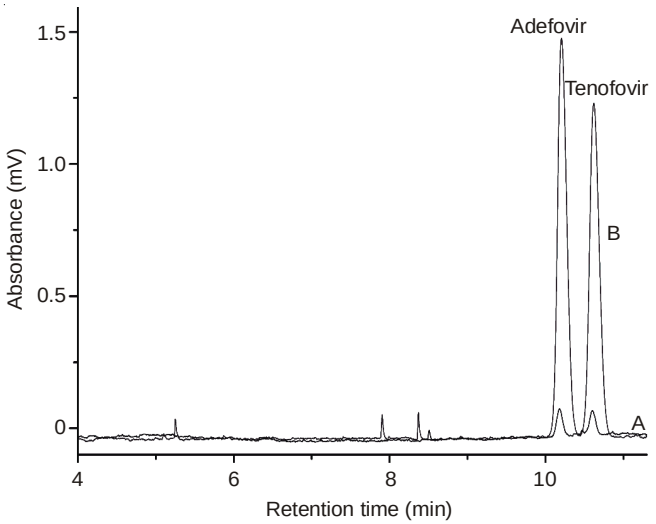

Fig. 6. Separation of the analytes under (A) non-stacking and (B) FASS conditions. Separation buffer: 80 mM NaH_2PO_4 with 0.3 % HPMC; sample electrokinetic injection: 20 s with 14.6 kV; water-plug injection time: 15 s at cathode end by gravity with 14 cm height difference; separation voltage: 18.0 kV. Sample solution: (A) 5 μg mL^{-1} adefovir and tenofovir injected by gravity for 10 s. (B) 500 ng mL^{-1} adefovir and tenofovir by FASS mode

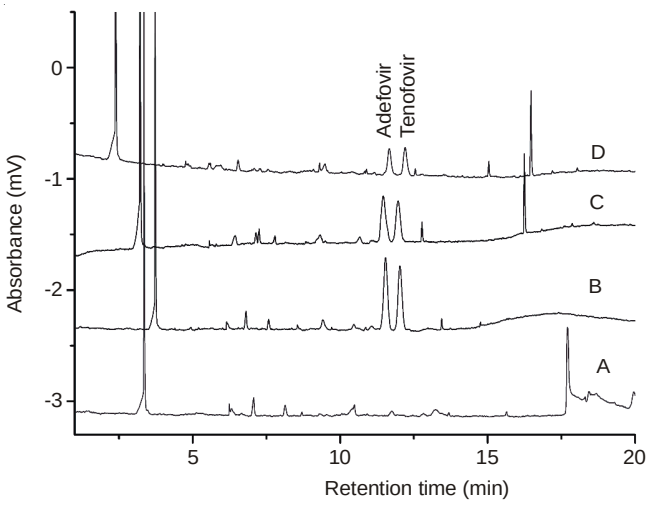

Fig. 7. Electropherograms of bovine serum under cathode-end FASS using the optimized conditions. Sample solution: (A) 100-fold dilute of original bovine serum; the solution for (B), (C) and (D) is obtained by spiking 0.40 μg mL^{-1}, 0.25 μg mL^{-1}, 0.10 μg mL^{-1} adefovir and tenofovir in the solution of (A), respectively

we can see that the peak amplitudes of the two compounds with 100-time dilution of original bovine serum were higher than that of 50-time dilution due to less ionic matrix interferences. Therefore, in the following experiment, we diluted the serum to 100 times for real sample analysis. Also, the regression equations (Table-1) given above were not appropriate

for serum samples. Since the sample had a dilute treatment, we have given the other linear equations that can be applied to the bovine serum sample after the dilute treatment. Linearity of the calibration curve in serum samples was assessed over a concentration range from 0.05 to 0.5 μg mL^{-1} of adefovir and tenofovir. The linear correlations were found to be y = -8.64626 + 1.77007x (R = 0.99548) for adefovir and y = 12.46259 + 1.45932x (R = 0.99636) for tenofovir. The limits of detection in serum sample were 4.36 ng mL^{-1} for adefovir and 5.28 ng mL^{-1} for tenofovir at a signal-to-noise ratio of 3.

Recovery experiments were performed in order to study the accuracy of the method. The spiked serum samples were prepared by adding different amounts of adefovir and tenofovir standard solution into the 100-time diluted blank serum samples to make the concentrations of adefovir and tenofovir in each serum sample of 0.40, 0.25, 0.10 μg mL^{-1}, respectively (Fig. 7). As shown in Table-2, the average recoveries ranged from 92 to 110 % and the RSDs were 3.5 %-14.3 % (n = 4) for matrix influences and handy manipulations (Table-2). Using an automatically controlled capillary electrophoresis instrument instead of manual manipulations or adding an internal standard substance into the sample solution, further improvements in repeatabilty and RSDs can be expected since the uncertainty from manual operations should be significantly reduced.

TABLE-2
ANALYTICAL RESULTS OF THE SPIKED SERUM SAMPLE AND THE RECOVERIES IN FASS METHOD (n = 4)

Analytes	Added amount (μg mL^{-1})	Detective amount (μg mL^{-1})	Recovery (%)	RSD (%)
Adefovir	0.40	0.38 ± 0.03	95.0	9.04
	0.25	0.24 ± 0.01	96.0	3.50
	0.10	0.11 ± 0.01	110.0	14.3
Tenofovir	0.40	0.39 ± 0.02	97.5	4.59
	0.25	0.23 ± 0.01	92.0	5.45
	0.10	0.11 ± 0.01	110.0	10.5

Conclusion

A simple, sensitive and reliable method of capillary electrophoresis combining with FASS has been successfully established for the analysis of adefovir and tenofovir. This analytical method has been successfully used to determine the adefovir and tenofovir in bovine serum and is suitable for pharmacokinetic studies. The proposed method can be used to determine the above-mentioned analytes at ppb level, which is about the same detection level as the expensive mass spectrometry. The characteristics of this technique are highly sensitive, cost-effective, relatively simple and time-saving compared with other methods, such as, LC/MS/MS, HPLC-UV, Fluorescent derivatization-HPLC and SPME-HPLC. Due to the equipment limitations in this laboratory at the movement, with manual manipulations the RSDs of this method are still somewhat limited. However, the repeatabilty and accuracy of the method should be significantly improved by using an automatic sampler and adding some proper internal standards.

ACKNOWLEDGEMENTS

The authors are grateful to the financial support from National Major Scientific Instruments and Equipments Development Special Funds (No. 2011YQ030113), National Recruitment Program of Global Experts (NRPGE), the Hundred Talents Program of Sichuan Province (HTPSP) and the Startup Funding of Sichuan University for setting up the Research Center of Analytical Instrumentation.

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