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A three-step stacking capillary electrophoresis of field-amplified sample injection, sweeping, and micellar collapse for determination of dabigatran and its active metabolite in human plasma

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Abstract

A three-step stacking capillary electrophoresis (CE) composed of field-amplified sample injection, sweeping, and analyte focusing by micellar collapse (FASI-sweeping-AFMC) was developed to determine dabigatran (D) and its major active metabolite, dabigatran acyl-beta-d-glucuronide (DAG), in human plasma. After optimization and validation, this novel approach was further applied to monitor 5 real samples, and the 25.2–186.8 ng mL⁻¹ D could be observed among those. Based on these results, the novel CE stacking strategy was successfully applied for the analysis of D and DAG in human plasma and could be served as a tool for clinical assays.

Keywords: Anticoagulant, Dabigatran, Dabigatran acyl-beta-d-glucuronide, FASI-sweeping-AFMC, Human plasma

1. Introduction

Antithrombotic therapy plays an important role in the prevention and treatment of thromboembolic disorders. However, currently available agents such as warfarin and oral vitamin K antagonists show numerous complex drug and food interactions, resulting in unpredictable pharmacokinetic outcomes [1]. Additionally, some antithrombotic agents such as low molecular weight heparin or fondaparinux are required with parenteral administration, resulting in a decrease of patients' compliance. For the above reasons, oral

active thrombin inhibitors possessing few interactions with drugs or food would offer some potential advantages over these agents. In 2010, dabigatran etexilate (DE) (Pradaxa®), a novel direct oral anticoagulant (DOAC), was approved for the prevention of stroke and thrombosis in patients with non-valvular atrial fibrillation.

DE is a prodrug and further metabolized by esterase to its active metabolite, dabigatran (D), *in vivo* [2,3], and then this could be further metabolized to form a glucuronide conjugate possessing pharmacological activity, dabigatran acyl-beta-d-glucuronide (DAG), which is the final metabolite in

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humans [4]. Although D and DAG both have pharmacological activity, D is only used as an anti-coagulant for clinical use, and DAG is generally regarded as a popular research subject in enzymology. Due to infrequent monitoring of the clotting tendency of blood in the treatment of D, it can be offered as an alternative therapy for warfarin [5]; however, the U.S. Food and Drug Administration (FDA) unexpectedly issued warnings in 2011 about the bleeding risk of DE. Furthermore, related researches exhibited a relationship between plasma concentration of D with the risk of bleeding (plasma concentration of D > 200 ng mL⁻¹) and thrombosis (plasma concentration of D < 50 ng mL⁻¹) [6,7]. For these above-mentioned arguments, a suitable detective approach for the determination of D and DAG levels in human plasma is necessary for clinical assays.

Up to now, coagulation tests have been widely utilized to assess the safe dosage range of antithrombotic drugs in clinical settings; for example, the prothrombin time (PT) and activated partial thromboplastin time (aPTT) have been evaluated for the safety of D, although due to numerous interferences from other coagulant drugs, endogenous changes in coagulation factors or variations in responses to drugs among the individuals, detection of PT or aPTT is difficult for assaying the safety of D in humans. Because of these reasons, some approaches such as HPLC-MS/MS, UPLC-UV, and UPLC-MS/MS have been developed for detection of the real D level *in vivo* to overcome these drawbacks. Among these approaches, UPLC-MS/MS has been frequently applied for the determination of D levels in human plasma due to its specificity and sensitivity while showing satisfactory outcomes (about 0.2 ng mL⁻¹) [8,9]. Unfortunately, most laboratories in hospitals and clinics can't afford the cost of the UPLC-MS/MS apparatus and additionally, such instruments require an operator with specialized experience; consequently, a specific approach with easy operation and inexpensive property is urgently needed for the determination of D and DAG levels in human plasma.

Capillary electrophoresis (CE) has become an attractive analytical technique due to its high separation efficiency, short analysis time, less consumption of materials, and its facility to be widely applied in different practical fields, including medicine, pharmaceuticals, chemistry, and biology [10–20]. However, the sensitivity of CE is comparatively low due to the minor amount of sample injection and the short optical length of capillary. To overcome this problem, various on-line preconcentration approaches have been developed to increase the sensitivity of CE, such as field amplified sample

stacking (FASS), field amplified or enhanced sample injection (FASI) [21,22], sweeping [23,24], large volume sample stacking (LVSS) [25,26], dynamic pH junction [27], analyte focusing by micelle collapse (AFMC) [28,29] and micelle to solvent stacking (MSS) [30–32], etc. These on-line preconcentration approaches have more favorable traits such as ease of manipulations without complicated modifications of commercial CE instrumentation and the significant enhancement of sensitivity. Among these stacking approaches, FASI utilizes the electrokinetic injection of samples in a diluent with conductivity at least 10 times lower than the background solution (BGS). In other words, it shows that the electric field strength in the sample zone is higher than that in the BGS zone, and as a result, the electrophoretic velocity of ions in the sample zone is faster. Ions migrate rapidly during electrophoresis and slow down abruptly when they encounter the BGS zone. The ions can be concentrated and stacked at the boundary between the BGS and sample zone or water zone [33]. Sweeping relies on the interaction of analytes with the micellar phase that penetrates the sample zone and creates a concentrated sample zone by physicochemical properties. The AFMC mechanism is based on the use of micellar pseudophase in the sample matrix, and analytes covered with micelles can collapse into a diluted zone of micelles to stack in the interface due to low concentration of micelles resulting from the difference of electric field strength between the water and BGS plug zone. The accumulated analytes are subsequently separated by electrophoresis [34]. The enhancement of sensitivity is limited when only a single stacking approach is performed in a study. In order to obtain further enhancement of sensitivity in CE, numerous studies indicate the merging of more than two stacking approaches into one CE separation. Currently, much of the literature has demonstrated the efficacy of a three-step stacking approach in CE is better than that of a single stacking approach [33,35]. For this reason, the above-mentioned stacking approaches were combined in this study to develop a novel three-step stacking CE approach.

Accordingly, a novel and convenient CE on-line three-step stacking approach called FASI-sweeping-AFMC, including FASI, sweeping, and AFMC was designed and developed for the determination of D and DAG levels in human plasma for clinical assays. To our best knowledge, few studies have reported the simultaneous determination of D and DAG levels in human plasma by CE, so this sequential three-step stacking (FESI, sweeping, and AFMC) approach should be the first article delineating the determination of D and DAG levels in human plasma in the field of CE.

2. Materials and methods

2.1. Chemicals and consumables

All of the analytes were analytical grade. D and DAG were purchased from Cayman Chemical (Ann Arbor, MI, USA). Clozapine as an internal standard (IS), verapamil, amiodarone, propranolol, digoxin, sodium dihydrogen phosphate (NaH_2PO_4), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH), hydrochloric acid (HCl), and sodium hydroxide (NaOH) were obtained from E. Merck (Darmstadt, Germany). Perchloric acid (HClO_4) was provided from Honeywell Fluka (Muskegon, MI, USA). Sudan III was purchased from Katayama Chemical (Osaka, Japan). The ultrapure water used for the preparation of buffer and other aqueous solutions was produced from Millipore Ultra-Pure water system (Bedford, MA, USA).

2.2. CE system

Beckman PA800 MDQ (Fullerton, CA, USA) electrophoresis instrument equipped with a UV detector was used for the determination of D and DAG levels in human plasma. The detective wavelength was set at 214 nm. An uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 50 cm total length (40 cm to the detector) was used. Before utilization of analysis, each new capillary should be rinsed with MeOH, ultrapure water, 1 M HCl, ultrapure water, 1 M NaOH, and ultrapure water, and each step was set at 30 psi for 10 min. The temperature of capillary was maintained at 25 °C, and the data obtained from tests were collected and analyzed by using MDQ 32 Karat software from Beckman.

2.3. Preparation of the plasma samples

All stock solutions of analytes including IS were prepared with the acidic water of pH 3.0 at a concentration of 1 mg mL^{-1} . Patient plasma samples were obtained from patients (P1–P5) with non-valvular atrial fibrillation taking DE during antithrombotic therapy. Additionally, the plasma obtained from five healthy volunteers was used as the plasma matrix in tests. Ethical approval for this study was obtained from the Institutional Review Board (IRB) of the Chung-Ho Memorial Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan (Number of IRB: KMHIRB-E(I)-20190422). All samples used in the tests were stored at -20 °C before use.

The 250 μL plasma sample was prepared in the plasma matrix with an expected concentration of D, DAG, and IS. Subsequently, 10 μL of 70% (V/V) HClO_4 was added into this plasma sample for precipitation of protein with vortex for 2 min. The mixture was then centrifuged at 10000 rpm for 15 min. When centrifugation was completed, the supernatant was transferred into a 2 mL eppendorf tube and then further extracted by solid phase extraction (SPE). In the SPE procedure, the Oasis HLB extraction cartridge (Waters, Wexford, Ireland) was utilized for pretreatment of the supernatant, with the procedure described as follows. Initially, the SPE cartridge was conditioned with 2 mL methanol and 2 mL ultrapure water respectively, and the supernatant was then loaded into the cartridge, and following the loading, the SPE cartridge was washed with 2 mL ultrapure water and 2 mL 10% (V/V) methanol respectively. The SPE cartridge then was eluted with 1 mL methanol and the eluent was collected, evaporated to dryness under vacuum, and finally reconstituted with acidic water (pH 3.0). The 100 μL reconstituted solution with 0.5 $\mu\text{g mL}^{-1}$ IS was analyzed by the developed CE system. The real plasma samples from the patients taking DE were also pretreated in the same procedure without the addition of D and DAG.

2.4. FASI-sweeping-AFMC electrophoresis

Before separation, the capillary was rinsed with the BGS (pH = 2.5) composed of 100 mM NaH_2PO_4 at 30 psi for 10 min. Subsequently, the micellar solution (MS) (pH = 2.5), composed of the BGS containing 50 mM SDS, was injected into the capillary at 0.5 psi for 20 sec. Next, the BGS plug solution (pH = 2.5) of 100 mM NaH_2PO_4 was injected into the capillary at 0.5 psi for 90 sec, and then ultrapure water was injected into the capillary at 0.5 psi for 60 sec. After the above steps, the analytes were electrokinetically injected into the capillary at 10 kV for 300 sec and then separated at 20 kV. In this study, the UV detector was set at the cathode side, detective wavelength was set at 214 nm, and the running temperature during analysis was maintained at 25 °C.

2.5. Method validation

In order to establish the calibration curves for quantification of D and DAG levels in human plasma, five different concentrations of D (2.5, 10, 100, 250, 1000 ng mL^{-1}) and DAG (50, 100, 375, 500, 1000 ng mL^{-1}) were spiked into the plasma matrix

and analyzed by this developed CE approach. All of the plasma samples spiked with known concentrations of D and DAG were also pretreated according to the procedures of section 2.3. The calibration curves were established by comparison of the corrected peak area ratio of each analyte to IS (Y axis) with the concentrations of each analyte in ng mL^{-1} (X axis). In the analysis of the precision and accuracy, three different concentrations of D (5, 50, and 500 ng mL^{-1}) and DAG (75, 250, and 750 ng mL^{-1}) were spiked into the plasma matrix and subsequently determined, with the results being evaluated by values of relative standard deviation (RSD) and relative error (RE) from the three different spiked concentrations in the analyses of both intra-day and inter-day. The outcome of intra-day was defined as three repeated analyses ($n = 3$) in a single day, and the outcome of inter-day was defined as the same analysis over five different days ($n = 5$). In this study, the limit of detection (LOD) was determined in the plasma matrix using the diluted standard solution where the ratio of signal to noise was equal to 3 ($S/N = 3$).

2.6. Stability and selectivity

The stability of our approach in CE during analysis was evaluated over 24 hours at room temperature. The RSD value of the peak area ratio (peak area of analyte/peak area of IS) ($n = 3$) was utilized for evaluation of the stability, and it was considered as stable when the RSD value was within $\pm 5\%$. Besides, the selectivity of our approach in CE was assessed by a comparison of the plasma matrix containing $1 \mu\text{g mL}^{-1}$ D, DAG, $0.5 \mu\text{g mL}^{-1}$ IS, and $1 \mu\text{g mL}^{-1}$ four antiarrhythmic drugs (verapamil, amiodarone, propranolol, digoxin) chosen from the viewpoint of participants under the treatment suggested by physicians [36] and the corresponding controls.

Initially, the plasma matrix samples in this study were pretreated and analyzed by the above-mentioned procedure. Subsequently, the migration time of any single peak was compared with that of the corresponding controls to demonstrate the selectivity of our approach in CE.

2.7. Sensitivity enhancing factors

In order to realize the sensitivity enhancement of the FASI-sweeping-AFMC, a typical injection CZE as a control group was performed to calculate the sensitivity enhancement according to the literature [31]. In the system of the typical injection CZE, the analytes were injected into the capillary at 0.5 psi for 5

sec, and the separated voltage and the buffer solution were the same as those in the FASI-sweeping-AFMC. However, in the consideration of the low sensitivity for the typical injection CZE, the concentrations of the analytes used in the typical injection CZE were 10 fold higher than those used in the FASI-sweeping-AFMC, and results would be calibrated at the end of the calculation. Based on the equation described from these researches [31,37], the sensitivity enhancement factors (SEFs) of the FASI-sweeping-AFMC were calculated. The equation was as following: $\text{SEFs} = [\text{corrected peak area in the FASI-sweeping-AFMC}/\text{corrected peak area in the typical injection CZE}] \times [\text{concentration in the typical injection CZE}/\text{concentration in the FASI-sweeping-AFMC}]$. The corrected peak area described in the equation was peak area of the analyte/migration time of the analyte.

3. Results and discussion

3.1. Mechanism of the FASI-sweeping-AFMC approach

In this study, the stacking CE techniques, including field-amplified sample injection, sweeping, and analyte-focusing by micellar collapse were integrated into a three-step stacking CE procedure (FASI-sweeping-AFMC) to determine the levels of D and DAG in human plasma. The principle of this developed CE approach is illustrated in Fig. 1. Firstly, the fused silica capillary was filled with BGE, micellar solution, BGS plug, and water sequentially (Fig. 1A); secondly, the sample was injected electrokinetically into the capillary; and finally, when the injected voltage was applied (anode was set at the inlet), the anionic micelles and cationic analytes would respectively migrate to the anode and cathode electrophoretically. During electrokinetic injection, it was noted that the stacking of FASI could create a concentrated sample zone between the water zone and the BGS plug due to the difference of electric field strength (Fig. 1B). Subsequently, as the separation voltage (normal polarity, 20 kV) was applied, the anionic micelles would penetrate the sample zone and sweep the analytes toward the anode to form the second stacking (Fig. 1C). At this moment, the direction of effective electrophoretic mobility for analytes was reversed from cathode to anode due to charge change. However, when the micelles carrying the analytes moved to the water zone, the concentration of micelles would decrease and become lower than the critical micelle concentration (CMC), resulting in the collapse of the micelles. Hence, the micelles would release the

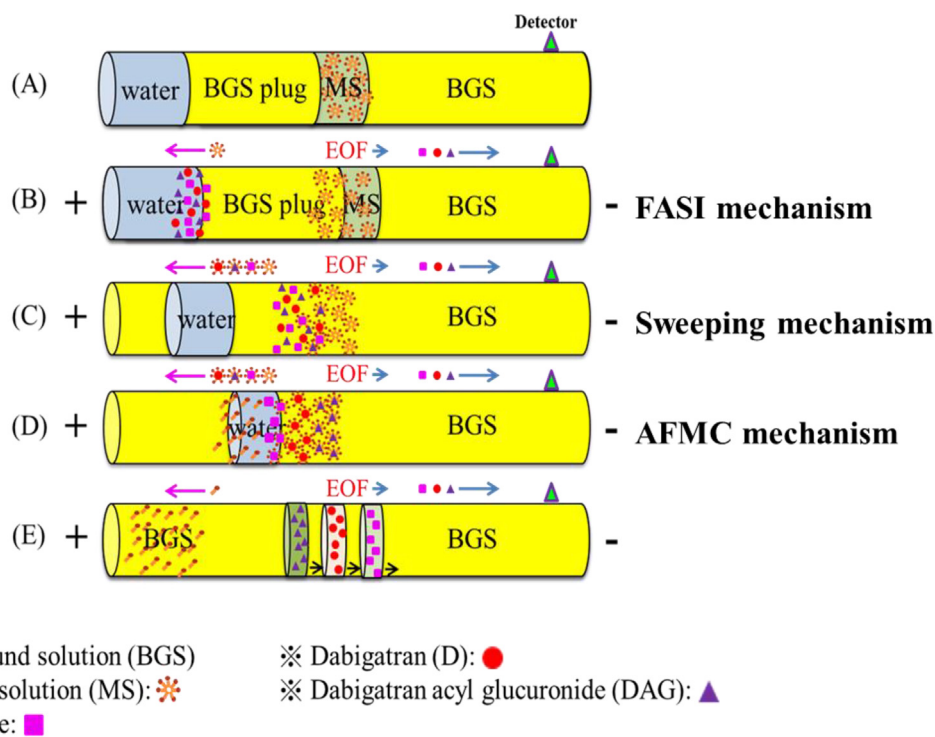


Fig. 1. Schematic diagrams of the FASI-sweeping-AFMC approach. (A) The starting situation, (B–D) injection, and the stacking process. (E) The separation. The details of this mechanism have been explained in the results section.

analytes in the dilution zone to accomplish the third stacking of the analytes. At this moment, the direction of effective electrophoretic mobility for analytes was reversed again, and analytes would migrate toward the cathode (Fig. 1D). Finally, the separation of analytes was determined by their charge and mass for separation, and the analytes were determined by UV detection at 214 nm wavelength (Fig. 1E). Our approach provided three stacking techniques, including FASI, sweeping, and AFMC in the one-pot procedure, to achieve sensitivity enhancement in CE analysis.

3.2. Optimization of the FASI-sweeping-AFMC approach

In order to obtain the highest efficiency of stacking and separation in the FASI-sweeping-AFMC approach, several parameters in CE analysis including the pH value of the buffer system, the concentration of BGS, the concentration of SDS, the concentration of BGS plug, and sample injection time were investigated as follows.

3.2.1. The pH value of the buffer system

In the CE system, the electrophoretic mobility is dependent on the ionized level of analytes, and the electroosmotic flow (EOF) is controlled by the silanol

groups (SiOH) existing in the anionic form (SiO⁻). Both of them were greatly affected by the pH values of the buffer system, so pH values play a key role in the CE system. In this study, the buffer system was composed of BGS, MS, and BGS plug solution, and the pH values of the three solutions were investigated from 2.0 to 3.5 (Fig. 2). According to the results, there was a noise peak presented at pH 2.0, and it did not interfere with the separation of analytes. When the pH value was higher than 2.5, the efficiency of stacking and separation was similar; however, high protonation of analytes (pH 2.0) would cause the analytes to strongly interact with SDS micelles, and the strong interaction would affect the release of analytes from the SDS, resulting in poor efficiency of stacking at pH 2.0 (Fig. 2A). Additionally, although in the use of pH 3.5 (Fig. 2D), the interfering peaks were fewer than others in the electropherograms, it consumes more time to separate. Separation time and interfering peaks are both significant factors for us to find the optimal condition. Although fewer interferences at pH 3.5, the separation time at pH 2.5 was shorter than that, and interferences at pH 2.5 would not affect the resolution of D and DAG. Due to these reasons, pH 2.5 was selected as the optimal condition of the pH value in the buffer system. Under such pH value (pH 2.5), the mobility of the EOF is $3.27 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$.

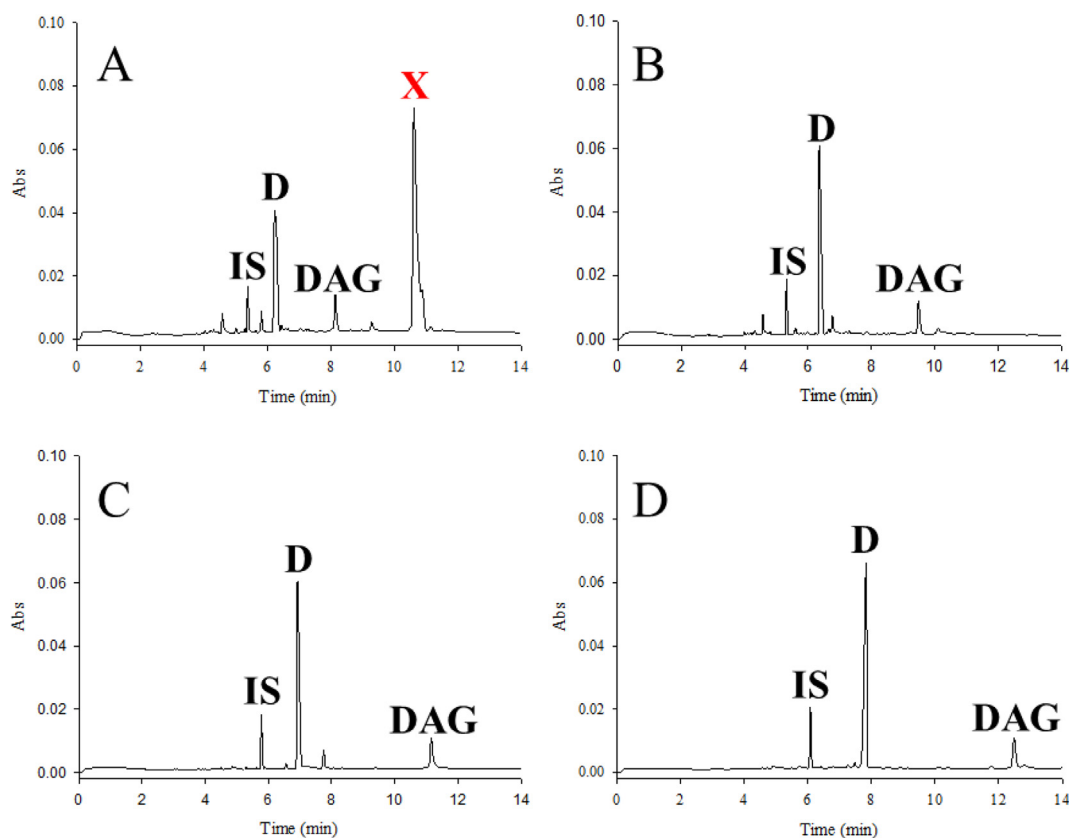


Fig. 2. Effect of pH ((A) pH 2.0; (B) pH 2.5; (C) pH 3.0; (D) pH 3.5) of buffer system on stacking and separation efficiency by the FASI-sweeping-AFMC approach. The symbol x represents the signal of interference from plasma matrix. Sample concentrations: $1 \mu\text{g mL}^{-1}$ D; $1 \mu\text{g mL}^{-1}$ DAG; $0.5 \mu\text{g mL}^{-1}$ IS. CE conditions: BGS, 100 mM NaH_2PO_4 ; MS, BGS contains 50 mM SDS; BGS plug, 100 mM NaH_2PO_4 ; Sample injection, 300 sec (10 kV); Separation voltage, 20 kV; Detection wavelength, 214 nm.

3.2.2. The BGS concentration

In this study, the BGS concentration was investigated from 75 to 150 mM (Fig. 3). When the BGS concentration was 75 mM (Fig. 3A), the peak heights of D and DAG were short. That was because the ionic strength was not high enough for focusing the D and DAG in the capillary. As the BGS concentration increased, although the migration time of analytes was prolonged due to the low electric field strength at a higher concentration of BGS, the higher concentrations of BGS could make the D and DAG peaks taller and sharper. However, when the BGS concentration reached 150 mM, the peak shape of analytes became broad and crotched (Fig. 3D). This was because higher BGS concentration has higher ionic strength, which generates a higher current (up to 120 μA) to cause Joule heating, resulting in poor efficiency of stacking. Finally, 100 mM BGS solution was utilized in this CE approach.

3.2.3. The SDS concentration

In the AFMC mechanism [38], proper SDS concentration would make micelles collapse easily at

the boundary between the water zone and the BGS plug. The research indicated that the larger difference of electric field strength or conductivity between both could obtain more improvement of stacking efficiency. As the surfactant plays a key role to improve sensitivity and resolution, the anionic surfactant, SDS, was investigated from 0 to 100 mM (Fig. 4). When the SDS concentration reached 100 mM, the SDS micelles were difficult to collapse, and the analytes could not be further accumulated in the interface between the water zone and the BGS plug, so the poor efficiency of stacking could be observed at 100 mM of SDS (Fig. 4E). For the use of 25, 50, and 75 mM SDS (Fig. 4B–D), the efficiency of separation and stacking were similar. An increase of SDS concentration was beneficial to sweeping, but it also resulted in the insufficiency of collapsed micelles. When 25 mM SDS was used, there were several noise peaks closed to IS (Fig. 4B). Finally, 50 mM SDS was selected as the optimal condition. Additionally, in the use of 0 mM SDS (Fig. 4A), poor efficiency of stacking was observed, so this indicated that sweeping of the SDS micelles could improve the stacking of analytes.

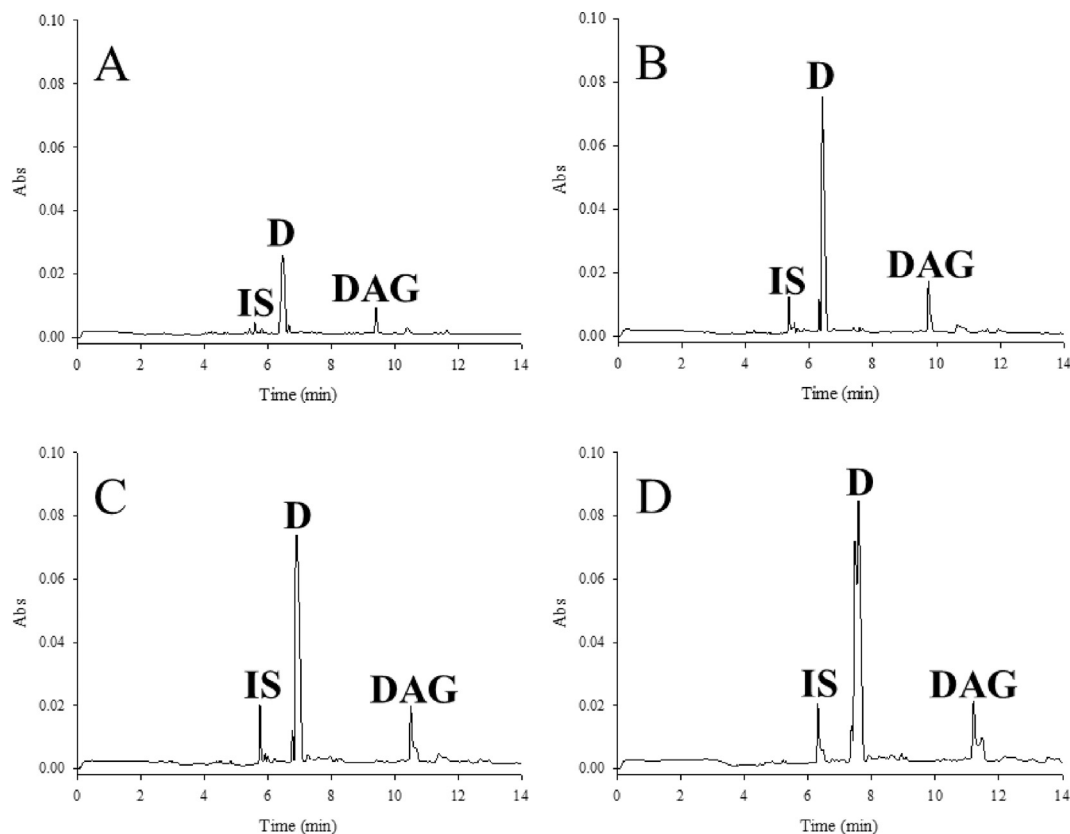


Fig. 3. Effect of BGS concentration ((A) 75 mM; (B) 100 mM; (C) 125 mM; (D) 150 mM) on stacking and separation efficiency by the FASI-sweeping-AFMC approach. Sample concentrations: $1 \mu\text{g mL}^{-1}$ D; $1 \mu\text{g mL}^{-1}$ DAG; $0.5 \mu\text{g mL}^{-1}$ IS. CE conditions: BGS, NaH_2PO_4 , pH 2.5; MS, BGS contains 50 mM SDS, pH 2.5; BGS plug, 100 mM NaH_2PO_4 , pH 2.5; Sample injection, 300 sec (10 kV); Separation voltage, 20 kV; Detection wavelength, 214 nm.

3.2.4. The BGS plug concentration

In order to obtain better efficiency of stacking in the FASI mechanism, the BGS plug was introduced to produce the difference of electric field strength between the water zone and the BGS plug. Additionally, the BGS plug also affects the migration of micelles sweeping toward the analytes. In this study, the BGS plug concentration was investigated from 50 to 200 mM (Fig. S1). As the concentration of the BGS plug reached 150 and 200 mM (Fig. S1C-D), the efficiency of stacking became worse, illustrating that a high concentration of the BGS plug could result in slow migration of micelles in the BGS plug, which might cause the insufficiency of collapsed micelles or poor sweeping. In the use of 50 mM BGS plug, poor efficiency of stacking was also observed. This was because that the difference of electric field strength between the water zone and the BGS plug was not high enough to produce the stacking of analytes in the interface during the electrokinetic injection. Finally, this approach was performed by using a 100 mM BGS plug.

3.2.5. The injection time of analytes

When analytes were electrokinetically injected into the capillary (10 kV), the amount of analytes introduced into the capillary was dependent on the injection time. Therefore, the injection time was investigated from 240 to 420 sec in this study (Fig. S2). When the condition was set at 360 sec or 420 sec, the efficacy of stacking decreased obviously, and the peak shape became broad and crotched (Fig. S2C-D). This showed that the length of the sample zone was too long to stack effectively. For this reason, injection time was set at 300 sec as the optimal condition.

3.3. Method validations

In this study, the LOD ($S/N = 3$) values of the D and DAG in the FASI-sweeping-AFMC approach were 0.25 ng mL^{-1} and 10 ng mL^{-1} respectively. Additionally, the calibration curves of the D and DAG were linear over the range of $2.5\text{--}1000 \text{ ng mL}^{-1}$ and $50\text{--}1000 \text{ ng mL}^{-1}$ respectively (data are shown in

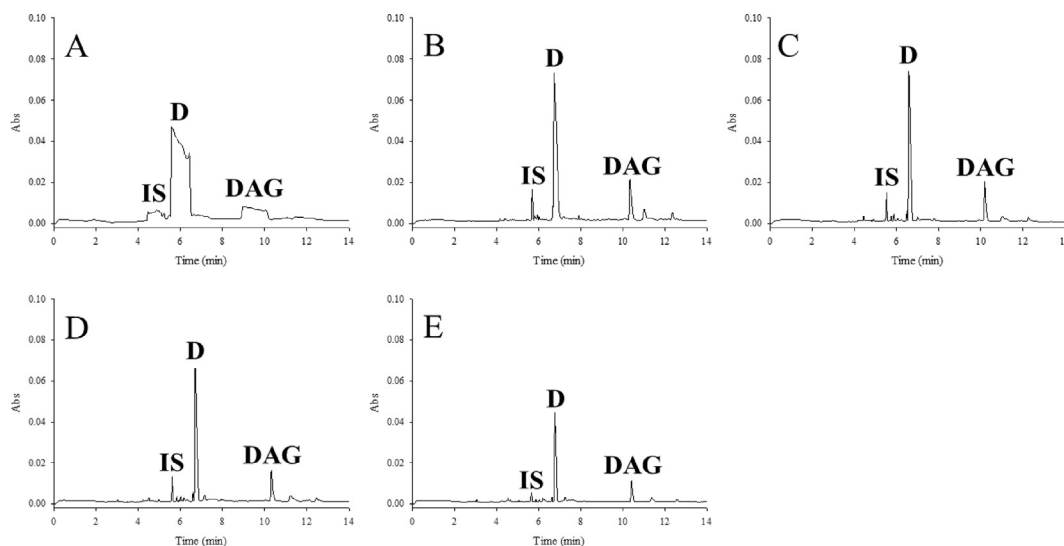


Fig. 4. Effect of SDS concentration ((A) 0 mM; (B) 25 mM; (C) 50 mM; (D) 75 mM; (E) 100 mM) on stacking and separation efficiency by the FASI-sweeping-AFMC approach. Sample concentrations: $1 \mu\text{g mL}^{-1}$ D; $1 \mu\text{g mL}^{-1}$ DAG; $0.5 \mu\text{g mL}^{-1}$ IS. CE conditions: BGS, 100 mM NaH_2PO_4 , pH 2.5; BGS contains SDS, pH 2.5; BGS plug, 100 mM NaH_2PO_4 , pH 2.5; Sample injection, 300 sec (10 kV); Separation voltage, 20 kV; Detection wavelength, 214 nm.

Table 1). In the analysis of intra-day and inter-day, the correlation coefficients (r) of D and DAG were all above 0.999, indicating a good linear response of this FASI-sweeping-AFMC approach. In the analysis of precision and accuracy, three different concentrations of D (5, 50, and 500 ng mL^{-1}) and DAG (75, 250, and 750 ng mL^{-1}) were spiked into the plasma matrix and then determined. The values of RSD and RE could be obtained to evaluate the precision and accuracy respectively, and according to **Table 2**, the good precision and accuracy of our approach were obtained. All values of the RSD and RE obtained from the analysis of intra-day and inter-day were less than 5%, and it showed good applicability of our approach for the analysis of biological samples.

3.4. Stability and selectivity

In order to evaluate the stability of our approach during analysis, the study of stability was executed for 24 hours at room temperature. In this study, the stability was presented as the RSD value of the peak area ratios for 24 hours (data shown in **Fig. S3**). During the 24 hour analysis, the RSD values of the peak area

ratios of D and DAG were 3.7% and 3.4% respectively. This data validated the excellent stability of our approach and ensured stable outcomes for the quantification of D and DAG levels in plasma over 24 hours. Additionally, the selectivity of our approach was confirmed by a comparison of the plasma matrix containing $1 \mu\text{g mL}^{-1}$ D, DAG, $0.5 \mu\text{g mL}^{-1}$ IS, and $1 \mu\text{g mL}^{-1}$ four antiarrhythmic drugs (verapamil, amiodarone, propranolol, digoxin) chosen from the viewpoint of participants under treatment suggested by physicians [32] and the corresponding controls. The data (**Fig. S3B**) showed that these analytes could be well-resolved under interferences resulting from the plasma matrix and interaction of these antiarrhythmic drugs. In summary, the results of this study indicated the high selectivity of this approach towards the identification of the target analytes, and four other antiarrhythmic drugs were confirmed.

3.5. The comparison with other stacking CE methods

In this research, the various stacking CE strategies and the typical injection CZE method were also

Table 1. Regression analysis for the determination of D and DAG levels.

| Analytes | Linear range (ng mL^{-1}) | Regression equation | Correlation coefficient (r) |
|-------------------------------------|--------------------------------------|---|---------------------------------|
| Intra-day (n = 3) | | | |
| Dabigatran | 2.5–1000 | $Y = (0.0092 \pm 0.0002) X + (0.2219 \pm 0.0513)$ | 0.9991 |
| Dabigatran acyl-beta-d- glucuronide | 50.0–1000 | $Y = (0.0035 \pm 0.0005) X + (0.0175 \pm 0.0548)$ | 0.9995 |
| Inter-day (n = 5) | | | |
| Dabigatran | 2.5–1000 | $Y = (0.0090 \pm 0.0002) X + (0.1295 \pm 0.0616)$ | 0.9995 |
| Dabigatran acyl-beta-d- glucuronide | 50.0–1000 | $Y = (0.0034 \pm 0.0002) X - (0.0418 \pm 0.0534)$ | 0.9993 |

Table 2. Analysis of the precision and accuracy for the determination of D and DAG levels.

| Analytes | Concentration known (ng mL ⁻¹) | Concentration found (ng mL ⁻¹) | RSD (%) | RE (%) |
|-------------------------------------|--|--|---------|--------|
| Intra-day (n = 3) | | | | |
| Dabigatran | 5 | 5.14 ± 0.11 | 2.08 | 2.79 |
| | 50 | 50.68 ± 2.15 | 4.24 | 1.37 |
| | 500 | 505.17 ± 6.31 | 1.25 | 1.03 |
| Dabigatran acyl-beta-d- glucuronide | 75 | 78.05 ± 1.02 | 1.30 | 4.07 |
| | 250 | 251.28 ± 0.79 | 0.31 | 0.51 |
| | 750 | 761.03 ± 3.15 | 0.41 | 1.47 |
| Inter-day (n = 5) | | | | |
| Dabigatran | 5 | 5.05 ± 0.11 | 2.22 | 1.04 |
| | 50 | 50.12 ± 0.82 | 1.63 | 0.25 |
| | 500 | 502.00 ± 6.35 | 1.26 | 1.01 |
| Dabigatran acyl-beta-d- glucuronide | 75 | 76.32 ± 1.97 | 2.59 | 1.76 |
| | 250 | 252.89 ± 3.00 | 1.19 | 1.16 |
| | 750 | 753.74 ± 2.59 | 0.34 | 0.50 |

performed to compare with the FASI-sweeping-AFMC by using the standard analytes. The data was as shown in Fig. S4. In the FASI-sweeping-AFMC (Fig. S4A), the D and DAG were well separated and stacked. When the separation was performed in the mode of the FASI-sweeping (Fig. S4B), the D and DAG were coupled. In the FASI-sweeping mode, the separation was set in the reverse polarity (The anode is set in the outlet), and the SDS (50 mM) micelles set in the inlet and outlet reservoir were continuously introduced into the capillary to sweep the analytes. The data indicated the continuous anionic SDS micelles under such conditions could sweep the analytes to the detector (anode), but could not resolve D and DAG. However, in the mode of the FASI-sweeping-AFMC, the separation was set in the normal polarity (The detector and cathode are in the outlet) and the SDS (50 mM) was present in a short plug (0.5 psi, 20 sec, about 1.3 cm of 50.0 cm total capillary length). Theoretically, the analytes would migrate to the inlet of the capillary due to the sweeping of the micelles, but the D and DAG could also be detected. Additionally, the mobility of the SDS micelles ($3.40 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$) was about ten-fold higher than that of EOF ($3.27 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$) under pH 2.5, and the analytes could not be pushed to the detector by EOF in the presence of the SDS micelles. Therefore, that meant the anionic micelles would be collapsed during separation, and the analytes were released to the free solution and moved to the cathode by EOF and their cationic charge. Second, in this study, the SDS (50 mM) micelles only occupied 2.6% of the total capillary (0.5 psi, 20 sec, about 1.3 cm of 50.0 cm total capillary length), and the SDS concentration would be decreased gradually due to distribution in the BGS plug and the water zone as micellar dilution zone (MDZ) during analysis. Finally, it would be low than

CMC to be collapsed and release the cationic analytes. If the micelles were not collapsed, the analytes would migrate to the inlet by the sweeping of the anionic micelles, and the signals of analytes were not detected by the detector in the cathode. In the FASI mode (Fig. S4C), the stacking efficiency was very poor for D and DAG, although they were well separated. In the comparison of the FASI-sweeping-AFMC and the FASI, it could be found the migration time became longer in the FASI-sweeping-AFMC due to the transient sweeping of the analytes to inlet by the short plug of SDS, and that also demonstrated the sweeping of the anionic SDS micelles were much helpful for sacking D and DAG.

3.6. Sensitivity enhancing factors

In order to know the sensitivity enhancement of the FASI-sweeping-AFMC, a typical injection CZE was also performed (Fig. S4D), and the sensitivity enhancement factors (SEFs) of the FASI-sweeping-AFMC were calculated. In the comparison of the typical injection CZE as the controlled group, the SEFs of the FASI-sweeping-AFMC were obtained. Based on the results (Table S1), the sensitivity was increased significantly in the FASI-sweeping-AFMC, and the SEFs of the FASI-sweeping-AFMC for D and DAG were 1572 and 634, respectively. The data has demonstrated that there was a sensitivity improvement by using the FASI-sweeping-AFMC for the determination of D and DAG when compared to the typical injection CZE.

3.7. Evaluation of SDS micelles collapse by using Sudan III

In this approach, the sweeping and the collapse of micelles played important roles for stacking the

Table 3. Characteristics and detected results of five patients in this study.

| Patient No. | Sex | Age (yr) | Weight (kg) | Dosage (mg/kg/day) | eGFR (mL/min/1.73 m ²) | D level ^a (ng mL ⁻¹) (n = 3) | DAG level (ng mL ⁻¹) (n = 3) |
|-------------|-----|----------|-------------|--------------------|------------------------------------|---|--|
| P1 | F | 85 | 60 | 3.7 | 61.7 | 186.8 ± 17.4 | ND ^b |
| P2 | M | 77 | 61.3 | 3.6 | 66.3 | 170.4 ± 14.2 | 57.6 ± 2.1 |
| P3 | M | 78 | 79 | 2.8 | 49.0 | 110.6 ± 9.5 | <50.0 ^c |
| P4 | F | 59 | 78.2 | 2.8 | 90.0 | 184.7 ± 8.9 | <50.0 ^c |
| P5 | M | 69 | 59.4 | 5.1 | 84.7 | 25.2 ± 1.6 | 74.5 ± 3.4 |

^a Normal therapy range: 50–200 ng mL⁻¹.

^b ND, not detected. The value is lower than LOD (<10 ng mL⁻¹).

^c The value was higher than LOD (>10 ng mL⁻¹), but lower than LOQ (<50 ng mL⁻¹).

analytes. In order to further confirm the collapse of SDS micelles in the use of such SDS concentrations, the Sudan III was utilized as a marker to evaluate the collapses of SDS micelles. Sudan III is utilized as a micellar marker, because it would be totally included into the micelles. In this study, the separation was set in the normal polarity (20 kV), and the detector was set in the cathode (the cathode is in the outlet). Therefore, the Sudan III was used as the analyte to perform this investigation under the same experimental setting (section 2.4) and conditions (BGS, 100 mM NaH₂PO₄, pH 2.5; MS, BGS contains SDS, pH 2.5; BGS plug, 100 mM NaH₂PO₄, pH 2.5; Sample injection, 300 sec, 10 kV; Separation voltage, 20 kV; Detection wavelength, 214 nm.) as those for the determination of D and DAG in this study, and the data was as shown in Fig. S5. The various SDS concentrations from 0 mM to 100 mM were tested (Fig. S5B–F). The concentration of Sudan III utilized in the experiment was 300 µg mL⁻¹ (about 0.85 mM) which was much less than the SDS concentrations and could be totally included by the SDS micelles. When no SDS was used, the peak of the Sudan III was broad (Fig. S5B), and it also displayed the Sudan III carried the positive charge. As soon as increasing the SDS concentrations from 25 to 100 mM, the peak of the Sudan III became sharper. That is meaning the sweeping of the SDS micelles would be beneficial for stacking. However, in such normal polarity (20 kV), the SDS micelles possessing the large negative charge would move toward the inlet (anode). In the consideration of the EOF, the mobility of the SDS micelles ($3.40 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$) was about ten-fold higher than that of EOF ($3.27 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$) under pH 2.5, and the analytes could not be pushed to the detector by EOF in the presence of the SDS micelles. Nevertheless, the Sudan III could be detected in such conditions, meaning the micelles must be collapsed. If the SDS micelles were not collapsed, the Sudan III would be totally included by the SDS micelles and migrate to the inlet (the anode is in the inlet, and the detector (cathode) is in the outlet). Therefore, the data demonstrated the Sudan III was firstly swept by the SDS micelles to make the sharp peak and then

released by the collapse of the SDS micelles, and finally moved to the detector (cathode) by its electrophoretic mobility and EOF. Additionally, in the use of the 100 mM SDS, the peak of the Sudan III became small. That could be supported the SDS micelles were more difficultly collapsed under such high SDS concentration, and a portion of the Sudan III was lost by the including of the micelles without collapsing. In the use of 50 mM SDS (Fig. S5D) which was the optimal condition in this research, the peak area was almost the same as that in the use of no SDS, meaning the SDS micelles were almost completely collapsed. The data could demonstrate the sweeping and the collapse of the SDS micelles were both helpful for stacking analytes in this research.

3.8. Application

In this study, 5 patients with nonvalvular atrial fibrillation participated in this experiment and provided their plasma after taking DE. All blood sampling time was within 3 hours after taking DE. After the pretreatment of the sample and the analysis in CE, different D and DAG levels could be observed in plasma samples of five different patients (as shown in Table 3). These real plasma samples from the patients were also pretreated in the same procedure (section 2.3) without spiking D and DAG. Although 250 µL plasma was used and then 100 µL solution was reconstituted, the calibration curves were also established according to the same procedure. According to this uniformity of pretreated procedures, the concentrations of D and DAG in the real samples could be directed quantified through the calibration curves. The electropherograms of the plasma samples obtained from two patients (P2 and P5) are as shown in Fig. 5, and the data indicated that this developed CE approach could be utilized for the detection of D and DAG levels in human plasma for clinical evaluation. In the electropherograms, there was an interference next to the peak of D. It's worth noting that the interference next to D is also increased after spiking. In the comparison of the electropherogram by using standard solutions

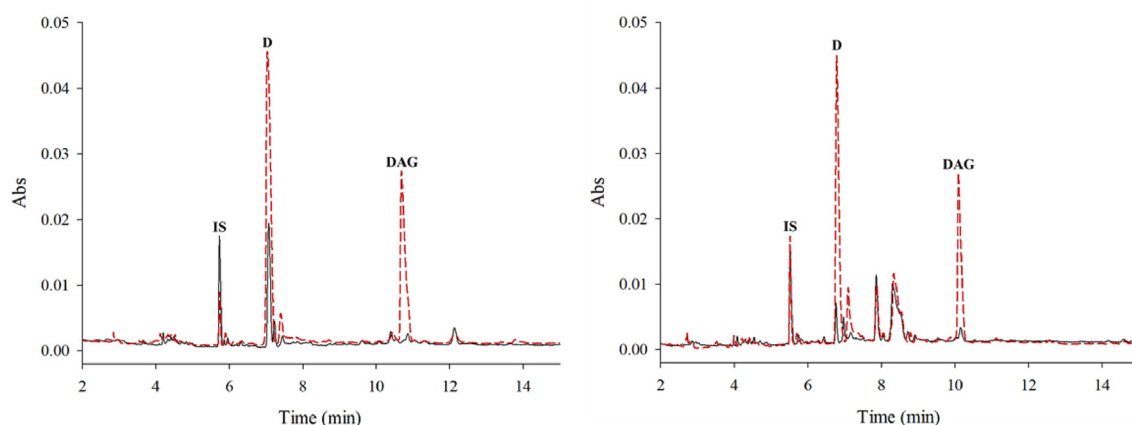


Fig. 5. Electropherograms of D and DAG levels in the plasma samples obtained from two (P2 and P5) patients. (A) P2, black line: real patient's plasma sample; red dotted line: patient's plasma sample spiked with standard solutions (B) P5, black line: real patient's plasma sample; red dotted line: patient's plasma sample spiked with standard solutions.

as analytes (Fig. S4A), that could be found the interference peak resulted from plasma. The signal increase after spiking was supported from the electrokinetic injection in this mode, and the unknown interaction between this endogenous interference and target analytes. However, the interference would not affect the separation and quantification of D.

Among these data (Table 3), the D level of one patient (P5) was lower than the normal therapeutic range (50–200 ng mL⁻¹) in human plasma after intake of high DE dosage (150 mg BID). After tracking this patient's records, it was found that his medication compliance was very poor. Additionally, his DAG level was the highest among the 5 plasma samples, indicating that the sampling time of this patient was inaccurate, resulting in the low level of D and the high level of DAG. Although the plasma DAG levels in the P3 and P4 were less than the LOQ, those in P2 and P5 could be successfully quantified according to the calibration curves. Until now, only few studies have mentioned the DAG, but they did not define the appropriate plasma level in clinical. Based on the results of this approach, physicians could offer individual suggestions of treatment to patients. Furthermore, the different DAG

levels in this study, which might result from the variety of glucuronosyltransferase activity in the population, could offer fresh insight for clinicians and investigators specializing in pharmacokinetics and gene polymorphism.

Until now, numerous approaches have been established for the determination of D levels in human plasma or serum. In comparison with previous researches (Table 4), although analytical time and consumption of plasma samples in this study were little more than some of those, this approach still possessed the advantages, such as the similar detection range of D in plasma without the use of mass spectrophotometry, simple instrumental configuration, less consumption of materials and organic solvent, and inexpensive cost of the instruments. All results demonstrated that the FASI-sweeping-AFMC approach in CE was a better tool for the determination of D and DAG levels in human plasma and reference in precision medicine.

4. Conclusions

In this study, a novel and convenient CE on-line three-step stacking approach called the FASI-sweeping-AFMC, including FASI, sweeping, and AFMC approaches was successfully established for

Table 4. The comparison of the approaches for determination of D levels in human plasma.

| Method | Linear range (ng mL ⁻¹) | LOD (ng mL ⁻¹) | Analyte | Ref. |
|---------------------------------|-------------------------------------|----------------------------|---------|---------------|
| UHPLC-MS/MS | 2.4–384 | 0.24 | D | [8] |
| SPE-UHPLC-MS/MS | 0.5–900 | 0.18 | D | [39] |
| UHPLC-MS/MS | 2.0–500 | 0.63 | D | [40] |
| UHPLC-UV | 20.0–1000 | 4.00 | D | [41] |
| UHPLC-MRM-MS | 0.8–800 | 0.21 | D | [9] |
| FASI-sweeping-AFMC ^a | 2.5–1000 | 0.25 | D | This research |
| | 50.0–1000 | 10.00 | DAG | |

^a A three-step capillary electrophoresis of field-amplified sample injection, sweeping, and analyte focusing by micelle collapse.

the determination of D and DAG levels in human plasma. This is the first time that a sequential three-step stacking approach in CE used for the determination of D and DAG levels in human plasma has been developed and applied. After the validation of this approach, these data demonstrated good linearity, precision, accuracy, stability, and selectivity of this approach for analysis of D and DAG in plasma matrix samples. This approach was further applied for the determination of D and DAG levels in the plasma samples of five patients. All results showed that this approach is feasible for the clinical determination of D and DAG levels in human plasma. Most importantly, it possesses superior properties compared to previous UPLC-MS/MS works such as the similar detection range of D and DAG, good sensitivity, simple experimental design, less consumption of materials, and inexpensiveness. In contrast with similar research [33,35], the experimental process could be simplified due to the operation without the switch of electrodes during the analysis. Furthermore, valuable application in clinical use has been demonstrated according to the results of this research. For the above-mentioned arguments and results, this developed approach

was feasible for the determination of D and DAG levels in human plasma and could serve as a tool for clinical assays.

Conflicts of interest

The authors announce that there are no competing financial interests or personal relationships influencing the work reported in this paper.

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Appendix A. Supporting information

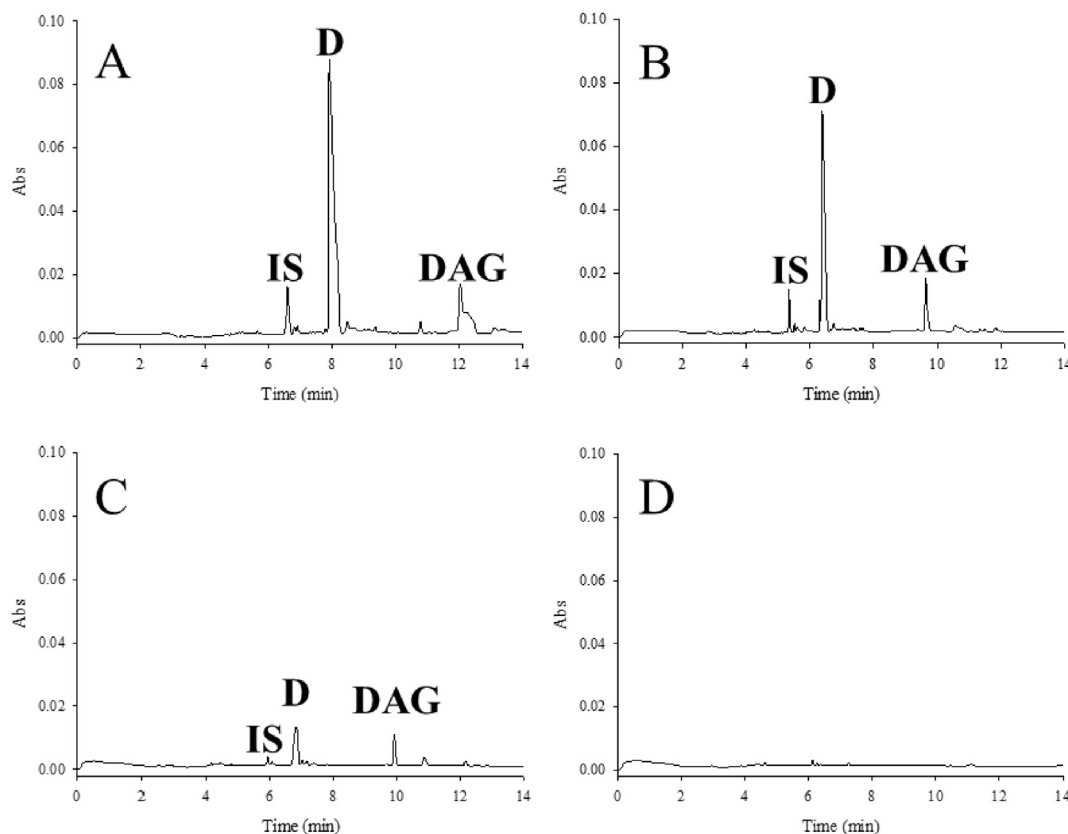


Fig. S1. Effect of BGS plug concentration ((A) 50 mM; (B) 100 mM; (C) 150 mM; (D) 200 mM) on stacking and separation efficiency by the FASI-sweeping-AFMC approach. Sample concentrations: $1 \mu\text{g mL}^{-1}$ D; $1 \mu\text{g mL}^{-1}$ DAG; $0.5 \mu\text{g mL}^{-1}$ IS. CE conditions: BGS, 100 mM NaH_2PO_4 , pH 2.5; MS, BGS contains 50 mM SDS, pH 2.5; BGS plug, NaH_2PO_4 , pH 2.5; Sample injection, 300 sec (10 kV); Separation voltage, 20 kV; Detection wavelength, 214 nm.

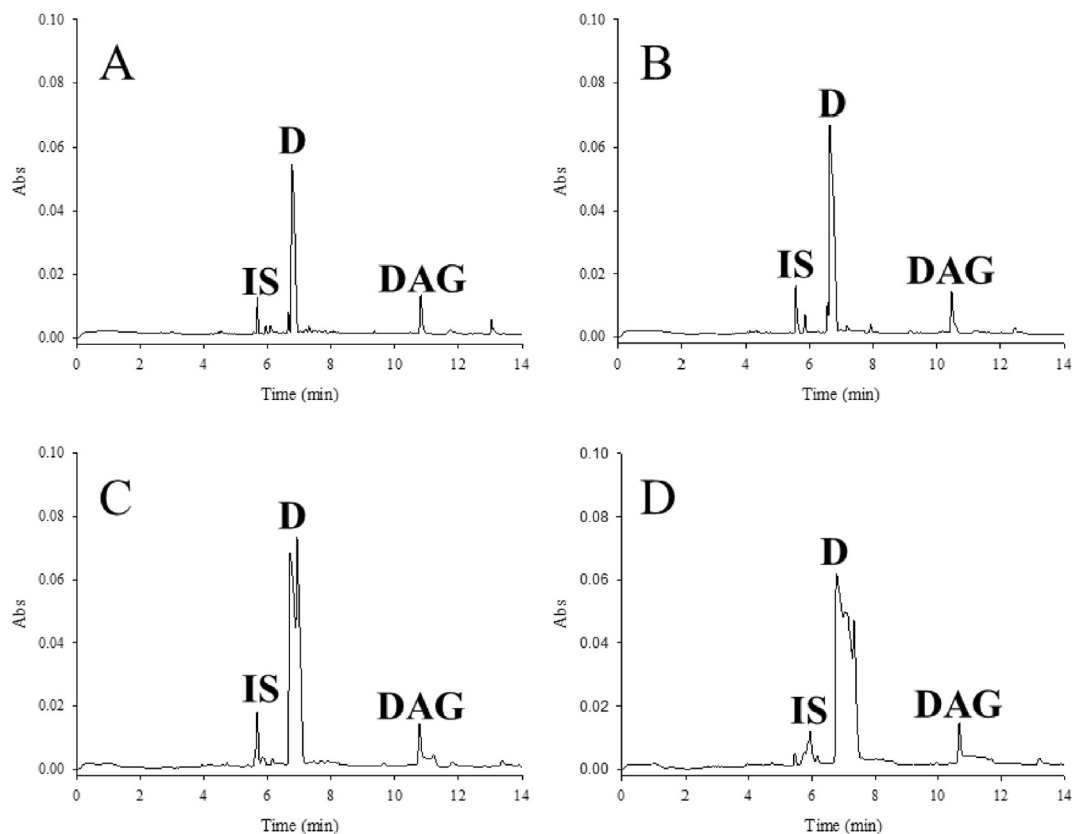


Fig. S2. Effect of injection time ((A) 240 sec; (B) 300 sec; (C) 360 sec; (D) 420 sec) of analytes on stacking and separation efficiency by the FASI-sweeping-AFMC approach. Sample concentrations: $1 \mu\text{g mL}^{-1}$ D; $1 \mu\text{g mL}^{-1}$ DAG; $0.5 \mu\text{g mL}^{-1}$ IS. CE conditions: BGS, $100 \text{ mM NaH}_2\text{PO}_4$, pH 2.5; BGS contains 50 mM SDS , pH 2.5; BGS plug, $100 \text{ mM NaH}_2\text{PO}_4$, pH 2.5; Sample injection, 10 kV ; Separation voltage, 20 kV ; Detection wavelength, 214 nm .

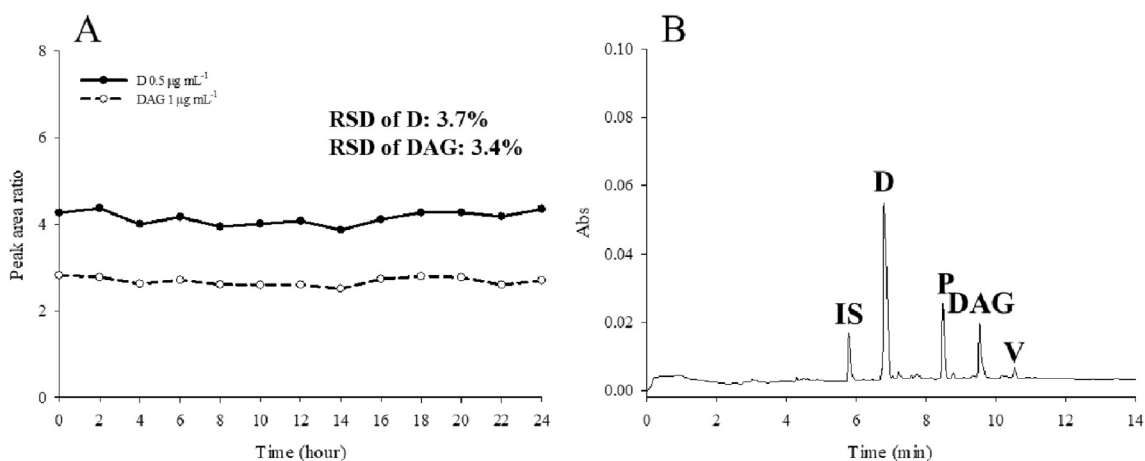


Fig. S3. Summary of stability and selectivity of the FASI-sweeping-AFMC approach in human plasma. (A) Results of RSD for the stability of the developed approach over 24 hours at room temperature. (B) Electropherogram of the plasma sample spiked with $1 \mu\text{g mL}^{-1}$ D, DAG, $0.5 \mu\text{g mL}^{-1}$ IS, and $1 \mu\text{g mL}^{-1}$ four antiarrhythmic drugs (V: Verapamil, A: Amiodarone, P: Propranolol, Di: Digoxin).

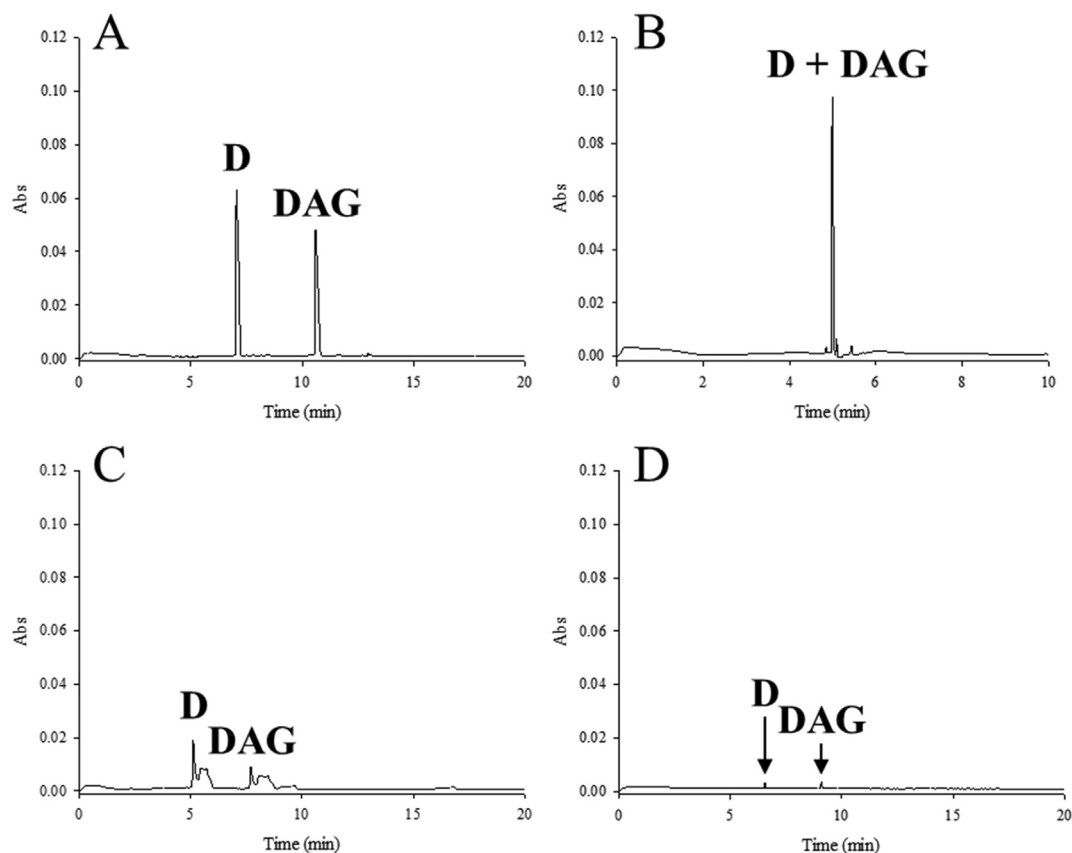


Fig. S4. Electropherograms of the (A) FASI-sweeping-AFMC; (B) FASI-sweeping; (C) FASI and (D) typical injection CZE for the analysis of D and DAG. (A) FASI-sweeping-AFMC conditions could be referred in section 2.4; (B) FASI-sweeping conditions were as following: BGS, 100 mM NaH_2PO_4 (pH 2.5); water plug, 0.5 psi for 5 sec; Sample injection, -10 kV for 300 sec; sweeping buffer, 50 mM SDS in BGS; Separation, -20 kV; (C) FASI conditions were as following: BGS, 100 mM NaH_2PO_4 (pH 2.5); water plug, 0.5 psi for 5 sec; Sample injection, 10 kV for 300 sec; Separation, 20 kV; (D) typical injection CZE conditions were as following: BGS, 100 mM NaH_2PO_4 (pH 2.5); Sample injection, 0.5 psi for 5 sec; Separation, 20 kV. All of the detection was accomplished at 214 nm. All of the analytes were the standard solution.

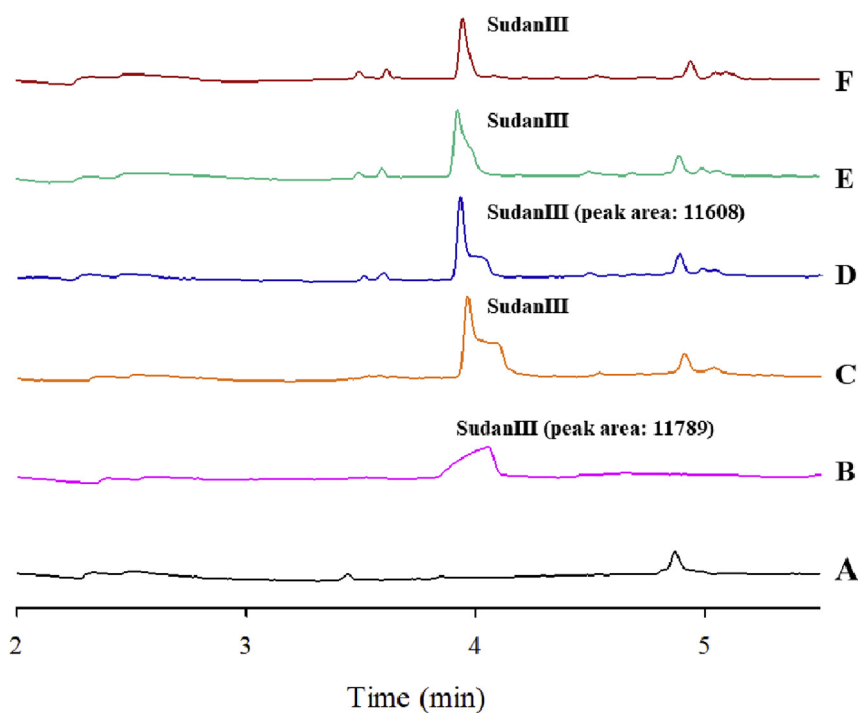


Fig. S5. The electropherograms of Sudan III in the FASI-sweeping-AFMC mode by using the (A) 50 mM SDS without Sudan III (blank); (B) 0 mM SDS; (C) 25 mM SDS; (D) 50 mM SDS; (E) 75 mM SDS and (F) 100 mM SDS. Sample: Sudan III, $300 \mu\text{g mL}^{-1}$ (about $0.85 \text{ mM} \ll \text{SDS}$ concentration used in this study). Other conditions are the same as those shown in Fig. 4.

Table S1. The sensitivity enhancement factors (SEFs) of the FASI-sweeping-AFMC compared to the typical injection CZE

| Method (n = 3) | FASI-sweeping-AFMC ^a | | Typical injection CZE ^b | |
|---|---------------------------------|-------|------------------------------------|-----|
| | D | DAG | D | DAG |
| Concentration ($\mu\text{g mL}^{-1}$) | 1 | 1 | 10 | 10 |
| Migration time (min) | 6.7 | 9.5 | 6.5 | 9.4 |
| Corrected peak area | 98725 | 33450 | 628 | 528 |
| SEFs ^c | 1572 | 634 | | |

^a FASI-sweeping-AFMC: 1 $\mu\text{g mL}^{-1}$ standard solution of each analyte in acidic water of pH 3.0; injection at 10 kV for 300 sec.

^b Typical injection CZE: 10.0 $\mu\text{g mL}^{-1}$ standard solution of each analyte in acidic water of pH 3.0; injection at 0.5 psi for 5 sec.

^c SEFs = [corrected peak area in the FASI-sweeping-AFMC/corrected peak area in the typical injection CZE] \times [concentration in the typical injection CZE/concentration in the FASI-sweeping-AFMC].

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