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Short communication

On-line concentration and determination of all-*trans*- and 13-*cis*- retinoic acids in rabbit serum by application of sweeping technique in micellar electrokinetic chromatography

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Abstract

A simple and rapid micellar electrokinetic chromatography (MEKC) method with UV detection was developed for the simultaneous separation and determination of all-*trans*- and 13-*cis*-retinoic acids in rabbit serum by on-line sweeping concentration technique. The serum sample was simply deproteinized and centrifuged. Various parameters affecting sample enrichment and separation were systematically investigated. Under optimal conditions, the analytes could be well separated within 17 min, and the relative standard deviations (RSD) of migration times and peak areas were less than 3.4%. Compared with the conventional MEKC injection method, the 18- and 19-fold improvements in sensitivity were achieved, respectively. The proposed method has been successfully applied to the determination of all-*trans*- and 13-*cis*-retinoic acids in serum samples from rabbits and could be feasible for the further pharmacokinetics study of all-*trans*-retinoic acid.

Keywords: All-*trans*-retinoic acid; 13-*cis*-Retinoic acid; Sweeping; Micellar electrokinetic chromatography

1. Introduction

Two of the most clinically useful vitamin A derivatives are all-*trans*-retinoic acid (all-*trans*-RA) and 13-*cis*-retinoic acid (13-*cis*-RA) [1]. All-*trans*-RA can be metabolized through stereoisomerization to 13-*cis*-RA in vivo. Acute promyelocytic leukemia (APL) is the first neoplasm to be successfully treated with all-*trans*-RA, resulting in 80% of patients achieving a complete remission [2]. However, relapse almost widely occurs during continuous treatment with all-*trans*-RA in APL. The study suggests that the clinical relapse and resistance are associated with the progressive reduction of the all-*trans*-RA concentration in plasma [3]. As a less toxic derivative among retinoids, 13-*cis*-RA has been used clinically in the treatment of severe acne [4]. Unfortunately, not only 13-*cis*-RA but also excessive doses of vitamin A are shown to have adverse effects such as hypervitaminosis A and teratogenic potential [4–6]. The toxicity studies of vitamin A have shown that the RA and its metabolites other than retinol may serve as biomarkers for evaluating the risk for vitamin A toxicity [7–9]. Therefore, whether the effectiveness and safety of the clinical application of RA or adequate vitamin A supplementation for patients, pregnant females and healthy people, it is of great importance to establish a rapid and reliable analytical method for determining RA in the biological matrices.

Up to now, several analytical methods for the determination of RA have been reported, including gas chromatography (GC) [10], high-performance liquid chromatography (HPLC) [11] and capillary electrophoresis (CE) [12]. CE is a widely used technique in separation science due to its high separation efficiency, rapid separation, low operational costs and small amounts of reagents or samples required. Nevertheless, the low sensitivity prevents the applicability of CE for determination of low levels

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of RA in biological fluids. Sweeping is a simple and convenient on-line concentration method for either charged or neutral analytes in micellar electrokinetic chromatography (MEKC) [13]. It was first described by Quirino and Terabe [14]. So far, this method has been widely applied in the on-line concentration and determination of metabolites in biological fluids [15] and active components in Chinese herbal medicines [16].

The aim of this work was to develop a simple and sensitive on-line sweeping technique for the determination of all-*trans*-RA and 13-*cis*-RA in the serum sample. To the best of our knowledge, there are no reports published on the simultaneous determination of the RA isomers in the biological fluids using CE.

2. Experimental

2.1. Chemicals and reagents

All-*trans*-RA and 13-*cis*-RA were obtained from Sigma (St. Louis, MO, USA). SDS was purchased from Amresco (Solon OH, USA). Tretinoin Tablets (Shandong Liangfu Pharmaceutical Group Ltd., Shandong, China) were purchased from Shaanxi Provincial People's Hospital. Other chemicals and reagents were analytical or HPLC grade. Deionized water was prepared by a Milli-Q system (Millipore, New Bedford, MA, USA). All solutions and samples were filtered through a 0.22 μm nylon-membrane filter prior to analysis.

2.2. Apparatus

All experiments were carried out on a Hewlett-Packard^{3D} capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detection (DAD) system. The pH of solution was measured by an Orion 250A pH meter (Orion research, Beverly, USA). Conductivities were measured using a sensION378 conductivity meter (HACH, Loveland, CO, USA). Centrifugation was performed on a Sigma 3K30 centrifuge (Sigma, Osterode, Germany).

2.3. Animals and blood sampling

In order to prevent photoisomerization and degradation of RA, all sample manipulations involving RA were performed in dark rooms under dim yellow light.

Three healthy New Zealand white rabbits, weighing about 2.0–2.5 kg, were obtained from the Laboratorial Animal Center of Xi'an Jiaotong University. All animal manipulations were strictly in accordance with *the Guide for the Care and Use of Laboratory Animals* (National Research Council of USA, 1996). Before drug administration, rabbits were fasted for at least 12 h and given free access to water. Tretinoin Tablets were suspended in 0.5% (w/v) carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution and administered a dose of 15 mg/kg body weight by intragastric gavage. Blood samples (1.0 mL) were withdrawn from the marginal ear vein at 2.0 h after administration of Tretinoin Tablets, and then immediately centrifuged at 3000 rpm for 15 min at 4 °C. The serum layer was collected and stored in the amber Eppendorf tubes at −20 °C until analyzed.

2.4. Sample preparation

Stock standard solutions of all-*trans*-RA (220 mg/L) and 13-*cis*-RA (250 mg/L) were prepared in methanol. The working standard solutions were prepared daily by diluting the stock standard solutions to obtain appropriate concentrations, and finally contained 20 mM borate buffer (pH 8.5), 15 mM NaCl and 15% (v/v) methanol. All solutions were protected from light exposure and stored at −20 °C before analysis.

The serum samples were 6:4 (v/v) diluted with methanol, and then mixed for 1 min using a vortex mixer, finally centrifuged for 15 min at 10,000 rpm. Subsequently, a 150 μL aliquot of supernatants was transferred into amber Eppendorf tubes and diluted with water and borate buffer to obtain 600 μL of sample solution comprising 20 mM borate buffer and 15% (v/v) methanol with pH 8.5.

2.5. Electrophoresis procedure

All the separations were performed on a fused-silica capillary of 48.5 cm (40 cm effective length) × 75 μm I.D. (Yongnian Optical Conductive Fiber Plant, Hebei, China) at 20 °C with a voltage of +15 kV. Detection wavelength was set at 350 nm. The running buffer (conductivity, 1.2 mS/cm) consisted of an aqueous solution with 30 mM borate buffer (pH 8.5), 30 mM SDS and 15% methanol (v/v). The sample matrix (conductivity, 0.25 mS/cm) contained 20 mM borate buffer (pH 8.5), 15 mM NaCl and 15% (v/v) methanol. Sample was introduced by pressure (50 mbar, 1 mbar = 100 Pa) injection. New capillaries were conditioned by rinsing with methanol (5 min), followed by 1.0 M NaOH (30 min), 0.1 M NaOH (15 min), purified water (20 min) and finally by the running buffer (20 min). To ensure repeatability between consecutive analyses, the capillary was preconditioned for 1 min by flushing with methanol, followed by 1 min with 0.1 M NaOH and 2 min with purified water, finally by rinsing with running buffer for 5 min. The running buffer was renewed after every three runs.

3. Results and discussion

As a simple and versatile method, sweeping can occur when the sample has a conductance that is lower, similar or higher than the background solution (BGS) and no pseudostationary phase (PS) exists [17]. A unique focusing effect is caused by partitioning or interaction between analytes and PS when charged PS penetrates the sample zone during the application of voltage. The injected length of an analyte zone is theoretically narrowed by a factor equal to $1/(1+k)$ (k, retention factor) and the concentration can be increased approximately by a factor, $1+k$ [18]. The greater the value of k, the greater the concentrating effect will be. In practical applications, several experimental parameters could also interfere with the concentrating effect and their influences are systematically investigated and optimized in the following section.

3.1. Optimization of separation and concentration conditions

In theory, a high concentration efficiency in sweeping can be obtained for opposite charged analytes and the PS under suppressed electroosmotic flow (EOF) conditions due to high k values caused by strong interaction of the analytes with the PS. However, strong interactions between the analytes and the PS may also result in poor resolution (R_s) [19]. All-*trans*-RA and 13-*cis*-RA are hydrophobic weak acids which have a hydrophobic interaction with the anionic SDS micelle in either acidic or basic environments. In this study, two different pH values (2.5 and 3.5) were firstly used. Under these conditions, the EOF was suppressed significantly and the RA isomers remained neutral. The results showed that the peaks of the RA isomers were coalesced, which may be due to similar chemical structures and strong hydrophobic interaction between the SDS and neutral RA. It was also found that the peak heights rapidly decreased in consecutive analysis. The experimental results indicated that the RA isomers were labile at the pH values (2.5 and 3.5) which were unsuitable for determination of the RA isomers. However, considering the strong electrostatic and hydrophobic interaction between the cationic micelles and negatively chargeable RA in the basic environments, it may not be easy to select cationic micelles for separation of the RA isomers. Although the k values of negatively chargeable compounds in MEKC with SDS were small due to the electrostatic repulsion between the anionic analytes and SDS, Monton et al. [20] used SDS for the sweeping of negatively chargeable analytes under the basic condition, and obtained the relatively low limit of detection. Therefore, using SDS as anionic micelles, the buffer pH was investigated by changing pH from 8.0 to 9.25. The two isomers were well separated in the selected range. Finally, the pH 8.5 buffer, which provided the highest theoretical plate numbers (N), was selected for further studies.

Next, a series of buffer solutions containing 20, 25, 30, 35 and 40 mM SDS were examined, respectively. It was found that with the increase of SDS concentration, both the migration times and N were increased. This was because the increase in concentration of SDS could lead to increase in the k values for the analytes, allowing hydrophobic interaction of the RA with the SDS, which would also enhance the concentration efficiency. However, when SDS concentration exceeded 35 mM for 13-*cis*-RA and 30 mM for all-tans-RA, N was decreased. Finally, a concentration of 30 mM SDS was selected for subsequent studies.

As serum contains high concentration of salt, the influence of NaCl concentration (ranging from 10 to 25 mM) was investigated. Finally, The NaCl concentration of 15 mM was selected because of the highest N. It was reported that the total salt concentration of plasma was about 150 mM [21]. When the rabbit serum samples were treated as described in Section 2.4, the salt concentration was about 15 mM, which was just the optimized concentration.

The organic modifiers are an important parameter influencing the separation selectivity, efficiency, R_s and k values. First, methanol, ethanol and acetonitrile were added at a percentage of 15% (v/v) to the buffer solution, respectively. The results indicated that the buffer containing methanol provided the highest N and a relatively short analysis times. Second, the concentration of methanol was optimized and 15% methanol was finally selected.

Keeping the other conditions unchanged, the borate concentration of running buffer was evaluated in the range 20–35 mM. The data indicated that with increasing borate buffer concentration, both the migration times and N increased. However, when the buffer concentration exceeded 30 mM, the peaks began to broaden and N decreased. Therefore, a buffer concentration of 30 mM was selected as optimum value.

The injection time will affect the sensitivity of the method. However, excessive injection time also results in remarkable loss of peak R_s. Therefore, the injection time was investigated over the range of 3–130 s at 50 mbar. It was found that with the increase in the injection time, the peak heights increased, and correspondingly the sensitivities also increased. However, when the injection time is longer than 60 s, the peak heights slightly decrease and peak zone becomes broad. This can be explained by the fact that sweeping is limited by k, wherein the length of the injected zone can only be narrowed by a factor equal to $1/(1+k)$ and the concentration could be increased approximately by a factor $1+k$. At the same time, the R_s gradually deteriorated with increase in the injection time. When the injection time was 130 s, no baseline separation was obtained. Giving an overall consideration of both the sensitivity and R_s, an injection time of 60 s at 50 mbar was selected for serum sample analysis.

In the case of the separation voltage optimization, the R_s, migration time and peak height were considered as the main evaluation factors. The results indicated that the RA isomers could be separated completely in the selected voltage, and a 15 kV of applied voltage was selected considering the appropriate migration times (less than 17 min) and the highest peak heights.

3.2. Sensitivity enhancement of the sweeping method

The sensitivity enhancement factor in terms of peak height (SEF_{height}) can be calculated by comparing the peak height using the conventional injection relative to the sweeping injection. The standard solutions were analyzed under different injection conditions. The typical electropherograms of the RA isomers are shown in Fig. 1A. It indicated that, even when 20% of the efficient capillary length was filled with the sample solution, the RA isomers were well separated and concentrated within 17 min with R_s (2.6) and 18- and 19-fold sensitivity enhancements for 13-*cis*-RA and all-*trans*-RA, respectively.

3.3. Quantification of the method

Quantification was performed using the external standard method for calibration under optimized conditions. The linear regression analysis, using peak area as the function of the concentration, was constructed by injecting series of standard

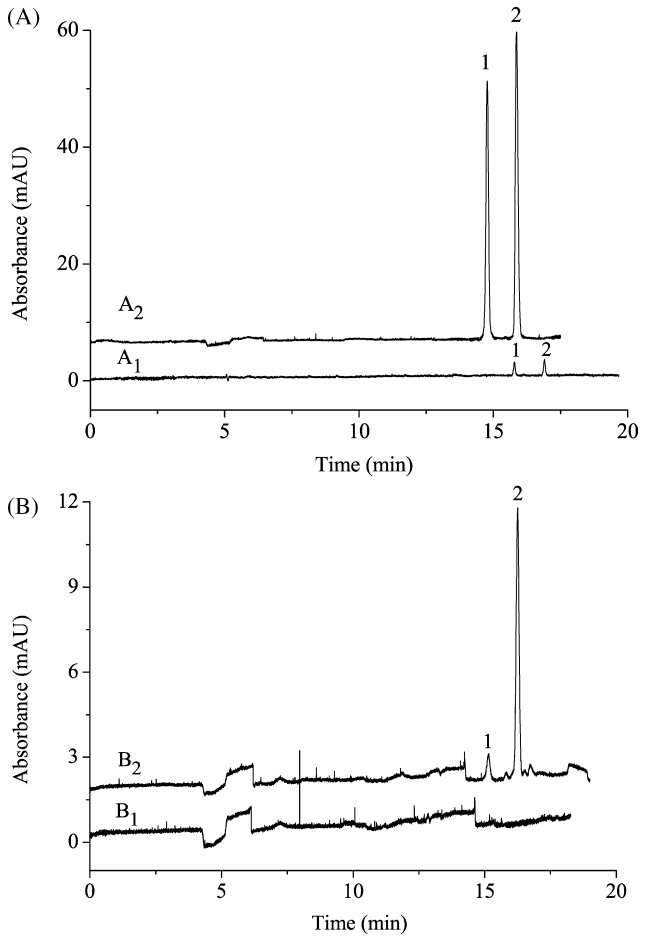

Fig. 1. The typical electropherograms of the standard mixture solutions and serum samples: (A) the standards mixture under the conventional injection (A_1) and the optimum sweeping injection (A_2) conditions; (B) blank serum (B_1) and the serum obtained from a rabbit at 2 h after oral administration of 15 mg/kg (B_2) under optimum conditions. The concentrations of the standards mixture were 2.2 and 2.5 μg/mL for all-*trans*-RA and 13-*cis*-RA, respectively. Conditions: uncoated fused-silica capillary, 48.5 cm (40 cm to detector) × 75 μm I.D; buffer, 30 mM borate containing 30 mM SDS with15% (v/v) methanol at pH 8.5; sample matrix, 15 mM NaCl, 15% (v/v) methanol and 20 mM borate at pH 8.5; conventional injection, 50 mbar 3 s (A_1), sweeping injection, 50 mbar 60 s; applied voltage, +15 kV; temperature, 20 °C; detection wavelength, 350 nm. Peaks: (1) 13-*cis*-RA; (2) all-*trans*-RA.

solutions. The linear regression equations were $y = 170x - 4$ (correlation coefficient, $r = 0.998$) and $y = 122x - 5$ ($r = 0.996$), with the linear ranges of 0.029–2.2 and 0.033–2.5 μg/mL for all-*trans*-RA and 13-*cis*-RA, respectively. The limits of detection (LOD) were determined at signal-to-noise ratio equal to 3 (S/N = 3) with the standard solutions. The LOD for all-*trans*-RA and 13-*cis*-RA were 0.026 and 0.030 μg/mL (S/N = 3), respectively, and the limits of quantification (S/N = 5) in serum were 0.36 and 0.49 μg/mL, which were caused by a 10-fold dilution in the serum preparation process for all-*trans*-RA and 13-*cis*-RA, respectively. The results were slightly higher compared with the reported HPLC-UV method [11]. The relative standard deviation (RSD) values of the migration times and peak areas ($n = 5$) were 1.2, 1.9–2.5% (within assay), and 2.1–2.7, 2.4–3.3% (between assay, for a 3-day period), respectively.

3.4. Application to rabbit serum

The proposed method was applied to determine all-*trans*-RA and 13-*cis*-RA serum concentrations in the rabbit following a single dose oral administration of 15 mg/kg Tretinoin Tablets. The blood samples were taken at 2.0 h after oral administration and treated according to the procedure described in Sections 2.3 and 2.4. The typical electropherograms of blank serum and serum samples are shown in Fig. 1B. The peaks of analytes were identified by comparing the migration times and the UV spectra of the analytes in rabbit serum with those of standards and spiking the standards to the rabbit serum. It was found that the serum samples matrix did not interfere with the determination of the RA isomers. The analysis results of the serum were 5.81 ± 0.066 and 1.24 ± 0.059 μg/mL ($n = 3$) for all-*trans*-RA and 13-*cis*-RA, respectively. The recovery experiments based on three concentrations were determined to evaluate the accuracy of the method. The mean recoveries were found to be 76 and 85% in serum ($n = 5$) for all-*trans*-RA and 13-*cis*-RA, respectively, with the RSD lower than 4.5%.

4. Conclusions

A new sweeping method in MEKC for the separation and on-line concentration of all-*trans*-RA and 13-*cis*-RA in the rabbit serum has been developed. A satisfactory separation could be obtained for the RA isomers on an uncoated fused-silica capillary within 17 min with good repeatability and linearity. Compared with the conventional MEKC injection method, the 18- and 19-fold improvements in sensitivity were achieved, respectively. The proposed method could be feasible for further pharmacokinetics study of all-*trans*-RA and a promising alternative for therapeutic drug monitoring.

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