

Analysis of Repaglinide Enantiomers in Pharmaceutical Formulations by Capillary Electrophoresis Using 2,6-Di-*o*-methyl- β -cyclodextrin as a Chiral Selector

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This study used the general applicability of 2,6-di-*o*-methyl- β -cyclodextrin (DM- β -CD) as the chiral selector in capillary electrophoresis for fast and efficient chiral separation of repaglinide enantiomers. A systematic study of the parameters affecting separation was performed with UV detection at 243 nm. The optimum conditions were determined to be 1.25% (w/v) DM- β -CD in 20 mM sodium phosphate (pH 2.5) as the running buffer and separation voltage at 20 kV. DM- β -CD had the best enantiomer resolution properties under the tested conditions, whereas other β -cyclodextrins showed inferior performances or no performance. The proposed method had a linear calibration curve in the concentration range of 12.5–400 μ g/mL. The limit of detection was 100 ng/mL. The intra-day and inter-day precisions were 2.8 and 3.2%, respectively. Recoveries of 97.9–100.9% were obtained. The proposed method was fast and convenient, and was determined to be efficient for separating enantiomers and applicable for analyzing repaglinide enantiomers in quality control of pharmaceutical production.

Introduction

Enantiomer distinction is a fundamental phenomenon in nature and chiral recognition, and it is also important in chemical systems. Studies of enantiomers have great impacts on various chemical fields dealing with bioactive compounds, including drug discovery (1, 2), agrochemical development (3), food additives (4, 5), fragrances (6, 7) and chiral pollutants (8). Most newly developed drug candidates are chiral, and the individual enantiomer of chiral species usually exhibits distinct pharmacodynamic and/or pharmacokinetic profiles. Therefore, it is common to perform biological and toxicological tests on new drug entities with racemate and individual enantiomer. To solve the stereoselectivity issues, enantioselective liquid chromatography (LC) has become as one of the major workhorses in research (9).

Repaglinide, a carbamoylmethyl benzoic acid derivative, is the first of new oral antidiabetic agents, designed to normalize postprandial glucose excursions in patients with type 2 diabetes mellitus (10). The drug can lower the postprandial glucose excursions by targeting the early-phase insulin release, which is thought to be important in reducing long-term cardiovascular complications of diabetes (11). Repaglinide is the derivation of carboxamide-methyl benzoic acid (Figure 1A). A chiral carbon determines its stereoselectivity. The activity of the S(+) isomer is 100 times higher than that of the R(–) isomer. Only the S(+) isomer is used clinically. Therefore, it is critical to test and control the content of R(–) isomer in

repaglinide to assure the quality of tablet. The chiral chromatographic technique is the most efficient way to test the enantiomer of drugs; these techniques include high-performance liquid chromatography (HPLC) (12, 13), gas chromatography (GC) (14–16), capillary electrophoresis (CE) (17–19), thin-layer chromatography (TLC) (20) and supercritical fluid chromatography (SFC) (21). Some HPLC methods (22, 23) have been reported in repaglinide chiral separation by using different chiral columns and adjusting the mobile phase. These methods are relatively simple, highly sensitive and require no additional sample pretreatment. However, they are time-consuming, because the retention time is over 30 min and the cost is another issue. Therefore, they are not suitable for quality control in industries.

In CE, chiral separation is achieved by simply adding appropriate chiral selectors to the background electrolyte (BGE). The chiral selectors used in CE separation are primarily cyclodextrin (β -CD) and their derivatives (24). Some of them are widely used due to their abundance, including 2,6-dimethyl- β -CD (2,6-DM- β -CD), trimethyl- β -CD (TM- β -CD) and occasionally, methyl- β -CD (RM- β -CD). Indeed, because of their properties (low toxicity, high aqueous solubility and binding capacity and reasonable price), they are used as drug carriers (25) and chiral selectors (26) in the pharmaceutical industry and analytical chemistry, respectively. However, no reports have been made about the separation of repaglinide enantiomers by using CE with β -CD as a chiral selector. The lipophilicity of repaglinide enantiomers affect the separation when normal β -CD is used as chiral selector. Fortunately, many derived β -CDs are commercially available and currently popular, and the liposolubility of some is increased and therefore suitable for the analysis of fat-soluble compounds used as an additive in CE. Although the low sensitivity is a shortcoming of the CE method, fast analysis speed and short analysis time are prominent characteristics of the CE method. In addition to improving separation selectivity, chiral resolution can also be improved by adjusting other experimental variables. For the chiral separations of basic analytes with neutral CDs in uncoated fused-silica capillaries, compounds are positively charged at low buffer pH and migrate in the same direction as the electroosmotic flow (EOF), from anode to cathode (27).

In this study, we explored a series of derived β -CDs to determine the optimal chiral selector. Data showed that 2,6-di-*o*-methyl- β -cyclodextrin (DM- β -CD) demonstrated the best enantiomer resolution properties among the tested compounds. We also investigated the effects of buffer pH, borax

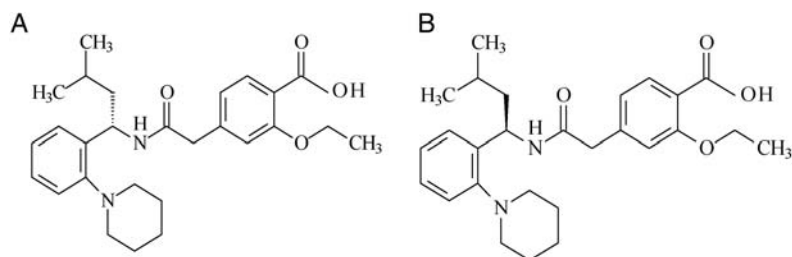


Figure 1. Chemical structures. Repaglinide (A); R-isomer (B).

and concentration of DM- β -CD on the separation of compounds. A fast and simple CE method was developed for the enantiomer analysis of repaglinide. The proposed method could be used in both repaglinide enantiomer tests of bulk drug and quality control of pharmaceutical production.

Experiments

Materials

Phosphates and β -CD were purchased from Sigma (St. Louis, MO) and used as buffer electrolyte and chiral additives, respectively. HPLC-grade methanol was purchased from EM Science. Repaglinide (S isomer) was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The racemic samples, bulk drugs and tablets of repaglinide were provided by Shijiazhuang Pharmaceutical Company of China (CSPC Pharma, Shijiazhuang, China). All other chemicals were of analytical grade unless stated otherwise.

Instrumentation and electrophoresis conditions

The experiments were carried out with a CE apparatus (Beijing Cailu Scientific Inc., Beijing, China), equipped with an ultraviolet (UV) detector. The UV signals were recorded at 243 nm. A fused silica capillary of 45 cm in length (effective length of 36 cm) and 50 μ m i.d. (Hebei Yongnian Optical Fiber Factory; China) was used as the separation tube.

Briefly, a new capillary was washed with methanol for 10 min, followed by ultrapure water for 5 min, 1M HCl for 10 min, ultrapure water for 5 min, 1M NaOH for 10 min and ultrapure water for 5 min. As a daily routine procedure, the capillary was rinsed with 0.1M NaOH for 10 min followed by a 10-min rinse with ultrapure water. Then it was flushed with running buffer for 3 min before sample injections. Before each run, the capillary was rinsed with running buffer for 3 min.

Running electrolytes were prepared by dissolving the appropriate phosphoric acid (20 mM) in ultrapure water and adjusting the pH to the desired pH (pH 2.5) with 1M sodium hydroxide. Subsequently, the DM- β -CD chiral selectors (1.25% w/v) were added to the BGE. To completely dissolve the electrolyte, the solution was sonicated at ambient temperature for 2 min and then filtered with a 0.45- μ m filter before use.

Samples were injected by gravity injection and the applied voltage was set at 20 kV. To achieve reproducible separations, all experiments were performed at 20°C.

Solution preparation

Stock and working solutions

Reference stock solution (1 g/L) of racemic samples and repaglinide (S isomer) reference were separately prepared with methanol and kept refrigerated. Working standard solutions were freshly prepared by diluting suitable aliquots of the stock solution with water.

Bulk drug sample

Bulk drug stock solution (1 g/L) of repaglinide was prepared with methanol and kept refrigerated. Working bulk drug solutions were freshly prepared by diluting suitable aliquots of the stock solution with water. The solution was filtered through a membrane (0.45 μ m) and the filtrate was introduced into the CE system for the separation.

Tablet drug sample

A total of 10 tablets were weighed, ground and mixed in a mortar. An appropriate amount of the powder equivalent to 100 mg of repaglinide was dissolved in 25 mL of methanol, then ultrasonicated for 3 min and diluted to 100 mL with methanol. The sample was filtered through a membrane (0.45 μ m) and 1mL of the filtrate was diluted to 10 mL with water. This solution was introduced into the CE system for the separation.

Analysis of bulk drug and pharmaceutical formulations

The validated method was applied to determine the repaglinide enantiomers in bulk drug and tablet drug samples. Figure 2 shows the typical electropherogram. Repaglinide isomers were not detected in either bulk drug or table drug samples.

Results and Discussion

Principle of separation

When β -CDs or modified β -CDs are put into the buffer, enantiomers bind with β -CDs based on the configuration, group character and molecule size. Therefore, diastereomer compounds emerge. Enantiomer separation is caused by their different electrophoresis characteristics (27, 28).

β -CD plays a very important role in the separation of chiral compounds using CE. The mode of action using β -CD as chiral selector includes: (i) β -CDs separately combine with enantiomers and form two different compounds; (ii) the hydroxy group of β -CDs interacts with enantiomers; (iii) Van der Waals

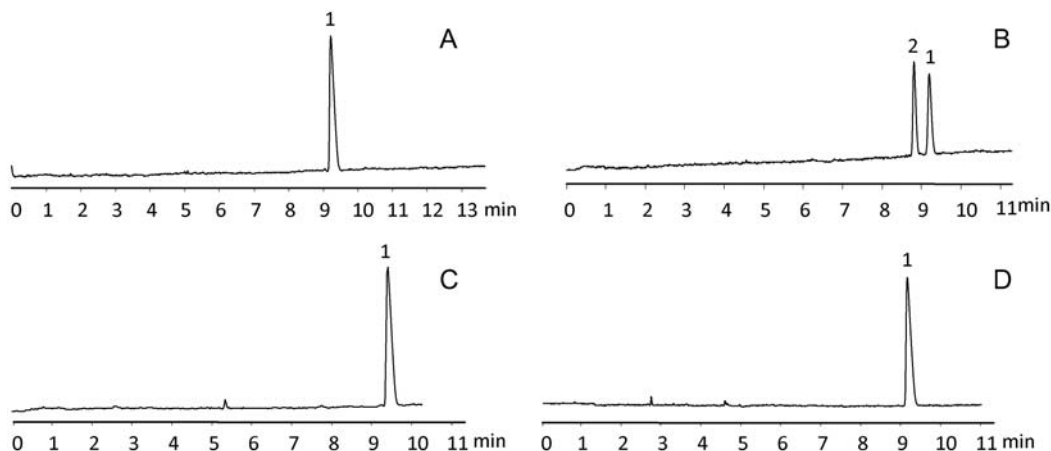


Figure 2. Chromatograms of reference solution (100 $\mu\text{g/mL}$) (A); racemic repaglinide solution (100 $\mu\text{g/mL}$) (B); bulk drug solution (100 $\mu\text{g/mL}$) (C) and pharmaceutical sample (100 $\mu\text{g/mL}$) (D) under most appropriate conditions: (1) repaglinide and (2) R (-) isomer.

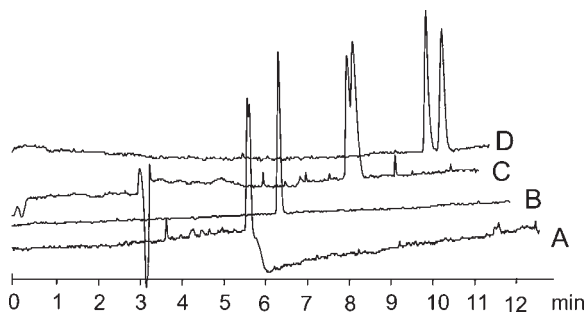


Figure 3. The electropherograms of repaglinide and its R (-) isomer with different types of CDs. Conditions: BGE; 20 mmol/L phosphate buffer (pH 2.5) with 1% of different of CDs; applied voltage; 20 kV. ME- β -CD (A); β -CD (B); SBE- β -CD (C) and DM- β -CD (D).

force. In the present study, DM- β -CD, which was a modified β -CD, was used as the optimal chiral selector. The mechanism may be related to the different influences by β -CDs. Therefore, different compounds form to obtain the separation of enantiomers (29). In our opinion, repaglinide and its enantiomer might associate with DM- β -CD. They might be entirely or partly embedded in the cavity of the DM- β -CD due to the different mechanisms. Therefore, the association between repaglinide and its enantiomer was different. Consequently, the mixture could be perfectly separated because of this different association or embedding.

Method development

Effect of β -CD type

We used β -CD and its derivatives as the chiral selectors in the buffer and evaluated the separation of repaglinide and its R(-) isomer. We found that could not be obtained when using β -CD and Me- β -CD as chiral selectors, but both compounds could be baseline separated by SBE- β -CD and DM- β -CD (Figure 3). This might be because the combination of β -CD with benzene ring and chain in enantiomers had different powers. In addition, DM- β -CD demonstrated a greater level than with SBE- β -CD in terms of the separation of repaglinide and its R(-) isomer. This

Table I

Effect of pH on Migration Time of the First Enantiomer (t_1), Resolution (R_s) and Current (A)

pH	2.5	5	7	9
t_1 (min)	11.1	9.8	6.7	3.2
R_s	1.8	1.2	0.8	0.4
A (μA)	32	19	23	35

might be related with the cavity size and degree, as well as the role of side-chain-related sites of repaglinide. We also investigated some other β -CDs, such as S- β -CD, HP- β -CD and CM- β -CD. However, the analysis was affected due to their low solubility in BGE. Therefore, DM- β -CD was finally selected as the chiral selector.

Effect of running buffer pH

The pH of running buffer is considered to be one of the most important parameters in CE because of its effect on the EOF and the overcharge of the analytes. Therefore, it is vitally important to optimize the optimum pH value of the running buffer. In this study, we investigated the effect of pH on the resolutions (with values ranging from values ranged from 2.5 to 9.0) and separation time using the buffer with a concentration of 20 mM. When the pH of running buffer was increased, the migration time of repaglinide and its R(-) isomer was decreased and the resolution between both analytes was diminished, as shown in Table I. Finally, pH 2.5 was selected as the optimum pH value of the running buffer because a suitable migration time and good resolution were obtained.

Effect of β -CD concentration

We also investigated the effect of β -CD concentration ranging from 0.25 to 1.5, w/v%. The DM- β -CD concentration was limited to 1.5 w/v%, considering the solubility of β -CD in water. Figure 4 shows that chiral separation of the two enantiomers was achieved with a DM- β -CD concentration of 0.50, w/v%.

When the β -CD concentration was further increased to 1.5 w/v%, the resolution of enantiomers was significantly increased. The elution time of the analyte was increased along with the increase of β -CD concentration. This might be

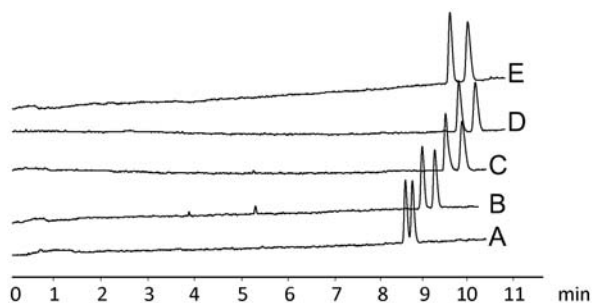


Figure 4. Comparison of electropherograms of repaglinide and its R (-) isomer with different DM- β -CD concentrations. Separation voltage: 20 kV; running buffer: 20 mM phosphate buffer (pH 2.5). 0.25% (A); 0.50% (B); 1.0% (C); 1.25% (D); 1.5% (E).

attributed to greater analyte-CD complexations. However, the solubility of DM- β -CD reached saturation when its concentration was increased to 1.5 w/v%. Finally, 1.25 w/v% was selected as the separation concentration, because with this concentration, satisfactory resolution with relatively short running time was obtained, and excessive β -CD consumption was avoided.

Effect of buffer concentration

The separation of enantiomers, which has complexation with BGE, generally requires a careful optimization of BGE composition. In this study, we examined the effect of buffer concentration on separation behavior of enantiomers ranging from 10 to 30 mM. As expected, an increase of buffer concentration from 10 to 30 mM resulted in a distinct improvement of enantioseparation. In CE, the buffer with increased concentration demonstrated high conductivity, leading to relatively high currents. This could be problematic because of excessive Joule heating. Data showed that the current was improved from 15 to 60 μ A with the increase of buffer concentration from 10 to 30 mM. When buffer concentration was less than 20 mM, separation efficiency ($R_s > 1.6$) was obtained with a current of 27 μ A, indicating that Joule heating was not critical. When the concentration was higher than 20 mM, peak shape distortion and serious baseline drift were observed and the analysis time was extremely extended. Therefore, 20 mM was selected as the optimal separation concentration.

Effect of applied voltage

Applied voltage greatly affects the migration time, current strength and resolution. When the applied voltage was increased from 10 to 30 kV, the migration time was decreased due to the increase of EOF. However, this induced poor peak resolution and a deteriorated baseline due to the much higher Joule heating and electric current at higher voltage. A voltage of 20 kV yielded the best compromise in terms of migration time, separation current and resolution, so it was used in all of the experiments.

Validation of the analytical methods

Selectivity

No interference from the formulation excipients was observed during the migration of enantiomers. The R-enantiomer

Table II

Recoveries from the Determination of Racemic Repaglinide in Placebos Containing Different Levels of Spiked Standards

Sample	Racemic (μ g/mL)	Recovery (%) (mean \pm SD)	
		R-enantiomer	Repaglinide
1	80	98.1 \pm 4.3	97.9 \pm 2.7
2	100	99.4 \pm 3.2	98.6 \pm 3.5
3	120	100.9 \pm 3.1	99.7 \pm 3.8

migrated first, and this was confirmed by spiking repaglinide standards.

Precision

Intra-day precision was performed by five consecutive standard injections of repaglinide solution. The inter-day precision was evaluated on three consecutive days. The relative standard deviation (RSD) of intra-day precision and inter-day precision were 2.8 and 3.2%, respectively.

Accuracy

The accuracy of the method was evaluated by recovery studies on placebos. Several aliquots of raceme at three different concentrations were added to an analytical placebo prepared from the excipients. Table II shows the results, and recoveries of 97.9–100.9% were obtained.

Linearity

To construct the calibration curve, the corrected peak area (A) was plotted as a function of analyte concentration (C) in μ g/mL. Six standard solutions containing 12.5–400 μ g/mL raceme were injected. The obtained linear regression equations were as follows: R-enantiomer $A = 318.56c + 2015.3$, $r^2 = 0.9948$; Repaglinide $A = 332.96c + 2568$, $r^2 = 0.9959$.

Limit of detection

Limit of detection (LOD) was assessed using a solution containing only a single enantiomer (repaglinide, 100 ng/mL).

CE versus HPLC

CE and LC are considered to be common techniques for analytical scale enantioseparations. The separation of repaglinide enantiomers by HPLC has been previously reported (22, 23). Although the proposed method has less sensitivity (higher LOD) than that of other methods, the resolution of the proposed method was better than other methods and the migration time was suitable for analysis. At the same time, the proposed method demonstrated excellent separation efficiency compared with other methods. Its theoretical plate numbers were higher than 10,000, which was significantly higher than that of the reported methods.

Conclusion

In the present study, we developed and proposed an easy, cost-effective and rapid CE method for the enantioselective separation of repaglinide enantiomers. The investigations were performed at different concentrations of electrolytes and employed various β -CD derivatives as chiral selectors, such as DM- β -CD, SBE- β -CD and Me- β -CD. We obtained very different

separation factors from the investigated compounds, not only the types of β -CD but also the solvents and the electrolytes. Depending on their structural features, we identified the most appropriate β -CD derivatives among the investigated substances. Significant advantages were provided by using DM- β -CD as a single chiral selector for the separation of repaglinide and its enantiomer. Furthermore, the newly developed method was more cost-effective than HPLC, and can be applied for bulk drug testing and quality control in industries.

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