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Epimerization study of the L,L- and L,D-diastereoisomers of the calpain inhibitor MDL 28170 by capillary electrophoresis

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Abstract

MDL 28170, Cbz-(L)–Val-(D,L)–Phe-H, which exists as a mixture of L,L- and L,D-diastereoisomers, is a calpain inhibitor currently investigated as a novel therapeutic agent for the treatment of ischemic stroke and head and spinal trauma. This report describes a capillary electrophoresis (CE) method that uses sodium dodecyl sulfate (SDS) micellar electrokinetic conditions for the separation of the L,L- and L,D-diastereoisomers of MDL 28170. The report also describes the applications of this CE method to the study of epimerization of the L,L- and L,D-diastereoisomers in pH 7.4 phosphate buffered saline solution (PBS), rat and human plasma at 37 °C. The relative percent-time courses obtained showed interconversion of the diastereoisomers in all three matrices studied. However, the epimerization process in rat and human plasma was found to be at least 50 times faster than that in PBS. The epimerization half-life of the L,L-diastereoisomer in rat plasma was approximately 30 min, which is about three-fold faster than the observed elimination half-life of the L,L-diastereoisomer reported in a pharmacokinetic study following intravenous bolus dosing.

Keywords: Epimerization; MDL 28170; Calpain inhibitor

1. Introduction

Calpain is a Ca^{2+}-activated neutral cysteine protease that plays a pivotal role in neurodegeneration caused by various injuries and diseases of the central nervous system [1–3]. The tensile forces created as a result of traumatic brain injury lead to a focal influx of Ca^{2+} that initiates a series of proteolytic processes; these post-injury processes cause irreversible damage over time. Over years of extensive research, scientists have developed cell-permeable calpain inhibitors targeting either the active site or an allosteric site of calpain for mediation of inhibitory action [4–11]. MDL 28170, Cbz-(L)–Val-(L,D)–Phe-H, is a synthetic calpain inhibitor formed by an N-protected dipeptidyl aldehyde, and exhibiting hydrophobic property at the N-terminal (Fig. 1). This potential drug has been shown to rapidly penetrate cell membranes and the blood–brain barrier in a dose-dependent manner [12–14]. MDL 28170 has been found to be therapeutic for ischemic stroke and head and spinal trauma [15–22].

MDL 28170 exists as a mixture of L,L- and L,D-diastereoisomers with an L,L/L,D ratio of 47/53. Potentially, these two diastereoisomers can epimerize through a transient enol intermediate. This paper describes a capillary electrophoresis (CE) method using micellar electrokinetic conditions for the separation of the L,L- and L,D-diastereoisomers of MDL 28170. The applications of this CE method to the study of epimerization of the L,L- and L,D-diastereoisomers in pH 7.4 phosphate buffered saline solution (PBS), rat and human plasma are also reported.

2. Experimental

2.1. Chemicals and solutions

MDL 28170 and its L,L- and L,D-diastereoisomers were supplied by Aventis Pharmaceuticals (Bridgewater, NJ, USA). Water was purified with a Millipore Milli-Q appa-

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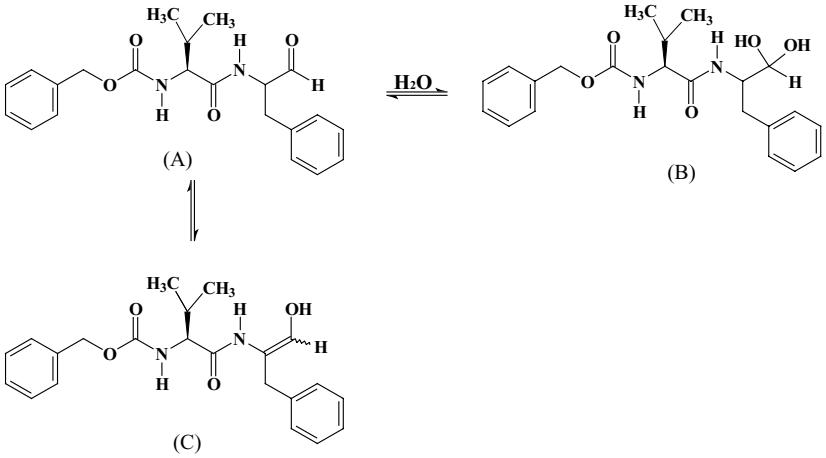

Fig. 1. Chemical structure of MDL 28170 ((A) keto form) and its equilibrium structures in aqueous solution ((B) hydrate form) and in epimerization ((C) enol form).

ratus (Milford, MA, USA). Sodium phosphate monobasic, sodium borate decahydrate, sodium dodecyl sulfate (SDS) and phosphate buffered saline, pH 7.4 (PBS) were obtained from Sigma (St. Louis, MO, USA). The CE capillary column was a polyimide coated fused silica capillary tubing purchased from Polymicro Technologies (Phoenix, AZ, USA). Acrodisc PVDF sample filter, 0.2 μm, was purchased from Pall Corporation (East Hills, NY, USA). Heparinized rat plasma was purchased from Pel-Freez Biologicals (Rogers, AR, USA) and human plasma was from Carolina Biological Supply Co. (Burlington, NC, USA). All other reagents and chemicals used were reagent grade and were used as received from the supplier.

2.2. Preparation of PBS samples

PBS was reconstituted with water to form a 10 mM PBS solution, pH 7.4. MDL 28170 and its L,L- and L,D-diastereoisomers were individually dissolved in PBS solution to a concentration of about 100 μg ml^{-1}, with the help of a rapid vortex and water bath sonication. The sample solutions were filtered through a 0.2 μm Acrodisc PVDF sample filter and immediately transferred to a 15 mm × 45 mm screw capped sample vial for incubation at 37 °C in a Fisher Isotemp incubator (Pittsburgh, PA, USA). The incubated PBS samples were directly analyzed by CE without any sample pretreatment.

2.3. Preparation of rat and human plasma samples

MDL 28170 and its L,L- and L,D-diastereoisomers were first dissolved in DMSO to a concentration of 20 mg ml^{-1}. The DMSO sample solutions were transferred to a 15 mm × 45 mm screw capped sample vial, diluted with heparinized plasma to a concentration of 1 mg ml^{-1}, and then immediately incubated at 37 °C. The incubated plasma samples, 200 μl, were transferred to 1.5 ml disposable micro centrifuge tubes and 200 μl of acetonitrile were added to precipitate the plasma proteins. After centrifugation (Eppendorf Centrifuge 5415C) at 44,000 × *g* for 5 min, 200 μl of the supernatant were removed and diluted with 100 μl of PBS, followed by transferring the supernatant solution to an Amicon micro concentrator (with membrane molecular weight cut-off at 10,000 Da, Beverly, MA, USA) and centrifuging at 44,000 × *g* for 15 min. Finally, the clear filtrate was pipetted to a 250 μl glass insert for CE analysis.

2.4. Instrumentation

The CE analysis was performed on a Beckman P/ACE 5000 CE system (Fullerton, CA, USA). The dimensions of the capillary column were 67 cm × 365 μm o.d. × 75 μm i.d.) with an effective length of 60 cm (the distance from the inlet of the capillary column to the detection window). The running buffer was prepared by mixing equal volumes of 7 mM sodium phosphate and 3 mM sodium borate. The pH of the running buffer was adjusted to 8.0 ± 0.5 with 0.1 M sodium hydroxide and SDS was added to a concentration of 30 mM. The column temperature was thermostated at 30 °C during the CE run and UV detection was set at 200 nm. Prior to each CE run, the column was consecutively rinsed with 1 M sodium hydroxide/1-propanol (50:50, v/v) mixed solution for 1 min, with water for 1 min, and with CE running buffer for 1 min, all at a pressure of 20 psi. Samples were injected with a pressure injection technique, 10 s at 0.5 psi for PBS samples and 5 s at 0.5 psi for plasma samples. The applied potential was set constant at 30 kV (∼50 μA) for all analyses.

3. Results and discussion

3.1. Analytical methodology

MDL 28170 is a dipeptidyl aldehyde with a protected hydrophobic Cbz moiety at the N-terminal. In aqueous solutions, the electrophilic carbonyl of the aldehyde functional

group at MDL 28170 molecules exists in equilibrium between the keto form and the hydrate form derived from water adduct (Fig. 1). In addition, a tautomeric equilibrium may also exist between the keto form and a transient enol form. This latter equilibrium, in principle, can lead to interconversion of the L,L- and L,D-diastereoisomers of MDL 28170. It is important to understand the epimerization kinetics because of potential differences in biological activity and pharmacokinetics of the resulting diastereoisomers.

Separation of the L,L- and L,D-diastereoisomers of MDL 28170 by conventional reversed-phase high performance liquid chromatography (HPLC) was found to be problematic, mainly because of co-existence of the keto and hydrate forms in the aqueous mobile phase, resulting in forming broad and tailing peaks for both the L,L- and L,D-diastereoisomers. On the contrary, CE with SDS micelles in the running buffer was able to completely separate the L,L- and L,D-diastereoisomers within 10 min with exceptional peak efficiency. The concentration of SDS micelle was found to have considerable effect on the migration time (t_R) of the L,L and L,D peaks as well as the CE background noise level, as depicted in Fig. 2. Both the migration time and the baseline noise level increased as SDS concentration increased from 20 to 75 mM. However, the peak resolution of the L,L- and L,D-diastereoisomers remained very similar under the same concentration range. The SDS concentration of 30 mM was chosen for this CE method instead of 20 mM because it was able to completely resolve the diastereoisomers and the plasma background peaks. Pretreatment of the plasma samples to remove proteins before CE analysis was required but the procedures were straightforward: one process of plasma protein precipitation with acetonitrile, followed by filtration with a micro concentrator. No significant interference from endogenous peaks was encountered, as illustrated in Figs. 3 and 4. Column-rinsing steps were needed before each CE run for maintaining high peak efficiency and reproducibility of migration time through the entire CE analysis.

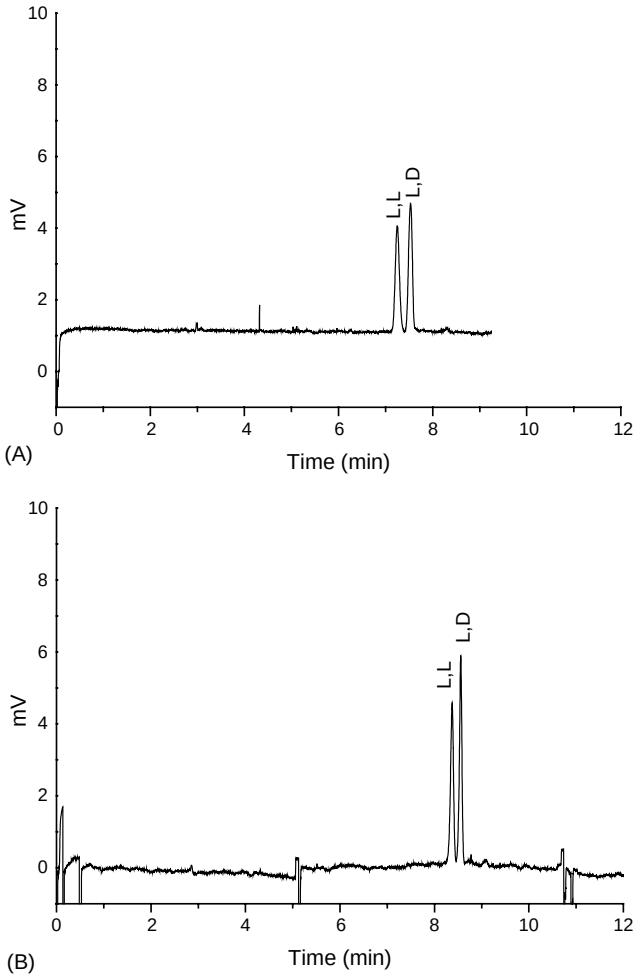

Fig. 2. Separation of the L,L- and L,D-diastereoisomers of MDL 28170 by CE with (A) 20 mM and (B) 75 mM SDS. CE conditions: capillary column, 67 cm × 365 μm o.d.× 75 μm i.d.; running buffer, 7 mM sodium phosphate/3 mM sodium borate (50:50, v/v), pH 8.0; detection, 200 nm; column temperature, 30 °C and applied voltage, 30 kV.

3.2. Epimerization

The samples of the L,L- and L,D-diastereoisomers of MDL 28170 used for epimerization study were found to contain trace amount (1–2%) of the diastereoisomeric impurity, as illustrated by the initial rat plasma samples shown in Fig. 5. The relative percentage of the L,L and L,D-diastereoisomers present in the incubated samples of PBS, rat and plasma was determined and their relative percent-time courses are illustrated in Table 1. It clearly shows a time de-

Table 1
Eperimization of the L,L- and L,D-diastereoisomers of MDL 28170 in PBS, rat and human plasma

Incubation time (h)	PBS		Rat plasma		Human plasma	
	LL/LD ratio of the L,L-diastereoisomer sample	LL/LD ratio of the L,L-diastereoisomer	LL/LD ratio of the L,L-diastereoisomer sample	LL/LD ratio of the L,L-diastereoisomer	LL/LD ratio of the L,L-diastereoisomer sample	LL/LD ratio of the L,L-diastereoisomer
0.25	98/2	1/99	85/15	18/82	84/16	20/80
0.5	98/2	2/98	69/31	25/75	79/21	29/71
1	97/3	2/98	60/40	35/65	59/41	38/62
3	96/4	3/97	51/49	45/55	52/48	44/56
24	85/15	13/87	47/53	47/53	47/53	47/53

Note: The initial L,L/L,D ratio of the L,L-diastereoisomer sample was 98/2, and the initial L,L/L,D ratio of the disatereoisomer was 1/99.

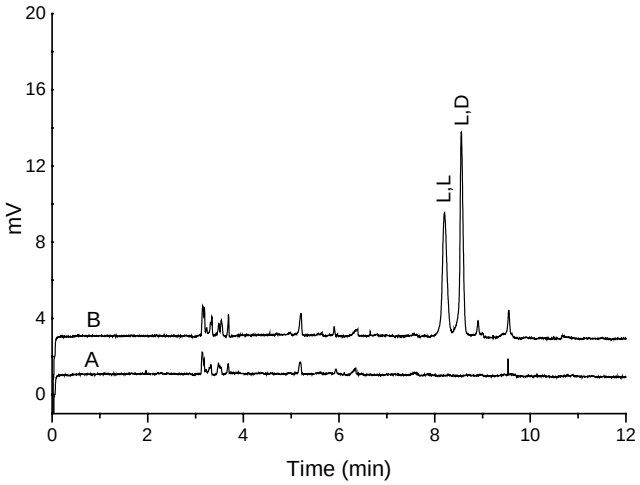

Fig. 3. Electropherograms of (A) rat plasma blank and (B) rat plasma sample containing 1.0 mg ml^{-1} of MDL 28170. CE conditions: capillary column, 67 cm × 365 μm o.d. × 75 μm i.d.; running buffer, 7 mM sodium phosphate/3 mM sodium borate (50:50, v/v), pH 8.0, 30 mM SDS; detection, 200 nm; column temperature, 30 °C and applied voltage, 30 kV.

pendent interconversion of the L,L-diastereoisomer to the L,D-diastereoisomer and vice-versa. The incubation in PBS was extended past 5 days, but an equilibrium plateau had not been observed, indicative of a slow epimerization process for both the L,L- and L,D-diastereoisomers in PBS. The data of 5-day-incubation in PBS showed an L,L/L,D ratio of 55/45 if started from the L,L-diastereoisomer and 40/60 if started from the L,D-diastereoisomer. Based on the relative percent-time course, the degrees of epimerization of the L,L- and L,D-diastereoisomers seem to be alike.

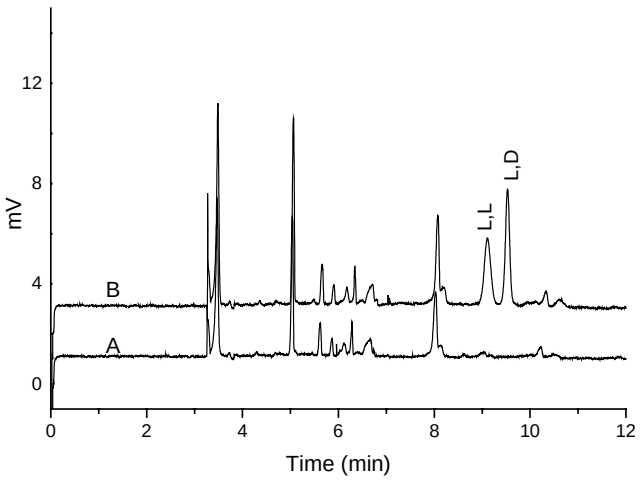

Fig. 4. Electropherograms of human plasma (A) blank and (B) sample containing 1.0 mg ml^{-1} of MDL 28170. CE conditions: capillary column, 67 cm × 365 μm o.d. × 75 μm i.d.; running buffer, 7 mM sodium phosphate/3 mM sodium borate (50:50, v/v), pH 8.0, 30 mM SDS; detection, 200 nm; column temperature, 30 °C and applied voltage, 30 kV.

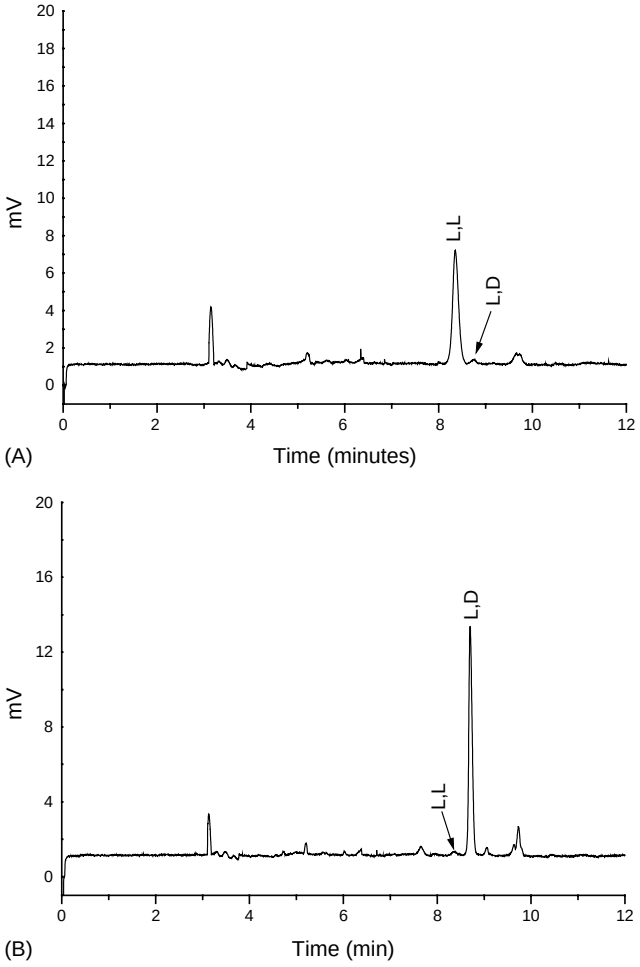

Fig. 5. The initial electropherograms of the L,L-and L,D-diastereoisomers of MDL 28170 incubated in rat plasma, 1 mg ml^{-1}. CE conditions: capillary column, 67 cm × 365 μm o.d. × 75 μm i.d.; running buffer, 7 mM sodium phosphate/3 mM sodium borate (50:50, v/v), pH 8.0, 30 mM SDS; detection, 200 nm; column temperature, 30 °C and applied voltage, 30 kV.

The epimerization process of the L,L- and L,D-diastereoisomers of MDL 28170 was found to be much faster in rat plasma than in PBS. L,L/L,D ratio was 51/49 for the L,L-diastereoisomer and 45/55 for the L,D-diastereoisomer with 3 h of incubation and eventually reached a 47/53 ratio at equilibrium. The kinetic half-life of the epimerization of the L,L-diastereoisomer in rat plasma was about 30 min, which is about three-fold faster than the elimination half-life for the L,L-diastereoisomer reported in a pharmacokinetic study following intravenous bolus dosing [23]. Following incubation in human plasma at 37 °C, epimerization of the L,L- and L,D-diastereoisomers was also observed and their kinetic interconversions were very similar to those found in rat plasma. The L,L/L,D ratio at equilibrium was also found to be 47/53. It was observed that the epimerization process of the L,L- and L,D-diastereoisomers in rat and human plasma had become slower by adding 25% of acetonitrile to the plasma before incubation as an example depicted

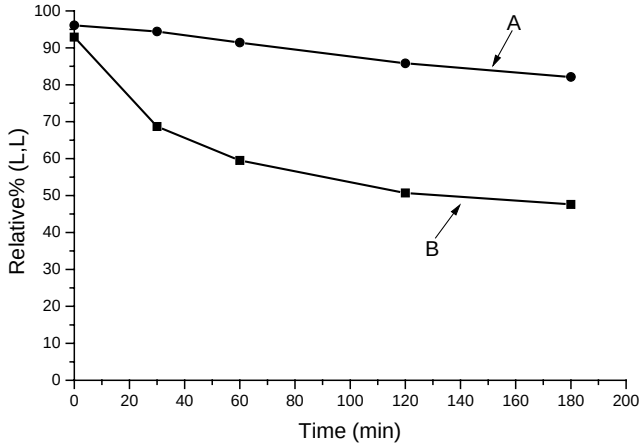

Fig. 6. Kinetics of epimerization of the L,L-diastereoisomer incubated in (A) denatured and (B) normal rat plasma. CE conditions: capillary column, 67 cm × 365 μm o.d. × 75 μm i.d.; running buffer, 7 mM sodium phosphate/3 mM sodium borate (50:50, v/v), pH 8.0, 30 mM SDS; detection, 200 nm; column temperature, 30 °C and applied voltage, 30 kV.

in Fig. 6, indicative of plasma enzymes enhancing the epimerization.

4. Conclusions

Methodology based on capillary electrophoresis using SDS micelles in the running buffer has been developed for the separation of the L,L- and L,D-diastereoisomers of MDL 28170. This methodology was applied to evaluate the epimerization of the diastereoisomers in PBS, rat and human plasma. The relative percent-time course indicates a time depending interconversion of the L,L-diastereoisomer to the L,D-diastereoisomer and vice-versa in all three matrices studied. However, the epimerization process of the L,L- and L,D-diastereoisomers in rat and human plasma is at least 50 times faster than that in PBS. When incubating the L,L- and L,D-diastereoisomers with rat and human plasma, the epimerization process reached equilibrium of 47/53 (L,L/L,D) ratio independent of the starting diastereoisomer. The epimerization half-life of the L,L-diastereoisomer in rat plasma was about 30 min, which is three-fold faster than the observed elimination half-life found in a pharmacokinetic study following intravenous bolus dosing of the L,L-diastereoisomer to rats.

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