DRUG FORMULATIONS AND CLINICAL METHODS

Rapid Enantiomeric Separation and Quantitation of Levetiracetam on α-Acid Glycoprotein (AGP) Chiral Stationary Phase by High-Performance Liquid Chromatography

Rouhollah Heydari
Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, PO Box 68149-89468, Khorramabad, Iran
Mojtaba Shamsipur
Razi University, Department of Chemistry, Kermanshah, Iran

A new, simple, and rapid chiral HPLC method was developed for enantioselective analysis of levetiracetam and its enantiomer [(*R*)-α-ethyl-2-oxo-pyrrolidine acetamide] in a pharmaceutical formulation and bulk material. Enantiomeric separation was achieved on a chiral-α_1-acid glycoprotein (AGP) column (150 × 4.0 mm, 5 µm) using an isocratic mobile phase of phosphate buffer (pH = 7) at a flow rate of 0.7 mL/min. The UV detector was set at 210 nm. Calibration curves were linear in the range of 1–100 µg/mL and 0.4–20 µg/mL for levetiracetam and the (*R*)-enantiomer, respectively. LOD and LOQ for the (*R*)-enantiomer were 0.1 and 0.4 µg/mL, respectively. The run time of analysis was less than 5.0 min.

Amino acids and sugars occur in nature as single enantiomer forms. Because these molecules are building blocks of enzymes and receptors in the human body, the latter can be seen as a highly chiral environment in which stereoselectivity will play a crucial role (1). Administering a racemic drug to a patient is therefore not justified in most cases. Therefore, regulatory authorities strongly recommend developing single enantiomer drugs when possible (2). In some cases, for example, when it is known that the enantiomer racemizes in vivo due to metabolization processes, the development of a racemate can still be approved.

Regarding legislation, three important guidelines are to be followed in the development of a chiral drug (3). The first is guideline ICH Q6A, which states that if the new drug substance is a single enantiomer form, a chiral identity test, a chiral assay, and a method able to determine the enantiomeric impurity are required for a drug substance. Only the latter two are needed for a drug product. When the drug substance is racemic, considering the need for verifying the chiral identity in drug substance release and/or acceptance testing is recommended. The second and third guidelines, Q3A and Q3B, concern impurities of regular drug substances. It is stated that enantiomeric impurities are excluded from these guidelines. This originates from the practical difficulties in achieving quantitation and identification thresholds, specified in Q3A and Q3B, for chiral impurities similarly as for nonchiral substances. Nevertheless, the principles written in these guidelines are expected to be applied. For drug products containing a nonchiral substance, guideline Q3B specifies that reporting thresholds of the impurities are 0.1 and 0.05%, depending whether more or less than 1 g of the drug is consumed/day, respectively.

Levetiracetam [(*S*)-α-ethyl-2-oxo-pyrrolidine acetamide] is a second-generation antiepileptic drug (AED), licensed in the United States in 1999 (4) and approved for clinical use in 2002 (5). Levetiracetam is a pyrrolidine derivative whose efficacy has been demonstrated in epilepsies with pharmaco-resistance to other AEDs. Levetiracetam is available in tablet form containing 250, 500, 750, and 1000 mg/tablet.

α-Ethyl-2-oxo-pyrrolidine acetamide possesses a single asymmetric carbon atom and therefore has two enantiomers (Figure 1). Only the (*S*)-enantiomer has anticonvulsant activity, and consequently, only this enantiomer has been developed and used as a new AED (6).

Separation and determination of the enantiomeric excess of levetiracetam in formulations have been performed by using LC (7) and capillary electrochromatography (CEC; 8). In the LC method, a Chiralpak AD-H column [amylose tris (3,5-dimethylphenylcarbamate) selector] was used in the normal phase (NP) mode to separate two enantiomers. In the optimized method, the retention times of the (*R*)-enantiomer and levetiracetam were 9.1 and 13.9 min, respectively, with a resolution above 7 using hexane–isopropanol (90 + 10, v/v) as the mobile phase. In the CEC method, separation was achieved in a shorter time with resolution about 5. Also, separation of levetiracetam and its enantiomer was reported with a cyclodextrin-modified microemulsion electrokinetic chromatography (CD-MEEKC) method (9) and a GC/MS method (10). However, in the CD-MEEKC method baseline separation could not be achieved.

α_1-Acid glycoprotein (AGP) is a very stable protein that tolerates pure organic solvents, high temperatures, and high and low pHs. The column is used in the reversed-phase (RP) mode. The chiral AGP column can be used for the resolution of an extremely broad range of chiral compounds, such as amines (primary, secondary, tertiary, and quaternary ammonium), acids, esters, sulfoxides, amides, and alcohols (11–22).

The purpose of this study was to develop a new, rapid, sensitive, and precise HPLC method using an AGP column

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Corresponding author's e-mail: rouhollahheydari@yahoo.com
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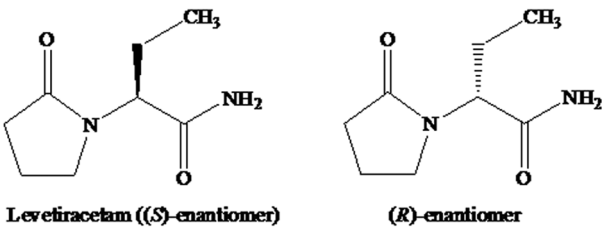

Figure 1. Chemical structures of levetiracetam and its enantiomer.

for the chiral separation and determination of chiral purity of levetiracetam in bulk form and a pharmaceutical preparation.

Experimental

Chemicals

Levetiracetam, its enantiomer, [(*R*)-enantiomer] and pharmaceutical raw material were obtained from Hetero (Hyderabad, India). Methanol, acetonitrile, isopropanol, sodium dihydrogen phosphate, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Biolepra tablets [containing 250 mg (*S*)-enantiomer of levetiracetam] were obtained from the Bakhtar Bioshimi Pharmaceutical Co. (Kermanshah, Iran).

Apparatus and Conditions

The KNAUER HPLC system was equipped with two Smartline 1000 pumps and a 2600 photodiode array detector (Berlin, Germany). Separation was carried out on a chiral-AGP column (ChromTech, Apple Valley, MN; 4.0 × 150 mm, 5 µm) using a mobile phase containing phosphate buffer (0.15 M, pH adjusted to 7.0 with sodium hydroxide solution, 0.1 M). The temperature of the column was maintained at 10°C. The mobile phase flow rate was kept constant at 0.7 mL/min. The solutes were detected at 210 nm. The injection volume was 5 µL.

Preparation of Stock and Working Standard Solutions

The stock solutions of (*S*)-levetiracetam and its enantiomer [(*R*)-enantiomer] were prepared by dissolving 10 mg of each compound in 10 mL of the mobile phase, separately. The corresponding working standard solutions were prepared by dilution of individual stock solutions with the mobile phase. From these solutions, serial dilutions were made to obtain concentration levels of 1.0, 2.0, 5.0, 10.0, 20.0, 30.0, 40.0, 80.0, and 100 µg/mL for (*S*)-levetiracetam, and 0.4, 0.8, 2.0, 4.0, 6.0, 8.0, 10.0, and 20.0 µg/mL for levetiracetam enantiomer [(*R*)-enantiomer].

Preparation of Sample Solutions

The sample solution was prepared by transferring 80 mg raw material [(*S*)-levetiracetam] to a 100 mL volumetric flask with addition of 100 mL of the mobile phase to obtain a sample solution containing 0.8 mg/mL of the drug.

Twenty tablets were powdered, and equivalent weight of tablet (Biolepra 250 mg) was transferred to a 100 mL volumetric flask and dissolved in mobile phase. In order to extract levetiracetam from the tablet matrix, the solution was ultrasonicated (Model VGT-1730 QTD, GT SONIC, China) for 30 min and centrifuged (Eppendorf, Model ALC 4232, Hamburg, Germany) for 25 min at 3000 rpm, and the supernatant was filtered with a 0.45 µm syringe filtration disk (Sartorius, Göttingen, Germany). Dilutions were made in the mobile phase to provide a concentration of 0.8 mg/mL (*S*)-levetiracetam. Finally, 5 µL of this solution was injected into the HPLC system.

Results and Discussion

The AGP column is an RP column, and both the retention and the enantioselectvity of analytes with this column were influenced by several parameters. The solutes are retained by three types of forces: ionic bonding (charge solutes), hydrophobic interaction and hydrogen bonding. The relative contribution of the different forces to the retention of the solutes depends on the nature of the analyte. Analytes containing charged groups, hydrogen binding groups, and hydrophobic parts can be retained by interaction with corresponding groups on the chiral selector. Thus, it is expected that the separation can be affected by pH, buffer concentration, type of organic modifier, and the organic modifier concentration (23).

Effect of pH on Enantioselectivity

The most important parameter in method development is the mobile phase pH. The reason is that by changing the pH, the net charge of the chiral selector as well as the charge of the solute can be changed, which affects the analyte interaction with the chiral selector. AGP has a low isoelectric point of 2.7. This means that net charge of the stationary phase at pH 2.7 is zero. With increasing pH of the mobile phase from 2.7 to 7, the degree of net negative charge of the chiral selector increases. Consequently, positively charged solutes are attracted toward the stationary phase which leads to increased retention of the solute. On the other hand, reducing the pH of the mobile phase towards the isoelectric point of AGP reduces the negative charge of the stationary phase, resulting in lower retention of the solute. A change of the net charge of the chiral selector strongly affects the interaction between the solute and the chiral stationary phase.

The influence of mobile phase pH on retention and enantioselectivity of the levetiracetam enantiomers on the chiral AGP column was studied by changing the pH of the mobile phase

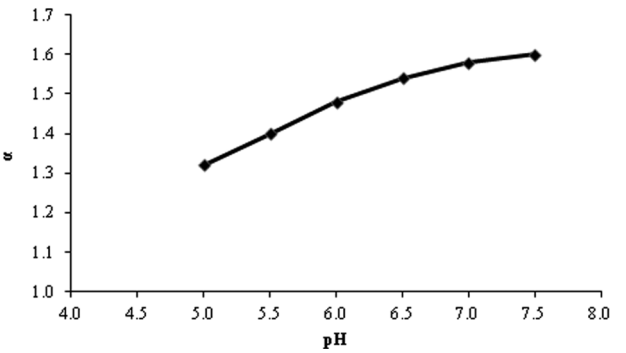

Figure 2. Effect of mobile phase pH on enantioselectivity. Conditions = flow rate, 0.7 mL/min; buffer concentration in the mobile phase, 0.15 M; and column temperature, 10°C.

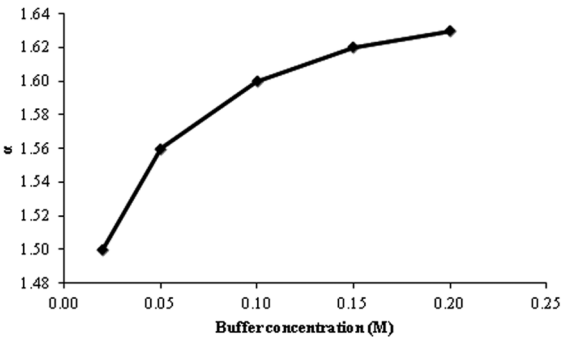

Figure 3. Effect of buffer concentration on enantioselectivity. Conditions = flow rate, 0.7 mL/min; column temperature, 10°C; and pH of the mobile phase, 7.

(0.15 M phosphate buffer) from 5.0 to 7.5. Figure 2 shows the effect of pH on the selectivity of the enantiomers. As is obvious, enantioselectivity increased considerably with increasing pH of the mobile phase from 5.0 to 7.5. However, because 7.5 is the highest limit of column pH and the enantioselectivity of the system changed only slightly as the pH was increased from the 7.0 to 7.5, pH 7.0 was selected as the optimum value for further studies. According to the manufacturer's manual, the silica-based column can be used in the pH range 4–7 (24). Use of the column at pH >7 or pH <4 for longer periods of time may decrease the column lifetime due to silica decomposition.

Effect of Buffer Concentration on Enantioselectivity

By changing the buffer concentration, it is possible to change both the retention and the enantioselectivity. The buffer concentration was varied in the range 0.02–0.20 M. It was found that the separation efficiency was affected by the concentration of buffer in the mobile phase. As shown in Figure 3, the separation efficiency of the two enantiomers was increased slightly by an increase in the buffer concentration from 0.02 to 0.20 M. Therefore, a 0.15 M solution of phosphate buffer was chosen as the mobile phase based on retention, selectivity, resolution, and maximum column life.

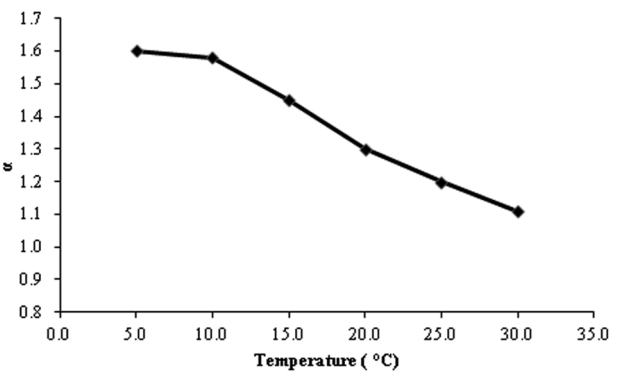

Figure 4. Effect of column temperature on enantioselectivity. Conditions = flow rate, 0.7 mL/min; buffer concentration in the mobile phase, 0.15 M; and pH of the mobile phase, 7.

Effect of Column Temperature on Enantioselectivity

The column temperature was also investigated as a possible parameter in improving the enantioseparation of levetiracetam enantiomers on the chiral AGP column. The effect of column temperature on enantioselectivity was investigated over the range of 5–30°C. The influence of column temperature on the enantioselectivity is illustrated in Figure 4. Generally, retention of the two enantiomers decreased as the column temperature was increased (25, 26). However, in the range 5–10°C, the variations of chiral selectivity were small. Therefore, a column temperature of 10°C was selected as the optimum temperature for further studies.

Influence of the Nature and Concentration of Organic Modifiers

It has been demonstrated that uncharged modifiers such as 1-propanol and acetonitrile are strongly adsorbed on the AGP phase (27). It was observed that the more hydrophobic uncharged modifiers such as 1-propanol were bound to the stationary phase with higher affinity. Adsorption isotherm studies showed that a monolayer was formed at 10 and 15% (v/v) for 1-propanol and acetonitrile, respectively (27). Also, the results demonstrated the formation of multilayers of solvent molecules on the protein surface at higher concentration of modifiers (27). The adsorption of the uncharged modifiers on the protein influences the column properties. Normally, both the enantioselectivity and the retention increase by decreasing modifier concentration in the mobile phase. However, for certain solutes it is possible to improve the chiral selectivity by increasing the modifier concentration (28).

Isopropanol, acetonitrile, methanol, and ethanol are the most frequently used organic modifiers. By changing from one organic modifier to another with different hydrogen bonding properties, i.e., from acetonitrile (hydrogen accepting properties) to isopropanol (hydrogen accepting and donating properties), it is possible that the enantioselectivity was changed strongly. Various organic modifiers such as isopropanol, methanol, and acetonitrile with different concentrations from 1 to 5% were studied using a 0.15 M phosphate buffer solution (pH 7.0) as the mobile phase at a column temperature of 10°C. Normally, higher modifier concentration reduces the retention

Table 1. Effect of various mobile phase organic modifiers on enantioselectivity (α)

Organic modifier	Percent in mobile phase, % (v/v)[a]	α
Isopropanol	1	1.21
	2	1.11
	5	1.00
Methanol	1	1.32
	2	1.16
	5	1.10
Acetonitrile	1	1.18
	2	1.12
	5	1.00

[a] Flow rate, 0.7 mL/min; buffer concentration in the mobile phase, 0.15 M; pH of the mobile phase, 7; column temperature, 10°C.

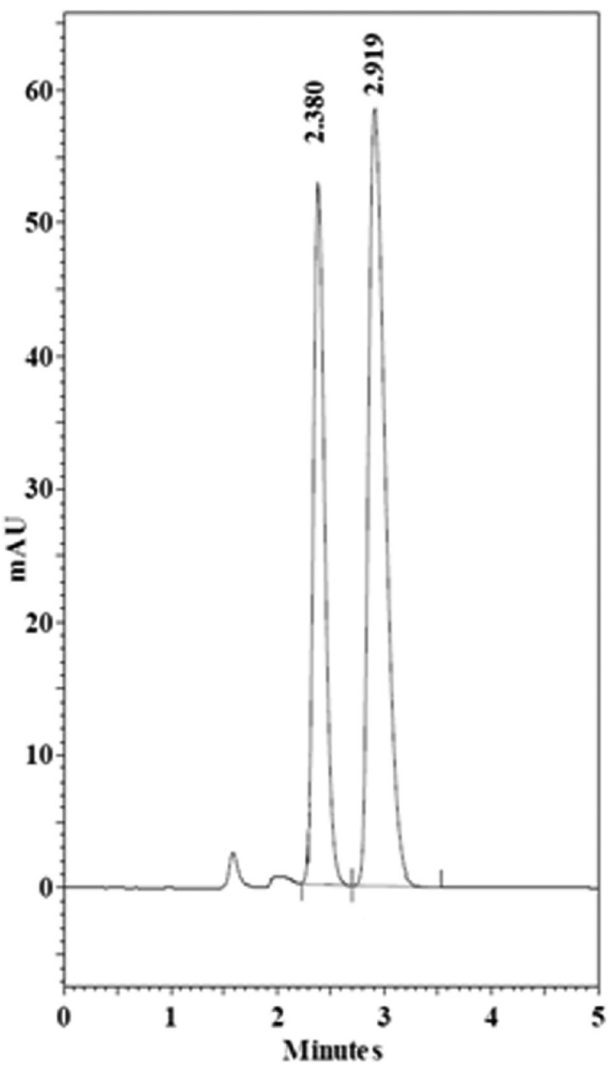

Figure 5. Typical chromatogram of (*S*)-levetiracetam (RT = 2.380) and its enantiomer (RT = 2.919). Conditions = flow rate, 0.7 mL/min; buffer concentarion in the mobile phase, 0.15 M; pH of the mobile phase, 7; and column temperature, 10°C.

and enantioselectivity. However, in this work addition of different organic modifiers into the mobile phase decreased the retention and destroyed the enantiomeric separation (Table 1). Therefore, a 0.15 M phosphate buffer solution of pH 7.0 without any organic modifier was used as the mobile phase for further studies. Figure 5 shows the chromatogram acquired using the AGP column under the optimized conditions. System suitability parameters under the optimized conditions are shown in Table 2.

Method Development and Evaluation

When a method has been developed it is important to evaluate it to confirm that it is suitable for its intended purpose. To ensure the ability of the proposed method to obtain test results that are directly proportional to the concentration of the analyte, a linear correlation was obtained between the peak area and concentration of levetiracetam and its enantiomer. The regression equation parameters for two analytes are shown in Table 3.

Table 2. System suitability parameters[a]

Compound	RT, min[b]	Rs[c]	N[d]	T[e]
Levetiracetam	2.380	—	1098	1.04
(*R*)-Enantiomer	2.900	2.0	2265	1.10

[a] Conditions as given in Table 1.

[b] RT = Retention time.

[c] Rs = Resolution.

[d] N = Number of theoretical plate.

[e] T = Tailing factor.

Table 3. Linear analytical response statistical summary for compounds using peak area

Compound	Calibration range, µg/mL	R^2	Intercept	Slope
Levetiracetam	1–100	0.9988	–16413.0	24245.0
(*R*)-Enantiomer	0.4–20	0.9981	4225.4	25004.0

Table 4. Results of determined amounts of levetiracetam and its enantiomer in real samples

Real sample		Results, %
Raw material	Levetiracetam	99.8
	(*R*)-Enantiomer	Not detected
Biolepra tablet	Levetiracetam	99.6
	(*R*)-Enantiomer	Not detected

The accuracy of the method was tested by analyzing freshly prepared solutions containing levetiracetam (800 µg/mL) and the (*R*)-enantiomer at different concentration levels (0.05% and 0.1% of the main peak, 0.4 and 0.8 µg/mL, respectively). The recoveries were found to be between 95.0 and 104.0%, and the average RSDs for three replicates were below 5.0%.

In order to investigate the repeatability of the proposed method under the optimized experimental conditions, solutions of known concentrations of levetiracetam and its enantiomer (typically 5 µg/mL) were injected into the HPLC system six times. The mean values of RSDs for levetiracetam and the (*R*)-enantiomer were 1.22 and 2.87%, respectively.

LOD (determined with an S/N of 3) and LOQ (determined with an S/N of 10) were calculated only for the (*R*)-enantiomer because levetiracetam exists as a major component in real samples. Results indicated that the values of LOD and LOQ were 0.1 and 0.4 µg/mL, respectively.

Finally, the proposed method was used for the analysis of commercial raw material and pharmaceutical preparation samples. The results are shown in Table 4. Figure 6 shows the HPLC chromatograms of the raw material (Figure 6A) and raw material spiked with the (*R*)-enantiomer of levetiracetam (20 µg/mL; Figure 6B).

The results of this study were compared with previous methods in Table 5. As observed from these data, run time, LOD, and LOQ of the proposed method were better than previous methods. In addition, this method is an RP-HPLC method that is more suitable than NP-HPLC methods. RP-HPLC is not only simpler, but also more cost-effective and green. By using water (or a water-based

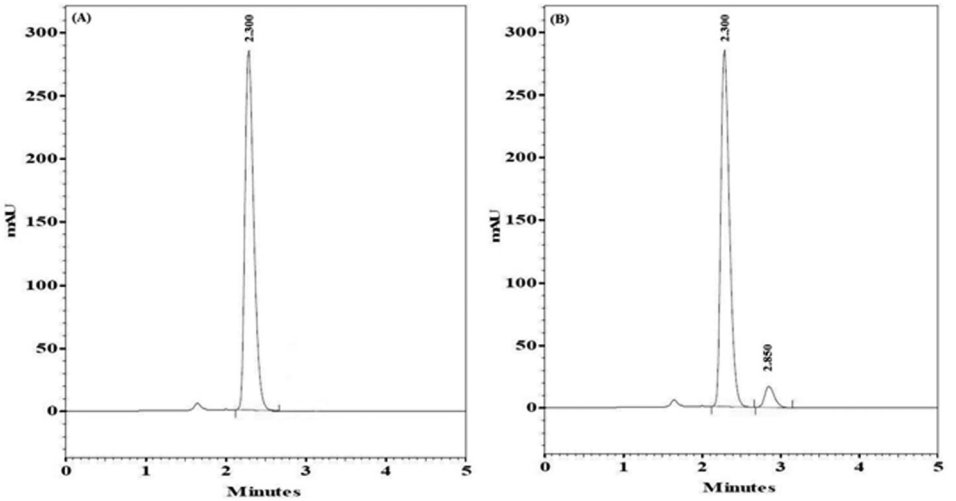

Figure 6. Chromatograms of (A) raw material (B) and raw material spiked with the (*R*)-enantiomer. Conditions as in Figure 5.

Table 5. Comparison of important parameters for the proposed method and several reported methods

Method		Rs	Run time, min	LOD	LOQ[a]	LOL[b]	Ref.
NP-HPLC	Chiralcel OD-H	3.3	18.0	—[c]	—	—	8
	Chiralcel OJ-H	1.8	12.0	—	—	—	
	Chiralpak AD-H	7.9	18.0	0.9 µg/mL	2.25 µg/mL	2.25–9.0 µg/mL	
CE		1.4	7.0	1.1 µg/mL	3.6 µg/mL	10–1000 µg/mL	9
CD-MEEKC		1.11	9.0	—	—	—	10
GC/MS		—	15.0	0.94 pmol	2.35 pmol	1.3–2.35 nmol	11
Proposed method		2.0	4.0	0.1 µg/mL	0.4 µg/mL	0.4–20 µg/mL	

[a] LOD and LOQ calculated only for the (*R*)-enantiomer.

[b] LOL = Limit of linearity of the calibration curve for the (*R*)-enantiomer.

[c] — = Not reported.

solvent) as the mobile phase, RP-HPLC eliminates the toxic and expensive solvents that are used in NP-HPLC.

Conclusions

We developed a simple and rapid method for determination of levetiracetam and its enantiomer in pharmaceutical formulation and raw material samples. The separation was completed in less than 5 min. The obtained results prove the repeatability, linearity, and sensitivity of the proposed method. This method was used for screening and determination of levetiracetam and its enantiomer in commercial bulk and pharmaceutical formulation samples, which shows the applicability of the method for use in QC laboratories.

Acknowledgments

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