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Enantiomeric separation of norgestrel by reversed phase high-performance liquid chromatography using eluents containing hydroxypropyl-beta-cyclodextrin in stereoselective skin permeation study

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Abstract

The chiral separation of norgestrel enantiomers using reversed-phase high-performance liquid chromatography (RP-HPLC) was studied with hydroxypropyl-beta-cyclodextrin (HP-beta-CD) as chiral mobile phase additive. The effect of mobile phase composition, concentration of HP-beta-CD and column temperature on enantioselective separation were investigated. The quantification properties of the developed RP-HPLC method were examined. A baseline separation of norgestrel enantiomers was achieved on a Agilent ZORBAX Eclipse XDB-C8 column (150 mm × 4.6 mm i.d., 5 μm). The mobile phase was a mixture of acetonitrile and phosphate buffer (pH 5.0, 20 mM) containing 25 mM HP-beta-CD (30:70, v/v) with a flow rate of 1.0 ml/min. The UV detector was set at 240 nm. Calibration curves were linear ($n = 8$) in the range of 0.2–25 μg/ml, the limit of detection and quantitation were 0.10 and 0.20 μg/ml, respectively, for racemic norgestrel. The values of RSD of repeatability and intermediate precision for spiked sample were less than 4.8%. The method was successfully applied to the enantioselective determination of this drug in stereoselective skin permeation study.

Keywords: Norgestrel; Enantiomeric separation; Hydroxypropyl-beta-cyclodextrin; HPLC

1. Introduction

Chirality is a fundamental characteristic of biological systems, and more than one-half of marketed drugs are chiral. Currently, the transdermal delivery of chiral species is an increasingly active and promising field. The stratum corneum, the rate-limiting barrier to percutaneous absorption, is principally made up of keratin and ceramides, which could potentially provide a chiral environment [1]. Differential binding of enantiomers to keratin or interactions with ceramide may give rise to differences in the permeation profiles of the enantiomers. As yet, the effects of stereoisomerism on percutaneous absorption of drugs are not well studied [2]. We are just beginning to realize that there are apparent differences between enantiomeric pairs or between the pure enantiomers and racemate of chiral drugs with respect to their percutaneous permeation. In our lab, stereoselective skin permeation of racemate and pure enantiomers of norgestrel (Fig. 1) was studied. Norgestrel, marketed as norgestrel racemic mixture and levonorgestrel, has only one of its isomers (the levonorgestrel isomer) biologically active, and which has twice its potency of the racemic form [3].

During the studies, a stereoselective analysis was needed. Concerning the enantiomeric separation of norgestrel, there have several reports. Native cyclodextrin (CD, cyclic oligosaccharides composed of six, seven or eight α-D-glucopyranose units (alpha-, beta-, gamma-CD, respectively) as chiral mobile phase additive was firstly studied and found feasible for separation of norgestrel enantiomers in RP-HPLC by gamma-cyclodextrin [4–8], and the separation method was optimized for enantiomeric and non-isomeric impurities testing of levonorgestrel

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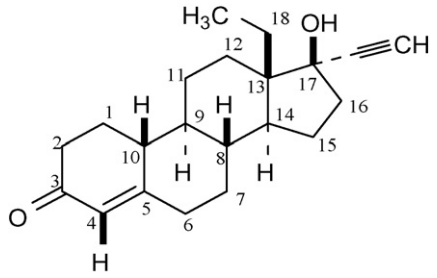

Fig. 1. The structural formula of norgestrel.

[7]. Afterward, it was reported that norgestrel enantiomers are separable in reversed-phase liquid chromatography using monolithic silica columns with beta-cyclodextrin (beta-CD) chemically bonded as a stationary phase [9] or by beta-CD as the chiral mobile-phase additive [10]. Another reported method is by capillary electrophoresis [11]. The predominating separation mechanism of CD for norgestrel was based on the phenomenon of guest-CD complexation, where a transient diastereomeric complex is formed between the CD and the analyte [10,12]. However, owing to the low solubility of native CD in water and organic solvent, baseline enantiomeric separation is hardly achieved for norgestrel [10]. Commonly, solubilizers for CD such as carbamide need to be added which can lead to column deterioration. So native CD used as mobile phase additive in HPLC for pharmaceutical analysis is limited. Hydroxypropyl-beta-cyclodextrin (HP-beta-CD) is a derivative of native beta-CD. Derivatization of the hydroxyl groups increases solubility and selectivity compared to the native beta-CD and the hydroxyl groups also undergo additional interactions with the analytes, thereby enhancing chiral recognition [13].

In this paper, the chiral separation of norgestrel enantiomers using reversed phase high-performance liquid chromatography (RP-HPLC) was studied with HP-beta-CD as chiral mobile phase additive. The effect of mobile phase composition, concentration of HP-beta-CD and column temperature on enantioselective separation were investigated, and the method for the determination of norgestrel enantiomers were validated and used for determining norgestrel enantiomers in stereoselective skin permeation study.

2. Experimental

2.1. Chemicals and reagents

D-(±)-Norgestrel (racemic norgestrel) and D-(−)-norgestrel (levonorgestrel) were provided by Yangzhou Pharmaceutical Co. (Jiangzu, China). HP-beta-CD (degree of substitution 4.9) was purchased from Xian Deli Biochemical Co. Ltd. (Xian, China). Sodium dihydrogen phosphate (NaH_2PO_4, analytical grade) was purchased from Shanghai Luyuan Fine Chemical Factory (Shanghai, China). Acetonitrile (Merk, Darmstadt, Germany) was of HPLC grade. Water was purified by double distillation. Sodium hydroxide (NaOH) and all other chemicals or solvents were analytical reagent and purchased from commercial sources.

2.2. Equipment and chromatographic condition

Chromatographic studies were performed on Agilent 1100 HPLCs (Agilent, Palo Alto, CA, USA) equipped with thermostated-column device and a variable-wavelength UV detector. The pH measurement was performed on a pH meter (LIDA, model PHS-3C, Shanghai, China). The separation of the analytes was achieved on a Agilent ZORBAX Eclipse XDB-C8 (150 mm × 4.6 mm i.d., 5 μm) column. The mobile phase was a mixture of acetonitrile and sodium dihydrogen phosphate buffer (pH 5.0, 20 mM) containing 25 mM HP-beta-CD (30:70, v/v) with a flow rate of 1.0 ml/min. The mobile phase was filtered through a 0.45 μm filter (Alltech Associates, Deerfield, IL, USA) and sonicated prior to use. The wavelength of UV detector was set at 240 nm and the column was operated at the temperature of 20 °C unless otherwise specified. Injection volume was 20 μL.

2.3. Preparation of stock and standard solution

Racemate and the D-(−)-enantiomer of norgestrel were accurately weighted, transferred to volumetric flasks and dissolved in methanol to make individual stock solutions of 500 μg/ml. The solution were stored at 4 °C and stable for at least 3 months. The stock solution was diluted with methanol or 40% (v/v) polyethylene glycol (PEG) 400 saline, a receptor medium used in permeation study, to certain concentration before use according to the analytical requirement.

2.4. Optimization of mobile phase parameters

The influence of mobile phase composition, concentration of HP-beta-cyclodextrin and column temperature on the high-performance liquid chromatographic separation of norgestrel enantiomers was studied. The enantiomeric separation ability was evaluated by resolution and retention time. The resolutions are based on the average of at least three independent determinations of each solute.

2.5. Application of the assay

The present assay has been used to quantify the concentrations of norgestrel enantiomers in skin permeation study. *In vitro* skin permeation study of norgestrel was conducted at 37 °C across the excised mouse dorsal skin mounted on the receptor compartment of a two-chambered Valia-Chien glass diffusion cell (fabricated by Zhejiang University Glass Company), while stirring at a constant rate of 500 rpm. Valia-Chien cells were composed of a receptor compartment and a donor compartment, with a volume of 4 mL for each compartment, and an effective diffusional area of approximately 0.72 cm^2. 0.5 mg/mL racemic norgestrel or levonorgestrel in ethanol–water solution (50:50, v/v) were used as donor medium, and 40% PEG 400 saline was used as a receptor medium. At specified intervals, 0.5–4 mL samples were withdrawn from the receptor compartment, and an equivalent amount of receptor medium was added to maintain the constant volume. The samples were analyzed under Section

Table 1
Effect of the concentration of HP-beta-CD on the resolution of norgestrel enantiomers

Concentration of HP-beta-CD (mol/L)	D-(+)-RT[a] (min)	D-(−)-RT (min)	Rs
15	39.4	41.6	1.02
20	35.4	37.6	1.29
25	31.6	33.9	1.5
30	27.4	29.8	1.64
40	25.8	28.2	1.75

Chromatographic conditions described in Fig. 2.
[a] RT, retention time.

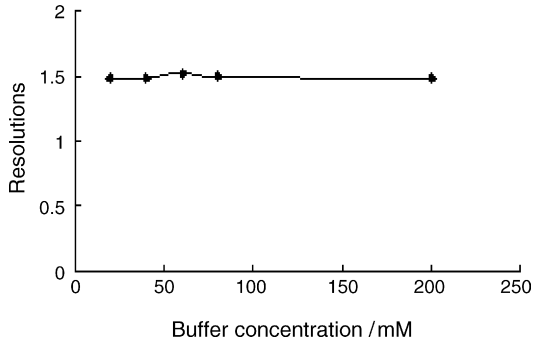

Fig. 3. Effect of concentration of buffer on the resolution of the enantiomers. Using 20–200 mM sodium dihydrogen phosphate at pH 5.0 with 25 mM HP-beta-CD containing 30% acetonitrile as mobile phase; other operating conditions as in Fig. 2.

2.2 described chromatographic conditions. Twenty microliters were injected directly into the liquid chromatography.

3. Results and discussion

3.1. Method development

In order to achieve the separation of norgestrel enantiomers, the influence of the concentration of HP-beta-CD on the resolution of enantiomers were examined during optimization. Addition of HP-beta-CD (15–40 mM) to the mobile phase composed of 70:30 (v/v) aqueous 50 mM phosphate buffer (NaH_2PO_4), pH 5.0 (adjusted with 10% NaOH)/acetonitrile gave a good enantiomeric resolution of norgestrel enantiomers. The results in Fig. 2 and Table 1 showed that an increase in the concentration of HP-beta-CD leads to both a concomitant decrease in retention time and an increase in resolutions, and the shortest retention and best resolution was achieved with 40 mM HP-beta-CD. However, the concentration of HP-beta-CD above 30 mM increase pressure obviously and has the trend to decrease column efficiency, so 25 mM HP-beta-CD was chosen for further study with which the baseline separation of norgestrel was achieved. HP-beta-CD concentration affects both enantiomer resolution and retention time. However, the interrelationships among the CD concentration, guest-CD complexes and efficiency of chiral resolution still lack theoretical bases. Additionally, using 6 mM beta-CD as chiral mobile phase additive was also investigated (the solubility of beta-CD in water at 15 °C is 9 mM), no enantioselectivity was observed. Gazdag et al. [7] also reported that beta-CD in the eluent at a concentration of 10 mM cannot resolve the enantiomers.

Optimization of the mobile-phase composition was achieved by testing the influence of buffer type, pH and concentration. Buffer type was found to be important for improvement of enantioselectivity. Acetate buffer and phosphate buffer solutions with different pH values were employed and evaluated, and the buffer of pH 5.0 sodium dihydrogen phosphate was finally chosen. The buffer concentration on the resolution was investigated for norgestrel enantiomers using 20–200 mM sodium dihydrogen phosphate at pH 5.0 with 25 mM HP-beta-CD containing 30% acetonitrile. The results are described in Fig. 3. No different resolutions were observed in those ionic strength ranges.

The pH of the buffer (NaH_2PO_4) on the enantioselectivity was examined by using 20 mM buffer solutions (pH 3.8–6.0, adjusted with 10% NaOH) with 25 mM HP-beta-CD containing 30% acetonitrile as the mobile phase as shown in Fig. 4. It is observed that the resolution of the enantiomers is pH-independent.

Acetonitrile was used as the organic modifier. The concentration of acetonitrile used in these experiments was 25–35%. The resolution decreased by increasing acetonitrile concentration (Fig. 5). The selectivity for norgestrel enantiomers was optimum at 30% considering resolution and retention time together.

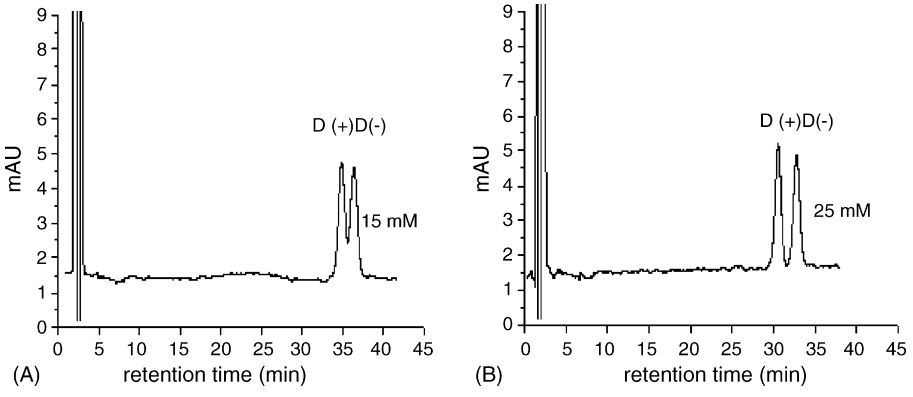

Fig. 2. Separation of D-(±)-norgestrel enantiomers at 15 and 25 mM concentration of HP-beta-CD. Chromatographic conditions: Agilent ZORBAX Eclipse XDB-C8 (150 mm × 4.6 mm i.d., 5 μm) column. Mobile phase: 70:30 (v/v) aqueous 50 mM phosphate buffer, pH 5.0 (adjusted with 10% NaOH)/acetonitrile. Flow rate: 1.0 ml/min. Injection volume: 20 μl. Wavelength used for UV detection: 240 nm. Column temperature: 20 °C.

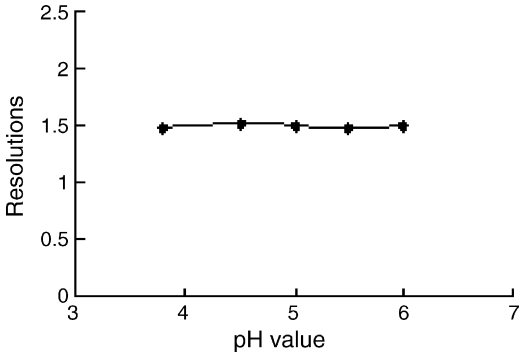

Fig. 4. Effect of pH on the resolution of the enantiomers: using 20 mM buffer solutions (pH 3.8–6.0, adjusted with 10% NaOH) with 25 mM HP-beta-CD containing 30% acetonitrile as the mobile phase; other operating conditions as in Fig. 2.

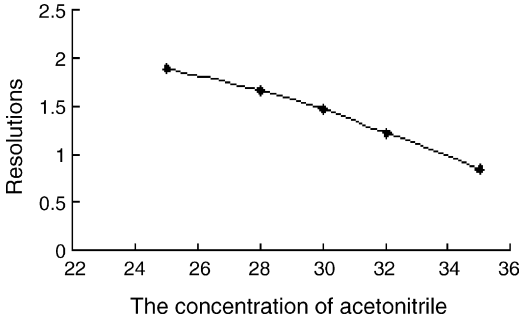

Fig. 5. Effect of concentration of acetonitrile on the resolution of the enantiomers. Using 20 mM buffer solutions (pH 5.0, adjusted with 10% NaOH) with 25 mM HP-beta-CD containing 25–35% acetonitrile as the mobile phase; other operating conditions as in Fig. 2.

Acetonitrile–water (30:70, v/v) containing 25 mM HP-beta-CD was applied as the mobile phase to study the effect of temperature on the enantioselectivity of D-(±)-norgestrel. The resolutions were measured over a wide range of column temperatures from 0 to 60 °C. The results in Table 2 showed that temperature does not only have a great effect on enantiomer resolution, but appears to cause retention times to alter such that separation is affected. Inferior results are obtained with increase temperature, and baseline separation is observed at 0–20 °C. The phenomenon that retention decreases with increase in temperature has been observed many times in both HPLC and GC. It can be easily explained by the faster migration of the solute molecules through the chromatographic column and their lower affinity to the stationary phase. At a molecular level, the enantioselectivity for this assay in the relative lower temperature ranges can probably be interpreted as being due to slower rotation of the guest and host molecules and hence a steric fit is possible [10]. The results presented here suggest that the predominant mechanism for retention is the formation of guest-beta-CD complexes in the mobile phase [10].

Table 2
The retention time (RT) and resolutions (Rs) for separation of norgestrel enantiomers at various temperature, other chromatographic conditions as in Fig. 2

Column temperature (°C)	D-(+)-RT (min)	D-(−)-RT (min)	Rs
60	24.8	25.9	0.77
45	26.5	28	1.03
35	28.4	30.2	1.25
20	30.3	32.5	1.48
10	33.4	35.8	1.61
0	39	41.8	1.8

3.2. Validation of the method

3.2.1. Assay specificity

The results showed in Fig. 6 indicated that the method was specific for determining norgestrel enantiomers under the chromatographic conditions employed. The D–(+)-norgestrel and D-(−)-norgestrel were achieved a baseline resolution with retention time of approximately 31 and 34 min, respectively. The peak locations of the two enantiomers were not interfered by the compounds in receptor medium.

3.2.2. Calibration curves

The calibration curve for the assay was constructed by analyzing a series of blank 40% PEG 400 saline spiked with racemic norgestrel in the concentration range from 0.2 to 25 μg/ml. The calibration curve of D-(+)-norgestrel and D-(−)-norgestrel were linear over the concentration ranges from 0.1 to 12.5 μg/ml by performing a regression linear analysis of the peak area (y) of each enantiomer versus the concentrations (x). The regression equation of the calibration curves were $y = 32.4x + 4.8$ ($r = 0.9997$) for D-(+)-norgestrel and $y = 32.6x + 5.0$ ($r = 0.9998$) for D-(−)-norgestrel. The limit of detection (LOD) was 0.1 μg/ml in receptor medium for racemic norgestrel in this assay, which was measured based on signal/noise (S/N)≥3). The limit of quantitation (LOQ) (S/N≥10) was 0.2 μg/ml for racemic norgestrel, and the relative standard deviation (RSD) was 5.6% ($n = 5$, the concentration measured in five replicates).

3.2.3. Recovery

The standard addition recoveries were carried out by adding a known amount of racemic norgestrel standard to the blank receptor medium (40% PEG 400 saline) at three different levels of 2.5, 5.0 and 10.0 μg/ml. Each level was repeated three times ($n = 3$) and the amounts of each enantiomers were found by the assay methods, as described under Section 2.2 (chromatographic condition). The recovery was calculated by dividing the amount of found by the added, then multiplied by 100%. The average recovery of assay was 93.6–101.6% for D-(+)-norgestrel and 97.6–105.6% for D-(−)-norgestrel, respectively.

3.2.4. Precision

Repeatability and intermediate precision of the HPLC method was studied. The drug-free 40% PEG 400 saline, spiked with racemic norgestrel at concentrations of 2.5, 5.0, 10.0 μg/ml were used for precision studies. The RSD (%) values of repeatability were ≤4.1% for D-(+)-norgestrel, and ≤3.2% for D-(−)-norgestrel, respectively. Two different analysts in two labs on two instruments performed intermediate precision experiments with separated mobile phase according to the assay method. Each sample solution was assayed in triplicate times. The RSD

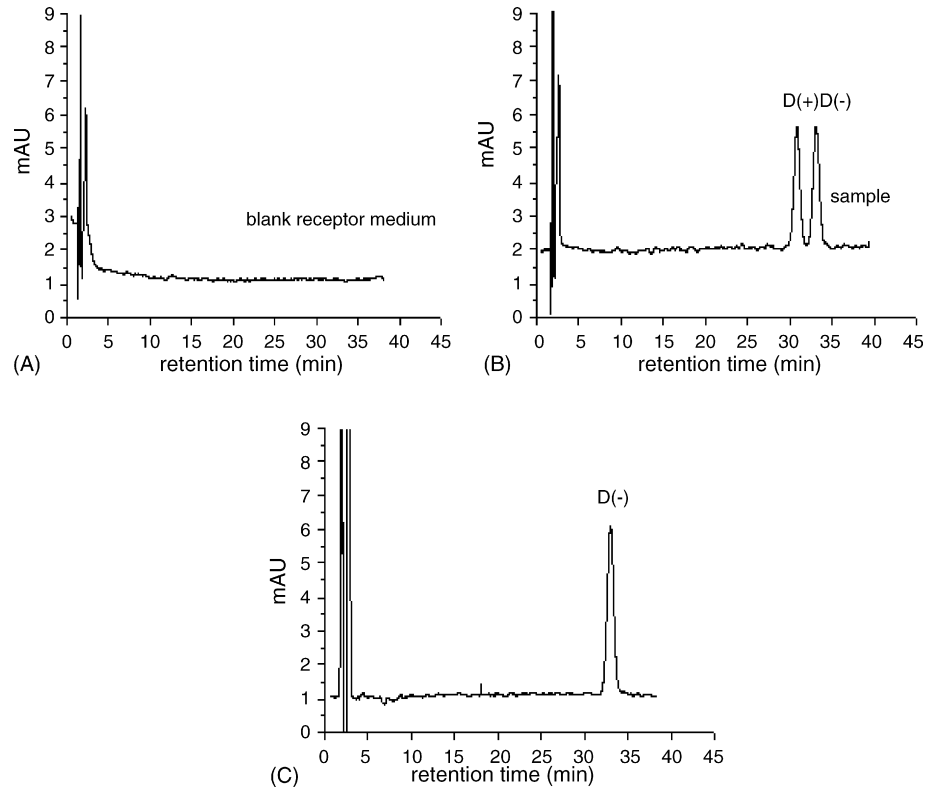

Fig. 6. Chromatograms of: (A) blank receptor medium, (B) blank receptor medium spiked with racemic norgestrel, (C) D-(−)-norgestrel. Mobile phase: 70:30 (v/v) aqueous 20 mM phosphate buffer, pH 5.0 (adjusted with 10% NaOH) with 25 mM HP-beta-CD/acetonitrile; other chromatographic conditions as in Fig. 2.

(%) values of intermediate precision were ≤4.2% for D-(+)-norgestrel and ≤4.8% for D-(−)-norgestrel, respectively. The results in Table 3 showed that the method is precise and accurate.

3.3. Application to pharmacokinetic study

Using the newly developed HPLC method, the permeation rate–time profiles of norgestrel racemate or enantiomers through mouse skins were then examined. The results in Fig. 7 showed that the permeation rate of D-(±)-norgestrel (1.43 ± 0.61 μg/cm^2/h, 48 h) through intact skin were significantly higher than those of D-(−)-norgestrel (0.84 ± 0.51 μg/cm^2/h, 48 h), which was consistent with previously reported results [14]. However, the permeation rate of two enantiomers has no significant difference when they delivered from the donor medium containing D-(±)-norgestrel in ethanol–water solution (50:50, v/v). The differences in permeation rate between D-(±)-norgestrel and levonorgestrel do not have to be related to stereoselective interactions, which may mainly contributed to the discrepancy in melting point (the difference was about 30 °C). The phenomena of differences in permeation rate

Table 3
Repeatability and intermediate precision of this assay in blank receptor medium ($\bar{x} \pm s\%$, $n = 3$)

	Concentration spiked (μg/ml)	Concentration found (μg/ml)		R.S.D (%)	
		D-(+)	D-(−)	D-(+)	D-(−)
Lab 1					
Analyst 1	2.5	1.21 ± 0.05	1.27 ± 0.04	4.1	3.2
	5.0	2.45 ± 0.09	2.5 ± 0.06	3.7	2.4
	10.0	4.91 ± 0.08	4.95 ± 0.08	1.6	1.6
Analyst 2	2.5	1.2 ± 0.04	1.27 ± 0.06	3.3	4.7
	5.0	2.44 ± 0.06	2.51 ± 0.06	2.5	2.4
	10.0	4.89 ± 0.08	4.96 ± 0.07	1.6	1.4
Lab 2					
Analyst 1	2.5	1.22 ± 0.04	1.24 ± 0.06	3.3	4.8
	5.0	2.46 ± 0.10	2.48 ± 0.06	4.1	2.4
	10.0	4.85 ± 0.15	4.85 ± 0.18	3.1	3.7
Analyst 2	2.5	1.18 ± 0.05	1.23 ± 0.04	4.2	3.3
	5.0	2.5 ± 0.07	2.51 ± 0.07	2.8	2.8
	10.0	4.94 ± 0.07	4.91 ± 0.08	1.4	1.6

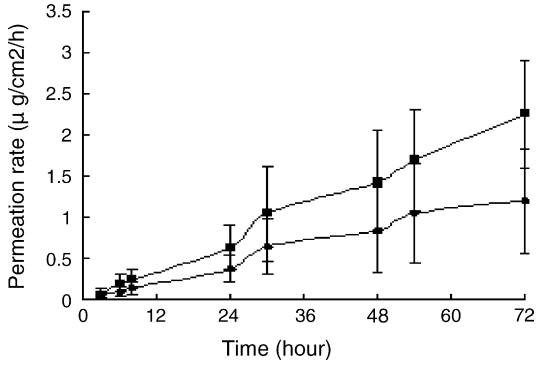

Fig. 7. Permeation rate–time profile of D-(±)-norgestrel and D-(−)-norgestrel through intact mouse skin (■: D-(±)-norgestrel, ♦: D-(−)-norgestrel, $\bar{x} \pm s\%$, $n = 7$).

between racemate and enantiomers had also been reported for other chiral drugs such as ketoprofen [15], ketorolac [16], a new antifungal Sch-39304 [17] and nivaldipine [18].

4. Conclusion

The HPLC method for resolution of the enantiomers of norgestrel was established and the assay of norgestrel in skin permeation fluid was developed and validated. HP-beta-CD has a good solubility in water and organic solvent, which make the chromatographic condition optimization easy, and increases selectivity for norgestrel enantiomers compared to the native beta-CD. This is the first report of enantiomeric resolution of norgestrel with HPLC using HP-beta-CD as chiral mobile phase additive. It was easy to perform, precise and accurate. The whole procedure also may be extended to the applications on quality control of commercial products.

Acknowledgements

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