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Determination of the enantiomer of a cholesterol-lowering drug by cyclodextrin-modified micellar electrokinetic chromatography

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Abstract: The development, optimization and application of a chiral CE (capillary electrophoresis) method for the determination of the enantiomer content of a new cholesterol-lowering drug (BMS-180431-09) is discussed. The chiral CE technique, cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC), was employed with hydroxypropyl β-cyclodextrin as the chiral selector in the run buffer. The detection limit of the unwanted enantiomer was about 0.06% w/w. The effect of various parameters on the separation, validation data and examples of the application of the chiral CE method are included.

Keywords: *Chiral; CZE; cyclodextrin; micellar electrokinetic chromatography; CD-MEKC; MEKC.*

Introduction

The development of an assay to determine the isomeric purity of a new chiral drug is required by the regulatory agencies during the drug development process. In addition, it is desirable that the assay possess satisfactory sensitivity, ruggedness, be cost and time efficient, and environmentally safe. To meet these requirements, a cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) method was developed to determine the enantiomeric purity of BMS-180431-09, a new cholesterol-lowering drug (Fig. 1). The chiral CE method was superior in terms of selectivity, resolution, simplicity, cost and time when compared with the chiral LC assay that was developed to determine the enantiomer content of the new drug. It was also adapted for use as a stereospecific identity test for the dosage form, an assay that is required by the Food and Drug Administration (FDA).

CD-MEKC, a variation of MEKC, was developed by Terabe [1–4]. The use of aqueous solutions containing micelles and cyclodextrins (CDs) as moving phases in capillary electrophoresis (CE) enables the separation of neutral as well as charged compounds and results in a selectivity that can be partially based upon the interaction of the analyte with the micelle as well as with the chiral cyclodextrin [1–4]. As described by recent review articles, there has been rapid development of the utility of cyclodextrins as chiral selectors in the field of chiral CE [5–7]. Cyclodextrins are cyclic oligosaccharides that can form stereoselective inclusion complexes with analytes [8, 9]. When used as a pseudophase in CE, high resolution can be obtained and difficult separations can be performed, particularly of enantiomers, and of other compounds of similar structure. Other types of additives such as proteins, enzymes, crown ethers, macrocyclic antibiotics, oligosaccharides and chiral surfactants are also used in CE as chiral discriminators [5, 10–12].

The reported method demonstrates that CD-MEKC can be successfully applied to the resolution and quantitation of the opposite enantiomer of a new cholesterol-lowering drug, BMS-180431-09 (Fig. 1). The effect of various parameters on the separation observed during method development as well as the validation data for the optimized method for the quantitation of the enantiomer are presented. The method also resolved an additional isomer, the 3R, 5R, 6E diastereoisomer (*cis*-diastereoisomer), and has the

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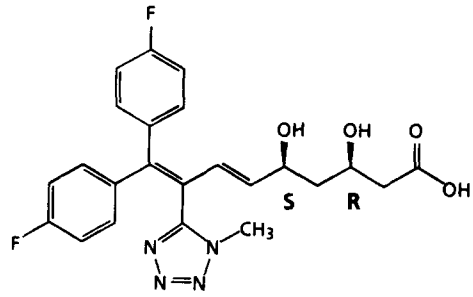
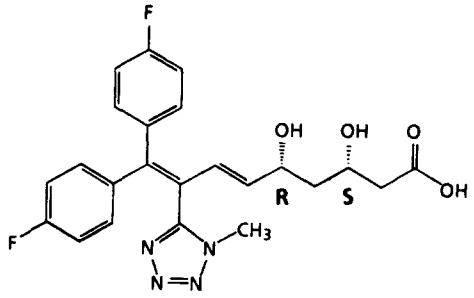
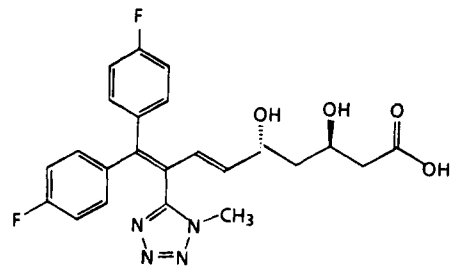


BMS-180431-09



Enantiomer



Cis-diastereoisomer

Figure 1
Structures of BMS-180431-09 and two stereoisomers of BMS-180431-09.

potential to quantify this possible impurity and, thus, reduce the number of assays currently used to determine the two stereoisomers of BMS-180431-09. Due to time constraints, method validation was not completed for the quantitation of the *cis*-diastereoisomer using the presented CD-MEKC assay. The other possible isomers were not available for investigation.

Experimental

Chemicals

Sodium dodecylsulphate (SDS), 99.0%, electrophoresis grade, was purchased from Mallinckrodt (Mallinckrodt Inc., Paris, KY, USA). Hydroxypropyl β-cyclodextrin, β-cyclodextrin and γ-cyclodextrin were obtained from Astec (Applied Separation Technologies, Astec, Whippany, NJ, USA) and heptakis (2,6-di-*O*-methyl)-β-cyclodextrin and tri-acetyl β-cyclodextrin from the Sigma Chemical Co. (St Louis, MO, USA). The sodium borate (ACS grade) was from Fisher Scientific (Pittsburgh, PA, USA). BMS-180431-09, its opposite enantiomer and the 3R, 5R, 6E diastereoisomer (*cis*-diastereoisomer) were obtained from the Bristol–Myers Squibb Pharmaceutical Research Institute. The chemical name for BMS-180431-09 is (3R, 5S, 6E)-9,9-bis(4-fluorophenyl)-3,5-dihydroxy-8-(1-methyl-1H-tetrazol-5-yl)-6,8-nonadienoic acid, L-arginine (1:1) salt. Distilled, deionized water was used throughout.

Apparatus

LC columns (Chiral AGP and Cyclobond I DMP) were obtained from Astec (Applied Separation Technologies, Astec, Whippany, NJ, USA). An HT-270A capillary electrophoresis system from Perkin–Elmer Applied Biosystems (PE-ABI, Perkin–Elmer Applied Biosystems, Foster City, CA, USA) was used. Much of the work was done using a 72-cm (effective length of 50 cm from the sample to the detector), 50 μm i.d. silica capillary from PE-ABI. The detection model was UV absorption at 200 nm. The applied voltage across the designated capillary was 15–20 kV and the capillary temperature was kept at 35°C. Integration was performed by a VG Multichrom Laboratory Automation System. Samples and run buffers were filtered through a 0.45-μm glass microfiber filter prior to use. New capillaries were conditioned by rinsing for 20 min each with 1 N sodium hydroxide, 0.1 N sodium hydroxide and run buffer. The CE instrument was programmed to rinse with run buffer for 3 min and to introduce the sample by vacuum for 3 s prior to each run. The run buffer was automatically replenished after about three to five sample injections to prevent ion depletion, and to maintain satisfactory reproducibility of response and migration time.

Results and Discussion

Chiral LC vs chiral CE

Initially, an α_1-acid glycoprotein LC column (chiral AGP) method was developed to resolve the opposite enantiomer of BMS-180431-09. Partial resolution with typical resolution

factors of 1.3–1.5 were obtained when using a mobile phase containing acetonitrile/phosphate buffer. The enantiomer eluted near or on the tail of the main peak (Fig. 2). A separate, coupled column method employing two 3,5-dimethylphenyl carbamate β-cyclodextrin columns (Cyclobond I DMP) was required to quantitate the *cis*-diastereoisomer (Fig. 3). The standard achiral columns were found not to be capable of resolving this diastereoisomer and a single Cyclobond I DMP column did not provide satisfactory resolution. The reported chiral CD-MEKC method was judged as a preferred substitute to the two LC assays primarily because it was found to baseline-resolve the *cis*-diastereoisomer as well as the enantiomer of BMS-180431-09 in a single run. In addition, the enantiomer eluted first with a resolution factor that was greater than 2.0 (Fig. 4). The sensitivity (detection limit of <0.1% w/w) of both LC and CE methods was equivalent for the quantitation of the enantiomer and *cis*-diastereoisomer when the sample concentration was increased from 0.1 to 0.3 mg ml^{-1} for the CE method. An electropherogram of BMS-180431-09 with added enantiomer and *cis*-diastereoisomer at a concentration of 0.1% w/w is shown in Fig. 5. Relative to the LC method, the CD-MEKC method had a shorter daily equilibration time for conditioned capil-

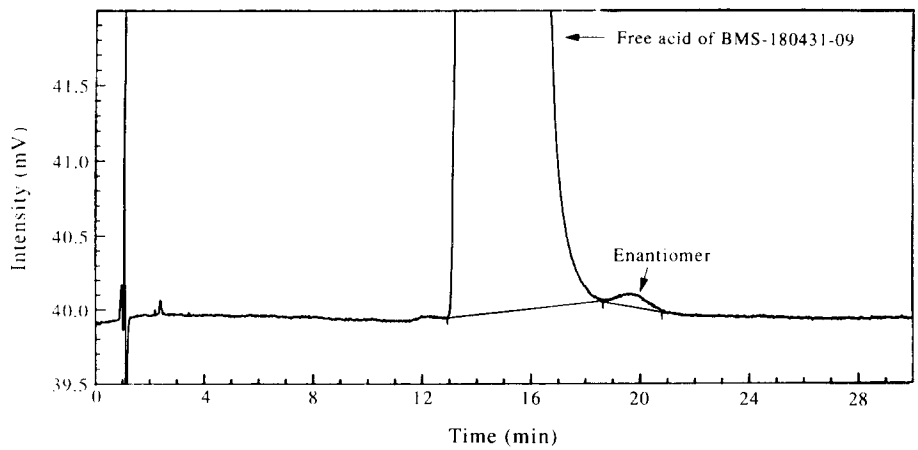


Figure 2
LC of BMS-180431-09 and added opposite enantiomer (0.1% w/w); conditions: chiral AGP column, 96% 10 mM potassium phosphate buffer (pH 4.0), 4% acetonitrile, 40°C column temperature, 1.0 ml min^{-1}, UV absorption detection at 296 nm, sample concentration was 0.1 mg ml^{-1} BMS-180431-09 with 0.1% w/w enantiomer added.

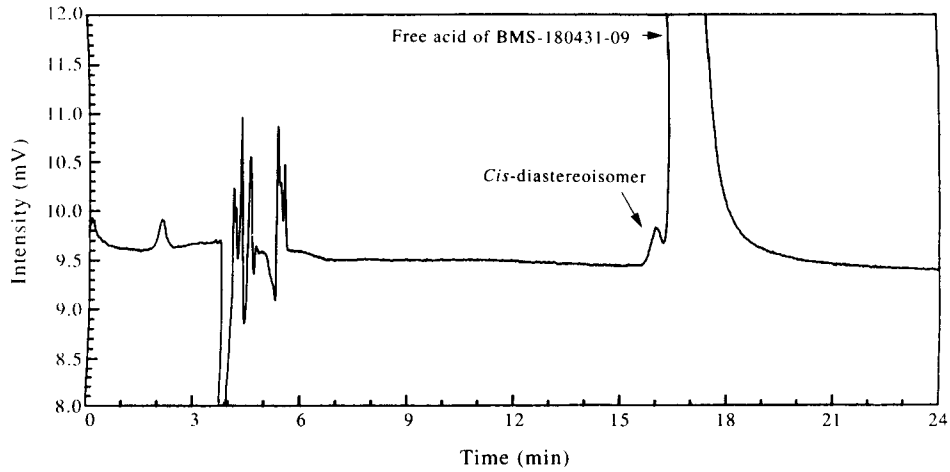


Figure 3
LC of BMS-180431-09 and added *cis*-diastereoisomer (0.1% w/w); conditions: cyclobond I DMP column, 99.95% methanol, 0.05% acetic acid, 1.0 ml min^{-1}, UV absorption detection at 296 nm, sample concentration was 0.1 mg ml^{-1} BMS-180431-09 with 0.1% w/w *cis*-diastereoisomer added.

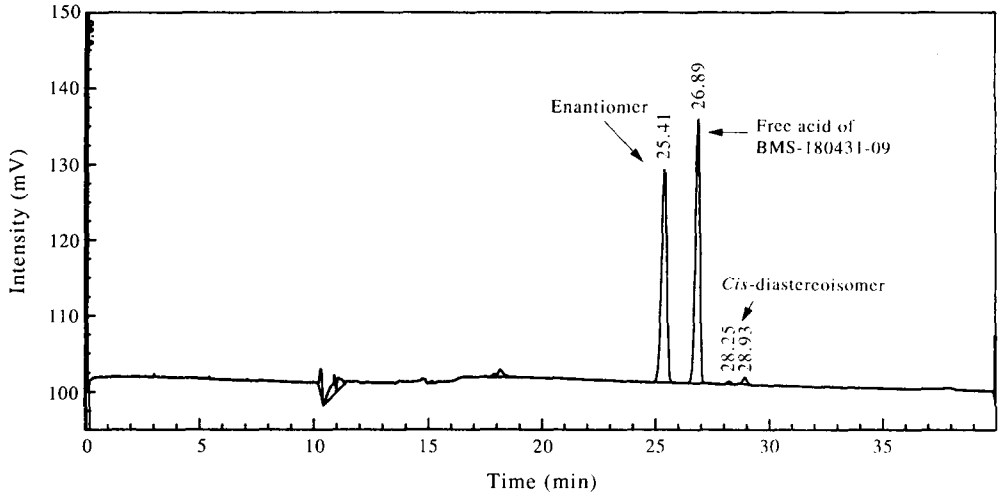


Figure 4
CD-MEKC of a racemic mixture of BMS-180431-09 with some trace *cis*-diastereoisomer added; conditions: 0.01 M (1.5 g/100 ml) hydroxypropyl β-cyclodextrin, 0.1 M sodium borate, 0.03 M sodium dodecylsulphate, pH 9.3, 20 kV, current ~65 μA, 50-cm effective capillary length, 50-μm capillary width, UV absorption detection at 200 nm, sample was 0.3 mg ml^{-1} in water.

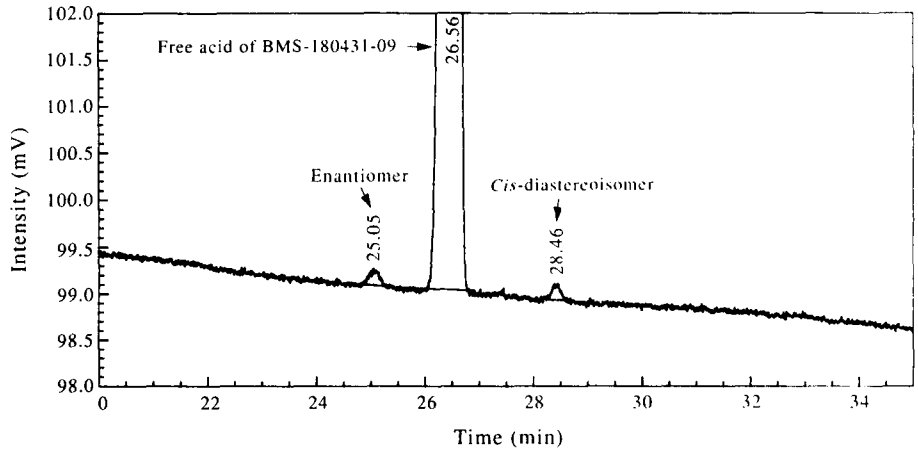


Figure 5
CD-MEKC of BMS-180431-09 with 0.1% w/w enantiomer and *cis*-diastereoisomer added; conditions are the same as listed in Fig. 4.

laries (5 min for a capillary compared to 1 h for the typical LC column). In addition, there was no loss in resolution with time, as occurs with aging LC columns, and capillaries cost less than chiral LC columns.

Development of the CDMEKC separation of the enantiomer: variation of system parameters

The first partial resolution of the racemic mixture of BMS-180431-09 by CD-MEKC was obtained by adding underivatized β-cyclodextrin to a borate/SDS run buffer. Since the resultant resolution factor for the enantiomer was 1.05 under the initial conditions (conditions described in Table 1), method development proceeded with the objective of optimizing the resolution. Other types of cyclodextrins were substituted for underivatized β-

Table 1
Effect of voltage on resolution (R_s) and migration

Voltage (kV)	Migration time enantiomer (min)	Migration time BMS-180431-09 (min)	R_s
10	25.64	26.03	1.05
15	15.77	15.97	1.01
20	10.48	10.58	1.01

*Conditions: 2.3 g β-cyclodextrin/100 ml, 0.02 M sodium dodecyl sulphate, 0.05 M sodium borate, pH 9.3, 72-cm silica capillary.

cyclodextrin. Under the same conditions, resolution was not observed with γ-cyclodextrin, tri-acetyl β-cyclodextrin, or heptakis (2,6-di-*O*-methyl)-β-cyclodextrin. Similar resolution was obtained when hydroxypropyl-β-cyclodextrin was used. Most of the development work was done using hydroxypropyl-β-cyclodextrin as the chiral selector because it is several times more water-soluble than underivatized β-cyclodextrin [8].

The effect of applied voltage on resolution was investigated when β-cyclodextrin was used as the chiral selector and is shown in Table 1. A decrease in resolution was observed upon increasing the voltage. Since an increase in voltage increases the rate of electroosmotic flow and migration times become shorter, the analytes may spend less time interacting with the chiral moving phase which would result in reduced chiral resolution. However, thermal effects due to Joule heating at higher applied voltages can also alter resolution. The effect of capillary length on the resolution factor is shown in Table 2. As expected, a longer capillary results in greater resolution and longer migration times. Thus, varying the applied voltage and capillary length produced minor changes and did not result in the desired baseline-resolution.

A significant effect on resolution of the enantiomer was obtained by adding SDS (Table 3). As the SDS concentration was increased from 0.02 to 0.04 M, the resolution factor increased from 1.2 to 2.9. There was also a significant increase in migration times with an increase in the SDS concentration. SDS monomers can have their hydrophobic tails co-included in the cyclodextrin cavity along with the analyte. This could change the nature of the analyte/CD interaction (its binding constant and stereoselective fit) and, consequently, the resolution. It is also likely that the increased fraction of the analyte partitioning into the SDS micellar phase at a higher surfactant concentration delays the migration of the analyte as well as affects the dynamics of the resulting enantiomer/CD complexation equilibria such that resolution is improved. When SDS was not used, the migration times were shorter and baseline resolution was also obtained (Table 3). In this case, the separation mechanism was solely based upon electrophoresis and the interaction of the analyte with the CD. Although SDS was not required to attain chiral resolution, its effects were used to develop the separation.

A large effect on resolution was obtained by increasing the borate concentration (Table 4). An increase in ionic strength increases the viscosity of the run buffer which slows the electroosmotic flow and increases migration times. Due to the extreme effect on resolution, it is suspected that there is an additional mechanism. Borate anion may complex with the vicinal hydroxyl groups of the analyte and play a critical role in the stereoselective interaction. It is well known that borate anions readily form a tetrahedral complex under alkaline conditions with compounds that have *cis*-hydroxyl groups [13, 14]. The analyte/borate complex would have a greater net

Table 2
Effect of the capillary length on resolution (R_s) and migration*

Length (cm)	Migration time enantiomer (min)	Migration time BMS-180431-09 (min)	R_s
72	14.36	14.43	1.04
122	34.07	34.34	1.13

*Conditions: 2.3 g hydroxypropyl β-cyclodextrin/100 ml, 0.02 M sodium dodecyl sulphate, 0.05 M sodium borate, pH 9.3, 20 kV.

Table 3
Effect of sodium dodecyl sulphate (SDS) concentration on resolution (R_s) and migration*

SDS (M)	Migration time enantiomer (min)	Migration time BMS-180431-09 (min)	R_s
0.00	31.9	32.6	3.0
0.02	34.1	34.4	1.2
0.03	38.0	38.6	2.3
0.04	44.7	45.6	2.9

*Conditions: 2.3 g hydroxypropyl β-cyclodextrin/100 ml, 0.05 M sodium borate, pH 9.3, 20 kV, SDS varied, 122-cm silica capillary.

Table 4
Effect of sodium borate concentration on resolution (R_s) and migration*

Sodium borate (M)	Migration time enantiomer (min)	Migration time BMS-180431-09 (min)	R_s
0.01	20.2	20.2	0
0.05	34.1	34.4	1.2
0.10	52.5	54.0	3.5
0.15	73.9	77.6	6.6

*Conditions: 2.3 g hydroxypropyl β-cyclodextrin/100 ml, sodium borate varied, pH 9.3, 20 kV, 0.02 M sodium dodecyl sulphate, 122-cm silica capillary.

negative charge (−2) and its motion would be retarded due to the increased electrophoretic attraction to the anode. When borate buffer was replaced by 0.05 M sodium phosphate at pH 9.5 and when the borate buffer concentration was low (0.01 M), chiral resolution was not observed. Thus, borate buffer appears essential for chiral resolution. Since cyclodextrins are composed of glucose units containing hydroxyl groups at asymmetric carbons, it is possible that a ternary complex between analyte, CD and borate is formed. Somewhat analogously, an LC column containing a chiral, borate-complexed polysaccharide polymer as stationary phase was shown to resolve the enantiomers of mandelic acid presumedly by the formation of a ternary complex and according to the principles of chiral ligand exchange chromatography [13].

The effect of hydroxypropyl β-cyclodextrin concentration on resolution and migration was also significant (Table 5). As the concentration of hydroxypropyl β-cyclodextrin increased, the migration times of the enantiomer and BMS-180431-09 decreased (Table 5). This is due to the increased fraction of analyte complexed by the neutral cyclodextrin which migrates faster than the analyte-containing, anionic micelle. Resolution increased with the concentration of hydroxypropyl β-cyclodextrin up to 1.5 g/100 ml (Table 5). At concentrations higher than this, resolution decreased. An optimum chiral selector concentration can be predicted mathematically and varies according to the affinity of each enantiomer for the chiral selector [15]. According to theory, at the optimum concentration of chiral selector, the apparent mobility difference of the two enantiomers (which is a function of the dynamic exchange of bound and unbound enantiomer) is maximized [15]. Without cyclodextrin, there was no chiral discrimination of the opposite enantiomer of BMS-180431-09. However, it was observed that the *cis*-diastereoisomer was resolved using ordinary MEKC with SDS and borate in the run buffer. The elution order of the *cis*-diastereoisomer and BMS-180431-09 were reversed in the MEKC system relative to the CD-MEKC system. The *cis*-diastereosiomer was not resolved by free zone CE using only a borate run buffer. To account for the stereoselectivity shown by the SDS micellar run buffer, it is apparent that these two diastereoisomers have different binding constants with SDS micelles.

The assay was optimized by selecting the run buffer that contained the smallest concentration of UV-absorbing components (least amount of SDS and hydroxypropyl β-cyclodextrin) to minimize the background noise and to optimize sensitivity. The capillary length was shortened to produce the fastest run times and the sharpest peaks for maximum sensitivity while maintaining baseline resolution. The optimized conditions are described in Fig. 4.

The sample diluent (100% water) was selected to maximize the peak response. Since the ionic strength of the sample diluent is less than that of the run buffer, the increased sharpness and height of the peaks due to "sample stacking" was utilized to enhance the sensitivity [16].

Assay validation

The response of BMS-180431-09 was linear at 200 nm in the concentration range from 0.15 to 0.40 mg ml^{-1}. A correlation coefficient of 0.9985 was obtained. A concentration of 0.3 mg ml^{-1} was selected as the sample concentration. The linearity of response of the enantiomer in the presence of BMS-180431-09 (at 0.3 mg ml^{-1}) was satisfactory with a correlation coefficient of 0.9998 when enantiomer was added to BMS-180431-09 (0.3 mg ml^{-1})

Table 5
Effect of varying the hydroxypropyl β-cyclodextrin concentration on resolution (R_s) and migration*

Hydroxypropyl β-cyclodextrin concentration (g/100 ml)	Migration time enantiomer (min)	Migration time BMS-180431-09 (min)	R_s
0.5	40.5	41.6	1.5
1.0	34.6	36.0	2.4
1.5	31.1	32.7	3.5
2.0	24.8	25.9	3.1

*Conditions: 0.10 M sodium borate, pH 9.3, 20 kV, 0.03 M sodium dodecyl sulphate, capillary length 72 cm, 20 kV.

Table 6
Percentage recovery of added enantiomer to BMS-180431-09

Percentage added enantiomer (w/w)	Area response of enantiomer	Area response of BMS-180431-09*	Percentage enantiomer found†	Percentage recovered‡
0.36	3714	982130	0.38	105.5
0.36	3435	999748	0.34	94.4
0.36	3876	993457	0.39	108.3
0.65	7283	1032236	0.71	109.2
0.65	6591	1028448	0.64	98.5
0.65	6400	1043272	0.61	93.8
0.80	9569	1197471	0.80	100.0
0.80	9315	1061963	0.88	110.0
0.80	9333	1118541	0.83	103.8
1.10	11417	1007060	1.13	102.7
1.10	11110	1014371	1.10	100.0
1.10	10956	997888	1.10	100.0

* BMS-180431-09 concentration = 0.3 mg ml^{-1}.
† Percentage enantiomer found = (area response of enantiomer ÷ area response of BMS-180431-09) × 100.
‡ Percentage recovered = (percentage enantiomer found ÷ percentage added enantiomer) × 100.

from 0.12% (w/w) to 1.1% (w/w). The detection limit (DL) was estimated to be 0.06% (w/w) by a statistical linearity method (DL = (3 × SD)/Slope). Recovery studies were performed by adding various amounts of enantiomer to 0.3 mg ml^{-1} aqueous solutions of BMS-180431-09. The results are shown in Table 6. The recovered amounts of added enantiomer ranged from 93.8 to 110% of theoretical which is satisfactory for the small amounts being quantitated and indicating that corrections for response differences due to the mobility differences of the two enantiomers were not required. Significant differences in the molar absorptivity of the two diastereisomeric inclusion complexes were also ruled out by the accuracy of the recovery studies. There was no evidence of enantiomer in any of the synthesized batches. Reproducibility of the main peak response was satisfactory. RSDs of between 1 and 2% were typical for repeated injections. The reproducibility was found to be improved by automatically changing the run buffer on the sample side more frequently (to as little as three injections per run buffer vial to obtain the lowest RSD).

Applications

Since the LC assays were developed and validated first, they were filed in the Investigational New Drug (IND) application and were used to analyse bulk and formulation samples for the stability studies. The chiral CE method was then used as a secondary, supportive assay. In particular, it was used at a time point in the stability study of the formulation to distinguish interfering degradant peaks from the presence of the enantiomer in some stressed samples. Various bulk batches of BMS-180431-09 were analysed to obtain supportive data, and there was good agreement between the LC and CE results. The separation was also found to be useful as a simple, stereospecific identity test since all available isomers were resolved using a single method. The migration times of a standard mixture of the isomers were compared to the migration time of BMS-180431-09 in the dosage form.

Conclusions

A CD-MEKC method utilizing hydroxypropyl β-cyclodextrin as the chiral selector for determining the enantiomer of BMS-180431-09, a new cholesterol-lowering drug, was developed. Resolution was affected most by borate buffer, hydroxypropyl β-cyclodextrin and sodium dodecylsulphate concentration. The method was judged preferable to the LC methods developed because of the decreased toxicity and smaller quantity of the solvents used, lower operational costs, simplicity, superior chiral resolution and decreased analysis time since two isomers can potentially be analysed in a single run. Validation of the

chiral CD-MEKC method showed it to be rugged and accurate. Unlike LC methods, there is no column-to-column variability and the capillary lasts indefinitely, provided it is rinsed with 0.1 N sodium hydroxide and is kept moist after use to prevent the precipitation of buffer components.

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