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Prediction on the Chiral Behaviors of Drugs with Amine Moiety on the Chiral Cellobiohydrolase Stationary Phase Using a Partial Least Square Method

Sun Ok Choi, Seok Ho Lee, and Hea-Young Park Choo[1]

Division of Biopharmaceutics, Department of Pharmacology, National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul 122-704, Korea and [1]School of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

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Quantitative Structure-Resolution Relationship (QSRR) using the Comparative Molecular Field Analysis (CoMFA) software was applied to predict the chromatographic behaviors of chiral drugs with an amine moiety on the chiral cellobiohydrolase (CBH) columns. As a result of the Quantitative CoMFA-Resolution Relationship study, using the partial least square method, prediction of the behavior of drugs with amine moiety upon chiral separation became possible from their three dimensional molecular structures. When a mixed mobile phase of 10 mM aqueous phosphate buffer (pH 7.0) - isopropanol (95 : 5) was employed, the best Quantitative CoMFA-Resolution Relationship, derived from the study, provided a cross-validated $q^2 = 0.933$, a normal $r^2 = 0.995$, while the best Quantitative CoMFA-Separation Factor Relationship, also derived from the study, yielded a cross-validated $q^2 = 0.939$, a normal $r^2 = 0.991$. When all of these results are considered, this QSRR-CoMFA analysis appears to be a very useful tool for the preliminary prediction on the chromatographic behaviors of drugs with an amine moiety inside chiral CBH columns.

Key words: QSRR, CoMFA, Resolution, Separation factor, Chiral separation

INTRODUCTION

Enantioseparation of chiral drugs can sometimes be laborious and time-consuming. Getting proper chiral stationary phases are often difficult. Therefore, with all of these practical aspects in mind, it is quite natural for us to look for an alternative way that could lead us to predict the chromatographic behaviors of chiral drugs before they are actually subjected to chiral separation. Were the mechanism of chiral separation on chiral stationary phase (CSP) known and the prediction of the chiral behaviors of drugs possible from its 3-dimensional molecular structure, it would be a very useful tool in evaluating chiral behaviors of drugs.

It has been well documented that, in general, single enantiomers of β-blockers contain at least one chiral center and an amine functional group, and most of them are marketed as racemic mixtures. Also, single enantiomers of β-blockers, as well as several other drugs, differ largely from their enantiomeric counterparts in terms of pharmacodynamic and pharmacokinetic profiles. Propranolol, one of the most prominent and intensively investigated β-blockers, is an excellent example for demonstrating such differences in pharmacodynamic and pharmacokinetic profiles between (*R*)- and (*S*)-enantiomers. (*S*)-propranolol is over 100 times more potent in its ability to block β-receptors than the corresponding (*R*)-enantiomer (Pharm-Huy *et al.*, 1995). Recently, a comparative pharmacodynamic study on (*R*, *S*)-atenolol demonstrated that a halved dose of (*S*)-enantiomer decreased heart rate and blood pressure to the same extent as the racemic mixture of the drug (Torgny *et al.*, 1997). Because of the differences in pharmacodynamic and pharmacokinetic profiles between enantiomers, many impressive results have been published about the chiral separation of β-blockers (Hanssan *et al.*, 1997; Lanchote *et al.*, 2000; Sallustio *et al.*, 1992; Balmer *et al.*, 1991). For example, studies with chiral separations

Correspondence to: Hea-Young Park Choo, School of Pharmacy, Ewha Womans University, 11-1 Daehyundong, Seodaemun-Ku, Seoul 120-750, Korea
Tel: 82-2-3277-3042, Fax: 82-2-3277-3051
E-mail: hypark@mm.ewha.ac.kr

of atenolol and metoprolol using cellobiohydrolase (CBH) columns (Hanssan *et al.*, 1997; Lanchote *et al.*, 2000) claim that CBH gives very large separation factors for almost all β-receptor antagonists (a group of amino alcohols), and therefore, appears to be the best choice for their separation. However, no further studies on the chiral separation of the above mentioned drugs with an amine moiety using CBH columns have been reported so far to allow us to generalize the principle and to apply it into practice. In this paper, eleven of the most popular β-blockers and other drugs that share some resemblance in their structures having an amine moiety were chosen as model drugs (Fig. 1), and a direct and simple high-performance liquid chromatographic (HPLC) method was described for the enantiomeric resolution of racemic drugs using the CBH chiral stationary phase.

The prediction of chiral behaviors of racemic drugs using a combination of experimental designs and artificial neural networks was attempted (Dohnal *et al.*, 1999). The search for conditions to yield optimal chiral separations, however, was also aided through the discovery of knowledge of the physicochemical constants such as analyte-chiral selector equilibrium constants, pK values, etc. Naturally, an effort to find optimal conditions should be facilitated if a suitable computer program exists. Another alternative is the experimental approach. To prepare experimental sets, a suitable experimental design is used, and the data is evaluated using some chemometric analyses like the partial least-squares (PLS) analysis or artificial neural networks.

The first fields of PLS, the quality control of pharmaceutical and foods (Blanco *et al.*, 1996; Jong *et al.*, 1991), ICP (Havel *et al.*, 1996), kinetics (Blanco *et al.*, 1996), etc. are characterized by their production of multivariable data and their lack of physical models. PLS is a multivariate regression analysis that provides an overview of large data sets. But there have been no known studies on the prediction of chromatographic resolution or separation factor of the drugs containing an amine moiety using the CoMFA software. In order to predict the chiral separation behaviors in a chiral CBH column of several drugs containing an amine moiety, the CoMFA software was used. This study includes the Quantitative CoMFA-Resolution (Separation Factor) Relationships analyses, using the partial least square method. Through this study, a prediction of the extent of the chiral separations of drugs with an amine moiety was attempted by evaluating their three dimensional molecular structure.

MATERIALS AND METHODS

Materials and reagents

Racemic atenolol was a gift from Il-Dong Pharmaceutical (Seoul, Korea), albuterol was purchased from Sigma (St. Louis, Missouri, USA), betaxolol was bought from Bu-Kwang (Seoul, Korea), dobutamine was obtained from Eli Lilly (Seoul, Korea), fenfluramine was bought from Sigma, metoprolol was purchased from Astra, Pindolol was a gift from Sandos Pharmaceutical, Sotalol was a gift from A-Zu Pharmaceutical (Seoul, Korea), terbutaline was a gift from Yuhan (Seoul, Korea), and norepinephrine and epinephrine were purchased from Sigma. All solvents were of HPLC grade from E. Merck (Darmstadt, Germany), and all other chemicals wer of Analytical-Reagent grade.

HPLC conditions

The HPLC system was employed, which was equipped with Shimadzu SIL-10Advp pumps (Kyoto, Japan), a SPD-M10Avp diode array detector, a DGV-14A Degasser, and a CTO-10Avp column oven. Injections were performed using a SIL-10Advp autoinjector. Chromatograms were recorded and processed with a Class-VP software (Shimatzu, Kyoto, Japan).

A CBH column (15 cm×4.0 mm I.D.; Regis, Morton Grove, IL, U.S.A.) with a guard column (1 cm×3.0 mm I.D.) containing cellobiohyolrolase was used in this study.

Optimization of mobile phases in analytical reversed phase HPLC

Reversed phase HPLC was carried out on a CBH column (15 cm×4.0 mm I.D.) at a flow rate of 0.9 mL/min at ambient temperature. Racemic atenolol, albuterol, betaxolol, dobutamine, fenfluramine, pindolol, sotalol, terbutaline, metoprolol, norepinephrine, and epinephrine solutions (100 μg/mL in the mobile phase used) were chromatographed (10 μL injection) in the system. From the obtained chromatograms, the chromatographic parameters, such as the separation factor (α) and the resolution factor (Rs), were calculated using the standard procedure.

Peak assignments

The chromatographic peaks corresponding to (+)- and (-)-enantiomers of the racemic drugs were monitored by using the photodiode array detector (Shimadzu, Kyoto, Japan). Then, the peaks were identified by the use of circular dichroism (CD) (Jobin-Yvon CD 6, U.S.A.) and polarimetry (Perkin Elmer 243 polarimeter, Germany).

Quantitative structure/resolution (separation factor) relationships computational methods

All molecular modeling and statistical analyses were performed using SYBYL 6.5 molecular modeling software from Tripos Inc. (Saintlouis, Missouri, USA) on Silicon Graphics Origin 300 (IRIX 6. 5).

The 2D structure of each compound was built using the SYBYL Build program with the default SYBYL settings.

The 2D structure was converted to a 3D structure using Concord 4.0. The structural energy minimization was performed using the SYBYL energy minimizer (Tripos Force Field) and Gasteiger-Huckel charge, with a 0.005 kcal/M energy gradient convergence criterion. Low energy conformation was searched by geometry optimization after rotating the bond between aromatic ring and chiral center every 30° of single bond from 1° to 330° of tortional angle. All of the structures generated were aligned into a lattice box by fitting with benzene as a common structure.

Calculation of CoMFA descriptors

Conventional CoMFA was performed with the Quantitative Structure Activity Relationship (QSAR) option of SYBYL. The steric and electrostatic field energies were calculated using sp^3 carbon probe atoms with +1 charge. Maximum energy cutoff for steric and electrostatic energies was 30 kcal/M. The CoMFA grid spacing was 2.0Å in all three dimensions within the defined region. The partial least squares (PLS) method was used for fitting the 3D structural feature descriptors and their biological activities. The optimum number of components in the final PLS model was determined by the q^2 value, which was obtained from the leave-one-out cross validation technique.

Molecular alignment

Using albuterol as the template molecule, superposition of test compounds was performed with the common benzene existing in all compounds.

RESULTS AND DISCUSSION

Mobile phase parameters, influencing on the enantioselective retention

CBH is one of the cellulose-degrading enzymes (cellulases) produced extracellularly by the mold *Trichoderma reesei* and is an acid glycoprotein that has an isoelectric point 3.6. The structure of CBH contains three regions: a catalytically active core, an inter-connecting region, and a cellulose-binding domain consisting of 36 amino acids that form two disulfide-bridged loops (Heriksson *et al.*, 1996). The major chiral separation mechanisms of the CBH column have been explained by hydrophobic and electrostatic interactions between CBH and the chiral molecule. The dominating chiral binding site has been known to be located within the core of CBH, and the other enantioselective site was found in the cellulose binding domain (Fornstedt *et al.*, 1996). On the other hand, the chiral separation mechanism has more complexed effects

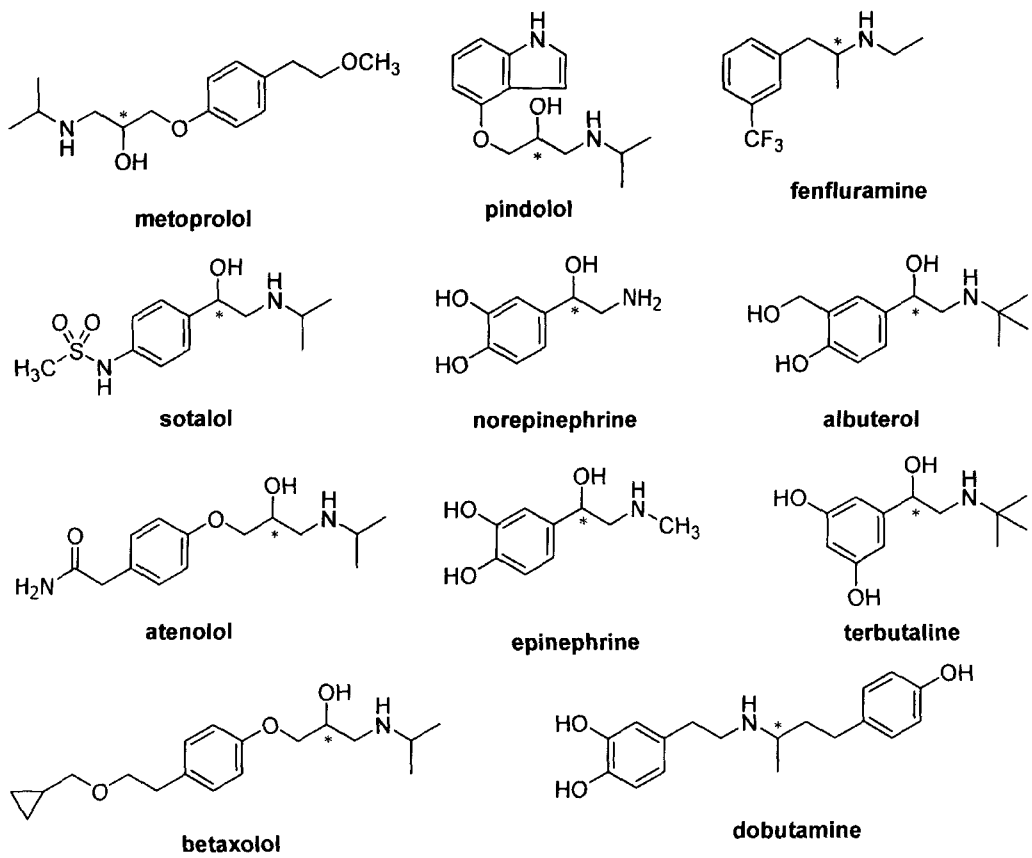

Fig. 1. Chemical structures of chiral drugs with amine moieties

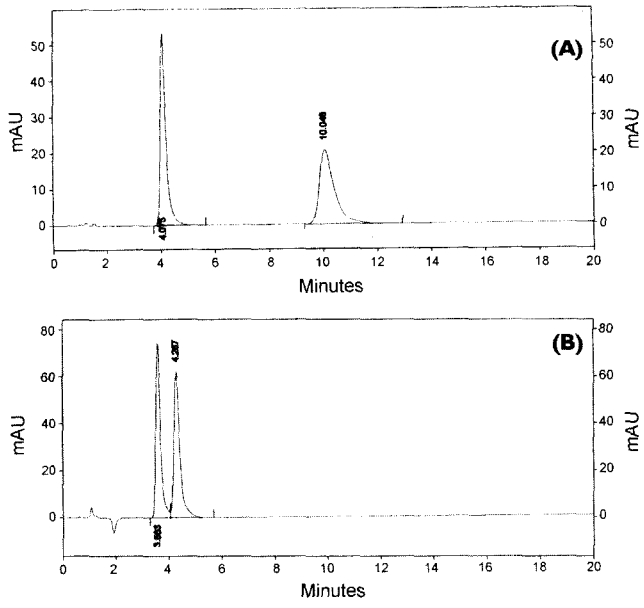

Fig. 2. Representative chromatograms of drugs with amine moieties on chiral CBH column chromatography. Mobile phase: 10 mM phosphate buffer (pH 7.0)-isopropanol (95 : 5) (A) Metoprolol and (B) Atenolol.

rather than being a simple mechanism, and therefore, it is very difficult to predict the chiral behavior in the chiral column before actually performing the experimental work.

In this paper, chiral separation was carried out on CBH chiral stationary phases for eleven drugs containing an amine moiety (Fig. 1) in order to figure out the mechanism of enantioseparation and to predict the degree of chiral separation from the differences in their three dimensional molecular structures. Fig. 2 shows representative chromatograms that were obtained from some of these drugs in the mobile phase, made of 10 mM phosphate buffer (pH 7.0) : isopropanol with a (v/v) ratio of 95:5. The influence of several mobile phase parameters on the enantioselective retention was evaluated; e. g., types of organic modifier (2-propanol, acetonitrile), concentrations of organic modifier, mobile phase buffer pH, and column temperature. The following parameters on the mobile phase were varied to optimize conditions that were chosen for the chiral-CBH column employed in the study : 3-5% (v/v) of 2-propanol or acetonitrile, 2-propanol or acetonitrile as organic modifiers, pH 5.0-7.0 for the mobile phase, and the column temperature of 25-40°C.

The presence of organic modifiers in the mobile phase was studied to see if there were any appreciable effects on the chromatographic parameters of the enantiomeric separation of racemic drugs. The results are summarized in Table I.

Changing the organic solvent from isopropanol to acetonitrile gave higher values of both α and Rs for drugs with an amine moiety in the mobile phase of 10 mM phosphate buffer (pH 7.0) : organic modifier (95:5, v/v).

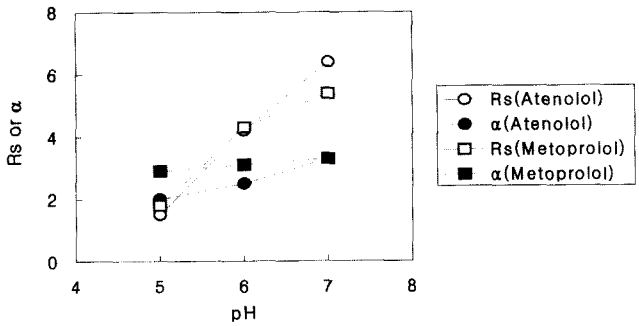

Fig. 3. Effect of the pH of molile phase on resolution factor (Rs) and separation factor (α); Mobile phase : 10 mM phosphate buffer (pH 7.0)-isopropanol (95 : 5)

Table I. Effect of organic modifiers on the resolution factor (Rs) and separation factor(α) on chiral CBH columns

Solute	A		B		C		D	
	Rs	α	Rs	α	Rs	α	Rs	α
Albuterol	1.40	1.44	1.60	1.49	1.33	1.40	1.33	1.37
Atenolol	6.40	3.30	6.80	3.50	6.4	2.79	6.17	2.77
Betaxolol	0	1.00	0	1.00	0	1.00	0	1.00
Dobutamine	1.67	1.65	1.72	1.75	1.45	1.33	1.57	1.71
Epinephrine	0	1.00	0	1.00	0	1.00	0	1.00
Fenfluramine	1.56	1.45	2.0	1.52	1.18	1.26	1.15	1.26
Metoprolol	5.39	3.30	8.51	3.85	8.29	3.65	7.38	2.79
Norepinephrine	0	1.00	0	1.00	0	1.00	0	1.00
Pindolol	0	1.00	0	1.00	0	1.00	0	1.00
Sotalol	1.45	1.33	1.71	1.39	1.33	1.34	1.33	1.30
Terbutaline	1.80	1.50	1.87	1.68	1.54	1.64	1.78	1.56

Column temperature: 40°; Mobile phase A: 10 mM phosphate buffer (pH 7.0)-isopropanol (95:5), B: 10 mM phosphate buffer (pH 7.0)-isopropanol (97:3), C: 10 mM phosphate buffer (pH 7.0)-acetonitrile (97:3), D: 10 mM phosphate buffer (pH 7.0)-acetonitrile (95:5)

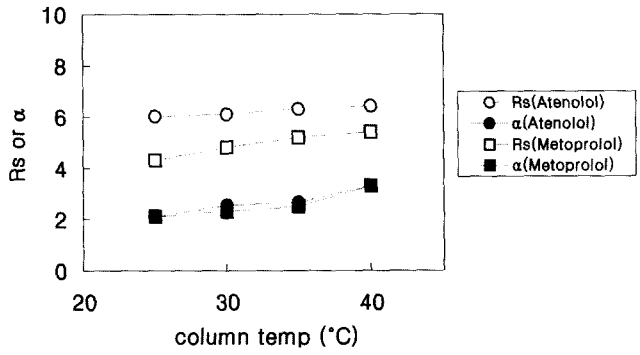

Fig. 4. Effect of column temperature on the resolution factor (Rs) and separation factor (α); Mobile phase : 10 mM phosphate buffer (pH 7.0)-isopropanol (95 : 5)

Both α and Rs increased as the content of the organic modifier decreased.

In this study, as the pH of the phosphate buffer changed, chromatographic parameters were markedly affected in several cases. Fig. 3 shows the effect of pH of the phosphate buffer on the chromatographic parameters. The retention factors as well as the enantioselectivity of amines were strongly dependant on pH. When the pH of the mobile phase decreased, enantioselectivity was also decreased.

Fig. 4 shows the effect of column temperature on the chromatographic parameters. In the mobile phase of 10 mM phosphate buffer (pH 7.0) : organic modifier (95 : 5, v/v), optimum column temperature was 40°C. Also, optimum buffer strength was found to be 10 mM with the phosphate buffer.

Quantitative structure-resolution relationships

The QSAR/CoMFA analysis developed by Cramer *et al.* (Gasteiger *et al.*, 1980; Cramer *et al.*, 1988) is a simple extension of the standard structure-activity relationship correlation tables with physicochemical parameters such as clogP and CoMFA. Very recent approaches have been made to predict the chiral behaviors in chiral stationary phases (De Boer *et al.*, 1999; Lammerhofer *et al.*, 1997; Zhou *et al.*, 2003; Van Eeckhaut *et al.*, 2000). In this study, we tried to correlate the CoMFA field to the Resolution (Separation factor) in the chiral column. The statistical results of the CoMFA are summarized in Tables II and III. The CoMFA and molecular refractivity (CMR) were used as descriptors, and the resolution was used as a dependent column. The statistical results of the CoMFA analyses, a cross validated value q^2, which was obtained as a result of the PLS analysis, served as a quantitative measure of predictability of the CoMFA model. The cross validated value q squared appears to be a good indicator of the accuracy of the predictions. In general, a comparative field analysis of any molecular property using PLS methodology that exhibits $q^2 \geq 0.5$ is indicative of the probability of a chance correlation between the molecular property and the CoMFA field examined to be ≤ 5%. It is believed that a CoMFA with a $q^2 = 0.5$, a point that is halfway between no possible model and a perfect model, is likely to be helpful in the decision making as to. Therefore, a model with $q^2 \geq 0.5$ is generally regarded as

Table II. Startistical results of resolution factor predictions in the PLS analysis

	Model I Mobile phase A	Model II Mobile phase B	Model III Mobile phase C	Model IV Mobile phase D
q^{2a}	0.933	0.928	0.934	0.928
ONC[b]	4	4	3	3
r^{2c}	0.995	0.996	0.991	0.989
SEE[d]	0.174	0.2	0.274	0.275
F[e]	777.3	1007.3	675.8	563.7
SF[f]	39.4 %	38.7%	36.9%	37.2%
EF[g]	60.6%	61.3%	63.1%	62.8%

Mobile phase : A, 10 mM phosphate buffer (pH 7.0)-isopropanol (95 : 5, v/v %); B, 10 mM phosphate buffer (pH 7.0)-isopropanol (97 : 3, v/v %); C, 10 mM phosphate buffer (pH 7.0)-acetonitrile (97 : 3, v/v %); D, 10 mM phosphate buffer (pH 7.0)-acetonitrile (95 : 5, v/v %)

[a] Cross validated r^2

[b] Optimum number of component

[c] Non-cross validated r^2

[d] Standard error estimate

[e] Fraction of explained versus unexplained variance

[f] Steric field contribution

[g] Electrostatic field contribution

Table III. Statistical results of separation factor predictions in the PLS analysis

	Model V Mobile phase A	Model VI Mobile phase B	Mode VII Mobile phase C	Model VIII Mobile phase D
q^{2a}	0.939	0.928	0.908	0.915
ONC[b]	3	3	3	3
r^{2c}	0.991	0.989	0.987	0.987
SEE[d]	0.084	0.110	0.105	0.081
F[e]	694.8	548.3	439.9	450.8
SF[f]	37.2%	37.3%	36.2%	38.2%
EF[g]	62.8%	62.7%	63.8%	61.8%

Mobile phase : A, 10 mM phosphate buffer (pH 7.0)-isopropanol (95 : 5, v/v %); B, 10 mM phosphate buffer (pH 7.0)-isopropanol (97 : 3, v/v %); C, 10 mM phosphate buffer (pH 7.0)-acetonitrile (97 : 3, v/v %); D, 10 mM phosphate buffer (pH 7.0)-acetonitrile (95 : 5, v/v %)

[a] Cross validated r^2

[b] Optimum number of component

[c] Non-cross validated r^2

[d] Standard error estimate

[e] Fraction of explained versus unexplained variance

[f] Steric field contribution

[g] Electrostatic field contribution

internally predictive.

QSRR analysis using CoMFA-resolution and CoMFA-separation factor are shown in Tables II and III, respectively. All eight models that were generated using 4 different mobile phases (A-D) showed good statistical results. The best Quantitative CoMFA-Resolution Relationship model (model I) derived from our study has a cross-validated (predictive) q^2 of 0.933 and a normal r^2 of 0.995. The best Quantitative CoMFA-Separation factor Relationship derived (model V) has a cross-validated (predictive) q^2 of 0.939 and a normal r^2 of 0.991, when the mobile phase of 10 mM phosphate buffer (pH 7.0) - isopropanol (95 : 5) was chosen. These cross-validated q^2 and conventional r^2 values supported the correlation between the descriptors and each of their activities and gave reliability to the prediction of the chiral separation of the test compounds. In model I, the relative contributions of steric and electrostatic field were 39.4% and 60.6%, respectively. When CMR was added as a descriptor, the analysis result was slightly improved, and the relative contributions of the steric field, the electrostatic field, and CMR were 34.9%, 54.5%, and 10.6%, respectively. The contribution of the electrostatic field was more important than steric fields, and molecular refractivity gave minor effect. The lipophilicity of the compounds (logP) did not enhance the correlation to activity and was excluded from QSAR analysis.

The lipophilicity of model drugs was calculated using the CLOGP program in order to calculate the Quantitative Lipophilicity-Resolution Relationships. Between the lipophilicity and the degree of resolution or separation factor, no correlation appeared to exist. In particular, metoprolol and atenolol illustrated very similar behavior on the chromatographic parameters such as resolution, but possessed very different cLogP properties. Therefore, in the chiral separation of the drugs bearing an amine moiety on chiral CBH columns, their conformations appear to exert great influence on their resolution that lead to chiral separation, rather than their lipophilicity.

Table IV indicates the comparison of the experimental and calculated values of the resolution in the mobile phase of 10 mM phosphate buffer (pH 7.0)-isopropanol (95 : 5, v/v %) using a CBH column. The predictive residual sum of squares (PRESS) of the training set was 0.51, which indicates a good predictive model.

The major steric and electrostatic features of the 3D QSAR derived from the CoMFA study were illustrated in Fig. 5 as three-dimensional transparent surfaces. Steric contours indicate the location of a sterically less bulky group that enhances resolution in yellow color in this series of compounds. The green color region indicates that the sterically bulky group enhanced the resolution. Electrostatic contours indicate the location of the electropositive character on the phenyl substituent in blue

Table IV. Comparison of the measured and calculated values of resolution in the mobile phase of 10 mM phosphate buffer (pH 7.0)-isopropanol (95:5, v/v %)

R or S	Compound	CoMFA	Rs		
			Measured	Calculated	Residue
S	Albuterol	112	1.40	1.41	-0.01
	Atenolol	128	6.40	6.46	-0.06
	Betaxolol	166	0	0.12	-0.12
	Dobutamine	144	1.67	1.72	-0.05
	Epinephrine	86	0	0.32	-0.32
	Fenfluramine	106	1.56	1.44	0.12
	Metoprolol	140	5.39	5.44	-0.05
	Norepinephrine	74	0	-0.08	0.08
	Pindolol	112	0	0.20	-0.20
	Sotalol	132	1.45	1.59	-0.14
	Terbutaline	102	1.80	1.42	0.38
R	Albuterol	118	1.40	1.51	-0.11
	Atenolol	138	6.40	6.47	-0.07
	Betaxolol	164	0	0	0
	Dobutamine	146	1.67	1.70	-0.03
	Epinephrine	78	0	-0.01	0.01
	Fenfluramine	92	1.56	1.48	0.08
	Metoproplol	142	5.39	5.16	0.23
	Norepinephrine	70	0	-0.26	0.26
	Pindolol	114	0	-0.07	0.07
	Sotalol	124	1.45	1.56	-0.11
	Terbutaline	106	1.80	1.76	0.04

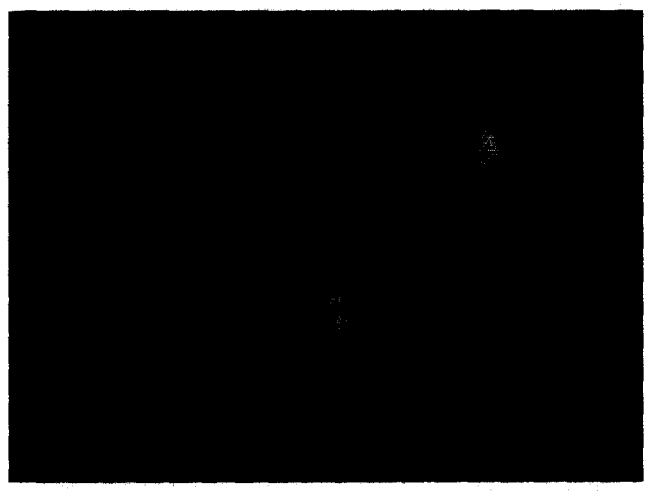

Fig. 5. Field graph and PLS results between CoMFA and resolution in chiral CBH column in the mobile phase of 10 mM phosphate buffer (pH 7.0)-isopropanol (95 : 5)

that enhances resolution. The red color on the region of the amino group showed that the electronegative group enhanced the resolution. Compounds with the amidomethyl group on phenyl, e.g. atenolol and metoprolol, showed high resolution.

Using the Quantitative CoMFA-Resolution (Separation factor) Relationships study combined with the partial least

square method, predictions on the extents of chiral separations of drugs containing an amine moiety were possible from their three dimensional molecular structures.

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