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Journal of Chromatography A, 773 (1997) 321–329

JOURNAL OF CHROMATOGRAPHY A

Improved chiral separation using achiral modifiers in cyclodextrin modified capillary zone electrophoresis

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Received 10 December 1996; revised 21 February 1997; accepted 25 February 1997

Abstract

The influence of achiral modifiers on the chiral separation of propranolol is examined by cyclodextrin modified capillary zone electrophoresis. The improved chiral separation of propranolol is by molecules previously identified in our group as forming ternary complexes with cyclodextrin and pyrene. The polarity, chain size and heteroatom composition of the functional groups on the comodifiers was systematically varied in order to study the influence of these variables on the separation of propranolol. The improved chiral separation is accompanied by a decrease in retention time. The decrease in retention time is suggestive of a decrease in the association of β-cyclodextrin (β-CD) with propranolol which was verified by calculation of apparent association constants using fluorometric methods.

Keywords: Enantiomer separation; Achiral modifiers; Propranolol

1. Introduction

Propranolol is a drug belonging to a class of compounds known as β-blockers, which are used to treat hypertension, angina pectoris and arrhythmia. The D-form of propranolol is approximately 40 times more potent in treating hypertension and antiarrhythmic activity than the racemic mixture, while only the L-form is beneficial in treating angina pectoris [1]. Several methods have been developed to determine the enantiomeric purity of propranolol as well as other types of chiral compounds. Some of these methods include high-performance liquid chromatography (HPLC) [2–4], nuclear magnetic resonance (NMR) [5], gas chromatography (GC) [6], thin layer chromatography (TLC) [7], capillary electrophoresis (CE) [8–10] and supercritical fluid chromatography [11].

In CE as well as in HPLC, cyclodextrins (CDs) are commonly used as chiral discriminators to achieve chiral separation [12,13]. Cyclodextrins are chiral cyclic oligosaccharides containing several D-(+)glucopyranose units with a shape which is usually depicted as a truncated cone. The most common forms of cyclodextrins are α, β and γ which contain 6, 7 and 8 glucopyranose units, respectively. Cyclodextrins have a hydrophobic inner cavity and a hydrophilic exterior, and are able to form inclusion complexes with other compounds based on size, geometry, hydrophobicity and hydrogen-bonding

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PII S0021-9673(97)00196-9

ability of the analytes. The hydroxyl groups on the cyclodextrins are bound to asymmetric carbons of the glucose and interaction with these hydroxyl groups are partly responsible for chiral separation.

Many papers have been published demonstrating the effect of parameters such as concentration of CD, type of counterion, column temperature, applied voltage, pH, type of cyclodextrin, use of derivatized cyclodextrins and effect of additives on the chiral separation of different types of chiral compounds [14–21]. Additives to the buffer solution such as surfactants, organic solvents, urea, other chiral compounds and alkylhydroxyalkylcelluloses have been used to improve peak shape, resolution and enhance enantioselectivity [8,9,16–18]. Urea is used to increase the solubility of CD, thus improving the inclusion complex formation, which can lead to improved chiral separation [8,12]. An increase in concentration of CD changes the dynamic equilibrium and increases the binding of analyte to CD [20]. Surfactants such as sodium dodecyl sulfate, which move against the electroosmotic flow in CE, are sometimes used to increase the retention time of the analyte, thus enhancing the chiral selectivity [18]. Organic solvents and alkylhydroxyalkylcelluloses serve to increase the retention times by decreasing the electroosmotic flow and changing the viscosity of the buffer solution [8,17,18]. The addition of other chiral compounds improves resolution by coinclusion of the chiral additive inside the CD cavity [18]. This leads to stereoselective interactions between both molecules and can lead to improved selectivity and a strong inclusion complex between one enantiomer and the additive. All of the above methods used to improve resolution often do so at the expense of separation time.

In the study presented here a systematic evaluation of several achiral modifiers for improved chiral separations is undertaken. We have previously examined many of these modifiers for improved cyclodextrin separation of achiral analytes [22]. In this manuscript, we desired to see if these modifiers would also affect chiral separations with cyclodextrins. These compounds were added to the buffer solution and an increase in chiral resolution was observed with a decrease in retention time and formation constants. The advantages of this type of comodifier are obvious, i.e., shorter analysis times with better resolution can be achieved.

2. Experimental

2.1. Capillary electrophoresis procedure

The CE experiments were performed on a CES I capillary electrophoresis system (Dionex, Sunnyvale, CA, USA). Data were collected by use of an AI-450 chromatography workstation. An uncoated fused-silica capillary, purchased from Polymicro Technologies (Phoenix, AZ, USA), 50 cm×50 mm I.D. was used as the separation column. The buffer solutions contained 10 m*M* β-cyclodextrin (β-CD), 50 m*M* sodium monophosphate and 40 m*M* comodifier. The pH of the buffer was adjusted to 2.5 with phosphoric acid. The buffer solutions were filtered through a 0.45 μm membrane filter prior to use. The propranolol solutions were at 0.01 mg/ml ($3.4 \cdot 10^{-5}$ *M*) and gravity injection was used to introduce samples into the capillary. Separations were performed at +15 kV with UV detection at 214 nm.

2.2. Fluorescence procedure

Steady state fluorescence studies were performed on a SPEX-Fluorolog Model F2T21I spectrofluorometer. A 1 cm quartz cell was used at ambient temperature with both excitation and emission slit widths set at 6.8 nm. The excitation wavelength was 288 nm and the fluorescence emission was scanned from 310 to 450 nm. The solutions contained 50 m*M* sodium phosphate monobasic, 40 m*M* comodifier and 0.01 mg/ml *R*- or *S*-propranolol with various amounts of β-CD. 50 ml of 2 *M* acrylamide solution was added to 5 ml of sample just prior to analysis and mixed thoroughly using a Vortex-2 Genie Model G-560 (Scientific Industries, Bohemia, NY, USA).

2.3. Materials

The *tert.*-butyl acetate, *tert.*-butyl formate, *tert.*-butylacetic acid, N-(*tert.*-butoxycarbonyl)-glycine, *tert.*-butyl N-hydroxy-carbamate, *tert.*-butyl carbamate, urea, urethane, 2-ethylbutyric acid and ammonium carbamate were purchased from Aldrich (Milwaukee, WI, USA). The monobasic sodium phosphate was purchased from Sigma (St. Louis, MO, USA). The *tert.*-butyl alcohol was purchased from Mallinckrodt (Paris, KY, USA). The acrylamide

was purchased from Amresco (Solon, OH, USA). The β-cyclodextrin was donated by the American Maize-Products Company. All chemicals were used as received.

3. Results and discussion

3.1. Effect of achiral comodifiers on the CE separation of propranolol

Improved chiral separation of propranolol was achieved by the addition of achiral modifiers to the buffer solution in cyclodextrin modified capillary zone electrophoresis (CD-CZE). Table 1 lists the comodifiers used in this study. Four of the comodifiers studied showed a significant increase in resolution. All four of these modifiers had a *tert.*-butyl moiety with an ester group attached to functional groups of varying polarity and heteroatom composition, $(CH_3)_3C$-O-CO-R.

Husain et al. [22] have reported the effects of some of these comodifiers on the reversed-phase liquid chromatographic separation of pyrene with β-CD in the mobile phase. In that study, a 59% (v/v) methanol–water solution was used as the mobile phase. Husain et al. found that, without addition of the comodifiers, pyrene exhibited little or no interaction with β-CD. It was also reported that addition of the comodifiers caused an increase in the formation constants between pyrene and β-CD with increasing chain length and heteroatom composition. This effect was attributed to the formation of a ternary β-CD–pyrene–comodifier complex. The opposite trend, a decrease in formation constants, was observed in this study with propranolol in aqueous solution. This observation will be discussed later in Section 3.2.

The formation of a β-CD–pyrene–tertbutyl alcohol complex has been extensively studied using fluorescence [23] and proton NMR [24] by Munoz de la Pena and coworkers. Results from that study suggest that the hydrophobic *tert.*-butyl moiety would be associated with the hydrophobic interior of the cyclodextrin cavity and that the hydroxyl group of the alcohol would hydrogen bond with the OH groups lining the periphery of the cyclodextrin rim.

Husain et al. used the first six comodifiers listed in Table 1 in HPLC studies. The purpose of choosing those compounds was to systematically vary the polarity, chain size and heteroatom composition of the functional groups in order to study the influence of these variables on the retention of pyrene under reversed-phase conditions. Those compounds were chosen for similar reasons in this study: to determine what effect, if any, these compounds would have on the enantiomeric separation of propranolol. The other compounds in Table 1 were chosen to investigate the role of the *tert.*-butyl group and the ester on complex formation and chiral separation.

The first comodifier studied was N(*tert.*-butoxycarbonyl)-glycine (TBCG). The concentration of TBCG was varied to find the optimum concentration. As shown in Fig. 1, the optimum concentration that yielded the greatest resolution with the shortest retention time was about 1%, which is approximately 40 m*M*. All comodifiers showed approximately the same optimum concentration. The improvement in resolution is accompanied, at least initially, by a decrease in retention time. The decrease in retention

Table 1
List of comodifiers used

Compound name	Structure
tert.-Butyl alcohol	$(CH_3)_3C$-OH
tert.-Butyl formate	$(CH_3)_3C$-O-CO-H
tert.-Butyl acetate[a]	$(CH_3)3_C$-O-CO-CH_3
tert.-Butyl carbamate[a]	$(CH_3)_3C$-O-CO-NH_2
tert.-Butyl(N-hydroxy)-carbamate[a]	$(CH_3)_3C$-O-CO-NH-OH
N-(*tert.*-Butoxycarbonyl)-glycine[a]	$(CH_3)_3C$-O-CO-NH-CH_2-COOH
tert.-Butyl acetic acid	$(CH_3)_3C$-CH_2-COOH
Ammonium carbamate	H_2N-O-CO-NH_2
Urea	H_2N-CO-NH_2
Urethane	H_2N-CO-O-CH_2-CH_3

[a] Comodifiers that improved chiral separation of propranolol.

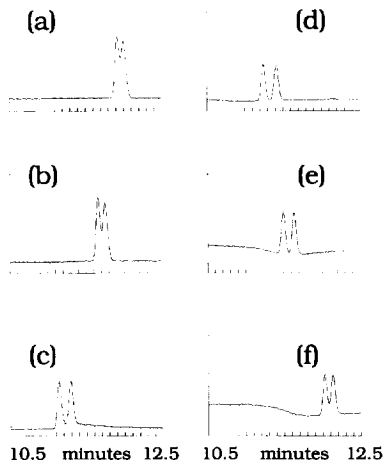

Fig. 1. Optimization of comodifier concentration (TBCG). Buffer solution contained 10 m*M* β-CD, 50 m*M* sodium monophosphate at pH 2.5, with the following amounts of TBCG (a) 0.05%, (b) 0.1%, (c) 0.5%, (d) 1%, (e) 2.5%, (f) 5%.

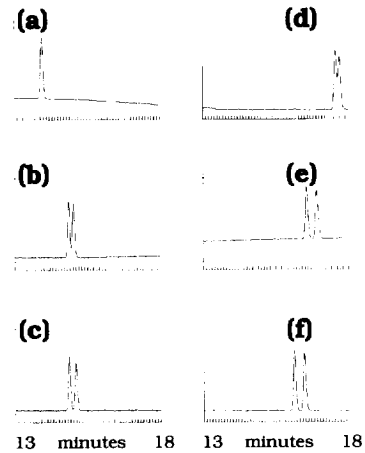

Fig. 2. Improved chiral separation of propranolol with and without various comodifiers. (a) Without β-CD or comodifier, (b) 40 m*M* TBAC, (c) 40 m*M* TBCM, (d) 10 m*M* β-CD and no comodifier, (e) 40 m*M* TBCG, (f) 40 m*M* TBHC.

time is due, at least in part, to a decrease in the association constant. At higher concentrations of comodifier the retention times increase. This increase in retention times is probably due to an increase in viscosity. The electrosmotic flow at the pH used (pH 2.5) is negligible. Therefore, any change in retention times would have to be due to either changes in the binding of propranolol to β-CD or a change in the viscosity of the bulk solution.

A dramatic improvement in resolution was achieved with the addition of 40 m*M* of the achiral modifiers *tert.*-butyl(N-hydroxy)-carbamate (TBHC) and N(*tert.*-butoxycarbonyl)-glycine (TBCG), as shown in Fig. 2. The resolution values (R_s) listed in Table 2, are 1.82 and 1.63, respectively. The addition of *tert.*-butyl carbamate (TBCM) and *tert.*-butyl acetate (TBAC) also improved the resolution, but not as dramatically, with respective R_s values of 1.2 and 1.0. The improvement in resolution was accompanied by a decrease in retention times, as compared to buffer solutions containing β-CD without any comodifiers. The reduced retention times are probably due, at least in part, to a decrease in the formation constants of β-CD and propranolol which was verified by our fluorescence discussed later in Section 3.2.

In order for chiral recognition between chiral compounds and cyclodextrins to occur, an inclusion complex must be formed and there must be a relatively tight fit between the complexed moiety and the cyclodextrin. There must also be an interaction between the chiral center of the chiral analyte and one of the chiral centers on the cyclodextrin. The chiral center on propranolol is on the carbon containing the OH group, which is separated from the naphthalene moiety by an oxygen and a carbon bond. It has been shown that the chiral center on propranolol interacts with the OH groups on the rim of the cyclodextrin which are connected to chiral carbons [4].

Propranolol contains three heteroatoms, the oxygen of the OH group on the chiral center and two other heteroatoms, an oxygen and a nitrogen. The oxygen and nitrogen are separated from the chiral center by a methyl group on either side. This gives three possible hydrogen binding sites on the propranolol with one on the chiral center. It is believed that these hydrogen binding sites as well as those on the comodifiers contribute to the improved chiral recognition observed in this study. The hydrogen-bonding of the heteroatoms between the comodifier and propranolol probably leads to a more rigid ternary complex with increased stereoselectivity, as compared to the binary complex of β-CD and propranolol. This increase in stereoselectivity can thus lead to increased chiral recognition. The importance of

Table 2
Calculated factors for CE separation of propranolol and association constants for fluorometric studies

					Calculated apparent association constants		Linear fit for Benesi–Hildebrand plots	
	T_1	T_2	R_s	$\alpha=T_2/T_1$	S	R	S	R
No comodifier	3.05	13.15	<1.0	1.01	166±13	138±26	0.9996	0.9986
TBHC	12.80	13.07	1.82	1.02	17±22	14±5	0.9999	0.9999
TBCG	12.40	12.66	1.63	1.02	19±10	21±19	0.9894	0.9993
TBAC	11.72	11.85	1.00	1.01	49±32	65±82	0.9998	0.9945
TBCM	11.18	11.34	1.20	1.01	19±6	11±5	0.9991	0.9997

hydrogen bonding is also evident in the improved resolution observed with increasing heteroatom number (Fig. 3). *tert.*-Butyl carbonyl glycine which is an exception to this observation has five heteroatoms compared to four in TBHC. The resolution is slightly less for TBCG than TBHC but this is probably due to charge interactions of the protonated propranolol and charges on TBCG.

The formation of a precipitate was observed with the introduction of the comodifiers *tert.*-butyl formate and *tert.*-butyl acetic acid. In order to study these compounds, 4 *M* urea was added to the buffer to increase the solubility of the complex. Urea without the addition of the comodifiers showed no apparent effect on the complex formation of β-CD and propranolol. However, urea did adversely interfere with the chiral separation since chiral recognition was completely lost. This was true even using the comodifier TBCG which gave excellent results in the absence of urea. It seems, however, that complex formation still occurred which is evident from the shorter retention times.

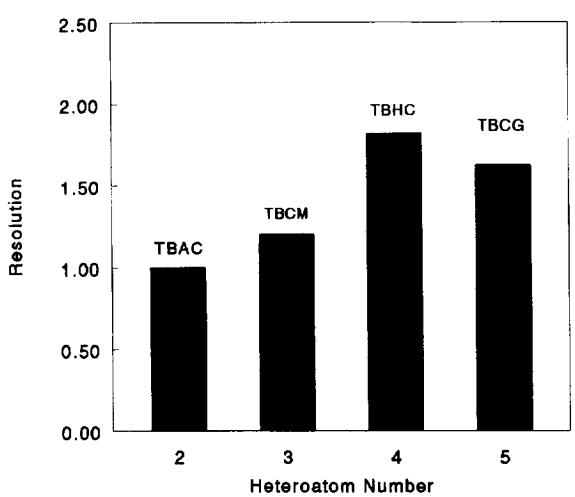

Fig. 3. Influence of heteroatom number on resolution of propranolol.

In order to determine the role of the *tert.*-butyl group, urethane and ammonium carbamate were added to the buffer solutions. Both of these compounds contained the same functional group as in *tert.*-butyl carbamate, an ester with a terminal amine ($R\text{-}O\text{-}CO\text{-}NH_2$). In the case of urethane, an ethyl group replaces the *tert.*-butyl and an amine in ammonium carbamate. No improvement in chiral separation was observed with the addition of urethane, compared to β-CD without a modifier, and only a small change in the observed retention times. Ammonium carbamate produced complete loss of chiral recognition with significantly shorter retention times. The role of the *tert.*-butyl group seems to be two-fold. In one role, the *tert.*-butyl group, which is hydrophobic, increases the formation constant of β-CD and the comodifier by entering the hydrophobic cyclodextrin cavity [22]. In another role, it acts as spacer in the cavity, limiting the possible orientations of propranolol within the cyclodextrin cavity [23,24].

Urea, which is structurally very similar to ammonium carbamate, was also tested to investigate the role of the ester in complex formation. The difference between urea and ammonium carbamate is that urea is a ketone and ammonium carbamate is an ester. The shorter retention time of ammonium carbamate is evidence of possible complex formation. Urea, however, seemed to have very little effect on the complex formation of propranolol and β-CD. This would indicate that the ester plays an important role in the complex formation, probably through hydrogen bonding with the OH groups on the rim of

the cyclodextrin. The role of the ester can also be seen in a comparison between the behavior of *tert.*-butyl alcohol and *tert.*-butyl acetate. *tert.*-Butyl alcohol shows no observable affect on the chiral selectivity of the complex while *tert.*-butyl acetate shows significant improvement in the resolution of the two enantiomers.

The comodifiers in this study decrease the formation constant of both the *R* and the *S* forms of propranolol but they decrease the *S* form more than the *R* form, thus yielding an improvement in chiral recognition. The *R* form eluted second for all the comodifiers used in this study. In order to verify the decrease in formation constants of propranolol–β-CD with the addition of the comodifiers, the apparent formation constants were calculated using fluorescence studies.

3.2. Fluorescence studies of the influence of achiral comodifiers on the apparent formation constants of β-CD and propranolol

Calculation of the apparent formation constants by use of fluorescence studies is based on differences in the fluorescence intensities between the complexed and the uncomplexed form of the fluorescent analyte. In the case of propranolol–β-CD, this difference is very small. Due to the small differences in the fluorescence of the complexed and uncomplexed form of propranolol, it is very difficult to calculate the formation constants using conventional fluorescence techniques. A quencher was therefore used to increase the difference between the complexed and uncomplexed forms.

While several quenchers were tried, acrylamide was chosen because it yielded the greatest difference in fluorescence. The optimum concentration of acrylamide was determined by plotting the difference in fluorescence intensity of propranolol with β-CD and intensity without β-CD versus concentration of acrylamide, (see Fig. 4). At 20 m*M* acrylamide the bottom curve, representing the difference in the fluorescence intensities, with and without β-CD, leveled off. This concentration represents the lowest concentration of acrylamide that can be used and yet produce the greatest change in intensity. This concentration was thus chosen as the optimum concentration.

The quencher had to be chosen very carefully since it should not interfere with the formation of the ternary complex. To verify that acrylamide would not interfere with the ternary complex formation of

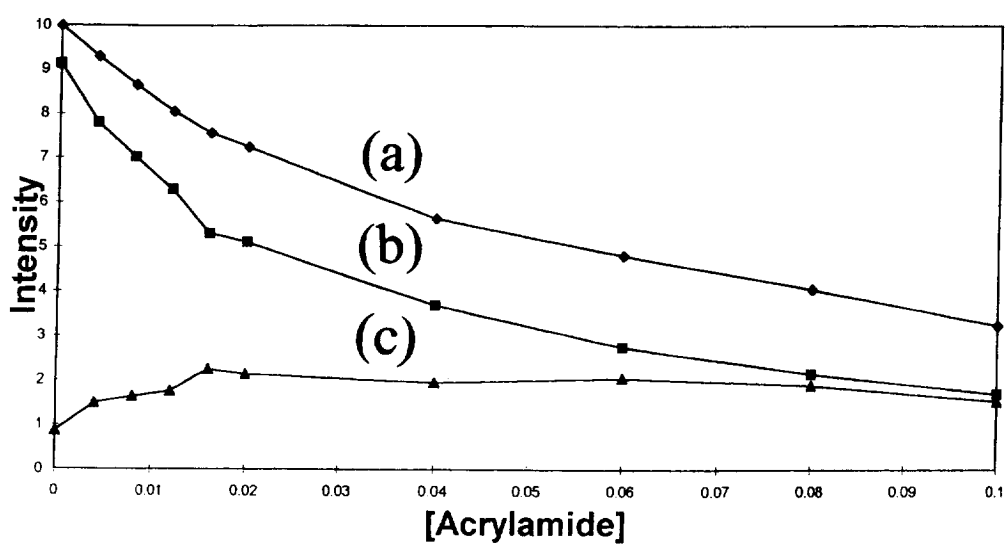

Fig. 4. Effect of acrylamide concentration on the fluorescence quenching of propranolol with and without β-CD. (a) 10 m*M* β-CD, (b) without β-CD, (c) difference in (a) and (b).

propranolol–comodifier–β-CD, the CE studies were repeated using the same concentration of quencher in the buffer as was determined to be of optimum concentration in the fluorescence studies, 20 m*M*. Fig. 5 shows that the addition of acrylamide had minimal effect on the ternary complex formation and thus the chiral separation.

Table 2 lists the calculated apparent association constants for β-CD–propranolol without a comodifier and with the four comodifiers that yielded improved chiral separation. The apparent association constants were calculated using double reciprocal Benesi–Hilderand plots [25]. These numbers, however, should not be used for anything other than confirming the fact that the addition of the comodifiers decrease the binding of β-CD to propranolol. The calculated apparent association constants are too small and the errors associated with them are very large. The intercept of the Benesi–Hildebrand plots were very close to zero and small changes in the slope thus caused large relative changes in the intercept. Therefore, the only reasonable conclusion about these numbers is that they are probably of the right order of magnitude. These computed binding constants confirm the hypothesis that the increase in chiral recognition with the addition of comodifiers is accompanied by a decrease in the binding of propranolol to β-CD. This decrease in binding is what is responsible, at least in part, for the shorter retention times.

The decrease in retention times with decrease in apparent association constants is due to the fact that β-CD acts as a pseudo stationary phase in the CE separation of propranolol. The migration of the positively charged propranolol is based on the electrophoretic mobility as well as the electroosmotic flow in the capillary. At a pH of 2.5, the electroosmotic flow is insignificant. Therefore, the movement of the analyte through the column is based primarily on the electrophoretic mobility. The electrophoretic mobility is based on the charge as well as the size of the analyte. Since β-CD is neutral, the movement of the uncomplexed molecule through the column is dependent primarily on the electroosmotic flow, which in this case is insignificant. When propranolol binds with cyclodextrin the movement through the column is retarded based on the fact that the complexed form is much larger than the uncomplexed form with the same charge. The fluorescence studies were done to confirm the fact that the differences in retention times were based on a decrease in association constants rather than some other phenomena such as a decrease in viscosity with the addition of the comodifiers, or formation of a multiply charged complex with increased association constants. The decrease in calculated apparent association constants support this view.

As indicated earlier, the decrease in association constants with addition of comodifiers observed in this study are in contrast to the results reported by Husain et al. [22]. In that study, the addition of the comodifiers increased the association constants between pyrene and β-CD. In the case of pyrene, a 2:1 complex is formed between pyrene and β-CD. That would not be the case with propranolol where the nature and bulkiness of the *R*-group of propranolol attached to the naphthalene ring would prevent a 2:1 complex from forming. Propranolol also has several heteroatoms which could hydrogen bond to the modifiers where pyrene contains no heteroatoms. Some of our previous studies have shown that comodifiers tend to decrease the association constants of heterocyclics with cyclodextrin [26].

4. Conclusions

Improved chiral separation can be achieved by the use of achiral additives to the buffer solution in cyclodextrin modified capillary electrophoresis. The improved chiral separation is accompanied by a decrease in retention time. The decrease in retention time is due, in part, to a decrease in the apparent association constant of β-CD and the chiral compound, propranolol. Moreover, the improvement in chiral recognition is probably due to a more rigid ternary complex of comodifier–β-CD–propranolol compared to the binary complex of β-CD–propranolol. This increase in rigidity of the complex is believed to be due to hydrogen bonding between the propranolol, β-CD and the achiral comodifier. We believe this method offers an interesting approach to chiral separation since other methods that improve the chiral separation using cylcodextrin modified capillary zone electrophoresis will usually achieve improved chiral separation at the expense of time.

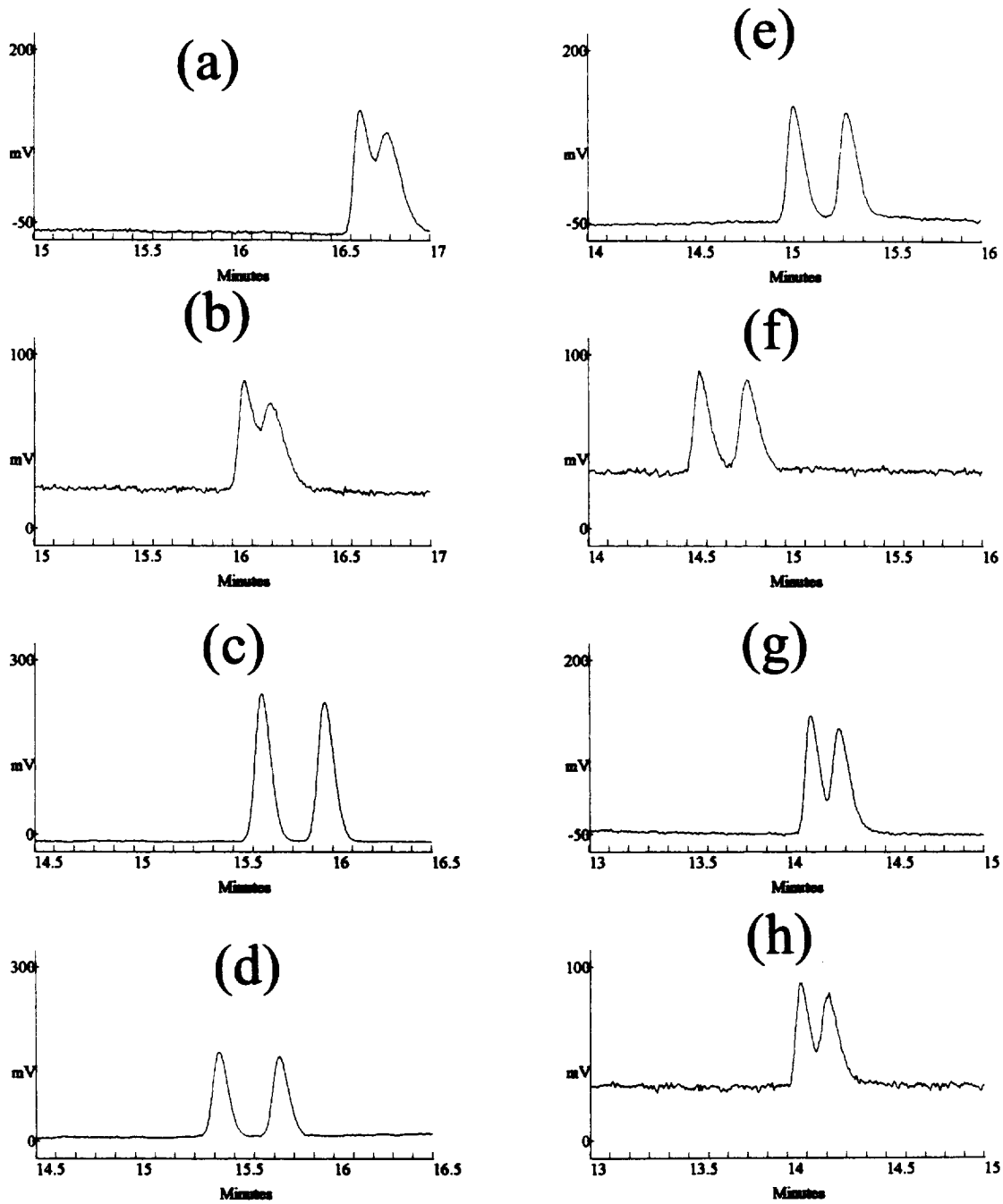

Fig. 5. Effect of quencher (acrylamide) on chiral separation and ternary complex formation with and without various comodifiers. (a) No comodifier and no quencher, (b) no comodifier with quencher, (c) TBHC without quencher, (d) TBHC with quencher, (e) TBCG without quencher, (f) TBCG with quencher, (g) TBAC without quencher, (h) TBAC with quencher.

Acknowledgments

We thank G.A. Reed of American Maize Products for the donation of the β-CD used in this study. This work was supported in part by a grant from the National Institute of Health (GM 39844). Isiah M. Warner also acknowledges the Philip W. West endowment for partial support of this research.

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