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Evaluation of a methacrylate bonded cyclodextrins as a monolithic chiral stationary phase for capillary electrochromatography (CEC)-UV and CEC coupled to mass spectrometry

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Abstract

Glycidyl methacrylate bonded β -cyclodextrin (GMA- β -CD) is synthesized as a new chiral monomer by direct chemical bonding with GMA using a fast and a simple alternative procedure. Very, rigid and homogenous monolithic columns were prepared by polymerization of GMA- β -CD monomer with ethylene dimethacrylate (EDMA), in the presence of commonly used porogens and a charged achiral monomer to form a versatile chiral monolith. This is the first report in which a preparation procedure for a methacrylate-bonded CD is introduced for chiral separations in CEC. The degree of substitution (DS) of GMA- β -CD monomer and mobile phase parameters were optimized to achieve highest enantioselectivity and plate number. To evaluate the GMA- β -CD monolithic column, different classes of chiral compounds were screened. Under the optimized β -CD monolith phase and the optimum mobile phase conditions, 30 neutral and basic chiral compounds and two acidic compounds could be separated. The high chemical and mechanical stability, homogenous microflow and no loss of material at the interface allows for the first time the feasibility of applying this polymer-based monolithic column for CEC coupled to ESI-MS. Compared to CEC-UV, CEC-ESI-MS showed higher sensitivity and lower resolution. However, resolution greater than 1.0 can still be obtained for majority of the select tested compound in CEC-ESI-MS with at least three out of seven compound providing $R_s \geq 1.5$. The results reinforce the potential of GMA- β -CD monolithic columns for chiral separations with high sensitivity in CEC-ESI-MS. Finally, using hexobarbital as model chiral analyte, the monolithic column demonstrated excellent stability and reproducibility of retention time and enantioselectivity.

Keywords

Enantiomeric separation; GMA- β -CD; monolithic column; CEC-UV; CEC-ESI-MS

1 Introduction

Cyclodextrins (CDs) and their derivatives are the most popular chiral selectors in enantiomeric separation owing to their great chiral recognition power. A literature search of review articles reveal that this group of cyclic oligosaccharides compounds been widely used as chiral stationary phases for gas [1], liquid [2], sub- and supercritical fluid chromatography [3], as well as mobile phase additives in liquid chromatography [4],

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capillary zone electrophoresis [5], or cyclodextrin modified micellar electrokinetic chromatography [6].

Capillary electrochromatography (CEC), a hybrid method of CE and HPLC, has obtained increasing attention for chiral separation. The use of CDs in CEC can be subdivided into three versions: open-tubular CEC [7, 8], CEC with packed capillaries [9, 10] and CEC with monolithic capillaries [11, 12]. Compared to conventional open-tubular CEC, tapered packed columns [13] are beneficial for achieving better enantiomeric selectivity as well as higher sample loading capacity. On the other hand, monolithic columns provide all the benefits of tapered packed columns with an added advantage of less prone to bubble formations. In addition, due to the unique properties of monolith materials, such as the availability of various functional monomers, the use of monolithic column in CEC has attracted much interest recently [14, 15].

According to the nature of the monolith, there are two distinct types of enantioselective monolithic approaches to utilize native CDs (α , β , γ) and modified CDs as monolithic chiral stationary phase. The first one is silica-based monolith prepared by sol-gel technology in which the gelled monolith is either coated with permethylated β -CDs [16] or covalently linked to γ -CD [17]. However, the column-to-column reproducibility was found to be unsatisfactory [11, 16]. Wistuba and Schurig functionalized CD silica particles followed by gluing together via sol gel technique [18]. The second approach is based on organic based polymer monolith, which is prepared in two steps: (i) polymerization of organic achiral monomer and crosslinker in the presence initiator and porogens (ii) the resulting monolith is modified with the CD selector either by copolymerization or by physical adsorption into the continuous bed [11]. Furthermore, it is worth mentioning that compared to organic polymer monolith, the preparation of silica monolith is usually more time-consuming and its procedures are more sophisticated. The instability in high pH range is another disadvantage of the silica-based material. Organic polymer monolith includes homogeneous gel [19–21] and rigid rod polymer monoliths [22, 23]. The former often encounters undesirable durability, while the latter is rigid with higher stability and tolerant of extreme pH. Thus, in all of the aforementioned approaches chiral selector is linked to the monolith either prior to or after the formation of monolithic column.

Very recently, the use of rigid polymer monoliths, polyacrylate monolithic column has been attracting a lot of interests [22, 24]. However, most of the CD modified polyacrylate monolithic columns were prepared using a post modification multiple-step method, where the monolithic column was modified with a CD selector matrix after the formation of the monolith. This is not only time consuming but it is also difficult to control the whole process, often providing unsatisfactory reproducibility. In this research, to avoid this problem, an alternative method was used to prepare the polymer based β -CD monolithic column.

In this work we propose a simpler approach to prepare methacryloyl bonded β -CD in few hours, which can be conveniently copolymerized with commonly used charged monomers (aminopropyl sulfonic acid, AMPS), crosslinker (ethylene dimethacrylate) in the presence of porogens (butanediol, propanol and dimethylsulfoxide) to form a rigid rod and a versatile chiral monolithic column. After the optimization of the degree of substitution of the bonding reaction and polymerization conditions on enantioselectivity, the mobile phase parameters of MS-compatible mobile phase were optimized via CEC-UV.

Enantiomeric separation and detection of chiral compounds using low molecular weight CDs as chiral selector in running buffer in capillary electrophoresis (CE)-mass spectrometry (MS) is one of the challenging areas, which limits the use of this valuable hyphenated

technology for chiral analysis. This is mainly because the use of charged chiral selector when present in the running CE buffer often interferes and competes with the ionization of the analytes in ESI-MS. To overcome this problem of analyte ion-suppression in electrospray, we proposed the potential of a homogenous fixed bed chiral stationary phase (i.e., polymer-based GMA- β -CD monolithic column), which is tightly anchored to the inner walls of the capillary column. The CEC-ESI-MS runs were performed using the same buffer composition optimize for CEC-UV conditions. To the best of our knowledge, this is the first report on the use of polymer-CD based monolithic column, for chiral CEC-MS.

2 Experimental

2.1 Chemicals and materials

The reagents ethylene dimethacrylate (EDMA), 1-propanol and 2,2'-azobisisobutyronitrile (AIBN) were from Aldrich (Milwaukee, WI, USA). Dimethyl sulfoxide (DMSO), β -cyclodextrin, γ -methacryl-oxypropyltrimethoxysilane, 2-acrylamido-2-methylpropane sulfonic acid (AMPS), 1,8-diazabicycloundec-7-ene (DBU), *N,N*-dimethylformamide (DMF) and all the chiral analytes (listed in Table 2) were obtained from Sigma (St. Louis, MO, USA). The porogen, 1, 4-butanediol and glycidyl methacrylate (GMA) were purchased from Fluka (Buchs, Switzerland). All the reagents were used as received except for the EDMA, which was purified by distillation under vacuum prior to use. Fused silica capillary (OD 375 μ m, ID 100 μ m) was obtained from Polymicro Technologies Inc. (Phoenix, AZ, USA).

2.2 Synthesis of glycidyl methacrylate- β -cyclodextrin (GMA- β -CD)

The synthetic scheme for the bonding reaction of GMA with β -CD resulting in the synthesis of GA- β -CD is shown in Figure 1 (Step 1). Briefly, β -CD (more than 5g) is dried under vacuum at 40 °C for 12 hrs to remove any moisture. 5g dried β -CD is dissolved in 15 mL of anhydrous DMF. 0.05 g 1,8-diazabicycl[5,4,0]undec-7-ene and GMA are added rapidly to this solution. Next, the reaction mixture is stirred for 2.5 h at 98 °C. The mixture is then cooled at room temperature and a small amount of solid precipitate is filtered off. About 200 mL of toluene is added to the filtrate. The GMA- β -CD, which precipitates in toluene is collected and washed with 200 mL of toluene and then twice with 500 mL of acetone. The precipitates are finally dried under vacuum at 40 °C for 12 hours [25]. Finally, the purity and degree of substitution (DS) value were verified by ^1H NMR spectroscopy (supplemental section Figure 1).

2.3 Preparation of monolithic columns

For the preparation of stationary phases, the inner walls of the capillaries were vinylized with 3-(trimethoxysilyl)propyl methacrylate. The procedure can be found elsewhere [26, 27]. Subsequently, various concentrations (w/w) of GMA- β -CD, EDMA, 1-propanol, 1,4-butanediol, DMSO and AIBN were mixed ultrasonically into a homogenous solution and purged with nitrogen for 10 min. Several bare-fused silica capillaries with a total length of 40 cm were filled with the various ratios of the components of the polymerization mixture to a length of 28 cm, sealed with rubber septum, and then placed in a GC oven to polymerize for 20 h at 60 °C (Figure 1, step 2). The ratio of components used to prepare optimized CEC-UV monolithic column is mentioned in section 3.1.3. For preparation of CEC-ESI-MS column, the same optimized components of polymerization mixture was used except that 53 cm total length of the silanized capillary was filled to a length of 28 cm for polymerization under the same conditions as CEC-UV monolithic columns. After the polymerization was completed, the monolithic columns are washed with acetonitrile using a HPLC pump to remove any unreacted monomers and porogens. For the monolithic column used in CEC-UV, an on-column detection window was made next to the polymer bed using a thermal

wire stripper. Finally, the column was cut to 35 cm with an effective length of 25 cm. On the other hand, for CEC-ESI-MS, the column was cut to 50 cm with an effective length of 25 cm. Hence, 10 cm and 25 cm segments were left empty on the inlet side of the CEC-UV and CEC-MS, column respectively due to instrument constraints.

2.4 Morphology characterization

The microscopic morphology of the monoliths was evaluated using scanning electron microscope with the aid of a Hitachi X-650 (Hitachi, Japan) SEM apparatus at 7.5 kV and a filament current of 40 mA. Monolithic column samples were fractured, cut to a length of 2 mm, and placed on an aluminum stub with a double sided carbon tape. Next, they were sputter-coated with a gold/palladium alloy using a SPI Sputter (SPI Supplies Division of Structure Probe, West Chester, PA, USA) for 1 min at 30 mA to prevent charging.

2.5 CEC-UV and CEC-ESI-MS instrumentation

All of the CEC-UV experiments were carried out using an Agilent HP^{3D}CE system (Agilent Technologies, Palo Alto, CA) equipped with an autosampler, a diode-array detector, 0–30-kV high-voltage power supply and Chemstation software (V9.0) for system control and data acquisition. For CEC-ESI-MS instrumentation, all experiments were performed on an Agilent HP^{3D}CE, which was interfaced to an Agilent 1100 series MSD quadrupole mass spectrometer equipped with a CE-MS adaptor kit and a sprayer kit. Sheath liquid was delivered by an Agilent 1100 series HPLC pump equipped with a 1/100 split flow.

2.6 CEC-UV and CEC-ESI-MS conditions

Mobile phase composition for all the chiral separation containing variable ACN concentration was combined with 5mM NH₄OAc containing 0.3% (v/v) TEA (pH was adjusted with HOAc). All of the mobile phases and sheath liquids were degassed for 30 min before use. Before the first CEC run, column was preconditioned with the desired mobile phase using the HPLC pump (Lab Alliance, State College, PA, USA) at ~2000 psi for 2 h followed by further conditioning on the CEC instrument for another 1 h. An external pressure of 6 bar was applied to inlet and outlet buffer vials to suppress bubble formation. For CEC-ESI-MS, separation voltage was set at 30 kV along with the application of 12 bar pressure at the inlet end. The following conditions were used unless otherwise stated: sheath liquid, MeOH/H₂O (80:20, v/v) containing 5 mM NH₄OAc, flow rate, 5.0 µL/min; capillary voltage, +2000 V; drying gas flow rate, 5L/min; drying gas temperature, 200 °C; nebulizer pressure, 5 psi. The selective ion-monitoring (SIM) mode was set at a corresponding polarity to monitor the desired protonated ([M+H]⁺) or deprotonated ([M-H][−]) molecular ions for each investigated analyte.

2.7 Calculation of apparent retention factor, resolution and plate number

The retention factor (*k*) describe here is a simply "apparent retention" that not only includes the the affinity of the analyte to the monolithic phase but also the contribution of the EOF and an electrophoretic migration of charged analytes.

Apparent retention factor *k* was calculated using equation (1)

$$k = \frac{(t_R - t_0)}{t_0} \quad (1)$$

where *t_R* is the retention time of analytes, *t₀* is the elution time of EOF marker. Because thiourea was strongly retained on this stationary phase, baseline perturbation was used as the marker of EOF [28].

The resolution (R_s) and plate number (N) were calculated by the Chemstation software (Agilent Technologies, Palo Alto CA). The plate number is reported as plates/m.

3 Results and Discussion

3.1 Preparation and characterization of GMA- β -CD and monolithic column

3.1.1 Preparation of GMA- β -CD—Figure 1 (step 1) demonstrates the synthetic scheme of GMA- β -CD. The bonding of methacrylate groups to β -CD units is particularly suitable by reaction with GMA. Because the primary hydroxyl groups of β -CD are more nucleophilic, more basic, and less sterically hindered than the secondary hydroxyl groups of β -CD, the primary groups exhibit greater reactivity with the epoxy group of GMA [21]. By comparing the signal areas in the ^1H -NMR spectrum of β -CD and substituted methacrylate group of GMA- β -CD, the reaction could be confirmed by the existing of double bond proton (around 5.7 ppm) grafted to β -CD ring. The DS could be determined by the ^1H -NMR spectroscopy (see supplemental information, Figure 1). By changing the molar ratio of GMA and β -CD, GMA- β -CD with different DS could be obtained and detailed information is shown in Table 1. The DS value indicates how many methacrylate molecules are bonded on average per glucose unit of β -CD molecule and can simply be calculated by taking the ratio of integrated peak areas of double bond proton (around 5.7 ppm) of GMA and hydroxyl anomeric proton at position 1 (around 5.0 ppm) of pyranose ring of β -CD. This GMA- β -CD contains bonded methacrylate-terminated polymerizable groups, which make possible its direct copolymerization with crosslinkers (e.g. EDMA) in the presence of commonly used porogens (propanol, DMSO and butanediol) and initiator (AIBN) for the preparation of rigid polymer based monolith, Figure 1 (step 2).

3.1.2 Effect of DS of GMA- β -CD—To study the effect of DS of GMA- β -CD on the property of monolithic column, GMA- β -CD with DS of 1.0, 2.0 and 3.0 were prepared, respectively as shown in Table 1. Figure 2 shows the typical scanning electron micrograph (SEM) cross-sectional images of these three monolithic columns. It is obvious that there is no significant difference on the morphological property, which means that the DS of GMA- β -CD has no obvious effect on the porosity of the monolith. Nevertheless, from the SEM images, it can be found that no large voids between the capillary inner face and the monolith. In addition, it can be seen that the monolith consists of large through-pores (1–2 μm) and tightly connected microspheres cluster. Compared to previous works involving polyacrylate monolithic columns [26, 27], this GMA- β -CD monolithic column contains much smaller microspheres than the typical methacrylate based polymer monolithic column.

Another study was conducted to investigate the effect of DS on the chiral separation property of the prepared monolithic column. Figure 3A–C shows the separation properties (apparent retention factor k , resolution R_s and the average plate number N of the two enantiomers) of the monolithic columns on neutral and basic chiral compounds. Because the plate numbers of the two enantiomer peaks were similar to each other, the average N values of the two enantiomers were used to evaluate the separation efficiency throughout this study. The GMA- β -CD-2 monolithic column prepared with DS=2.0 provided the highest R_s for most of the analytes. For N , except for miconazole, column GMA- β -CD-2 demonstrated the highest separation efficiency. For k , except for aminoglutethimide, column GMA- β -CD-2 showed the highest apparent retention. Overall, GMA- β -CD-2 showed the best chiral separation performance, and hence this column was used for further optimization.

3.1.3 Preparation and optimization of GMA- β -CD-2 monolithic column—After optimizing the DS of GMA- β -CD, several factors, including composition of polymerization mixture, kinds and concentration of porogens, reaction temperature and reaction time, which

could affect the separation capability of GMA- β -CD-2 monolithic column was evaluated. Following the preliminary study on the aforementioned polymerization factors (data not shown), the concentration of monomers and porogens were found critical to the performance of the GMA- β -CD-2 monolithic column.

Specific attention was paid to the design of the porogen mixtures for preparation of the GMA- β -CD monolithic column. Frequently used porogens, such as 1-propanol, 1,4-butanediol, decanone, dodecanone, DMF, DMSO, and water in their various proportions were tested as porogens. The results showed that the ternary porogen system containing 20% (w/w) 1-propanol, 30% (w/w) 1,4-butanediol and 30% (w/w) DMSO, was most suitable for the GMA- β -CD-2 monolith, which provides: (1) a homogeneous, single phase polymerization mixture; (2) control porosities over a broad range; (3) facile initial washing and equilibration of the capillary column.

In addition, extensive studies of the concentration of monomer, crosslinker, initiator and charged monomer were conducted, considering the permeability, homogeneity and porosity of the monolith bed, which may significantly impact the enantiomeric separation property. Finally, the optimized composition of monomer, crosslinker, initiator and charged monomer were found as follows: 15% (w/w) GMA- β -CD, 5% (w/w) EDMA, 0.4% (w/w) AIBN and 0.4% (w/w) of AMPS in the polymerization mixture.

3.2 Effect of mobile phase parameters using GMA- β -CD-2 monolithic column in CEC-UV

It is well established that the mobile phase conditions usually have significant effects on chiral separation in CEC. This is because separation conditions may change the electrophoretic mobility and electroosmotic flow (EOF) due to changes in the surface property of the monolithic stationary phase, subsequently affecting the elution order and enantioseparation of the analytes. To achieve better separation, the pH of the mobile phase, organic modifier and capillary temperature were studied, respectively.

3.2.1 Effect of mobile phase pH—Different chiral analytes have different pK_a , which affect their effective charge and consequently electrophoretic mobility under different pH conditions. Separation properties (k , R_s and N) of six representative compounds (Figure 4 A-C) including three basic compounds, one moderately acidic compound, one weakly acidic compound and one neutral compound were studied in the pH range of 3.0–6.0. For the most studied compounds, retention time was very long (e.g. longer than 80 min) when pH was higher than 6.0. Considering the practicality of the separation method, pH higher than 6.0 was not pursued. The k trend of the first eluting enantiomer as a function of pH is shown in Figure 4A. The moderately and weakly acidic compound, hexobarbital and catechin becomes partially ionized (negatively charged) upon increasing the pH, but the charge is reduced at lower pH of 3.0, resulting in decreasing retention. Note that k of hexobarbital and catechin shows a substantial increase with increasing pH from 5.0–6.0. On the other hand, basic compounds such as Tröger's base are more ionized (positively charged) at lower pH, resulting in faster elution. Increasing the pH, reduces the positive charge on this compound, which behave more like neutral at pH 6.0, thereby increasing the apparent retention. On the other hand, aminoglutethimide ($pK_{a2} = 11.1$) showed a drop in k with increasing pH from 3.0 to 6.0 suggesting that the effective positive charge is retained for this compound and drop in apparent retention is primarily due to the increase binding with the negatively charged monomer, AMPS. The k of miconazole first increase in the pH range of 3.0–4.0 then levels off, suggesting the compounds becomes essentially neutral at pH 6.0 ($pK_a \sim 6.5$). The neutral compounds such as benzoin are only transported by EOF, the change of the pH does not affect their apparent retention time and the k . Nevertheless, the pH of the mobile phase had a significant effect on the k of chargeable enantiomers, which were transported

both electrophoretically and electroosmotically as well as due to their affinity for the monolithic stationary phase. Nevertheless, with basic or acidic analytes the k may also depend on additional interactions such as the hydrophobic and the hydrogen bonding interaction as well as their ability to form inclusion complex with GM- β -CD-stationary phase. For example, miconazole should be positively charged at low pH (pH 4.0) and hexobarbital should be neutral. On the other hand, at pH 6.0 the former is neutral and the latter is partially negative. Hence, at some point the k are very similar (i.e., pH 6.0, Figure 4A).

The addition of AMPS as charged monomer during the preparation of GMA- β -CD-2 made the monolithic column negatively charged. In addition, β -CD has a pK_a of 12.2 [29]. Thus in the operating pH range of 3.0–6.0, β -CD is essentially neutral and the EOF was produced only by AMPS. The resolution trends of the selected six chiral analytes are shown in Figure 4B. The resolution of each pair of neutral enantiomers (benzoin) and acidic enantiomers (catechin and hexobarbital, which also remain essentially neutral) was optimum at pH 4.0. The R_s drops more substantially at pH higher than 4.0 for catechin and hexobarbital as these analytes become negatively charged and are repelled by the negative charge on AMPS. On the other hand, for benzoin increasing the pH to 4.0 showed only a slight increase (probably due to the slight increase in k) followed by again only slight changes in R_s . A trend of R_s for miconazole enantiomers is similar to the one observed for benzoin, whereas for the other two basic enantiomers (aminoglutethimide and Tröger's base) the resolution continues to drop from pH 3.0–6.0 with the optimum observed at pH 3.0.

The trend in average N of the enantiomeric pairs of hexobarbital and catechin in the pH range of 3.0–4.0 is similar to R_s trends. In contrast, the average N of benzoin drops substantially upon increasing the pH from 3.0–4.0, even though there is only a slight increase in k and R_s . Interestingly, the average N of the three basic enantiomers does not change substantially over the pH range of 3.0–6.0.

3.2.2 Effect of ACN concentration in the mobile phase—In CEC using reversed-phase monolithic columns, the addition of organic solvents tends to decrease k [18]. The effects of ACN on the R_s , k , and average N were studied by increasing the concentration of ACN in the mobile phase from 20 to 50% (v/v) (Figure 5). Clearly, with the increase of %ACN, k of the analytes decreases (Figure 5A). This suggests that the GMA- β -CD monolithic column exhibits hydrophobic interactions under the partially aqueous separation conditions. With the increase of ACN in the mobile phase, the difference in hydrophobicity between the mobile phase and stationary phase gets smaller, and thus the solutes are eluted faster as the binding with GMA- β -CD monolith is decreased.

As shown in Figure 5B, the lower % (v/v) ACN shows a general trend of better R_s . This is because the lower concentration of ACN is favorable for the formation of inclusion complex thereby increasing the binding between the analytes and the β -CD cavity. For the average plate number of two enantiomers (Figure 5C), under higher concentration of ACN, the monolith showed better separation. At lower concentration of ACN, the peak, especially the second peak of the enantiomeric pair, turned broad and this resulted in lower plate numbers.

3.2.3 Effect of column temperature—Column temperatures from 15 to 40 °C have been studied to investigate the temperature on the chiral separation property, and hexobarbital was used as a model compound. As shown in Figure 6A, with the increase of temperature, there was an almost linear increase in EOF because the viscosity of the mobile phase decreased. The k of hexobarbital (assumed to be neutral at pH 4.0, 0.3% TEA, 50% (v/v) ACN), shows more or less decreasing trend with increasing EOF. The two exceptions were seen in the range of 15–20 °C and 35–40 °C where a slight increase in k was observed. While, the former increase in k in the lower temperature range, could be related to non-

equilibration of the column at lower temperature range, we hypothesized that the later increase in k could be attributed to the increase in temperature induced ionization of hexobarbital. For the resolution and separation efficiency of hexobarbital (Figure 6B), the obvious effect of decreasing R_s and N only was seen in the higher temperature range of 25–40°C. For hexobarbital, 25°C was a good compromise between of k vs. R_s or N .

3.3 Chiral screening with GMA- β -CD-2 monolithic column

To extensively investigate the enantioselectivity and resolving power of the GMA- β -CD-2 monolithic column, a wide range of chiral compounds from neutral to basic as well as few acidic compounds were screened. A total of 32 chiral compounds listed in Table 2 were completely or partially resolved on the GMA- β -CD-2 monolithic column. The analytes were first classified into neutral, basic, and acidic compounds in solution phase and then discussed in group based on their structural similarity. The analyte classification is based on what type of charge are present in the analyte in the solution phase. For example, most of the basic compounds due to presence of protonated amine group are positively charged under the pH conditions studied as well as the gas phase in electrospray. The only exception was parahydroxyamphetamine, which exist as zwitterionic species in the CEC mobile phase. The acidic compounds (e.g., hexobarbital and catechin may exist as neutral compounds (due to presence of unionizable phenolic group) in solution phase at the optimized CEC mobile phase pH of 4.0 (Table 2, last two rows). However, in electrospray, the same neutral compounds usually exist as negatively charged compound in the gas phase. Nevertheless, mobile phase conditions listed for each compound were optimized taking into considering of α , R_s and N .

For the neutral analytes, 5 out of 10 analytes could be resolved with resolutions in the range of 1.0–1.4. In particular, high resolution (i.e., $R_s > 1.5$) of flavanone and chlorthalidone could be easily achieved within 20 minutes with 50% ACN in the mobile phase. Although benzoin provided a resolution value of 1.03, but three of its derivatives were easily baseline separated when lower concentration of ACN was used. The relatively lower selectivity and resolution of benzoin than those of its alkylated derivatives suggest that the substitution group linked to the chiral center is favorable for chiral recognition. Hence, steric interaction is important for the formation of inclusion complex between the analytes and the β -CD cavity.

The GMA- β -CD-2 monolithic column also showed good chiral resolution and selectivity for acidic and basic analytes. Very high resolution of two acidic compounds, hexobarbital and catechin (in the neutral form), were resolved with $R_s > 2.5$. Baseline resolution of several basic compounds such as aminoglutethimide, pseudoephedrine, Tröger's base, propranolol and prilocaine was easily observed. On the other hand, hydroxymethamphetamines and micanazole could get separations with resolution higher than 1.0. However, there are 16 out of 20 basic chiral analytes, which could be only partially resolved with resolution lower than 1.0. Note that pseudoephedrine with two chiral centers exist as four stereoisomers providing baseline resolution of only enantiomers of pseudoephedrine but its diastereomeric pairs of ephedrine were only slight resolved. For β -blockers (except for propranolol), it appears that baseline separation could not be easily achieved under the reverse phase mode. However, our recent studies have shown that under non-aqueous mobile phase conditions, baseline resolution could be obtained. The results under non-aqueous conditions are on-going and will be reported at a later date.

3.4 Application of GMA- β -CD-2 monolithic column in CEC-ESI-MS

From the discussion in previous sections and the data shown in Table 2, clearly GMA- β -CD-2 monolithic column is a versatile stationary phase for a broad range of chiral analytes

in CEC. In this section, its application in CEC-ESI-MS is discussed. Direct infusion of several representative chiral compounds was first conducted to obtain optimum fragmentor voltage, which produce the most abundant protonated ($[M+H]^+$) or deprotonated ($[M-H]^-$) molecular ions. Under the optimum fragmentor voltage, each of the analyte was detected SIM at the selected m/z (providing highest abundance) for the on-line CEC-ESI-MS in either positive or negative ion mode.

The ESI-MS parameters for each studied compounds are listed in Table 3. Figure 7 shows comparison of CEC-UV and CEC-ESI-MS chromatograms of the acidic and basic chiral compounds. Several points are worth mentioning. First, note that racemic mixture of hexobarbital and non-racemic mixture of catechin are acidic compounds but remains neutral under the mobile phase separation conditions. However, when these compounds are ionized in the gas phase in ESI, they exist as negatively charged enantiomers. Second, flavanone and Tröger's base are neutral compound under the mobile phase conditions used for separation, but are ionized as abundant ($[M+H]^+$) ion in the gas phase in electrospray. Overall, CEC-UV provided higher resolution and shorter retention times but CEC-MS provides higher S/N . This is because the configuration used in CEC-ESI-MS system is different from column used in CEC-UV. In case of CEC-UV, the effective length (25 cm) of the monolithic bed is the same as CEC-MS, however, there was an empty segment of 20 cm from the inlet side of the CEC-MS column, which may have contributed to some band broadening and resolution loss in CEC-ESI-MS. To understand the difference between UV and ESI-MS on sensitivity, the S/N ratios are also marked in Figure 7. Although the ESI parameters have not been extensively optimized, CEC-ESI-MS still showed 2–32 folds higher S/N than CEC-UV for all of the generated chromatogram of each analytes.

3.5 Column stability and reproducibility

Column stability and reproducibility are crucial to demonstrate the ruggedness of a new chiral stationary phase. Hexobarbital was used as a model compound for the evaluation of the column separation stability and reproducibility. A total of thirty injections of hexobarbital were performed for two consecutive days on GMA- β -CD-2 monolithic column. The detailed reproducibility data shown in Table 4 revealed that the intra-day RSD for retention time was 3.1–6.4% and the inter-day RSD was 3.4–4.7%. The reproducibility of selectivity is also excellent with intra-day and inter-day RSD s lower than 2.3% and 1.6%, respectively. Compared to other parameters such as R_s and α , the % RSD of plate number N is slightly high (10–13%), but it is still considered satisfactory for practical applications. Therefore, the results clearly demonstrated the good separation stability and reproducibility of the optimized GMA- β -CD-2 monolithic column.

4 Concluding remarks

Instead of typical two-step method reported in the literature, a simple alternative copolymerization method was presented to prepare polymer based monolithic column, which combines the chiral recognition ability of β -CD and versatility of monolithic material. Based on the effect of DS of GMA- β -CD on the enantioselectivity and separation efficiency, we found that GMA- β -CD monolithic column prepared with DS of ~ 2.0 demonstrated the best chiral separation performance. Mobile phase parameters such as pH of the buffer, organic modifier content were also systematically optimized to manipulate the hydrophobic inclusion between the analytes and chiral stationary. Additionally, chiral screening was conducted for a wide range of chiral compound under the optimized conditions. A total of 32 chiral compounds could be baseline or partially resolved using the proposed GMA- β -CD monolithic column, suggesting that this chiral monolith appeared to be more versatile in comparison with the previously reported chiral monolithic columns in CEC.

Furthermore, chiral analysis is also possible using the β -CD monolith in CEC-ESI-MS, which not only showed high sensitivity, enantioselectivity and efficiency, but also exhibited homogenous microflow and no loss of material at the CEC-MS interface. This is because the GMA- β -CD monolith was tightly anchored to the capillary wall. In addition, GMA- β -CD monolithic column demonstrated excellent stability (60 injections in 2 days) and separation reproducibility with an inter-day % RSD of 3.4–4.7% for retention time, 1.7% for selectivity and 4.2% for resolution. Hence, the development of this new kind of CD-bonded monolithic column has great potential in extending the preparation of CD modified monolithic column for separation of even more classes of chiral compounds using other classes of charged monomers and crosslinker, which will provide even higher selectivity and versatility to cover even broader range of chiral analytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DS	degree of substitution
EDMA	ethylene dimethacrylate
GMA-β-CD	glycidyl methacrylate- β -cyclodextrin

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Step 1: **Step 2:** **Figure 1.**

Synthetic scheme for the preparation of GMA- β -CD (step 1), and copolymerization with EDMA and AMPS to form a chiral monolithic column.

Figure 2. Scanning electron micographs (SEMs) of monolith columns. column GMA-β-CD1: DS=1.0; GMA-β-CD2: DS=2.0; GMA-β-CD3: DS =3.0. Detailed information of the polymerization mixture composition for the monolith is described in Table 1.

Figure 3.

The effect of degree of substitution (DS) of GMA- β -CD on the chiral separation properties: (A) Resolution R_s , (B) apparent retention factor k for the first elute of the two enantiomers, and (C) average plate number N of the two enantiomers. Conditions: mobile phase, 50% ACN in 5 mM ammonium acetate buffer containing 0.3% TEA, pH 5.0; applied voltage, +20 kV; detection, 214nm; electrokinetic injection, 5 kV, 3 s of 0.5 mg/mL of each racemic compound; capillary temperature 25°C.

Figure 4.

The effect of pH of buffer on the (A) apparent retention factor k , (B) the resolution R_s and (C) average plate number N of the enantiomeric pairs. Conditions: column, GMA- β -CD, mobile phase, 50% ACN in 5 mM ammonium acetate buffer containing 0.3% TEA, pH was adjusted using acetic acid; applied voltage, +20 kV; detection, 214nm; samples concentration, 0.5mg/mL for each racemic compound; electrokinetic injection, 5 kV, 3 s; capillary temperature 25°C.

Figure 5.

The effect of acetonitrile content in the aqueous triethylamine acetate buffer on (A) apparent retention factor k , (B) the resolution between the two enantiomers R_s and (C) average plate number N of the two enantiomers. Conditions: column, GMA- β -CD-2; mobile phase, different concentration of ACN in 5 mM ammonium buffer containing 0.3% TEA, pH 4.0; applied voltage, +20 kV; detection, 214nm; samples concentration, 0.5mg/mL for each racemic compound; electrokinetic injection, 5 kV, 3 s; capillary temperature 25°C.

Figure 6.

The effect of capillary temperature on the (A) EOF and apparent retention factor k of the first-eluting enantiomer; (B) the resolution R_s and average plate number N of the two enantiomers of hexobarbital. Other conditions are same as mentioned in Figure 5.

Figure 7. CEC-ESI-MS and CEC-UV separation of hexobarbital, catechin (**non-racemic mixture**), flavanone, pseudoephedrine, aminoglutethimide, Tröger's base, and prilocaine chiral compound on GMA- β -CD-2 monolithic column. Mobile phase conditions are the same as shown in Table 2; applied voltage, +30 kV for CEC-ESI-MS, +20 kV for CEC-UV; detection, 214nm; samples concentration, 0.5mg/mL for each racemic compound; electrokinetic injection, 5 kV, 3 s; capillary temperature 25 °C. ESI-MS parameters are shown in Table 3 and the experimental section.

Table 1 Preparation of GMA- β -CD with different concentration of GMA and β -CD

	GMA: β -CD ^a	DS ^b
GMA-CD1	1.4:1	1.0
GMA-CD2	2.8:1	1.8
GMA-CD3	5.6:1	2.6

^a Molar ratio of GMA and β -CD used in the preparation of GMA- β -CD.^b degree of substitution.

Table 2

Chiral screening results: Apparent retention factor (k), selectivity (α), resolution (R_s) and plate number (N , plates/m) of neutral, basic and acidic chiral compounds. Mobile phase conditions are the optimal conditions for each analytes. Other conditions are the same as mentioned in Figure 6.

Analytes	Mobile phase		Separation properties			
	pH	%ACN	k	α	R_s	N (plates/m)
Neutral analytes						
Flavanone	4.0	50	0.39	1.08	2.53	133000
Chlorthalidone	4.0	50	0.49	1.08	1.96	65000
Benzoin	4.0	20	5.72	1.04	1.03	40000
Hydrobenzoin	4.0	20	4.71	1.08	1.65	31000
Benzoin methyl ether	4.0	20	8.22	1.07	1.65	60000
Benzoin ethyl ether	4.0	20	8.89	1.06	1.43	24000
PTH-leucine	4.0	50	0.36	1.03	1.31	136000
PTH-isoleucine	5.0	50	0.18	1.03	0.55	21000
PTH-valine	4.0	50	0.24	1.01	0.57	174000
5-Methyl-5-phenylhydantoin	4.0	30	3.08	1.03	0.53	36000
Basic analytes						
Terbutaline	5.0	50	0.60	1.03	0.46	46000
Doxylamine	4.0	40	4.96	1.09	0.62	4000
Ketamine	5.0	50	1.48	1.01	0.21	12000
Hydroxymethamphetamine	4.0	40	1.85	1.05	1.22	32000
Pseudoephedrine	3.0	50	0.87	1.14	2.71	24000
Ephedrine	4.0	50	1.01	1.01	0.10	45000
Atenolol	5.0	50	0.88	1.02	0.61	36000
Alprenolol	4.0	40	3.20	1.04	0.82	41000
Metoprolol	4.0	40	3.16	1.02	0.60	75000
Propranolol	3.0	50	1.29	1.04	1.57	101000
Troger's base	3.0	50	1.09	1.15	2.81	35000
Bupivacine	4.0	40	1.34	1.04	0.62	29000
Prilocaine	4.0	50	1.77	1.09	2.47	50000
Ipratropium bromide monohydrate	4.0	50	2.83	1.03	0.43	26000

Analytes	Mobile phase		Separation properties			
	pH	%ACN	k	α	R_s	N (plates/m)
laudanosine hydrobromide	5.0	50	0.68	1.02	0.40	13000
1-(1-Naphthyl)ethylamine	4.0	50	1.32	1.07	0.87	12000
PTH-arginine	4.0	30	3.09	1.02	0.41	69000
PTH-histidine	3.0	50	0.79	1.02	0.62	76000
Miconazole	4.0	50	4.53	1.12	1.04	6000
Aminoglutethimide	3.0	50	3.50	1.11	1.81	31200
Acidic analytes						
Hexobarbital	4.0	50	1.92	1.07	2.73	107000
Catechin	4.0	50	1.21	1.07	2.77	132000

Table 3

ESI/MS conditions for chiral CEC separations of acidic and basic analytes

Analytes	ESI mode	SIM ions (m/z)	Fragmentor voltage (v)
hexobarbital	negative	235[M-H] ⁻	72
catechin	negative	289 M-H] ⁻	104
flavanone	positive	225 [M+H] ⁺	64
pseudoephedrine	positive	166 [M+H] ⁺	80
Troger's Base	positive	251 [M+H] ⁺	108
aminoglutethimide	positive	245 [M+H] ⁺	100
prilocaine	positive	237 [M+H] ⁺	100

Table 4

Intra-day and inter-day reproducibility of retention times, selectivity, resolution and efficiency for hexobarbital on GMA- β -CD2 monolithic column in CEC. Separation conditions are the same as mentioned in Figure 6.

Day	Total Injection	Average retention time		Average selectivity (RSD, %)	Average resolution (RSD, %)	Average efficiency (plates/m) (RSD, %)
		(min) (RSD, %)	t_{R2}			
1	30	22.5 (3.1)	23.7 (4.8)	1.07 (2.3)	2.65 (5.3)	112,000 (10)
2	30	21.2 (5.7)	22.6 (6.4)	1.06 (1.6)	2.71 (4.9)	104,000 (13)
Overall	60	21.9 (3.4)	23.2 (4.7)	1.07 (1.7)	2.68 (4.2)	108,000 (10)