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Journal of Chromatography A, 955 (2002) 125–131

JOURNAL OF CHROMATOGRAPHY A

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New triazine spectroscopic reagent for the separation of DL-amino acids by micellar electrokinetic chromatography

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Received 30 August 2001; received in revised form 28 February 2002; accepted 28 February 2002

Abstract

An approach to the chiral separation of racemic mixtures of amino acids by means of micellar electrokinetic chromatography after derivatization with a new triazine spectroscopic reagent, 3-(4,6-dichloro-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine (DTDP), has been evaluated. It was found that the derivatives of the aliphatic amino acids such as serine, valine and arginine, could produce a strong UV absorption at 282 nm, whose apparent molar absorptivities are of 10^4 M^{-1} cm^{-1}, and thus the concentration of the amino acids down to 3×10^{-7} M can still give a detectable signal ($S/N=3$). β-Cyclodextrin (β-CD) added to the buffer system was used as a chiral selector, and separation conditions were optimized. The presence of an organic modifier (2-propanol) was also a prerequisite for the chiral separation. The best results for the chiral separation of DTDP-amino acids were achieved in a mixed sodium dodecylsulfate–β-CD–borate–2-propanol medium at pH 9.0. Compared to some of the commonly used derivatization methods, the present one offers a relatively stable derivative and strong UV absorption for the spectroscopically inert amino acids, thus enabling amino acids to be separated and detected by CE even with a simpler UV detector. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, electrophoresis; Enantiomer separation; Triazines; Amino acids; Dichlorotriazinylamino-dimethylaminomethylphenazine

1. Introduction

Optically active compounds are an integral part of most biological systems. Amino acids are found as a single enantiomeric form within a biogenic macromolecule. For example, proteins consist of L-amino acids, while natural sugars are D-enantiomers. Since amino acids are an important group of substances, much effort has been devoted to their analysis. Capillary electrophoresis (CE), due to its high separation efficiency, short analysis time, and compatibility with small sample volumes, has been receiving much research interest for enantiomeric separation [1–11].

The lack of a strong chromophore for aliphatic amino acids has been one of the limitations in their analysis by CE. With the current CE techniques for chiral separation, therefore, the derivatization of amino acids with a strong chromophore or suitable fluorophore has become the most prevalent methodology to improve sensitivity. An ideal derivatiza-

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PII: S0021-9673(02)00230-3

tion reagent should fulfil several requirements [2]. It must contain or produce a strong chromophore or fluorophore for spectroscopic measurements. The reagent should be stable and give rapid reactions in high yields at low temperatures. The reaction products should be sufficiently stable, and excess reagent or by-products from the reaction should not disturb the separation. A variety of reagents have been proposed for precolumn derivatization in CE [3]. Commonly employed derivatization reagents with strong UV chromophores include 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns), 9-fluorenylmethyl chloroformate (FMOC-Cl), 2,4-dinitrofluorobenzene, *o*-phthaldialdehyde (OPA), naphthalene-2,3-dicarboxaldehyde, phenyl isothiocyanate, etc. Among these derivatization reagents, dansylation is the most commonly applied derivatization method [4–6]. Various separation systems providing chiral selectivity have been used for the separation of these types of derivatives. To increase further resolution, many buffer additives have been introduced, such as surfactant [7,8], cyclodextrin (CD) [9–12], crown ether [13] and bile salt [14], etc. It has proven effective that they can provide additional selectivity over that obtained with traditional electrolytes for the separation of complicated underivatized and derivatized amino acids.

Micellar electrokinetic chromatography (MEKC) has been a successful approach to improve the separating power of CE for derivatized amino acids. Selectivity in MEKC is based on the differential partitioning of an analyte between the aqueous phase and pseudostationary micelle phase. The mechanism of partitioning in MEKC is complex and becomes even more intricate for ionic species, such as amino acids, where the analytes migrate by a combination of their own electrophoretic mobility and that induced by the micellar phase which makes separations difficult to predict [15]. To achieve chiral separation, cyclodextrins are often added to the running electrolyte since they can offer separation systems of high separation efficiency and reasonable selectivity, and do not interfere with UV-photometric detection. The combination of cyclodextrins and MEKC has proven extremely successful in the resolution of derivatized amino acid enantiomers. The use of chiral surfactants (bile salts), β-cyclodextrin and γ-cyclodextrin with sodium dodecylsulfate (SDS) in CD-modified MEKC have been reported for the enantiomeric separation of dansylated amino acids [16,17].

In connection with a program of investigating new spectroscopic probes, a series of triazine reagents have been prepared in our laboratory [18,19], one of which is 3-(4,6-dichloro-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine (DTDP) containing a strong UV absorption group. Although chiral monohalo-*s*-triazine reagents were reported for the liquid chromatographic resolution of DL-amino acids [20], so far triazine reagents have not been applied to chiral separation of DL-amino acids by CE. In this work, the application of DTDP to the chiral separation of DL-amino acids was evaluated. The effects of time and temperature on the derivatization reaction, the concentration of surfactant (SDS) and pH on the resolution and the selectivity were investigated. Further, the effects of chiral selector (β-CD) and organic modifier were also studied.

2. Experimental

2.1. Apparatus

MEKC was carried out with an ISCO Model 3850 capillary electropherograph (Lincoln, NE, USA) equipped with uncoated fused-silica capillary tubing (50 μm I.D., Hebei, China), 65 cm in total length. On-column UV detection was performed at 282 nm at a position 45 cm from the inlet. The applied high voltage was 18 kV. Samples were introduced by electromigration at the anodic end of the capillary for 5 s under the same voltage. The separation was carried out at room temperature (25 °C). New capillaries were conditioned with 0.2 *M* sodium hydroxide for 2 h before use. Prior to each sample injection, the capillary was first rinsed with 0.1 *M* NaOH for 5 min, then with water for 5 min and finally conditioned with the running buffer for 10 min. UV–visible spectra were recorded in 1-cm cells with a Model 8500 UV–Vis spectrophotometer (Techcomp, Shanghai, China).

2.2. Reagents

The amino acids were obtained from Sigma. SDS

was obtained from Serva. β-CD, borate and phosphate were purchased from Beijing Chemical Factory. Following a similar procedure [18,19], the new triazine reagent DTDP (Fig. 1) was synthesized by treating equimolar portions of cyanuric chloride and neutral red at 0–5 °C for 1 h in the presence of NaOH (pH 8–9), and characterized by MS analysis (m/z: 400$[M+H]^+$) and elemental analysis (calculated for $C_{18}H_{15}N_7Cl_2 \cdot H_2O$: C, 51.72; H, 4.10; N, 23.45%; found: C, 52.03; H, 4.36; N, 21.78%). Other reagents for preparation of buffers were of at least analytical grade. Doubly distilled water was used to prepare all solutions. Buffers were prepared daily and filtered through a 0.45 μm-porous filter before use.

2.3. Derivatization procedure

For the study of chiral separation, several DL-amino acids were selected here as examples according to representative side-chain features, such as serine (Ser, hydroxymethyl), glutamic acid (Glu, acidic) and valine (Val, neutral, sterically hindered). Standard solutions of selected DL-amino acids (100 m*M*) were prepared in 1 *M* HCl media. To 20 μl (2 μmol) of standard solution, 50 μl of 1.0 *M* Na_2CO_3 solution and 2 ml (2 μmol) of DTDP solution (1 m*M*) were added and the mixture was heated for 0.5 h at 40–45 °C. Then the reaction solution was diluted to 4 ml with water, and this sample was ready for introduction.

3. Results and discussion

3.1. Optimization of derivatization procedure

It is well-known that cyanuric chloride has three chlorine atoms that are readily controlled for stepwise substitutions, depending upon temperature and pH of the reaction medium. The second chlorine atom is still rather active and often reacts at room temperature after the replacement of the first one [21,22]. Therefore the reaction of the synthesized DTDP with the nucleophiles such as amino acids can take place under a mild condition. Moreover, the resulting derivatization products would have a strong UV absorption since DTDP contains a large chromophore (phenazine group). This would render the basis for sensitive detection of amino acids. Fig. 2 shows the UV spectra of DTDP and one of its amino acid derivatives, illustrating that the absorption peak of DTDP is located at 282 nm with a molar absorptivity (ε) of 4.4×10^4 M^{-1} cm^{-1}, and the spectrum change is rather small after derivatization. The apparent molar absorptivities of these compounds are about 10^4 M^{-1} cm^{-1}, and thus the concentration of the amino acid derivatives down to 10^{-7} *M* can still give a detectable signal ($S/N=3$) even with a simpler UV detector.

To optimize the derivatization condition, the effects of heating time and temperature were studied. It was found that the first chlorine atom replacement of DTDP by amino acids proceeded smoothly at 30–

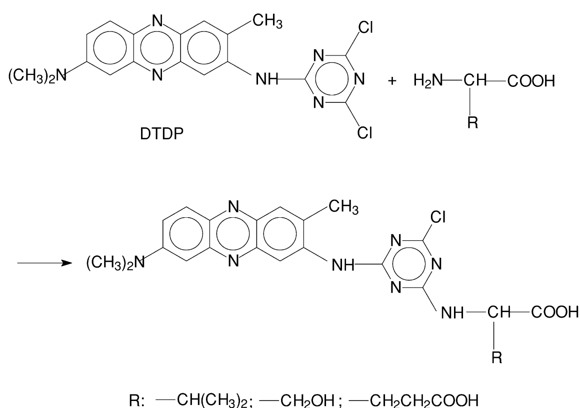

Fig. 1. Reaction of 3-(4,6-dichloro-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine (DTDP) with amino acids.

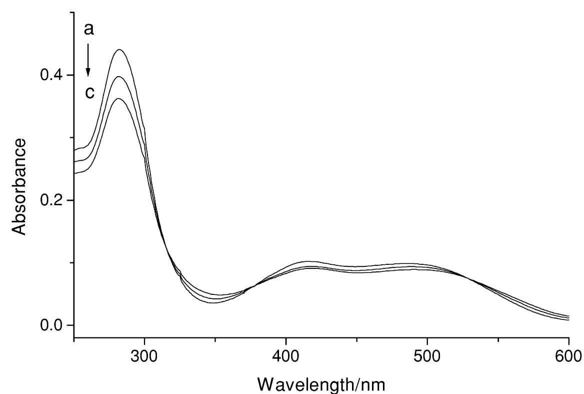

Fig. 2. The UV spectra of DTDP and DTDP-Ser. The molar ratio of serine to DTDP is 0, 1, 2 for curves a, b, and c, respectively. The concentration of DTDP is 1×10^{-5} *M*.

45 °C, and a single reaction product was formed based on TLC. Above 50 °C, however, very complex derivatization products were yielded, and it was difficult to distinguish and separate them. To achieve the chiral separation and decrease the disturbance of the by-products, a heating temperature of 40–45 °C was chosen. It was also found that as the heating time increased, the absorbance of derivatives increased too, and it had a relatively flat maximum after 0.5 h (Fig. 3). This time could thus be chosen for the derivatization time. At room temperature, the derivatives were stable for at least 24 h by monitoring their UV absorption. The yield of the reaction of DTDP with amino acids was estimated through CE analysis, i.e. through comparing the peak height of DTDP with that of the remaining reagent after reaction with amino acids ($(H_0 - H)/H_0$, H_0, peak height of DTDP; H, peak height of the remaining DTDP). It was found that under the optimal condition (heating at 40–45 °C for 0.5 h), a yield of 90% could be obtained at a concentration of 2 μ*M* of the reactants. We also checked the reactivity of DTDP with other common nucleophiles such as phenol, ethanol and *p*-toluidine. The results showed that the presence of these nucleophiles, not more than 25 times of amino acids, did not affect the yield of DTDP-amino acids, suggesting that the present condition is the most favorable one for derivatizing amino acids. In addition, it is better to use about equimolar quantities of amino acids and DTDP for the derivatization, since if a large excess of DTDP was employed (e.g. more than twofold of amino acids) it would interfere with the separation of the derivatives.

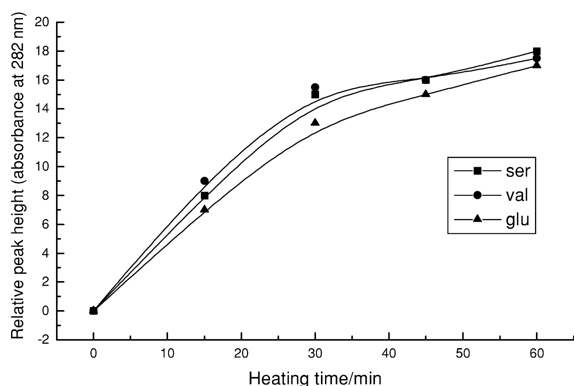

Fig. 3. Effect of the heating time on the peak height of the DTDP-amino acids.

3.2. Optimization of the separation conditions of DTDP-amino acids by MEKC

In CE, a number of variables have to be considered when optimal conditions are sought. For the present system, chiral separation of DTDP-amino acids may be influenced by many factors such as the concentration of SDS, β-CD, organic modifier and pH. The proper choice of key separation parameters may result in favorable resolutions.

3.2.1. Effects of β-CD, SDS and pH on the chiral separation of DTDP-amino acids

For the separation of DL-amino acids derivatized with DTDP, β-CD used as a chiral selector was added to the borate buffer system, and several key factors (pH, β-CD and SDS concentrations) were optimized in detail. The separations were characterized by resolution (R_s) values [5]. The tested ranges of the factors were pH 7.0 to 9.5, β-CD concentration 10 to 60 m*M* and SDS concentration 5 to 80 m*M*, respectively. A mixture of three different DTDP-amino acids (Ser, Glu and Val) was selected as a sample.

β-CD has a limited solubility in water [1], but this can be increased by adding alcohols or urea to the buffer solution [11]. Moreover, it was reported that the introduction of alcohols and urea could not only improve the resolution but also prevent the adsorption of analytes on the capillary wall [1]. So, 50 m*M* of urea was added to the buffer. In this case, 30 m*M* of β-CD was found to be suitable. However, if its concentration exceeded this value, the migration solution became unstable, readily producing a precipitate.

Different amino acids have different optimal SDS concentrations. For example, serine, the first eluted amino acid, required a relatively high SDS concentration, ca. 30 m*M*. In contrast, for the last eluted amino acid, glutamic acid, the SDS concentration may be down to 5 m*M*. Higher SDS concentration (>30 m*M*) could result in increased resolution for the three examined amino acids as a whole, but prolonged the migration times; e.g. the use of 60 m*M* SDS gave a migration time of 12.4 min for DTDP-

Ser (D-form). A moderate SDS concentration of 30 m*M* was thus employed in this study. pH is often an important factor affecting the separation. However, the presence of β-CD could decrease the susceptivity of the system to pH, i.e. pH change from 7.0 to 9.5 hardly affected the resolution of the DTDP-amino acids. So, a pH of 9.0 may be used in this work.

3.2.2. Effect of organic modifier on chiral separation

Addition of organic solvents often changes the inclusion complex formation constant, thus affecting the optimum concentration of β-CD and the critical micelle concentration of surfactant; separation may thereby be improved or worsened [27]. In this work, the introduction of two different organic modifiers, acetonitrile and 2-propanol, was examined. The results showed that 2-propanol was better than acetonitrile for chiral separation of DTDP-amino acids by β-CD-modified MEKC. Perhaps the reason for this is that 2-propanol resulted in greatly decreased electroosmotic flow as compared with acetonitrile. Penn et al. have discussed the merit of small amounts of an organic modifier required for the moderation of the analyte-β-CD interaction in the buffer [28], and in this work only a low concentration of 2-propanol (10%) was used. The experimental results confirmed again that the presence of 2-propanol was a prerequisite for the chiral separation of DTDP-amino acids with β-CD-MEKC, just like the case for FMOC-amino acids [2,9]. Fig. 4 shows a typical electropherogram of the chiral separation of a mixture of DTDP-amino acids under the determined optimal conditions, indicating that a baseline resolution is nearly achieved (Ser, $R_s = 0.90$; Val, $R_s = 1.05$; Glu, $R_s = 1.0$).

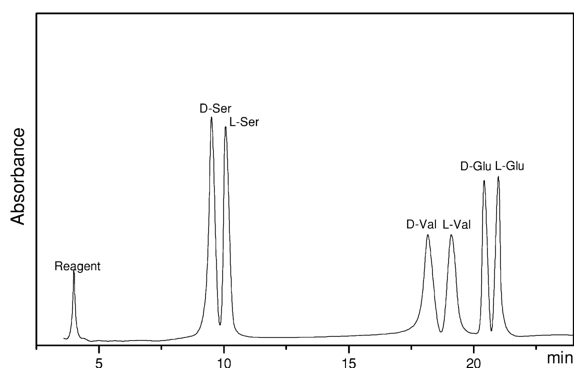

Fig. 4. Electropherogram of a mixture of three DTDP-amino acids. Conditions: 30 m*M* SDS, 50 m*M* urea, 20 m*M* borate, 30 m*M* β-CD and 10% 2-propanol at pH 9.0.

The separation mechanisms of the chiral separation of derivatized amino acids when using cyclodextrins as chiral selectors have been discussed by several researchers [29,30]. In the present β-CD-MEKC system, the mechanism of chiral separation should be a direct one, because the introduction of a chiral selector β-CD led to the enantiomeric separation of DTDP-amino acids [2,15]. The analytes are distributed among the β-CD, the micelles and the aqueous phase. The partitioning of the analytes among these three phases is strongly influenced by the presence of an organic modifier. It seems that 2-propanol can influence interactions either by means of a dynamic modification of the CD-hydroxyls and/or by inclusion in the cavity, thus modifying the cavity size. It should be noted that such cavity size modification could be achieved also by inclusion of SDS monomers. β-CD itself migrates at a velocity identical to the electroosmotic flow. Consequently, when a solute tends to interact with a β-CD through the formation of an inclusion complex, its migration time becomes shorter. The difference in migration time for a solute with β-CD is strongly dependent on the degree of complexation of the solute with β-CD. Therefore, addition of β-CD to the micellar solution can change the migration selectivity for the solutes in MEKC [30]. As shown in Fig. 4, the D-form of amino acids was first eluted, thus indicating that the D-form is more strongly bound to β-CD than the L-form. In addition, this elution order may be beneficial in the determination of large enantiomeric excess for natural L-amino acids, since the D-enantiomer is usually considered as an impurity and its elution prior to the large peak favors quantitation. It should be pointed out that β-CD was the only CD explored and only three selected amino acids demonstrated in this work. Further studies involve examining other CD and more DL-amino acids.

3.3. Comparison with other derivatization methods

A comparison of DTDP with other derivatization reagents with UV chromophores is summarized in Table 1. Some reagents produce fluorescent deriva-

Table 1
Comparison of derivatization reagents for UV detection of amino acids

Reagent	ε (M^{-1} cm^{-1})	λ (nm)	Comments	Ref.
2-(9-Anthryl)ethyl chloroformate (AEOC)	1.8×10^5	256	pH 8.9, borate buffer, derivatization at room temperature for 5 min, form stable derivatives with both primary and secondary amino groups. Remove the excess reagent by extraction with pentane	[23]
5-Dimethylaminonaphthalene-1-sulfonyl chloride (Dns)	1.4×10^4	254	pH 8.5, phosphate–borate buffer, derivatization at room temperature in the dark for 5 h	[4–6]
9-Fluorenylmethyl chloroformate (FMOC-Cl)	$\sim1.0\times10^4$ [a]	265	pH 9.3, boric acid–sodium hydroxide buffer, derivatization for 1 min, rapid reaction with primary and secondary amines to form stable derivatives. Excess FMOC-Cl and its hydrolyzed products have to be removed by extraction with pentane, since they overlap with the derivatized amino acids in the chromatogram	[24]
o-Phthaldialdehyde (OPA)	– [b]	260	Rapid and selective derivatization with primary amino acids, and excess reagent is not fluorescent. But the derivatives are not stable	[25]
1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent)	3×10^4	340	Derivatization in 0.5 *M* sodium bicarbonate solution at 40 °C for 90 min. High sensitivity but time-consuming	[26]
DTDP	4.4×10^4	282	Derivatization at 40–45 °C for 0.5 h, stable derivatives. Moderate reaction rate	This work

[a] Determined in acetone in this work (interference was observed from its strong fluorescence).
[b] Unavailable.

tives after reaction, giving a high sensitivity. However, UV detection is more popular. Table 1 also shows that excess reagents need to be removed in some methods, while the others have a relatively unstable derivative or a long derivatization time. These data show that each reagent has its own characteristics, including advantages and disadvantages. The major features of DTDP are the relatively high molar absorptivity and stable derivatives, though its derivatization rate is moderate. Therefore, DTDP might provide a new choice for the derivatization and especially detection of amino acids with a more common and simpler UV detector by CE.

Acknowledgements

The authors gratefully acknowledge the financial support from the NNSF (No. 20175031, No. 20035010) of China and from CAS (CMS-CX200104).

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