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Enantiomer separation of *N*-protected amino acids by non-aqueous capillary electrophoresis and high-performance liquid chromatography with *tert*.-butyl carbamoylated quinine in either the background electrolyte or the stationary phase

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Abstract

A non-aqueous CE method was developed for evaluating the chiral discrimination potential of cinchona alkaloids and different kinds of carbamoylated derivatives of quinine and quinidine type chiral selectors towards acidic analytes, in particular a series of various Bz (benzoyl), DNB (3,5-dinitrobenzoyl) and DNZ (3,5-dinitrobenzyloxycarbonyl) amino acid derivatives. In this study, the enantioselectivity values obtained in non-aqueous CE with *tert*.-butyl carbamoylated quinine as chiral additive have been compared with the values found for the same series of selectands in HPLC using the same selector immobilized onto silica as chiral stationary phase. Similarly to the background electrolyte used in CE an ethanol–methanol mixture (60:40, v/v) containing 100 m*M* octanoic acid and 12.5 m*M* ammonia has been selected as HPLC mobile phase. Under these conditions, a good correlation ($r=0.954$) between the enantioselectivities observed with the two techniques has been obtained. Thus the non-aqueous CE method can be applied as a screening tool for the rapid evaluation of the chiral discrimination potential of a large set of newly developed chiral selectors derived from quinine and related alkaloids.

Keywords: Enantiomer separation; Background electrolyte composition; Non-aqueous capillary electrophoresis; Chiral stationary phases, LC; Chiral selectors; Amino acids; *Tert*.-butylcarbamoylquinine; Alkaloids; Cinchona alkaloids

1. Introduction

Quinine has been used successfully for enantiomer separation in high-performance liquid chromatography (HPLC) as a chiral ion-pairing agent in the mobile phase and as a chiral stationary phase (CSP) ligand [1–4]. In the last years, CSPs based on the use of carbamoylated derivatives of quinine and quinidine as selectors were found to be highly stereoselective for the direct resolution of chiral acids using mixtures of aqueous buffers and methanol or acetonitrile as mobile phases [5–16]. These new chiral selectors are classified as weak anion-exchangers, due to the presence of a tertiary amino group within the quinuclidine ring which is protonated at the usual working pH of the mobile phase. This primary ionic interaction between the anionic solutes (selectands, SAs) and the cationic selector (SO) is significantly accompanied by additional intermolecular interac-

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tions as hydrogen bonding, dipole–dipole, charge transfer (π–π), hydrophobic and steric interactions.

A CE method was developed for screening the chiral discrimination potential of this kind of derivatives as selectors and studying the interactions between these selectors and various kinds of analytes [12]. Due to the limited solubility of the quinine and quinidine derivatives in aqueous buffers, non-aqueous background electrolytes (BGE) were investigated. In a first study, the potential of quinine (QN) and *tert*.-butyl carbamoylated quinine (tBu-CQN) as chiral SOs for the enantiomer separation of *N*-protected amino acids was evaluated [17]. A buffer made of 12.5 m*M* ammonia, 100 m*M* octanoic acid and 10 m*M* SO in an ethanol–methanol mixture (60:40) was found to give the best compromise in terms of selectivity, resolution, efficiency, peak symmetry and analysis time. Using these experimental conditions and the reversed polarity mode, the free SO^+ and SA^- species exhibit countercurrent-like electrophoretic migration and have their overall velocity influenced by the cathodic electroosmotic flow. Thus, the free and complexed (ion-pair) SA species show significantly countercurrent mobilities, a fact that gives rise to high enantioselectivity. The principal contributions to the enantiomer separation are the differences in the ion-pair formation constants for (R)- and (S)-enantiomers of the SAs, based on enantioselective intermolecular interactions with the chiral SO. Due to the high molar absorptivities of the cinchona alkaloids derivatives, the electrolyte solution introduced in the reservoir at the anodic (detector) side was devoid of the UV absorbing chiral SO. When the separation started, the SO^+ was migrating towards the cathodic (injection) side so that its high absorbance background was leaving the detection window before the analytes were reaching this window [18]. This countercurrent separation technique revealed to be favourable in comparison with the total filling or the partial filling technique also studied [14,19–22].

The selected countercurrent CE system was applied for the rapid screening of different kinds of cinchona alkaloids and monomeric or dimeric forms of quinine and quinidine derivatives tested as SOs with regards to their enantioselectivity with a series of various N-protected amino acid derivatives as chiral acidic SAs, including Bz (benzoyl), DNB (3,5-dinitrobenzoyl) and DNZ (3,5-dinitrobenzyloxycarbonyl) amino acids (see Fig. 1) [18,23–25].

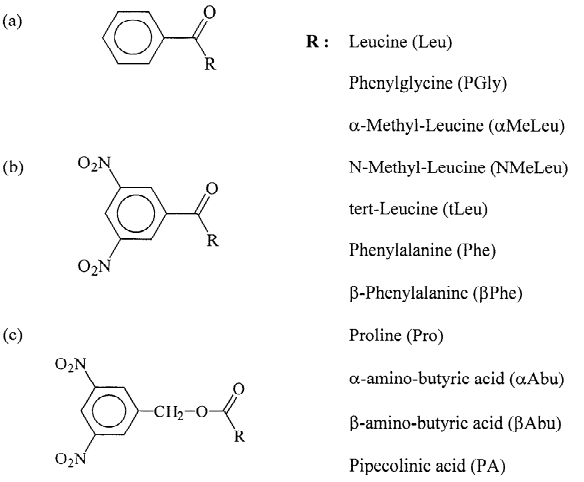

Fig. 1. *N*-protected α-amino acid derivatives: (a) Bz (benzoyl), (b) DNB (3,5-dinitrobenzoyl), (c) DNZ (3,5-dinitrobenzyloxycarbonyl).

In this study, the enantioselectivity values obtained in CE with tBu-CQN as chiral additive (see Fig. 2a) have been compared with the values found for the same series of selectands in high-performance liquid chromatography using tBu-CQN immobilized onto silica as chiral stationary phase (see Fig. 2b).

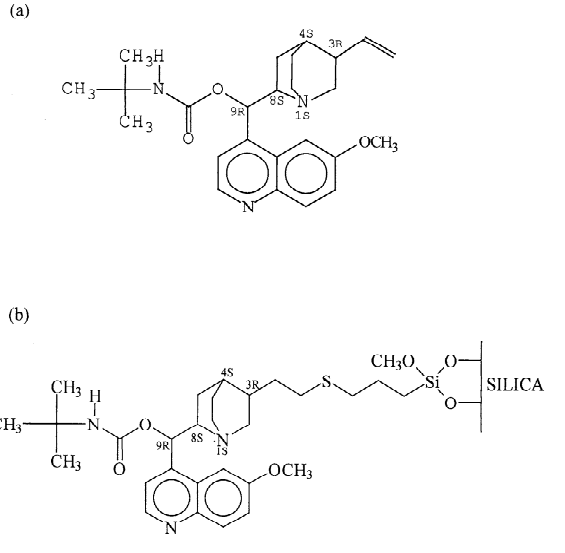

Fig. 2. (a) Chiral selector: *tert*.-butyl carbamoylated quinine (tBu-CQN). (b) tBu-CQN chiral stationary phase.

Similarly to the BGE used in CE a mixture ethanol–methanol (60:40, v/v) containing 100 m*M* octanoic acid and 12.5 m*M* ammonia has been selected as HPLC mobile phase.

2. Experimental

2.1. Chemicals

The organic solvents were of HPLC grade:ethanol absolute from Merck (Darmstadt, Germany) and methanol from Fisher Scientific (Leicestershire, UK). Octanoic acid and benzyl alcohol were from Sigma (St. Louis, MO, USA) and 25% ammonia solution from Carlo Erba (Rodano, Italy). tBu-CQN was synthesized according to a standard procedure described elsewhere [26]. The racemic and enantiomerically pure amino acids were purchased from Sigma. Bz and DNB derivatives were synthesized according to standard derivatization procedures [10] except DNB-Leu and DNB-PGly obtained by Sigma. To synthesize the DNZ compounds, aqueous solutions of amino acid were derivatized with 3,5-dinitrobenzyl chloroformate [11]. The sample solutions were prepared by dissolving each amino acid derivative at a concentration of 50 μg/ml in methanol. These sample solutions and the CE buffers were filtered through a Polypure polypropylene membrane filter (0.2 μm) from Alltech (Laarne, Belgium) before use.

2.2. CE experimental conditions

All experiments were performed on a SpectraPHORESIS 1000 CE instrument (Spectraphysics, San Jose, CA, USA) equipped with an autosampler, an UV–visible detector (190–800 nm) and a temperature control system (15–60 °C). Electrophoretic separations were carried out with uncoated fused-silica capillaries, 44 cm (37 cm to the detector) × 50 μm I.D., provided by Supelco (Bellefonte, PA, USA). A column cartridge was obtained from Spectraphysics.

The buffer was made of 100 m*M* octanoic acid, 12.5 m*M* ammonia and 10 m*M* of tBu-CQN (see Fig. 2a) in ethanol–methanol (60:40). At the beginning of each working day, the capillary was washed with ethanol–methanol (60:40, v/v) for 5 min and with the running buffer for 10 min, while after each injection the capillary was washed with the solvent mixture for 1 min and was equilibrated with the buffer mixture for 10 min. The injections were made at the cathodic side and the applied voltage was −25 kV (reversed polarity mode). The UV detection (at the anodic side) was performed at 214 nm. Injections were made in the hydrodynamic mode for a period of 5 s (corresponding to 14.5 nl) and the capillary was thermostated at 15 °C.

The resolution (R_s) and the plate number (N) were calculated according to the standard expressions based on peak width at half-height [27]. The selectivity (α) was calculated according to $\alpha = \mu_{e1}/\mu_{e2}$ where $\mu_e = \mu_a - \mu_{EOF}$ (μ_e is the effective mobility, μ_a is the apparent mobility and μ_{EOF} is the electroosmotic mobility).

2.3. HPLC experimental conditions

HPLC experiments were performed with a Merck–Hitachi HPLC system (Darmstadt, Germany) equipped with a L-6200A intelligent pump, an AS-2000A autosampler, a L-4250 UV–visible detector, a D-6000 interface and a L-5025 column thermostat.

The chiral stationary phase was synthesized according to the standard procedure described elsewhere [6] by immobilizing the tBu-CQN selector on Kromasil 100-5 μm spherical silica particles (EKA Nobel, Bohus, Sweden). The modified chiral sorbent with a selector coverage of about 0.32 mmol/g silica was packed into a 150 mm × 4.6 mm I.D. column at the Forschungszentrum Seibersdorf (Austria). The structure of the CSP is given in Fig. 2b.

Except otherwise stated, the mobile phase was a mixture of ethanol–methanol (60:40) containing 100 m*M* octanoic acid and 12.5 m*M* ammonia. This mobile phase was filtered through a Nalgene nylon membrane filter (0.2 μm) from Nalge (New York, NY, USA) and degassed during 10 min in an ultrasonic bath Sonicor SC-100-22TH (Copiague, NY, USA) before use. The flow-rate of mobile phase was 1 ml/min and the column was thermostated at 15 °C. The UV detection wavelength was 254 nm and the volume of injection was 10 μl.

3. Results and discussion

3.1. Preliminary correlation of α_{HPLC} and α_{CE}

In HPLC, the enantiomer separation of the series of selectands (see Fig. 1) had already been studied (except DNZ-βPhe not available) using the tBu-CQN chiral stationary phase and a mobile phase made of a mixture methanol–0.1 *M* ammonium acetate (80:20) adjusted to the apparent pH 6.0 with acetic acid (see Ref. [12] for DNZ derivatives and Ref. [7] for DNB and Bz derivatives).

Under the selected operating conditions for CE using the tBu-CQN as chiral additive (see Section 2.2), the migration times (t), the enantioselectivity (α) and the resolution (R_s) for the two enantiomers of all the amino acid derivatives were presented in Ref. [18].

In order to determine a preliminary evaluation of the correlation of the two techniques the enantioselectivity values measured in those HPLC conditions (α_{HPLC}) were compared with the values found in CE (α_{CE}). The corresponding plot of α_{HPLC} vs. α_{CE} for 23 amino acid DNB, DNZ and Bz derivatives is presented in Fig. 3.

This correlation line ($n=23$) has the following equation: $\alpha_{\mathrm{HPLC}}=20.55\ (\pm 1.54)\cdot\alpha_{\mathrm{CE}}-19.65\ (\pm 1.78)$. The standard error is $s_e=1.38$ and the correlation coefficient is $r=0.946$.

This first coefficient appeared to us relatively low. This could be due to the different composition of the mobile phase in HPLC (mixture methanol–water containing ammonium acetate) and of the BGE used in CE (mixture methanol–ethanol containing octanoic acid and ammonia) and also due to the difference of temperature (15 °C in CE and 25 °C in HPLC).

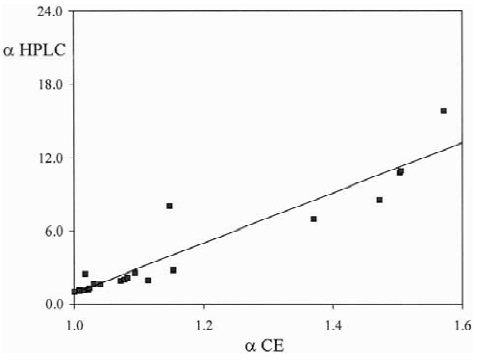

Fig. 3. Comparison of HPLC and CE enantiomer separations of the amino acid DNB, DNZ and Bz derivatives. Conditions as described in Section 3.1 ($n=23$).

For these reasons and in order to improve the value of comparison, the enantioselectivity of each derivative was studied in HPLC using exactly identical solvents and electrolytes as those used in CE: a mixture of ethanol–methanol (60:40) containing 100 m*M* octanoic acid and 12.5 m*M* ammonia as mobile phase.

3.2. Correlation using identical experimental conditions

The enantiomer separation of all the amino acid derivatives was studied in HPLC with tBu-CQN immobilized onto silica as chiral stationary phase

Table 1
Enantiomer separation of α-amino acid derivatives by HPLC[a]

Analyte	t_1 (min)	t_2 (min)	α	R_s
DNB-Leu	5.63	98.00	22.65	15.0
DNB-PGly	8.31	67.55	9.49	14.0
DNB-tLeu	5.60	54.53	12.46	14.7
DNB-αMeLeu	5.96	8.20	1.49	2.8
DNB-NMeLeu	5.81	6.32	1.11	<0.7
DNB-Phe	7.09	89.47	15.34	14.9
DNB-Pro	7.21	–[b]	–[b]	–[b]
DNB-αAbu	6.13	75.73	15.49	14.9
DNB-βAbu	9.13	83.52	10.56	13.0
DNZ-Leu	7.03	23.03	3.82	8.5
DNZ-αMeLeu	6.17	6.77	1.12	<0.7
DNZ-NMeLeu	7.01	8.39	1.24	1.6
DNZ-Phe	10.16	23.55	2.52	6.3
DNZ-βPhe	8.19	17.67	2.39	5.9
DNZ-Pro	9.88	12.28	1.28	1.6
DNZ-αAbu	7.96	23.2	3.31	7.7
DNZ-βAbu	6.07	15.64	3.03	7.8
DNZ-PA	9.95	11.51	1.18	1.2
Bz-Leu	4.23	9.63	2.88	6.9
Bz-Phe	6.01	11.25	2.12	5.2
Bz-βPhe	5.16	8.40	1.85	4.2
Bz-αAbu	4.76	9.59	2.42	6.0
Bz-βAbu	3.80	6.65	2.17	4.8
Bz-PA	5.79	6.20	1.09	<0.7

[a] Conditions as described in Section 2.3.
[b] No enantiomer separation observed ($R_s<0.5$).

using the experimental conditions described in Section 2.3. For each amino acid DNB, DNZ and Bz derivatives, the chromatographic retention data (t_R and k') as well as the separation data (α and R_s) of the corresponding enantiomers are listed in Table 1. The measured t_0 was 1.35 min.

The enantiomer separation of DNB-Leu is illustrated in Fig. 4a in comparison with its enantiomer separation obtained previously by CE illustrated in Fig. 4b.

Using these new conditions, a lower retention was observed in comparison with the previous results obtained in HPLC using as mobile phase a mixture methanol–0.1 *M* ammonium acetate (80:20) adjusted to the apparent pH 6.0 (see Ref. [12] for DNZ derivatives and Ref. [8] for DNB and Bz derivatives). The obtained enantioselectivity was higher for all the selectands tested. The new HPLC experimental conditions were then revealed favourable for enantioselectivity. However, the resolution was less good due to the reduced efficiency (at least two times lower) in comparison with the previously used experimental conditions. For example, the numbers of plates observed for the enantiomers of DNZ-Leu

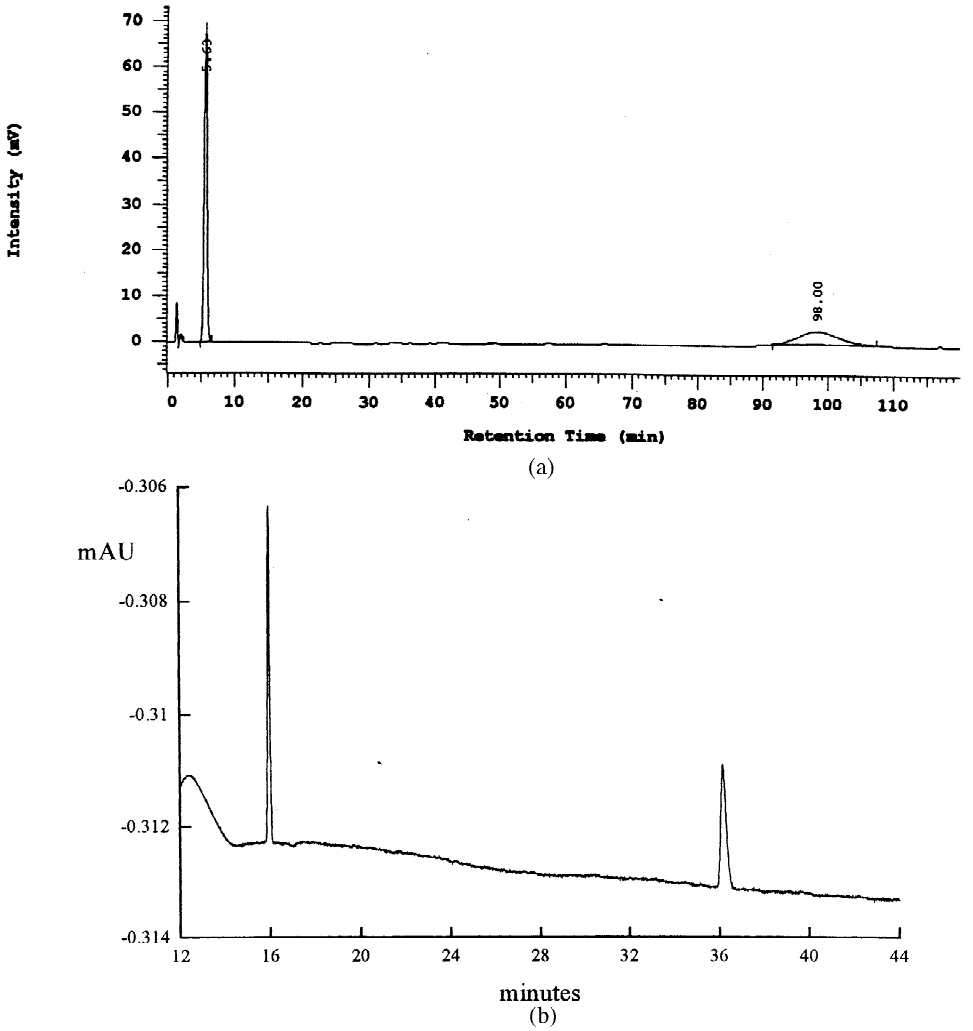

Fig. 4. Enantiomer separation of DNB-Leu (a) by HPLC and (b) by CE. Mobile phase and background electrolyte: 100 m*M* octanoic acid and 12.5 m*M* ammonia in methanol–ethanol (40:60). Other conditions as described in Sections 2.2 and 2.3.

were 1420 and 940 instead of 3160 and 3050 observed previously. The control of the elution order for DNB-Leu showed that the (R)-enantiomer migrates before the (S)-enantiomer.

The best enantioselectivities were obtained for DNB-Leu, DNB-αAbu, DNB-Phe and DNB-tLeu in the same order than the values obtained in CE (see Ref. [18]). The DNB derivatives are favourable (with respect to enantiodiscrimination capability of tBu-CQN) in comparison with DNZ and Bz derivatives. For example, the α values for the leucine derivatives were: 22.65 for DNB-Leu, 3.82 for DNZ-Leu and 2.88 for Bz-Leu. The enantiomers of DNZ-Pro were separated but not the enantiomers of DNB-Pro. All these observations had already been made previously in CE with the exception of the partial Bz-PA enantiomers separation ($R_s < 0.7$) not obtained in CE [18].

The enantioselectivities (α) and resolutions (R_s) observed for the 24 compounds in HPLC were compared to the values obtained previously in CE [18] using tBu-CQN as chiral selector in the BGE of the same composition than the mobile phase.

The corresponding plot of α_{HPLC} vs. α_{CE} ($n=24$) is presented in Fig. 5.

This correlation line ($n=24$) has the following equation: $\alpha_{HPLC} = 30.20\ (\pm 2.02) \cdot \alpha_{CE} - 29.38\ (\pm 2.33)$. The standard error is $s_e = 1.82$ and the correlation coefficient is $r = 0.954$. This new correlation was slightly better than the previous one, but the modification of the experimental conditions in HPLC did not cause a significant increase of the correlation coefficient: $r = 0.954$ instead of $r = 0.946$.

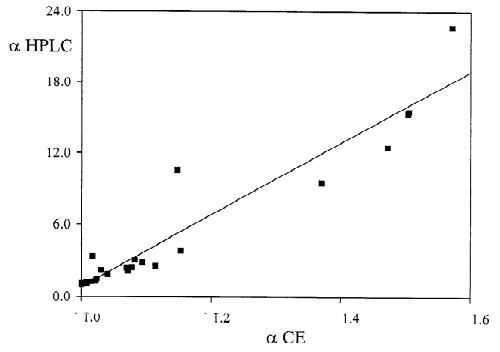

Fig. 5. Comparison of HPLC and CE enantiomer separations of the amino acid DNB, DNZ and Bz derivatives. Conditions as described in Section 3.2 ($n=24$).

In Fig. 5, it was observed that three points corresponding to DNB-Leu, DNB-βAbu and DNZ-αAbu deviate manifestly from the line. In order to detect if these points can be consider as outliers, the Grubbs' test was performed as proposed by the ISO norm 5725-2 [28]. According to this norm, the single Grubbs' test permitted only to detect a straggler (DNB-Leu) and the points suspected as outliers in the Fig. 5 could not be eliminated.

The use of the same composition of HPLC mobile phase and CE background electrolyte lead thus to a final $r = 0.954$ for the 24 amino acid derivatives studied. This value illustrates the correlation of the enantioselectivity observed with tBu-CQN in HPLC and CE under the developed analytical conditions.

4. Conclusions

A non-aqueous CE method developed for evaluating the chiral discrimination potential of cinchona alkaloids and different kinds of carbamoylated derivatives of quinine and quinidine type chiral selectors was selected for the enantiomer separation of a series of various Bz (benzoyl), DNB (3,5-dinitrobenzoyl) and DNZ (3,5-dinitrobenzyloxycarbonyl) amino acid derivatives. The enantioselectivity values obtained with a CE method employing tBu-CQN as chiral additive were compared with the values found for the same series of selectands in HPLC using the same selector but immobilized onto silica as chiral stationary phase. Similarly to the BGE used in CE an ethanol–methanol mixture (60:40, v/v) containing 100 m*M* octanoic acid and 12.5 m*M* ammonia was selected as HPLC mobile phase. Under these conditions, the obtained correlation between the two techniques was reasonably good ($r = 0.954$). This comparison of the results proved that a relationship exists between enantioselectivity values obtained with CE and HPLC methods and that the HPLC enantiomer separations can be predicted from the CE results. Thus the non-aqueous CE method can be applied for a fast evaluation of the chiral discrimination potential of the non-immobilized new quinine and quinidine derivatives as chiral SOs. This screening tool may thus be used early in the development

and the optimization of new chiral HPLC stationary phases.

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