Enantioselective separation of racemates using CHIRALPAK IG amylose-based chiral stationary phase under normal standard, non-standard and reversed phase high performance liquid chromatography

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Abstract

We have previously reported on the solvent versatility of immobilized amylose and cellulose-based chiral stationary phases in enantioselective liquid chromatographic separation of racemates. The studies were mainly focusing on the tris substituted 3,5-dimethylphenylcarbamate polysaccharide-based chiral stationary phases namely CHIRALPAK IA® [Amylose tris (3,5-dimethylphenylcarbamate)] or ADMP and CHIRALPAK IB® [Cellulose tris (3,5-dimethylphenylcarbamate)] or CDMP. Here we focus on the application of the recently introduced amylose tris (3-chloro-5-methylphenylcarbamate) or ACMPC and brand name CHIRALPAK IG® with a chlorine substituent replacing the methyl group in CHIRALPAK IA®. This was investigated for the enantioselective separation of different classes of pharmaceuticals namely β- and α-blockers, anti-inflammatory and antifungal drugs, norepinephrine-dopamine reuptake inhibitor, catecholamines, sedative hypnotics, anti-histaminics, anticancer drugs, antiarrhythmic drugs, flavonoids, amino acids, alpha-2 adrenergic agonist, adrenaline and miscellaneous. A brief comparison between CHIRALPAK IG® and CHIRALPAK IA® under normal standard, non-standard and reversed mobile phase is demonstrated. The results revealed the versatility of the CHIRALPAK IG® column, its compatibility with a wide ranges of solvent and operation modes and its ability to separate chiral compounds not separated with other amylose based chiral stationary phases.

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1. Introduction

Many pharmaceutics and herbicides are chiral. They exist as two incongruent stereoisomers called enantiomers. As optical isomers, they rotate linearly polarized light in opposite directions although they are generally known to have similar physical properties (eg, melting point, hydrophobicity, etc) and they can behave quite differently to one another in a chiral asymmetric environment. Since biological processes tend to involve chiral chemicals (eg, enzymes), chirality constitutes an important topic in drug development [1]. The United States Food and Drug Administration (FDA) requires toxicology testing for racemates only, regardless of industry plans to market a single isomer. In case of unexpected or significant toxicity is found in the racemate, FDA suggests querying the agency on whether similar studies are required for individual enantiomers. In such case, the FDA requires that only the active drug enantiomer (the eutomer) is produced by an enantioselective access (eg., via asymmetric synthesis, resolution via diastereomers, kinetic resolution, enzyme catalysis or chirality pool approach). The inactive enantiomer (the distomer) constitutes ‘isomeric ballast’ or it may be highly toxic. In the case of thalidomide, one enantiomer possessed the required therapeutic effect, while the other was eventually shown to be teratogenic causing birth defects in the unborn babies. While the use of enantiochemically pure drugs may appear to be a viable solution to such a problem, configurationally unstable stereoisomers like thalidomide may interchange (known variously as enantiomerization, enantiomeric inversion or racemisation) [2]. The thalidomide tragedy was entirely avoidable, had the physiological properties of the individual thalidomide forms been identified, separated and tested prior to commercialization.

Enantioselective chromatography has been well documented as a powerful, contemporary and practical technique for the chiral separation of racemic drugs, food additives, agrochemicals, fragrances and chiral pollutants [1,2]. This technique is several steps ahead of other previously reported methods to access pure
enantiomers; including synthesis from a chirality pool, asymmetric synthesis from pro-chiral substrates and the resolution of racemic mixtures [3]. The separation of racemic mixtures has been considered as the most feasible method for industrial applications compared to the time consuming and expensive synthetic approaches [4]. Remarkable developments have occurred in enantioselective chromatography since the first chiral separation of enantiomers using optically active stationary phase in the mid-sixties [5]. Following this development, several subclasses have emerged as well established chromatographic techniques with outstanding applications in chiral separation like electrophoresis (EC), supercritical fluid chromatography (SFC), counter current chromatography (CCC), gas chromatography (GC), and high performance liquid chromatography (HPLC) [6]. The chiral selectors used as stationary phases in liquid chromatography play a crucial role in the separation efficiency and the column backpressure governing the entire separation [1].

Most enantioselective separations are performed by direct resolution using a chiral stationary phase (CSP) where the chiral selector is adsorbed, attached, bound, encapsulated or immobilized to an appropriate support to make a CSP. The enantiomers are resolved by the formation of temporary diastereomeric complexes between the CSP and the analyte. Yet, thousands of CSPs have been reported, with more than one hundred commercialized [7]. Among the existing CSPs, those prepared from polysaccharides such as cellulose and amylose, attract more attention due to their powerful separation capability [8–18]. In general, the developments of chemically post-modified polysaccharides are the mainstream trend in the commercial and non-commercial chiral stationary phases. Out of the commercially available polysaccharide-based chiral stationary phases, cellulose and amylose were adsorbed, bonded, encapsulated or immobilized [19–26]. Of the amylose derivatives, the coated tris (3,5-dimethylphenylcarbamate) known as CHIRALPAK AD® has been widely and effectively used in chiral separation. However, it is not compatible to all eluents solvents, in particular, non-standard organic solvents such as ethyl acetate (EtOAc), tetrahydrofuran (THF), methyl tert-butyl ether (MtBE), dichloromethane (DCM) and chloroform, in which the polysaccharide derivatives can be dissolved or swollen. To widen the selection of solvents, the polysaccharide derivatives have been immobilized/bonded onto a silica matrix and have been extensively used as chiral stationary phases in non-standard organic solvents. Such immobilization of the polymeric chiral selector is considered as an efficient approach to confer a universal solvent versatility [27–32]. Several immobilized phases have been commercialized (Fig. 1). For examples CHIRALPAK IA®: Amylose tris (3,5-dimethylphenylcarbamate); CHIRALPAK IB®: Cellulose tris (3,5-dimethylphenylcarbamate); CHIRALPAK IC®: Cellulose tris (3,5-dichlorophenylcarbamate); CHIRALPAK ID®: Amylose tris (3-chlorophenylcarbamate); CHIRALPAK IE®: Amylose tris (3,5-dichlorophenylcarbamate) and CHIRALPAK IF®: Amylose tris (3-chloro-4-methylphenylcarbamate) have been extensively studied and proved to be solvents versatile in the enantiomeric separation of racemates [3,4]. Most recently CHIRALPAK IG®: Amylose tris (3-chloro-5-methylphenylcarbamate) with a chlorine substituent replacing the methyl group in CHIRALPAK IA® was introduced. Here we focus on the solvents versatility of CHIRALPAK IG® and the enantioselective separations of racemates (Fig. 2) under non-standard organic solvents and reversed phase chromatographic conditions. A brief comparison with CHIRALPAK IA® showing the effect of chlorine substituent in CHIRALPAK IG® on the enantiomeric separation of racemates is also demonstrated.

2. Materials and methods

2.1. Instrumentation

Conventional HPLC analysis was carried out using a Prominence Shimadzu System that consists of an LC-20 AD VP pump (Kyoto, Japan), SIL- 20AHT auto sampler, a GL Science UV-vis detector model MU 701 UCVIS (Tokyo, Japan), and a Shimadzu CDM-20A communications bus module (Kyoto, Japan). All analyses were performed at room temperature. CHIRALPAK IG® (4.6 mm ID × 250 mm, 5 µm silica gel) was supplied by Daicel (Tokyo, Japan).

2.2. Chemicals and reagents

All solvents were HPLC grade purchased from Sigma-Aldrich (St. Louis, MO, USA). Most of the tested compounds (Fig. 2) were also purchased from Sigma-Aldrich (St. Louis, MO, USA) namely Propanolol 1, Naproxen 3, Flurbiprofen 4, Indoprofen 5, Miconazone 8, Nomifensine 10, Arterenol 11, Normetanephrine 12, Ilosafamide 15, Tocainide 16 Propafenone 17, Glutamic acid monohydrate 20, Tyrosin 21, Phenylalanine 22, α-Methyl DOPA 23, Epinephrine 24, 1-Acenaphthenol 25 and 4-Hydroxy-3-methoxymandelic acid 26. On the hand, Naftopidil 2 was purchased from Boehringer Mannheim (Mannheim, Germany), Cizolirtine 6 was purchased from American Custom Chemicals Corp., (San Diego, CA, USA).
Carprofen 7 and Sulconazole 9 were purchased from AK Scientific (Union, CA, USA), Aminogluthethimide 13 was purchased from CIBA GEICY (Basel, Switzerland), Chlorphenamine 14 was purchased from Research Biochemicals International (Natick, MA, USA). Flavanone 18 and 6-Hydroxyflavanone 19 were purchased from Alfa Aesar (Ward Hill, MA, USA). 1-Indanol 27 was purchased from Fluka Chemical (Milwaukee, WI, USA). 1-Phenyl-2,2,2-trifluorethanol 28 was purchased from Sigma-Aldrich Switzerland.

Classification of the investigated racemates and their purities are as listed below:

| Classification                  | Durg                                           | Purity & Supplier       |
|--------------------------------|------------------------------------------------|-------------------------|
| β-blocker                      | Propranolol 1                                 | 99%, Sigma-Aldrich, USA |
|                               | Naftoprilol 2                                 | NA, Sigma-Aldrich, USA  |
| Anti-inflammatory drugs        | Naproxen 3                                    | NA, Sigma-Aldrich, USA  |
|                               | Flurbiprofen 4                                | NA, Sigma-Aldrich, USA  |
|                               | Indoprofen 5                                  | NA, Sigma-Aldrich, USA  |
|                               | Cizolirtine 6                                 | NA, American Custom     |
|                               | Carprofen 7                                   | 98%, AK Scientific, USA |
| Antifungal drugs               | Miconazole 8                                  | 98% Sigma-Aldrich, USA  |
|                               | Sulconazole 9                                 | NA, AK Scientific, USA  |
| Norepinephrine-dopamine reuptake inhibitor | Nomifensine 10                               | NA, Sigma-Aldrich, USA  |
| Catecholamines                 | Arterenol 11                                 | 97%, Sigma-Aldrich, USA |
|                               | Normetanephrine 12                            | 98%, Sigma-Aldrich, USA |
| Sedative hypnotic              | Aminogluthethimide 13                         | NA, CIBA GEICY, Switzerland |
|                               | Chlorphenamine 14                             | NA, Research Biochemicals International, USA |
| Anti-histaminic                | Carprofen 7                                   | 98%, Sigma-Aldrich, USA |
| Anticancer drug                | Ifosamide 15                                  | 98%, Sigma-Aldrich, USA |
| Antiarrhythmic drugs           | Tocainide 16                                  | 98%, Sigma-Aldrich, USA |
| Flavonoids                     | Flavanone 18                                  | 98%, Alfa Aesar, USA    |
|                               | 6-Hydroxyflavanone 19                         | 98%, Alfa Aesar, USA    |
| Amino acids                    | Glutamic acid monohydrate 20                  | 98%, Sigma-Aldrich, USA |
|                               | Tyrosin 21                                    | 99%, Sigma-Aldrich, USA |
|                               | Phenylalanine 22                              | 99%, Sigma-Aldrich, USA |
|                               | a-Methyl DOPA 23                              | NA, Sigma-Aldrich, USA  |
| Adrenaline                     | Epinephrine 24                                | NA, Sigma-Aldrich, USA  |
| Miscellaneous                 | 1-Acenaphthenol 25                            | 99%, Sigma-Aldrich, USA |
|                               | 4-Hydroxy-3-methoxymandelic acid 26           | 98%, Sigma-Aldrich, USA |
|                               | 1-Indanol 27                                  | 98%, Fluka Chemika, USA |
|                               | 1-Phenyl-2,2,2-trifluorethanol 28             | 98%, Sigma-Aldrich, Switzerland |

2.3. Sample preparations

Stock solutions of the racemic analytes at concentrations of 1 mg/mL in filtered HPLC-grade 2-propanol were prepared, filtered through Sartorius Minisart RC 15 0.2-μm pore size filters (Goettingen, Germany) and further used for analysis without dilution; the injection volume was 1 μL.

2.4. HPLC conditions

The enantioselective analyses were conducted using standard normal mobile phase comprised of n-hexane in combination with 2-propanol (2-ProH) or ethanol (EtOH) and non-standard normal phase namely tetrahydrofuran (THF), dichloromethane (DCM) and methyl tert-butyl ether (MtBE). Reversed mobile phase consisted of acetonitrile (ACN) and water (H2O) mixture. The additives TEA and TFA were added in both normal and reversed mobile phases. UV analyses were performed at fixed wavelength (254 nm) for all compounds.

Fig. 2. Chemical structures of a set of racemates investigated for their enantioselective separation on CHIRALPAK IC®.
3. Results and discussion

The well-known coated amylose tris (3,5-dimethylphenylcarbamate) ADMPC (CHIRALPAK AD®) in which the amylose derivative is physically coated on 5 or 10 μm silica particles has been widely and effectively used in chiral separation of racemates in high performance liquid chromatography. Its immobilized version namely CHIRALPAK IA® introduced ten years ago showed excellent solvent versatility and enantioselectivity in normal standard and non-standard organic mobile phases [27–32]. More recently, this phase showed promising enantioselectivity under HILIC and reversed phase modes as well [33]. In CHIRALPAK IA®, the chiral selector is immobilized/bonded onto 5 μm silica particles. The replacement of one donating methyl group with a withdrawing chlorine substituent of the ADMPC has resulted in the commercialization of a new immobilized phase namely amylose tris (3-chloro-5-methylphenylcarbamate) known as CHIRALPAK IG® or ACMPC. Here we demonstrate the solvent versatility and enantioselectivity of the new phase CHIRALPAK IG® under normal standard and non-standard organic phase as well as reversed phase chromatographic conditions. A brief comparison with CHIRALPAK IA® showing the effect of the donating methyl vs withdrawing chlorine substituent in amylose derivatives on the enantioselectivity is briefly demonstrated.

3.1. Chiral separation under normal standard and non-standard organic mobile phase

The initial mobile phase selected for the enantioselective separation of racemates 1–28 (Fig. 1) was a binary mixture of standard organic solvents consisting of n-hexane/ethanol screened from 90:10 to 10:90 v/v at 1 ml/min flow rate on CHIRALPAK IG® at fixed UV detection 245 nm. Out of the twenty eight compounds screened, fifteen compounds namely 1, 3–8, 10, 12, 14, 16–19 and 25–28 were baseline separated under either 90:10 or 80:20 v/v n-hexane/ethanol, respectively (Table 1 and Fig. 3). No baseline separation was achieved for 2, 9, 11, 13, 15 and 20–24. Replacing ethanol (EtOH) with 2-propanol (2-ProH) resulted in the baseline separation of 1, 4, 6, 7, 8, 10, 12, 14–19, 25 and 27 under either 90:10, 80:20, 70:30 or 60:40 v/v n-hexane/2-ProH. Comparing 2-ProH with ethanol in mobile phase composition and in terms of enantioselective separation, resolution Rs and separation factor α, ethanol in mobile phase composition was superior than 2-ProH. Thus, 1, 3, 5, 14, 26 and 28 were all separated under n-hexane/ethanol which wasn’t the case in n-hexane/2-ProH implying that ethanol works better with the 3-chloro substituted amylose in amylose tris (3-chloro-5-methylphenylcarbamate) or CHIRALPAK IG®. It is noteworthy that the retention is generally shorter with ethanol than 2-ProH or when using higher alcohol contents in relation to n-hexane in mobile phase composition (Table 2 and Fig. 3). To widen the choice of solvents in an attempt to enhance the separation or resolve the unresolved compounds under standard solvents above: dichloromethane (DCM), tetrahydrofuran (THF) or methyl tert-butyl ether (MtBE) were used before combination with standard organic solvent. The addition of non-standard solvents in mobile phase composition enhanced the resolution Rs and separation factor α of several tested racemates (Table 1). For example, in case of 6, the resolution Rs jumped from Rs 1.47 and separation factor α 1.38 in standard solvents namely n-hexane/2-ProH 90:10 v/v, respectively and Rs 3.83 and α 1.19 in n-hexane/EtOH 90:10 v/v to Rs 5.13 and separation factor α 3.35 when using non-standard solvent in excess in mobile phase composition (MtBE 98% v) in combination with ethanol (EtOH 2% v) or MtBE/EtOH 98:2% v/v. Of particular interest, compound 11 which wasn’t resolved under any standard solvents ‘combination investigated in this study was baseline separated under excess of
Table 1
The resolution $Rs$ and separation factor $\alpha$ for the enantioselective separation of racemates under normal standard and non-standard mobile phase condition ($Rs < 1$ = not separated, $Rs > 1$ = separated).

| IG | Standard solvents | Normal solvents | Nonstandard solvents | Additives | IA |
|----|-------------------|-----------------|----------------------|-----------|----|
|    | $Rs$ | $\alpha$ | n-Hexane | 2-ProH | EtOH | THF | DCM | MtBE | TEA | TFA | $Rs$ | $\alpha$ |
| 1  | 0.621 | 1.137 | 80 | 20 | 0.1 | NS | NS |
| 1  | 1.202 | 1.152 | 90 | 10 | 0.1 | NS | NS |
| 1  | 0.711 | 1.12 | 80 | 20 | 0.1 | NS | NS |
| 2  | 0.278 | 1.12 | 80 | 20 | 0.1 | NS | NS |
| 3  | 1.243 | 1.122 | 80 | 20 | 0.1 | NS | NS |
| 3  | 1.232 | 1.119 | 90 | 10 | 0.1 | NS | NS |
| 3  | 3.196 | 1.236 | 80 | 20 | 0.1 | NS | NS |
| 4  | 5.411 | 1.384 | 90 | 10 | 0.1 | NS | NS |
| 4  | 1.745 | 1.091 | 80 | 20 | 0.1 | NS | NS |
| 4  | 2.799 | 1.151 | 90 | 10 | 0.1 | NS | NS |
| 5  | 0.678 | 1.149 | 80 | 20 | 20 | 98 | NS | NS |
| 5  | 2.035 | 1.634 | 2 | 98 | NS | NS |
| 5  | 2.341 | 1.129 | 90 | 10 | 0.1 | NS | NS |
| 5  | 1.469 | 1.134 | 70 | 30 | 0.1 | NS | NS |
| 5  | 2.434 | 1.125 | 90 | 10 | 0.1 | NS | NS |
| 5  | 1.652 | 1.104 | 80 | 20 | 0.1 | NS | NS |
| 5  | 1.367 | 1.054 | 60 | 40 | 0.1 | NS | NS |
| 5  | 9.623 | 4.803 | 80 | 20 | 0.1 | NS | NS |
| 5  | 10.05 | 1.507 | 90 | 10 | 0.1 | NS | NS |
| 5  | 8.423 | 1.462 | 80 | 20 | 0.1 | NS | NS |
| 5  | 8.214 | 1.533 | 80 | 20 | 0.1 | NS | NS |
| 5  | 9.202 | 1.931 | 90 | 10 | 0.1 | NS | NS |
| 5  | 0.798 | 1.132 | 80 | 20 | 0.1 | NS | NS |
| 5  | 1.15 | 1.338 | 50 | 0.2 | 50 | NS | NS |
| 5  | 3.361 | 1.497 | 80 | 20 | 0.1 | NS | NS |
| 5  | 2.793 | 1.275 | 60 | 40 | 0.1 | NS | NS |
| 5  | 1.413 | 1.347 | 2 | 98 | NS | NS |
| 6  | 1.47 | 1.38 | 90 | 10 | 0.1 | NS | NS |
| 6  | 2.361 | 1.134 | 80 | 20 | 0.1 | NS | NS |
| 6  | 3.835 | 1.19 | 90 | 10 | 0.1 | NS | NS |
| 6  | 5.131 | 3.354 | 2 | 98 | NS | NS |
| 6  | 2.391 | 1.684 | 60 | 40 | NS | NS |
| 7  | 0.897 | 1.141 | 90 | 10 | 0.1 | NS | NS |
| 7  | 1.454 | 1.15 | 80 | 20 | 0.1 | NS | NS |
| 7  | 0.399 | 1.121 | 80 | 20 | 0.1 | NS | NS |
| 7  | 0.647 | 1.347 | 60 | 40 | 0.1 | NS | NS |
| 7  | 1.435 | 1.173 | 80 | 20 | 0.1 | NS | NS |
| 7  | 1.247 | 1.16 | 90 | 10 | 0.1 | NS | NS |
| 7  | 0.778 | 1.067 | 80 | 20 | 0.1 | NS | NS |
| 7  | 1.813 | 1.131 | 90 | 10 | 0.1 | NS | NS |
| 7  | 3.058 | 1.218 | 80 | 20 | 0.1 | NS | NS |
| 7  | 4.197 | 1.247 | 90 | 10 | 0.1 | NS | NS |
| 7  | 1.289 | 1.198 | 50 | 0.2 | 50 | NS | NS |
| 7  | 1.123 | 1.095 | 70 | 30 | NS | NS |
| 8  | 1.141 | 1.053 | 80 | 20 | 0.1 | NS | NS |
| 8  | 3.018 | 1.224 | 80 | 20 | 0.1 | NS | NS |
| 8  | 4.758 | 1.282 | 90 | 10 | 0.1 | NS | NS |
| 8  | 3.397 | 1.292 | 80 | 20 | 0.1 | NS | NS |
| 8  | 5.268 | 1.293 | 90 | 10 | 0.1 | NS | NS |
| 9  | 2.503 | 1.242 | 80 | 20 | 0.1 | NS | NS |
| 9  | 1.964 | 1.281 | 60 | 40 | 0.1 | NS | NS |
| 10 | 4.116 | 1.372 | 80 | 20 | 0.1 | NS | NS |
| 10 | 3.74 | 1.672 | 70 | 30 | NS | NS |
| 10 | 4.65 | 1.454 | 80 | 20 | 0.1 | NS | NS |
| 10 | 2.528 | 1.399 | 60 | 40 | 0.1 | NS | NS |
| 10 | 2.615 | 1.772 | 80 | 20 | 0.1 | NS | NS |
| 11 | 1.248 | 1.28 | 60 | 40 | NS | NS |
| 12 | 1.14 | 1.104 | 80 | 20 | 0.1 | NS | NS |
| 12 | 0.671 | 1.604 | 80 | 20 | 0.1 | NS | NS |
| 12 | 0.891 | 1.041 | 90 | 10 | 0.1 | NS | NS |
| 13 | 5.136 | 2.121 | 2 | 98 | 6.94 | 3.429 |
| 14 | 3.327 | 1.286 | 80 | 20 | 0.1 | NS | NS |
| 16 | 0.415 | 1.123 | 80 | 20 | NS | NS |
| 16 | 0.648 | 1.104 | 70 | 30 | NS | NS |
| 16 | 0.737 | 1.132 | 80 | 20 | 0.1 | NS | NS |
| 16 | 5.02 | 1.152 | 60 | 40 | 0.1 | NS | NS |
| 16 | 1.677 | 1.101 | 90 | 10 | 0.1 | NS | NS |
| 16 | 1.338 | 1.083 | 80 | 20 | 0.1 | NS | NS |
| 16 | 2.369 | 1.119 | 90 | 10 | 0.1 | NS | NS |
| 16 | 0.944 | 1.188 | 80 | 20 | 0.1 | NS | NS |
| 16 | 1.084 | 1.149 | 80 | 20 | NS | NS |
Table 1 (Continued)

| IG | Standard solvents | Nonstandard solvents | Additives | IA |
|----|-------------------|----------------------|-----------|----|
|    | Rs    | α     | n-Hexane | 2-ProH | ETOH | THF | DCM | MTBE | TEA | TFA | Rs | α |
| 17 | 2.392 | 1.518 | 60       | 40     |      |      |      |      |      |      |      |    |
| 18 | 7.2   | 1.907 | 90       | 10     |      |      |      |      |      |      |      |    |
| 19 | 1.453 | 1.115 | 90       | 10     |      |      |      |      |      |      |      |    |
| 20 | 0.998 | 1.146 | 70       | 30     |      |      |      |      |      |      |      |    |
| 21 | 14.749| 2.029 | 80       | 20     |      |      |      |      |      |      |      |    |
| 22 | 20.526| 2.304 | 80       | 20     |      |      |      |      |      |      |      |    |
| 23 | 2.536 | 2.022 | 80       | 20     |      |      |      |      |      |      |      |    |
| 24 | 1.919 | 1.141 | 70       | 30     | 0.1  |      |      |      |      |      |      |    |
| 25 | 1.339 | 1.821 | 80       | 20     |      |      |      |      |      |      |      |    |
| 26 | 1.306 | 1.101 | 80       | 20     |      |      |      |      |      |      |      |    |
| 27 | 1.195 | 1.035 | 90       | 10     |      |      |      |      |      |      |      |    |

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non-standard organic solvent (MtBE 98% v) in combination with ethanol (EtOH 2% v) or MtBE/EtOH 98:2 v/v, respectively with resolution Rₐ 5.13 and separation factor α 2.12. Similarly compound 13 was only separated under non-standard organic mobile phase composition consisting of MtBE/EtOH 98:2 v/v. Better separations were achieved in non-standard solvents when compared to similar separation under standard organic solvents for compounds 3, 6 and 16 (Table 1 and Fig. 3). One can conclude that polarity plays a role in the chiral recognition of CHIRALPAK IG®. For example, ethanol with polarity index 5.2 works well in combination with n-hexane or MtBE while 2-ProH with polarity index 3.9 is less sensible in terms of enantioseparation under standard and non-standard organic solvents. Another factor might be the amendment of the stereo environment of the chiral cavities in amyllose derivatives is favourable in presence of ethanol for the enantioseparation of the investigated racemates.

3.2. Chiral separation under reversed phase

Although the use of reversed phase in amyllose and cellulose-based as CSPs in enantioselective liquid chromatography is limited, there are few recently reported studies [22,34–39]. The choice of reversed phase was based on its economic and environmental benefits. Thus, the enantioselective separation was investigated using reversed phases including acetonitrile (ACN) and water (H₂O) mixture ranging from 10–90% v/v (Table 2 and Fig. 4). Few baseline separations were achieved under acetonitrile condition for compounds 4, 8, 12, 14, 16, 17, 19, 25, 26 and 27. Of particular interest compound 4 was baseline separated with unprecedented resolution of Rs 16.80 and separation factor α 2.63 under ACN/H₂O 60:40 v/v in presence of 0.1% TEA in mobile phase composition. Similarly, in case of 8, Rs 5.83 and separation factor α 1.46 were superior to other separations achieved under standard and non-standard organic solvents (Table 2 and Fig. 4). Compound 12 which was moderately separated under standard and non-standard solvents, was baseline separated under reversed phase condition (ACN/H₂O/TEA 60:40:0.1% v/v) with superior Rs 1.35 and α 1.46.

3.3. Methyl vs chlorine substituent in CHIRALPAK IA® vs CHIRALPAK IG®

In an attempt to study the effect of the introduction of the withdrawing chlorine group instead of donating methyl group in the third position of amyllose (3,5-dimethylphenylcarbamate) or ADMPC known as CHIRALPAK IA® to make the amyllose trim (3-chloro-5-methylphenylcarbamate) known as CHIRALPAK IG®, a brief comparison between CHIRALPAK IA® vs CHIRALPAK IG® took place under standard, non-standard and reversed phase mobile phase composition for the enantioselective separation of selected racemates (Fig. 2). As previously demonstrated above, in terms of the resolution Rs and separation factor α, n-hexane/EtOH 90:10 v/v mixture was the best performing mixture of standard solvents in mobile phase composition. This mobile phase mixture was chosen in the comparison study for the enantioselective resolution of racemates under CHIRALPAK IA®, comparing with CHIRALPAK IG®, under similar condition, only compounds 2 (Rₛ 0.31, α 1.11), 18 (Rₛ 15.61, α 2.32), 25 (Rₛ 1.04, α 1.07) and 27 (Rₛ 1.65, α 1.13) were partially or base-line separated under n-hexane/EtOH 90:10 v/v mobile phase. Moving to n-hexane/2-ProH 90:10 v/v instead of n-hexane/EtOH 90:10 v/v in mobile phase composition resulted in the partial or base-line separation of 18 (Rₛ 1.64, α 1.11), 19 (Rₛ 0.59, α 1.17), 25 (Rₛ 1.51, α 1.09), 27 (Rₛ 1.89, α 1.13) and 28 (Rₛ 0.90, α 1.10). In terms of resolution Rs and separation factor α, CHIRALPAK IG® was superior than CHIRALPAK IA® when operating under n-hexane/EtOH 90:10 v/v or n-hexane/2-ProH 90:10 v/v in mobile phase composition. When using non-standard solvent in excess in mobile phase composition e.g., n-hexane/MtBE 2/98% v/v, only two compounds were separated on CHIRALPAK IA® namely compounds 18 (Rₛ 1.51, α 1.46) and 27 (Rₛ 0.68, α 1.17). Moving to MtBE/EtOH 98/2% v/v, only four compounds were separated under CHIRALPAK IA® namely 13, 18, 19 and 26 comparing to seven compounds separated under CHIRALPAK IG® (3, 5, 6, 13, 18, 19, 25 and 26). It is noteworthy that the resolution Rs and separation factor α were all better on CHIRALPAK IG® (Table 1). The results align with previous finding about the chiral recognition of the regioselective substituted polysaccharide derivatives [8]. The different chiral recognition abilities may be ascribed to the electronic effect of substituents namely the withdrawing chlorine group versus the donating methyl group which in turn can alter the polarity and the 3D structure of the polymer.

Under reversed mobile phase composition namely ACN/H₂O/TEA 60:40:0.1% v/v, respectively, only compounds 2 (Rₛ 1.42, α 1.09), 13 (Rₛ 2.27, α 1.26), 18 (Rₛ 10.91, α 1.67), and 19 (Rₛ 5.56, α 2.82) were baseline separated on CHIRALPAK IA® comparing to compounds 4, 8, 12, 14, 16, 17, 19, 25, 26, and 27.

### Table 2
The resolution Rs and separation factor α for the enantioselective separation of racemates under reversed mobile phase condition.

| Reversed mobile phase | Rs    | α    | ACN/H₂O/0.1TEA | Rs    | α    |
|-----------------------|-------|------|---------------|-------|------|
| IG                    |       |      |               |       |      |
| 2                     | NS    | NS   | 60/40         | 1.429 | 1.097|
| 4                     | 16.801 | 2.637| 60/40         | NS    | NS   |
| 8                     | 12.384 | 2.719| 80/20         | NS    | NS   |
| 12                    | 5.831  | 1.461| 60/40         | NS    | NS   |
| 13                    | 1.325  | 1.179| 60/40         | NS    | NS   |
| 14                    | 0.965  | 1.094| 60/40         | 2.275 | 1.285|
| 16                    | 1.443  | 1.09 | 80/20         | NS    | NS   |
| 17                    | 1.496  | 1.109| 80/20         | NS    | NS   |
| 18                    | 4.549  | 1.399| 60/40         | NS    | NS   |
| 19                    | 4.216  | 1.385| 60/40         | NS    | NS   |
| 25                    | 1.125  | 1.055| 40/60         | NS    | NS   |
| 26                    | 1.117  | 1.247| 60/40         | NS    | NS   |
| 27                    | 1.857  | 1.133| 40/60         | NS    | NS   |
| 28                    | 0.906  | 1.07 | 60/40         | NS    | NS   |
separated on CHIRALPAK IG®. It is noteworthy to mention that compounds 2, 13, 18 were not previously separated on CHIRALPAK IG® under similar conditions (Table 2).

The enantioselective separation under non-standard solvents’ mobile phase revealed that the combination of n-hexane with MtBE works best where ten compounds (3, 5, 6, 11, 13, 16, 18, 19, 25 and 27) were separated comparing with n-hexane/THF with eight compounds separated (3, 4, 5, 16, 17, 19, 25 and 27) and n-hexane/DCM with only four compounds separated (4, 7, 25 and 27).

4. Conclusions

The solvents versatility of CHIRALPAK IG® has been demonstrated. The results revealed that solvents known as prohibited non-standard LC solvents such as MtBE, DCM and THF in which the amyllose derivatives CSP can be dissolved/swollen can be used as eluents in mobile phase compositions. The addition of these solvents will be also beneficial when used as diluents to directly monitor organic reactions online. Several tested racemates that were not separable under normal standard organic solvents were separated under non-standard organic solvents in mobile phase composition. The use of reversed phase consisting of ACN/H₂O broaden the application of CHIRALPAK IG® with enhanced resolution Rₚ and separation factor α comparing to similar separation under standard and non-standard organic solvents. Compared with CHIRALPAK IA® and in terms of resolution Rs and separation factor α, CHIRALPAK IG® appears to be superior under standard and non-standard solvents for the tested compounds. Overall, for the tested compounds, CHIRALPAK IG® appears to be superior to CHIRALPAK IA® and it may offer an alternative to CHIRALPAK IA®.

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