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REVIEW

Recent Trends in Chiral Separations by 2D-HPLC

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Abstract
2D-HPLC is an important development in chiral separations due to its excellent capability to resolve the enantiomers. This article describes the latest update in the chiral resolution of racemates using 2D-HPLC. The various aspects of the chiral resolution such as validation, optimization, and applications are discussed in both biological and environmental matrices. Some latest developments of nano-2D-HPLC are also included. Besides, efforts are made to discuss the challenges and future perspectives. During the preparation of this article, it was observed that much work has not been done in this direction and only a few papers describe the application of 2D-HPLC in the chiral separation. There is an urgent need to develop more 2D-HPLC methods for a variety of racemates. This article will be useful to the investigators, academicians and industry persons in the future to improve 2D-HPLC for the chiral resolution of the various racemates.

Keywords 2D-HPLC · Chiral separations · Applications · Challenges and future perspectives

Introduction

The separation is very important in chemical science as it is being used to separate many complex mixtures. Separation science has great importance in the enantiomeric separation. The separation mechanism is very difficult to be understood in the case of chiral drugs, because chiral drugs exist in more than one form. Another most important point to be noted is the 40% existence of the chiral drugs among the drugs available in the market. Chiral drugs exist in two or more forms, in which only one form is biologically important, while another is not [1–4]. The separation of racemic mixture is very difficult in the achiral environment, but it can be achieved in the chiral environment. USA, Japan, and some other European countries revised the main features with medicinal productions on the chiral medicinal products [5, 6]. Although European countries have entirely excluded the promotion of enantiomeric drugs, these are frequently given and marketed in Asia [7, 8]. The separation method is the best one to get a pure biologically active enantiomeric form. Hence, it can play a significant role in the economic production of optically active medicines. Hence, the chiral separation method is being used to obtain single enantiomers. Besides, many procedures have been established for the enantio-separation of the enantiomeric forms, but no other technique is as good as chromatography due to numerous advantages [9–23]. We have many chromatographic modalities, but multi-dimensional high-performance liquid chromatography (2D-HPLC) has gained its unique position and became a choice of every researcher working in the field of chromatography. Multicomponent combinations with astonishing multifaceted nature required separation approaches offering expanded settling control, as the whole barrier of the complex mixture is not completely achieved by 1D chromatography (ODC). 2D liquid chromatography involves two types of modalities, i.e., (i) achiral and (ii) chiral. Both modes give advanced discrimination. The advantages of

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the multi-dimensional (MD) methodology are significant as there is no possibility for co-eluting sample components in 2Ds (two columns). The exchange of effluent is the most important phenomenon in 2D-HPLC, which occurs when components enter in the second column (2D) from the first one (1D). A difference between 2D-HPLC and ODC is made by only this exchange of effluent. A fitting interface is an associate with the columns in the two dimensions in 2D-HPLC. Therefore, in chiral resolution, 2D-HPLC has a special place, that is why the pharmaceutical companies are demanding it. Owing to the significance of the enantio-separation and abilities of 2D-HPLC, the current review article is written to be used for industrial persons, academicians, researchers, and government establishments. Hence, the presented paper designates the importance of 2D-HPLC in the separation of enantiomers.

Significance of the Separation of Enantiomers

We have already stated in the introductory part of the paper if one or two chiral-centered molecules are there, only one enantiomer is important and others might be inactive or unsafe, which is a serious problem. In the case of multi-chiral-centered medicines, the situation is more serious. It is fascinating that some racemates show a good application in therapy and cannot be ignored. Therefore, the knowledge of stereo-selective importance of the racemic forms of the chiral drugs and the chiral separation is important. Chiral drugs have superiority over their racemates as the former bind with hormones, enzymes, and diverse proteins stereo-selectively [24–26]. Therefore, the therapeutic actions of some important multi-chiral racemates/drugs are reduced. Two chiral-centered, diltiazem is a very important drug to cure angina pectoris, hypertension, and arrhythmia. Additionally, diltiazem is prescribed for headaches. Besides, relaxing of coronary vascular smooth muscles is another function of diltiazem by blocking calcium ion influx [27]. Two chiral centered formoterol is considered as the important medication for asthma assault [28, 29]. In the same way, four chiral centered nebivolol is prescribed to treat hypertension. Nebivolol is also assumed as a β_1-receptor blocker when it is prescribed with NO (nitric oxide) [30]. Another two chiral centered molecule labetalol shows β_2-opponent exercise. Labetalol is also prescribed for the treatment of hypertensive crises, bounce back hypertension, and pheochromocytoma-related hypertension [21]. On the other hand, three chiral centered molecule, nadolol [31] has been permitted by Food and Drug Administration for the handling of angina pectoris. In brief, we would like to share only one thing that the enantio-separation is very important in environmental studies, drug development, and other sectors such as agriculture. The use of only a single biologically active enantiomer may save a huge quantity of manpower; establishment of a country budget.

Importance of 2D-HPLC

2D-HPLC is a technique involving separation with two columns of two different orthogonality. This is also referred to as LC × LC technique. Two dissimilar HPLC columns are connected in a categorization way, and the mobile phase from 1D is transferred to 2D [32]. This sort of HPLC is better to resolve complex molecules, which cannot be separated by routine HPLC. 2D-HPLC is better suited in case of the chiral separations. The major benefit of LC × LC over 1D-liquid chromatography is improved peak capacity (nc), which is maximized by choosing different mobile phases and different stationary phases. 2D-HPLC is becoming more widely accepted among researchers and industry persons to solve challenges and difficulties in the separation. Augmented accessibility and advances in the commercially existing equipment in recent years are lowering the obstruction to broader use. Therefore, 2D-HPLC has achieved great attention during the last few years, especially in separating complex mixtures. It is important to mention here that the second phase must be fast in 2D-HPLC.

The researchers working on the analysis of complex samples have given their priority to use 2D-HPLC. The reason behind it is that ODC is unfit to get a satisfactory separation. Enantio-separation needs specific requirements, because it is a very difficult task. The enantio-separation may be achieved easily with multi-dimensional chromatography. 2D-HPLC allows the mixing of at least two independent steps of separation, increasing the power of separation. Some reviews and books have been appeared in the literature, describing many parts of 2D-HPLC [33–40]. Of course, HPLC is a versatile technique in chiral separation. HPLC can be applied for chiral separation, either by chiral additives in the mobile phase or CSP. Selectivity is established by chiral stationary phases, but it is not particular for a sample in which other impurities are present. Therefore, to resolve such problems, the multi-dimensional method is very useful and named as multi-dimensional high-performance liquid chromatography (MD-HPLC).

2D-HPLC in the Chiral Separations

Of course, the chiral resolution is not as easy as it is thought, because it needs a chiral environment to get enantiomers resolved. The researchers have to face many problems during the development and validation of the method of enantio-separation, but 2D-HPLC eased all those troubles. Many

researchers have used 2D-HPLC in the separation of enantiomers of the chiral compounds. Sheng et al. [41] characterized and identified the components present in Denzhan Shenmai (DZSM) by 2D-HPLC. Separation of DZSM was done using $C_8 \times C_{18}$ HPLC column, and most of them identified and characterized within 75 min. In multiple heart-cutting ^{2}D-LC systems, $C_8 \times$ Chiral HPLC column system was used for enantiomeric analysis.

Cannazza et al. [42] used a multi-dimensional HPLC (sf-MD-HPLC) system to study the effect of pH on the enantiostability and hydrolysis of (±)-7-chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide [(±)IDRA21] (Fig. 1). The primary and secondary columns used were C_{18} column and chiral stationary phase, respectively. Woiwode et al. [43] used two types of columns in 2D-HPLC for the chiral separation of amino acids. In ^{1}D, O-9-(2,6-diisopropylphenylcarbamoyl) quinine (DIPPCQN), CSP was used as stationary phase, while in ^{2}D, tert-butylcarbamoylated (t-BuCQN) CSP as a chiral stationary column at 360 nm UV detection. In both dimensions, the mobile phase used was MeOH/CH_3COOH/CH_3COONH4 (98/2/0.5, v/v) with a flow rate of 0.1 mL/min.

Furthermore, Woiwode et al. [44] used the heart cut 2D-UHPLC technique for chiral separation of some

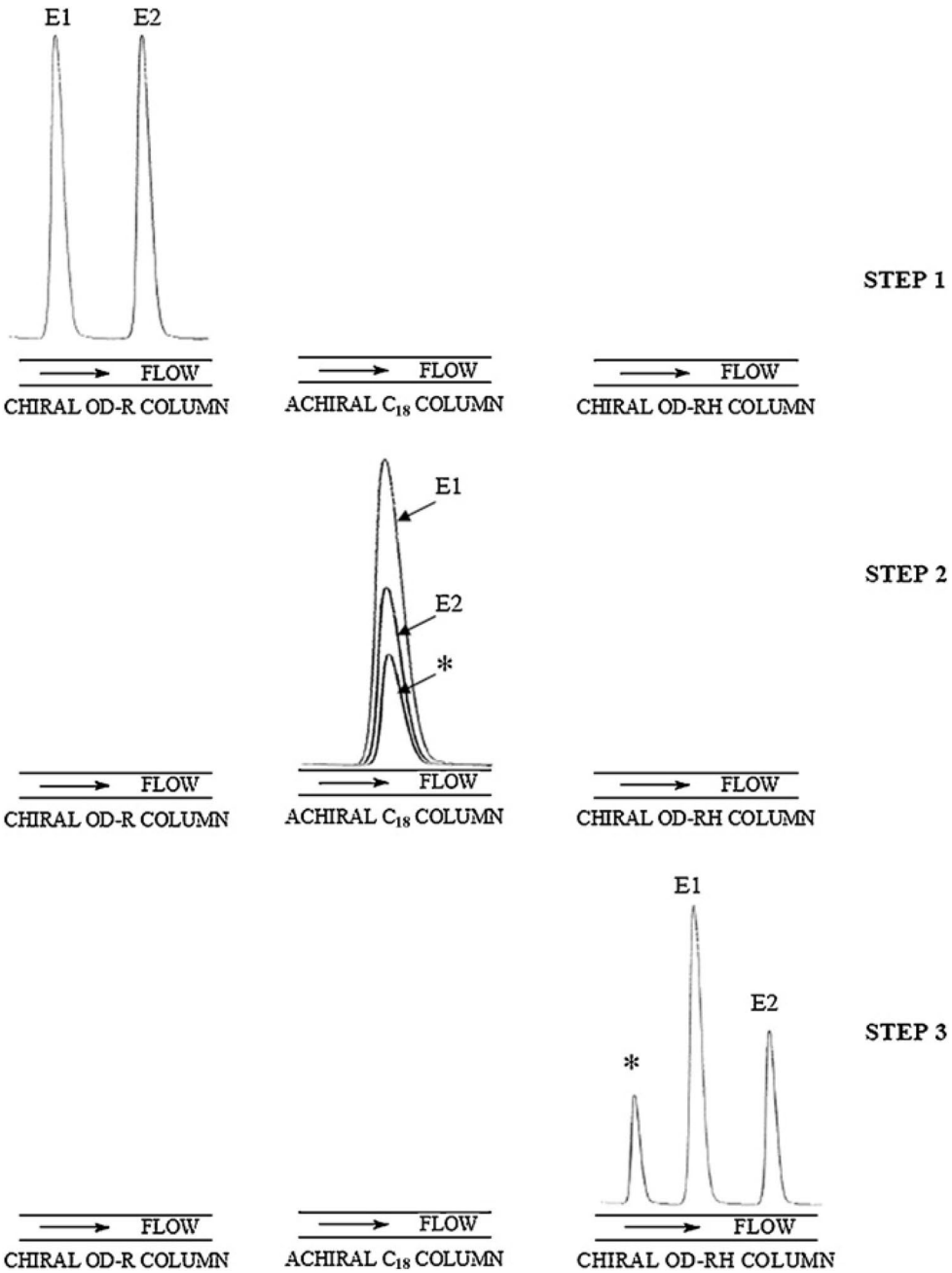

Fig. 1 The chromatographic detection (±)-7-chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide [(±) IDRA21] [42]

amino acids with UV detection (Fig. 2). The primary and secondary columns used were 1.8 µm C_{18} column (100 mm × 2.1 mm) and a *tert*-butylcarbamoylquinine-based 2.7 µm Core–shell column (50 mm × 3 mm), respectively. The derivatizing agent used was Sanger's reagent. Guillen-Casla et al. [45] also initially enantio-separated some amino acids with detection at 260 nm UV (Fig. 3). The primary and secondary columns used were C_{18} and teicoplanin chiral columns, respectively. The mobile phase used was CH_3COONH_4 buffer (20 mM, pH 6) (94%) and MeOH (6%). In the same series, the same group [46] used chiral LC × chiral LC with *tert*-butylcarbamoyl quinidine and *tert*-butylcarbamoyl quinine chiral selectors in the first and second columns for the enantiomeric resolution of D- and L-amino acids form peptide hydrolysates. FMOC was used as a derivative agent. The authors observed orthogonality between 1D and 2D chiral stationary phases, which mainly depends on their chirality. The authors reported the successful resolution of amino acids in that paper.

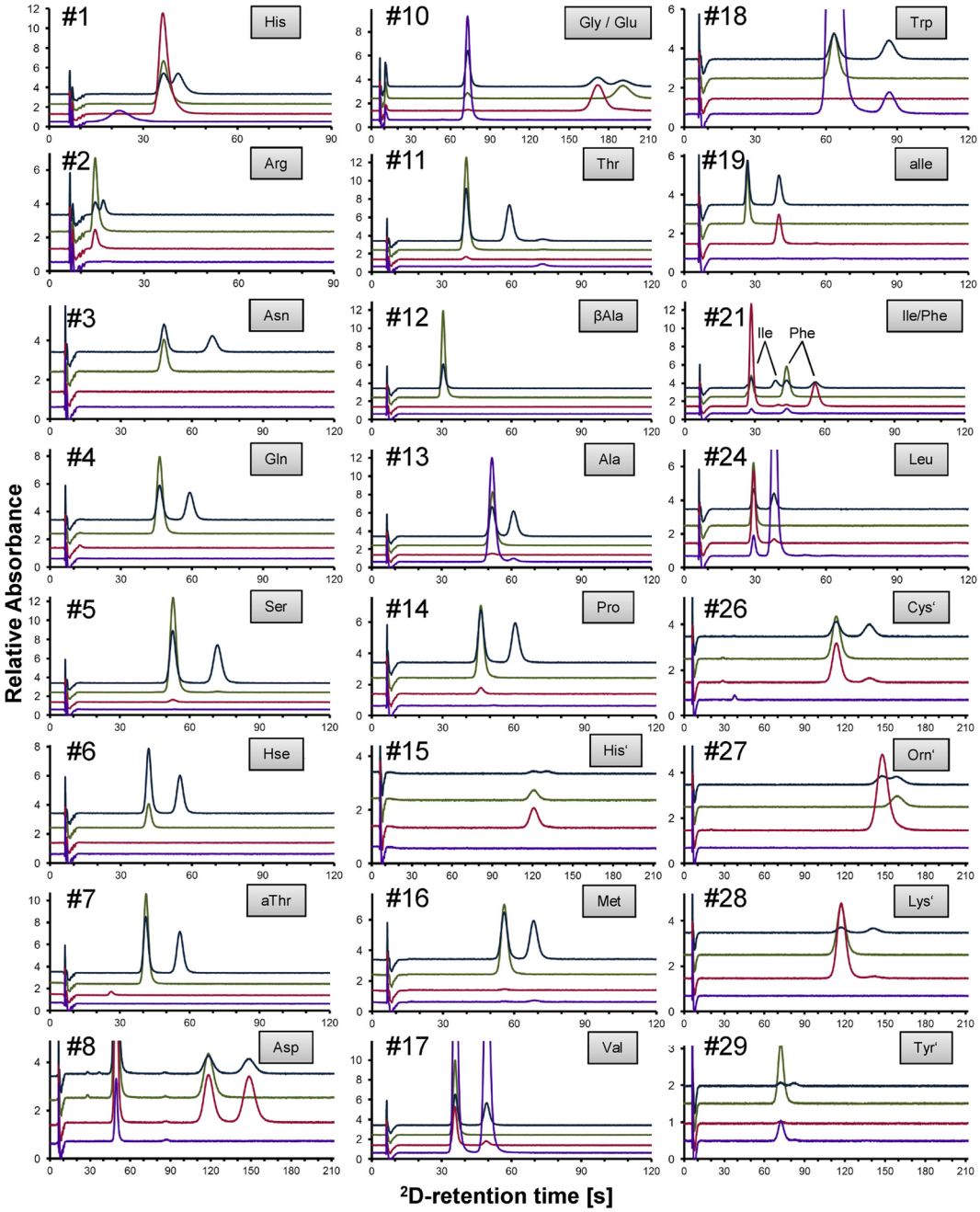

Fig. 2 Enantio-separation of amino acids in 2D-HPLC [44]

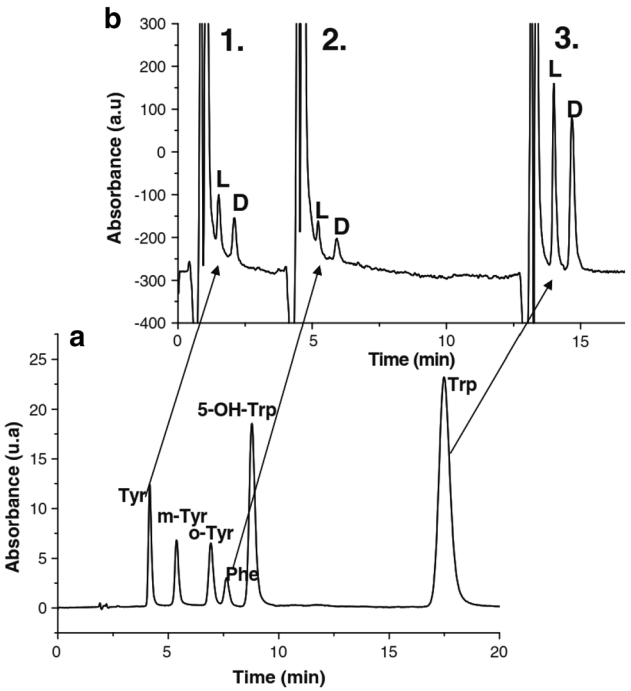

Fig. 3 Heart cut two-dimensional high-performance liquid chromatograms from achiral–chiral coupling. **a** Achiral separation of 20 mg/L of each amino acid enantiomer and 20 mg/L of m-tyrosine, o-tyrosine, and 5-OH-triptophan. **b** Chiral separation: (1) enantiomeric separation of L- and D-tyrosine, (2) enantiomeric separation of L- and D-phenylalanine, and (3) enantiomeric separation of L- and D-tryptophan adapted from Ref. [45]

Koga et al. [47] used an ODS microbore monolithic column (0.53 mm i.d. × 100 cm) as primary and narrow bore enantio-selective columns (1.5 mm i.d. × 150 or 25 cm) as a secondary column in 2D-HPLC for the identification of NMDA (*N*-methyl-D-aspartic acid). Besides, in the pre-column condition, the derivatizing agent used was 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F).

Hamase et al. [48] analyzed eight chiral amino acids and five non-chiral amino acids using a 2D-HPLC system (Fig. 4). An ODS capillary monolithic column in 1D and Pirkle type enantio-selective method was used. The derivatizing agent used was 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). In ^{1}D, amino acids were separated, while in ^{2}D, enantio-separation was done. For the investigation of D-amino acids, Han et al. [49] used an entirely automatic 2D-HPLC system. The primary and secondary columns were monolithic ODS column and micro-Chiralpak QD-1-AX column, respectively. ACN-CF_3COOH-H_2O (9:0.05:92, V/V) was used as the mobile phase for ^{1}D; 10 mmol/L citric acid in MeOH:ACN (50: 50, v/v) was used as the mobile phase for ^{2}D. The derivatizing agent used was NBD-F. Barhate et al. [50] used two columns, i.e., in ^{1}D with chiral selectors in ^{2}D, for the analysis and separation of pharmaceuticals. The mobile phase used was 0.1% H_3PO_4/ACN in both dimensions. Ishii et al. [51] developed a 2D-HPLC–MS/MS system for the study of five major D-amino acid residues (Fig. 5) under numerous circumstances. The primary and secondary columns were a capillary monolithic ODS column, ML-1000, and KSAACSP-001S, respectively. In ^{1}D,

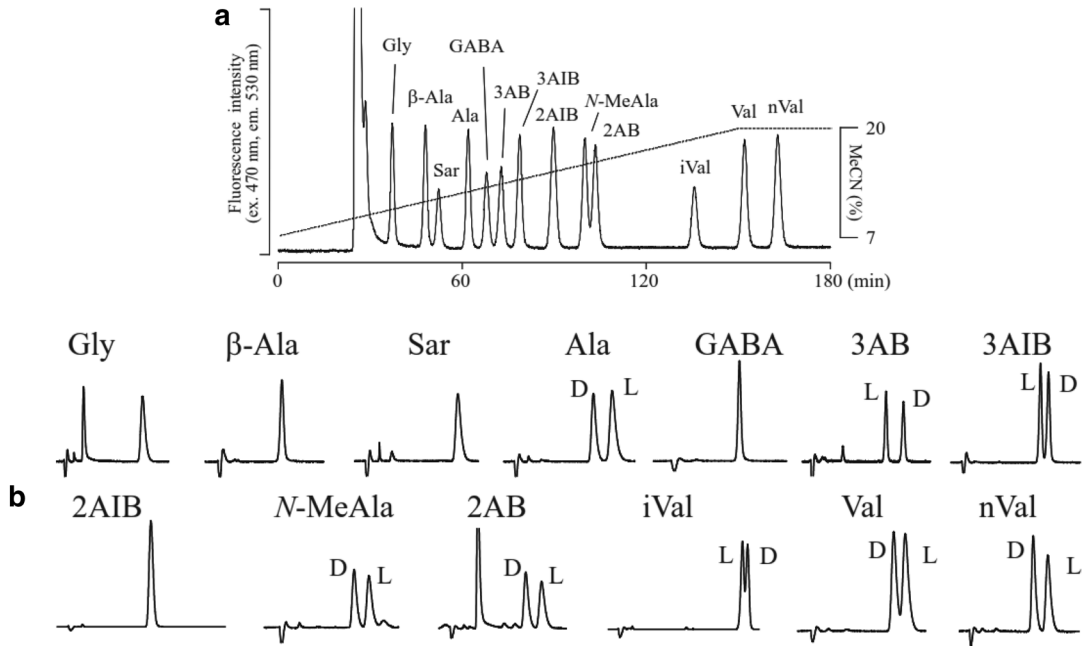

Fig. 4 Enantio-separation of eight amino acids in 2D-HPLC. **a**: separation of NBD-amino acids as their D plus L mixtures using a monolithic ODS column; **b**: chromatograms of extraterrestrial amino acid enantiomers as their NBD derivatives (second dimension) [48]

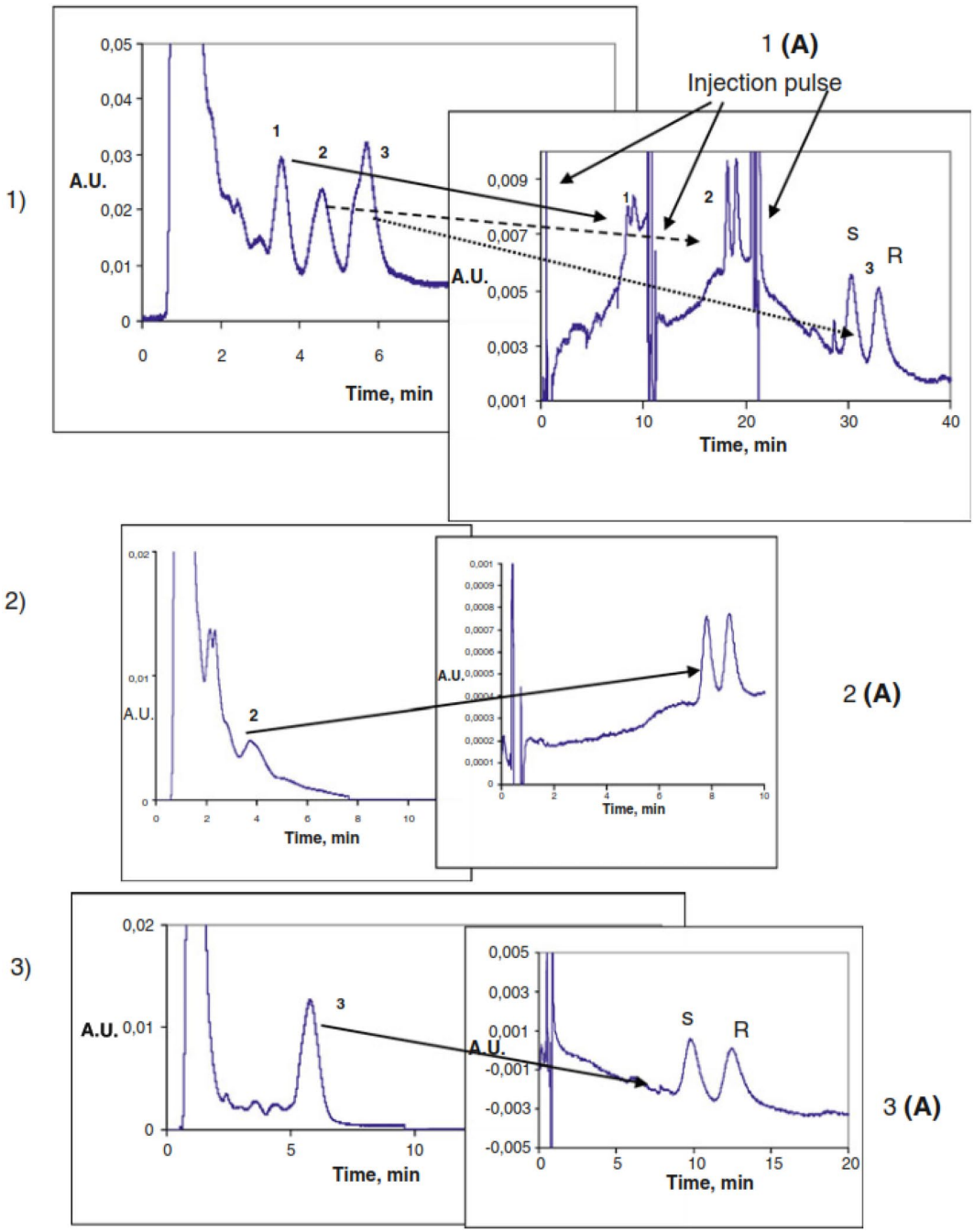

Fig. 5 Enantio-separation of cardiovascular drugs in 2D-HPLC adapted from reference [59] 1(A) and 2(A): achiral separation and 3(A): chiral separation

the NBD-L- and D-amino acids were separated at 40 °C using aqueous 5–18% MeCN solutions as a mobile phase. In ^{2}D, the chiral separation was done at 25 °C. Goel et al. [52] synthesized a chiral drug having three chiral centers. After that, the enantiomeric forms of that drug were enantio-separated using 2D-HPLC. Liu et al. [53] determined the enantiomers in a sample mixture using 2D-LC. The primary and secondary columns used were –NH_2 column and the chiral column, respectively. The mobile phase used was 70% *n*-C_6H_{14} and 30% isopropanol; detection: 365 nm; flow rate: 1.0 mL/min; temperature: 25 °C. The enantiomeric metabolites of hydroxychloroquine were determined by Iredale and Wainer [54] using 2D-HPLC. The primary and secondary columns used were Ultremex cyano-bonded phase and immobilized AGP, respectively. The mobile phase used was (1) ammonium acetate buffer (pH 4.5) with flow rate of 0.6 mL/min and (2)

0.03 M Na_3PO_4 buffer (pH 7.0) with detection at 320 nm. Peak purity for a compound of chiral pharmaceutical interest was determined by Lee et al. [55] using 2D-HPLC. The primary and secondary columns used were Waters Symmetry Shield RP-18 and Chiralpak AD-H, respectively. Deionized H_2O with 0.1% (v/v) H_3PO_4 [A] and HPLC grade ACN [B] was used as a mobile phase with detection: 225 nm; flow rate: 0.8 mL/min and temperature: 22 °C. The enantiomers of verapamil were enantio-separated at room temperature by Oda et al. [56] using 2D-HPLC. The primary and secondary columns used were inertial ODS-2 and Ulton ES-OVMG chiral columns, respectively. The mobile phase used was ACN/water/TFA (25:75:0.1–50:0.1); K_3PO_4 buffer (5 mM, pH 7.5); potassium dihydrogen phosphate with ethanol and water; detection: 280 nm in 1D and 230 nm in 2D; flow rate: 1 mL/min. The enantiomeric excess of 2-phenyl-2,3-dihydro-4-quinolone was determined at room temperature by Liu et al. [57] using 2D-HPLC. The primary and secondary columns used were -NH_2 and ODH Chiral, respectively. *n*-C_6H_{14} and isopropanol (7:3) were used as the mobile phase with a flow rate of 1 mL/min at detection 365 nm. Cheng and Liao [58] determined the amino acid sequence of short peptides using 2D-HPLC. The primary and secondary columns used were Astec ChiroBiotic™ T and the ligand-exchange chromatography (LEC) column, respectively. The mobile phase used was MeOH/H2O (30:70) solution; detection: 210 nm; flow rate: 1 mL/min at 40 °C.

Biological Analysis

Besides the applications mentioned above, 2D-HPLC has also been used in drug analysis in biological matrices. Yang et al. [59] applied a heart-cut 2D-LC strategy for the enantiomeric separation of cardiovascular drugs in the urine (Fig. 5). The primary and secondary columns were an essential column (Kinetex™ HILIC) and a vancomycin chiral column (Chirobiotic™ V), respectively. In ^{1}D, the mobile used was methanol:acetonitrile:CH_3COONH_4 buffer (5 mM, pH 6) (90:5:5, v/v) with 0.40 mL min^{-1} flow rate. In ^{2}D, the mobile phase used was methanol:CH_3COONH_4 buffer (2 mM, pH 4) (97:3, v/v) with 0.50 mL min^{-1} flow rate at UV detection of 227 nm. Hamase et al. [60] used 2D-HPLC system having a microbore monolithic ODS column (^{1}D) a narrow bore-enantio-selective column (^{2}D) for the analysis of enantiomers of amino acid and the non-chiral amino acid in biological matrics. The derivatizing agent used was NBD-F.

Karakawa et al. [61] used 2D-HPLC for the investigation of the effect of the possible factors regulatory of the enantiomers of D-Ala, D-amino acid oxidase (DAO), and intestinal bacteria. The primary and secondary columns used were a reversed-phase column and a chiral column, respectively.

Iguiniz et al. [62] described on-line selective comprehensive 2D-RP-HPLC and SFC (sRPLC × SFC) for the analysis of pharmaceutical racemates (Fig. 6). The effects of injection volume and injection solvent were also studied on the chiral SFC. The columns used were Acquity BEH C_{18} and a Chiralpak IC. The phenobarbital and the enantiomer of methylphenobarbital in human plasma were determined by Ceccato et al. [63] using 2D-HPLC. The primary and secondary columns used were LiChrospher RP-18 ADS and Chiralcel OJ-R, respectively. The mobile phase used was 50 mM phosphate buffer and ACN; detection: 225 nm; flow rate: 1.0 mL/min. The stereoisomers of leucovorin and 5-methyltetrahydrofolate in plasma were determined by Silan et al. [64] using 2D-HPLC. The primary and secondary columns used were bovine serum albumin (BSA) and a C_{18} column, respectively. The mobile phase used was (0.10 M Na_3PO_4, pH 5.l); detection: 310 nm; flow rate: 1 mL/min at 40 °C. The discrimination effects in phenprocoumon metabolism were studied by Kammerer [65] using 2D-HPLC. The primary and secondary columns used were C_{18} column and a Chira Grom 2 column, respectively. The mobile phase used was H_2O/ACN/HCOOH (48/52/0.5) for chiral analysis and CH_3COONH_4 buffer (5 mM, pH 3.9) and MeOH for achiral analysis. Salbutamol, salmeterol, and atenolol in urine samples were determined by Yang et al. [59] using 2D-HPLC. The primary and secondary columns used were Kinetex™ HILIC and a vancomycin chiral column, respectively. The mobile phase used was MeOH:acetonitrile:CH_3COONH_4 buffer (5 mM, pH 6) (90:5:5, v/v) for 1D and methanol:CH_3COONH_4 buffer (2 mM, pH 4) (97:3, v/v); detection: 227 nm; flow rate: 0.4–0.5 mL/min. The enantiomers of 1,4-dihydropyridine calcium antagonists in the plasma of dogs were determined by Fujitomo et al. [66] using achira/chiral 2D-HPLC methods. The primary and secondary columns used were an anion exchange and Ultron ES-OVM, respectively. The mobile phase used was 0.05 M KH_2PO_4:MeOH (55:45) for 1D and 0.05 M KH_2PO_4:MeOH (56:44) for 2D; detection: 345 nm; flow rate: 0.8 mL/ min; at room temperature. R- and S-propafenone were analyzed by Lamprecht and Stoschitzky [67] in plasma using 2D-HPLC. The primary and secondary columns used were a cation exchanger and an enantioselective Chiralcel column, respectively. The mobile phases used were eluent A = 0.025 M KCl + 0.03 M H_3PO_4, adjusted to pH 2.4 with KOH Eluent B = ACN, eluent C = MeOH:H_2O (30:70, v/v); detection: 211 nm; flow rate: 1.3 mL/min at room temperature. For bioequivalence purposes, carvedilol was determined in plasma samples by 2D-HPLC [68]. The primary and secondary columns used were Chromolith Performance RP-18e column and guard cartridge Chiralcel ®OD-RH, respectively. The mobile phase used was (65%, v/v) aqueous 50 mM CH_3COONa buffer at pH 6 and 35% acetonitrile; detection: 285–355 nm; flow

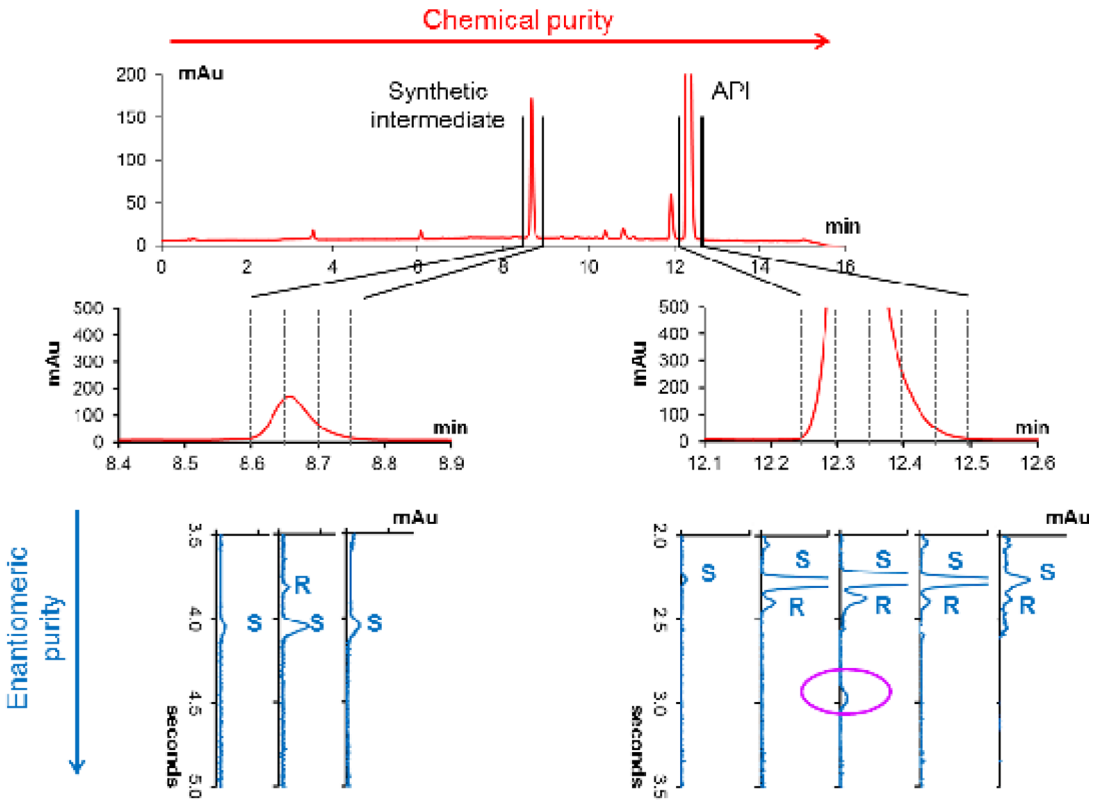

Fig. 6 Enantio-separation of pharmaceutical racemates [62]

rate: 2 mL/min. In blood serum, enzymatic hydrolysis of diperodon enantiomers was determined by Hroboňová et al. [69] using 2D-HPLC. The primary and secondary columns used were C_{18} and teicoplanin, respectively. The mobile phase used was MeOH/ACN (45/55, v/v) for 1D and 2D; detection: 240 nm; flow rate: 1 mL/min. Paracetamol and the enantiomers of ketorolac in human plasma were determined by Ing-Lorenzini et al. [70] using 2D-HPLC. The primary and secondary columns used were C_{18} and a polysaccharide-based chiral column, respectively. The mobile phase used was acetonitrile/0.1% HCOOH (50:50, v/v) at 1 mL/min flow rate. Lopes et al. [71] determined norfluoxetine (N-FLU) and fluoxetine (FLU) in colostrum and mature milk using a 2D-LC coupled to triple quadrupole tandem mass spectrometer (2D-LC–MS/MS). The primary and secondary columns used were phenyl-hexyl column and C_{18} column, respectively. The mobile phase used was buffer NH_4OAc (10 mmol/L; pH 5.8):acetonitrile:methanol (40:25:35, v/v); detection: 280 nm; flow rate: 20 µL/min; temperature: 50–60 °C. Wang et al. [72] separated and detected free D- and L-amino acids in tea by off-line 2D-LC. The primary and secondary columns used were Gemini C_{18} and Chiralpak® IC-3 column, respectively. The mobile phase used was ACN:TFA in water (0.05%, v/v); detection: 265 nm; flow rate: 1 mL/min at 40 °C. Alvim-Jr et al. [73] quantified the enantiomers norfluoxetine (NFLX) and fluoxetine (FLX) in human milk using a 2D-LC system by direct injection of samples. The primary and secondary columns used were an octadecyl column (RAM-$BSAC_{18}$) and a chiral column based on the antibiotics, respectively. The mobile phase used was 10 mM aqueous CH_3COONH_4 (pH 6.8)/C_2H_5OH (7.5:92.5, v/v); flow rate: 400 µL/min at room temperature. In the shoots of Rubus idaeus 'Glen Ample', the analysis of polyphenols and simple phenols was done by Kula et al. [74]. The primary and secondary columns used were an octadecyl C_{18} silica column and pentafluorophenyl column, respectively. The mobile phase used was ACN:MeOH:H_2O:TFA; detection: 280 nm; flow rate: 1 mL/min; temperature: 20 °C. The enantiomeric separation of ibutilide and artilide in plasma was determined by 2D-HPLC [75]. The authors used primary and secondary columns C_{18} column and a Pirkle's, respectively. The mobile phase used was MeOH-H_2O (40:60, v/v) for the ibutilide assay, MeOH-H_2O (35:65, v/v) for the artilide assay; flow rate: 1 mL/min.

Environmental Analysis

2D-HPLC has got tremendous success in the environmental sample analysis. Dugo et al. [76] used 2D-HPLC for the investigation of the enantiomeric distribution of

furocoumarins and coumarins in various citrus oils. The primary and secondary columns were a micro-silica column and a cellulose-based column, respectively. In citrus important oils, the outcomes confirmed the separation of chiral molecules. The most important thing to be noted was the strong occurrence of one of the two enantiomers. Chlorodibenzo-p-dioxin (CDD), by-side chlorodibenzofuran (CDF), Chlorobiphenyl (CB), and chloronaphthalene (CN) homologue cluster and congener alignment, and concentrations have been quantified [77]. The porous graphitic carbon as a primary column and pyrenyl silica as a secondary column have been used. The oxidized sterols were identified by Woods et al. [78] as an important constituent of dissolved organic matter, using 2D-HPLC. A silica column was used as a primary column, while as a secondary column, unbound silica stationary phase. A 95% ACN/5% 100 mM deuterated CH_3COONH_4 was used as a mobile phase.

Nano 2D-HPLC

Nano 2D-HPLC is a new development with some applications in the literature. 2D online nano-LC/MS was developed that substituted the inserted salt step gradient with an optimized semi-continuous pumped salt gradient. The feasibility of that process was also confirmed in the outcomes of a relative investigation of a composite tryptic digest of the yeast proteome using the inoculated salt solution process and the semi-continuous pump salt process [79]. 8-Iso-prostaglandin F2α was measured from human urine [80]. A microchip-based nano-HPLC was also used. Obviously, we have many techniques around us, but multi-dimensional protein identification technology (MudPIT) allows the online isolation of extremely complex peptide mixes coupled with mass spectrometry-based identification. Hence, they presented a dissimilarity of the old-style MudPIT protocol, joining extremely searching chromatography using a liquid chromatography system based on nanoflow (nano-LC) with a 2D-pre-column in a vented column setup [81].

Challenges and Future Perspectives

It is important to mention here that 2D-HPLC is gaining importance in the chiral separation, because sometimes, racemates are present at low concentrations or in low amounts. Some papers on chiral separation by 2D-HPLC are in Table 1. A limited number of applications indicate that 2D-HPLC could not be utilized fully in this area of separation and need more attention. It is due to certain limitations and challenges. The most significant challenges include the ideal combinations of stationary phases and the mobile phase. The paraphernalia also needs to be improved; especially the column switching method. It is significant to mention here that the speed of separation on the second mobile phase must be greater than the first column in online separation and, sometimes, it becomes hard to achieve. Generally, the chromatographs are using temperature increment to increase the separation in 2D, which is one of the biggest challenges in 2D-HPLC for the chiral resolution. In addition to this, the paraphernalia of 2D-HPLC is more costly than 1D-HPLC and it is a little bit difficult to operate 2D-HPLC at an economic level. Nano-2D-HPLC is the latest development and it will be the most applicable technique in the chiral resolution in the future.

Despite many challenges, 2D-HPLC has a bright future in chiral separations. This modality of chromatography may be useful for samples having low concentrations of the racemates. It was observed that in most of the research papers, the first phase is achiral, while the second phase is chiral. Therefore, the first stationary phase should be optimized using the column with various functionalities like reversed-phase, hydrophilic, size exclusion, ion exchange, etc. Similarly, the second column should be optimized with various chiral selectors like polysaccharides, cyclodextrins, proteins, crown ether, a macrocyclic glycopeptide antibiotic, Pirke type, ligand exchange, etc. Moreover, the best and large-scale chiral separations may be archived by coupling first and second both the same chiral columns. Also, the two chiral columns may be different, i.e., having different chiral selectors. Instead of increasing the temperature of the second phase, it is better to use a suitable mobile phase to speed up the separation on the second column. There is an urgent need for advancement in 2D-HPLC in the economic and fast chiral separations. In this direction, much work is needed to advance up the paraphernalia of nano-2D-HPLC. The other most important aspects to be controlled are the column dimensions, the different combinations of the mobile phases compatibility, column regeneration, and flow rates.

Conclusion

2D-HPLC is being used for the separation of complex mixtures since its development in the 1970s. Later on, it has been applied to the chiral separations by some researchers. A thorough search of the literature indicates only a few publications in this area. Some limitations have been identified for the chiral resolution by 2D-HPLC. Briefly, the efforts are made to deliberate the position of the enatio-separations and 2D-HPLC, its application in biological and environmental matrices and nano-2D-HPLC. Besides, the challenges and future perspectives are also discussed. The present review will be very useful in the improvement of 2D-HPLC for the chiral resolution of the numerous racemates, and especially

Table 1 Chiral separations using 2D-HPLC

Racemates	Experimental conditions		Refs.
	Mobile phase in (1D)	Mobile phase in (2D)	
(±)-7-Chloro-3-methyl-3,4dihydro-2*H*-1,2,4benzothiadiazine1,1-dioxide [(±)IDRA21	H_2O:ACN (60:40) or 0.05 M $NaClO_4$ Buffer (pH 2): ACN (70:30, v/v) in C_{18} Colum	H_2O:ACN(60:40) or 0.05 M $NaClO_4$ Buffer (pH 2): ACN (70:30,v/v) in Chiral OD-R	[42]
Amino acids	(1A)water + 0.05% formic acid, (1B)ACN + 0.05% formic acid(v/v) in C18 column	(2A)MeOH/H_2O(98/2,v/v) with 100 mM formic acid + 100 mM CH_3COONH_4, (2B)MeOH/H_2O (98/2,v/v) in t-BuCQN	[43]
Coumarins and furocoumarins	H_2O/ MeOH /THF (85:10:5) (solvent A) and MeOH /THF (95:5) (solvent B) in a micro-silica column	98%of *n*-hexane and 2% propanol in a cellulose-based column	[76]
D- and L-Phe, D- and L-Trp, D- and Tyr	CH_3COONH_4 buffer(20 mM, pH6)(94%) and MeOH (6%) in C_{18} Column	CH_3COONH_4 buffer(20 mM, pH6)(94%) and MeOH (6%) in teicoplanin-based column	[45]
Salmeterol, atenolol and salbutamol	MeOH:ACN:CH_3COONH_4 buffer(5 mM, pH6) 90:5:5 in Kinetex™ column	MeOH:ACN:CH_3COONH_4 buffer(5 mM, pH6) 90:5:5 in Chirobiotic™ V column	[59]
Amino acids	6% MeCN and 0.06% TFA in ODS-Microbore-monolithic column	2.5 mM citric acid in the mixed solution of MeOH–MeCN (25:75) for Glu and Asp; 0.5 mM Citric acid in MeOH for His; pure MeOH for Arg in Pirkle's type enantio-selective column	[48]
N-Methyl-D-aspartic acid	MeCN-TFA-H2O (6:0.05:94) in ODS-Microbore-monolithic column	1.5 mM citric acid mixed in MeOH–MeCN (50:50) or 0.8% formic acid mixed MeOH–MeCN(20:80) in narrow bore-enantio-selective columns	[47]
Amino acids	7%MeCN 0.05%TFA in water and (B) 20% MeCN 0.05%TFA in water in ODS Capillary monolithic column	MeOH:MeCN (90:10) with 0.375% formic acid or with 0.02% formic acid in pirkle-type enantio-selective column	[48]
D-Ala, D-amino acid oxidase	THF-TFA-MeCN-H_2O (1:0.02:10:89,v/v/v/v) in reversed-phase column	5 mM citric acid in MeOH for chiral column	[61]
Warfarin and hydroxy warfarin	0.1% H_3PO_4/ACN in ZORBAX RRHD Eclipse Plus C_{18} column	0.1% H_3PO_4/ACN in vancomycin based column	[50]
Amino acids	5–18% MeCN solutions containing TFA in ODS capillary monolithic column	5–18% MeCN solutions containing TFA in an enantio-selective column, KSAACSP-001S	[51]
Metabolites of hydroxychloroquine	0.02 M *N*,*N*-DMOA-0.06 M CH_3COONH_4 (40:60), (pH 4.5) in ultremex cyano-bonded phase column	0.03 M sodium phosphate buffer pH 7.0-C_2H_5OH-ACN (79:20: 1) with 0.005 M N,N- DMOA in immobilized AGP column	[54]
Verapamil	ACN/water/TFA (25:75:0.1–50:0.1); K_3PO_4 buffer (5 mM, pH 7.5); KH_2PO_4 with C_2H_5OH and H_2O in inersil ODS-2 column	ACN/water/TFA (25:75:0.1–50:0.1); K_3PO_4 buffer (5 mM, pH 7.5); KH_2PO_4 with C_2H_5OH and H_2O in ulton ES-OVMG chiral column	[56]
2-Phenyl-2,3-dihydro-4-quinolone	70% *n*-hexane and 30% isopropanol in NH_2 column	70% *n*-hexane and 30% isopropanol in ODH Chiral column	[57]
Amino acid sequence	MeOH/H_2O (30/70, v/v) in Astec ChiroBiotic™ T column	MeOH/H_2O (30/70, v/v) ligand-exchange chromatography	[58]
Methylphenobarbital enantiomers	50 mM phosphate buffer and acetonitrile in LiChrospher RP-18 ADS column	50 mM phosphate buffer and acetonitrile in Chiralcel OJ-R	[63]
Stereoisomers of leucovorin and 5-methyltetrahydrofolate	0.10 M sodium phosphate, pH 5.1 in C_{18} column	0.10 M sodium phosphate, pH 5.1 in bovine serum albumin based column	[64]
Phenprocoumon metabolism	CH_3COONH_4 buffer (5 mM, pH 3.9) (solvent A) and methanol (solvent B) in C_{18} column	H_2O/ACN/formic acid (48/52/0.5) in Chira Grom 2 column	[65]

Table 1 (continued)

Racemates	Experimental conditions		Refs.
	Mobile phase in (1D)	Mobile phase in (2D)	
Salbutamol, Salmeterol and Atenolol	MeOH:ACN:ammonium acetate buffer (5 mM, pH 6) 90:5:5) in Kinetex™ HILIC	MeOH:ammonium acetate buffer (2 mM, pH 4) 97:3 in vancomycin chiral column	[59]
Enantiomers 1,4 = dihydropyridine	0.05 M KH_2PO_4:MeOH (55:45) in an anion exchange column	0.05 M KH_2PO_4:MeOH (56:44) in ultron ES-OVM	[66]
R- and S-propafenone	Eluent A = 0.025 M KCl + 0.03 M H_3PO_4, (pH = 2.4), Eluent B = ACN, Eluent C = MeOH/H_2O (30/70, v/v) in a silica-based strong acid cation exchanger	Eluent D = 0.25 M $HClO_4$ + 0.05 M H_3PO_4 + 44% ACN, (pH = 2.5) in Chiralcel column	[67]
Carvedilol	65% (v/v) aqueous 50 mM CH_3COONa buffer at pH 6 and 35% ACN in a single monolithic Chromolith Performance RP-18 column	65% (v/v) aqueous 50 mM CH_3COONa buffer at pH 6 and 35% ACN in Guard Cartridge Chiralcel ®OD-RH	[68]
Enzymatic hydrolysis of diperodon enantiomers	MeOH/ACN 45/55 v/v in C_{18} column	MeOH/ACN 45/55 v/v in teicoplanin-based column	[69]
Ketorolac enantiomers and paracetamol	ACN/0.1% HCOOH 50:50 (v/v) in C_{18} column	ACN/0.1% HCOOH 50:50 (v/v) in a polysaccharide-based chiral column	[70]
Fluoxetine and norfluoxetine	NH_4OAc (10 mmol/L; pH 5.8):ACN:MeOH (40:25:35, v/v/v) in phenyl-hexyl column	NH_4OAc (10 mmol/L; pH 5.8):ACN:MeOH (40:25:35, v/v/v) in C_{18} column	[71]
Amino acids	(A) ACN and (B) TFA in water (0.05%, v/v) in Gemini C_{18}	(A) ACN and (B) TFA in water (0.05%, v/v) in CHIRALPAK® IC-3 column	[72]
Fluoxetine and norfluoxetine	10 mM NH_4OAc (pH 6.8)/C_2H_5OH (7.5/92.5) in octadecyl column	10 mM NH_4OAc (pH 6.8)/C_2H_5OH (7.5/92.5) in an antibiotic-based chiral column	[73]
Polyphenols and simple phenols	ACN:MeOH:H_2O:TFA in octadecyl C_{18} silica column	ACN:MeOH:H_2O:TFA in pentafluorophenyl column	[74]
Ibutilide and artilide	MeOH:H_2O 40:60 (v/v) in C_{18} column	MeOH:H_2O 40:60 (v/v) in a Pirkle column	[75]

for those who are working in the field of separation science the researchers, academicians, and industry persons.

Declarations

Conflict of interest The authors declare no conflict of interest.

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