Development of Fast and Selective Analytical Methods of Pharmaceuticals and Herbal Medicines by High-Performance Liquid Chromatography and Capillary Electrophoresis

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Abstract
Pharmaceuticals are essential for the medical treatment of various diseases. From the herbal medicines (crude drugs), synthetic medicines to biopharmaceuticals, to assure its efficacy and safety, the production procedures and the quality control methods of pharmaceuticals are important. These are strictly regulated by Good Manufacturing Practice (GMP) and various guidelines. The key is to develop the “Specifications and testing methods” of the pharmaceutical scientifically and rationally according to the production process. There are many testing items such as identity tests, purity tests, assay, etc. in “Specifications and testing methods”. Among them, assay and purity test are core testing items. Optical purity evaluation is required for the single enantiomer pharmaceutical. Chromatographic methods and its detection methods are useful for the purpose. High-performance liquid chromatography (HPLC), thin layer chromatography (TLC) and gas chromatography (GC) are commonly used because the target analyses are almost a mixture. In this review, results of the development of the quality evaluation methods of relatively low molecular pharmaceuticals and herbal medicines by HPLC and capillary electrophoresis (CE) we have been investigated are summarized.

Keywords: Pharmaceuticals; Enantiomers; HPLC; Capillary electrophoresis; Micellar electrokinetic chromatography

1. Introduction
There are two major types of analysis in the development process of pharmaceuticals. One is the quality control analysis of active pharmaceutical ingredients (APIs, drug bulk substance) and those formulations. Various purity tests and assay methods of APIs and tablets etc. are developed according to the synthetic or production process and its law materials. The other is the clinical analysis. The so-called ADME (absorption, distribution, metabolism, excretion) analysis is performed to clarify the drug properties. From the physicochemical properties and ADME analysis, the dosage form of the pharmaceuticals is decided.

Among various analytical methods, separation analysis, especially chromatographic methods and its detection methods are important and useful for the purpose. High-performance liquid chromatography (HPLC), thin layer chromatography (TLC) and gas chromatography (GC) are well adopted because the target analyses are almost a mixture. In this review, results of the development of the quality evaluation methods of relatively low molecular pharmaceuticals and herbal medicines by HPLC and capillary electrophoresis (CE) we have been investigated for more than 40 years are summarized.

To assure efficacy and safety, the production procedures and the quality control methods of pharmaceuticals are important. These are strictly regulated by Good Manufacturing Practice (GMP) and ICH guidelines [1]. As for the quality assurance, the key is the development of the “Specifications and testing methods” of the pharmaceutical according to its production process. There are many testing items such as identity tests, purity tests, assay, etc. which should be included in the testing methods of pharmaceuticals by regulation [2,3]. Various stability studies are also important to assure the quality of
pharmaceuticals, leading to the determination of the expiry date and the final package form.

Among various testing items, assay and purity tests are core testing items. After the problem of harmful side effects of thalidomide (in the 1950s), APIs existing enantiomers are developed as a single enantiomer. As in theICH quality guidelines mentioned above, the enantiomer purity test comes to be essential. A minor enantiomer is regarded as a major impurity of APIs. Chromatographic methods are quite effective for the purpose. Through various analytical techniques such as the chiral derivatization, use of chiral columns etc., optical purity of pharmaceuticals can be evaluated and controlled well. However, in the 1980s, this was a challenging theme for chromatographers.

At the beginning, the HPLC enantiomer separation of pharmaceuticals developed by Tanabe Mitsubishi pharmaceutical company (the author belonged until 2008) is mentioned. Next, a simultaneous HPLC separation of many ingredients (APIs) contained in formulations, employing a micellar solution (micellar LC), and HPLC evaluation methods of herbal medicines (crude drugs) are shown. Continuously CE separation of pharmaceuticals, and CE enantiomer separation are summarized. Finally, separation analysis by HPLC with core-shell type columns is described. Chemical structures of optically active pharmaceuticals mainly targeted in this study are shown in Fig. 1.

**Fig. 1.** Chemical structures of optically active pharmaceuticals investigated in this study.

2. **Enantiomer separation of pharmaceuticals by HPLC and its application to optical purity test**

As mentioned above, the development of enantiomer separation methods becomes to be quite important, however, there is no universal and useful methods in the 1980s. As a direct evaluation method, only a Pirkle-type chiral column was available [4], except the optical rotation measurement method in those days. Therefore, a development program of the novel chiral derivatization reagent started in Tanabe. As a chiral moiety, natural L-amino acids, and a phenyl or a naphthalene skeleton as a UV chromophore, several chiral derivatization reagents were synthesized and applied for the optical purity test of diltiazem hydrochloride (HER, Herbesser®) (see, Fig. 1). HER has two asymmetric carbons, leading to four optical isomers. Among them, a (2S,3S)-form is active and developed as a Ca-channel blocker. In the new drug approval to FDA authorities, optical purity evaluation by the chiral derivatization method was applied. Some of chiral reagents developed or used for the optical purity tests of pharmaceuticals (Fig. 1) are summarized in Fig. 2. β-Naphtysulfonyl-L-prolyl chloride (β-NSPC) was successful for the separation of four isomers of HER [5]. In the derivatization of HER, after the hydrolysis of HER by NaOH, its deacetyl-form (OH residue) was reacted with β-NSPC in dichloromethane and pyridine. Corresponding each derivative (diastereomer) was separated (Rs > 1.5) within 20 min in an elution order (2S,3S) (HER), (2S,3R), (2R,3R), and (2R,3S) by a Zorbax Sil column (4.6 mm ID x 15 cm, flow rate 1.0 mL/min, room temperature, 254 nm) under the adsorption mode (mobile phase, CHCl3/CH3Cl/CH3OH/diethylamine (DEA) = 200/50/30/0.1). Limit of detection (LOD) of the minor enantiomer was around 0.1% at signal to noise (S/N) = 3. Other than HER, enantiomers of denopamine (DP, Kalgut®), a cardioprotective drug were successfully separated (Rs > 1.5) by the derivatization with β-NSPC under the same adsorption mode (Zorbax Sil (4.6 mm ID x 15 cm), flow rate 1.0 mL/min, mobile phase CH3Cl/CH3OH/DEA = 95/5/1). The elution order was S-form (minor enantiomer) and R-form (DP). LOD of the minor enantiomer at S/N =3 was 0.1% [6]. β-NSPC was useful for the separation of 18 DL-amino acids by TLC and adsorption HPLC with a Zorbax Sil column (4.6 mm ID x 15 cm) [7].

β-NSPC can be applicable for an amino and a hydroxy residue, next, chiral derivatization reagents for a carboxy residue were investigated. Among various chiral amino compounds, a chiral amine L-alanine-β-naphtylamide hydrobromide (L-Ala-β-NA, see Fig. 2) was successfully applied for the enantiomer separation of naproxen (NX, Naixan®, a nonsteroidal anti-inflammatory drug) [8], imidapril hydrochloride (TAL, Tanatril®, an ACE-I inhibitor) [9], a drug candidate, acetyl-L-carnitine (L-AC) [10]. Derivatization of NX was performed in chloroform and N,N’-dicyclohexylcarbodiimide (DCC), and derivatives were separated by a Zorbax Sil column (4.6 mm ID x 15 cm, 40°C, flow rate 1.0 mL/min, 254 nm) with the mobile phase CHCl3/CH3OH = 500/3 within 10 min (Rs 6.6, R-form > S-form (NX)). LOD of the minor enantiomer was ca. 0.2%.

In TAL, derivatization was performed in chloroform with pyridine and DCC. Complete separation (Rs 10.5) was
obtained by a Zorbax Sil column (5 μm, 4.6 mm ID x 15 cm, 40°C, flow rate 1.0 mL/min, 254 nm) with the mobile phase CHCl3/CH3OH/C2H5OH/DEA = 600/10/2/0.1 within 15 min. SSS-form (TAL) eluted faster than the minor enantiomer RRR-form. LOD of the minor enantiomer was 0.05%. In case of L-AC, derivatives were separated by a C18 column (5 μm, 4.6 mm ID x 15 cm, 40°C, flow rate 1.0 mL/min, 254 nm) with the mobile phase of 0.05 mol/L phosphate buffer (pH 2.5)/acetonitrile (ACN)/tetrahydrofuran (THF) = 85/9/6. A typical chromatogram is shown in Fig. 3A. Four derivatives including DL-carnitine derivatives were successfully separated within ca. 21 min [10]. For a fast enantiomeric purity test of L-AC, a mixture of the same buffer mentioned above and 23% ACN was used as the mobile phase. DL-AC derivatives were separated within 10 min with Rs 1.94. The elution order was the minor enantiomer (D-AC) > L-AC. LOD of the minor enantiomer was 0.05%. The structural analysis of the derivatives in the above investigations were performed by NMR and MS analysis after the preparative process.

**Fig. 2.** Chiral derivatization reagents investigated in this study.

In general, enantiomer separation by the adsorption HPLC mode seems to be effective for the distinction of the diastereomer. However, for samples dealing in the biological analysis such as blood and urine, the reversed-phase HPLC mode has many advantages compared with the adsorption HPLC mode, because these samples can be directly injected to the HPLC apparatus after some pretreatments such as deprotonation. Since the 1990s, chiral stationary phases (CSPs) that can be used in the reversed-phase mode have become commercially available. Among them, an ovomucoid (OVM) column [11,12] where a protein is immobilized, showed a wide enantioselectivity for HER and its analogues. As shown in Fig. 4, enantiomers of HER and its main decomposed and/or impurity, the deacetyl form (totally four substances) were simultaneously separated by an OVM column (Ultron ES-OVM, 4.6 mm ID x 15 cm) within 20 min employing 0.02 M phosphate buffer (pH 6.0)/ACN = 88/12 as the mobile phase [13]. Rs value of the enantiomers of HER was 6.41. This OVM column turned out to be useful for the enantiomer separation of synthetic intermediates of HER [14]. Further the separation of enantiomers of 8-chloroderivative of HER (clentiazem) was successful by the OVM column [13]. Other than protein immobilized CSPs, enantiomers of TAL were separated by a ligand-exchange type CSP, a CHIRALPAK WH column [9], enantiomers of L-AC were separated by a SUMICHLAL OA-6100 column (4.6 mm ID x 15 cm, 40°C, flow rate 1.0 mL/min, 254 nm), employing a bulky acid (0.5 mol/L NaClO4) and 2 mmol/L Cu2+ as the mobile phase [15] (see, Fig. 3B). Enantiomers of DP were also successfully separated by a β-cyclodextrin (β-CD) derivative immobilized column, Ultron ES-CD with an aqueous mobile phase [16].

**Fig. 3.** (A) Separation of DL-carnitine derivatives and DL-AC derivatives by a C18 column (Cosmosil ODS ARII). (B) Direct enantiomer separation of DL-AC by the ligand-exchange HPLC.

**Fig. 4.** Direct enantiomer separation of (A) HER and its deacetyl form (OH-form) by an OVM column. (B) HER API spiked ca. 0.1% minor enantiomer and ca. 0.1% main impurity (OH-form).

On the other hand, when a direct reversed-phase
Chromatography

Enantiomer separation of drugs is unsuccessful, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isocyanate (GITC) chiral derivatization reagent was powerful for amino pharmaceuticals. GITC derivatives of DP and trimetoquinol hydrochloride (AQ, Inolin®; a bronchodilator) were separated with a C18 column [17,18].

Recently many kinds of CSPs immobilized polysaccharide derivatives have been developed and commercially available. Among them, a CHIRALPAK AS-3R (3 μm, 4.6 mm ID x 15 cm) that can be used in the reversed-phase mode showed a remarkable separation ability for NX enantiomers and impurities. As shown in Fig. 5, evaluation of related substances including the minor enantiomer (R-form) as an impurity was achieved with the mobile phase ACN 60% within 15 min. The retention time of S-form was around 5 min with Rs between the enantiomers of 2.55 [19]. Results of the purity test including optical purity were summarized in Table 1. Seven impurities A-F including the minor enantiomer R-form were detected. Main impurity was the R-form (0.21-0.78%). Table 2 summarized a content uniformity test of NX tablets together with a chromatogram.

Next, to widen the applicability of chiral crown ether moiety, we synthesized a CSP immobilized a chiral crown ether, (+)-(R)-18-crown-6 tetracarboxylic acid ((+)-18C6H4), which has been used as a useful chiral selector in the CE enantiomer separation [21]. A schematic illustration of the novel CSP is shown in Fig. 6. Some successful enantiomer separation of highly hydrophobic compounds is shown in Fig. 7.

Fig. 5. Chromatograms of the optical purity test and related substances of NX APIs by the CHIRALPAK AS-3R column. (A) API from Tanabe, (B) API from A company.

Fig. 6. A novel chiral stationary phase developed for hydrophobic amino acids.
Direct enantiomer separation of DL-Ala-β-NA and RS-1-(1-naphthyl)ethylamine (1-NEA) was successful within 15 min [22,23]. Further, chiral recognition mechanism of this CSP was investigated by NMR [24,25] and X-ray crystallographic analysis of the complex composed of the R-1-NEA and (+)-18C6H4 [26-30]. These information were referred to the following CSPs design.

Fig. 7. Direct enantiomer separation of amino compounds (A) DL-Ala-β-NA and (B) 1-NEA. Mobile phase, ACN/0.01 mol/L HClO4 = 50/50; column temperature, 25°C; detection, 254 nm.

3. Quality evaluation of pharmaceuticals and herbal medicines (crude drugs) by HPLC

As for the HPLC quality evaluation methods of pharmaceuticals, fast and robust methods are recommended. Though addition of a surfactant such as sodium dodecyl sulfate (SDS) to the mobile phase in the reversed-phase mode, so-called micellar LC, simultaneous HPLC determination of five APIs, which are neutral (predonisolone, crotamiton, glycyrrhetinic acid) and basic (dibucaine, chlorhexidine), in the ointments was successful in an isocratic mode within 18 min [31]. The HPLC conditions are as follows: column, Zorbax RX-C8 (5 μm, 4.6 mm ID x 15 cm); column temperature, 40°C; detection, 270 nm; mobile phase, 0.05 mol/L phosphate buffer (pH3.0) containing 0.3% SDS/2-propanol = 60/40. In this case, retention of three neutral APIs was manipulated by the concentration of an organic solvent. By increasing SDS concentration, retention of both chlorhexidine and dibucaine increased, although that of other three neutral APIs did not change. Manipulation of the retention of two basic APIs was successful through the concentration of SDS. In this HPLC conditions (micellar LC), crotamiton, which is used as a scabicide, insecticide, and antipruritic agent, having cis- and trans-isomers in approximate proportions of 5:95, eluted as one peak, which will be better as a quality control method of formulations. Micellar LC with a C18 column and SDS was also successfully applied for the separation of three APIs (phenylpropanolamine, tipepidine, chlorpheniramine) in the cold medicine [32]. These were separated in an isocratic mode within 14 min by the mobile phase of a mixture of 0.2% SDS in 0.1% phosphoric acid and ACN = 55/45. As for the separation of cis-trans isomers of crotamiton, because that ratio is needed for quality control of crotamiton, THF gave the best separation [31]. Other than the separation of crotamiton isomers, THF was quite effective for the separation of dihydrocodeine and codeine (the only difference is one double bond), berberine and palmatin [32,33].

As for the quality evaluation of herbal medicines (crude drugs), Conventionally identification by TLC analysis and microbiological tests have been performed as the quality test. Therefore, investigation on the marker substance in Kampo and formulations such as drinkable preparations was performed by employing HPLC. First, marker substances for the identification of Astragalus Radix (Ougi) were investigated. As a result of analysis, one of the main components was clearly detected even in the fluid extracts, leading to a good marker substance. This component was identified to be calycosin [34]. Some chromatograms are shown in Fig. 8. In case of Cistanches Herba (Nikujyuyou), echinacoside and acteoside were found as useful marker substances [35]. Lumbricine (a unique amino acid found in lumbricus (Jiryū) [36]. An isocratic HPLC determination method of some saponins (ginsenoside Rb1, Rb, Re, Rg1) by a C8 column, and an ion-exchange HPLC (Asahikagakum 502N, 7.6 mm ID x 10 cm, 40°C, 215 nm) analysis of β-oxalo-L-α,β-diaminopropionic acid (β-N-ODAP) were developed and applied for the content determination of these components in Panax notoginseng (Denhichininjin) [37]. Further, as a method for the antioxidative activity of methanol extracts of herbal medicines, 1,1-diphenyl-2-pircyhydrazyl (DPPH) radical scavenging activities and electrochemical detection (ECD)-HPLC were investigated. A correlation (r² = 0.76) between DPPH radical scavenging ability and total peak areas detected by ECD-HPLC was obtained [38]. For the HPLC evaluation of five fungi such as Poria cocos (Bukuryyou), Grifola frondosa (Maitake), polysaccharide marker substances for the identification was investigated by gel permeation chromatography (GPC). As a result, different GPC patterns and polysaccharide contents were observed [39]. Other than plants herbal medicines, ICP-AES analysis was performed for the evaluation of mineral herbal medicines such as Fossilia ossis mastodi (Ryukotsu), Gypsum fibrosum (Sekkou), and Ostreae testa (Borei) [40,41]. For animal herbal medicine, Cervi parvum cornu (Rokujo), DNA analysis was performed [42]. Results obtained and mentioned above have been applied for the quality evaluation of NANPAO capsules (Tanabe), where 31 herbal medicines are compounded.
4. Analysis of pharmaceuticals by CE

Since the 1980s, capillary electrophoresis (CE) and its related techniques have been noticed because of fast separation and high resolution capability [43-45]. Various modes of separation, from capillary gel electrophoresis (CGE) to micellar electrokinetic chromatography (MEKC) have been developed. Among them, capillary zone electrophoresis (CZE) mode and MEKC mode where a micelle is used as a pseudo-phase, allowing the separation of electrically neutral compounds, have been successfully used. Further, addition of chiral compounds such as CDs and bile acids to the CE background electrolytes offers the separation of drug enantiomers.

First, separation capability of CE (CZE) and MEKC was investigated. Separation of water soluble vitamins (eleven mixture) [46], β-lactam antibiotics (seven penicillin antibiotics and nine cephalosporin antibiotics) [47] were successfully separated within 20 min by MEKC with a fused-silica capillary tube (effective length 57 cm x 0.05 mm ID, applied voltage +20 kV) and an anionic surfactant, SDS solution or an N-lauroyl-N-methyltaurate (LMT) solution. Compared with a simple CZE mode with a buffer solution of pH 9.0, MEKC showed good selectivity and high theoretical plate numbers of around 300000. With an increase of the surfactant concentration, a large increase of the migration time of cationic drugs such as vitamin B1 and hydrophobic drugs was observed. An ion-pair formation (electrostatic interaction) between the analyte and the micelle seems to contribute its behavior. Then, effect of further additives such as tetraalkylammonium (TAA) salts to the SDS solution was investigated. As a result, addition of TAA salts to the SDS solution affected the migration times of cationic or anionic compounds, leading to the improvement of selectivity [48].

Next, as for the surfactant structure, five anionic linear type surfactants including SDS and LMT, were employed for the separation of twelve APIs used in the cold medicine [49]. Different separation selectivity was obtained from each surfactant. Employing ethyl p-hydroxybenzoate as an internal substance (IS), assay of APIs (acetaminophen, caffeine, ethenzamide) in commercial preparations was successful with average content almost 100% and C.V. below 3%.

In the MEKC separation with these five surfactants, basic or hydrophobic APIs such as noscapine, chlorpheniramine, and tipepidine migrated at a migration time of the micelle. To reduce the strong interaction between the surfactant and drugs, addition of urea [50], or CD [51] (so-called CD-MEKC mode) was investigated. Successful separation of corticosteroids (eight mixture) and aromatic hydrocarbons such as toluene, naphthalene, fluorene etc. (nine mixture) was achieved through the addition of β-CD or γ-CD and high concentration (2-8 mol/L) urea to the SDS solution. Other than additives, for the separation of these drugs showing strong interaction with the micelle, bile salts were employed in MEKC. By employing bile salt micelles, successful separation of cold medicines (fourteen mixture), corticosteroids (eight mixture) and benzothiazepin analogues including HER and its related substances (twelve mixture), was achieved with a high resolution [52,53].

Separation example is shown in Fig. 9. Under the optimum conditions (0.1 mol/L sodium cholate (SC) in pH 8.0 buffer), the purity test of HER API and its tablets was performed. As a result, 0.22-1.14 % of deacetyl form (OH-form) was detected. Assay of HER tablets was performed by the IS method. The average assay value for six replicate analyses was 101.5 % and the C.V. was 1.7% [53]. Topsym® cream containing 0.05 % of fluocinonide was also assayed by the IS method employing MEKC with 0.1 mol/L SC in a pH 9.0 buffer. The average assay value for six replicate analyses was 100.9 % and the C.V. was 4.1% [53].

Other than separation analysis, plasma proteins such as albumin and globulin, which might interfere with drug analysis, are solubilized by the micelle, hence these elute later than the drugs. Consequently, a direct plasma sample injection can be performed without any pretreatment such as deprotonation or extraction in MEKC [54]. Determination of aspoxicillin (ASPC, Doyle®, a penicillin antibiotic) in human plasma was successfully performed by MEKC with an SDS solution within 20 min through a direct sample injection and the IS method [55]. LOD of ASPC was 1.3 μg/mL at S/N =3 which covers the clinical blood concentration level of ASPC.
Further, separation of GITC derivatives of DL-amino acids was investigated by MEKC with an SDS solution. 19 of 21 derivatized DL-amino acids, except DL-aspartic acid and DL-glutamic acid, were enantioseparated within 50 min (capillary, effective length 57 cm and 0.05 mm ID; applied voltage, +20 kV) [56]. Recently oligonucleotide pharmaceuticals have been actively developed. For the separation of nucleotides, CGE is effective. As a sieving gel media, dextran, dextrin, pullulan, and poly(ethylene glycol) were employed under the concentration below 30%. Polydeoxythymidyllic acids (p(dT)11-20) were used as a test sample. Employing 30% dextran solution, successful separation of p(dT)11-20 was achieved within 35 min [57].

MEKC shows a powerful separation capability for herbal medicines. Calycosin in drinkable preparations was determined successfully within 5 min by MEKC with a 0.05 mol/L SDS (capillary, effective length 20 cm x 0.075 mm ID; applied voltage, +20 kV) [58]. Atractylenolide III in Astragali Radix (Ougi) extracts was determined within 10 min by MEKC with a 0.05 mol/L SDS (capillary, effective length 20 cm x 0.075 mm ID; applied voltage, +20 kV) [59]. Compared with HPLC analysis, CE separation showed a high resolution.

5. Enantiomer separation of pharmaceuticals by CE

As mentioned above, an optical purity test of pharmaceuticals becomes essential in the development of optically active drugs [1]. However, all of them are not evaluated by the conventional chromatography. Therefore, many researches have continued the development of the novel chiral evaluation method. In these situations, since the 1990s, CE techniques such as CZE and/or MEKC having a high resolution capability, have developed into an important enantiomer separation technique. We investigated the enantiomer separation of pharmaceuticals by employing a fused-silica capillary tube of 0.05 or 0.075 mm ID and effective length of 20-57 cm. In the usual run, +20 kV was applied for the separation under the ambient.

Fig. 9. Separation of fourteen APIs used in the cold medicine by MEKC with (A) 0.1 mol/L SDS, (B) 0.1 mol/L SC, (C) 0.05 mol/L SDC in 0.02 mol/L phosphate-borate buffer (pH 9.0). Capillary, effective length 57 cm and 0.05 mm ID; applied voltage, +20 kV; temperature, ambient; detection, 210 nm. Peak Number: 1, caffeine; 2, acetaminophen; 3, sulpyrin; 4, AQ; 5, guaifenesin; 6, NX; 7, ethenzamide; 8, phenacetin; 9, isopropylantipyrine; 10, noscapine; 11, chlorpheniramine; 12, tipepidine; 13, dibucaine; 14, triprolidine.

Fig. 10. Enantiomer separation of HER and its analogues by MEKC with 0.05 mol/L STDC. Capillary, effective length 50 cm x 0.05 mm ID; applied voltage, +20 kV; temperature, ambient; detection, 210 nm.

Bile acids which show excellent separation selectivity for highly hydrophobic drugs, turned out to be useful for the enantiomer separation. Carboline derivatives (drug candidates) were successfully enantioseparated within 17 min (α, 1.06-1.17; theoretical plate numbers, 350000-550000) by four bile salts, namely sodium cholate (SC), sodium taurocholate (STC), sodium deoxycholate (SDC), and sodium taurodeoxycholate (STDC), employing a 0.05 mol/L phosphate-borate buffer (pH 7.0) containing 0.05 mol/L each bile salt [60]. Enantiomer separation of AQ, tetrahydropapaveroline (α 1.03-1.07), HER and its deacetyl form, its chloroderivative (α 1.04-1.05) was successful with only a STDC solution (pH7.0, 0.05 mol/L), showing the structure of bile salts affected the enantioselectivity [60,61].
Enantiomer separation of HER, its OH-form, 6-chloroderivative of HER, and its OH-form by MEKC with a 0.05 mol/L STDC (0.02 mol/L phosphate-borate buffer pH 7.0) is shown in Fig. 10. Further, enantiomer separation of AQ, its related compounds, laudanosine, laudanosoline, and norlaudanosoline was investigated. Except laudanosoline showing a fast migration time, three enantiomers were separated. 1% of the minor enantiomer of AQ (S-form) was directly detected by the method [62].

Other than MEKC with bile salts, addition of chiral additives (chiral selectors) to the buffer used in CZE or MEKC is useful. CDs are attractive as chiral selectors, because there are three type CDs, namely α-CD, β-CD and γ-CD, further these many derivatives are also commercially available. In MEKC with an SDS solution, so-called CD-MEKC mode, γ-CD was effective for the enantiomer separation of barbiturates such as thiopental and pentobarbital, hydrophobic compounds such as 2,2′-dihydroxy-1,1′-dinaphthyl etc. The buffer used for CD-MEKC was a 0.02 mol/L phosphate-borate buffer (pH 9.0) containing 0.05 mol/L SDS and 0.03-0.06 mol/L γ-CD [63]. Two hydrophobic compounds were enantioseparated with a decrease of the migration time as shown in the separation of corticosteroids mentioned above. Further chiral additives such as d-camphor-10-sulfonate or l-methoxyacetic acid to this CD-MEKC improved the enantioselelctivity. As an example, enantiomer separation of two barbiturates by CD-MEKC is shown in Fig. 11.

![Fig. 11. Enantiomer separation of two barbiturates by CD-MEKC. Buffer, 0.05 mol/L SDS with 0.03 mol/L γ-CD and d-camphor-10-sulfonate in 0.02 mol/L phosphate-borate buffer (pH 9.0).](image)

The CD-MEKC mode is useful for the enantiomer separation of uncharged or electrically neutral drugs. On the other hand, addition of electrically neutral chiral selectors (for example, neutral CDs) to the CZE mode (CD-CZE) is powerful for the enantiomer separation of ionic drugs. In case of basic drugs, these migrate to the negative end (the detector side) by electrophoresis under the acidic buffer (low electroosmotic flow (EOF)). Under this CD-CZE mode, enantiomers of AQ, DP and primaquine etc. (totally eleven solutes in 30 enantiomers) were successfully separated by 2,6-di-O-methyl-β-CD (DM-β-CD) among four electrically neutral CDs (β-CD, DM-β-CD, heptakis(2,3,6-tri-O-methyl)-β-CD (TM-β-CD), γ-CD) [64,65]. Separation example is shown in Fig. 12. In this experiment, DM-β-CD used was obtained from the commercial source, it was found that resolution between enantiomers (reproducibility) differed by manufacturers. Therefore, the purity of the positional isomerism of DM-β-CD was investigated by employing HPLC and MALDI-TOFMS [66]. As a result, β-CD having more than two methyl residues gave the better enantiomer separation. Under the optimum conditions, the content uniformity test with the optical purity test of DP tablets was performed within 10 min [66].

![Fig. 12. Enantiomer separation of AQ and DP by CD-CZE. Capillary, effective length 37 cm x 0.05 mm ID. 0.05 mol/L DM-β-CD in 0.02 mol/L phosphate-borate buffer (pH 2.5).](image)

Other than CDs, electrically neutral polysaccharides such as dextrin and dextran have been applied to the enantiomer separation of pharmaceuticals by CZE [67,68]. As a result, enantiomers of many basic drugs such as HER were successfully separated by CZE with a 0.02 mol/L phosphate-borate buffer of pH 2.5 containing 3-15 % dextrin (JP grade, 4000 Da). Separation of HER, its OH-form and 8-chloroderivative by CZE with dextrin is shown in Fig. 13. Three enantiomers were separated through the addition of dextrin to the buffer. In this case, interaction of OH-forms with dextrin is larger compared with HER, leading to the long migration time. By employing CZE with 9% dextrin and the IS method...
(chlorpheniramine as IS) under a short capillary tube (effective length 20 cm, + 20 kV), fast content uniformity test of HER tablets including optical purity test was performed within 5 min [69]. β-CD polymer was also useful for the enantiomer separation of AQ etc. [70,71].

Further, ionic CDs such as CD-phosphates [16,72-74], CD-sulphates [73-76], carboxymethyl (CM)-CDs, carboxyethyl (CE)-CDs [73,74], ionic polysaccharides such as dextran sulfate [77] and DEAE-dextran [78], and aminoglycosidic antibiotics such as streptomycin sulfate and fradiomycin [77] were successfully employed as chiral selectors in electrokinetic chromatography (EKC) mode. Further, employing these various type chiral selectors, manipulation of the migration order of enantiomers (S-form and R-form) could be easily performed through changing buffer pH (low, high), applied voltage (positive, negative), and type of capillary tube such as uncoated fused-silica capillary, polyacrylamide coated capillary tube, or amine capillary tube [73].

Biologically natural materials such as chondroitin sulfate C (sodium salt) and heparin (sodium salt), both are known as mucopolysaccharides, were also successfully employed for the enantiomer separation of pharmaceuticals. Enantiomers of HER, its 8-chlorodervative, AQ, its isomer etc. were separated by the mucopolysaccharide-mediated EKC [71,79,80] (Fig. 14). 0.2 % of the minor enantiomer was detected by the method. Chiral amino drugs such as octopamine, DOPA, baclofen etc. were enantiomer separated by CE with 18C6H4 under the acidic conditions [21]. Enantiomer separation of Dl-amino acids by an indirect photometric detection was successful by CZE with (+)-18C6H4 and chrysoidine (a cationic dye) [81].

6. Development of fast and selective separation methods of pharmaceuticals and herbal medicines by core-shell type columns

In the development of pharmaceuticals, to obtain high efficiency and improvement of productivity, high throughput analysis is required. To correspond these requirements, ultra-HPLC (UHPLC), where small particle size (around 2 µm) packing materials are employed, has been progressing rapidly [97,98]. Higher performance (higher theoretical plate number, lower theoretical plate height) and fast analysis can be obtained by the UHPLC, compared with the conventional HPLC with the usual size packing materials (5 µm). However, small particle size leads to a dramatic increase of the column pressure drops. Therefore, UHPLC needs a special HPLC apparatus. In the conventional HPLC conditions (column: 4.6 mm ID x 15 cm, 5 µm, 40°C, flow rate 1.0 mL/min), column pressure drops are below 100 kg/cm². On the other hand, totally porous 2.2 µm column (3 mm ID x 5 cm, 40°C, flow rate 0.7 mL/min, MeOH 70 %) gave 300 kg/cm² [99]. Therefore, core-shell (CS) type packing materials, where relatively high performance is obtained with a relatively low column pressure drop, have drawn attention [100,101]. In the CS type packing materials, a solid core (around 1.5 µm) is encircled by a roughly 0.5 µm porous layer. Samples distribute to the shell part, not the core part. High performance is obtained from the thin shell layer. Schematic illustrations of conventional totally porous particles (5 µm) and CS type particles are shown in Fig. 15. Depth of the shell layer is various among the manufacturers. For example, Kinetex® has a shell layer of 0.35 µm and a core size of 1.9 µm. The CS type packing materials show much lower column pressure drops compared with sub-2 µm totally porous packing materials (for UHPLC), leading to
the usableness of the conventional HPLC apparatus after optimizing cell volume, sampling time of the detector, inner diameter of the tube connecting after the column etc.

![Solute](Image)

**Fig. 15.** Schematic illustrations of conventional packing materials and core-shell (CS) type packing materials.

Separation of HER and its main impurity and the decomposed product (deacetyl-form) was successful within 100 s, employing a CS-type C18 column (2.6 μm, 4.6 mm x 10 cm, MeOH 65%, 40°C, flow rate 1.0 mL/min) [102-104]. Closely related compounds, for example, codeine and dihydrocodeine [33], berberine and palmatin were baseline-separated by a CS-type C18 column (2.6 μm, 4.6 mm x 10 cm, 40°C, flow rate 1.0 mL/min), within 4 min employing THF as an organic solvent. Fast separation of corticosteroids (eight mixture), cyanocobalamin (vitamin B₁₂), its photo-degraded product and hydroxocobalamin, cold medicines (twelve mixture), nonsteroidal anti-inflammatory drugs (NSAIDs, eleven mixture) was also successful [104].

![Schematic illustration](Image)

**Fig. 16.** Schematic illustrations of various CS type reversed-phase columns. A : C18, B : Biphenyl, C : Cholesterol, D : Phenyl-hexyl.

Other than the usual C18 column, various reversed-phase CS type columns having a planar moiety such as phenyl and biphenyl etc. have been developed as shown in Fig. 16. For the separation of HER, its 8-chloroderivative (clentiazem), those main impurities (corresponding deacetyl-forms), and trans-forms (diastereomers), four CS type reversed-phase columns shown in Fig. 16 were employed with ACN as an organic solvent (40%). Among four columns, a Cholesterol column gave the best selectivity between trans-form and cis-form (HER), and the separation between the main peak and those deacetyl-forms (main impurity) was excellent in a Biphenyl column and a Phenyl-hexyl column [103]. From these results, a Biphenyl column was selected and applied for the actual analysis. Fast purity test of APIs and formulations (tablets (60 mg) and injections (10 mg)), assay and content uniformity test of tablets were achieved within 90 s (one analysis time) as shown in Fig. 17. Sufficient separation selectivity was obtained by the planar type CS columns with ACN giving low column pressure drops, compared with MeOH generally having a good selectivity. Further, a CS type reversed-phase packing material having a phenyl moiety was found to be effective for the separation of a mixture of corticosteroids [105]. Separation of corticosteroids by CS type reversed-phase columns was successful as shown in Fig. 18. For the separation of eight corticosteroids (triamcinolone (1), triamcinolone acetonide (2), cortisone (3), hydrocortisone (4), cortisone acetate (5), flusinonide (6), flusinolone acetonide (7), dexamethasone (8)), separation of 3 and 4 was unsuccessful in ACN 40%, 2 and 7 was unsuccessful in MeOH 55%. On the other hand, separation of 3 and 4 was successful in MeOH 55%, and THF was effective for the separation of 2 and 7. From these results, a mixture of water, MeOH and THF solution was used in a C18 column. A successful separation of eight corticosteroids was obtained even in use of ACN 40%, when a perfluorophenyl (5F) column was employed.

![Schematic illustration](Image)

**Fig. 17.** Separation of (A) HER and its related compounds by a CS type C18 column (Sunshell C18, 2.6 μm, 4.6 mm ID x 10 cm) with MeOH 65%, flow rate 1.0 mL/min, and (B) by a CS Biphenyl column with ACN 40%, flow rate 1.5 mL/min. (B-1) purity test and (B-2) assay by the IS method (IS: iPr, isopropyl paraben).
Meanwhile, CS type columns have great merits for the separation of herbal medicines, where complex compounds are included, because of its high performance. Separation of berberine (BE) and palmatin (PA) was separated by a CS-type C18 column (2.6 µm, 4.6 mm ID x 10 cm, flow rate 1.0 mL/min) within 4 min. Examples are shown in Fig. 19. By employing THF as an organic solvent, resolution between BE and PA was much improved without addition of SDS, and PA eluted before the main impurity. Further, sennoside A and sennoside B both contained in senna and daio (Fig. 20), six active ingredients contained in ginger, processed ginger, and magnolia bark (gingerols, shogaols, honokiol, and magnolol) (Fig. 21), were successfully separated within a short time, compared with the conventional official methods [106], indicating the separation power of the CS type column.

Fig. 18. Separation of eight corticosteroids by CS type reversed-phase columns. (A) Sunshell C18 (2.6 µm, 4.6 mm ID x 10 cm), water/MeOH/THF = 5/4/1, (B) Kinetex 5F (2.6 µm, 4.6 mm ID x 10 cm), ACN 40%.

Fig. 19. Separation of berberine and palmatin. (A) the official method (Japanese pharmacopoeia 17th method), COSMOSIL 5C18 (5 µm, 4.6 mm ID x 25 cm), phosphate buffer containing 0.2% SDS/ACN = 50/50, (B) Sunshell C18 (2.6 µm, 4.6 mm ID x 10 cm), a diluted acetic acid/THF = 80/20, 40°C, flow rate 1.0 mL/min, 345 nm.

Fig. 20. Separation of sennoside A and sennoside B in the powder of senna. (A) the official method (Japanese pharmacopoeia 17th method), Kinetex 5C18 (5 µm, 4.6 mm ID x 15 cm), acetate buffer (pH 5.0)/ACN = 17/8 (ACN 32%) containing 5 mmol/L [CH₃(CH₂)₆NBr and (B) a Cholesterol column (2.6 µm, 4.6 mm ID x 10 cm), a diluted acetic acid (1→80)/ACN = 4/1, 40°C, flow rate 1.0 mL/min, 340 nm.

Fig. 21. Separation of six active ingredients in (A) Kampo products (extract granules, Hangeshashin-to) and (B) Shoga-to (ginger food). Column, Biphenyl; mobile phase, 50% ACN; column temperature, 40°C; flow rate, 1.0 mL/min; detection, 210 nm.
7. Development of HPLC separation methods for highly hydrophilic pharmaceuticals

Reversed-phase HPLC with a C18 column has been successfully employed for the assay and purity tests of various pharmaceuticals. However, recently peptide type, protein type pharmaceuticals (so called biopharmaceuticals) have been widely used in the clinical field, because of its high efficacy. Further, metabolome analysis deals with various hydrophilic compounds such as organic acids, amino acids, sugars etc. The reversed-phase mode does not suit for the separation of these compounds because of low retention. For example, as shown in Fig. 22 [107], water soluble vitamins such as L-ascorbic acid (vitamin C, VC) and nicotinamide (NAA) eluted around t0 with no or low organic modifier (1%). On the other hand, vitamin B12 (riboflavin) and vitamin B12 were successfully separated by the reversed-phase HPLC with an isocratic mode of ACN 15% using a CS type C18 column [104].

In such cases, ion-exchange HPLC is powerful. Various ion-exchange HPLC methods are adopted for the process analysis (for example, purification) of biopharmaceuticals (His-tag® etc.) Recently multimode stationary phases have been developed and commercially available. Other than ion-exchange HPLC, normal-phase HPLC (NP-HPLC) is used for the highly hydrophilic compounds. In the NP-HPLC mode, Hydrophilic Interaction Chromatography (HILIC) mode, where a mixture of water and water mixable organic solvents such as ACN is used as the mobile phase, is effective for the separation of these compounds [108-110]. Schematic illustrations of various HILIC stationary phases employed for the separation of highly hydrophilic pharmaceuticals are shown in Fig. 23.

First, separation of seven water soluble vitamins by a dual mode column (reversed-phase + anion-exchange) was examined. A typical chromatogram is shown in Fig. 24 with a separation of five paraben mixture (ACN 60%). Except B1 (no retention) and NA (long retention), separation of five water soluble vitamins was successful. Especially, VC and its analogues (isoascorbic acid (isoVC) and ascorbic acid 2-glucoside (VC-Glu)), those are not retained in the reversed-phase mode, were separated within 7 min. Effects of salt (ammonium acetate) concentration and organic modifier concentration on sample retention was explained by both ion-exchange mode and reversed-phase mode.

Next, HILIC mode was applied for the separation of water soluble vitamins. Among various HILIC mode columns, successful separation was achieved except a bare-silica type column. Typical separation chromatograms of seven water soluble vitamins by an amide type CS column (Sunshell HILIC-Amide, 2.6 μm, 4.6 mm ID x 10 mm) were shown in Fig. 25. Among those, vitamin B12 (cyanocobalamin), MeB12, mecobalamin, OHB12, hydroxocobalamin, and vitamin B12 were successfully separated within 7 min.

Fig. 22. Separation of water soluble vitamins by reversed-phase HPLC. (A) Kinetex C18 (2.6 μm, 4.6 mm ID x 10 cm), phosphate buffer (pH 5.2) containing 1% ACN, (B) C30 column (Develosil XG-C30M-3, 5 μm, 4.6 mm ID x 15 cm), 0.1% trifluoroacetic acid, (C) Kinetex C18, 15% ACN, and (D) Kinetex C18, 18% ACN. Column temperature, 40°C; detection, (A) (B), 210 nm and (C) (D), 254 nm; flow rate, 1.0 mL/min. Samples: VC; NA, nicotinic acid; NAA; B1, vitamin B1; B6, vitamin B6; B2, vitamin B2; B2-P vitamin B2; B12, vitamin B12 (cyanocobalamin); MeB12, mecobalamin; OHB12, hydroxocobalamin; t0, void volume.

Fig. 23. Schematic illustrations of HILIC mode and dual mode stationary phases employed in this study. (A) amide, (B) diol, (C) five-OH, (D) triazole, (E) CD, (F) bare silica, and (G) dual mode.

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cm) and a diol type column (COSMOSIL HILIC, 5 μm, 4.6 mm ID x 15 cm) are shown in Fig. 25. Vitamin C and its related compounds (three mixture) were successfully separated by the HILIC mode column.

Other than water soluble vitamins, some organic acids used as a counter-ion of active pharmaceutical ingredients (APIs) were successfully separated with the corresponding base-form by the HILIC mode (Fig. 26). This method leads to the both assay of counter-ions and corresponding base-forms simultaneously.

8. Conclusion
Fast and selective analysis of pharmaceuticals has been successfully performed by HPLC and CE techniques. The key of analytical methods in the quality evaluation section (for example, CMC (Chemistry, Manufacturing and Control) research laboratory etc.) is simple and fast, leading to the high throughput analysis. The development of robust methods enables the speed up of the pharmaceutical development process. Further contributions are expected by the new emerging separation technology.

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Conflict of interest
The author declares no conflicts of interest, financial, or otherwise.
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