New Trend in the LC Separation Analysis of Pharmaceuticals – High Performance Separation by Ultra High-performance Liquid Chromatography (UHPLC) with Core-shell Particle C18 Columns –

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This article presents a mini-review of the recent results in the ultra high-performance liquid chromatography (UHPLC) separation of pharmaceuticals by our group. High performance UHPLC separation employing core-shell particle C18 columns was demonstrated. High performance (high theoretical plate number of approximately 20000/10 cm, low theoretical plate height of 5 μm) was obtained without any specific devices in the conventional HPLC apparatus, only through changing detector sampling times and the inner diameter of the connecting tube. High theoretical plate numbers with low column back pressure obtained by the core-shell particle columns enabled fast separation of the analytes. Methanol, which gives high column pressure drops in the reversed-phase mode HPLC compared with acetonitrile, can be used without any trouble. One analysis of the purity testing of diltiazem hydrochloride was performed within 100 s. One analysis in the photostability testing of mecobalamin (vitamin B12 analogue) was successful within 180 s.

Keywords Ultra high-performance liquid chromatography (UHPLC), separation, core-shell particle, pharmaceuticals, diltiazem

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1 Introduction

The most versatile analytical method used in the quality evaluation of pharmaceuticals is chromatography, especially HPLC. From the 1970s, excellent reversed-phase packing materials such as μBondapak®C18 or Nucleosil®C18 have been available, leading to rapid progress in HPLC analysis.1 In those

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days, 10 and 7 μm particles for the reversed-phase mode, named 10C18 and 7C18 respectively, were the most popular packing materials. Since then, 5 μm (SC18) has become the popular particle size in HPLC analysis. For example, “The testing methods and the specifications” of most pharmaceuticals in The Japanese Pharmacopoeia 16th edition (JP16), which lists a large number of important or well-used pharmaceuticals, adopts HPLC purity testing (so-called related substance testing) and HPLC assay by the 5C18 columns.2

Among with the HPLC investigations, development of high performance (high theoretical plate number (N), low theoretical plate height (H)), high selectivity, and high sensitivity (low detection limit) systems has progressed. For high performance, smaller particle size of the packing materials gives a lower H value, according to the van Deemter equation3 (see below). In 2004, an ultra high-pressure HPLC system with smaller particle packing materials (< 2 μm) was introduced to the market, based on the above concept.4 Small particle size leads to a dramatic increase of the column pressure drops. This system is called ultra performance liquid chromatography (UPLC)4 or ultra fast liquid chromatography (UFLC) depending on the system maker. At the present time, ultra high-performance liquid chromatography abbreviated as UHPLC is accepted worldwide, high speed and high performance separation analysis has been demonstrated by UHPLC.

The use of packing materials with around 2 μm particle size leads to high column pressure drops. For example, particle size of 2.2 μm (3 mm φ x 5 cm) under the following conditions: a mobile phase mixture of methanol (MeOH)/water = 60/40, flow rate of 1 mL/min, and column temperature of 40°C results in a pressure drop of about 300 kg/cm². Compared with 50–100 kg/cm² in the conventional analytical columns (5 μm, 4.6 mm φ x 15 cm), very high pressure was monitored. High pressure is not good for the packing materials and the apparatus, even though both are usable under high pressure conditions.

Recently, new packing materials called core-shell particles have appeared on the market.5 Core-shell particles enable high performance separation with lower column pressure drops, compared with the smaller totally porous particle packing materials previously employed in UHPLC. Core-shell particles are superficially porous silica particles, which were developed by Kirkland in 1969, and were called pellicular type silica in those days.6 In that paper, Kirkland reported novel particles composed of a 30-μm solid core encircled by a roughly 0.5-μm porous layer. However, due to low performance (low N) and low sample loadability, this pellicular type material was not used widely and converted to the totally porous 10C18 particle. In 2000, Kirkland et al.7 reported 5 μm core-shell particles composed of a 4-μm solid core encircled by a roughly 0.5-μm porous layer. Then in 2007, this group developed 2.7 μm core-shell particles composed of a 1.7-μm solid core encircled by a roughly 0.5-μm porous layer.8 This particle column shows high N values obtained by the sub-2 μm totally porous particle with much lower column pressure drops. This column has drawn attention in recent UHPLC analysis activities.

Since 2010, reports on the applications using this type of core-shell particle column have increased. For example, a fast analysis of amines and amino acids was successfully performed within 7 min.9 In this mini-review, some of our group’s recent results regarding the fast analysis of pharmaceuticals by employing core-shell particle C18 columns are shown.10–16 Our group uses a conventional HPLC apparatus with changes to the connecting tubes and some other parameters of the detector, other than the columns (i.e., core-shell particle). In line with the trend toward the use of smaller particle packing materials, columns for chiral separation and conventional HPLC having a small particle size (3 μm) have become commercially available. High performance enantiomer separations have been successfully achieved using 3 μm chiral columns.17

2 UHPLC Separation

2-1 UHPLC separation by the core-shell particle columns

The structure of a core-shell particle is shown in Fig. 1. This particle reported by Kirkland in 2007,7 has a 1.7-μm solid core encircled by a roughly 0.5-μm porous layer. Particle size distribution of this particle is quite low (standard deviation (SD), approximately 6%), compared with conventional commercially available packing materials (SD approximately 20%).18

Separation performance in HPLC can be described by the van Deemter’s equation, which shows a relationship between H and linear velocity (u): 

\[ H = Ad_p + B(D_p/u) + C(d_p^2/D_a)u. \]  

(1)

H, d_p, u, and D_a are the theoretical plate height, particle diameter
of packing materials, linear velocity of the mobile phase, and diffusion coefficient of an analyte in the mobile phase, respectively. A is a term regarding eddy diffusion, B is a term concerning diffusion into a column axis direction, and C is a term concerning mass transfer between the stationary phase and the mobile phase, and that in the particle inside. According to this equation, $d_p$ in A and C terms largely influences $H$, i.e., the smaller $d_p$, the smaller $H$ values (higher $N$ values).

Core-shell particles of 2.6 $\mu$m shown in Fig. 1, which are much smaller in size than the conventional 5 $\mu$m totally porous particles, give low $H$ values as shown in van Deemter's equation. Other than this small $d_p$ effect, an important factor in the core-shell particle, the narrow distribution range of the particle size largely contributes to low $H$ values. A narrow distribution range of the particle size enables minute packing, leading to narrow gaps between the particles. Therefore, eddy diffusion is limited, and consequently a small A term is obtained. On the other hand, the particle having a wide particle size distribution can not be packed minutely and shows a large A term. Furthermore, a non porous solid core, where an analyte can not diffuse inside, contributes to the small diffusion in the core-shell particle. As mentioned above, the B term is diffusion to the column axis direction and the C term is a term of the mass transfer in the particle. Diffusion in both terms is concerned in a thin layer (i.e., superficially porous 0.5 $\mu$m layer) of the core-shell particle. This thin layer gives a short transfer time or short transfer distance, leading to smaller B and C terms. Higher performance of the core-shell particle compared with the totally porous particle of the same size can be ascribed to the above mentioned factors.

### 2-2 HPLC system for UHPLC separation

To show the high performance of UHPLC, it is important to properly set HPLC parameters other than the columns. The inside diameter (i.d.) of the connecting tubes between the injection port and the column, and those between the column and the detector should be smaller than that of the conventional ones. Use of low cell volume (micro-cell) in the detector is desirable. The detector’s sampling time also should be optimized (fast sampling time). Apparatus for exclusive UHPLC use, these devices and parameters are loaded from the first time. However, our group used a conventional HPLC apparatus, the Prominence (Shimadzu, Kyoto, Japan), which can be used up to 40 MPa. We used the device with some modification. A photodiode array detector (PDA) was used for the detection and the monitoring of the UV spectra of each analyte. Samples of 0.1% concentration (1 mg/mL) were prepared by MeOH and mixed or diluted with a mobile phase or water to the desirable concentration (usually 0.01%). Approximately 5 - 10 $\mu$L was injected by an auto-injector SIL-20AC.

First, connecting tubes between the injection port and the column, and the column and the detector were changed to 0.1 mm i.d. Then, performance of the system was investigated by changing the detector response (time constant, default 640 ms, available 25 - 2000 ms) and sampling cycle (default value 640 ms, available 25 - 2000 ms). The slit width used was 8 nm. The samples used were a mixture of methyl (Me), ethyl (Et), propyl (nPr), isopropyl (iPr), and butyl (Bu) paraben. The separation was performed by the core-shell column (Kinetex C18, 4.6 $\mu$m $\times$ 10 cm, 2.6 $\mu$m) with MeOH/water = 70/30 as a mobile phase. The detector cell used was the normal cell (cell volume, 10 $\mu$L). The obtained $N$ values of Bu paraben (latest eluted peak at around 2 min) were 9500 at 640 ms and 19000 at 80 ms. Very low $H$ around 5 $\mu$m was obtained. Satisfactory high performance was obtained even in the normal cell, although it is possible to obtain higher performance and a very fast analysis when a micro-cell is used. In conclusion, we decided to use the conventional HPLC with a normal cell after changing both detector response and sampling time from 640 to 80 ms, considering the versatile usage of the system. Resolution ($Rs$), $N$ and $H$ were calculated by using the software equipped with the apparatus (LC solution, Shimadzu).

### 2-3 Comparison between the conventional columns (5 $\mu$m) and the UHPLC columns (<<3 $\mu$m)

Performance of the conventional column with totally porous particle (Phenomenex Gemini-NX C18, 4.6 $\mu$m $\times$ 15 cm, 5 $\mu$m) and the UHPLC column with totally porous particle (Shim-pack XR-ODS, 3 mm $\phi \times$ 5 cm, 2.2 $\mu$m) was compared by using a mixture of five parabens as a test sample and the mixture of MeOH/water = 60/40 as a mobile phase. The HPLC apparatus and the parameters used were the same as those mentioned above. Results are shown in Table 1. Data indicates that almost the same performance ($Rs$, $N$, and $H$) was obtained in one-third the analytical time by the UHPLC column. However, column back pressures were 75 kg/cm² at the Phenomenex Gemini-NX C18 column, and 280 kg/cm² at the Shim-pack XR-ODS column.

Next, the same analysis was performed employing core-shell particle columns (Kinetex C18, Sunshell C18, 4.6 $\mu$m $\phi \times$ 10 cm, 2.6 $\mu$m). The same performance as with 2.2 $\mu$m particle was obtained by the core-shell column judging from the Rs between two parabens (iPr and nPr) (Table 1). Typical separation of parabens by HPLC and UHPLC is shown in Fig. 2. Column back pressures of 280 kg/cm² for 2.2 $\mu$m particle at 40°C was reduced to 230 kg/cm² at 50°C.

### Table 1: Comparison of the performance between the conventional columns and the UHPLC columns

| Column | $d_p/\mu$m | $\phi/\mu$m | Column length/cm | Mobile phase MeOH, % | $N$ of Bu paraben | $\frac{N}{R}$ of Bu paraben/min | Rs (iPr vs. nPr) | Pressure/ kg cm⁻² | Fig. 2 |
|--------|-----------|------------|-----------------|---------------------|------------------|-----------------------------|----------------|-----------------|-------|
| Total A | 5         | 4.6        | 15              | 60                  | 9400             | 7.97                        | 1.77            | 75 (A)         |
| Total B | 2.2       | 3          | 10              | 65                  | 7200             | 2.30                        | 1.57            | 280 (B)        |
| Core A  | 2.6       | 4.6        | 10              | 60                  | 10600            | 3.17                        | 1.55            | 180 (C)        |
| Core B  | 2.6       | 4.6        | 10              | 60                  | 13600            | 4.49                        | 2.13            | 180 —          |
| Core C  | 2.6       | 4.6        | 10              | 60                  | 15300            | 3.90                        | 1.99            | 220 —          |

a. Total A, Phenomenex Gemini C18; Total B, Shim-pack XR-ODS; Core A, Sunshell C18; Core B, Kinetex C18. Conditions: column temperature, 40°C; flow rate, 1.0 mL/min.
3 Application to the Separation of Pharmaceuticals

3.1 Separation of diltiazem and its related compounds

Separation of diltiazem and its analogues was investigated by employing core-shell particle columns. Diltiazem (Herbesser®) is a Ca-channel blocker used for the treatment of hypertension in more than 100 countries. Columns used were Kinetex C18 and Sunshell C18 (4.6 mm i.d. × 10 cm, 2.6 μm) under the following conditions: flow rate 1.0 mL/min, column temperature 40°C, and mobile phase of phosphate buffer (pH 3.0)/MeOH = 35/65; column temperature, 40°C; detection, UV 254 nm. Results of the purity testing are summarized in Table 2. Separation of the desacetyl-form of diltiazem and diltiazem was successful within 100 s, resulting in a fast purity testing. Samples, active pharmaceutical ingredients (API), and some formulations, were stored in colorless glass bottles at room temperature for over 5 years. This indicates that diltiazem and its formulations are very stable.

![Fig. 3 Separation of diltiazem and its related compounds and purity testing of diltiazem API by UHPLC with 2.6 μm core-shell particle column (Sunshell C18, 4.6 mm i.d. × 10 cm). Conditions: flow rate, 1.0 mL/min; mobile phase, 0.05 mol/L phosphate buffer (pH 3.0)/MeOH = 35/65; column temperature, 40°C; detection, UV 254 nm.](image)

Desacetyl diltiazem, diltiazem, desacetyl chlorodiltiazem, chlorodiltiazem.
Diltiazem API is included in the JP16 and the specification of the related substances is not more than 0.3%. The method employed in JP16 is the reversed-phase HPLC with 5C18. According to the JP16 method, one analysis needs around 20 min. In the case of the quality evaluation test (both HPLC related substances and HPLC assay) for five lots of newly produced tablets, many solutions need to be injected. For example, standard solutions, sample solutions, solutions for the system suitability test, etc. A total of 30 analyses are required. This means 30 times 20 min = 600 min (10 h). In this case, the quality control laboratory at the tablet maker usually operates the HPLC system all-night. Checking of the raw data (chromatograms), summarizing of the results etc., are performed the following day. However, with the use of the developed UHPLC method, 30 times 2 min = 60 min (1 h). Analysis time required will be reduced to one tenth, enabling completion of the test within one day. First, the HPLC apparatus is operated, then the solutions are prepared and injected, and this is followed by the data collection and documentation to be finished within the same day. There is no need to operate the system all-night. Furthermore, one analysis can be completed in 1 min using a core-shell particle (2.6 μm) column of 5 cm in length for much faster analysis.12

### 3·2 Separation of water-soluble vitamins

Separation of 10 water soluble vitamins was investigated by employing core-shell particle columns. Columns used were Kinetex C18, Sunshell C18, and Capcellcore C18 (4.6 mm φ × 10 cm, 2.7 or 2.6 μm) under the following conditions: flow rate, 1.0 mL/min; mobile phase, (A) ACN 15%, (B) ACN 18%; column temperature, 40°C; detection, UV 254 nm.

![Fig. 4](image)

**Fig. 4** Separation of (A) vitamin B₁₂, vitamin B₁₂, and its analogues (a standard solution) and (B) OHB₁₂ and B₁₂ (just after the dissolution of MeB₁₂ API) by UHPLC with 2.6 μm core-shell particle C18 column. Conditions: Kinetex C18 (4.6 mm φ × 10 cm); flow rate, 1.0 mL/min; mobile phase, (A) ACN 15%, (B) ACN 18%; column temperature, 40°C; detection, UV 254 nm.

Diltiazem API is included in the JP16 and the specification of the related substances is not more than 0.3%. The method employed in JP16 is the reversed-phase HPLC with 5C18. According to the JP16 method, one analysis needs around 20 min. In the case of the quality evaluation test (both HPLC related substances and HPLC assay) for five lots of newly produced tablets, many solutions need to be injected. For example, standard solutions, sample solutions, solutions for the system suitability test, etc. A total of 30 analyses are required. This means 30 times 20 min = 600 min (10 h). In this case, the quality control laboratory at the tablet maker usually operates the HPLC system all-night. Checking of the raw data (chromatograms), summarizing of the results etc., are performed the following day. However, with the use of the developed UHPLC method, 30 times 2 min = 60 min (1 h). Analysis time required will be reduced to one tenth, enabling completion of the test within one day. First, the HPLC apparatus is operated, then the solutions are prepared and injected, and this is followed by the data collection and documentation to be finished within the same day. There is no need to operate the system all-night. Furthermore, one analysis can be completed in 1 min using a core-shell particle (2.6 μm) column of 5 cm in length for much faster analysis.12

![Fig. 5](image)

**Fig. 5** Photostability of mecobalamin (MeB₁₂). Sample solution was injected at every 5 min interval. Square symbol indicates MeB₁₂ and circle symbol indicates OHB₁₂, which is a photo-decomposed product of MeB₁₂.

### Table 2 Results of the purity testing of diltiazem API and its formulations by UHPLC with the core-shell particle column

| APIa | Formulation (tablets)a |
|------|------------------------|
| Lot No. | Impurity, %b | Lot No. | Impurity, %b |
| A     | 0.28                | A     | 0.29                |
| B     | 0.19                | B     | 0.42                |
| C     | 0.18                | C     | 0.38                |

a. Stored at room temperature for more than 5 years.
b. Only desacetyl form was detected.

![Graph](image)
chromatograms of the separation of vitamin B$_2$ (B$_2$), vitamin B$_12$ (B$_{12}$), vitamin C, and its analogues are shown in Fig. 4. Mecobalamin (MeB$_{12}$) is well known as an unstable vitamin for light irradiation. As shown in Fig. 4, even just after dissolution, the decomposed product hydroxocobalamin (OHB$_{12}$) was observed. Within 1 h under irradiation by room light (about 3000 lux), almost all B$_{12}$ transferred to OHB$_{12}$ as shown in Fig. 5. One analysis can be finished within 180 s, the method enables frequent sampling (sampling interval 5 min) of the sample solution. The other five highly hydrophilic water soluble vitamins, including vitamin C, mentioned above were successfully separated within 5 min by employing a C30 column (3 μm, 4.6 mm φ × 15 cm) (chromatogram not shown). The mobile phase was a diluted trifluoroacetic acid (1 → 1000).14

3-3 Separation of NSAIDs and cold medicines

The separation of 12 cold medicines and nonsteroidal anti-inflammatory drugs (NSAIDs) was investigated by employing core-shell particle C18 columns.15 Columns used were Kinetex C18, Sunshell C18, and Capcellcore C18 (4.6 mm φ × 10 cm, 2.7 or 2.6 μm) under the following conditions: flow rate 1.0 mL/min, column temperature 40°C, and mobile phase of phosphate buffer (pH 2.5)/MeOH. Selectivity in MeOH was found to be better than ACN. The mobile phase used for 6 cold medicines was MeOH 30%, and that for six NSAIDs was MeOH 70%. All 12 drugs were separated by employing a gradient elution mode within approximately 8 min. Separation examples are shown in Fig. 6.16 A mixture of 11 NSAIDs (Fig. 6) was also successful within roughly 6 min except co-elution of indomethacin and dicrofenac. In the separation of parabens and diltiazems, no difference was found in separation selectivity. However, retention of chlorpheniramine, a basic cold medicine (antihistamine), differed among three core-shell particle columns as sometimes experienced in the use of the conventional reversed-phase columns.

4 Conclusion and Perspectives

In the HPLC separation analysis of pharmaceuticals, by employing core-shell particle columns or small 3 μm particle columns in the conventional HPLC apparatus with some modifications, it was found that high performance (high N or low H) can be obtained, leading to fast analysis. High performance with low column back pressure, compared with the smaller particle size and totally porous UHPLC columns,18 is the largest merit of the core-shell particle column. Low column back pressure enables the use of the conventional HPLC apparatus. From the viewpoint of the routine (cost) or versatility of analysis, core-shell particle columns with the conventional HPLC apparatus seem to be the most suitable because high performance can be obtained without any special devices, such as microcell or special UHPLC system. At this time, however, the variety of core-shell particle columns is limited to the C18 types. Especially, core-shell particle chiral columns are not available at present. However, chiral columns of 3 μm particle size became commercially available a few years ago.19 As an example, separation of enantiomers of naproxen, one of the NSAIDs, by a reversed-phase chiral column with 3 μm particle is shown in Fig. 7.17 Baseline separation of enantiomers was successful within approximately 7 min, leading to fast optical purity testing. Resolution (Rs) between the enantiomers was 2.55. Enantiomer separation is an important problem for pharmaceutical companies. Development of novel chiral columns is still ongoing.20 Markedly high performance and high speed enantiomer separation could be obtained with chiral core-shell particle columns with around 3 μm particle size. Therefore, development of chiral core-shell columns is highly expected. In the near future, columns used for the routine analysis of pharmaceuticals is expected to shift from the totally porous 5C18 to core-shell particle C18 with particle size of
around 3 μm. A paper on the characteristics of the core-shell particle published recently provides additional information.21

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