Contribution of Chromatography and Hyphenated Technology to Production Process Development for Pharma Chemicals

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"Chromatography" is an indispensable technique for treating small organic molecules, and "Hyphenated Technology", which means a combination of two different techniques (in this case, chromatography and mass spectrometry), is a powerful tool for quick on-line identification of trace amount impurities in processes. During production process development for active pharmaceutical ingredients (APIs) and reactive intermediates, these techniques are mainly utilized for confirmation of synthesized target molecules, by-products and impurities, and also for their identification. This paper describes the contribution of these techniques to process study acceleration and product quality improvement, while showing some examples.

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Introduction

For the pharma-chemical business of our company's Fine Chemicals Sector, the speed of synthesis process development and product quality are critical factors in the broad expansion of business. However, it is not easy to maintain high quality while accelerating process development. Particularly, in regard to APIs, because product quality is directly connected to drug safety, detailed examination is essential for process development.

Impurities are the factor of greatest concern in the effort to maintain product quality. Of all impurities, genotoxic impurities (GTIs) have the potential to cause health damage even when present in trace amounts. Therefore, it is necessary to use extra caution so as to avoid by-production and the contamination of such impurities in designing for the synthesis process of APIs.[1), 2)] Accordingly, at research and development sites it is crucial to accurately detect any impurities that are present in each process, elucidate their molecular structures, and incorporate measures for the elimination of impurities into the process design. These analytical techniques for impurities play a significant role in accelerating this process.

Chromatography is the core technique for impurity analysis used in the area of synthetic pharma-chemicals handled by our sector. Because most pharma-chemicals are small organic molecules with wide ranged polarities, liquid chromatography (LC) is generally used. In the field of LC, in addition to the widely disseminated high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography (UHPLC) has recently been utilized, contributing to the reduction of analysis times and the further improvement of separation.

Chromatography is also useful as a method for impurity isolation and refinement. Substance isolation can be quickly conducted through the use of flash chromatography, which separates a g-volume sample by medium pressure, and preparative chromatography, for which a column packing material for HPLC is used for the preparative column. Furthermore, due to the high dispersion of supercritical fluid, supercritical fluid chromatography, which utilizes supercritical fluid as the mobile phase, has enabled the fractionation of mg-volume samples with the analysis column at high speed in addition to its applicability for rapid analysis.

However, the core techniques for structural elucidation are nuclear magnetic resonance (NMR) and LC-MS, in which latter case mass spectrometry (MS) is combined with LC. LC-MS became widespread once the connection with LC became easier due to the development of atmospheric pressure ionization (API), which began in 1990. Currently, LC-MS enables the estimation of molec-

ular weight, molecular formulas and substructures through high sensitivity, high resolution, and the fragmentation technique. Thus it has become an indispensable system for the analysis of trace amounts of impurities. Moreover, regarding NMR, which is an essential item for researchers of organic synthesis, measurement and analysis using trace amounts are now possible. At the same time, the performance of LC-NMR, in which NMR is combined with LC, has rapidly improved, thus enabling its application to trace-amount ingredient analysis.

Hyphenated technology is a generic name for techniques in which two different techniques are combined. Although the advancement of the analysis technique has always led the advancement of such hyphenated technology that combines LC and the analysis technique,[3] it can be expected that its sensitivity and speed will improve even more as the latest LC technology is implemented. Therefore, an approach from the LC side has become more popular in recent years.

This paper will introduce our efforts to improve the speed of analysis in the development of pharma-chemical production processes through the use of the latest chromatography technology, an approach by which to improve the hyphenated technology from the chromatography side, and the application of the technology to the aforementioned production process development.

Efforts to Improve LC Analysis for High Throughput

1. Dissemination of UHPLC

(1) What is UHPLC?

Although the term "UHPLC" is generally referred to as the "LC technology that makes it possible to attain high speed and separation through the use of a fine particle packing column," actually it doesn't have a clear definition. With respect to LC analysis, the smaller the particle size is, the better the separation will be. However, at the same time the column pressure will become higher as it becomes inversely proportional to the square of the particle size, thus requiring a system with higher pressure resistance when ultra-fine particle columns are used. In 2004 the W company manufactured and released a packing column for the particle size of 1.7 μm and a dedicated LC system with the maximum withstand pressure of 100 MPa, which garnered considerable attention. Because other LC system manufacturers and column manufacturers didn't follow the trend immediately, for a period of time the product name of the W company's LC was commonly used as a moniker for the ultra-high-speed LC. However, a year later other LC system manufacturers finally started launching their products with high levels of pressure resistance. It seems that since then the term "UHPLC"—which had previously been used as an abbreviated name for ultra-high-pressure liquid chromatography—has been used as a generic name for this new LC technology.

Although initially columns having particle sizes ranging from 1.7 μm to 1.9 μm were mainstream, several manufacturers began launching columns having the particle sizes exceeding 2 μm in attempt to achieve the equivalent high separation at lower pressures. In particular, the S company—the largest domestic LC system manufacturer—introduced a column having a particle size of 2.2 μm and a dedicated UHPLC system with a maximum pressure resistance of 35 MPa. Following the S company, another leading domestic column manufacturer released a column having a particle size exceeding 2 μm. According to those particle sizes, the columns with particle sizes less than 2.0 μm were classified as sub-2 μm columns and the ones not less than 2.0 μm were classified as sub-3 μm columns. The former makes the most of the system's high pressure resistance property, while the latter competes with the former in high speed and high resolution, assuming the application for the standard type HPLC (conventional LC). In addition to these columns, other products that enable high speed and high resolution at lower pressures (e.g., the porous monolithic column and the dual-structure column packing material called "fused core"[4] (with a particle size of 2.7 μm)) have been released, thus expanding the selection of columns for each system environment. Moreover, in regard to the LC system with high pressure resistance, assuming the use in conventional LC, a compatible type (which can also be used with the conventional 5 μm column) has in recent years become the mainstream.

(2) Application to Process Development

Impurity management is the important key to the pharma-chemical production process. LC is the most effective technique for process analysis. As previously described, GTIs have garnered attention recently, thereby increasing the level of importance of impurity management in the production process. Normally, the properties of such impurities range widely from high polarity to low polarity. Therefore, when simultaneously detecting all existing impurities under the conventional LC

conditions, the analysis tends to require a longer time. This tendency not only causes analytical problems (such as diminished sensitivity due to the expansion of the peak width), but also other problems such as prolonged data acquisitions. Particularly, when complying with the Good Manufacturing Practice (GMP) stipulated by the government during the API production process, the system suitability test (SST) required for the process testing alone will take a substantial amount of time. For these reasons it is hoped that introducing an analysis technique that allows us to achieve high separation within a shorter period of time will greatly contribute not only to the process development but also to the greater efficiency of the production process.

From 2006 we have started collecting information and examined the possibility of a UHPLC introduction. The details of our examinations are described below.

(i) Model Selection and Limitation

Because LC technology has already been widely utilized in the pharma-chemical production process, from development through production and quality control, upon the introduction of UHPLC it is necessary to consider compatibility with the conventional LC conditions. In order to fulfill this requirement, we chose the UHPLC system by the S company, basically aiming to apply a sub-3 μm column, in consideration of compatibility with the existing system in each section. The system was created based on the conventional LC system by increasing the pressure resistance of the sample injection unit, and also by decreasing the diameter of the liquid transfer tube running from the sample injection unit to the column, the gradient mixer volume, and the cell volume of the absorption detector. The system can be used as a

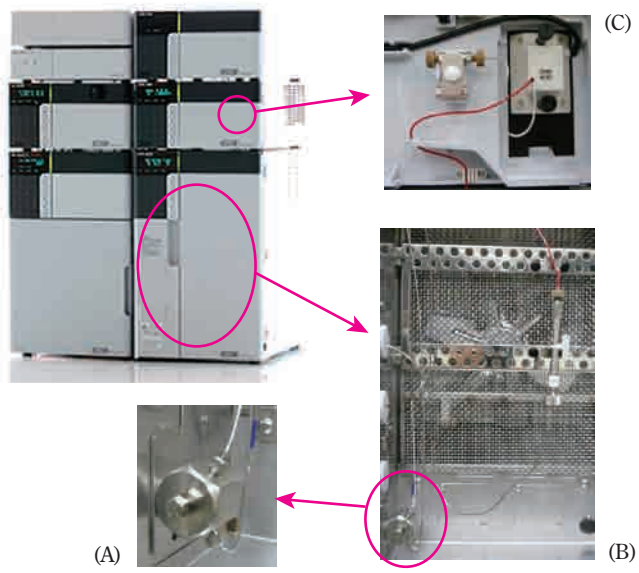


Fig. 1 UHPLC system (Prominence UFLC®)
(A) mixer (100μL volume)
(B) SUS tube (0.1×600mm)
(C) semi micro cell (2.5μL volume)

conventional LC system by replacing each unit (**Fig. 1**). However, because the maximum pressure resistance of this UHPLC system was 35 MPa, which was low for UHPLC system, one could assume that the range of usable columns would be limited.

However, as **Fig. 2** shows, the maximum column pressure of each major commercialized sub-3μm column is lower than 30 MPa when using acetonitrile-water, which is commonly used as a general reverse-phase system mobile phase, thus confirming that many types of columns could be used.

(ii) Column Evaluation

Judging by the column pressure, we were able to confirm that many of the commercially available sub-3 μm columns were usable. Thus we continued the investiga-

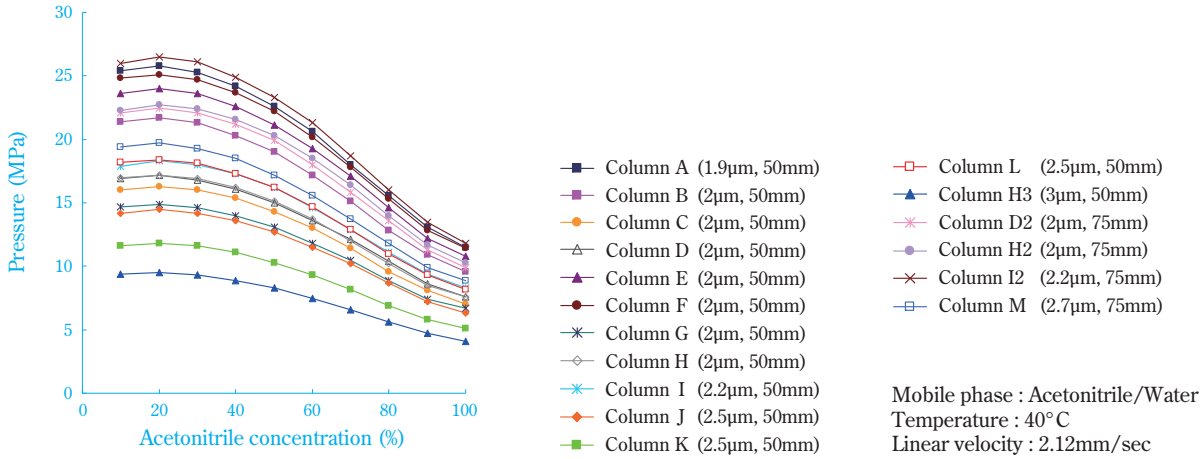


Fig. 2 Pressure of various UHPLC columns

Table 1 Evaluation of various ODS columns for UHPLC

Column	Particle size (μm)	Length (mm)	Efficiency vs Pressure*1)			Hydrophobicity	Pyridine
			Efficiency(N)	Pressure(MPa)	N/MPa	α *2)	Tailing factor *3)
Column A	1.9	50	5,446	22.6	241	1.437	2.591
Column B	2(1.8)	50	7,858	19.3	407	1.495	1.840
Column C	2(2.2)	50	6,216	13.9	447	1.508	1.887
Column D	2(2.2)	50	6,168	15.0	411	1.472	1.955
Column E	2	50	6,707	21.3	315	1.469	2.090
Column F	2	50	6,654	22.1	301	1.416	no data
Column G	2(2.2)	50	5,940	13.8	430	1.480	1.227
Column H	2	50	6,510	15.0	434	1.464	1.345
Column H2	2	75	11,300	20.2	559	1.465	no data
Column I	2.2	50	6,243	15.9	393	1.507	2.053
Column I2	2.2	75	9,637	21.7	444	no data	no data
Column J	2.5	50	6,121	12.6	486	1.480	2.259
Column L	2.5	50	6,153	16.2	380	1.488	> 2
Column M	2.7	75	10,853	17.1	635	1.506	1.960

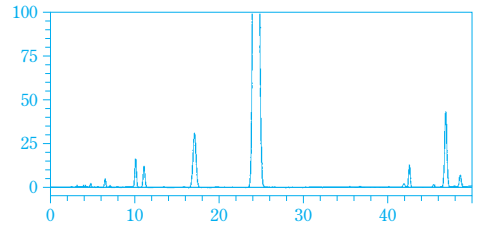
*1) Mobile phase: Water/Acetonitrile=50/50 Temperature: 40°C Linear velocity: 2.12mm/sec Sample: Biphenyl
*2) Mobile phase: Water/Acetonitrile=40/60 Temperature: 40°C Linear velocity: 2.12mm/sec Sample: Uracil(t_0), Toluene, Ethyl benzene
*3) Mobile phase: Water/Methanol=70/30 Temperature: 40°C Linear velocity: 1.06mm/sec Sample: Pyridine, Phenol

tion on the separation capabilities of various columns. The columns were evaluated using three parameters: the number of theoretical plates, hydrophobic interaction, and the peak shape of basic compounds which are generally utilized for the ODS column evaluation. **Table 1** shows the evaluation results. Based on these results, high separation can be expected in these columns within a short period of time. It has also been confirmed that there are a variety of columns with diverse properties. It indicates that we can choose an appropriate column for UHPLC analysis in the same manner as the conventional LC.

(3) Efforts for Dissemination

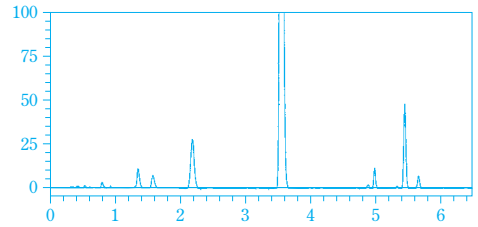
To promote the application of UHPLC to process development, we gathered the ideas of our laboratory researchers regarding the target process, for which a reduction in analysis time was desired, and devised an UHPLC analysis method accordingly. Taking into account the separation of impurities, the target analysis time was determined to be a maximum of 15 minutes. By using the newly devised analysis method, the analysis time was reduced by approximately one-third to one-eighth of that of the current analysis method, as exemplified in **Fig. 3**. In this example the analysis time was reduced from 60 minutes to eight minutes without disturbing the separation. Based on those results, we explained the usability of UHPLC to staff members at the laboratory and manufacturing plant, thereby promoting the implementation of UHPLC. First we installed the

(A) Conventional LC



Conditions
Column : ODS(6.0 × 150mm, 5μm)
Mobile phase : [A] 0.1% TFA in water [B] Acetonitrile
Gradient : 50%B(0-20min)
50-90%B(20-50min)
Temperature : 40°C
Flow rate : 1.0mL/min
Detection : UV at 254nm
Analytical time : 60min

(B) UHPLC



Conditions
Column : ODS(3.0 × 50mm, 2.2μm)
Mobile phase : [A] 0.1% TFA in water [B] Acetonitrile
Gradient : 45%B(0-2min)
45-90%B(2-6min)
Temperature : 40°C
Flow rate : 1.0mL/min
Detection : UV at 254nm
Analytical time : 8min

Fig. 3 Reduction of analytical time by UHPLC

system in the research room, where the researchers showed a certain degree of interest. We then asked them to use the system for the actual process research and development. As a result, most of the feedback we received was positive, such that abundant data was acquired within a short period of time.

When UHPLC can only be partially introduced, we need to establish an analysis method for the conventional LC. In that case, it can be quickly established at first by UHPLC, following that the analysis conditions are converted to that for the conventional LC by theoretical calculation. Although it is ideal to use the same packing materials as a prerequisite, several major column manufacturers already have column product lines with different particle sizes for the same packing material, and additionally some LC manufacturers provide column screening units. Therefore, it seems that UHPLC is widely used in many analysis method development departments. In 2009, we at our laboratory also introduced another UHPLC system from the D company, which is capable of column screening. Through this introduction we aimed to establish a quick system for the development of an analysis method in parallel to the development of the UHPLC technology.

2. High-throughput on Conventional LC

(1) LC Parameters that Affect Separation

In the past, when the 5μm-column was most commonly used, a short-time LC analysis that used a short column having a length of approximately 50 mm gathered attention under the name "fast LC." At that time we tried this method with the conventional LC system by the S company, but it was not usable due to the insufficient separation. However, in this present study, when we were examining the implementation of UHPLC, we inadvertently realized that some parameters would greatly affect separation. Although these parameters cause no problem during a long-time analysis with high flow rate, they will affect separation during a short-time, low-flow analysis.

The parameters that particularly affect separation are as follows:

(i) Dwell Volume

The dwell volume is also referred to as the gradient delay volume. It is the volume between the starting point of mobile phase mixing and the column top. It is the total volume of the following parts: the tube running from the pump head to the gradient mixer; the mixer; the tube running from the mixer to the injector; the injector; and the tube running from the injector to the column.

(ii) Extra-Column Volume

The extra-column volume is the total volume of the area between the injection point and the detection point of the system, excluding the void volume of the column. The extra-column volume is divided into two: the pre-column volume and the post-column volume. The former refers to the injection volume and the volume of the tube running from the injector to the column entrance. The latter refers to the volume of the tube running from the column exit to the detector and the cell detecting point.

(iii) Sample Solvent and Injection Volume

Fig. 4 shows the dwell volume and the range of extra-column volume in an LC system. Additionally, regarding company S's conventional LC system and the UHPLC system, **Table 2** indicates the specifications and volumes of the parts related to the dwell volume and extra-column volume.

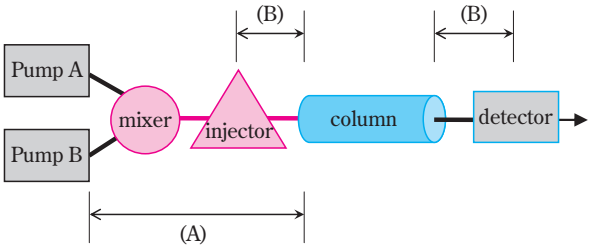


Fig. 4 Dwell volume (A) and extra-column volume (B)

Table 2 Constituents of dwell volume and extra-column volume

	LC system		
	Conventional system		UHPLC system
	Type A	Type B	
mixer volume (mL)	2.6(1.7,0.5)	2.6(1.7,0.5)	0.1
sample loop (μL)	50	100	100
Tube (injector-column)	0.3×600mm	0.25×600mm	0.1×600mm
Tube (column-UV cell)	0.25mm ID	0.25mm ID	0.13mm ID
UV cell volume (μL)	8(5)	12(2.5)	2.5
dwell volume (mL)	3~5 (at mixer volume 2.6, 1.7mL) 0.6~1.0 (at mixer volume 0.5mL)		0.4

The dwell volume induces a delay in a gradient program of mobile phase concentration, and the excessive mixer volume will cause departure from the set gradient curve. Their allowances are thought to be less than

10% of the gradient volume (flow rate of the mobile phase × gradient time).[5] Because the standard mixer volume and the dwell volume of company S's conventional LC system are 2.6 mL and in excess of 4 mL respectively, it is difficult to accommodate a short-time, low-flow analysis. However, it is expected that this can be improved with a reduction in mixer volume by changing the flow channel (0.5 mL) or by using a small-volume mixer suitable for UHPLC (volume of 0.1 mL). With regard to the extra-column volume, while there is no clear standard for the pre-column volume, the effect of the pre-column volume can be ignored under the condition in which the elution strength of the sample solvent is either equivalent to or less than that of the mobile phase solvent, even though the column has an internal diameter of 2.1 mm or less, as long as the volume is 10 µL or less.[5] Furthermore, regarding the post-column volume, although diffusion can be a problem when the cell volume of the detector exceeds approximately 10% of the void volume of the column,[5] in an actual case, in which a column having an inside diameter of 2.1 mm and a length of 50 mm or longer is used, it seems there is no major problem in using company S's standard cell (volume 8, 12 µL). When the elution strength of sample solvent is stronger than that of the mobile phase solvent, the sample solvent will continue to diffuse even after it reaches the column, thereby causing peak diffusion. When the elution strength of the sample solvent is equivalent to that of the mobile phase solvent, peak deformation can be controlled by maintaining the injection volume at less than 15% of the peak volume (flow rate × peak detection time).[5] When the elution strength of the sample solvent is weaker than that of the mobile phase solvent, the sample will become concentrated at the column tip, thereby improving the peak shape (**Fig. 5**).

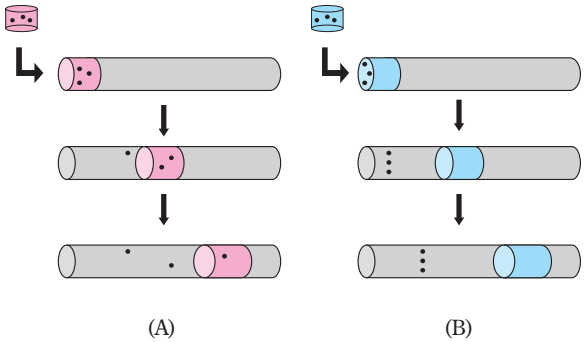


Fig. 5 Mechanism of peak broadening at solvent front
(A) Strong solvent (Methanol)
(B) Weak solvent (Water) (•): analyte

(2) Column Selection

Based on the discussion of the above parameters, it was presumed that the LC system was the cause of the problem in separation during the fast LC procedures. Paradoxically, this means that by reducing the dwell volume or the extra-column volume we can use columns in which high separation could not previously be obtained, thus expanding the column selection significantly. To date, virtually all column manufacturers have 3 µm column product lines in addition to 5 µm lines. It can be assumed that the current configuration of the conventional LC system of the S company is not fully bringing out the 3 µm column performance. However, if the system structure is modified, in some cases high-throughput may be achieved in the analysis using the 3 µm column. We are currently investigating this method as a supplement to UHPLC, for which a system introduction will require some time.

3. Supercritical Fluid Chromatography

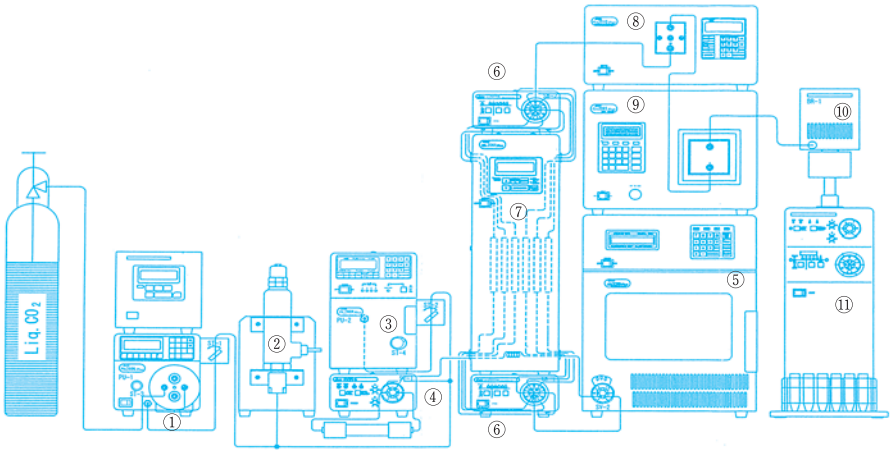
Supercritical fluid chromatography (SFC) is a form of chromatography that utilizes CO_2 in its supercritical state as the primary mobile phase solvent. Because the polarity of supercritical CO_2 is similar to that of hexane, it is generally used as an alternative to normal-phase LC. Epecially, due to its low viscosity and high diffusion coefficient it is commonly used as a high-speed, high-separation analysis method for polymers and chiral organic compounds. Regrettably, although over 1,000 units are in operation in Europe and the U.S., fewer than 20 units have been introduced in Japan because the system is subject to the regulations stipulated by the High-Pressure Gas Safety Act, thus requiring notifications and applications to the competent authorities.

Chiral analysis plays an important role in APIs in terms of the management of pharmaceutical product efficacy. Although we have been using the dual-phase (normal phase and reverse phase) LC chiral column screening system developed 10 years ago by our company's Organic Synthesis Research Laboratory, upon the renewal of the normal-phase system we introduced the SFC system for the purpose of achieving high-throughput screening. **Fig. 6** shows the structure of the SFC system thus introduced.

This system has a CO_2 delivery pump with the cooling function and a back-pressure control valve located at the detector exit, as well as all other parts that the standard LC system structure has. As many as six types of normal-phase chiral columns are arranged in a row, thus en-

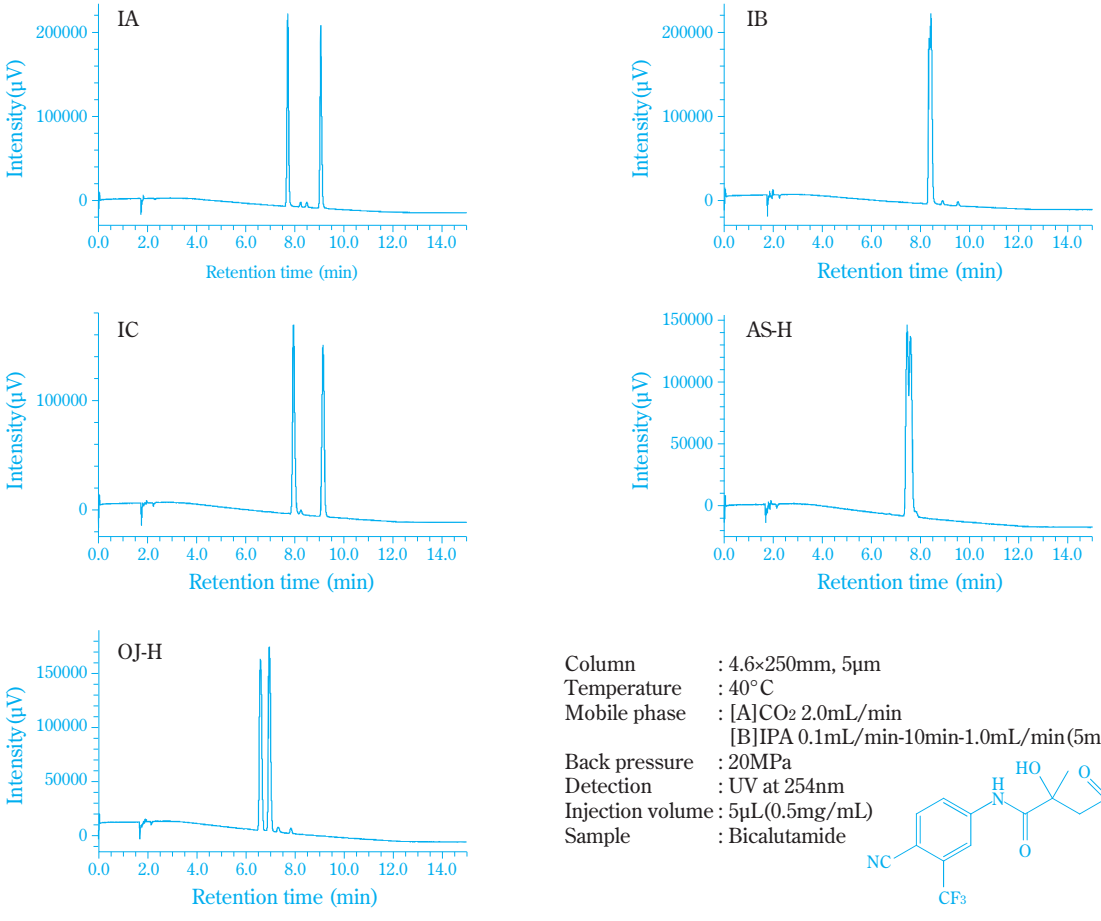
abling auto-injection with column switching. In this system a screening cycle can be completed within approximately three hours, whereas with the old screening system a period of twelve hours was required. The conventional LC function has also been kept to this system in order to convert the SFC conditions to the conventional LC conditions, following optimizing them, after screening, thus achieving the rapid development of the analysis method. **Fig. 7** shows examples of actual applications. In these examples separations were confirmed in five types of columns, and favorable separations were observed in the IA and IC columns.

Moreover, taking advantage of the high diffusibility of supercritical CO_2, the quick fractionation of the ingredients is possible using an analytical column. In this system an mg-volume sample — which usually requires a



① CO_2 delivery pump
② Safety valve
③ Modifier delivery pump
④ SFC/LC line selector valve
⑤ Autosampler
⑥ Column selector valve
⑦ Column oven
⑧ Diode array detector
⑨ Circular dichroism detector
⑩ Automatic back pressure regulator
⑪ 6-way valve

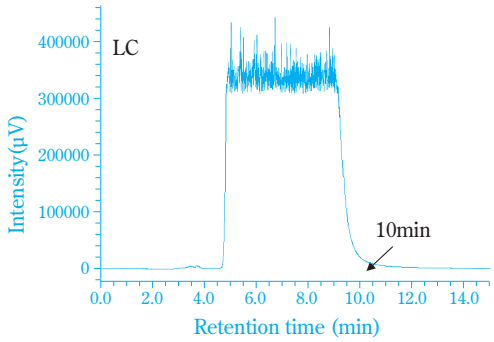
Fig. 6 SFC system



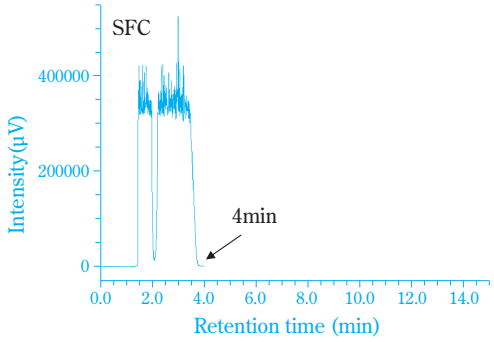
Column : 4.6×250mm, 5μm
Temperature : 40°C
Mobile phase : [A]CO_2 2.0mL/min
[B]IPA 0.1mL/min-10min-1.0mL/min(5min)
Back pressure : 20MPa
Detection : UV at 254nm
Injection volume : 5μL(0.5mg/mL)
Sample : Bicalutamide

Fig. 7 Chiral column screening with SFC

preparative column in the standard LC system — can be separated using the analytical column in a short time. Additionally, sample concentration after fractionation will be easier because CO_2 will become vaporized and thereby eliminated after the pressure release. **Fig. 8** depicts an example of ingredient fractionation using SFC. While ingredient fractionation at a load of 7 mg is difficult under the conditions of the conventional LC, separation is achieved in a short period of time under the SFC conditions.



Column : IA(4.6×250mm, 3μm)
Temperature : 40°C
Mobile phase : n-Hexane/IPA = 67/33(v/v)
Flow rate : 1.0mL/min
Detection : UV at 254nm
Column pressure : 7.2MPa



Column : IA(4.6×250mm, 3μm)
Temperature : 40°C
Mobile phase : CO_2/Methanol = 67/33(v/v)
Flow rate : 3.0mL/min
Detection : UV at 254nm
Back pressure : 15MPa
Column pressure : 23.7MPa (⊿P = 8.7MPa)

* Bicalutamide 7mg injected

Fig. 8 Preparative SFC separation

Impurity Analysis

1. Flow of Impurity Analysis

The structural information of impurities observed during the production-process development for pharmachemicals is one of the key items of information for the rapid progress of development. **Fig. 9** shows the procedure for the structural analysis of unknown impurities detected by LC. The procedure is composed of two steps. Online measurement and analysis are mainly conducted in Step 1.

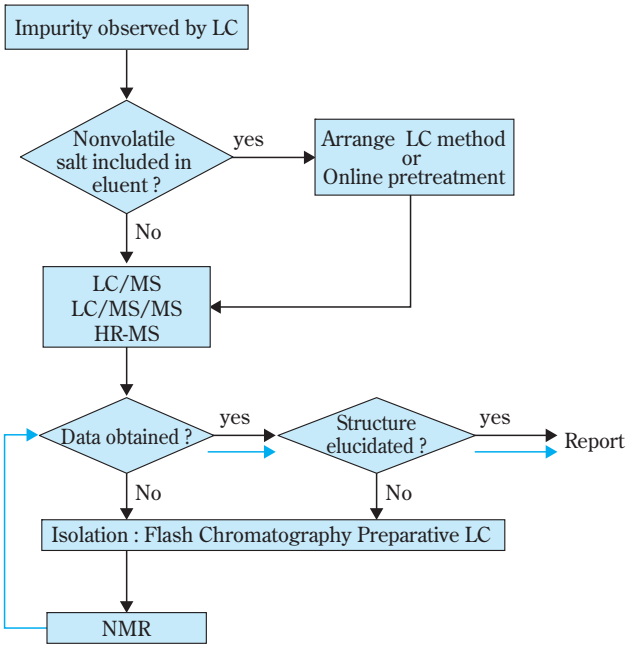


Fig. 9 Typical approach for structure elucidation of trace level impurities observed during synthesis process development

If its structure cannot be elucidated in this step, isolation is performed in Step 2 and then detailed analysis is conducted using NMR. Flash chromatography and preparative chromatography are used for isolation. Particularly, recycling preparative chromatography has made it possible to obtain isolated substances having high purity. Currently, a timeframe goal for Step 1 is within one week after the application of analysis, and that for Step 2 is one to two weeks after Step 1 is completed. However, in order to achieve fast analysis it is important to efficiently conduct the analysis in Step 1.

2. Hyphenated Technology

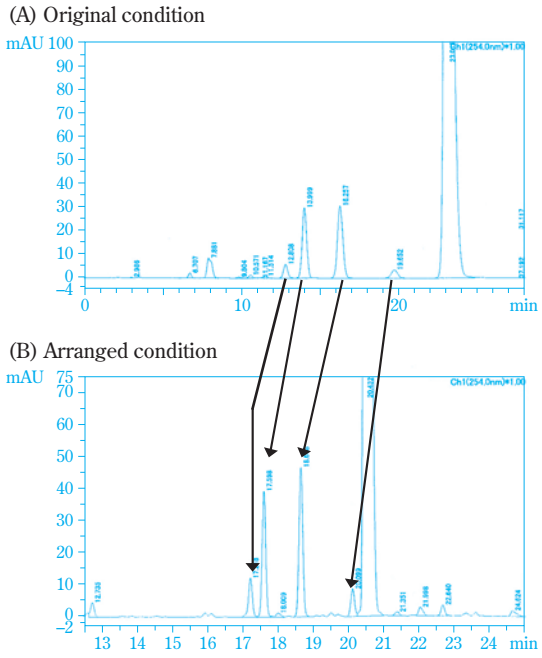
(1) LC-MS

At our section, we have installed two ion-trap type LC-MS units by which MS/MS measurement can be performed, mainly through the eletrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) methods, thereby conducting the structural analysis of impurities. The MS/MS measurement is the method by which to investigate the partial structure of a substance by generating product ions through the application of energy, with focus on a specific peak. The ESI and APCI

are mild ionization methods that mainly produce molecular ion peaks, thus allowing us to obtain estimated molecular weight data. However, not all substances will become ionized through these methods. Particularly, in most cases low-polarity substances do not become ionized at all. Moreover, highly volatile substances cannot be measured due to the system structure that eliminates such substances upon the removal of the mobile phase solvent. Furthermore, in some occasions adduct ions and fragment ions are already visible from the beginning. Therefore, it is necessary to conduct structural analysis using synthetic knowledge as well. Additionally, a molecular composition formula can be estimated through precision MS measurement, which utilizes the high-resolution system. For this measurement we have established a system by which to ask other sections of our company to conduct the measurement when need arises or to immediately outsource the measurement to an analysis company.

The first question, upon LC-MS measurement, concerns the applicability of the LC conditions to the LC-MS system at the time of analysis. Normally, in LC-MS, mobile phase solutions that contain nonvolatile salts cannot be used. In such cases it is necessary to examine an alternative analysis method that utilizes volatile salts. Generally, it is relatively easy to replace the phosphoric acid and phosphate used in LC analysis with formic acid, acetic acid and its ammonium salt. On some occasions, separation can be achieved without using any salt. However, in most cases when an ion-pair reagent is used for the analysis, merely replacing the reagent with a volatile ion-pair reagent will not solve the problem. Most of the samples we handle are basic, and therefore in most case it is effective to replace with trifluoroacetate (TFA). However, because the separation pattern and/or the retention time can change, it is necessary to carefully determine the peak position of the target substance. Moreover, it is known that TFA serves to suppress ionization, which affects the sensitivity of MS measurement, thus requiring extra caution. **Fig. 10** indicates changes in the LC conditions.

When the target subject cannot be detected under the alternative conditions after several attempts, the following system is effective: Conduct desalination and concentration online; separate through LC; lastly, conduct the MS measurement. The S company's Co-Sense® for microanalysis has a structure whereby two LC systems are connected in tandem through a sample loop and trap columns. In this system, when a semi-micro column is used for the secondary-side LC, the response is four times greater than at the primary-side LC with a conventional column. The fraction containing the target subject separated by the primary-side LC is fractionated into the sample loop. Subsequently, the target subject is injected into a trap column while being diluted, usually by water, to approximately ten times its original volume, whereupon it is tentatively retained. The retained target subject is then guided into the secondary-side LC system



Conditions
Mobile phase : [A] 5mM Sodium dodecyl sulfate (pH3.0)
[B] Acetonitrile
Gradient : 40%B (0-25min)
Temperature : 30°C
Flow rate : 1.0mL/min
Detection : UV at 254nm

Conditions
Mobile phase : [A] 0.1% TFA in water
[B] Acetonitrile
Gradient : 15-40%B (0-30min)
Temperature : 30°C
Flow rate : 1.0mL/min
Detection : UV at 254nm

Fig. 10 Arranging LC condition to apply for LC-MS

by the gradient elution. Subsequently, the observed peak is measured through the MS measurement method. **Fig. 11** shows the system diagram. The key factor in this system is to retain the target subject in a trap column. Particularly, when the mobile phase of the primary-side LC contains an ion-pair reagent, the dilution by water alone may not achieve the retention. If this is the case, adding TFA to water for dilution may solve the retention problem. However, this solution is not always effective, thus requiring confirmation. **Fig. 12** shows an

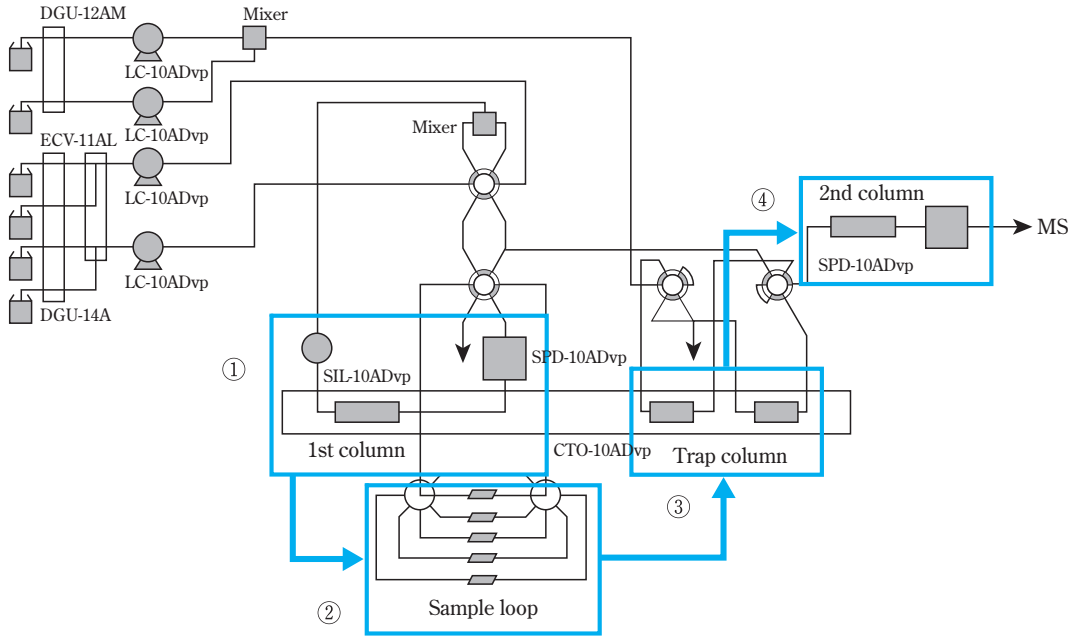
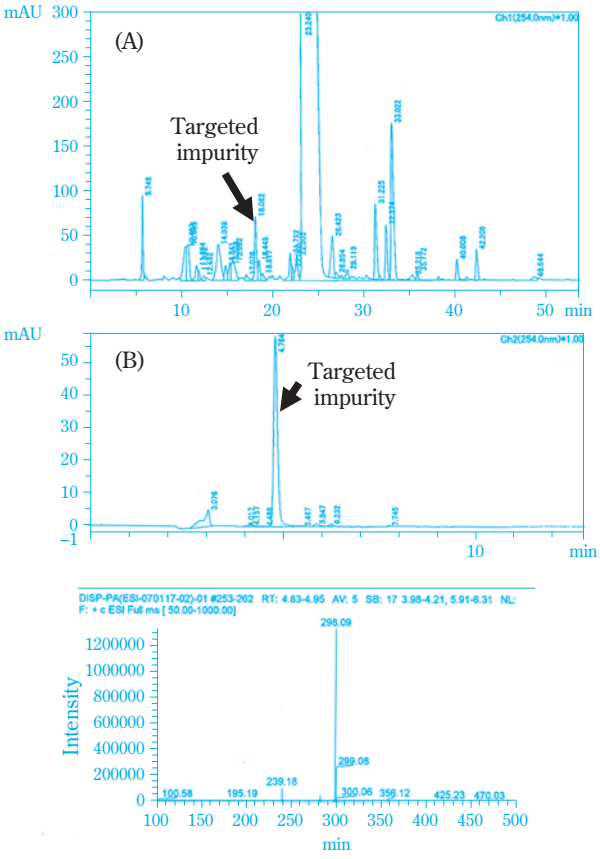


Fig. 11 Online sample pretreatment system (Co-Sense® for Microanalysis)



(A) 1D Analytical conditions
Column : ODS(6.0×150mm, 5μm)
Mobile phase : [A] 5mM 1-Heptanesulfonic acid and 20mM Potassium dihydrogen phosphate in water pH3.0 adjusted by phosphoric acid
[B] Acetonitrile
Gradient : 20%B(0-5min)
20-35%B(5-35min)
35%B(35-50min)
Flow rate : 1.0mL/min
Temperature : 35°C
Detection : UV at 254nm
Injection volume : 10uL

Online trapping conditions
Column : ODS(2.0×5.0mm, 5μm)
Mobile phase : [C] 0.1%TFA in water *For fractionated sample delivery
[D] 0.1%TFA in water *For online dilution
Total flow rate : 1.0mL/min([C]0.1mL/min, [D]0.9mL/min)
Dilution factor : 10fold
Trapping time : 6min

(B) 2D Analytical conditions
Column : ODS(2.1×150mm, 5μm)
Mobile phase : [A] 0.1% TFA in water
[B] Acetonitrile
Gradient : 20-90%B(0-20min)
Flow rate : 0.2mL/min
Temperature : 35°C
Detection : UV at 254nm

Fig. 12 LC-MS analysis utilizing Co-Sense® for Microanalysis

example of successful analysis achieved through the addition of TFA. Thanks to the recent advancement in column technology, the selection of trap columns has been expanded. Accordingly, it is expected that the range of application will further expand in the future.

The LC-MS sensitivity increases as the flow velocity decreases. Also, the sharper the peak is, the better the LC-MS sensitivity will be because the sensitivity is affected by the volume of the target subject per unit of time. Accordingly, sensitivity can be improved by replacing the columns with ones having small diameters and the same packing materials, thereby decreasing the mobile phase flow rate in proportion to the cross section. Moreover, even higher sensitivity can be expected when it is connected to UHPLC. **Fig. 13** indicates the UHPLC application example. In this example the MS intensity increased 1.6 times toward a half of the sample injection amount. Because the MS system is expensive but difficult to upgrade to a more sensitive one, it is crucial to have such an approach from the LC side.

(2) LC-NMR

Besides LC-MS, LC-MNR is also effective for the structural analysis of organic compounds having low molecular weights. However, unlike LC-MS the NMR system is still extremely expensive. Consequently, NMR is used as an LC-dedicated detector only in a small number of cases. It is therefore more common practice to replace the system with NMR alone. Because the frequency of the single use of NMR is high, LC-NMR tends to have a time limit unlike LC-MS, although they both employ the same hyphenated technology. Therefore, we apply LC-NMR only to cases in which structural analyses and separations are difficult to achieve merely through LC-MS (e.g., the substance decomposes during the separation process; due to the high volatility, even though it becomes separated, it doesn't concentrate, and complete separation is difficult).

The LC-NMR system we are currently using belongs to the National Institute of Biomedical Innovation.[6] **Fig. 14** indicates the system flowchart. It is a high-perfor-

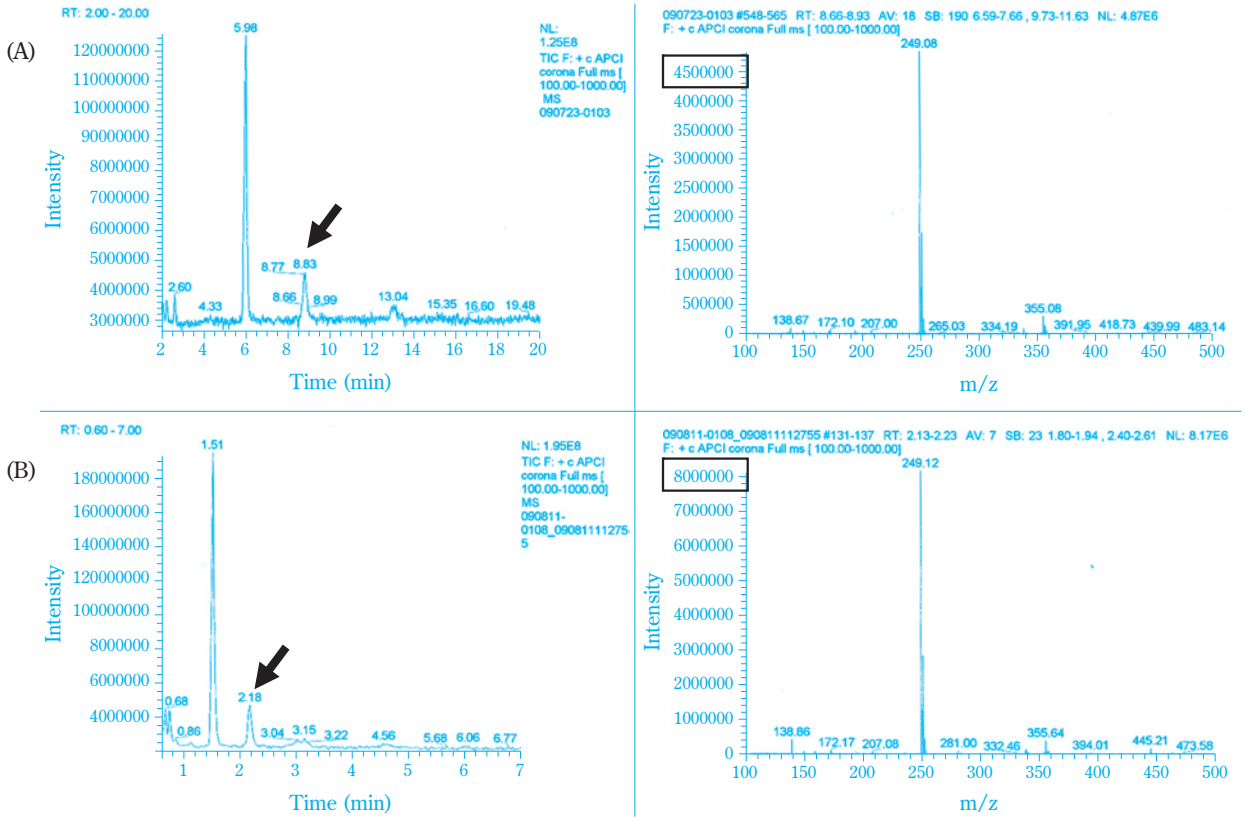


Fig. 13 Comparison of MS sensitivity between conventional LC and UHPLC
(A) Convetional LC Column: ODS (4.6×150mm, 5μm)
Injection volume: 10μL
(B) UHPLC Column: ODS (3.0×75mm, 2.2μm)
Injection volume: 5μL
Mobile phase : 10mM Ammonium Acetate in water/Acetonitrile=30/70(v/v) Flow rate: 1.0mL/min

mance system having a resonance frequency of 800 MHz. Although there is a certain timeframe for the use of this system as an LC-NMR, it can be used on a daily basis. With this system the ^{1}H spectrum (one-dimensional, two-dimensional) and ^{13}C spectrum can be measured. Therefore, in our experiment the amounts of samples required for the measurement were inspected using the model compounds. As a result, the absolute amounts of samples injected into LC were 1 µg for the one-dimensional ^{1}H spectrum, 20 µg – 50 µg for the two-dimensional ^{1}H spectrum and 100 µg for the ^{13}C spectrum, thus proving that the system could measure trace amounts. **Fig. 15** indicates the actual measurement examples.

To improve LC-NMR sensitivity, it is a critical question of how abundantly the sample can be packed and retained in the NMR flow cell having a capacity of only 60 µL. For this process also, the most important approach from the LC side is simply to produce as sharp a peak as possible. The sensitivity can be improved by reducing column diameters, by using UHPLC columns and by shortening the retention time.

Regarding the problems associated with data analysis, it is difficult to identify the ^{1}H signal at the high magnetic field side of the sample when the concentration level is low because it overlaps the signal derived from the solvent. Furthermore, in the flow of our impurity analysis (**Fig. 9**), the LC-NMR system can demonstrate its effect on rapid analysis if it is used in Step 1. However, in reality it is difficult to use LC-NMR as a

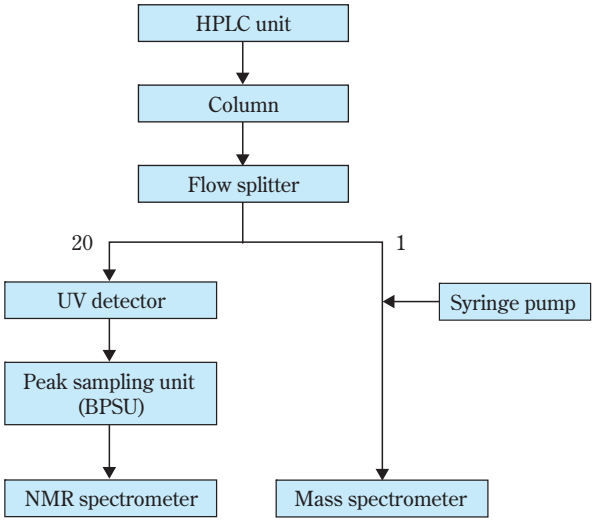


Fig. 14 Flow Diagram of LC-NMR/MS system

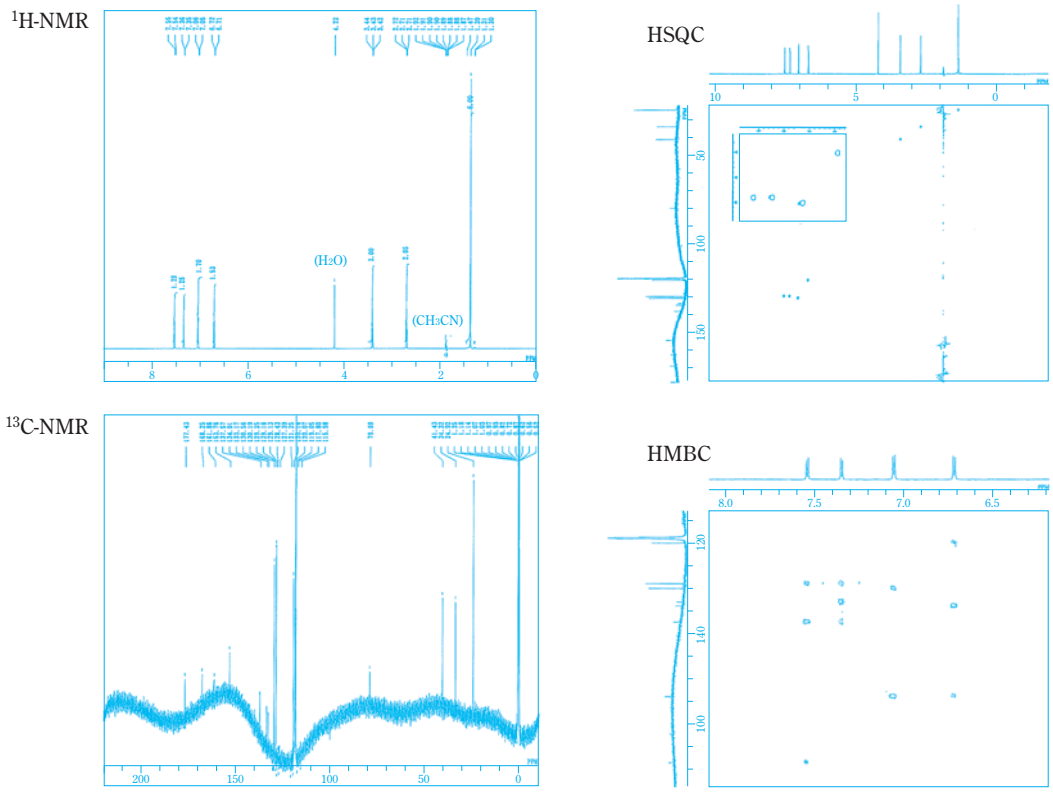


Fig. 15 NMR spectrum by LC-NMR
Mobile phase : 0.1% TFA-d in D_2O/Acetonitrile-d_3 = 55/45 (v/v)
Injection volume : 100µL (2mg/mL)

dedicated system due to its high cost. Thus we hope to see the emergence of an exclusive system that is compact and inexpensive.

Conclusion

We have introduced the contributions of chromatography and the hyphenated technology to production-process development for pharma-chemicals. Basically, chromatography is a mature technology. Therefore, in cases where only organic compounds having low molecular weight are concerned, there already are sufficient product lines that can fulfill the requirements of current systems and columns. However, given the progress in the production and distribution of the system black box, product lines have increased as well. This will raise the concern that we may lack the knowledge and experience to make good use of its features. Due to the GMP management, it is difficult to change the analysis conditions once they have been set in pharma-chemical production sites. Therefore, with the development of analysis it is important to set appropriate conditions including not only separation conditions but also the condition of analysis time. In that sense, developers of analysis methods are required to have broad knowledge of chromatography and the ability to apply such knowledge to their developments.

Regarding the application of the hyphenated technology to the analysis of small organic compounds, it can also be said that LC-MS systems are nearly mature in terms of apparatus. Apparatus manufacturers have already shifted their attention to various bio-related fields, and LC-MS is at the stage of "how we can use the system to its full extent" at sites of pharma-chemical production-process development. Despite the fact that GC-MS (in which a mass spectrometer is connected to the gas chromatography system) is already at the stage in which anyone can easily use it, LC-MS still requires technology and knowledge of ionization, including that of the LC side, as well as that of spectrum analysis. Consequently, LC knowledge is crucial in order to improve the sensitivity of LC-MS. With respect to LC-NMR, although the further advancement of MNR technology depends upon apparatus manufacturers, efforts by users are also required for the utilization of LC. In that sense one can expect that the importance of chromatography in the hyphenated technology will continue to grow.

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