Chem. Pharm. Bull. 57(12) 1396—1399 (2009) Vol. 57, No. 12

Time-Dependent Changes of Oxytocin Using ^{1}H-NMR Coupled with Multivariate Analysis: a New Approach for Quality Evaluation of Protein/Peptide Biologic Drugs

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Received September 2, 2009; accepted September 29, 2009; published online October 5, 2009

A new method that combines ^{1}H-NMR and principal component analysis (PCA) was employed to obtain the quality evaluation of biopharmaceuticals, with regard to their quality, consistency, and differences in protein modification patterns. To assess the feasibility of the method, three ^{1}H-NMR spectra of oxytocin (OXT) were collected every 7 d (at Day 0, 7 and 14), and time-dependent changes in the spectra were found by PCA of the ^{1}H-NMR signals from 0.5—9.0 ppm, excluding the region around the water signal (4.6—5.0 ppm). Although the three OXT spectra seemed similar by simple visual inspection, time-dependent differences among the three spectra were clearly distinguished by a PCA scores plot. Peak changes indicating both OXT decomposition and the emergence of new OXT decomposition products within the timeframe of the experiment were also observed by a PCA loading plot. The results demonstrate that this method can evaluate the consistency of biopharmaceutical quality.

Key words quality evaluation; biologic drug; principal component analysis; ^{1}H-NMR; oxytocin

The biotechnology industry has grown significantly in the past decade and continues to grow at a rapid rate. Biopharmaceuticals such as oxytocin (OXT), insulin, and somatropin are large, complex molecules that are receiving increased attention as therapeutics in humans, particularly since this class of molecules can potentially exert pharmacological effects that are unattainable by synthetic chemical products. However, while biologics show great potential value in medicine, many technical hurdles must be overcome before such treatments are made practical. In particular, since biologic drugs are typically derived from living sources such as microorganisms, plants, or human or animal cells, the production and use of such material introduces certain hazards that are not presented by small molecule drugs manufactured through chemical synthesis.[1)] In addition, to fully understand the mechanism of action of a protein/peptide drug, not only the primary amino acid sequence but also the folding, post-translational processing, and multimerization properties of the biologic within the cell must be considered. Moreover, different cell types or cell growth conditions may yield different protein modification patterns, as well as different impurities, into the desired product.[2,3)] Therefore, all of these complications may potentially influence the intended pharmacological effect of biologic drugs. With respect to safety and efficacy concerns surrounding biologics, the consistency of biologics between production lots, including maintenance of tight quality control specifications, is an important consideration for manufacturing biologic drugs.

To date, many analysis methods, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),[4,5)] capillary electrophoresis (CE),[6)] mass spectrometry (MS), tandem MS (MS/MS),[7)] liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC)[8)] and nuclear magnetic resonance (NMR), are employed to evaluate physicochemical characteristics and purity, and thus determine the quality of biopharmaceuticals.[2,3,9,10)] However, characterizing the full complexity of biologics by present examination methods is still currently difficult. Therefore, new analytical techniques that provide more detailed evaluation of biologic quality are necessary. Among the various analytical techniques, we focused on NMR profiling as the most suitable tool for rigorous quality evaluation because this approach can provide structural information on all compounds contained in product lots, and can distinguish structural differences. Principal component analysis (PCA) is often useful for profiling and classifying sample groups, and to characterize the most effective valuables in separation compounds.[11,12)] Therefore, small differences in product quality, *e.g.* a structural change or the appearance of a decomposition product, are thought to be appropriately evaluated by the combination of PCA using multivariate statistics and ^{1}H-NMR.

Herein, we demonstrate that ^{1}H-NMR spectroscopy coupled with PCA can provide a molecular fingerprint to precisely characterize a specific protein/peptide, using the determination of time-dependent changes of OXT as an example of this method. The results suggest that this new methodology can be useful for the quality evaluation of a manufactured protein/peptide biologic drug.

Experimental

Chemicals and Reagents All reagents used for ^{1}H-NMR experiments were purchased from Wako Chemicals, were of analytical grade (purity >99%), and were used without further purification. Deuterium oxide (D_2O, isotopic purity 99.9%) containing 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) was purchased from Aldrich (St. Louis, MO, U.S.A.). TSP was used as an internal standard at a chemical shift (δ) of 0.0 ppm for ^{1}H-NMR measurements.

Sample Preparation and ^{1}H-NMR Spectroscopic Analysis OXT (5 mg) was dissolved in 60 μl of D_2O containing 0.75% TSP, 30 μl of 0.2 M phosphate buffer (pH 6.2), and 510 μl of ultrapure water to produce a 600 μl solution for NMR measurements. The sample was introduced into an NMR test tube, and nuclear Overhauser effect spectroscopy (^{1}H-NOESY) spectra were recorded every 7 d at 25 °C using a Varian 600 MHz NMR spectrometer equipped with a coldprobe. Thirty-two free induction decays (FIDs) with 77 K data points per FID were collected using a spectral width of 9615.4 Hz, an acquisition time of 4.00 s, and a total pulse recycle delay of 2.02 s. The

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water resonance was suppressed using by presaturation during the first increment of the NOESY pulse sequence, with irradiation occurring during the 2.0 s relaxation delay and also during the 100 ms mixing time. Prior to Fourier transformation (FT), the FIDs were zero-filled to 128 K and an exponential line broadening factor of 0.5 Hz was applied.[12,13] All peak intensity values (in arbitrary units) were expressed as the means of three separate experiments (n=3). Following each spectrum acquisition, the sample was stored at 4 °C and protected from light.

NMR Data Reduction and Preprocessing All ^{1}H-NMR spectra were phased and baseline corrected by Chenomx NMR Suite 5.0 software, professional edition (Chenomx Inc., Canada). Each ^{1}H-NMR spectrum was subdivided into regions having an equal bin size of 0.04 ppm over a chemical shift range of 0.5—9.0 ppm (excluding the region around the water signal; 4.6—5.0 ppm), and the regions within each bin were integrated. The integrated intensities were then normalized to the total spectra area, and the data was converted from the Chenomx software format into Microsoft Excel format (*. xls). The resultant data sets were then imported into SIMCA-P version 12.0 (Umetrics AB, Umeå, Sweden) for multivariate statistical analysis.

Multivariate Data Analysis PCA was performed to examine the intrinsic variation in the data set.[14,15] The quality of the models was described by R^2x and Q^2 parameters, which indicate the proportion of variance in the data explained by models and goodness of fit. R^2x represents the goodness of fit of the PCA model, and Q^2 reveals the predictability of the PCA model.[16]

Results and Discussion

^{1}H-NMR spectra of OXT obtained at Days 0, 7 and 14 are shown in Fig. 1. While a simple visual inspection suggests that the analysis of a qualitative and quantitative changes might be difficult in three spectra, real spectral differences may be detected if changes can be represented as points in a multidimensional space and examined using PCA. As such, PCA of each OXT spectrum was performed. As a result, distinct differences among the three ^{1}H-NMR spectra were readily detected by both the scores of principal component 1 (PC1) and principal component 2 (PC2), and can be clearly depicted as three separate points as shown in Fig. 2. The PCA modeling revealed R^2x and Q^2 values of 0.80 and 0.49 for PC1, indicating 80% of variance and 49% predictability in the multidimensional space, respectively (Fig. 2). On the other hand, PC2 was explained with a low contribution ratio of 20%. This result suggests a high contribution rate for PC1, indicating that each of the two spectra collected at 7 and 14 d are considerably different from the spectrum collected at Day 0. The differences of the data points on PC1 likely reflect the progress of time-dependent changes of the OXT sample.

The loading plot of all ^{1}H-NMR signals evaluated is shown in Fig. 3. This loading plot reveals the contributions of particular variables (integral regions, in this study) towards either an increase or a decrease in integrated intensities over time. In this case, each variable represents a peak at a particular chemical shift in the ^{1}H-NMR spectral region shown in Fig. 1. From the score and loading plots, the components responsible for increasing or decreasing time-dependent changes can be identified. In addition, the variables at the chemical shifts associated with the largest changes in integrated intensity can be found farther away (to either the left or to the right) from the center of the PC1 coordinate axis.

Eight variables showing typical fluctuations in Fig. 3 were identified, and time-dependent changes of the integrated intensities associated with these variables are shown in Fig. 4. The tendency of the intensity of each of these variables to either increase or decrease is evident, and suggests the formation of new degradation products (Fig. 4A) or the decomposition of the original OXT sample (Fig. 4B).

An increase of the negative value along the PC1 axis in Fig. 3 of four variables (δ 0.82, 2.34, 6.82 and 7.06 ppm) is associated with larger increases in integrated intensity over time (Fig. 4A), and an increase of the positive value along the PC1 axis in Fig. 3 of four variables (δ 0.90, 7.22, 2.06

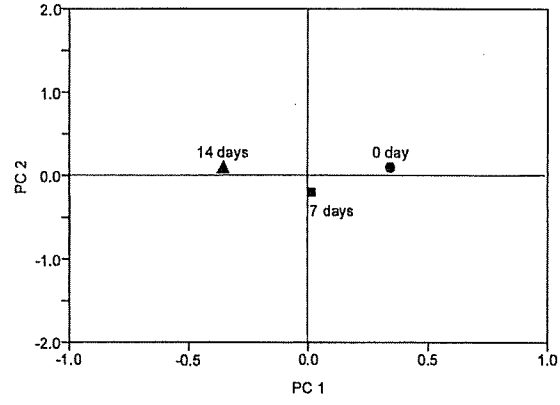

Fig. 2. The PCA Scores Plot Derived from the ^{1}H-NMR Spectra Data of OXT

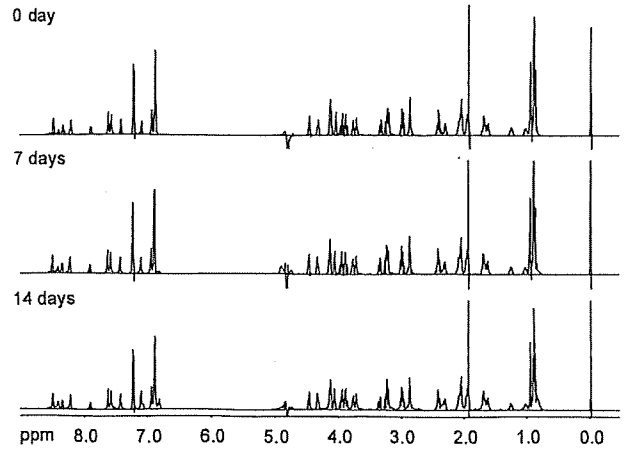

Fig. 1. ^{1}H-NMR Spectra of OXT in 10 mM Phosphate Buffer (pH 6.2) at 25 °C, Collected Every 7 d

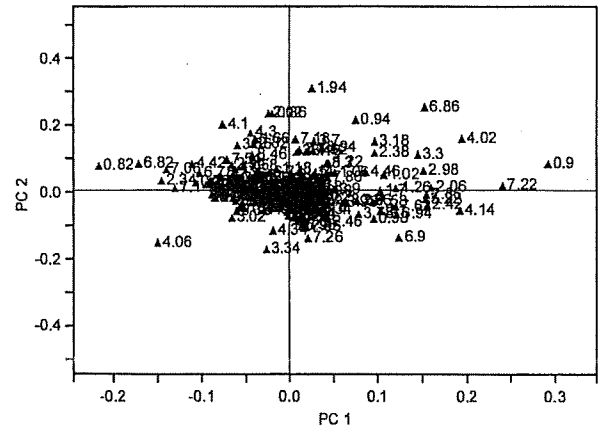

Fig. 3. The PCA Loading Plot Derived from the ^{1}H-NMR Spectra Data of OXT

The variables are shown in chemical shifts, ppm.

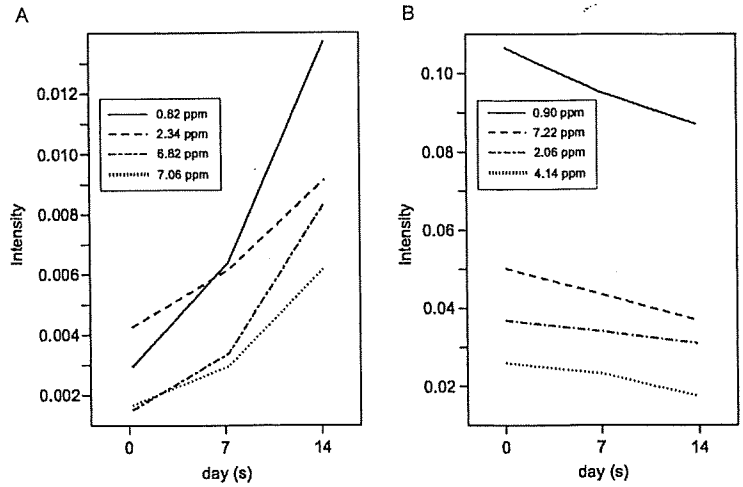

Fig. 4. Time-Dependent Changes in the OXT ^{1}H-NMR Spectrum Showing the Formation of New Decomposition Products (A) and the Decomposition of OXT (B)

and 4.14 ppm) is associated with larger decreases in integrated intensity over time (Fig. 4B). In addition, while the time-dependent increases in intensity for each variable in Fig. 4A are surmised to be due to new peaks from OXT decomposition products of OXT, identifying individual peaks in Fig. 1 associated with these decomposition products is difficult, as these peaks are minor signals. The peaks corresponding to these particular variables were too small to analyze further. On the other hand, peaks for each variable in Fig. 4B could be identified: the variable at 0.90 ppm was attributed to both the δH (δ 0.88) and γ'H (δ 0.89) of Ile and to the δ'H (δ 0.90) of Leu; the variable at 7.22 ppm to the 2′, 6′H (δ 7.22) of Tyr; the variable at 2.06 ppm to the γH (δ 2.05) of Pro and to the βH (δ 2.08) of Gln; and the variable at 4.14 ppm to the αH (δ 4.14) of Gln and to the αH (δ 4.16) of Ile. Therefore, some amino acids for each variable in Fig. 4B are inferred to be associated with OXT decomposition, although proposing a specific decomposition mechanism for OXT is difficult owing to the complexity of protein/peptide systems.

Recently biotechnology-derived drugs for medical treatment are increasingly receiving attention, but many problems associated with the quality, efficacy, and safety of biologics persist. Almost all biologics are designed to mimic human proteins to better predict pharmacological effects, and are thus produced from recombinant or non-recombinant cell-culture expression systems. Preserving the consistency of these complex products during the production is important, as well as reducing or eliminating molecular heterogeneity and higher-order structural aggregates. In addition, since raw materials derived from animals or humans may be used during production,[1] consideration of possible viral contamination is also crucial. To date, the quality of manufactured biologics is evaluated by measuring biological activity of the biologics, rather than through analysis of physicochemical information. In fact, the product complexity and purity for quality control of biologics are quite difficult to evaluate by current physicochemical methodologies, such as SDS-PAGE, CE, MS, and HPLC.[2—10]

As a new approach for assessing the quality of biopharmaceuticals, we examined a method to distinguish time-dependent changes of OXT ^{1}H-NMR spectra by introducing PCA of NMR signals. We initially found that simple visual inspection was insufficient to distinguish whether the three ^{1}H-NMR spectra of OXT, gathered every 7 d for 14 d, showed time-dependent changes. However, the PCA scores plot of these same spectra clearly revealed time-dependent changes. In addition, peak changes associated with both new decomposition products and the decomposition of OXT were also observed by the PCA loading plot. Therefore, a combination of ^{1}H-NMR and PCA techniques can provide a molecular fingerprint capable of precisely identifying a protein/peptide biologic, and can represent a powerful new approach for assessing the quality of protein/peptide biologic drugs. The study concerning the feasibility and the limitation of this method in terms of the molecular size is currently under way.

Acknowledgements We thank Mr. H. Watanabe and Mr. K. Kushida (Varian Technologies Japan Ltd.) for their technical assistances in measuring NMR spectra. This work was supported by a Grant-in-Aid for Scientific Research (No. 20390038) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Current Topics

New Era of Glycoscience: Intrinsic and Extrinsic Functions Performed by Glycans

The Significance of Glycosylation Analysis in Development of Biopharmaceuticals

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Received October 24, 2008

Many glycoproteins and glycosaminoglycans are approved for clinical use. Carbohydrate moieties in biopharmaceuticals affect not only their physicochemical properties and thermal stability, but also their reactivity with their receptors and circulating half-life. Modification of glycans is one target of drug design for enhancement of efficacy. Meanwhile, there have been reports of serious adverse events caused by some carbohydrates. It is crucial to maintain the constancy of carbohydrate moieties for the efficient and safe use of glycosylated biopharmaceuticals. On the other hand, for scientific, safety-related, and economic reasons, changes in the manufacturing process are frequently made either during the development or after the approval of new biopharmaceuticals. Furthermore, the development of biosimilar glycoprotein products has been attempted by different manufacturers. Changes in pharmaceutical manufacturing processes possibly cause alteration of glycosylation and raise concerns about alteration of their quality, safety, and efficacy. In this review we provide some current topics of glycosylated biopharmaceuticals from the viewpoints of efficacy, safety, and the manufacturing process and discuss the significance of glycosylation analysis for development of biopharmaceuticals.

Key words glycoprotein; glycosaminoglycan; biopharmaceutical; efficacy; safety; manufacturing process

1. INTRODUCTION

Many biological molecules containing carbohydrates are approved for clinical use in Japan (Table 1). In the beginning, most therapeutic glycoconjugates were naturally occurring glycoproteins and glycosaminoglycans (GAGs) derived from human and healthy animal tissues, such as gonadotropins from human urine, and heparins from the porcine intestine. Recombinant glycoproteins, including erythropoietin and tissue plasminogen activator (tPA), have been developed as copies of native human glycoproteins since the early 1990s in Japan. In many cases their carbohydrate moieties were different from the original human ones. Carbohydrate moieties in glycoproteins affects not only their physicochemical properties and thermal stability but also their reactivity with their receptors and circulating half-life.[1] Modification of carbohydrate moieties is one target of drug design to enhance the efficacy of the original ones. Meanwhile, there have been reports from around the world of serious adverse events caused by carbohydrate-related drugs. For the efficient and safe use of glycoprotein/GAG products, it is necessary to maintain the structures and heterogeneity of carbohydrate moieties in glycosylated biopharmaceuticals.

On the other hand, carbohydrate moieties in biotechnology-derived drugs are variable when the manufacturing process is changed. For scientific, safety-related and economic reasons, it has become common for companies to change the manufacturing process for their approved products. Furthermore, biosimilar glycoprotein products, which are manufactured by different companies, have been developing in many regions.[2,3] One of the main issues for the devel-

Table 1. Glycosylated Biopharmaceuticals in Japan

Origin	Japanese accepted name
Granulocyte-colony stimulating factor	Lenograstim
Granulocyte macrophage colony-stimulating factor	Mirimostim
Interferon	Interferon Alfa (NAMALWA), Interferon Alfa (BALL-1), Interferon Beta, Interferon Beta-1a, Interferon Gamma-n1
Erythropoetin	Epoetin Alfa, Epoetin Beta, Darbepoetin Alfa
Monoclonal antibody	Ibritumomab Tiuxetan, Basiliximab, Infliximab, Rituximab, Cetuximab, Gemtuzumab Ozogamicin, Palivizumab, Tocilizumab, Trastuzumab , Bevacizumab, Adalimumab
Receptor	Etanercept
Follicle stimulating hormone	Follitropin Alfa, Follitoropin Beta
Gonadotropin	Human Menopausal Gonadotropin, Human Chorionic Gonadotropin, Serum Gonadotropin
Factor VII	Eptacog Alfa (Activated)
Factor VIII	Octocog Alfa, Rurioctocog Alfa
Thrombomodulin	Thrombomodulin Alfa
Urokinase	Urokinase
Pro-urokinase	Nasaruplase
Tissue-plasminogen activator	Alteplase, Monteplase, Pamiteplase
Enzymes	Kallidinogenase, Agalsidase Alfa, Agalsidase Beta, Alglucosidase Alfa, Alglucerase, Idursulfase, Imiglucerase, Laronidase, Galsulfase
Heparins	Heparin Sodium, Heparin Calcium, Parnaparin Sodium, Dalteparin Sodium, Enoxaparin Sodium, Reviparin Sodium
Hyaruronate	Sodium Hyaruronate
Chondroitin sulfate	Chondroitin sulfate

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opment of biosimilar products is how to assure the similarity of carbohydrate moieties between the biosimilar products and the reference products.

In this review we provide some current topics of glycosylated biopharmaceuticals in terms of efficacy, safety, and manufacturing process and discuss the significance of glycosylation analysis in the development of biopharmaceuticals.

2. ROLE OF CARBOHYDRATES ON EFFICACY

Glycosylation in some biopharmaceuticals is crucial for their biological activity. Lysosomal storage diseases are characterized by deficiencies of lysosomal enzymes that degrade the glycoconjugates, such as mucopolysaccharides and glycolipids, and consequent cellular damages by their accumulated metabolites.[4] For use in enzyme-replacement therapy against lysosomal storage diseases, several recombinant glycoprotein products have been approved, namely *agalsidase alfa* and *agalsidase beta* for Fabry's disease, *alglucosidase alfa* for Pompe's disease, and *laronidase*, *idursulfase*, and *galsulfase* for mucopolysaccharidosis I, II, and VI, respectively.[4—8] These drugs contain *N*-linked oligosaccharides attached to mannose 6-phosphate (M-6-P), and the secreted enzymes are transported to an acidified prelysosomal compartment through the M-6-P receptor.[9,10] The carbohydrate residue is essential for lysosomal targeting and to exhibit complete enzyme activity in lysosomes. *Imiglucerase* is an analog of human β-glucocerebrosidase, which is used for the treatment of Gausher's disease. This glycoprotein is produced by recombinant DNA technology and exoglycosidase treatment to expose mannose residues in *N*-linked oligosaccharides. The carbohydrate modification facilitates incorporation of this drug into macrophages through mannose-binding receptors.[11] These recombinant lysosomal enzymes have achieved dramatic therapeutic effects against the lysosomal storage diseases.

Several human glycoprotein analogs whose carbohydrates are modified to enhance their efficacy have been developed in recent years. Erythropoietin is a glycoprotein containing three *N*-glycans and one *O*-glycan, and sialylation on its nonreducing ends is closely associated with its circulating half-life.[12] *Darbepoetin alfa* is an erythropoietin analog to which two additional *N*-glycans are attached by replacement of five amino acid residues.[13] The modification of glycosylation prolongs the half-time of this analog compared to its native form. Similar genetic engineering for improvement of half lives has been successfully attempted in antithrombolytic drugs. T-PA consists of finger, epidermal growth factor (EGF), kringle1, kringle2 and catalytic domains, and three *N*-glycans. High-mannose type oligosaccharides at the kringle1 domain and EGF domain are related to the short circulation half-life of t-PA.[14] Extension of half-life in blood has been achieved by eliminating the kringle1 domain from human t-PA and replacing one amino acid residue (*pamiteplase*).[15] These improvements have contributed to reducing the frequency and dose of administration.

Recombinant monoclonal antibodies, which have been successfully used in the treatment of cancers and immune diseases, might be the next target of drug design by glyco-engineering. Several anti-tumor monoclonal antibodies that contain a constant region derived from immunoglobulin (Ig) G have antibody-dependent cellular cytotoxicity (ADCC), and removal of a fucose (Fuc) residue from *N*-linked oligosaccharides at the constant region causes the enhancement of ADCC.[16,17] A non-fucosylated IgG-derived antibody is expected to improve the therapeutic effects of these antitumor pharmaceuticals.

Glycosylation has also been used for the site-selective modification of proteins with polyethylene glycol (PEGylation).[18] In the GlycoPEGylation method, sialic acid covalently substituted with polyethylene glycol (PEG) can be enzymatically transferred to *O*-glycans at serine or threonine positions in proteins produced in *Escherichia coli*. This strategy has overcome the problems of the previous PEGylation, which provided a heterogeneous mixture of PEG positional isomers. There is increasing interest in utilization and modification of glycans in the development of biopharmaceuticals.

3. IMPACT OF CARBOHYDRATES ON SAFETY

Some glycans have caused serious adverse events in clinical stages. Heparin is a highly sulfated GAG composed of a disaccharide unit, β1-4 linked α-D-glucosamine and α-D-iduronic acid or β-D-glucuronic acid (averaging 2.5 sulfate groups per disaccharide). In 2007—2008, a serious adverse event associated with *heparin sodium*, including over eighty deaths, occurred in the United States (US).[19] Over-sulfated chondroitin sulfate (OSCS), which consists of fully sulfated β1-3 linked α-D-*N*-acetylgalactosamine (GalNAc) and a β-D-glucuronic acid unit, was identified as the contaminant in the heparin sodium that had caused hypersensitivity reactions.[20,21] It has been reported that OSCS activated the kinin-kallikrein system and induced generation of C3a and C5a, potent anaphylatoxins derived from complement proteins. The contaminated heparin sodium was distributed to at least twelve countries and raised concern about a shortage of heparin products. For ensuring the safety of heparin products, pharmacopoeias in Japan, the US and EU immediately amended their heparin sodium monograph to confirm the absence of OSCS by ^{1}H-NMR and/or capillary electrophoresis (Fig. 1A). This adverse event has left concerns about the safety of GAGs and motivated the development of a sensitive and selective analytical method for GAGs products all over the globe (Fig. 1B).

An alternative concern for a carbohydrate-related adverse event is immunogenicity of nonhuman glycan motifs. The galactose-α1,3-galactose (Gal(α1-3)Gal) motif is known as one of the major problems in the transplantation of organs from pigs to humans.[22] Mouse myeloma cell lines, which are often used for production of recombinant glycoproteins, also expresses the Gal(α1-3)Gal motif in *N*-linked oligosaccharides. Recently, a high prevalence of hypersensitivity reactions was reported in patients who had been injected with *cetuximab*, which is produced in mouse myeloma cells.[23] This chimeric mouse-human antibody is attached to *N*-linked oligosaccharide containing the Gal(α1-3)Gal motif at the Fab region.[24] It is reported that IgE antibodies against the Gal(α1-3)Gal motif had been present in serum before therapy, and these antibodies caused hypersensitive reactions after cetuximab treatment. Another immunogenic nonhuman glycan is *N*-glycolylneuraminic acid (NeuGc), a sialic acid.[25]

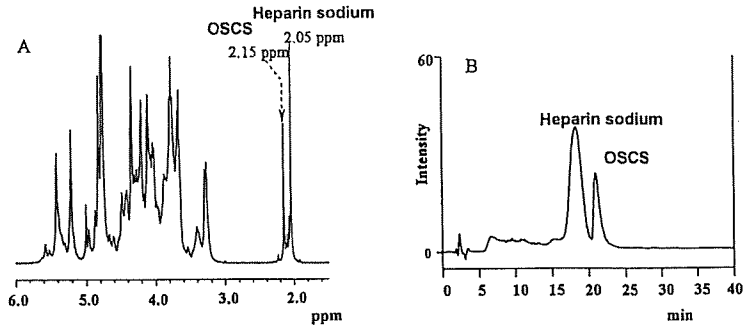

Fig. 1. ^{1}H-NMR Spectra (A) and Anion-Exchange Chromatogram (B) of Heparin Sodium Containing 10% OSCS

^{1}H-NMR spectrum was obtained at 298 K using a 500 MHz JEOL JNM-ECA500 instrument equipped with a 5-mm filed gradient tunable probe with standard JEOL software. Chemical shifts were referenced to the signal of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) as internal standard (0.00 ppm). Anion-exchange chromatogram was obtained using TSKgel DEAE-5PW (21.5 mm I.D.×15 cm, 10 μm) at a flow rate of 1.0 ml/min at 40 °C. The UV detector was set at 230 nm. GAGs were eluted by a linear gradient of 0—100% B buffer (buffer: A, 20 mM Tris–HCl (pH 8.0); B, 20 mM Tris–HCl (pH 8.0) containing 2 M NaCl) within 40 min.

Since Varki *et al.* reported the incorporation of NeuGc into human embryonic stem (ES) cells through the animal serum and the feeder layer, contamination of cell therapy products with NeuGc has become a serious issue in clinics.[26] These reports imply the significance of glycan analysis in recombinant glycoprotein products and risk assessment of nonhuman glycans.

4. ALTERATION OF CARBOHYDRATES BY CHANGES IN EXPRESSION SYSTEM AND MANUFACTURING PROCESS

In addition to recombinant technology using mammalian cells, a transgenic technique has been also focusing on large-scale production of therapeutic glycoproteins.[27,28] Antithrombin III is an anticoagulant factor, and a freeze-dried preparation of human antithrombin III has been approved for treatment of disseminated intravascular coagulation and thrombogenic tendencies. In 2006, the European Medicine Agency (EMEA) authorized the marketing of *antithrombin alfa*, a recombinant human antithrombin III produced from the milk of transgenic goats. Detailed structural analysis revealed that these two glycoproteins were structurally identical except for a difference in glycosylation.[27] An alternative approach for the large-scale and low-cost production of biopharmaceuticals is the use of transgenic plants.[29] Plant cells express nonhuman glycans, such as Fuc (α1-3) *N*-acetylglucosamine (GlcNAc), xylose (Xyl) (β1-2) GlcNAc and Gal (β1-3) GlcNAc at the reducing-end of complex-type *N*-linked oligosaccharides. Currently, plant glyco-engineering, including modification of glycosyltransferases, is developing to overcome limitations in the production of glycoprotein products.[30]

For various scientific, safety-related and economic reasons, changes in manufacturing processes for biopharmaceuticals are often attempted during the development phase and after marketing authorization. Meanwhile recombinant glycoprotein products that have been claimed to be similar to a reference medical product already authorized (biogeneric/biosimilar/follow-on biologics) have been approved by different manufacturers. The changes in the manufacturing process possibly cause the alteration of glycosylation in the glycoprotein products and consequent changes in quality, efficacy and safety. The first biosimilar glycosylated pharmaceutical, *epoetin alfa*, was approved in the EU recently. According to the Japanese guidelines for the biosimilar products, the applicants have to submit some efficacy and safety study data but not all if they can show the data on structural properties and physicochemical/biological similarity between the authorized and biosimilar products. One of the challenging issues in the development of biosimilar glycoprotein products is a comparison of glycosylation between the authorized products and the new entry biosimilar products.

Using some *epoetin* products, we have studied the possibility of several analytical methods for comparison of the glycosylation between closely related biopharmaceuticals. Commercially available *epoetin* products (products A—D) were electrophoresed, and *N*-linked oligosaccharides were released from bands at 30 kDa by an in-gel glycopeptidase F digestion (Fig. 2A). The resulting oligosaccharides were reduced with $NaBH_4$ and subjected to LC/MS. In Fig. 2, *International nonproprietary names* (*INN*) of *epoetin*s contained in the products A—C and D are tentatively named as *epoetin* α and β, respectively. Products A and B are marketed in country X, while products C and D are manufactured and distributed in country Y. Figure 2B shows the total ion chromatograms of *N*-linked oligosaccharides released from four *epoetin* products, and the mass spectra acquired from the most intense peaks (peak z_{1_4}) are shown in Fig. 2C. The *m/z* values of the most intense ions (*m/z* 1226.8) and a series of triply charged ions with *m/z* 14 spacing pattern reveal that the most abundant glycan in *epoetin* products are a tetrasialyl fucosylated-tetraantennary oligosaccharide in common but there are tangible differences in acetylation of sialic acids between products A—C (*epoetin* α) and product D (*epoetin* β) (Fig. 2C). Even among *epoetin* α products there were some significant differences, such as the non-fucosylated oligosaccharides that were found in *epoetin* α products in country X (products A and B) but not in country Y (product C). The latest analytical technology allows us to evaluate the similarity of the glycosylation of closely related biopharmaceuticals.[31,32]

5. CONCLUSION

As described above, glycosylation in most biopharmaceuticals affects their efficacy and safety, and the glycosylation is dependent on the manufacturing process and the expres-

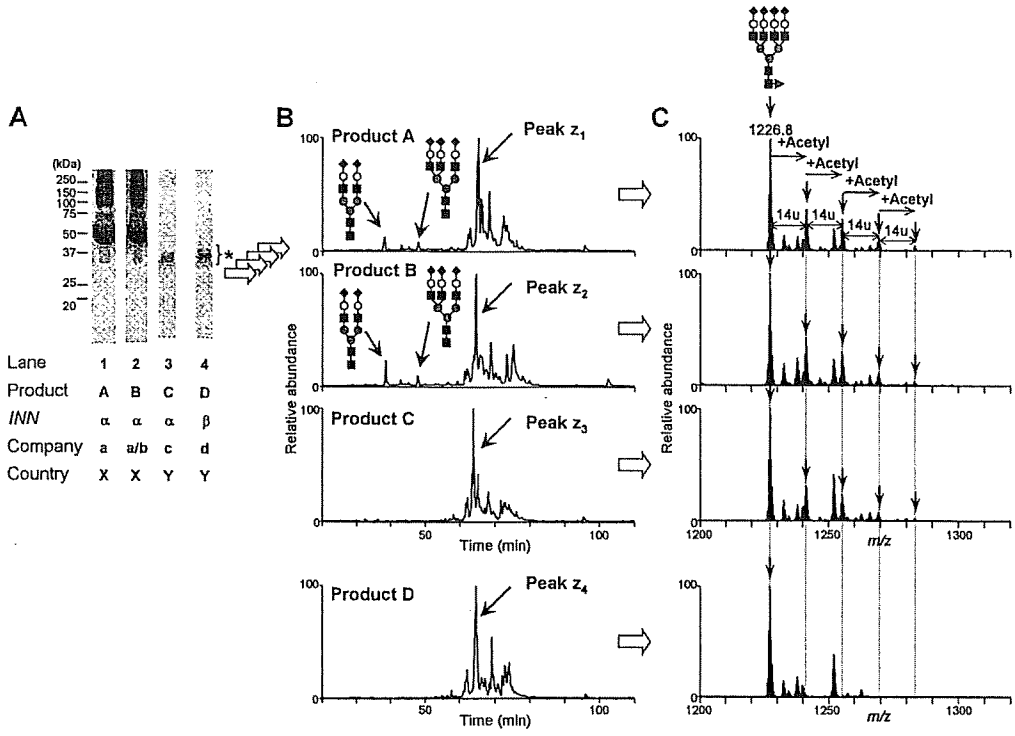

Fig. 2. Glycosylation Analysis of *Epoetin* Products

(A) SDS-PAGE images. Sample: lane 1, product A; lane 2, product B; lane 3, product C; lane 4, product D. (B) *N*-glycan profiles of products A—D acquired by LC/MS in the negative ion mode. (C) Mass spectra of peaks z_{1-4}. Product: A, an *epoetin* α product manufactured/distributed by company a in country X; B, an *epoetin* α product manufactured by company a and distributed by company b in country X; C, an *epoetin* α product manufactured/distributed by company c in country Y; D, an *epoetin* β product manufactured/distributed by company d in country Y. *INN* of products A—C, and D are tentatively named as *epoetin* α and *epoetin* β, respectively. Symbols: ●, Man; ○, Gal; ■, GlcNAc; ▲, Fuc; ◆, NeuAc. * Bands of *epoetins*. LC: instrument, nanoFrontier nLC system (Hitachi High-Technologies Corporation); column, graphitized carbon (0.075×150 mm, ThermoFisher Scientific); flow rate, 200 nl/min; buffer A, 5 mM ammonium acetate with 2% acetonitrile (pH 9.6); buffer B, 5 mM ammonium acetate with 80% acetonitrile (pH 9.6); gradient condition, 5—35% B (110 min). MS: instrument, LTQ-TF (ThermoFisher Scientific); electron voltage, 2.0 kV (negative ion mode).

sion system. Physicochemical and biological characterization of such glycosylation is crucial at various stages, namely the development of new biotherapeutic glycoproteins, the establishment of changes in the manufacturing process, and the development of biosimilar products. Appropriate glycan testing must be adopted if the carbohydrate moiety influences safety and efficacy of the pharmaceutical. Furthermore, an *in vivo* assay could be replaced by the glycan test if the glycan profile is strongly associated with *in vivo* activity. Advances in analytical techniques for carbohydrate moieties are expected to facilitate the development of biopharmaceuticals.

Acknowledgments The work was supported in part by a Grant-in-Aid from the Japanese Ministry of Health, Labor, and Welfare.

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AAPS PharmSciTech, Vol. 10, No. 3, September 2009 (© 2009)
DOI: 10.1208/s12249-009-9291-5

White Paper

FIP Position Paper on Qualification of Paddle and Basket Dissolution Apparatus

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Received 5 May 2009; accepted 9 July 2009; published online 16 July 2009

Abstract. The qualification process for ensuring that a paddle or basket apparatus is suitable for its intended use is a highly debated and controversial topic. Different instrument qualification and suitability methods have been proposed by the pharmacopeias and regulatory bodies. In an effort to internationally harmonize dissolution apparatus suitability requirements, the International Pharmaceutical Federation's (FIP) Dissolution/Drug Release Special Interest Group (SIG) reviewed current instrument suitability requirements listed in the US, European, and Japanese pharmacopeias and the International Conference on Harmonization (ICH) Topic Q4B on harmonization of pharmacopoeial methods, in its Annex 7, Dissolution Test General. In addition, the SIG reviewed the Food and Drug Administration (FDA) Draft Guidance for Industry, "The Use of Mechanical Calibration of Dissolution Apparatus 1 and 2—Current Good Manufacturing Practice (CGMP)" and the related ASTM Standard E2503-07. Based on this review and several in-depth discussions, the FIP Dissolution/Drug Release SIG recommends that the qualification of a dissolution test instrument should be performed following the calibration requirements as indicated in the FDA (draft) guidance. If additional system performance information is desired, a performance verification test using US Pharmacopeia Reference Standard tablet or an established in-house reference product can be conducted. Any strict requirement on the use of a specific performance verification test tablet is not recommended at this time.

KEY WORDS: basket apparatus; chemical qualification; dissolution; mechanical qualification; paddle apparatus; performance verification test.

INTRODUCTION

Over the last four decades, the dissolution test has evolved into a powerful method for characterizing oral drug products. It is an important tool for assessing lot-to-lot quality of a drug product, guiding development of new formulations and ensuring continued product quality and performance after post-approval changes in formulation, manufacturing process, site of manufacture, and scale-up of the manufacturing process. This has been possible only because of our increased knowledge and understanding of the science behind dissolution test methodology, and continuous improvement of the instrumentation.

The engineering of dissolution testing instruments has evolved over the years. This has resulted in the availability of precise, rugged, and reliable dissolution apparatuses. Because dissolution is not an absolute method, no definitive standard is available against which to verify the performance of the apparatus. Thus, qualification of dissolution instruments needs to include a complete description of the instrument dimensions and setup to ensure meaningful dissolution results.

Currently, the qualification process for ensuring that a paddle or basket apparatus is suitable for its intended use is a highly debated and controversial topic. Different instrument qualification and suitability methods have been proposed by various pharmacopeias and regulatory bodies. For example, chapter 711 of the *United States Pharmacopeia* (USP) describes mechanical calibration specifications and the use of performance verification reference tablets, historically known as calibrator tablets, to establish instrument suitability (1). The Food and Drug Administration (FDA) has issued a draft guidance for industry, "The Use of Mechanical Calibration of Dissolution Apparatus 1 and 2—Current Good Manufacturing Practice (CGMP)," recommending that a properly executed rigorous mechanical calibration will satisfy CGMP requirements for dissolution apparatus calibration in lieu of performance verification with specified tablets (2). The European Pharmacopoeia (Ph. Eur.) recommends mechanical calibration for instrument qualification and suggests that the performance of the dissolution test instrument may be monitored by the selection and testing of an appropriate reference product (3). The Japanese Pharmacopoeia states

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that the fundamental system suitability of the dissolution apparatus must include conformance to the dimensions and tolerances stated in chapter 6.10 Dissolution Test, but specific requirements on performance verification of the apparatus are not given (4). Hence, the International Conference on Harmonization (ICH) Topic Q4B on harmonization of pharmacopoeial methods, in its Annex 7, Dissolution Test General Chapter, notes that the harmonized dissolution test apparatus should be calibrated to ensure compliance with regional good manufacturing practice (GMP) requirements (5).

This article offers a review of the current instrument qualification proposals and provides recommendations from the International Pharmaceutical Federation's (FIP) Dissolution/Drug Release Special Interest Group (SIG) on how to ensure that the dissolution apparatus is appropriately qualified for its intended use.

HISTORY/BACKGROUND

A significant step towards standardizing the dissolution methods and resolving lab-to-lab result discrepancies occurred in the 1980s (6). The specifications and acceptance criteria for the USP calibrator tablets (prednisone and salicylic acid) were established from collaborative study results organized by the Pharmaceutical Manufacturer's Association (PMA)/Pharmaceutical Research and Manufacturers of America (PhRMA). The dissolution values from six individual units had to comply with an established range for %-dissolution to qualify the instrument for routine operation (7). Originally, calibrator tablets were adopted to detect the influence on dissolution results due to improper alignment of the instrument, vibration in the instrument, failures in the drive chains and belts, and deaeration (8). Thus, the calibrator tablet became an important check on operating procedures, especially in terms of consistency between laboratories on an international basis (9). The testing with USP calibrator tablets is currently described in USP 711 as the Performance Verification Test. The precise engineering of dissolution instrumentation and the ability to accurately measure the instrument's mechanical operations has caused the industry to question the USP's performance verification requirement utilizing the historical calibrator tablet practice.

In 2000, PhRMA published results from a collaborative study to evaluate the performance of the then current USP 50-mg prednisone and 300-mg salicylic acid[1] reference tablets and a 10-mg prednisone tablet from the FDA Division of Pharmaceutical Analysis (DPA) known as NCDA#2 (8). Their recommendations included enhanced mechanical calibration testing on each dissolution bath and a reduction in reliance on the testing of USP reference standard (calibrator) tablets. This approach was endorsed by the FDA Pharmaceutical Science Advisory Committee in October 2005 (9). The American Society for Testing and Materials (ASTM), a voluntary consensus standard-setting organization, created a standard for mechanical calibration of basket and paddle dissolution apparatus building on these recommendations (10).

[1] The requirement for use of the salicylic acid tablets will be eliminated at the end of calendar year 2009.

CURRENT INSTRUMENT QUALIFICATION PROPOSALS

According to USP 32 NF 27 2009, the suitability of the dissolution paddle or basket assembly is determined by conformance to the dimensions and tolerances stated in its chapter 711. Dissolution medium volume, temperature, and shaft rotation speed need to be monitored during use. In addition, the USP requires a performance verification test with reference standard tablets, formerly called calibrator tablets. For apparatuses 1 and 2, disintegrating prednisone reference standard tablets are used to establish system suitability.

FDA's CGMP regulations require that laboratory apparatuses are calibrated at suitable intervals according to established written procedures and specifications (6). Recently, the FDA issued a draft guidance for industry—The Mechanical Calibration of Dissolution Apparatus 1 and 2—Current Good Manufacturing Practice (CGMP). The draft guidance recommends a more rigorous mechanical calibration of the paddle and basket apparatus as a suitable alternative to the USP Performance Verification Test (PVT). In the spirit of continuous improvement, this change in qualification procedure has been proposed since the wide acceptance range of the Performance Verification Test results makes it difficult to assess the suitability of the dissolution apparatus.

ASTM Standard E2503-07 (10) provides guidance for basket and paddle dissolution apparatus setup and calibration to ensure reproducibility of results without specifying how to perform dissolution testing. This standard takes a more detailed approach to instrument setup than is currently outlined in the harmonized pharmacopeial chapters by providing quantitative criteria for shaft wobble. Shaft and vessel verticality are new parameters not currently addressed in the pharmacopoeial chapters. The ASTM standard also has tighter criteria for shaft/vessel centering (vessel offset), rotational speed, and basket wobble than that given in the Pharmacopoeial Discussion Group (PDG) harmonized dissolution test. This change in calibration methodology, from the reliance on less stringent methodology and use of tablets to rigorous mechanical calibration, will reduce the bias and variation in measurement systems. Hence, FDA states that properly executed rigorous mechanical calibration will satisfy the CGMP requirements for dissolution apparatus calibration in lieu of chemical tablet calibration.

Ideally, knowledge of how the product is affected by each source of instrument-related variability will allow tighter control of those variables and result in more meaningful decision making from any dissolution data that are generated. In the absence of such knowledge, rigorous mechanical setup criteria will ensure less instrument contribution to test method variability.

The European Pharmacopoeia (3) recommends that the qualification of the dissolution test instrument has to consider the dimensions and tolerances specified for the apparatus. Parameters such as dissolution medium temperature and volume, rotation speed, and sampling probes need to be monitored periodically during use. The following general statement is made in reference to a performance test: "The performance of the apparatus may be monitored by testing a

Table I. Mechanical Calibration Parameters: Dissolution Rotating Basket Apparatus

Calibration parameter	PDG harmonized pharmacopeial specifications (USP, EP, JP)	FDA recommendations based on ASTM standard
Shaft wobble	Rotates smoothly without significant wobble	≤1.0 mm total runout
Shaft verticality	N/A	Bubble must be with-in the lines of bubble level (≤0.5° from vertical)
Basket wobble	±1 mm runout	≤1.0 mm total runout
Vessel/shaft centering	≤2.0 mm from centerline.	≤1.0 mm from centerline measured at an upper and lower position
Vessel verticality	N/A	≤1.0° from vertical
Height check/basket depth	25±2 mm	25±2 mm
Rotational speed	±4% from target	±2 rpm from target

reference product that is sensitive to the hydrodynamic conditions. Such tests may be performed periodically or continuously for comparative reason with other laboratories (3)." Based on this Ph. Eur. recommendation, individual laboratories can independently determine if a reference product test is needed, and if so, the laboratories are responsible for the selection and qualification of an appropriate reference product for performance verification.

The Japanese Pharmacopoeia (4) states that the fundamental system suitability of the dissolution apparatus must include conformance to the dimensions and tolerances stated in chapter 6.10 Dissolution Test. In addition, critical test parameters, such as rotation speed and volume and temperature of the dissolution medium, must be monitored periodically during use. The JP also states that apparatus performance should be monitored periodically, but specific requirements on performance verification of the apparatus are not given.

For comparison, Tables I and II detail the harmonized PDG and FDA mechanical calibration requirements for basket and paddle apparatus, respectively.

In 1997, the FIP Dissolution Working Group issued a guideline on the Dissolution Testing of Solid Oral Products (11). In the guideline, FIP states that dissolution apparatus qualification should include conformance to the geometrical and dimensional specifications and verification of operational parameters such as test medium, temperature and volume, and rotation speed during periods of use. FIP acknowledged that apparatus suitability testing is an important aspect of qualification, and while the USP calibrator tablets were acknowledged to be controversial at that time, the FIP still supported the use of these calibrators since they were the only standards available and had been helpful in identifying system and operator failures. In the same guidelines, FIP also proposed that, since some drug products might reveal similar or even higher sensitivity to apparatus variability than the USP calibrator tablets, "in-house" standards were considered to be an acceptable alternative to the USP calibrator tablet.

NEW FIP INSTRUMENT QUALIFICATION RECOMMENDATION

In the spirit of continuous improvement, the FIP SIG on Dissolution/Drug Release supports the more stringent mechanical calibration approach. In an effort to internationally harmonize dissolution apparatus suitability requirements, the need and type of performance verification tests should be determined by the individual laboratories based on the type of testing they are performing. Any strict requirement on the use of a specific performance verification test tablet is not recommended at this time.

Any product established as an "in-house" performance verification reference product should be well characterized, sensitive to critical parameters of the dissolution test such as different hydrodynamic conditions, and representative of the products currently being tested in that laboratory. For most marketed products, extensive dissolution studies are conducted during product development, method validation, and laboratory-to-laboratory method transfers prior to final approval of the product. Analysis of method development, transfer, and validation data, as well as registration stability data, can insure confidence in the characterization of the "in-house" performance verification product and facilitate the establishment of acceptance criteria. The recommended acceptance criteria should include mean value, standard

Table II. Mechanical Calibration Parameters: Dissolution Rotating Paddle Apparatus

Calibration parameter	PDG harmonized pharmacopeial specifications (USP, EP, JP)	FDA recommendations based on ASTM standard
Shaft wobble	Rotates smoothly without significant wobble	≤1.0 mm total runout
Shaft verticality	N/A	Bubble must be with-in the lines of bubble level (≤0.5° from vertical)
Vessel/shaft centering	≤2.0 mm from centerline	≤1.0 mm from centerline measured at an upper and lower position
Vessel verticality	N/A	≤1.0° from vertical
Height check/paddle depth	25±2 mm	25±2 mm
Rotational speed	±4% from target	±2 rpm from target

deviation, and stability. Gage repeatability and reproducibility studies can be useful to determine the mean and variability of a dosage form and for improving equipment variability (12). If different from the product(s) to be tested, a reference product should be sensitive to the same variables that affect the products tested in that laboratory.

The FIP recommends that the qualification of a dissolution test instrument should be performed following the calibration requirements as indicated in the FDA (draft) guidance. If additional system performance information is desired, performance verification test using USP reference standard tablet or an established in-house reference product can be conducted.

In the future, improvements in instrument technology, performance verification standards, and the ability to measure hydrodynamic variables may change this recommendation.

CONCLUSIONS

The dissolution test procedure is well established, reliable, and reproducible, and it is a valuable tool for characterizing a drug product at different stages in its lifecycle. A thorough understanding of all sources of variability within dissolution laboratory systems will minimize uncertainty when examining or acting on results. Qualification of the dissolution system should include verification of the dimensions and tolerances of the apparatus. Critical test parameters such as rotation speed, media temperature, and volume need to be monitored periodically during use. Overall system performance can be monitored by running a performance verification test by testing a well characterized dosage form, such as USP performance verification tablets or an in-house product, with sufficient knowledge of the mean, variability, and stability. As a standard practice, laboratory scientists are encouraged to critically evaluate dissolution data variability within and between laboratories to determine if the variability is product-related *vs* laboratory system-related.

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生薬ソヨウの成分含量測定法とペリルアルデヒドの安定性に関する検討

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Examination of Component Determination Method for Perilla Herb and Stability of Perillaldehyde

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(Received April 9, 2009)

A method for component determination for Perilla Herb was examined. A new method was applied to evaluate the quantity of perillaldehyde, which was thought to be effective to avoid distribution of low quality Perilla herb in the Japanese market. The stability of perillaldehyde in various solvent solutions, storage conditions and loss of perillaldehyde of Perilla herb during the drying process were also examined. In conclusion, we found that some imported Perilla herbs in the Japanese market contained a small amount of perillaldehyde and, on the other hand, a large amount of α-asarone.

Keyword: ***Perilla frutescens*, Pharmacopoeia, component determination test, perillaldehyde, asarone**

1. 緒　言

生薬ソヨウ（蘇葉）は，第十五改正日本薬局方 (JP15) においてシソ科（*Labiatae*）のシソ*Perilla frutescens* Britton var. *acuta* Kudo又はチリメンジソ*Perilla frutescens* Britton var. *crispa* Decaisneの葉および枝先と規定されている．[1] また漢方における配合処方としては香蘇散，柴朴湯，参蘇飲，神秘湯，半夏厚朴湯，茯苓飲合半夏厚朴湯などがある．

薬用に用いるシソは，葉が赤紫色で，シソの特有の香りであるペリルアルデヒド (perillaldehyde：以後PAと略す) 臭が強いものが良品とされているが，近年中国産のソヨウにペリルアルデヒド臭のしない粗悪なものが紛れ込んでいる．現在JP15ではその確認試験法に無水酢酸と硫酸を用いたテルペン類の呈色反応を採用しているが，本確認試験法のみではそのような粗悪な生薬の流入を防ぐことができないため，早急に新たな規定作りが求められている．

シソは精油の主成分によりいくつかに系統分類がされている．すなわちペリルアルデヒドを主精油成分とするperillaldehyde (PA) 型の他，perilla-ketone (PK) 型，elsholtziaketone (EK) 型, citral (C) 型, phenylpropanoid (PP)

型，perillene（PL）型がある．[2] またシソにはPAの他の生理活性物質としてロスマリン酸 (rosmarinic acid) を含む．ソヨウは精油成分を多く含む葉類生薬であり，それらの収穫後の加工調製には精油成分を失わないようにするために注意を要する．低い温度での乾燥は生薬の色調を悪くし，逆に高い温度では精油の損失を招く．そこで収穫後の乾燥条件がこれらの成分含量に与える影響を調べた．

さらに高速液体クロマトグラフィー法を用いた成分含量測定法の検討を行った．試験法の設定に際しPAの溶液中での安定性を調べた．

2. 材料および方法

2.1 検討用ソヨウ試料：独立行政法人医薬基盤研究所薬用植物資源研究センター（以後センターと略す）筑波研究部産の他，中国産７種類 (No.1-7)（山東省産１種，広東省産３種，河北省産１種，産地不明２種），国内産（栃木）３種類 (No.8-10) を用いた．No.1-7 の中国産はすべて市場流通品である。アサロンの単離には中国産（産地不明）(No.11) を用いた．GCMS 検討用試料においては京都大学大学院薬学研究科伊藤美千穂先生よりご供与された PA 型，PP 型ソヨウ試料を一部比較のため用いた．

2.2 試薬：PA は条件検討用にはアルドリッチ社製 (92 %) を用いたが，最終的なソヨウの成分含量測定においては和光純薬工業社製生薬試験用 *S*-(-)-perillaldehyde（試作品）を用いて行った．ロスマリン酸はアルドリッチ社製 (97 %) を用いた．アサロン(*E*-体) (α-asarone) はシグマ社製を用いた．TLC はメルク社製（5715）シリカゲルガラスプレートを用いた．

2.3 測定機器：HPLC はウォーターズ社製 HPLC システム（1525 バイナリポンプ，2487UV 検出器，717plus オートサンプラー），および島津製作所社製 LC-10ADvp HPLC システムを用いた．NMR は日本電子社製 JEOL alpha-500，UV は島津製作所社製 UV mini 1240 を用いた．GCMS は島津製作所社製 QP-5050 に SUPELCO 社製 SLB-5ms カラム（30 m x 0.25 mm x 0.2 μm film thickness）を接続して測定した．GCMS 測定温度条件は各項目に記載した．

2.4 TLC によるロスマリン酸の確認方法

本品の粉末 0.3 g をとり，メタノール 3 ml を加え，10 分間振り混ぜた後，遠心分離し，上澄液を試料溶液とした．別にロスマリン酸 3 mg をメタノール 5 ml に溶かし，標準溶液とした．試料溶液および標準溶液 10 μl ずつを TLC 上にスポットした．次に酢酸エチル／水／ギ酸／酢酸（100）混液（120 : 2 : 1 : 1）を展開溶媒として約 10 cm 展開した後，薄層板を風乾した．これに紫外線（主波長 365 nm）を照射して，標準溶液から得た青紫色の蛍光を発するロスマリン酸のスポットと色調及び *Rf* 値が等しいスポットを観察した．

2.5 HPLC による成分含量測定法の実験方法

本品から新たに調製した粉末約0.2 gを精密に量り，共栓遠心沈殿管に入れ，メタノール20 mlを加えて10分間振り混ぜた後，遠心分離し，上澄液を分取した．残留物にさらにメタノール20 mlを加え，同様に操作した．全抽出液を合わせ，メタノールに溶かして正確に50 mlとし，試料溶液とした．別にPA標準品約10 mgを精密に量り，メタノールに溶かして正確に100 mlとし，標準溶液とした．試料溶液および標準溶液10 μlずつを正確に取り，次の条件で液体クロマトグラフィーにより試験を行い，それぞれの液のPAのピーク面積*At*および*As*を測定した．

PAの量（mg）＝*Ws* x (*At*/*As*) x (1/2)

Ws：成分含量測定用PAの秤取値（mg）

HPLC 試験条件

検出器：ＵＶ検出器 230 nm

カラム：内径4.6 mm，長さ15 cmのステンレス管に5 μmの液体クロマトグラフィー用オクタデシル化シリカゲルを充填する．

カラム温度：30℃付近の一定温度

移動相：アセトニトリル／水混液（35 : 65）

流速：1.0 ml/min

本検討においては，TSK-gel ODS 80TM (4.6 mm i.d. x 150 mm)（東ソー株式会社製）を 用いた．

また，PA の検量線は，36.8 μg/ml (40μg/ml の 92%純度換算) とその 5 倍希釈（7.36 μg/ml），25 倍希釈（1.472 μg/ml）を用いて２回引いたところ，いずれも高い直線性を示した（$R^2 \geqq 0.99$）．

また，ソヨウ試料中に混在するα-アサロン（α-asarone: 以後 AS と略す）に関しては，別途以下のように標準試料を調製し，検量線作成後に定量を行った．

AS 20 mg を精密に秤量し，メタノール 100 ml に溶解し，それを原液として希釈し検量線を作成した．

2.5.1 抽出溶媒の検討

粉砕直後のソヨウ（中国産）粉末 0.2 g を **2.5** の方法に準じアセトニトリル，およびメタノールにて抽出し，上記 HPLC 条件でソヨウ中の PA 含量を測定した．結果はメタノール抽出では 0.52 mg/200mg dry wt.，アセトニトリル抽出では 0.44 mg/200 mg dry wt.であった．

2.5.2 ソヨウ粉砕時の PA の損失率の検討

後述の AS をほとんど含まないソヨウ（中国山東省産）を試料として用い，粉砕直後にメタノール抽出（１時間以内に操作）を行った抽出液と，粉砕後２日間デシケーター中（塩化カルシウム存在下）で放置した試料の PA 含量を測定した．

2.6 乾燥温度の検討

センターにて栽培し収穫したソヨウを収穫後 30，50，70℃でそれぞれ 4，8，24，48 時間乾燥した 12 サンプルについて上記条件での TLC，HPLC による分析を行った．それぞれのサンプルについて乾燥減量を求め PA 含量値の補正を行った．乾燥減量値は局方に従い，分析後の残りの試料について 105℃，6 時間乾燥後にシリカゲルを入れたデシケーター中に放冷し，精密に秤量して求めた．

2.7 PA の安定性に関する検討

2.7.1 UV による検討

PA をメタノール，アセトニトリルにそれぞれ約 1 mg/100ml の濃度となるように溶解し，室温における UV 吸収の経時変化を調べた．タイムスキャンプログラムにより，10 分おきに 230 nm での吸光度を測定し，最終的に 6550 分まで測定した．

メタノール溶液　(濃度 0.91 mg/100ml)　0 min/A 0.842, 6550 min/A 0.812　減少率 3.6 %.

アセトニトリル溶液　(濃度 1.0 mg/100ml)　0 min/A 0.900, 6550 min/A 0.884　減少率 1.8 %.

2.7.2 NMR による検討

重水素化メタノールに PA 40mg を溶解し，封管後に一定時間毎に ^{1}H-および ^{13}C-NMR を測定した．

^{13}C-NMR (0 hr) δ: 20.9, 22.5, 27.5, 32.8, 42.0, 110.0 (exomethylene), 142.3 (olefinic), 149.8 (olefinic), 152.8 (olefinic), 195.7 (aldehyde). ^{13}C-NMR (96 hr) δ: 21.0, 24.5, 28.4, 31.3, 42.5, 107.6, 109.3, 126.8, 135.5, 150.7 (新たに出現したピークのみ)

^{1}H-NMR (0 hr) δ: 1.75 (3H, s), 4.74 (1H,s), 4.77 (1H,s), 6.93 (1H, t, *J*=2.9Hz), 9.39 (1H, s).

^{1}H-NMR (96 Hr) δ: 1.72 (s), 4.49(s), 4.70 (s), 4.71 (s), 5.84 (br s) （新たに出現したピークのみ).

2.7.3 GCMS による検討

PA 標品 20 mg をメタノール 100 ml に溶解後，サンプル管に密封して室温放置．6 日後と溶解直後に GCMS を以下の条件で測定した．

気化室温度 230℃，インターフェース温度 230℃，昇温プログラム：90℃（15 分），90℃→120℃（15 分），120℃（30 分），120→150℃（15 分），150℃（35 分）で行った．

溶解直後における PA 保持時間： 18.9　min, 6 日後における PA 保持時間：18.6　min, 変化体ピークの保持時間：27.4 min.　EI-MS *m/z*:　PA 150 $[M]^{+}$, 135 $[M\text{-}CH_3]^{+}$, 122 $[M\text{-}CO]^{+}$, 107, 91, 67, 53. 変化体 196 $[M]^{+}$, 181 $[M\text{-}CH_3]^{+}$, 165 $[M\text{-}OCH_3]^{+}$, 149, 133, 121$[M\text{-}(CH_3O)_2\text{-}CH]^{+}$, 105, 91, 75.

2.7.4 HPLC による検討

PA を，メタノール，90%メタノール，アセトニトリルにそれぞれ溶解し，スクリュー管に密封して 5℃，室温にて２ヶ月間保存した．**2.5** の方法に従い HPLC 分析を行い，各々の溶液中の１か月ごとの PA の面積を用事調製を行ったアセトニトリル中の PA の面積との面積比でグラフを作成した．

またソヨウメタノール抽出液の PA の安定性を調べるために，ソヨウ(中国産 No.1 を粉砕後デシケーター中で数日間保存したもの)抽出液をスクリュー管に分け，保存温度を 5℃，室温の２種類の条件で３ヶ月間保存し，各月でのソヨウ中の PA の含量 (mg) を測定した．

2.8 ソヨウ中の AS の確認

2.8.1 中国産ソヨウから AS の単離

中国産市場流通品（2004 年 12 月入手，18.2 g）を粉砕後にメタノール 120 ml を加えて室温下で 10 分間振り混ぜ，遠心分離後に得られた上澄み液を Mega BondElut C18 (Varian) にチャージし，アセトニトリル／水混液（1：1）で 50 ml 溶出後に 100%アセトニトリルで溶出した．100%アセトニトリル分画を HPLC（カラム：東ソーTSK-gel ODS-80TM 21.5 mm x 300 mm，移動相：アセトニトリル／水混液 35:65, 流速：8.0 ml/min）にて精製し，化合物 **1** を得た．**1 (α-asarone)** ^{1}H-NMR ($CDCl_3$) δ: 1.86 (3H, dd, *J*=6.3, 1.5 Hz), 3.80, 3.84, 3.86 (3H, s), 6.08 (1H, dq, *J*=16.1, 6.3 Hz), 6.47 (1H, s), 6.63 (1H, dd, *J*=16.1, 1.5 Hz), 6.92 (1H,s).

その ^{1}H-NMR スペクトルは AS 標品のスペクトルと完全に一致した．

2.8.2 GCMS による AS の確認

2.8.1 における中国産ソヨウについて，AS 標品と以下の条件にて GCMS で保持時間と質量スペクトルの比較を行

った.

気化室温度 220℃, インターフェース温度 230℃, 昇温プログラム：90℃（5分), 90℃→200℃（55分), 200℃（40分）で行った.

標品における AS の保持時間：37.1 分, 中国産ソヨウのメタノール抽出液における AS の保持時間：37.0 分.

EI-MS *m/z* AS: 208 $[M]^+$, 193 $[M\text{-}CH_3]^+$, 165, 150, 137, 119, 105, 91, 69, 65, 53.

その結果ソヨウのメタノール抽出液において, 保持時間37分付近に現れるピークにおいてAS標品の保持時間と質量スペクトルが完全に一致した.

3. 結　果

3.1 PA の安定性に関する検討

PA は精油成分であるため, 定量分析において精密に秤量することが困難であるが, さらなる問題としてはその安定性が挙げられる. PA の反応性については, 光反応に関しての報告例があるが[3], 溶液中における安定性についてはほとんど報告がない. PA はその分子内にアルデヒドを有するためにアルコール存在下において容易にジメチルアセタールへと変化してしまう可能性がある. アセタールに変化するとUVにおける極大吸収が変化してしまうため, 正確な定量ができなくなる. そこで本化合物の成分特性を確認する上で PA の安定性を検討した.

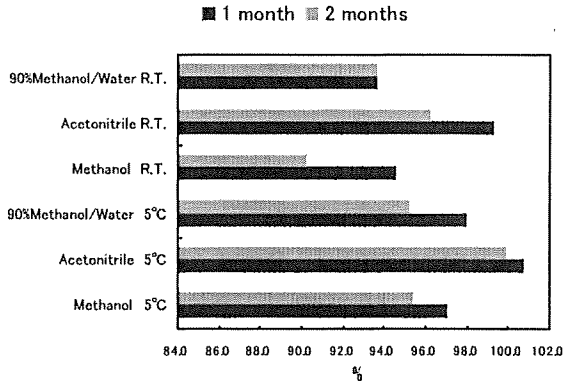

Fig.1 Comparing of PA stability in methanol, 90%methanol and acetonitrile solutions. Relative area ratio (%) of PA peak toward freshly prepared acetonitle solution on HPLC.(R.T.: room temperature)

メタノール, アセトニトリル, 90%メタノールのそれぞれの溶液中での長期間（2ヶ月間）での安定性を検討した結果, メタノール中で最も PA の減少率が大きく, アセトニトリル中では比較的安定であることがわかった. また室温中では減少率が大きくなった **(Fig.1)**.

また, 短期間のメタノール中とアセトニトリル中での安定性を確認するため, それぞれの溶媒中での UV における吸光度の経時変化を検討した結果, 4 日半（6550 分）においてのメタノール中での減少率は約 3.6%であったのに対し, アセトニトリル中では約 1.8%であった **(Fig. 2,3)**.

また, GCMS で確認したところ, 溶解して時間が経つと保持時間27.4分に分子量196の新たなピークが現れることが確認された**(Fig.4)**.

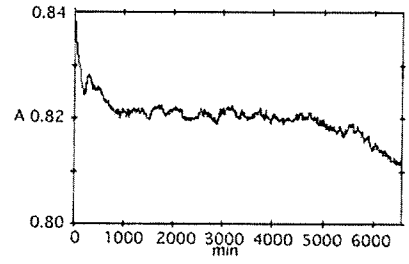

Fig. 2 Time course of PA decreasing in methanol solution (UV 230 nm).

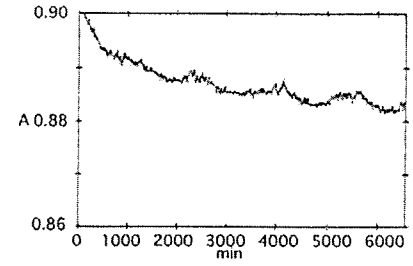

Fig. 3 Time course of PA decreasing in acetonitrile solution (UV230 nm).

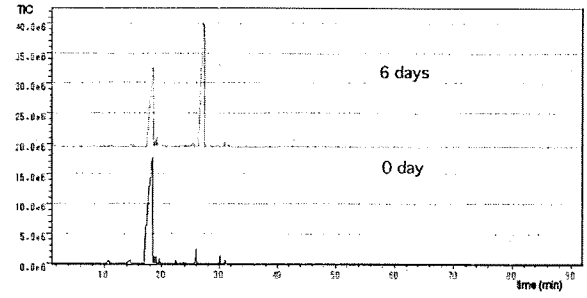

Fig. 4 Gas chromatography of freshly prepared (upper) and after 6 days (lower) PA solution in methanol.

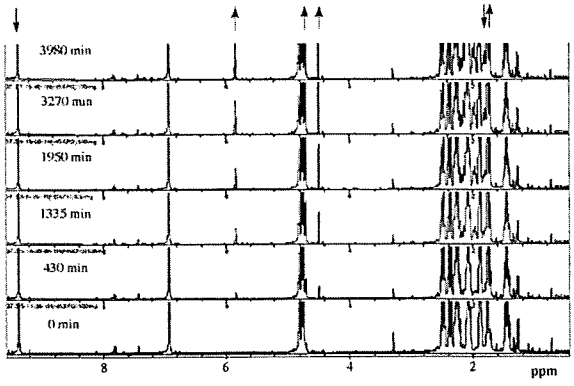

Fig. 5 Time course of PA conversion in deuterated methanol solution. (measured on NMR spectrum) (in CD_3OD). Arrows indicate enhanced signals (↑) and reduced signals (↓).

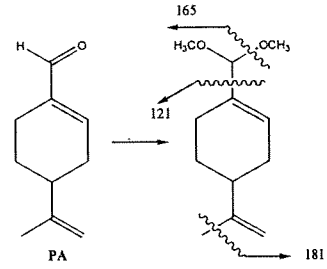

Fig. 6 EI-MS fragmentation pattern of converted compound of PA in methanol.

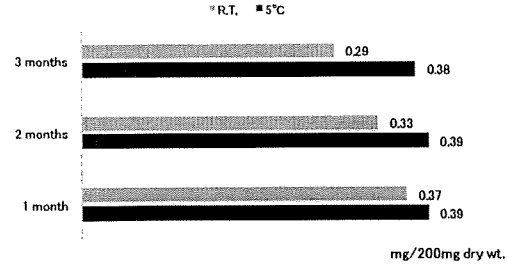

Fig. 7 PA content (mg) in methanol extract of Perilla herb in various storage conditions (data for 3 months).(R.T.: room temperature)

重水素化メタノールに溶解し，一定時間ごとにNMRを測定したところ，**Fig. 5**に示すようにシグナルの経時的な変化が見られた．^1Hおよび^{13}C-NMRにおいて溶解直後と96時間後のチャートを比較した結果，9.38 ppmのアルデヒド基由来のシグナル，共役オレフィン由来のδ_H 6.93 ppm (δ_C 95.7) のシグナルが減少し，新たに5.84, 4.49, 4.70, 4.49, 1.72 ppmにシグナルが出現した．各種二次元NMRで確認したところ，ジメチルアセタール化により共役オレフィンが孤立オレフィンとなりδ_H 5.84 ppm (δ_C 126.76) へ高磁場シフトし，新たにアセタールのカルビニル由来のシグナルがδ_H 4.49 ppm (δ_C 107.6) に現れたことを確認した．ただし重水素化メタノール中での反応であるため新たに出現したと思われる２つのメトキシル基の存在はNMR上では確認できないが，メタノール溶液中のGCMSで分子量196を示し，そのMSでの分裂様式からもジメチルアセタール体の構造を確認した (**Fig. 6**)．本化合物はメタノール中酸触媒存在下で工業的に製造できることが報告されている．[4)]

また，ソヨウのメタノール抽出液の安定性を調べるために，抽出液を5℃，室温の2種類の条件（いずれも遮光条件）で３ヶ月間保存し検討した．その結果，いずれもPAは5℃では安定であり，室温においては減少が著しかった (**Fig. 7**)．

これらのことは，PAはアルコール中でのアルデヒド基に対するアセタール化のみが変換の主要因であり，アプロティックな溶媒や低温中では比較的安定であることが示された．

3.2 HPLCによる成分含量測定の方法について

ソヨウにおけるPAの成分含量測定法の設定を行うにあたり，ソヨウの抽出溶媒の検討を行った．逆相系カラムを用いたHPLC法を適用することを考え，移動相に主に用いられるアセトニトリルとメタノールの２種類について検討した結果，メタノール抽出でのPA含量はアセトニトリル抽出時の約２割程度抽出効率が良いことがわかった．よって抽出溶媒はメタノールで行うのが良いと判断した．逆相系カラムを用いたHPLC分析における移動相は，アセトニトリル／水混液が最も良い分離を示した．後述のように一部の中国産試料での近傍ピークの重なりは，移動相の溶媒混合比率をアセトニトリル／水混液 (35:65) にしたところ，二つのピークは完全に分離した．また標準溶液の調整において，**3.1**で検討したように，PAはメタノール中では速やかに分解してしまうため標準溶液の調整は用時に行うのが望ましいと考えられた．またPAは精油成分であり，葉の裏側の腺鱗中に含まれているため，粉砕して粉末にすると揮発してしまい損失が大きくなるため，粉砕後速やかに溶媒で抽出する必要がある．粉砕後二日間デシケーター中で放置した場合，粉砕直後（１時間以内に操作）にメタノール抽出をした場合と比較し，損失は約半分

(57%) に及んだ. また抽出液中であっても前述のように, メタノールにより徐々に PA のアセタールへの変換は起こるため, 抽出後に速やかに分析を行うことも必要である.

3.3 一部の中国産ソヨウの PA 含量と AS の含有について

逆相系カラムを用いた HPLC 分析における移動相は, アセトニトリル／水混液が最も良い分離を示したが, 当初溶媒混合比率 (2:3) にて市場品の含量を調べたところ, 一部の中国産の PA 臭が全くしない試料において, PA が高含量を示すという矛盾した結果を与えるに至った. そこで溶媒混合比率を(35:65) にしたところ PA のピーク付近にもう一つのピークが現れ, フォトダイオードアレイ (PDA) を用いた分析を行ったところ, それは PA とは全く異なる紫外線吸収スペクトルを示すことが分かった**(Fig. 8)**. 本ピークは GCMS および, 単離後の NMR 解析により, AS (α-asarone; (*E*)-asarone)であると決定した. [5] 国内産の phenylpropanoid を多く含有するタイプの PP 型ソヨウ試料と GCMS パターンを比較したところ, 全く異なる精油パターンを与えた**(Fig. 9)**.

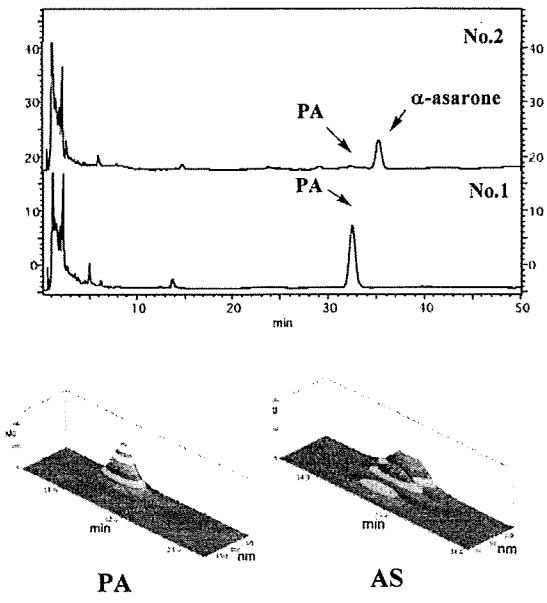

Fig. 8 HPLC chart of methanol extract of Perilla herb (No.1 and 2) and PDA detection of PA and AS.

3.4 ソヨウの収穫後の乾燥条件について

センターにて栽培し収穫したソヨウを収穫後の乾燥温度条件における PA の損失度合を検討するため, 収穫後 30, 50, 70℃でそれぞれ 4, 8, 24, 48 時間乾燥した 12 サンプルについて TLC, HPLC による分析を行った. HPLC による PA 含量については, **Fig. 10** に示すように 70 ℃乾燥ではほとんど失われてしまう. 30℃, 50℃条件においても 2 日間の乾燥では損失が大きいことがわかった. また TLC の結果では, ソヨウ中の別の生理活性物質であるロスマリン酸は 50 ℃での乾燥試料では 4 時間の乾燥条件を除きいずれにも明確に確認できるが, 高温の 70℃での乾燥条件では全く確認されない. また 30℃でも 2 日間乾燥を行った試料以外は確認できなかった**(Fig. 11)**. このことは酵素が活発に働く温度条件とも関係あると思われた. また高温条件 (70℃) では分解されてしまい含量が減るとも考えられる. 一般に精油成分を多く含む薬物生薬をこのような高温で乾燥させることは考えにくいが, これらの結果から乾燥条件には注意が必要であることがわかった.

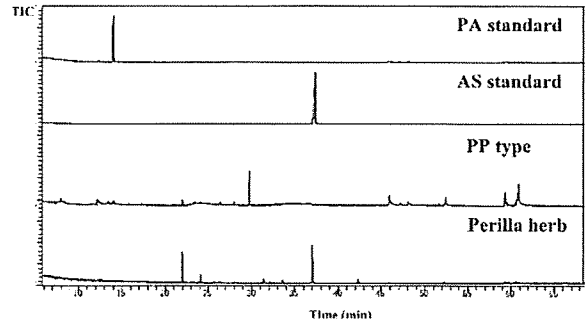

Fig. 9 Gas chromatography chart of Perilla herb (PP type, No.11) extracts, PA and AS.

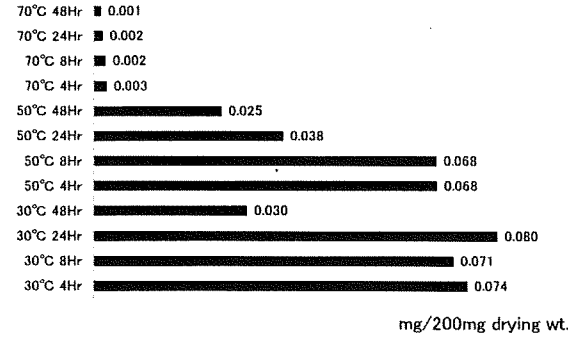

Fig. 10 PA content of Perilla herb (collected in Ibaraki Prefecture) extracts in various drying conditions.

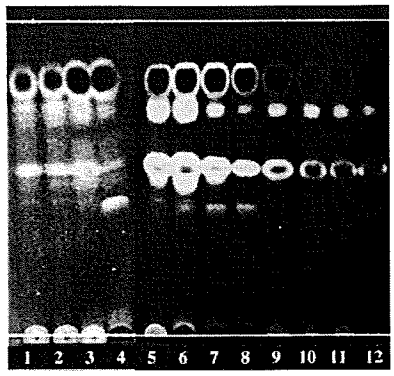

Fig. 11 TLC chromatogram of Perilla herb extract in various drying conditions. Developing solvent system: Ethylacetate/water/formic acid/acetic acid (100) (120:2:1:1), detect: UV 365 nm Sample drying conditions (temperature, times) are as follows: No.1: 30°C, 4 hrs., No.2: 30°C, 8 hrs., No.3: 30°C , 24 hrs., No.4: 30°C , 48 hrs., No.5: 50°C, 4 hrs., No.6: 50°C, 8 hrs., No.7: 50°C, 24 hrs., No.8: 50°C, 48 hrs., No.9: 70°C, 4 hrs., No.10: 70°C, 8 hrs., No.11: 70°C, 24 hrs., No.12: 70°C, 48 hrs.

最終的にこの成分含量測定の方法によりソヨウ（中国産市場品 7 種類，日本産 3 種類）について PA 含量の検討を行った結果，**Fig. 12** に示すような結果となった．中国産においては PA をほとんど含まないものが多く，そのようなものは AS を代わりに多く含有することが分かった．逆に日本産のものでは AS を含むものがなく，いずれも PA を含む．

4. 考察および結論

今回のソヨウの成分含量測定法の検定において，HPLC 法による PA 含量の測定法を検討し，**2.5** のように提案した．しかしその検討過程において，精油成分である PA は，メタノール中では不安定であることが分かった．また，その後の検討で，一部の中国産ソヨウに PA 臭の全くしない試料が HPLC 分析により高い PA 含量を示したことから，PA 保持時間上に重なりがあると予想され，その後 PDA, GCMS, NMR による分析によりα-アサロン(AS)であると決定した．AS は phenylpropanoid 系化合物であるが，国産のいわゆる PP 型のソヨウには認められず，文献上にも一部の *Perilla* 種（種名不明）に検出されたとの報告が有るのみであり，[6] ソヨウからの単離報告はこれが始めてである．また，今回検出したアサロンは(*E*)-体であるが，(*Z*)-体には変異原性が報告されており，米国においては食品添加物としては FDA が禁止している化合物である．市場に流通する多くの中国産に含まれていたことは本報が初めての報告である． 以上の結果，成分含量測定法についてソヨウ特有の香気成分である PA の含量を規定した．ソヨウは粉砕後に速やかに揮発してしまうため，試験方法には「本品から新たに調製した粉末～」と記載することとした．

これらの方法により，近年市場に流通している粗悪な中国産ソヨウの国内流通を防ぐことができると考えられた．

また，本論文では詳細は割愛するが，システム適合性（分離度，再現性）についてはいずれも成分含量測定法に適する結果を与えた（分離度の設定に AS と PA を用いた場合の両者の分離度は，3 種類のカラムを用い，いずれも 1.9 以上，PA のピーク面積での相対標準偏差は 1.5%以下）．し

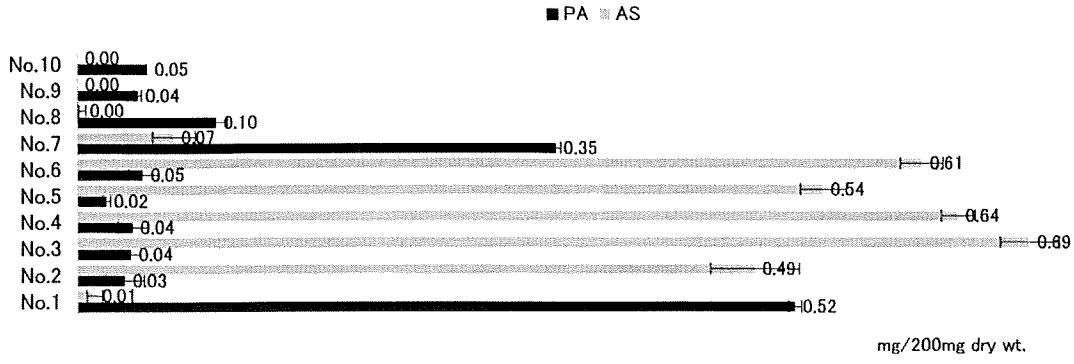

Fig. 12 PA and AS content (mg) of Perilla herb distributed in Japanese market. (No.1-7; imported (China), No.8-10; domestic). Each value is the mean ± SE (n=3)