Sample Preparation for Headspace GC Analysis of Residual Solvents in Hyaluronic Acid Derivative Fiber

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Hyaluronic acid (HA) is a linear polysaccharide built from repeating disaccharides of alternating D-glucuronic acid and N-acetyl-D-glucosamine.[1] HA can be isolated from biological sources such as vitreous humor, human umbilical cord and rooster and hen comb.[2] HA has biocompatibility and its average molecular weight is usually several million.[3] HA is very viscous in aqueous solution because of its molecular conformation.[4] Due to its viscoelastic properties, HA is used in the medical applications as ophthalmology, dermatology, osteoarthritis, urology and wound healing.[5]

HA derivative has an improved rheological property without losing natural biocompatibility of HA. Because HA derivative is water-insoluble or slowly dispersible in human body, it lasts much longer than HA in human body.[6] Therefore, HA derivatives have been widely developed as post-operative adhesion-preventing films or gels,[7] materials for wrinkle treatment,[8] materials for soft tissue augmentation,[9] materials for the arthritis treatment,[10] vehicles for drug delivery,[11,12] etc. In this study, a HA derivative fiber is obtained from amide reaction of chitosan and HA using *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) hydrochloride and *N*-hydroxysuccinimide (NHS).[13] HA derivative fiber is turned into HA derivative gel in water.

Headspace gas chromatography (HS-GC) method has been used for the determination of residual solvents in pharmaceutical compounds.[14-24] Direct injection of analytes evaporated through equilibration between liquid (or solid) phase and gas phase to GC system minimized the contamination of GC system and the deterioration of GC column.[25] In addition, the automation of equilibrium and injection procedure reduced analysis time and improved reproducibility in injection procedure.

In this study, HA derivative fiber was hydrolyzed by hyaluronidase (HAse) in buffer solution for the minimization of variation in the viscosity of HA derivative fiber in water resulting in the higher extraction of residual solvents from the gel matrix. HAse is an enzyme that randomly hydrolyzes the linkage between the *N*-acetyl-D-glucosamine and the D-glucuronate residues in HA.[26] The hydrolysis rate is dependent on pH and concentration of HAse. In this study viscosimetric assay was applied because it was a simple and rapid method for the measurement of activity of HAse.

The aim of this study is to develop efficient sample preparation method for HS-GC analysis of residual solvents in HA derivative fiber. Compared to direct extraction of residual solvents from HA derivative fiber, the extraction through the hydrolysis of HA derivative fiber by HAse gave more complete and higher reproducible quantification of residual solvent. To validate HS-GC analysis method of residual solvents, specificity, limits of detection and quantification, linearity, accuracy and precision are investigated in the study.

Experimental Section

Sample and standards. Ethanol was obtained from Merck (Merck 64271, Darmstadt, Germany). Isopropanol, acetone and 2-methyl-2-propanol (*t*-butanol) were obtained from Aldrich (Sigma-Aldrich 14508, St. Louis, MO, USA). All solvent were of ≥ 99.5% purity. Water was purified by MilliQ-plus (Millipore, Bedford, MA, USA). Sodium phosphate monobasic anhydrous and sodium hydroxide were purchased from Aldrich and hydrochloric acid was obtained from Wako (Wako pure chemical Industries, Ltd. Osaka, Japan) to prepare buffer solutions. HAse used to cleave HA derivative was obtained from Sigma.

Headspace gas chromatography. The analyses were performed on an Agilent 6890 gas chromatography (Agilent, Palo Alto, CA, USA) equipped with an Agilent 7694 headspace sampler and a flame-ionization detector. The injector temperature was 140 °C and detector temperature was 240 °C. Column was HP-1 (100% dimethyl polysiloxane, 30 m × 0.53 mm ID, 3.0 μm d.f. Capillary). Split ratio of injection was 1 : 10. Oven temperature was maintained at 40 °C for 15 min, then raised at a rate of 20 °C/min to 240 °C, maintained for 5 min. Total run time was 30 min. Helium gas was used as a carrier gas at a constant flow rate of 1.5 mL/min.

Headspace oven temperature was 90 °C. The temperatures of the sample valve and transfer line were 110 °C and 130 °C, respectively. GC cycle time was 38 min. The times of equilibrium and pressure in the headspace vial were 20 and 0.2 min, respectively. Both times of sample loop fill and loop equilibrium were 0.05 min. Sample injection time was

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1.0 min. The sample unit was equipped with a 1.0 mL of injection loop. The headspace sampler units, a sample loop, needle assembly and transfer line were made of fused silica-mined stainless steel to avoid a common problem such as carryover from the previous injection.

Procedure. Standard stock solutions of residual solvents were prepared by diluting standard of residual solvents in water. Standard solutions of residual solvents were prepared by diluting standard stock solutions of residual solvents using HAse solution. Internal standard solution was prepared by diluting t-butanol in water. To prepare HAse solution, HAse was dissolved to the concentration of 0.5 mg/mL in 0.2 M phosphate buffer solution of pH 5.0. Sample solution without residual solvents for accuracy study was prepared by swelling HA derivative fiber in HAse solution by 2.0 mg/mL followed by incubating it in water bath at 37 ± 0.1 °C for 2 hours.

Real sample solution was prepared: the real sample of HA derivative fiber in 20 mL of headspace vial was swelled by 9.0 mL of HAse solution to 2.0 mg/mL (w/v) and then 1.0 mL of internal standard solution was added. The resulting real sample was incubated in water bath at 37 ± 0.1 °C for 2 hours.

Results and Discussion

Method validation. The method validation was done by evaluating specificity, limit of detection and quantitation, linearity, accuracy, repeatability, average content and method precision of residual solvents as was indicated in the International Conference on Harmonization (ICH) guideline Q2B 'Validation of Analytical Procedures: Methodology'.[27]

For the specificity, ethanol, acetone and isopropanol were used as residual solvents in HA derivative fiber while *t*-butanol was used as an internal standard. The baseline separation of residual solvents and internal standard was done by the HS-GC conditions (results not shown).

The limits of detection (LODs) of residual solvents in HA derivative fiber were determined based on signal-to-noise ratio of 3 : 1 : ethanol 0.02% (w/w), acetone 0.005% and isopropanol 0.01%.

Table 1 shows limits of quantitations (LOQs), linearity and accuracy. LOQs of residual solvents were determined based on signal-to-noise ratio 10 : 1. The linearity was determined at seven levels in the range between LOQ and 8.0% (w/w). Three replicates were performed at each level. The calibration curves were obtained with the average of peak area ratios of three replicates. All of the correlation coefficients (R^2) were higher than 0.9999 and the calibration curves were linear within the range. The accuracy was evaluated by the recoveries of residual solvents spiked in sample solution without residual solvents. The recovery results of solutions spiked at concentration level of 2.0, 4.0 and 6.0% (w/w) are also summarized in Table 1: the recoveries of residual solvents were ranged between 95.1 and 102.8%.

Repeatability was evaluated using 4.0% of sample spiked with residual solvents as shown in Table 2. The relative standard deviations (RSDs) were: 1.7% for ethanol, 0.4% for acetone, and 1.5% for isopropanol which were below the USP mandated limit of 15% RSD for organic volatile impurities analysis.[28] Method precisions were also evaluated by RSD values of the residual solvent in a real sample (Table 2). The estimated content values of isopropanol and acetone were below LODs while the contents of ethanol were higher. It was supposed that the contents of isopropanol and acetone were lower because they were used in the middle of reaction of HA with chitosan and EDC/NHS while ethanol was used to wash HA derivative fiber in a final step of processes. From comparison with method I, method II gave the higher

Table 1. Linearity and accuracy of residual solvents

Solvent	Linearity				Accuracy (n = 18)		
	Range (%)[a]	R^2	Slope	Intercept	Recovery (%)	Average	RSD (%)
Ethanol	0.08-8.0	0.99996	174.56	1.2471	95.1-101.1	98.7	2.0
Acetone	0.02-8.0	0.999995	61.22	0.0041	97.8-102.6	99.5	1.2
Isopropanol	0.04-8.0	0.999991	93.27	0.3741	97.4-102.8	100.1	1.3

[a]The range of linearity was LOQ –8.0% based on a sample prepared at a concentration of 2 mg/mL.

Table 2. Repeatability in spiked sample and average content and method precision of residual solvents in real sample

Solvents	Repeatability (n = 10)	Average content and method precision (w/w %, n = 6)						
		Method I[a]		Method II[b]				
		0 day		0 day		7 day later		Intermediate precision RSD (%)
	RSD (%)	Average Content (%)	RSD (%)	Average Content (%)	RSD (%)	Average Content (%)	RSD (%)	
Ethanol	1.7	1.4	23.4	2.2	7.7	2.1	5.3	3.7
Acetone	0.4	< 0.005	–	< 0.005	–	< 0.005	–	–
Isopropanol	1.5	< 0.01	–	< 0.01	–	< 0.01	–	–

[a]Method precision without cleavage of real sample. [b]Method precision with cleavage of real sample.

Table 3. The residual solvent contents in real samples (w/w %, n = 2)

Sample	I	II	III	IV	V	VI
Ethanol	2.8 ± 0.06	3.2 ± 0.01	2.9 ± 0.03	2.3 ± 0.01	2.7 ± 0.01	2.4 ± 0.08
Acetone	< 0.005[a]	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Isopropanol	< 0.01[b]	< 0.01	< 0.01	< 0.04[c]	< 0.04	< 0.04

[a b]below limit of detection. [c]below limit of quantitation.

content of ethanol and the much lower RSD. It was supposed that hydrolysis of HA derivative gel by HAse lessened the viscoelastic property of HA derivative gel resulting in improved extraction of residual solvents from the gel matrix. Because HA derivative gel produced through mixing of HA derivative fiber with water in headspace vial had the diversity in the morphology and viscosity, the partition coefficients of residual solvents between the gel matrix and water were also diverse. Therefore, hydrolysis of HA derivative fiber by HAse was an efficient method for minimization of deviation in partition coefficients. The intermediate precision of 3.7% obtained from RSD values of 0 and 7 days in method II indicated that this method had good repeatability.

Real sample analysis. The residual solvents in six real samples were determined using the optimized method as shown in Table 3. The contents of ethanol were ranged from 2.3 and 3.2%. The contents of isopropanol and acetone were below LOD or LOQ as described above.

Conclusions

HA derivative fiber was hydrolyzed using HAse for headspace gas chromatographic analysis of residual solvents of ethanol, acetone and isopropanol in HA derivative fiber. This study showed that the developed method had specificity, linearity, accuracy and precision. In addition, it demonstrated that HS-GC coupled with matrix-breaking method such as hydrolysis was available for the determination of residual solvents in a matrix like HA derivative fiber.

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