

Determination of Methanol Increment in Mobile Phase Consisting of Methanol and Water by On-line UV Spectrometry in Reversed Phase Liquid Chromatography

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An on-line UV spectrometric method for the quantitative determination of methanol increment of methanol-water in the mobile phase (*i. e.*, of greater concentration than that of the mobile phase) by frontal analysis (FA) of insulin in reversed phase liquid chromatography (RPLC) was presented. When the methanol increment concentration ranged from 0.05% to 0.50%, $V(\text{CH}_3\text{OH})/V(\text{H}_2\text{O})$, a set of elution curves could be obtained at 198 nm by a diode-array detector in the presence of 47% methanol, $V(\text{CH}_3\text{OH})/V(\text{H}_2\text{O})$ containing 0.03% hydrochloric acid, $V(\text{CH}_3\text{OH}-\text{H}_2\text{O})/V(\text{HCl})$ in the mobile phase. The plateau height of the elution curves of the methanol increment was found to be proportional to the methanol increment in the mobile phase. The methanol increment could be determined on a quantitative basis. When the method was used to investigate the elution curve of insulin by FA in RPLC, a small plateau, being the methanol increment, was detected before the usual insulin plateau of each elution curve. In this case the methanol increment was found to vary with insulin concentration in the mobile phase. When that concentration was between 0.025 mg/mL and 0.30 mg/mL, the methanol increment could be determined in the range from 0.03% to 0.19% with a deviation of $\pm 0.02\%$ and a relative deviation of $\pm 10\%$. A nuclear magnetic resonance spectrometer (NMR) was also employed to confirm the obtained result. A methodology with a very rigorous experimental procedure for obtaining results of such accuracy is also included. The presented result may be used to prove that a displacement process definitely occurs as insulin is adsorbed by the RPLC stationary phase inFA.

Keywords reversed-phase liquid chromatography, retention mechanism, methanol increment, insulin

Introduction

Frontal chromatography (FC), an old branch in liquid chromatography (LC),¹ has been successfully employed in the solution of many theoretical as well as applied problems—such as measuring binding constant between components² and kinetic parameters of chemical reactions,³ adsorption behavior of components on various surfaces,⁴⁻⁶ binding constant between component and solid surface,⁷ adsorption isotherms,⁸⁻¹⁰ *etc.* Reversed-phase liquid chromatography (RPLC) has been proven to be the most popular method accounting for 75% in the total publications in LC. However, many theoretical problems in RPLC have not been fully resolved to this date. In a special volume of *Journal of Chromatography*, one of these challenging problems is stated as “Does sample retention cause displacement of organic solvent from the stationary phase?”¹¹

The stoichiometric displacement model for retention (SDM-R) in LC claims that a stoichiometric number of organic solvent molecules are necessarily released, or displaced at the contact region between solute and the stationary phase.¹² A fraction of the displaced solvent is solely from the stationary phase. If the displaced solvent could be quantitatively and directly determined, it could be used, not only to prove the SDM-R, but also to provide directly a definite answer to the above difficult question.

Many methods can be used for the quantitative determination of trace methanol in a solution. However,

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Received January 21, 2001, revised August 30, 2001; accepted September 10, 2001.

Project supported by the National Natural Science Foundation of China (Nos. 39880003 and 20175016).

they could not be employed to determine the "methanol increment", or extra methanol (*i. e.*, of greater concentration than that in the mobile phase), in the presence of methanol in high concentration. Thus the primary requirements are for a quantitative method with very high sensitivity which is suitable for the determination of the displaced organic solvent from the RPLC stationary phase. We are not aware of such a method having been presented in the literature yet. The aim of this study is to present a new quantitative method that satisfies the foregoing requirement. From the standpoint of experimental methodology, if the usual LC is used, the displaced solvent would display a pulse signal together with the usual solvent peak. It is impossible to distinguish the two from each other. Thus, frontal analysis (FA) should be used in this study. According to the SDM-R, the amount of the released organic solvent molecules is much greater from biopolymers than that from small solutes.^{12,13} Insulin of very high purity was selected as the solute and was pumped continuously to the RPLC column until the solute exited from the column, in which case the column would be saturated by insulin. Methanol and water, being the simplest and the most useful mobile phase in RPLC, were selected as the mobile phase.

Experimental

Equipment and chemicals

A Hewlett Packard 1090 liquid chromatograph (CA, USA) consisting of three pumps with a diode-array detector was used for the FC analysis with on-line UV spectrometry. A color Pro plotter from the same manufacturer was used for plotting the merged elution curves. A SynChrompak RP-P (100 × 4.6 mm, particle size 5.6 μm, pore diameter 30 nm) was purchased from SynChrom Inc (West Lafayette, IN, USA). RPLC column temperature was controlled at 25 ± 0.5°C with a water bath. A Gemini 200 nuclear magnetic resonance spectrometer (NMR) from Varian Co. (Palo Alto, CA, USA) and a NMR tube (Kontes, diameter 5 mm, grade 6, 528 pp) from Scientific Glassware/Instruments were used for NMR analysis.

Insulin (bovine pancreas, HPLC) was obtained from Sigma Co. Methanol was bought from EM Science (Gibbstone, NJ, USA). Hydrochloric acid (Ultrex, Ultrapure Reagent) was obtained from Baker J. T. Co.

Acetic acid (glacial, Fisher Chemical) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The pure water employed was double-deionized water.

The mobile phase consisting of 47.0% methanol-water solution, $V(\text{CH}_3\text{OH})/V(\text{H}_2\text{O})$, with 0.03% hydrochloric acid, $V(\text{CH}_3\text{OH}-\text{H}_2\text{O})/V(\text{HCl})$, was prepared in sufficient volume for accomplishing a set of FC analysis of insulin. It has two functions: (1) to act as the mobile phase for insulin adsorption and for preparing insulin solution. Its concentration was kept constant for a set of experiments, and will be referred as solution A-1; (2) to be employed for other purposes, such as weak solution for gradient elution of insulin desorption and column cleaning. The latter may vary and be referred to as "solution A-2". There were two solutions, 50% acetic acid-water solution, $V(\text{CH}_3\text{COOH})/V(\text{H}_2\text{O})$, named as solution B-1 and 90% methanol-water solution containing 0.03% hydrochloric acid, $V(\text{CH}_3\text{COOH}-\text{H}_2\text{O})/V(\text{HCl})$, named as solution B-2 to be the strong solutions for gradient solution to do column cleaning. Three pumps were necessary for the FC analysis in this investigation. The pump 1 was used to pump either the solution A-1 contained in the big bottle 1 (in Fig. 1) for insulin adsorption, or prepar insulin solution. The pump 2 was used to pump either the two B solutions, or solution A-2 to clean the connecting tubes. The pump 3 was used only to pump insulin solution contained in a 15 mL-plastic bottle 3 shown in Fig. 1. To avoid contaminations from each solution entering the other, each solution had to be contained in a separate bottle.

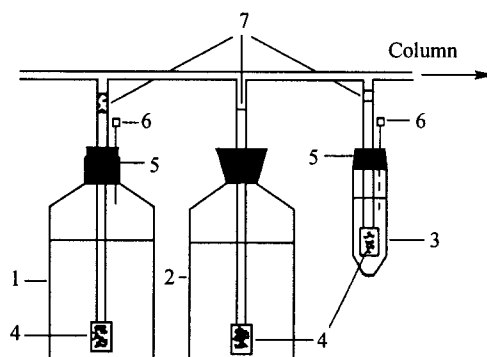


Fig. 1 Scheme of solution entering. (1) glass bottle containing storage solution A or solution A-1; (2) glass bottle containing strong solvent or solution A-2; (3) 15mL-plastic bottle containing only insulin solution; (4) filters; (5) stopper sleeve; (6) steel needle 6 #; (7) plastic connecting tubes.

When the same pump was used to pump different solutions, the bottle containing the other solutions was changed together with the attached filters 4, stoppers 5, steel needles 6, and plastic connecting tubes 7.

To keep the solution A-1 in bottle 1 invariable for a set of experiments, degassing was never used during insulin adsorption. Thus, the procedure was followed: (1) In order to completely isolate the insulin solution from the surrounding air, the plastic bottle 3 with a rubber stopper sleeve 5 for containing the insulin solution was used (Fig. 1); (2) A stainless steel needle 20 # denoted as 6 in Fig. 1 was inserted into the rubber stopper sleeve and immersed in the insulin solution. The needle not only prevented the formation of negative pressure inside the plastic bottle 3 during insulin adsorption, but also resulted in a reproducible evaporation loss during the process. The appearance of very fine air bubbles at the outlet of the needle was an indication that the FC system was performing well.

Column cleaning and baseline

To obtain a set of comparable data with the required accuracy and reproducibility, a very clean surface of stationary phase must be provided that can be reproduced easily. After completing each insulin adsorption, the RPLC column required thorough cleaning with gradient elution. Solution A-2 was the weak solution together with the strong solutions B-1 and B-2 to do the same non-linear gradient elution (100% solution A-2 for 20 min, then changed to 100% solutions B-1 firstly, and solution B-2 finally for 60 min). The procedure for testing the column cleanness was followed to mimic that for determining real methanol increment displaced by insulin (see below). The linearity, smoothness, slope, and the intercept of the obtained elution curve from the methanol increment of 1.0% (47.0% to 48.0%) provided the needed information to justify whether the column cleanness was satisfactory or not. This preferred testing procedure was done before each FC analysis of insulin, with 20 min delay at a flow rate of 1.0 mL/min.

The base line in this study was actually the blank in usual quantitative determination. The base line here was employed not only for the measurement by UV spectrometry, but also as the blank for NMR determination. To verify whether a small plateau existed before the plateau formed by insulin, it was absolutely necessary to

obtain a reproducible, smooth, and horizontal base line for each elution curve of insulin. Thus, a specially designed base line (see below) was required. The UV absorption of methanol at 198 nm is so sensitive that a good base line can be obtained only under some strict experimental conditions. To keep each component of solution A, such as methanol, water and hydrochloric acid approaching those three components in the insulin solution, it was necessary to take enough time to set up the equilibrium between the stationary and the mobile phases. Therefore, the length of the base line must be long enough to indicate that all the experiment conditions were normal. But, if it took too much time to accomplish this, some losses of methanol and/or water in solution A-1 would occur. The optimization of the specially designed base line was experimentally made up of two parts. The first was an "ideal" base line in the range of 0–7.50 min at a flow rate of 0.40 mL/min before insulin entering, or sampling. The second was the base line in the range from 7.50 min to the dead time of the FC system (the "real" base line, or the time between the start of sampling and the coming out time of the displaced methanol). The dead time of the FC system was measured with deuterium oxide to be 4.27 min at the same flow rate. Consequently, the actual length of the specially-designed base line in this study was 11.77 min. Whenever the two base lines were nearly horizontal straight lines, the obtained elution curve was referred to be of satisfactory accuracy.

FC analysis of insulin

After testing for column cleanness the following procedure was followed. (1) Disconnect the analytical column from the solvent delivery system. (2) Wash the connecting tube between the solvent mixer and the insulin solution container with 1.0 mL of insulin solution. (3) Use 15 mL of solution A-2 to wash the connecting tube between the solvent mixer and bottle 2. (4) Connect the solution A-1 in bottle 1 and the analytical column into the solvent delivery system and then use 60 mL of solution A-1 to wash it. (5) Dissolve insulin into the solution A-1 just taken out of bottle 1 and set up the distribution equilibrium between the mobile phase and the stationary phase at a flow rate of 0.40 mL/min for at least 40 min.

The insulin concentration was adjusted by the ratio

of the flow rate of insulin solution to that of solution A-1. The flow rate was 0.40 mL/min and the signal was recorded with an on-line UV spectrometer at 198 nm with a reference wavelength of 280 nm. The fraction of the eluate from 0.0 min to 11.77 min was collected as the blank for NMR determination. The fraction between 11.77 min and the point just before insulin exited was collected for the determination of the methanol increment with NMR. While the plateau height in this range of the elution curve was used for measuring the amount of the methanol increment displaced by insulin. To obtain a complete elution curve of insulin, the adsorption had to be allowed to proceed for 5 min to 10 min after breakthrough of insulin on the elution curve.

NMR measurement

The conditions for measuring protons are as follows: frequency, 200 MHz; special width, 3000 Hz; delay, 2.0; pulse width, 22 degrees; room temperature; No of repetitions 12 and double precision acquisition. Data processing: linear broadening, 0.1 Hz; FT size, 16; deuterium oxide as solvent.

Results and discussion

Elution curves of methanol at various wavelengths

Trifluoroacetic acid (TFA) is usually used as an ion-pairing agent for protein separation in RPLC. However, TFA not only has very strong UV absorbance, but can somehow be adsorbed by the stationary phase in RPLC. Its adsorption and desorption affect the quantitative determination with on-line UV spectrometry. Hydrochloric acid is often used for the separation of peptides in RPLC.^{14,15} It has only very weak UV absorbance and is not adsorbed by RPLC column. It is an ideal ion-pairing agent for this study.

Fig. 2 shows one set of the elution curves of the methanol increments obtained at three different wavelengths 280 nm (Fig. 2A), 254 nm (Fig. 2B), and 198 nm (Fig. 2C) with a reference wavelength of 550 nm. Each of these elution curves consists of six individual curves corresponding to the six methanol concentrations 47.05%, 47.10%, 47.20%, 47.30%, 47.40% and 47.50%. Together with methanol, 0.03% HCl was used as the ion-pairing agent in the

mobile phase. Comparing the three elution curves of Fig. 2 each other, it can clearly be seen that the profile of each curve is very close to that of a common solute. The sensitivity detected at wavelengths 280 nm and 254 nm was found to be almost identical, but the sensitivity

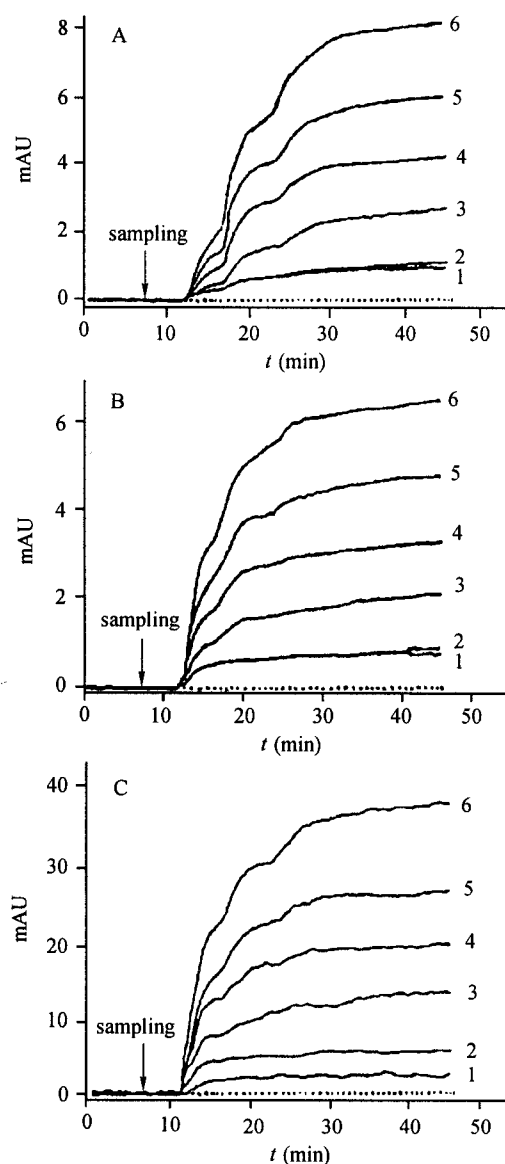


Fig. 2 Elution curves of methanol with various concentrations at A: 280 nm; B: 254 nm; C: 198 nm with reference wavelengths of 550 nm. SynChrompak, RP-C18; mobile phase, 47.0% to 47.5% methanol and water, $V(\text{CH}_3\text{OH})/V(\text{H}_2\text{O})$ containing 0.03% hydrochloric acid, $V(\text{CH}_3\text{OH}-\text{H}_2\text{O})/V(\text{HCl})$; methanol concentrations: (1) 47.05%, (2) 47.10%, (3) 47.20%, (4) 47.30%, (5) 47.40%, (6) 47.50%; flow rate: 0.40 mL/min; column temperature: 25 ± 0.50 °C; sampling time: 7.50 min.

obtained at wavelength 198 nm was much greater than that recorded at the other two wavelengths. With detection at 198 nm, the diode-array detector can still, very clearly, distinguish it from the common base line denoted by dash line, even for changes in methanol concentration over 0.05%.

Insulin was dissolved solely in solution A-1 at a concentration of 1.0 mg/mL. To prevent the insulin polymerization on standing, the insulin solution was prepared with strictly (bottle 3 in Fig. 1) controlled standing time maintained from the start to between three and a half and four and a half hours.

Insulin is known to have stronger UV absorbance at 280 nm than that at 250 nm. To diminish the possibly existed interference from insulin itself, a reasonable reference wavelength was chosen at 280 nm.

Fig. 3 shows a set of six elution curves of methanol increment detected at 198 nm under the same experimental conditions as shown in Fig. 2, but at a reference wavelength of 280 nm. Compared to the sensitivity shown in Fig. 2C, with a reference wavelength of 550 nm, that shown in Fig. 3 with the reference wavelength of 280 nm is lower by about 20%. It would be an advantage to offset some interference from insulin or impurities that have strong UV absorbance at 280 nm.

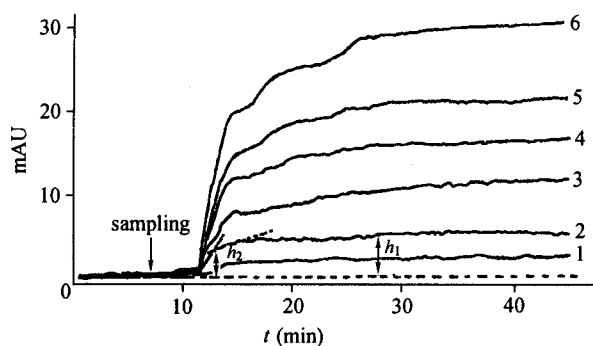


Fig. 3 Elution curves of methanol at 198 nm with reference wavelength of 280 nm. Methanol concentrations (1) 47.05%, (2) 47.10%, (3) 47.20%, (4) 47.30%, (5) 47.40%, (6) 47.50%; Other experiment conditions are the same as those shown in Fig. 2.

Calibration of methanol in UV spectrometry

The exact determination of the methanol increment with UV spectrometry depends on the accuracy of mea-

surement of the plateau height. Fig. 3 illustrates how the two plateau heights, h_1 and h_2 , of the elution curve of 47.10% methanol are determined. Specially, h_1 is defined as the average distance between the top line and the common base line denoted by dash line, and h_2 as the distance between the intersection of the two tangents of the elution curve to the common base line. From the plots of both h_1 and h_2 versus the real methanol concentration in the mobile phase, as shown in Fig. 4, two straight lines were obtained. These two straight lines can be employed for their calibration as expressed by the following two equations.

The calibration from h_1 :

$$\text{mAU}_{(1)} = -2796.67(\sigma = \pm 0.423) + 59.46(\sigma = \pm 1.08) C_{(\text{MeOH})} \quad r = 0.9983 \quad (1)$$

The calibration from h_2 :

$$\text{mAU}_{(2)} = -1756.17(\sigma = \pm 0.677) + 37.36(\sigma = \pm 1.74) C_{(\text{MeOH})} \quad r = 0.9983 \quad (2)$$

Because the constants and the standard deviation σ given in Eqs. (1) and (2) are the average values from three consecutive parallel measurements, they were considered to be sufficiently reliable. Eq. (1) is more accurate with a relative standard deviation of $\pm 1.8\%$, while Eq. (2) has a value of $\pm 4.0\%$. However, the accuracy for both plateau heights was considered sufficient in our study.

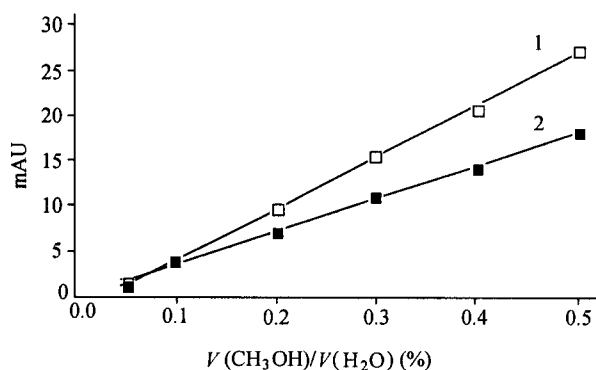


Fig. 4 Calibration of methanol increment in 47.0% $V(\text{CH}_3\text{OH})/V(\text{H}_2\text{O})$ aqueous methanol solution containing 0.03% hydrochloric acid, $V(\text{CH}_3\text{OH}-\text{H}_2\text{O})/V(\text{HCl})$ by UV spectrometry. (1) plateau h_1 ; (2) plateau h_2 . All data correspond to Fig. (3).

Determination of methanol with NMR

Because the exchanges between deuterium in deuterium oxide and hydrogen in both hydroxyl and methyl groups are very quick, it is an easy task to measure the amount of methyl and hydroxyl groups with NMR. Thus, any change of the concentration of either methanol or water can be quantitatively measured by the ratio of the amount of hydroxyl to that of the methyl groups, $R_{(\text{OH}/\text{CH}_3)}$. So long as the measured methanol-water solution does not contain any other substances having methyl and/or hydroxyl groups, the methanol released by insulin may be determined by NMR. When the changes in the methanol concentration were limited to a very narrow range, *i. e.*, 47.00% to 47.50% in the presence of 0.03% HCl, the calibration curve of real methanol concentration with a good linearity could be obtained and expressed with Eq. (3) as the following:

$$R_{(\text{OH}/\text{CH}_3),1} = (4.871 \pm 0.0001) - (0.05914 \pm 0.0014) C_{(\text{MeOH})}, \quad r = 0.9985 \quad (3)$$

From Eq. (3), the NMR determination can also distinguish the change in 0.10%, $V(\text{methanol})/V(\text{water})$. Its relative standard deviation was $\pm 2.8\%$.

Determination of the released methanol during insulin adsorption

Fig. 5A shows the whole elution curve of 0.025 mg/mL of insulin in a 47.0% aqueous methanol solution containing 0.03% hydrochloric acid. The plateau of insulin elution curve appears after 50 min. The curve displays a very good, almost linear base line between 0 min to 50 min. However, zooming-in to enlarge Fig. 5A by about 200 times but only taking the section of the graph from 0.0 mAU to 10 mAU, as shown in Fig. 5B, a small plateau appears in the range from 12 min to 48 min. To distinguish the small plateau from the large one formed due to insulin adsorption, the small plateau is called "first plateau", while the big one due to insulin is called "main plateau". The first plateau looks similar to the elution curve of methanol increment with concentration fixed at 47.05%, as shown in Fig. 3.

One may simply assume that the first plateau is due to impurities, including some varieties of insulin, small

solutes, *etc.*, which have no retention but strong absorption at 198 nm. But on the other hand, according to stoichiometric displacement model for retention (SDMR),^{12,13} methanol originally adsorbed by RPLC stationary phase would be displaced upon insulin adsorption. The desorbed methanol enters the mobile phase to increase the concentration of methanol in the mobile phase. Consequently, the mobile phase should now have some extra methanol, or methanol increment. The aim of this study was to investigate whether there is the existence of the methanol increment in the mobile phase. If there is, how much is it accurately?

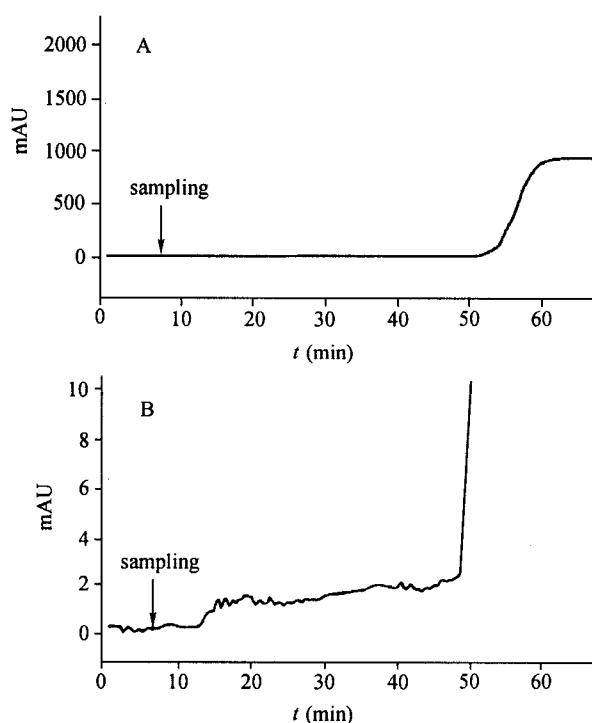


Fig. 5 Curve of the displaced methanol by insulin of 0.025 mg/mL. All experimental conditions are the same as those shown in Fig. 2, except its detection wavelength is 198 nm with reference wavelength of 280 nm. (A) original elution curve of insulin; (B) enlarging the elution curve of insulin by 200 times with zoom technique, but only taking the part of the range of 0 mAU to 10 mAU.

Comparison between UV-spectrometry and NMR result

Table 1 shows the comparison of the determined concentrations of methanol increment by on-line UV-absorbance and NMR for insulin adsorption at various concentrations. The experimental conditions in the FC anal-

ysis were the same as those shown in Fig. 5. The results in Table 1 are average values of two consecutive determinations. The listed standard deviations indicate that the reproducibility for both UV spectrometry and NMR was quite satisfactory. The results from UV spectrometry indicate higher sensitivity than those from NMR. The sensitivity of NMR, as pointed above, was 0.10% methanol. Thus, Table 1 only shows the results of the concentration of the displaced methanol higher than 0.10%. Though the results obtained by NMR are a little higher than those by UV-spectrometry, from the standpoint of analytical chemistry, both methods produce results are consistent. However, when the equilibrium concentration of insulin is beyond 0.30 mg/L, the displaced methanol decreases. On one hand, we need to explain the reason. On the other hand, we can conclude that the first plateau can not be primarily from impurities. Otherwise, the concentration of the possibly existing impurities would be proportional to the insulin equilibrium concentration in the mobile phase.

As can be seen from Table 1, the concentration of the methanol increment increases with insulin concentration, when the equilibrium concentration of insulin is not very high. This is because the rate of the displaced methanol due to insulin adsorption is more rapid in higher concentration solutions than that in lower concentra-

tions. In addition, because the composition and the thickness of the adsorbed layer on the stationary phase surface should have certain given values as the FC system is fixed, the sooner the methanol was displaced at the contact region between the stationary phase and the insulin molecules, the higher the first plateau and the shorter its length would be.

The decreases in methanol increment with very high insulin concentration may be attributed to the formation of the multilayer of insulin on the stationary phase. The interactions among insulin molecules may cause desolvation of the solvated insulin molecules. The insulin molecules should interact with the RPLC stationary phase by some amino acid sequences with strong hydrophobicity, and make some amino acid sequences with high hydrophilicity to face to the mobile phase. As a result, the displaced methanol in this case should be less than that when the insulin adsorption is monolayer.

Because NMR can be used to measure methanol directly, we can definitely conclude the presence of methanol increment in the fraction corresponding to the first plateau in Fig. 5B. The foregoing comparisons between results from UV-spectrometry and those from NMR further confirm the fact that the determined methanol increment was within sufficient accuracy.

Table 1 Comparisons of the amounts of methanol determined by UV spectrometry and NMR*

C_{Insulin} (mg/mL)	C_{Methanol} , (found) (% , V/V)			
	UV spectrometry		NMR	
	Real concentration	Methanol increment	Real concentration	Methanol increment
0.025	47.034 ± 0.001	0.034 ± 0.001	-	-
0.050	47.045 ± 0.003	0.045 ± 0.003	-	-
0.075	47.057 ± 0.004	0.057 ± 0.005	-	-
0.100	47.089 ± 0.010	0.089 ± 0.010	47.17 ± 0.04	0.17 ± 0.04
0.200	47.155 ± 0.011	0.155 ± 0.011	47.20 ± 0.04	0.20 ± 0.04
0.300	47.193 ± 0.012	0.193 ± 0.012	47.24 ± 0.02	0.24 ± 0.03
0.400	47.182 ± 0.010	0.182 ± 0.009	47.19 ± 0.02	0.19 ± 0.02

* Average values of three individual determinations for both UV spectrometry and NMR. SynChropak-RP C18, methanol/water [47% (V/V)] + HCl [0.03% (V/V)], 25 ± 0.5 °C.

Correction

The method for determination of the methanol increment with on-line UV spectrometry presented in this study is highly sensitive, highly accurate, and conve-

nient. However, it might be affected by many potential interferences. The method requires all chemicals to be very pure and the RPLC column to be cleaned very well throughout. In addition, although NMR spectrometer has very high selectivity and less interference, many other

factors may influence its overall accuracy. NMR is not an on-line determination method, so the measurement of the ratio of hydroxyl to methyl groups, $R_{(OH/CH_3)}$, must be done by using the collected eluate. Any kind of adsorption or desorption of either methanol or water on the surfaces of plastic or glass tubes, evaporation of both, or the molecular conformation changes of insulin which involve its solvation and/or desolvation, as the denatured insulin gradually renaturates, may affect the results. Of course, the reversed process may change the ratio also. Even though the experimental conditions are strictly controlled, some corrections are still necessary.

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(E0101211 SONG, J. P.; DONG, L. J.)