

Technical Note

Elution of Two Separated Peaks after Injection of a Small Sample Volume Using an Autosampler

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Abstract

Two separated peaks were detected when a small volume (approximately less than 0.5 μL) of one compound was injected into a HPLC system using an autosampler equipped with a 6-port valve. The origin of the two peaks was found to be injection by an autosampler that sucked the sample solution twice during the injection of each sample. First, the autosampler sucked in a sample solution to fill the needle, and then, after the rotation of the 6-port valve, the autosampler sucked in the sample solution again to fill the injection loop. Because the twice suck generated two separated sample zones in a 6-port valve and these sample zones were injected into the column separately, two separated peaks were observed. The increase in the sample volume induced disappearance of second peak and only one peak was observed. And also the same phenomenon was observed with a manual injector instead of the autosampler even when small volume of sample was injected.

Keywords: Autosampler; Peak shape; Small sample volume

1. Introduction

High-performance liquid chromatography (HPLC) is the most popular separation technique for analysis of mixture samples such as environmental, biomedical, and food samples, because it allows for the identification of multiple compounds in a sample with a single analysis [1-3]. Furthermore, many hot research areas such as proteome and metabolome analysis cannot be pursued without HPLC [4-6]. Many improvements to HPLC systems have been made up to now to reduce the sample volumes required for the analysis, increase the number of compounds that can be identified in a single run, and increase the reliability of the analytical results. For example, improvements such as a new pump that can deliver the mobile phase precisely in a low flow rate range, a new capillary column that is packed with a uniform stationary phase, and a new detector with a high sensitivity and selectivity have been developed [7-10]. Such improvement of HPLC systems has reduced the

sample volume required for analysis, and now less than 1 μL is required for a single analysis [11]. This reduction of the sample volume also reduces the subject (patient or volunteer) load necessary at the sampling time by reducing the volumes of samples collected from the subjects and makes it possible to perform analyses that would previously have required too large a sample volume. Hence, many demands for reductions in the sample volume still remain [12].

Automation is now used many areas to reduce human errors, costs, and tedious tasks. Sample injection is one of the most advanced processes that have been automated in HPLC analysis, and researchers and technicians can avoid manual sample injection by using an autosampler [13]. When sample solutions are provided to the autosampler, the autosampler injects a precise amount of a specified sample into the HPLC system in the exact order automatically, and finally the system reports the analytical results. Hence, the

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autosampler is an essential apparatus for the analysis of many samples using HPLC.

In this study, we report a phenomenon in which two separated peaks are eluted from one chemical compound when a small volume of sample solution is injected into the HPLC system using a commercially available autosampler and explain the reason for the phenomenon and a procedure for avoiding it.

2. Experimental

2.1. Chemical

Thiourea was purchased from Wako Pure Chemical Industries (Osaka, Japan). Phenylalanine, trypsin inhibitor type II-s, and hemoglobin were from Sigma-Aldrich (St. Louis, MO, USA). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. HPLC analysis

HPLC analysis was performed by using a MP 711 Vpumps (GL Science, Tokyo, Japan), a MI 709 autosampler (GL Science), a micro 21 UV-02 UV array detector (Jasco, Tokyo, Japan) and a HPLC System organizer (EZChrom Elite v.3.1.7J). The volume of 0.1 μL was recommended as a minimum injection volume of the autosampler by the supplier. A fused silica column (I.D. 25 μm , length 3.1 m) was obtained from Polymicro technology (Phoenix, AZ, USA). Water was used as mobile phase at the flow rate of 1.5 $\mu\text{L}/\text{min}$. The detection was performed at UV 200 nm. The typical injection volume was 0.05 μL . The C4 manual injector (VICI AG, Schenkon, Switzerland) attached with a 0.05 μL sample loop was used for manual injection.

3. Results and discussions

Two peaks, at 2.8 and 5.3 min of elution time, were observed when 50 nL of thiourea was injected into the

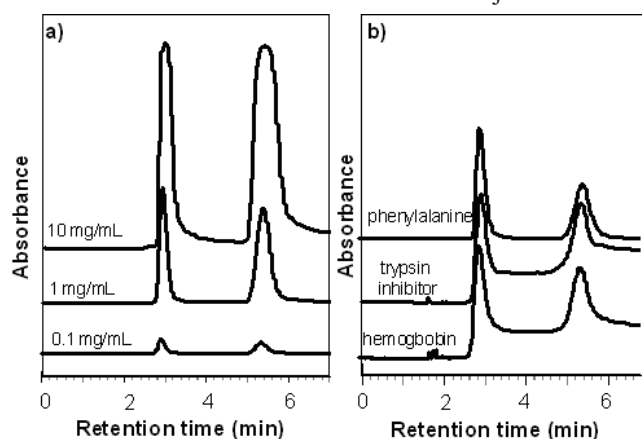


Fig. 1. Effect of sample concentration on the elution profiles of thiourea (a) and effect of compound on the elution profile for different compounds (b). Injection volume 50 nL, flow rate 1.5 $\mu\text{L}/\text{min}$.

HPLC system using the autosampler. To clarify the origin of the two peaks, elution profiles of three different concentrations (0.1, 1.0, and 10 mg/mL) of thiourea were examined (Fig. 1 a). Although the elution times of these peaks were not affected by the thiourea concentration, the heights and areas of both peaks increased with increasing thiourea concentration. To confirm whether this phenomenon, detection of two separated peaks from one compound, is observed only for thiourea or for other compounds as well, elution profiles of phenylalanine, trypsin inhibitor and hemoglobin were examined. Two separated peaks were also observed for each analyzed compound, and the elution times of both peaks were same for all compounds (Fig. 1 b). Hence, the elution of these two separated peaks did not originate from a specific property of thiourea, but was a common property of all compounds used in this study.

Because we assumed that these two peaks were derived from the autosampler in the HPLC system, we also used a manual injector instead of the autosampler for the injection of the sample into the HPLC system. Only one peak was observed when thiourea was injected using the manual injector (Fig. 2). The elution time of this peak was as the same as that of unretained compound. That is, it is correspond to the void volume of the HPLC system. This result indicates that the elution of two peaks was derived from the autosampler and not from another apparatus, such as a column or detector.

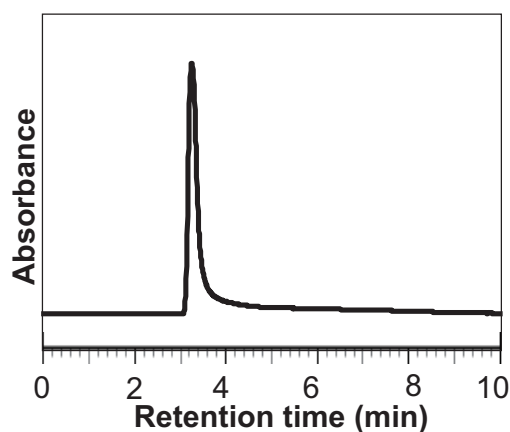


Fig. 2. Chromatogram of thiourea obtained using a manual injector.

To clarify the reason for the elution of two peaks much more, the effect of the injection volume on the elution profile of thiourea was examined. Although the elution times of the two peaks did not change when the injection volume was changed, the peak area of second peak increased with decrease in the injection volume (Fig. 3). On the other hand, area of the second peak decreased with increasing the injection volume and disappeared when the injection volume was more than 0.5 μL . The first peak did

not disappear under any of experimental conditions in this study. We paid attention to the volume of compound eluted between the two peaks, because it was almost constant for all experiments and was similar to that of the sample loop of the autosampler (about 5 μL). Furthermore, the change of the sample loop volume induced the change of the volume of between the two peaks. From these results, we suppose that the mechanism for the formation of the two peaks formation is as follows (Fig. 4).

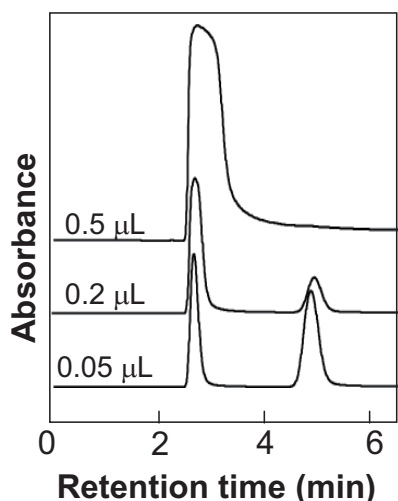


Fig. 3. Effect of injection volume on the elution profile of thiourea. Sample: 1mg/mL thiourea.

First, the autosampler equipped with a 6-port valve sucked in 5 μL of sample solution and filled the needle with the sample solution (preinjection time, a). Then, counter-clockwise rotation of the 6-port valve occurred (b). Next, the exact amount of sample was withdrawn into the sample loop of the autosampler (injection time, c), and finally clockwise rotation of the 6-port valve occurred to inject the sample solution into the HPLC system (d).

The second peak appeared when the injection volume was small, and it disappeared when the injection volume was more than 0.5 μL . As shown in Fig. 4c, when the injection volume is very small, the sample solution injected at the preinjection time still remains near port No. 3. This is due to such a small volume of sample solution moves very little. For example, when 50 nL of sample is injected into a sample loop with a volume and length of 5 μL and 10 cm, respectively, the distance the sample solution moves is approximately 1 mm. In that case, there is a possibility that a small amount of sample solution is present at port No. 3 of the 6-port valve because of diffusion or insufficient movement during the injection process. This indicates with dot line in Fig. 4c. Because the sample solution was present at both ends of the injection part after the rotation of the 6-port valve (Fig. 4d) and these solution volumes were injected into the column separately, two separated peaks were observed. The first and second peaks were derived from the sample solution zones at ports No. 5 and No. 2 of

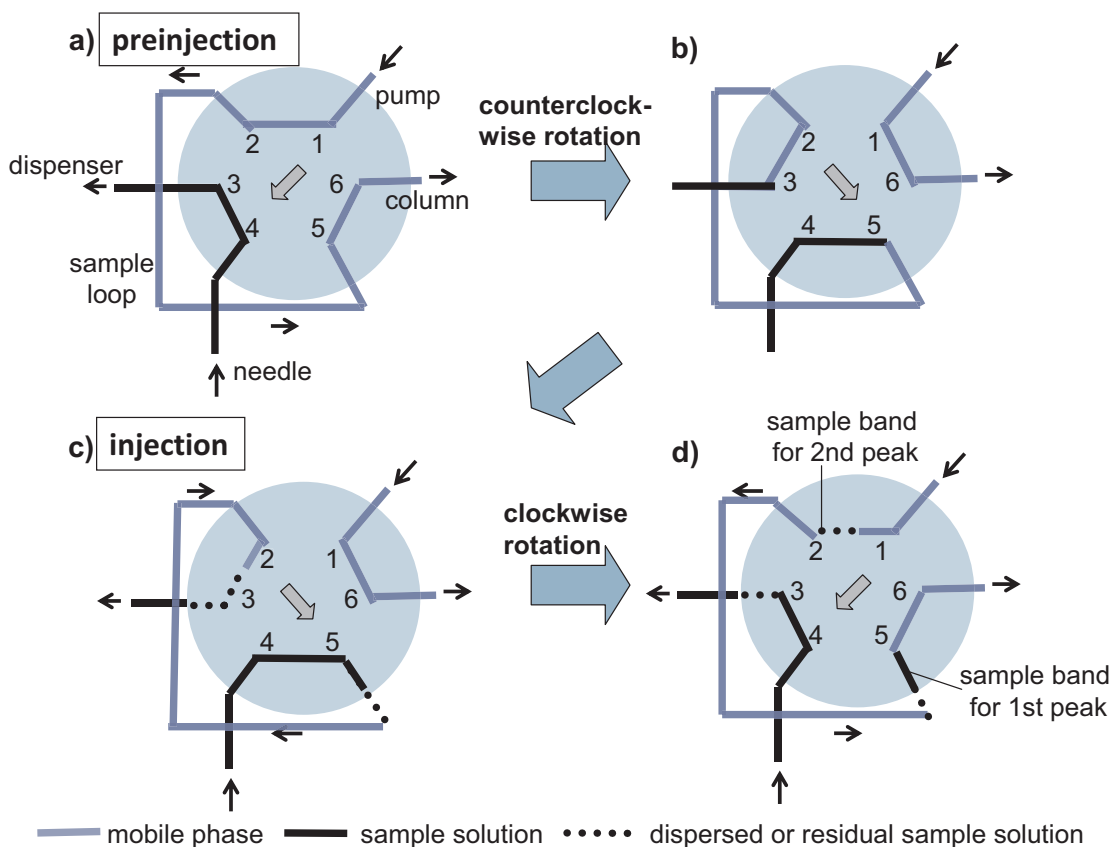


Fig. 4. Schematic image of production of the two peaks by the 6-port valve of the autosampler.

the 6-port valve, respectively. The second peak became large when the injection volume was small, because there was little movement of the solution at the injection time and the possibility of the sample solution remaining near port No. 3 of the 6-port valve became large. On the other hand, the second peak became smaller and finally disappeared when the injection volume was large, because there was a large movement of the solution at the injection time and the sample solution moved away from port No. 3 of the 6-port valve. The preinjection of the sample solution to fill the needle was the reason for the two separated peaks observed when the autosampler was used. In the case of the manual injector, the two-peak elution phenomenon did not occur, because the sample solution was directly injected into the sample loop from port No. 4 of the 6-port valve.

4. Conclusions

In this study, we found that two separated peaks were observed when a small volume of sample was injected into the HPLC system using an autosampler. It was found that the two peaks were produced by the autosampler during preinjection of the sample to fill the needle with sample solution. Because the use of an autosampler is popular for the injection of the sample into the HPLC system, this phenomenon occurs not only in our system but also in other HPLC systems. Two separated peaks are observed from one compound when autosampler was used for sample injection. The elution volume between the two peaks was similar to the volume of the sample loop and the retention factor of the sample was small, it should be suspected that the second peak was generated from the injection with autosampler.

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