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Determination of Lysozyme Chloride in Antiphlogistics by Reversed-Phase High-Performance Liquid Chromatography

KOU HAYAKAWA,* EIJI OKADA, HIDEAKI HIGASHIKUZE, and TAKESHI KAWAMOTO

Tokyo Research Laboratory, Shimadzu Corporation, 1–63–1 Shibasaki, Chofu 182, Japan

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An improved reversed-phase high-performance liquid chromatographic method is proposed for the determination of lysozyme. The protein was separated on a short column (4 mm i.d. × 50 mm) packed with octadecylsilane-bonded silica gel (Nucleosil $10C_{18}$) by linear gradient elution using 0.1 M sodium phosphate buffer, pH 2.0 (solvent A) and a mixture of isopropanol, ethylene glycol and solvent A (3 : 1 : 1), apparent pH 2.0 (solvent B). Monitoring was done with a ultraviolet detector. Under the elution conditions, lysozyme was recovered from the column in a high yield (97%). This method was found to be applicable to the determination of lysozyme chloride in antiphlogistics.

Keywords——reversed-phase high-performance liquid chromatography; UV detection; octadecylsilane-bonded silica; lysozyme; recovery test; antiphlogistics; tablet; capsule

Reversed-phase high-performance liquid chromatography (RP-HPLC) with octadecylsilane (ODS)-bonded silica gel packing materials has been widely employed for the analysis of samples of low molecular weight compounds by virtue of its unique separating properties and relative ease of handling.[1)]

Application of RP-HPLC to protein separation has been reported by several workers.[2a–c)] However, we could find few papers which described the recovery of the proteins from the column. We examined the determination range and the recovery of lysozyme and of ovalbumin by the method of O'Hare and Nice[2b)] and the method of Mönch and Dehnen.[2c)] In our preliminary tests, we found that the recoveries of lysozyme and ovalbumin were about 80 and about 0%, respectively, in the method of O'Hare *et al.* In the method of Mönch *et al.*, the recovery of ovalbumin was found to be about 20%. In this paper, we modified the method of Mönch and Dehnen to afford linear calibration curves of lysozyme and ovalbumin, and achieved 97 and 80% recoveries of lysozyme and ovalbumin, respectively.

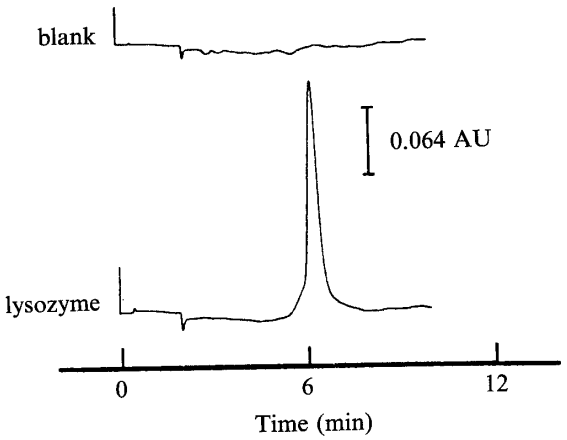

Fig. 1. Chromatogram of Standard Lysozyme Chloride

Lysozyme chloride (50 μg) was injected. The gradient rate was 10% per min. UV detection at 280 nm was used. Other conditions were as in Experimental.

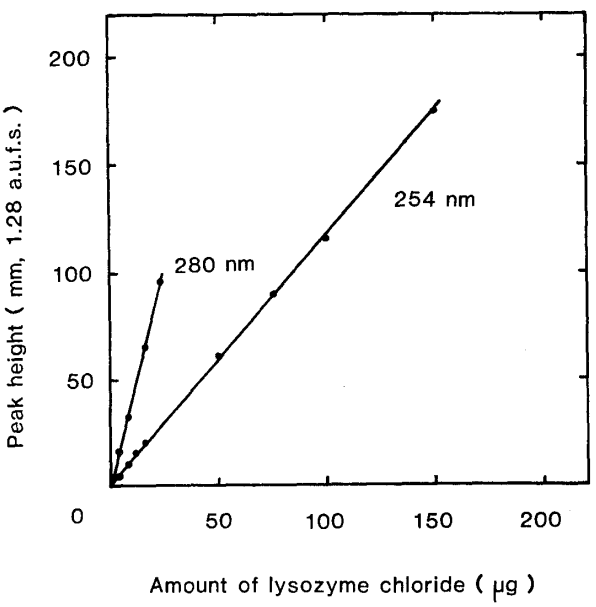

Fig. 2. Calibration Curves for Lysozyme Chloride Obtained by Our Method

Conditions for analysis were as in Fig. 1.

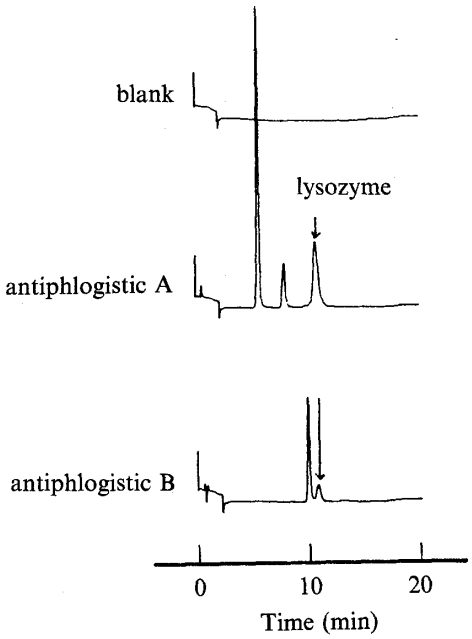

Fig. 3. Chromatograms of Antiphlogistics A and B

The gradient rate was 5% per min. Other conditions were as in Fig. 1.

TABLE I. Recovery of Lysozyme Chloride from the Column[a)]

Run number	Loading (mg)		
	1.97	3.80	7.80
1	1.95	3.50	7.64
2	1.98	3.93	6.71
3	1.92	3.68	7.88
4	1.77	3.66	—
Average	1.91 (97%)	3.69 (97%)	7.41 (95%)
S.D.	0.093	0.178	0.618
C.V. (%)	4.9	4.8	8.3

a) The amount of lysozyme chloride was determined by the method of Lowry *et al.*[3)] The gradient rate used in these experiments was 10% per min.

A chromatogram of standard lysozyme obtained by our method is shown in Fig. 1. The amount of protein injected into the RP-HPLC system and the observed peak height were linearly correlated, as shown in Fig. 2. We also obtained a linear calibration curve for ovalbumin.[4)] We chose ultraviolet (UV) detection at 280 nm in the following lysozyme determinations because of a higher sensitivity at 280 nm than at 254 nm. The detection limit was 1 μg (S/N=2). The repeatability of the method was evaluated by injecting 25 μg of lysozyme chloride 4 times and measuring the peak heights. The obtained values were 109, 113, 107 and 107 mm, the coefficient of variation being 2.6%.

When 1.97—7.80 mg of lysozyme chloride was injected, the recovery of lysozyme from the column was over 95% (Table I).

We applied this method to the determination of lysozyme chloride in antiphlogistics. Typical chromatograms are shown in Fig. 3. We used a gradient rate of 5% per min to

Table II. Determination of Lysozyme Chloride (mg) in Antiphlogistics by RP-HPLC

Exp. No.[a]	Antiphlogistic A[b]	Antiphlogistic B[c]
1	21.0	9.2
2	19.6	9.0
3	19.6	9.1
Average	20.1	9.1
S.D.	0.81	0.1
C.V. (%)	4.0	1.1

a) Three independent extractions were carried out. Averages of three determinations per sample are shown. Details of extraction procedures are given in Experimental. The gradient rate used in these determinations was 5% per min.
b) The weight of one capsule was 305 mg.
c) The weight of one tablet was 1.40 g.

Table III. Recovery of Lysozyme Chloride from Antiphlogistics[a]

Test No.	Antiphlogistic A[b] (add 10 mg)	Antiphlogistic B[b] (add 0.44 mg)
1	9.7	0.44
2	9.7	0.41
3	10.4	0.42
Average	9.9 (99%)	0.43 (96%)
S.D.	0.40	0.012
C.V. (%)	4.1	2.9

a) Conditions are given in Experimental. The gradient rate used in these tests was 5% per min.
b) Antiphlogistic A used was 150 mg. Antiphlogistic B used was 200 mg. Lysozyme contents in antiphlogistics A and B were 9.89 and 1.33 mg, respectively.

separate the lysozyme peak from the interfering peak, especially in antiphlogistic B. The quantitative values are shown in Table II.

From the results in Table II, we determined the recovery of lysozyme from antiphlogistics. As shown in Table III, the recoveries of this extraction method were 99% for antiphlogistic A and 96% for antiphlogistic B. Thus, this method was proved to be applicable to lysozyme determination in antiphlogistics.

Experimental

Chemicals and Reagents——Isopropyl alcohol, ethylene glycol, sodium dihydrogen phosphate, orthophosphoric acid, Folin–Ciocalteu reagent, cupric sulfate, and sodium tartrate were reagent grade products from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Lysozyme chloride from hen egg-white was purchased from Tokyo Kasei Co., Tokyo. Ovalbumin from hen egg-white was from Boehringer Mannheim. Antiphlogistics used were commercial products.

Lysozyme chloride was dissolved in solvent A (0.1 M NaH_2PO_4, adjusted to pH 2.0 by adding 6 ml of 85% orthophosphoric acid to 1 l of 0.1 M NaH_2PO_4 solution).

High-Performance Liquid Chromatography——The chromatograph assembly (Shimadzu Corp., Kyoto, Japan) consisted of a liquid chromatograph (model LC-2) equipped with a gradient elution unit (model GRE-2: proportioning-valve mixing method with one pump under high pressure), a sample injecting system (model SIL-1A: 200-μl stainless steel loop), and a variable-wavelength detector (model SPD-1). The column (stainless steel, 4.0 mm internal diameter, 50 mm length) was packed as follows: 0.5 g of Nucleosil 10 C_{18} (octadecylsilane-coated spherical silica gel particles of 10 μm average diameter; Macherey, Nagel & Co., Düren, G.F.R.) was weighed and suspended in

7 ml of isopropyl alcohol. The suspension was packed with an LC-3A pump (Shimadzu) at 200 kg/cm^2 for 1 h. The number of theoretical plates obtained was 1200 by using *n*-nonylbenzene (isocratic elution with methanol : water = 8 : 2, by volume). The column temperature was maintained at 15 °C with a water bath, and protein analysis was carried out by the use of a linear gradient from solvent A to solvent B (isopropyl alcohol : ethylene glycol : solvent A = 3 : 1 : 1 by volume ratio; adjusted to pH 2.0 by adding 14 ml of 85% orthophosphoric acid to 500 ml of the mixed solution) at 10 or 5% per min. The flow rate was 1.0 ml per min and the pressures were 20—100 kg/cm^2. Recovery to the initial condition occurred within 5 min. Eluted proteins were detected by UV-absorbance measurement at 280 nm and/or 254 nm.

Recovery of Lysozyme Chloride from the Column——Protein recovery experiments were carried out as follows: 2.0, 4.0, 7.9 mg of lysozyme chloride were injected and analysed, and the effluent fraction corresponding to the lysozyme peak was collected. A blank experiment omitting the protein was also carried out. The amount of protein in the fraction was measured by the method of Lowry *et al.*,[3] using lysozyme chloride as a standard protein. Absorption at 750 nm was measured with a double-beam spectrophotometer (Shimadzu, model UV-240).

Extraction of Lysozyme from Antiphlogistics——Five tablets or capsules were weighed and ground with a pestle and mortar. The pulverized antiphlogistics were precisely weighed (150 and 200 mg of antiphlogistic A and B, respectively). The powder was mixed with 5 ml of solvent A and allowed to stand for 30 min with occasional swirling. The solution was then centrifuged for 5 min at 2500 rpm, and the upper portion of the supernatant was filtered through a membrane filter (Fluoropore, pore size 0.3 μm, Sumitomo Denko Co., Osaka). The filtrate was injected into the HPLC system.

Recovery of Lysozyme Chloride from Antiphlogistics——Amounts of 10 mg and 0.44 mg of lysozyme chloride were added to 5 ml of solvent A containing antiphlogistic A (150 mg) and B (200 mg), respectively. Extractions were performed as described above. Comparisons of lysozyme analyses with added and non-added samples gave the recovery from the antiphlogistics.

References

1) C. Horváth, W. Melander, and I. Molnár, *J. Chromatogr.*, **125**, 129 (1976).
2) *a*) F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, **103**, 1 (1980); *b*) M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, **171**, 209 (1979); *c*) W. Mönch and W. Dehnen, *J. Chromatogr.*, **147**, 415 (1978).
3) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
4) K. Hayakawa, E. Okada, H. Higashikuze, and T. Kawamoto, *J. Chromatogr.*, **256**, 172 (1983).