A QUANTITATIVE ASSAY OF COVALENTLY BOUND CEPHALOSPORIN DERIVATIVES IN CEPHALOSPORIN-PROTEIN CONJUGATES

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β-Lactam antibiotic-protein conjugates have been prepared by many workers to study the immunological properties of penicillin[1] or cephalosporin derivatives.[2] The ratios of covalently bound β-lactam antibiotics to carrier proteins have been estimated by the penamaldate method,[3] UV absorption method,[2] the formol titration[4] and the dinitrophenylation method.[5] However, the obtained values of the ratio are not always satisfactory except the case of penamaldate method for penicillin derivatives.

The present paper deals with an alternative method to determine covalently bound cephalosporin derivatives quantitatively in cephalosporin-protein conjugates. This method includes a deamination reaction of the free ϵ-amino groups of lysine residues by nitrite in acidic medium, which is well known as the VAN SLYKE method.[6]

Ten to twenty milligrams of a dried cephalosporin derivative-protein conjugate* were dissolved in 5 ml of 80% acetic acid, then 1 ml of water and 1 ml of 30% $NaNO_2$ were added to the conjugate solution at 0~4°C. The reaction mixture (pH 3.4~3.8) was stirred for 2 hours at room temperature (20~25°C). After 2 hours, 1 ml of 30% $NaNO_2$ was further added and the mixture was stirred continuously for 2 additional hours under the same conditions. After the reaction was finished the mixture was diluted by adding 4 ml of water, and dialyzed against water for 24 hours. The deaminated and dialyzed conjugate was lyophylyzed and was dried in a desiccator.

The deaminated conjugate was then hydrolyzed for amino acid analysis by constant boiling hydrochloric acid at 100°C for 24 hours. In the analysis, the lysine content of the conjugate could be estimated as mole residues per mole of protein based on the content of other amino acids such as aspartic acid, glycine, alanine and phenylalanine, by comparing to the amino acid content previously obtained from the usual analyses of the carrier protein. It is obvious that the remaining lysine of the conjugate indicates the fraction of lysine protected from deamination by conjugation with antibiotic. Table 1 shows some results on certain cephalosporin-bovine γ-globulin (BGG) and bovine serum albumin (BSA) conjugates. In this table it was shown that cephalothin (CET), cephaloridine (CER) and cefazolin (CEZ) combined in ranges of 16~26 and 7~17 equivalents per mole of BGG (mol. wt. 169,000) and BSA (mol. wt. 70,000), respectively. In cephalexin (CEX) protein conjugates, only 5~6 equivalents per mole of each protein were bound. This might be due to the fact that cephalexin is more stable in alkaline medium compared to the other three antibiotics.[7]

Table 1. Moles of antibiotic bound determined by amino acid protected

Cephalosporins	Proteins			
	BGG		BSA	
	Equivalents per mole of BGG	Weight per cent of antibiotic	Equivalents per mole of BSA	Weight per cent of antibiotic
Cephalothin	26.0±0.8	6.1	16.9±0.2	9.2
Cephaloridine	14.5±0.7	3.6	11.6±0.3	6.8
Cephalexin	6.1±0.2	1.3	4.8±0.2	2.5
Cefazolin	16.6±0.4	4.5	7.2±0.3	4.7

* Conjugates were prepared by the method of LEVINE[1] at pH 8.5 except cephalexin-protein conjugate at pH 9.8.

Table 2. Lysine contents in BGG and BSA before and after the deamination

	Found lysine residues per mole of protein	
	Before deamination	After deamination
BGG	72.8 (100%)	4.31 (5.5%) 3.38 (4.3%)
BSA	54.6 (100%)	1.67 (3.6%) 1.71 (4.3%)

Table 3. A comparison in determination of bound cephalothin and cephalexin on BSA

Conjugate	eq./mole BSA		
	Deamination method	UV method*	DNP method**
CET	16.9±0.2	29.0±0.6	22.7±0.5
CEX	4.8±0.2	31.4±0.6	4.3±0.3

* For the standard solution, a solution of cephalosporoyl-ε-aminocaproic acid was used, which was prepared by treating of each cephalosporin derivative with ε-aminocaproic acid in an alkaline medium.

** Values were obtained from lysine amount in an acid hydrolysate of the each DNP-protein conjugate.

The deamination reaction described above proceeded almost completely even in the case of native proteins. As shown in Table 2, free ε-amino groups of lysine residues of native BGG and BSA were deaminated to the extent of 95~97%.

Further, results obtained by this analysis method were compared to the UV and dinitrophenylation (DNP) methods. Ratios of cephalosporin derivatives found in CET-BSA and CEX-BSA conjugates were also assayed by use of these methods. Table 3 shows that the UV method gave somewhat higher values than those by the deamination method. This indicates that cephalosporin derivatives which are absorbed on carrier proteins are measured together with the covalently bound antibiotics by the UV method. Since the UV absorption of cephalosporin derivatives may be distinguished from each other and those of the β-lactam ring opened-derivatives are more complicated, the UV method is not appropriate for the present purpose. In Table 3 it is also shown that ratios obtained by DNP method agreed with those from this deamination method whenever the combined amounts of the derivatives are relatively small. When the ratios of covalently bound derivatives, were relatively high, dinitrophenylation of free ε-amino groups of lysine residues on carrier protein by 2,4-dinitrofluorobenzene was not complete. This probably suggests that some steric hindrance by the covalently bound antibiotics might be present.

Under the deamination conditions described here, cephalosporin-protein conjugates were stable and no degradation was found. This was also confirmed by use of the model N-penicilloyl-ε-aminocaproic acid.

References

1) Levine, B. B. & Z. Ovary: Studies on the mechanism of the formation of the penicillin antigen. III. The N-(D-α-benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G. J. Exp. Med. 114: 875~904, 1961

2) Shibata, K.; T. Atsumi, K. Nishida & Y. Horiuchi: Immunological cross-reactivities of cephalothin and related compounds with benzylpenicillin (penicillin G). J. Allergol. (Japan) 16: 108~112, 1967

3) Levine, B. B.: N(α-D-Penicilloyl) amines as univalent hapten inhibitors of antibody-dependent allergic reactions to penicillin. J. Med. Pharm. Chem. 5: 1025~1034, 1962

4) Levy, M.: Equilibria in the formol titration. J. Biol. Chem. 99: 767~779, 1933

5) Sanger, F.: The free amino groups of insulin. Biochem. J. 39: 507~515, 1945

6) Van Slyke, D. D.: Eine Methode zur quantiotiven Bestimmung der aliphatischen Aminogruppen; einiger Anwendungen derselben in der Chemie der Protein, des Harns und der Enzyme. Ber. 43: 3170~3181, 1910

7) Yamana, T.; A. Tsuji, L. Kanayama & O. Nakano: Comparative stabilities of cephalosporins in aqueous solution. J. Antibiotics 27: 1000~1002, 1974