An Enzymatic Assay of Chloramphenicol Coupled with Fluorescence Reaction

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An alternative method for the assay of chloramphenicol using an enzymatic reaction coupled with a fluorescence detection system has been developed. Chloramphenicol was enzymatically acetylated by chloramphenicol acetyltransferase in the presence of acetyl-coenzyme A (acetyl-CoA) as the acetyl-donor, after which the liberated CoA-SH was derivatized with a fluorogenic reagent, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole. The assay was linear over the range of 2.5—40 μg/ml. Analytical recoveries of chloramphenicol at concentrations of 7.5 and 22.5 μg/ml, added to human serum, plasma, or a slightly hemolyzed serum, were in the range of 93.3 to 106.2%. The enzymatic assay was not affected by the presence of ten other antibiotics tested.

Keywords enzymatic assay; chloramphenicol; chloramphenicol acetyltransferase; CoA-SH; fluorometric determination; fluorogenic thiol reagent; 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

Introduction

Chloramphenicol had been extensively employed as an effective broad-spectrum antibiotic to treat infections including gram-positive and gram-negative bacteria and rickettsia. The toxic effects such as bone marrow suppression, aplastic anemia, and the "gray baby syndrome" have restrained its use.[1] Despite these serious side effects, however, chloramphenicol is still a potent drug for the treatment of life-threatening infections such as childhood meningitis. A combined therapy of chloramphenicol and ampicillin is chosen for the initial treatment of Haemophilus influenzae type b meningitis,[2] even though the arguments against it have been observed.[3,4]

Several methods for measuring the drug levels of chloramphenicol in serum have been described, including microbiological,[5] colorimetric,[6] fluorometric,[7] gas chromatographic,[8] high-performance liquid chromatographic,[9-11] and enzymatic[12-16] methods and an immunoassay.[17] Enzymatic methods are based on the acetylation of chloramphenicol by chloramphenicol acetyltransferase in the presence of acetyl-coenzyme A (acetyl-CoA) as the acetyl donor, and the detections are carried out with the measurement of radioactive chloramphenicol acetate that is produced[12-14] or with the determination of the liberated CoA-SH by a bioluminescent[15] or a spectrophotometric assay.[16]

Herein we describe an alternative approach to the enzymatic determination of chloramphenicol in which the enzymatic reaction followed by derivatization with a fluorogenic reagent for thiols, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) has been introduced.

Experimental

Materials Acetyl-CoA and chloramphenicol acetyltransferase (E.C. 2.3.1.28; from *Escherichia coli*) were purchased from Sigma Chemical Co., St. Louis. ABD-F was obtained from Dojin Laboratories, Kumamoto, Japan. Chloramphenicol, chloramphenicol palmitate, erythromycin, gentamicin sulfate, streptomycin sulfate and tobramycin were the products of Sigma Chemical Co., St. Louis. Ampicillin, cephalexin, cephalothin sulfate, kanamycin sulfate, penicillin G and tetracycline hydrochloride were from Wako Pure Chemical Industries, Ltd., Osaka. All other reagents were of analytical grade.

Apparatus Fluorescence intensity was measured with a Hitachi 204-S fluorescence spectrophotometer.

Procedures Solvent Extraction: An extraction of the chloramphenicol spiked to human serum, plasma, or a slightly hemolysed serum was made as follows. A 0.3 ml aliquot of each sample and 0.15 ml of 0.5 M acetate buffer (pH 5.0) were put into a centrifuge tube. 2.0 ml of ethyl acetate was added to the tube and mixed well. After standing for several minutes, the mixture was centrifuged at $700 \times g$ for 5 min and the supernatant was transfered to a test tube. As a result of the examination, two extractions using 2.0 ml of ethyl acetate were satisfactory. The upper organic phase was combined, and then evaporated at 40 °C under a stream of nitrogen. 0.3 ml of water was added to the residue before the enzymatic assay was undertaken.

Enzymatic Assay: 0.3 ml of sample chloramphenicol in water, 0.1 ml of 0.5 M Tris–HCl (pH 7.8) containing 5 mM ethylenediaminetetraacetic acid disodium salt (EDTA·2Na), and 0.05 ml of 3 mM acetyl-CoA were added to a test tube, successively. The mixture was incubated at 40 °C for 5 min. After incubation, 0.05 ml of chloramphenicol acetyltransferase (100 mU/ml, one unit will convert 1 μmol of chloramphenicol and acetyl-CoA to chloramphenicol 3-acetate and coenzyme A per min at pH 7.8 at 25 °C) was added and mixed, and then the mixture was incubated for 30 min at 40 °C. For the blank assay, 0.05 ml of water was added in place of the enzyme solution. 1.5 ml of 0.2 M borate buffer (pH 8.0) and 1.0 ml of 2 mM ABD-F solution were added to the reaction mixture and then incubated at 40 °C for a further 30 min. The fluorescence intensity was measured at excitation and emission wavelengths of 381 and 512 nm, respectively.

Results and Discussion

ABD-F, a fluorogenic reagent for thiols, was synthesized by Toyo'oka and Imai and has been used as the pre-column labelling reagent for high performance liquid chromatography (HPLC).[18] The ABD-F is an excellent reagent in terms of solubility in water, reactivity, selectivity to thiols, and stability of fluorophore, among its many characteristics. Also, the reaction of ABD-F with thiols was completed quickly and quantitatively at pH 8.0 and the resulting fluorophore was stable for over 60 min.[18] The ABD-F was not reacted with sample chloramphenicol in this instance because it was highly specific to thiols. The concentration of acetyl-CoA, however, had an influence on the fluorometric determination of the chloramphenicol on using the enzymatic reaction. An excess concentration of acetyl-CoA to that of chloramphenicol was required in the reaction mixture, whereas increasing the concentration of acetyl-CoA gave a higher blank value. As a compromise between progressing with the enzymatic reaction and minimizing the blank value, an adequate concentration of acetyl-CoA (300 μM in the reaction mixture) was chosen. The amount of enzyme in the reaction mixture affected the acetylation reaction of the chloramphenicol. The enzymatic acetylation of the chloramphenicol by chloramphenicol

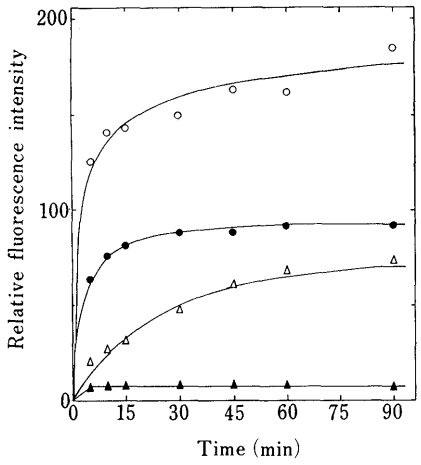

Fig. 1. Time Course of Chloramphenicol Acetylation under Various Concentrations of Enzyme

The quantity of chloramphenicol acetyltransferase added in the assay mixture was (○) 20 mU, (●) 5 mU, (△) 1 mU, and (▲) 0.01 mU.

acetyltransferase proceeded through the chloramphenicol 3-acetate to chloramphenicol 1,3-diacetate, though the rate of diacetylation was slower than that of monoacetylation.[19] Figure 1 shows the time course of chloramphenicol acetylation using several amounts of enzyme. The use of excess enzyme and the prolonged incubation accelerated the diacetylation of the chloramphenicol, whereas a lesser amount of enzyme needed a longer period of incubation. Using 5 mU of enzyme and an incubation for 30 min was adequate for the completion of the monoacetylation reaction. The result was similar to that reported by Lietman *et al.*[12] A linear standard curve was observed between fluorescence intensity and chloramphenicol concentration in the range of 2.5 to 40 μg/ml of sample in water, and a correlation coefficient (r) of 0.9989. The therapeutic range for chloramphenicol in serum is 10 to 25 μg/ml, therefore this assay permits the detection of the therapeutic levels of chloramphenicol. Analytical recovery was assessed by adding chloramphenicol at two levels of concentration to human serum, plasma, and a slightly hemolyzed serum. The recovery data of the spiked chloramphenicol, obtained with the solvent extraction and with the subsequent enzymatic assay, are shown in Table I. Reasonable recoveries (93.3—106.2%) were obtained by this assay. Analytical recoveries of known amounts of chloramphenicol added to human plasma, normal human serum or renal failure patient serum, were reported to be over the range from 94.4 to 106.1% for the HPLC method,[10] and from 98.4 to 106.8% for the enzymatic method.[16] Within-assay and between-assay precisions were determined by measuring chloramphenicol containing 15 μg/ml of serum, and the between-assay precision was estimated over a period of 3 weeks. The means and standard deviations were 15.9±0.7 μg/ml for 15 within-assays and 15.4±1.2 μg/ml for 12 between-assays. The relative standard deviations (R.S.Ds.) were 4.4 and 7.8%. The results were within an acceptable limit. Chloramphenicol palmitate, a 3-hydroxy drivative of chloramphenicol, was not acetylated by this assay and ten other antibiotics did not interfere with the enzymatic assay of chloramphenicol as shown in Table II.

The method proposed here is specific, accurate, and precise. The enzyme is commercially available and the assay can be achieved within 1 h. Although the serum volume of 0.6 ml, in which the half-volume will be subjected to the blank assay, was slightly large for pediatric or neonatal patients, the level of sensitivity for the assay was enough to monitor the drug in the clinical samples. The method needs pretreatment of the sample using an organic solvent, however, most of the other enzymatic methods and the HPLC method also require solvent extraction at either step of the analysis. Thus, the assay described here offers an alternative method for the determination of chloramphenicol in serum.

TABLE I. Analytical Recovery of Chloramphenicol

	Added (μg/ml)	Mean±S.D.[a] (μg/ml)	R.S.D.[b] (%)	Recovery (%)
Serum	22.5	23.9±0.3	1.3	106.2
Plasma	22.5	23.1±1.2	5.1	102.7
Hemolytic serum	22.5	22.4±0.7	3.2	99.6
Serum	7.5	7.7±0.2	2.1	102.7
Plasma	7.5	7.1±0.4	5.5	94.7
Hemolytic serum	7.5	7.0±0.5	6.7	93.3

a) Average of 5 determinations with standard deviation (S.D.). *b*) R.S.D., relative standard deviation.

TABLE II. Effect of Other Antibiotics on Enzymatic Assay

Antibiotics added	Concentration (μg/ml)	Chloramphenicol recovered (%)
None	—	100
Ampicillin	25	98.5
Cephalexin	50	98.6
Cephalothin	25	99.0
Erythromycin	5	99.0
Gentamicin	10	99.1
Kanamycin	20	99.5
Penicillin G	50	98.6
Streptomycin	50	102.7
Tetracycline	5	101.5
Tobramycin	20	101.3

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