J. Pharma. Pharmacol. 1989, 41: 136–137
Communicated February 22, 1988

Determination of cephalosporin-C amidohydrolase activity with fluorescamine

F. REYES, M.-J. MARTINEZ, J. SOLIVERI, *Departmento de Microbiologia Aplicada, Centro de Investigaciones Biológicas (C.S.I.C.), Velazquez 144, Madrid-28006, Spain*

Abstract—A spectrophotometric procedure for the assay of cephalosporin-C amidohydrolase activity, based on the determination of the 7-aminocephalosporanic acid (7-ACA) produced in the hydrolysis of cephalosporin-C by the enzyme, is described. This procedure can be used to detect 7-ACA over a range of 10 to 200 $\mu g\ mL^{-1}$. The same method can be used as a fluorometric procedure with a 100-fold greater sensitivity. At pH 4·5 7-ACA produces a strong fluorophor with fluorescamine, detectable spectrophotometrically at 378 nm and fluorometrically at an excitation of 378 nm and emission of 495 nm. At this pH the fluorophors formed with cephalosporin-C, proteins and aminoadipic acid present minimal absorbance values. The conditions for maximal detection of 7-ACA in the presence of proteins, cephalosporin-C and aminoadipic acid have been determined.

The usual chemical methods for testing penicillin amidase activity are based on the amount of 6-aminopenicillanic acid (6-APA) produced in the hydrolysis of penicillin substrates. The same methods have also been used for the determination of 7-aminocephalosporonic acid (7-ACA) or related compounds produced by the action of cephalosporin amidohydrolase (acylase) on different substrates (Marelli 1968; Ichikawa et al 1981).

A more sensitive fluorometric method, previously described for amino acids, proteins and amino sugars (Underfriend et al 1972; Chen & Mayer 1981; Jimenez & Weill 1982) based on the reaction of these substances with fluorescamine, has been used for the determination of 6-APA at pH 7 by Veronese et al (1981). Further, Baker (1984) reported that with fluorescamine 6-APA produces a stronger fluorescence intensity at pH 4 than at pH 7. The reaction presents a high specificity for 6-APA, since proteins and amino acids develop minimal fluorescence at pH 4.

Based on these findings we have established a spectrophotometric method for the detection of cephalosporin-C acylase activity by reacting 7-ACA with fluorescamine in the presence of cephalosporin-C, aminoadipic acid and proteins. The same method has been adapted for fluorimetric determination thereby increasing the sensitivity 100-fold.

Materials and methods

Cephalosporin-C, 7-ACA, aminoadipic acid, fluorescamine and bovine serum albumin were provided by Sigma Chemical Co. Ltd.

The fluorescence and absorbance were determined using a Fica 55 MK II Spectrofluorimeter and a Bausch and Lomb Spectronic 2000 Spectrophotometer, respectively.

The fluorescamine solution was prepared in AR acetone. The samples of 7-ACA and other compounds were prepared in 1 mL of 10 mM borate citrate phosphate buffer and were treated with 0·1 mL of fluorescamine.

Results and discussion

The purpose of this work has been to establish a sensitive method capable to detect 7-ACA produced on the hydrolysis of cephalosporin-C by cephalosporin-C amidohydrolase (Fig. 1).

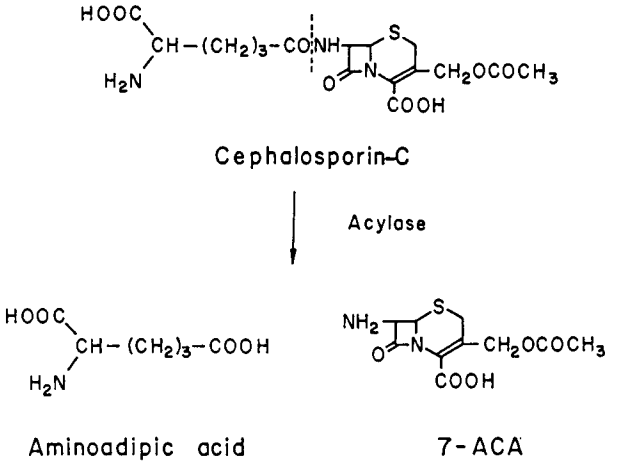

FIG. 1. Deacylation of cephalosporin-C by cephalosporin-C amidohydrolase.

Spectrophotometric results. The variations in the absorbance of the different fluorescamine-compounds (protein, cephalosporin-C, 7-ACA and aminoadipic acid) present after stopping the enzymic reaction with fluorescamine were studied separately (Fig. 2A) and together (Fig. 2B) as a function of pH. The enzyme was substituted by bovine serum albumin, as a standard protein. The complex of fluorescamine-7-ACA gave maximal absorbance at pH 4·5. At this pH, the other fluorophors have minimal absorbance values: aminoadipic acid (50 $\mu g\ mL^{-1}$) has an absorbance of practically nil, cephalosporin-C (100 $\mu g\ mL^{-1}$) has only 14% of the absorbance of 7-ACA at the same concentration and the standard protein (serum albumin at 100 $\mu g\ mL^{-1}$) has 36% of that value.

When serum albumin and cephalosporin-C are mixed (100 $\mu g\ mL^{-1}$ each) the absorbance after the fluorescamine reaction achieves a non-additive value which represents 21% of the 7-ACA fluorofor absorbance and not the 54% presumed. If cephalosporin-C, serum albumin and aminoadipic acid are mixed, the same results are obtained (Fig. 2B). Similar results were found when serum albumin was replaced by other proteins obtained from autolysed cultures of filamentous fungi. From these experiments we established the need for a control for the reaction with fluorescamine at zero time in the assay cephalosporin-C acylase activity. The increase of absorbance during the enzymic reaction after stopping the enzymic reaction with fluorescamine was due to the production of 7-ACA.

As well as pH (4·5), other parameters were also fixed for the reaction of 7-ACA with fluorescamine with an optimum of reaction time of 40 min and a fluorescamine concentration 100 $\mu g\ mL^{-1}$, but for economical reasons 50 $\mu g\ mL^{-1}$ was chosen. In these conditions the absorbance achieved maximal values at 378 nm; at pH 7·5 a small shoulder was also detected. The effect of

Correspondence to: F. Reyes, Departamento de Microbiologia Experimental, Centro de Investigaciones Biológicas (C.S.I.C.), Velazquez 144, Madrid-28006, Spain.

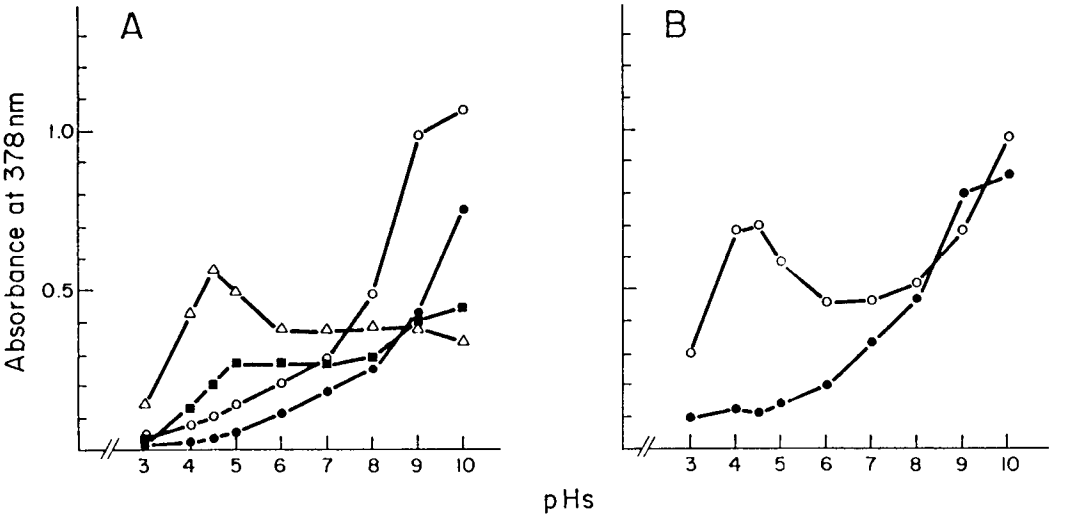

FIG. 2. Variation in the absorbance intensity as a pH function of different fluorescamine-compounds: 7-ACA (△A); serum albumin (■A); aminoadipic acid (●A) and cephalosporin-C (○A). Cephalosporin-C and serum albumin (●B). A mixture of cephalosporin-C and serum albumin plus 7-ACA and aminoadipic acid (○B). The concentrations of 7-ACA and aminoadipic acid were 50 μg mL^{-1}, and the concentrations of serum albumin and cephalosporin-C were 100 μg mL^{-1}.

Table 1. Absorbance of fluorescamine fluorophors produced with increasing 7-ACA concentrations and the variations of these absorbances in presence of fixed concentrations of bovine serum albumin (BSA, 500 μg mL^{-1}), cephalosporin-C (CpC, 500 μg mL^{-1}) and aminoadipic acid (AdA, 50 μg mL^{-1}).

7-ACA (μg mL^{-1})	7-ACA	7-ACA + BSA	7-ACA + CpC	7-ACA + AdA
2	0·065	0·115	0·095	0·095
5	0·120	0·170	0·145	0·145
25	0·395	0·425	0·400	0·400
50	0·670	0·700	0·685	0·680
100	1·030	1·055	1·040	1·037
250	1·300	1·320	1·317	1·320

fixed concentrations of serum albumin, cephalosporin-C or aminoadipic acid on increasing 7-ACA concentrations in the fluorescamine reaction is represented in Table 1. The absorbance of 7-ACA-fluorescamine was linear with increasing concentration between 10 and 200 μg mL^{-1} and the same results were obtained in the presence of a mixture of serum albumin and cephalosporin-C at 1 mg mL^{-1}; the influence of these substances being higher at low concentration of 7-ACA.

Fluorimetric results. In the established conditions we found that excitation and emission maxima for 7-ACA at 378 and 495 nm respectively. The same results were obtained at pH 7·5 but with a lower fluorescence intensity. The fluorescence intensity increases linearly with increasing 7-ACA concentrations between 0·05 and 1 μg mL^{-1}. The same experiments were made in the presence of aminoadipic acid, cephalosporin-C, serum albumin and a mixture of these substances. In all the cases the same linearity was observed, although a higher fluorescence intensity was developed because of the presence of cephalosporin-C and principally serum albumin, in accordance with the finding that at low 7-ACA concentrations, the influence of these substances is higher. The fluorimetric method is 100-fold more sensitive than the spectrophotometric method, although the control at zero time should be higher.

As a general remark, the enzymic reaction can be carried out at an appropriate pH, adjusting the pH to 4·5 before fluorescamine is added.

References

Baker, W. L. (1984) A sensitive procedure for the screening of microorganisms for the presence of penicillin amidase. Aust. J Biol. Sci. 37: 257–265

Chen, A. C., Mayer, R.T., (1981) Fluorescamine as a tool for amino sugar analysis. J. Chromatogr. 207: 445–448

Ichikawa, S., Murai, Y., Yamamoto, S, Shibuya, Y., Fujii, T., Komatsu, K., Kodaira, R. (1981) The isolation and properties of *Pseudomonas* mutants with an enhanced productivity of 7β-(4-carboxybutanaundo)-cephalosporanic acid acylase. Agric. Biol. Chem. 45: 2225–2229

Jimenez, M. H., Weill, C. E. (1982) A sensitive fluorimetric analysis of amino sugars. Carbohydr. Res. 101: 133–137

Marelli, L. P. (1968) Colorimetric method for determination of 7-aminocephalosporanic acid (7-ACA) and related compounds. J. Pharm. Sci. 57: 2172–2173

Underfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., Weigele, M. (1972) Fluorescamine: a reagent for assay of amino acids, peptides, proteins and primary amines in the picomole range. Science 178: 871–872

Veronese, F, Franchi, D., Boccu, E., Guerrato, A., Orsolini (1981) *E. coli* penicillin arylase: extraction, purification and kinetic characterization by improved methods. Il. Farm. Ed. Sci. 36: 663–670.