SUSCEPTIBILITY OF NATURALLY OCCURRING CARBAPENEM ANTIBIOTICS TO RENAL DEHYDROPEPTIDASE-I

AN EASY ASSAY USING A PLATE TECHNIQUE BASED ON A NOVEL CHROMOGENIC β-LACTAMASE SUBSTRATE

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A general screening procedure is described which allows the rapid determination of the susceptibility of naturally occurring carbapenem antibiotics to renal dehydropeptidase-I at the broth level. The procedure is based on the incubation of carbapenem-containing solutions with dehydropeptidase-I and the subsequent assay of residual β-lactamase-inhibiting/inactivating activity of carbapenems by means of a plate technique using the chromogenic β-lactamase substrate PADAC.

The last few years have seen an enormous proliferation in the number of naturally occurring carbapenem-type of antibiotics, β-lactam compounds possessing the dethiacarbapenem nucleus.[1,2] Presently, some 29 or so compounds are known.[3] The chances to isolate even more novel carbapenems are supported by the recent finding[4] that, besides actinomycetes, bacteria are also able to produce carbapenems. Even though carbapenem antibiotics generally possess a broad spectrum antibacterial activity against Gram-positive and Gram-negative bacteria[5,6] as well as β-lactamase-inhibitory/inactivating properties,[7] naturally occurring carbapenems suffer from a major disadvantage: as has been reported recently, carbapenems are metabolized in experimental animals and human beings to a significant extent by a renal enzyme identified as the membrane-bound dehydropeptidase-I resulting in poor urinary recoveries.[8,9] Therefore, the need for chemical or biochemical modifications of naturally occurring carbapenems has been suggested.[9] In view of the large structural diversity of the already known naturally occurring carbapenems, however, the possibility cannot be excluded that dehydropeptidase-I-resistant carbapenems are also being produced by microorganisms. In order to screen for such compounds the need for a simple assay is obvious by which dehydropeptidase-I-sensitive compounds could be recognized and thus eliminated at a very early screening stage.

This paper describes a simple technique to assess the susceptibility of carbapenem compounds at the broth level after exposure to dehydropeptidase-I. The procedure reported here makes use of the β-lactamase-inhibiting properties of intact carbapenems[7] which can conveniently be monitored using the chromogenic β-lactamase substrate PADAC.[10,11]

Materials and Methods

Materials

PADAC, 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridiniomethyl]-3-cephem-4-carboxylic acid, was the product of Calbiochem GmbH. Glycyldehydrophenylalanine was prepared in our own laboratories.

Isolation of Dehydropeptidase-I

Dehydropeptidase-I was purified from frozen hog kidney up to step 5 essentially as described by CAMPBELL.[12] This clear preparation of solubilized enzyme was used throughout this study.

Assay of Dehydropeptidase-I

The originally described assay conditions[12] using glycyldehydrophenylalanine in 25 mM Tris-HCl buffer pH 8.0 resulted in an unsatisfactory rate of hydrolysis for proper kinetic studies. Therefore, standard assay conditions were adopted from KROPP *et al.*[3] The assay mixture (770 μl) contained: 50 mM 3-*N*-morpholinopropane sulfonic acid buffer pH 7.1 and 60 μM glycyldehydrophenylalanine. The reaction was performed at 37°C and started by addition of 20 μl of the dehydropeptidase-I preparation. The decrease in absorption was recorded at 275 nm. Under these conditions the dehydropeptidase-I preparation hydrolyzed glycyldehydrophenylalanine (60 μM) at a rate of 0.4 μmol/minute per mg protein.

Susceptibility Assay of Carbapenems

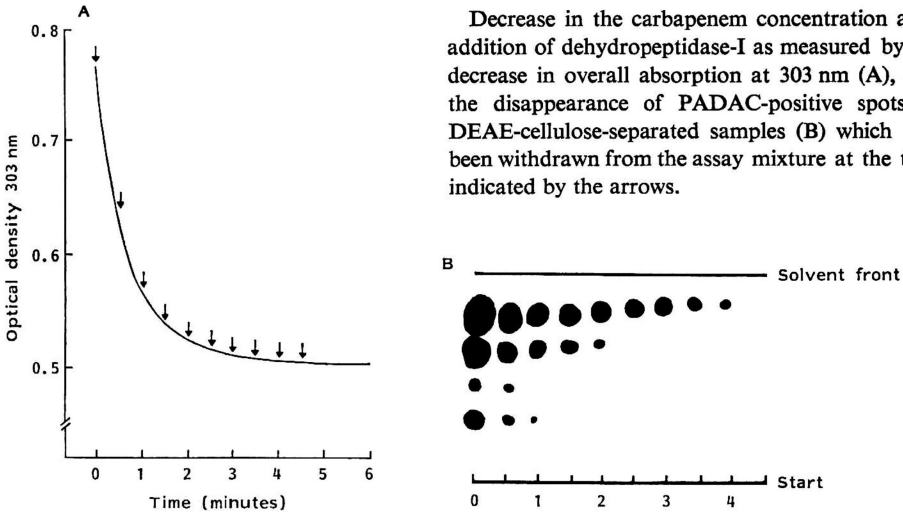
This was performed analogous to the assay for dehydropeptidase-I. Instead of glycyldehydrophenylalanine, a partially purified preparation of a mixture of carbapenem antibiotics produced by Streptomyces strain Y 5633 a/BL 36 was used as the "substrate", and reading of the decrease in absorption was done at 303 nm.

In order to correlate the hydrolysis of carbapenem constituents with the loss of β-lactamase inactivating properties of carbapenems, 10 μl samples were withdrawn from the assay mixture immediately prior to the addition of dehydropeptidase-I, and at intervals of 30 seconds after the start of the reaction. All samples were immediately spotted on a DEAE-cellulose plate (Polygram CEL 300 DEAE, Macherey & Nagel) and air-dried. The chromatogram was developed with 0.1 M NaCl in 0.5 M potassium phosphate buffer pH 7.0.[13] Localization of intact carbapenems was performed by placing the chromatogram on top of an agar plate (20×20 cm) containing β-lactamase from *Enterobacter cloacae* P99. After 15 minutes of incubation at room temperature the chromatogram was removed and carbapenem-inactivated β-lactamase was detected by overlaying with 50 ml of top agar containing the β-lactamase substrate PADAC. After an additional 10 minutes at room temperature the positions of intact carbapenems appeared as violet spots on a yellow background.[10]

Results

Streptomyces strain Y 5633 a/BL 36 produces a mixture of various olivanic acid-type carbapenems which effectively inactivate the β-lactamase from *E. cloacae* P99. The carbapenems can be separated on DEAE-cellulose chromatograms into four classes of compounds (Fig. 1B at time 0), and can subsequently be visualized by the PADAC plate technique as violet spots.[10,11] When a partially purified preparation of these compounds was incubated with dehydropeptidase-I under standard assay conditions, a partial loss in overall absorption at 303 nm was observed with time (Fig. 1A). This decrease in absorption was identical to that after addition of cysteine (data not shown). Since the susceptibility of carbapenems to cysteine at neutral pH-values is a well-known property of this type of β-lactam antibiotic,[13] this seemed to indicate that all of the carbapenems originally present had been hydrolyzed by dehydropeptidase-I. In order to establish this unequivocally, samples (10 μl) were withdrawn from the assay mixture before and (at the indicated intervals) after addition of dehydropeptidase-I. Separation of residual intact carbapenems by thin-layer chromatography on DEAE-cellulose and subsequent localization as violet spots by means of the PADAC plate technique (Fig. 1B) clearly shows that there is a close correlation between the disappearance of "PADAC-positive" spots and the decrease in absorption at 303 nm (Fig. 1A). In fact, at the time when no further change in absorption could be observed, no "PADAC-positive" spots were left, indicating that all carbapenems had been completely destroyed.

Fig. 1. Time-course of the hydrolysis of carbapenem antibiotics by dehydropeptidase-I.



Decrease in the carbapenem concentration after addition of dehydropeptidase-I as measured by the decrease in overall absorption at 303 nm (A), and the disappearance of PADAC-positive spots of DEAE-cellulose-separated samples (B) which had been withdrawn from the assay mixture at the time indicated by the arrows.

In case the chromatographic separation into single classes of carbapenems is not wanted, the time-dependent rate of hydrolysis of the carbapenem complex present can, of course, also conveniently be followed by simply spotting the probes onto DEAE-cellulose plates which immediately prevents the further degradation of carbapenems by dehydropeptidase-I. Thus, a whole set of individual probes can rapidly be screened for dehydropeptidase-I-resistant carbapenems on one single DEAE-cellulose plate.

Discussion

In a large-scale screening program for new antibiotics from microorganisms the early elimination of known compounds, or of compounds with undesirable properties is a major goal in order to reduce the amount of work necessary to identify active principles.

Despite their promising intrinsic antibiotic activity and potent β-lactamase-inhibitory activity, the clinical utility of naturally occurring carbapenems is hampered by their unexpected instability to dehydropeptidase-I as compared to penicillins and cephalosporins.[8]

Known assays to test the susceptibility of carbapenem antibiotics to dehydropeptidase-I comprise both microbiological[9] as well as spectrophotometric[5] methods. However, both assays are not entirely satisfactory: the microbiological assay is notoriously time-consuming and the direct spectrophotometric procedure based on the quenching of the UV absorption of carbapenems cannot routinely be applied to crude culture broths due to the high UV background of broths. Alternatively, since carbapenems can rapidly be detected and assayed based on their β-lactamase-inhibiting/inactivating activity the decrease in carbapenem content upon exposure to dehydropeptidase-I can easily be monitored with the help of chromogenic β-lactamase substrates as described previously.[7,10,11,14]

The experiments reported in this communication indicate that the time-course of hydrolysis of carbapenem antibiotics by renal dehydropeptidase-I as determined spectrophotometrically correlates exactly with the loss of β-lactamase-inhibitory activity. Since the detection and localization of carbapenems based on the PADAC plate technique can be applied to crude culture broths[10] the method described here clearly bears the potential to quickly select dehydropeptidase-I-resistant carbapenems produced by microorganisms.

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