Enzymatic Method for Rapid and Sensitive Determination of \( \beta \)-Lactam Antibiotics

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A rapid and sensitive procedure for the estimation of \( \beta \)-lactam antibiotics is described which makes use of the ability of these antibiotics to inactivate the R39 DD-carboxypeptidase. Depending on the values of the kinetic parameters which govern the reaction, the antibiotics fall into two groups. The lower limit for the quantitative estimation of the antibiotics of groups I and II is about 5 and 50 pmol/ml, respectively. The procedure has been adapted to biological fluids such as human sera and cows' milk.

\( \beta \)-Lactam antibiotics can be estimated by various procedures. The microbiological (agar diffusion) technique (2, 3, 11) permits detection of about 0.1 to 0.5 \( \mu \)g of antibiotic per ml, but it is time consuming. Chemical procedures (9) are faster but much less sensitive. High-pressure liquid chromatography has been used to estimate amoxycillin and ampicillin in sera and urines (12) and cephalosporin C in fermentation media (1); minimal concentrations of 0.5 \( \mu \)g of antibiotic per ml are necessary. Finally, an enzyme immunoassay (8) has been devised which detects ampicillin at concentrations as low as 10 ng/ml; \( \beta \)-lactam antibiotics other than ampicillin, however, were not investigated by this technique. The present report describes an enzymatic method through which \( \beta \)-lactam antibiotics in nanogram amounts can be rapidly estimated. The method makes use of the ability of \( \beta \)-lactam antibiotics to inactivate the 53,000-dalton R39 DD-carboxypeptidase and rests upon the determination of the residual enzyme activity after interaction with the antibiotic.

**Theoretical background.** The R39 enzyme (E) reacts with \( \beta \)-lactam antibiotics (I) according to the formula: E + I \( \stackrel{K}{\rightarrow} \) EI \( \xrightarrow{k_3} \) EI* \( \xrightarrow{k_4} \) E + P(s), where P(s) represents the degradation product(s) arising from the antibiotic, K is the dissociation constant of the first stoichiometric complex EI, EI* is a second complex of higher stability in which both the enzyme and the antibiotic molecules are modified, and \( k_3 \) and \( k_4 \) are first-order rate constants (7). The efficacy with which the antibiotics immobilize the R39 enzyme in the form of complex EI* depends on both the rate at which complex EI* is formed and the rate at which complex EI* breaks down. The degradation step EI* \( \xrightarrow{k_4} \) E + P(s), on the one hand, can be neglected if \( t_1 + t_2 << 0.69/ k_4 \) (where \( t_1 \) is the time of interaction between the enzyme and the antibiotic and \( t_2 \) is the time required for the estimation of the residual enzyme activity). With the R39 enzyme and all the \( \beta \)-lactam antibiotics tested so far, the \( k_4 \) term of the reaction is very low \( (k_4 < 3 \times 10^{-3} \text{s}^{-1}) \) so that breakdown of complex EI* has no effect on the quantitation of the antibiotic if \( t_1 + t_2 << 400 \) min (or more), a condition which is easily fulfilled. The rate of formation of complex EI*, on the other hand, is governed by the ratio \( k_3[I]/K \) (if \( I > K \), which is always the case). In this respect, the \( \beta \)-lactam antibiotics which have been studied fall into two groups (4, 7). The antibiotics of group I (ampicillin, benzylpenicillin, phenoxymethylpenicillin, cephalosporin C, cephaloylglycine, and cephalothin) characterize themselves by very high \( k_3/K \) values \((\geq 50,000 \text{M}^{-1} \text{s}^{-1} \text{at} 37^\circ \text{C})\). The antibiotics of group II (carbenicillin, methicillin, oxacillin, cloxacillin, and cephalaxin) characterize themselves by moderate \( k_3/K \) values \((\leq 5,000 \text{M}^{-1} \text{s}^{-1} \text{at} 37^\circ \text{C})\). Antibiotics of groups I and II will be examined separately (see below).

**MATERIALS AND METHODS**

Enzymes and coenzyme. The R39 enzyme (molecular weight, 53,000 \( \pm \) 1,000) was prepared as described previously (6) or purchased from UCB Bioproducts, Brussels, Belgium. Horseradish peroxidase, \( \beta \)-amino acid oxidase, and flavin adenine dinucleotide were purchased from Boehringer, Mannheim, West Germany. Rennet (in solution) was from Federa, Brussels.

**Substrate.** \( N^\alpha,N^\beta\)-Diacetyl-L-lysyl-L-alanyl-D-alanine (Ac-L-Lys-D-Ala-D-Ala) was obtained from UCB.

**Reagents.** \( o \)-Dianisidine was from E. Merck AG, Darmstadt, West Germany (no. 2963), and \( o \)-dianisidine sulphate was a gift from P.E. Reynolds, Department of Biochemistry, Cambridge, United Kingdom. Merck-oquant-peroxidase strips (no. 10011) were from Merck.

**\( \beta \)-Lactam antibiotics.** Benzylpenicillin was ob-
tained from Rhône-Poulenc, Paris, France, or from UCB; ampicillin and oxacillin were from Bristol Ben-
elux S.A., Brussels; carbenicillin, cloxacillin, methicil-
ilin, and amoxyccillin were from Beecham Research Laboratories, Brentford, United Kingdom; cephal-
olin, cephaloridin C, cephaloglycine, and cepha-
lexin were from Eli Lilly and Co., Indianapolis, Ind. Phenoxymethylpenicillin was a gift from H. Vander-

Estimation of residual enzyme activity. The reaction catalyzed is \( \text{Ac}_{2}-\text{L-Lys}-\text{d-Ala}-\text{d-Ala} + \text{H}_2\text{O} \rightarrow \text{Ac}_{2}-\text{L-Lys}-\text{d-Ala} + \text{d-Ala} \). The kinetic parameters are: \( K_m = 0.1 \text{ mM} \); \( V_{max} = 19 \mu\text{mol of tripeptide hydrolyzed min}^{-1} \) (mg of protein) \(^{-1} \). The released d-alanine was estimated by the d-amino acid oxidase-peroxidase-o-
dianisidine technique. The free d-alanine is trans-
formed into pyruvate and \( \text{H}_2\text{O} \) by the d-amino acid oxidase-flavin adenine dinucleotide system, and the o-
dianisidine is oxidized by \( \text{H}_2\text{O}_2 \) into a chromogenic
compound (at \( \lambda_{max} = 460 \text{ nm} \)) under the action of the
peroxidase. In some cases, the technique was per-
formed exactly as described previously (5), except that
because of the presence of \( \text{Mg}^{2+} \) ions in the reaction
mixtures, the potassium pyrophosphate buffer was
replaced by 0.1 M tris-(hydroxymethyl)aminomethane-
hydrochloride buffer, pH 8.3 (procedure A). In other
cases (procedure B), the procedure was further
modified as follows: (i) a twofold- and a fivefold-increased
amount of d-amino acid oxidase and peroxidase, re-
spectively, were used; (ii) the solution of o-dianisidine
in methanol was replaced by a solution of o-dianisidine
sulphate in water (5 mg/ml); and (iii) a 2% sodium
dodecyl sulphate solution in water (300 ml) was used
instead of the methanol-water solution just before
estimating the absorbance of the solution at 460 nm.
Finally, for semiquantitative assays, the amount of
\( \text{H}_2\text{O}_2 \) produced from d-alanine by the d-amino acid
oxidase-flavin adenine dinucleotide system was
roughly estimated with the help of Merckquant-per-
oxide strips.

RESULTS

Estimation of antibiotics of group I in
aqueous solution. The antibiotics of group I, in
amounts ranging between 0.5 and 2.5 pmol,
were estimated by using 2.5 pmol of R39 enzyme
in a final volume of 100 \( \mu\text{l} \) of 50 mM N\(^{-2}\)-hydroxethylpiperazine-N\(^{-2}\)-ethanesulfonic acid
(HEPES) buffer (pH 8.3) containing 0.1 M NaCl
and 5 mM MgCl\(_2\). At 37°C, formation of complex
EI* was complete within 10 to 15 min. Since \( I_0 \)
\( \leq E_0 \) (where \( I_0 \) and \( E_0 \) are the concentra-
tions of antibiotic and enzyme, respectively, at \( t = 0 \)),
the amount of complex EI* formed is virtually
equal to the amount of \( I_0 \), i.e., to \( E_0 (1 - A/A_0) \),
where \( A_0 \) and \( A \) are the initial and residual
enzyme activities, respectively. As an example,
Fig. 1 shows the results obtained with benzyl-
penicillin (for experimental details, see legend to
the figure). The whole operation is achieved in
about 30 min.

A concentration of 5 to 10 pmol (i.e., about 2
to 4 ng) of antibiotic per ml was the lower limit
for quantitative (and rapid) determinations.
However, the technique permits detection of the
antibiotics at much lower concentrations. To
illustrate the high sensitivity of the method, Fig.
2 shows the results obtained with samples con-
taining 0.05 to 0.25 pmol of various antibiotics
(in 100 \( \mu\text{l} \) of buffer containing 0.25 pmol of R39
enzyme). At these extremely low concentrations,
the assays remain specific but provide only
semiquantitative results. Moreover (see legend
to Fig. 2), the whole operation requires at least
180 min.

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**Fig. 1. Residual dd-carboxypeptidase activity of the R39 enzyme (0.12 pg) after reaction at 37°C with 0.2 to 1.0 ng of benzylpenicillin in 100 \( \mu\text{l} \) of 50 mM HEPES buffer (pH 8.3) containing 0.1 M NaCl and 5 mM MgCl\(_2\). Samples (90 \( \mu\text{l} \)) containing 2 to 10 ng of benzylpenicillin per ml (and 90 \( \mu\text{l} \) of water used as control) were supplemented with 10 \( \mu\text{l} \) of 500 mM HEPES buffer (pH 8.3) containing 1.0 M NaCl, 50 mM MgCl\(_2\), and 2.5 pmol (i.e., 0.12 pg) of R39 enzyme and incubated for 10 min at 37°C. The reaction mixtures were then supplemented with 75 nmol of \( \text{Ac}_{2}-\text{L-Lys}-\text{d-Ala}-\text{d-Ala} \) (5 \( \mu\text{l} \), in water) and incubated at 37°C for 10 min. The released d-alanine was finally
determined by the d-amino acid oxidase-peroxidase-
o-dianisidine technique (procedure A).**

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**Fig. 2. Residual dd-carboxypeptidase activity of the R39 enzyme (0.012 pg) after reaction at 37°C with 0.02 to 0.1 ng of benzylpenicillin (O), ampicillin (E),
cephaloridin C (●), and phenoxymethylpenicillin (■) in 100 \( \mu\text{l} \) of 50 mM HEPES buffer (pH 8.3) containing 0.1 M NaCl and 5 mM MgCl\(_2\). The experi-
mental conditions were the same as those described
in the legend to Fig. 1, except that both the time \( (t_1) \)
of interaction between the enzyme and the antibiotic
and the time \( (t_2) \) of incubation of the substrate with
the residual enzyme were 90 min (instead of 10 min).**
Estimation of antibiotics of group I in human sera and fresh cows' milk. The estimation of antibiotics of group I occurring in picomole amounts per milliliter of human serum and cows' milk requires prior treatments of the fluids. As observed previously (10), low quantities of control serum (0.5 to 2 µl) apparently enhance the Dd-carboxypeptidase activity of the R39 enzyme, whereas large quantities strongly inhibit the coupling D-amino acid oxidase-peroxidase system. Similarly, the activity of the R39 enzyme appears to be strongly inhibited by at least some milk samples.

Serum samples (50 µl) containing 10 to 50 pmol of antibiotic per ml (and a serum control without antibiotic) were supplemented with acetone (100 µl). After stirring, the mixtures were centrifuged for 15 min at 5,000 rpm, the supernatant fractions were rapidly evaporated under vacuum, and the residues were treated as described in the legend to Fig. 3. Similar results were obtained with antibiotics other than benzylpenicillin. A double-blind test was also performed on a series of 30 samples containing from 6 to 20 ng of benzylpenicillin per ml of serum. The average error was 12%, and the largest individual error was 34%.

Milk samples containing 25 to 250 pmol of antibiotic per ml (and a milk control) were supplemented with an equal volume of ethanol. After elimination of the precipitate by centrifugation, 40-µl samples of the supernatant fractions were evaporated under vacuum, and the residues were treated as described in the legend to Fig. 4. Alternatively, the milk samples (1.5 ml) were supplemented with 100 µl of the rennet solution. Clotting was achieved within 5 min at 37°C. After elimination of the precipitate, 20-µl samples of the supernatant fractions were treated as described in the legend to Fig. 4.

The usefulness of this method is not confined to these examples. They are meant to illustrate how the proposed enzymatic procedure can be adapted to estimate β-lactam antibiotics of group I occurring in sera and milk at concentrations as low as 5 and 25 ng/ml, respectively. Depending on the antibiotic concentrations and the precision required, the technique is susceptible to many variations, as shown by the following two cases.

Precipitation with acetone of serum samples containing 10 to 50 pmol of antibiotic per ml could be avoided for semiquantitative assays. Serum samples (50 µl) and a serum control were supplemented with 50 µl of 100 mM HEPES buffer (pH 8.5) containing 0.2 M NaCl, 10 mM MgCl₂, and 2.5 pmol (i.e., 0.12 µg) of R39 enzyme. The reaction mixtures were successively incubated for 20 min at 37°C, supplemented with 150 nmol of Ac₂-L-Lys-D-Ala-D-Ala (5 µl), incubated for 30 min at 37°C, supplemented with 60 µl of a solution containing 10 ng of D-amino acid oxidase and 3 µg of flavin adenine dinucleotide and 30 µl of a solution containing 10 ng of D-amino acid oxidase and 3 µg of flavin adenine dinucleotide without the individual enzyme

FIG. 3. Estimation of benzylpenicillin in human serum. Samples (50 µl) of control serum (without benzylpenicillin) and of sera containing 10 to 50 pmol of benzylpenicillin per ml were treated with acetone, and the supernatant fractions were evaporated (see text). The residues were dissolved in 40 µl of 50 mM HEPES buffer (pH 8.3) containing 0.1 M NaCl, 5 mM MgCl₂ and 2.5 pmol (i.e., 0.12 µg) of enzyme. The reaction mixtures were incubated for 10 min at 37°C, supplemented with 75 nmol of Ac₂-L-Lys-D-Ala-D-Ala (5 µl), and incubated at 37°C for 10 min. The amount of δ-alanine released was then estimated by the D-amino acid oxidase-peroxidase-o-dianisidine technique (procedure B).

FIG. 4. Estimation of benzylpenicillin in fresh cows' milk. Samples of control milk (without benzylpenicillin) and of milk containing 25 to 250 pmol of benzylpenicillin per ml were treated either with an equal volume of ethanol and 40-µl samples of the supernatant fractions were evaporated (see text), or with rennet (using 1 vol of rennet solution for 15 vol of the milk sample) and 20-µl samples of the supernatant fractions were collected (see text). The dry residues (after ethanol treatment) (Fig. 4A) and the 20-µl samples (after rennet treatment) (Fig. 4B) were successively (i) supplemented with 30 µl of 50 mM HEPES buffer containing 0.1 M NaCl and 50 mM MgCl₂ and then with 10 µl of a solution containing 0.16 µg of R39 enzyme, (ii) incubated for 10 min at 37°C, (iii) supplemented with 75 nmol of Ac₂-L-Lys-D-Ala-D-Ala (5 µl), and (iv) incubated for 30 min at 37°C. The released δ-alanine was finally estimated by the D-amino acid oxidase-peroxidase-o-dianisidine technique (procedure B).
in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.3); and, finally, incubated for 2 min at 37°C. A Merckoquant-peroxidase strip was then immersed in each solution for 2 to 3 s, removed, and maintained at room temperature for 5 to 10 s. With the control serum, the strips turned blue-grey. With sera containing 50 pmol or more of antibiotic per ml, the strips remained yellow-pink, indicating that all the enzyme had been immobilized by the antibiotic in the form of complex EI*. Sera containing less than 50 pmol but at least 10 pmol of antibiotic per ml gave rise to intermediate colors.

Evaporation of the supernatant fractions obtained after acetone precipitation of the serum samples could be avoided if these samples contained 0.6 to 3.0 nmol (i.e., 0.25 to 1.20 μg) of antibiotic per ml. Samples (1 to 5 μl) of the supernatant fractions were diluted to 90 μl with water, and the assays were performed as described in the legend to Fig. 1. Quantitative and reproducible results were obtained.

Estimation of antibiotics of group II. When compared with the antibiotics of group I, and as a consequence of the moderate $k_3/K$ values (5,000 M$^{-1}$ s$^{-1}$ at 37°C), formation of complex EI* with the antibiotics of group II should require, to be complete, a 10-fold increased incubation time ($t_i$) between the antibiotic and the R39 enzyme. However, since an upper limit is imposed on $t_i$ by the condition $t_1 + t_2 \leq 0.69/k_3$, the lower limit of detection of the group II antibiotics is, in practice, 10-fold higher. It thus follows that with the antibiotics of group II, $I_0 >> E_0$ and that the reaction is stopped long before formation of complex EI* is complete. Human sera containing 20 to 80 ng of oxacillin, cloxacillin, or cephalaxin, 40 to 200 ng of methicillin, or 80 to 400 ng of carbenicillin per ml were processed as described in the legend to Fig. 3, except that the incubation of the antibiotic with the enzyme was for 30 min, and the subsequent incubation with $Acp L-Lys-D-Ala-D-Ala$ was for 20 min. Figure 5 illustrates the results obtained with cephalaxin.

FIG. 5. Estimation of cephalaxin in human serum. For conditions, see text and legend to Fig. 1.

DISCUSSION
The enzymatic procedure described in this paper allows the estimation of nanogram amounts of β-lactam antibiotics and permits many simultaneous tests to be carried out in a short time. It is specific for β-lactam antibiotics, and its specificity can be easily checked by studying the effect of β-lactamase action on the samples to be analyzed. The assay, however, does not discriminate between various β-lactam antibiotics, and its sensitivity for a given antibiotic depends on the $k_3/K$ value (see above) which characterizes the interaction between the R39 enzyme and the antibiotic under consideration. It thus follows that depending on the $k_3/K$ value, the amount of antibiotic or the size of the sample to be analyzed must be adapted accordingly. This point has been illustrated by using antibiotics for which the $k_3/K$ value is very high (≥50,000 M$^{-1}$ s$^{-1}$) or moderate (about 5,000 M$^{-1}$ s$^{-1}$). Novel β-lactam compounds such as meccillinam and clavulanate have very low $k_3/K$ values (30 to 50 M$^{-1}$ s$^{-1}$ at 37°C) (J. Kelly, J. M. Frère, D. Klein, and J. M. Ghuysen, manuscript in preparation), so that the sensitivity of the assay is considerably decreased. In principle, however, microgram amounts of such compounds could also be estimated. Finally, difficulties may be encountered with complex biological fluids. The procedure has been applied to human serum and cow milk. When compared with the other DD-carboxypeptidases so far identified, the R39 enzyme characterizes itself by extremely high $k_3/K$ values for many β-lactam antibiotics and extremely low $k_3$ values for all the antibiotics tested, which make it especially suitable for the type of enzymatic assay discussed here.

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LITERATURE CITED


