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Novel Method for Detection of β-Lactamases by Using a Chromogenic Cephalosporin Substrate

CYNTHIA H. O'CALLAGHAN, A. MORRIS, SUSAN M. KIRBY, AND A. H. SHINGLER
Glaxo Research Ltd., Greenford, Middlesex, England

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A new cephalosporin with a highly reactive β-lactam ring was found to give an immediate color change in the presence of β-lactamases from many bacteria, including staphylococci, *Bacillus* species, *Enterobacteriaceae*, and *Pseudomonas*. The reaction is confined to organisms producing β-lactamases, but it is sufficiently sensitive to indicate the presence of this enzyme in small amounts in strains previously considered not to produce it. The compound has an unusual ultraviolet spectrum, and the color change can be followed quantitatively by measuring changes in absorption which occur in the 380- to 500-nm region, where cephalosporins normally have no absorption. The development of color is thought to be a consequence of the β-lactam ring being unusually highly conjugated with the 3-substituent. Although in the bacteria only β-lactamases produce this color change, it was found that serum and tissues from experimental animals also rapidly produced the colored breakdown product, which was then excreted in the urine. The mechanism of the mammalian breakdown was considered to be different from that found in bacteria.

There are several techniques available for the detection and estimation of β-lactamases (2). Some are normally used quantitatively, and others can also be applied as qualitative spot tests. None of these methods is entirely satisfactory and, for carrying out spot tests, there is a requirement for a new reliable and sensitive diagnostic agent. This report describes the properties of such a substance. In the course of a screening program, a new cephalosporin was made: 3-(2,4-dinitrostyryl)-(6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer. It will be referred to here under the code number 87/312, and has the structure shown in Fig. 1.

The compound is one of a group of related cephalosporins which undergo a distinctive color change when hydrolyzed by β-lactamases. It has been investigated to see whether it might be a useful reagent for the detection of β-lactamase-producing organisms and whether it could be of use for both qualitative and quantitative determinations.

MATERIALS AND METHODS

Cultures. A wide range of bacterial isolates was obtained from the London Hospital, St. Bartholomew's Hospital, St. Thomas's Hospital, the Postgraduate Medical School, London, and the Radcliffe Infirmary, Oxford, England. They were all maintained on Oxoid Nutrient Agar; cells for experimentation were grown either in Oxoid Nutrient Broth or on Oxoid Nutrient Agar at 37 C for 24 hr.

Spectrophotometric method for determination of β-lactamase activity. The method used was adapted from one described previously using cephaloridine as a substrate (9), in which the rate of breakdown of this substrate in the presence of β-lactamase was determined by measuring the rate of change of the ultraviolet absorption associated with the β-lactam ring. In aqueous solution at *p*H 7, 87/312 has two main absorption peaks, one at 217 nm and one at 386 nm (Fig. 2). The peak at 217 nm is associated with the 7-acyl group, and no change in absorbance occurred after the enzymatic hydrolysis. The peak at 386 nm decreased and, simultaneously, a new peak appeared at 482 nm. Using the Unicam SP 800 recording spectrophotometer (Pye-Unicam Ltd., Cambridge, England) with a 1-cm cell, we found the most convenient concentration of 87/312 to be 10^{-4} M, i.e., 51.6 μg/ml. At this concentration, the optical density (OD) at 386 nm decreased from 1.75 to 0.72, a change of 1.03, and the OD at 482 nm increased from 0.15 to 1.74, a change of 1.59, when 87/312 was completely destroyed by a β-lactamase (Fig. 2). There was also a change in spectrum and color when 87/312 was attacked by serum; in this case, the decrease at 386 nm was the same, but the new peak was formed at 510 nm instead of 482 nm. At this wavelength, the maximal change observed was from 0.12 to 1.74 for a solution of 10^{-4} M. The shift in wavelength is due to serum binding (Fig. 3).

The enzyme reaction was carried out at 37 C in a

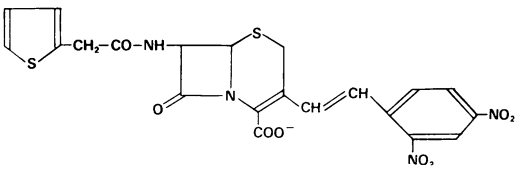

FIG. 1. *Structure of 87/312.*

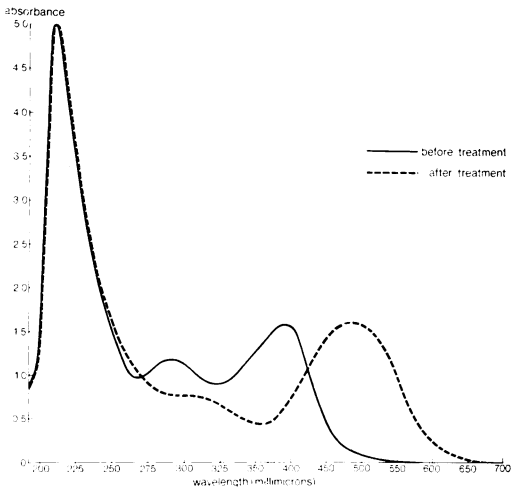

FIG. 2. *Absorption spectrum of 87/312 (10^{-4} M) before and after enzyme treatment.*

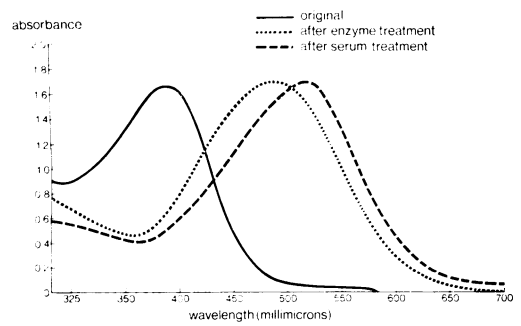

FIG. 3. *Effect of a β-lactamase and serum on the absorption of 87/312 (10^{-4} M).*

1-cm cell, which contained a total volume of 3 ml. This consisted of 0.05 M phosphate buffer at *p*H 7 containing 51.6 μg of 87/312/ml and a suitable concentration of enzyme. Most of the enzyme preparations were crude or only partially purified; they sometimes required dilution to enable the enzymatic hydrolysis to be carried out at a measurable rate. Enzyme concentrations at which the reaction was complete in about 5 min were found to be the most convenient, and the change in OD at either wavelength was measured over the first 2 min. Activity of the enzymes was defined as micromoles of substrate destroyed per minute per milliliter of enzyme at 37 C at *p*H 7.0. At 386 nm,

$$\text{enzyme activity} = \frac{x}{1.03} \times 0.3 \times v \ \mu\text{mole destroyed per min per milliliter of enzyme}$$

where x is the ΔOD/minute observed, 0.3 is the number of micromoles of 87/312 present, and v is the factor adjusting the enzyme volume to 1 ml. Similarly, at 482 nm,

$$\text{enzyme activity} = \frac{y}{1.59} \times 0.3 \times v ,$$

where y is the ΔOD/minute observed at 482 nm.

Michaelis constants and substrate specificity. Michaelis constants (K_m) and relative specific activities at substrate saturation were obtained from Lineweaver-Burk plots (6).

Preparation of enzymes. The enzyme produced by *Bacillus cereus* was a commercial sample (A.V.M. Laboratories Ltd., Wrexham, Denbighshire, U.K.). K1, P99, and TEM enzymes were partially purified as described previously for P99 (9) after the sonic treatment and ultracentrifugation stages; the enzymes from *Pseudomonas aeruginosa* and *Staphylococcus aureus* were passed down a Sephadex G-50 column but were not purified further.

Protein determination. Protein was determined by the method of Lowry (7).

RESULTS

Color produced by bacterial cultures. During routine investigation of the antibacterial activity of cephalosporin analogue 87/312, it was observed that broths of most organisms growing in subinhibitory concentrations of the substance were the normal pale-beige color. However, when known β-lactamase-producing gram-negative organisms grew in subinhibitory concentrations, the broths were colored red. The intensity of the color in the tubes was related to the original concentration of 87/312. No color was observed under these conditions with β-lactamase-producing gram-positive bacteria, but most of these organisms were inhibited by comparatively low concentrations of 87/312.

When a few drops of a solution of the compound at 500 μg/ml were added to a grown broth of a penicillinase-producing strain of *S. aureus*, a red color was produced at once, but no color was formed by strains known to be non-penicillinase-producing. The antibacterial activity of 87/312 and the correlation between β-lactamase activity of the organisms and production of color is given in Table 1.

It was observed that the red color could also be produced when a solution of 87/312 was dropped onto colonies of β-lactamase-producing

TABLE 1. *Antibacterial action of 87/312*

Organism	Minimal inhibitory concn (μg/ml)	Color change
Staphylococcus aureus 663	0.05	−
S. aureus 3452[a]	3.1	−[b]
Escherichia coli 573	62	±
E. coli TEM[a]	>250	++
Enterobacter cloacae P99[a]	>1,000	++++
Proteus mirabilis 431	125	−
P. morganii NCTC 235[a]	250	+++
Salmonella typhimurium 804	62	−
Pseudomonas aeruginosa 150[a]	>1,000	+++
Haemophilus influenzae 1184	31	−

[a] β-Lactamase-producing organism.
[b] Color produced when 87/312 was added to the grown culture.

organisms growing on agar plates. Color production was almost immediate with powerful β-lactamase producers, but a further period of incubation of some 30 min was required with weak producers. Unlike the case with broth cultures, the red color was fugitive and disappeared after 2 to 3 hr. When the colonies on the plate were a mixture of producers and nonproducers of β-lactamase, e.g., with *Enterobacter cloacae* P99 and a non-lactamase-producing mutant, the mutant was easily distinguished from its enzyme-producing parent by the difference in color of the two types of colony in the presence of 87/312.

Absorption spectrum of 87/312. In general, cephalosporins have an absorption maximum of ca. 260 nm, which is associated with the intact β-lactam ring. Cephalosporins with a thienylacetyl 7-acyl group also have an absorption associated with this moiety at ca. 237 nm. They do not usually have an absorption in other regions of the spectrum. Examination of 87/312 in a recording spectrophotometer showed that this cephalosporin had an extra absorption maximum at 386 nm, in addition to its normal absorption maxima in the ultraviolet. The absorption maxima associated with compounds related to dinitrostyrene are in the region of ca. 320 nm. The absorption of 87/312 at 386 nm was therefore attributed to the dinitrostyrene substituent at position 3, with a bathychromic shift due to the strong conjugation of the β-lactam ring and the double bond in the dihydrothiazine ring with the dinitrostyryl group (Fig. 2 and 3).

Effect of bacterial β-lactamase preparations on 87/312. A partially purified preparation of the enzyme from *E. cloacae* P99 (9) was incubated with 87/312 at 10^{-4} M in a recording spectrophotometer. As the red color developed, the peak at 386 nm disappeared; it was replaced by a new maximum at 482 nm. The same effect occurred when partially purified preparations from *S. aureus*, *Klebsiella aerogenes* (*K. pneumoniae*), or *P. aeruginosa* were used, or with centrifuged broken cell preparations from *Proteus morganii* or *Bacillus cereus* (Fig. 2).

Using the rate of decrease in absorption at 386 nm, Michaelis constants and reaction velocities were calculated for several enzymes (Table 2), but, since pure enzymes were not used, the specific activities are not truly representative.

The relationship between enzyme concentration and Δ OD at 386 nm and Δ OD at 482 nm is shown in Fig. 4. At 386 nm, the relationship is linear over the range from 0.01 to 0.1 unit and at 482 nm over the range from 0.006 to 0.2 unit. At lower concentrations, the reaction proceeds too slowly and at higher concentrations too quickly to allow accurate determinations to be made. Enzyme activity is expressed as micromoles of 87/312 destroyed per minute per milliliter of enzyme at 37 C and *p*H 7.0. At 386 nm, 1 unit causes a Δ OD of 1.0 per minute and at 482 nm a Δ OD of 1.7 per minute.

Figure 4 shows that 87/312 is more susceptible to the enzyme than is cephaloridine.

When selected enzymes were treated with known enzyme inhibitors of the penicillin or cephalosporin type, the color change was affected in the way expected for that enzyme. For example, cloxacillin and methicillin prevented the formation of red color with P99 enzyme and with TEM, but had no effect with K1. This is in agreement with the relative sensitivities of these enzymes to cloxacillin reported earlier (4), and is consistent with the color change being an immediate consequence of β-lactamase action on the compound.

TABLE 2. *Kinetics of hydrolysis of 87/312 by various β-lactamases*

Source of β-lactamase	K_m (μM)	V_{max}[a]
Enterobacter cloacae P99	303	148
Klebsiella aerogenes K1	55.5	81
Escherichia coli TEM	136	1.06
Pseudomonas aeruginosa DAL	83	0.89
P. aeruginosa SSA	116	0.77
Staphylococcus aureus PC28	116	6.2
Bacillus cereus A.V.M.	104	0.72

[a] Micromoles per minute per milligram of protein.

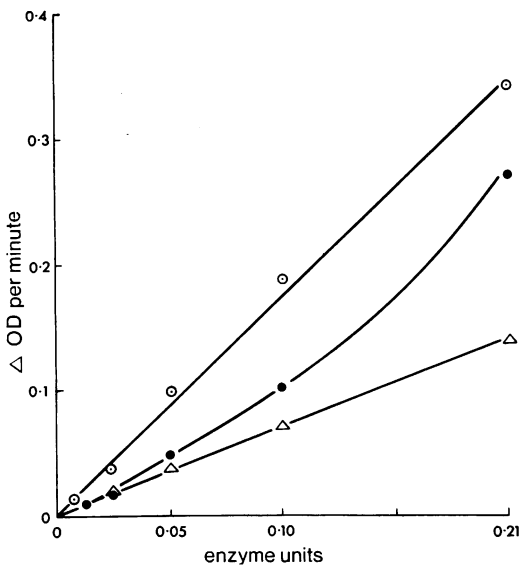

FIG. 4. *Relationship between change in absorption and enzyme concentration. Symbols:* ●, *change at 386 nm of 87/312;* ⊙, *change at 482 nm of 87/312;* ▲, *change at 255 nm of cephaloridine.*

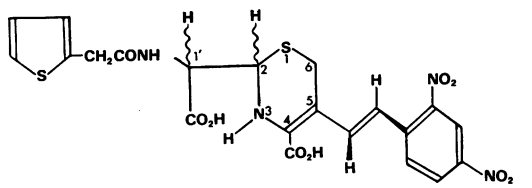

FIG. 5. *Structure of the red decomposition product of 87/312, isolated after pretreatment with a β-lactamase.*

Identification of the colored product. The colored compound was subsequently isolated and identified as the corresponding cephalosporanoic acid, with the structure shown in Fig. 5.

The structure assigned to the red compound was based on data from infrared spectrum, nuclear magnetic resonance spectrum, and microanalysis. This compound had very poor antibacterial activity.

A solution of 87/312 (500 mg) in 0.1 M phosphate buffer, *p*H 7 (450 ml), and dimethyl sulfoxide (50 ml) was incubated for 2 hr at 37 C with sufficient K1 enzyme to give 40 international units of activity. The resulting dark-violet solution was washed with ethyl acetate (250 ml), covered with ethyl acetate (250 ml), and acidified to *p*H 4 with 2 N hydrochloric acid. The organic phase was separated, the solvent was evaporated in vacuo, and the residue was triturated with ether (25 ml) to give a dark-red solid (213 mg), melting point, 140 to 148 C (decomposition); λ_{max} (0.1 M phosphate buffer, *p*H 6), 236 nm (ε 20,600) and 491 nm (ε 15,800), inflexion at 296 nm (ε 8,700). The infrared spectrum of this product indicated the absence of the β-lactam chromophore, and the nuclear magnetic resonance spectrum, in hexadeuterodimethyl sulfoxide, in addition to signals associated with the protons of the thienylacetamido- and *trans*-2,4-dinitrostyryl functions [at τ 1.33, 1.59, and 2.09 (−2,4-dinitrophenyl), 1.76 and 3.22 (*trans*-CH—CH, two doublets, J 16 N_z), 2.66 and 3.06 (thienyl), and 6.26 (CH_2CONH)], exhibited a two-proton singlet at 6.36 τ, assigned to the $C_{(6)}$-methylene protons of a 2,3-dihydro-6H-1,3-thiazine ring, and a two-proton multiplet centered on 5.3 τ, assigned to the $C_{(1')}$ and $C_{(2)}$ protons of the "cephalosporanoic acid" derivative shown in Fig. 5.

Microanalysis. Found: C, 46.0; H, 3.4; N, 9.9; S, 11.8. Calculated for $C_{21}H_{18}N_4O_9S_2$ 2/3 H_2O: C, 46.05, H, 3.55; N, 10.0; S, 11.7%.

Further evidence in support of this structure was provided by esterification of this product, with diazomethane, which yielded the corresponding dimethyl ester. This ester had a melting point of 210 to 216 C (decomposition); λ_{max} (ethanol), 237 nm (ε 22,300) and 447 nm (ε 15,600); infrared and nuclear magnetic resonance spectra were in agreement with the above structure.

Microanalysis. Found: C, 49.1; H, 4.1; N, 10.0; S, 11.2. Calculated for $C_{23}H_{22}N_4O_9S_2$: C, 49.1; H, 3.95; N, 9.95; S, 11.4%.

The nuclear magnetic resonance spectra of both the "cephalosporanoic acid" and the dimethyl ester were not sufficiently well resolved to permit determination of the configuration across the $C_{(1')}$–$C_{(2)}$ bond.

Effect of administration of 87/312 to experimental animals. Several A_2G female mice received one subcutaneous dose of 250 mg of 87/312 per kg. They were kept in metabolism cages arranged for the collection of urine and were observed at intervals. The exposed skin of the animals in the tail, feet, and mouth soon became noticeably more pink than in untreated mice, and the urine passed was deep red in color. The animals were killed and examined postmortem. The injection site was deeply colored, and the skin of the body wall was much deeper pink than in a control animal. All of the abdominal contents, apart from the stomach, were strongly colored, and the intestinal contents in all parts were a deep brownish-red. Metabolism to a colored compound had obviously occurred very soon after administration; it was observed that this was also being excreted in the bile.

Effect of serum on 87/312. Serum from mouse, rat, rabbit, horse and man was incubated with 10^{-4} M 87/312 (51.6 μg/ml). Even at a dilution of

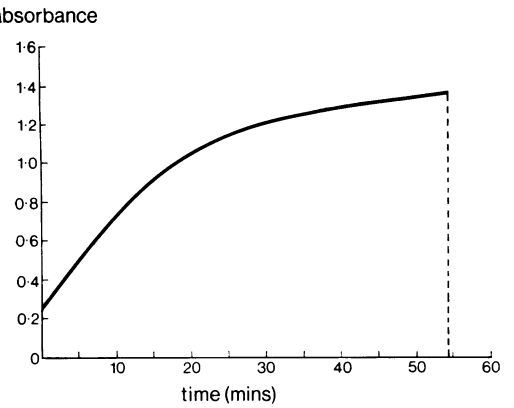

FIG. 6. *Change in absorption at 510 nm of 87/312 in the presence of rabbit serum (diluted 1:4).*

1:4 in 0.05 M phosphate buffer, the serum rapidly produced the red color. Under these conditions, serum has very little absorption in the 370- to 550-nm range, so that the change in absorption could be measured in a spectrophotometer. The absorption maximum characteristic of 87/312 at 386 nm rapidly diminished, and a new broader peak appeared at 510 nm (Fig. 3). The rate of reaction with rabbit serum at 1:4, with 87/312, was estimated by using the absorbance at 510 nm. The reaction proceeded rapidly at first, with 50% of the 87/312 being converted into the red compound in 13 min, but the conversion was not complete in 50 min (Fig. 6).

The serum reaction was also carried out in the presence of a number of penicillin and cephalosporin analogues known to be inhibitors of bacterial β-lactamases, to exclude the possibility that an enzyme of bacterial origin might be present in the serum. None of the inhibitors had any effect on the reaction of the serum with 87/312. Addition of serum to the purified red compound produced by β-lactamase action resulted in an immediate shift of the λ_{max} from 482 to 510 nm. The characteristics of this solution on gel filtration on Sephadex G-50 indicated that the red compound was strongly bound to serum proteins (G. W. Ross, *personal communication*).

Effects of other substances. A solution of 100 μg of 87/312 per ml was incubated with a variety of amino acids and chemical compounds at 1 mg/ml, and with various biological fluids. The substances tested and the results obtained are given in Table 3. Relatively few of the materials examined produced a color change; of the simpler chemical compounds, those which had an effect contained a free thiol group, although not all compounds with such a group could produce color. Reducing agents such as ascorbic acid and oxidizing agents such as hydrogen peroxide were also negative but color was produced by strong alkali at *p*H in excess of 10. This color was not stable, rapidly turning to purple and then black.

There was some effect from egg white, pasteurized milk, and denatured serum, but none from urine, egg yolk, or serum globulin.

TABLE 3. *Effect of various substances on 87/312*

Substance	Amt and rate of color production[a]
Whole serum	++++
Denatured serum	+++
Serum albumin	+++
Serum globulin	−
Egg white	+
Egg yolk	−
Milk	+
Urine	−
Amino acids (not cysteine)	−
Cysteine	+
Glutathione	++
Mercaptoethanol	+++
Dimercaprol	++++
Thioglycolic acid	trace
Ascorbic acid	−
Hydrogen peroxide	−
Alkali (*p*H 10)	++++
Acid (*p*H 2)	−
2-Mercaptopyridine	−
4-Mercaptopyrimidine	−
Thiophenol	−

[a] Symbols: ++++, color production rapid and intense; +, traces of color produced slowly; −, no color change.

DISCUSSION

The cephalosporin described is unusual in that it is a colored compound; the solid is yellow, and it gives a yellow solution when made up in water. It has an unusual absorption spectrum with an extra band of absorption with its λ_{max} at 386 nm; this is attributed to the high degree of conjugation in the substituent in the 3-position, and the further conjugation of this group with the β-lactam ring and the double bond in the dihydrothiazine ring. This conjugation is thought to make the β-lactam ring unusually reactive, and the compound thus rapidly breaks down in the presence of β-lactamases from a variety of bacteria. Even the enzyme from *S. aureus* rapidly attacked it, although most cephalosporins are highly resistant to staphylococcal β-lactamase (8).

The disruption of the β-lactam ring was accompanied by a change in color of the compound from yellow to red. Although rupture of the β-lactam ring in cephalosporins is always accompanied by loss of the ultraviolet absorption in the 260-nm region, a change in the spectrum occurring in the visible region is unusual. Other ceph-

alosporins conjugated in this way gave a similar effect, but if the 3-substituent did not carry a further chromophore, in this case two nitro groups, then the change in the spectrum was not visible and went undetected unless deliberately sought. Color production with a different type of cephalosporin has also been reported (5), but the reaction with that compound was much slower and less sensitive than with 87/312.

The reaction product is closely related to the starting material, the only difference being the addition of one molecule of water across the β-lactam ring. With 87/312, the group at position 3 was not immediately expelled from the molecule, unlike cephalosporins with a 3-substituent able to accept an electron, in which the 3-substituent is lost simultaneously with rupture of the β-lactam ring (10; O'Callaghan et al., *in preparation*). The color produced by bacterial β-lactamase action on 87/312 was found to be comparatively stable in aqueous solution, and the half-life of the isolated and purified reaction product was about 18 hr. There was, however, much more rapid fading of the color in contact with Nutrient Agar: the nature of this further reaction was not investigated.

Production of color in mice was unexpected, as was the color produced by serum of various species. The activity was associated with the albumin fraction, globulin having no effect. Other materials of biological origin such as egg white and milk could also react with 87/312, but the suspicion that the reaction was enzymatic seemed unlikely to be correct, when it was found that even boiled serum was effective and that other, much simpler substances could also produce the color. Simple thiols in particular could turn a solution red, but cysteine alone among the amino acids had this effect. A tripeptide, glutathione, produced the color and there is sufficient glutathione in animal serum and tissues to account for the reaction which was seen, but not to account for its velocity.

Not all thiols were effective, and several aromatic −SH compounds with strong nucleophilic activity were inactive. The reducing action of substances such as cysteine and glutathione could not be implicated, as strong reducing agents, e.g., ascorbic acid, had no effect. The action of the thiol amino acids did not appear to be connected with the action of β-lactamases or with serum, as there are few or no −SH groups in albumin and most of the β-lactamases so far analyzed did not contain cysteine (1, 3).

The possibility that animal and human tissues contain β-lactamase-like enzymes has been under investigation elsewhere for some time. It has recently been reported that albino mice possess such enzymes in their kidneys (11). Initially, it was hoped that 87/312 would facilitate such studies, but the nonspecificity of the compound in vivo limits its use in this field. The red derivative of 87/312 is strongly bound to human serum, and this may help to elucidate the nature of the complex that is formed between some other β-lactam compounds and the albumin (12).

The high susceptibility of the compound to all bacterial β-lactamases so far examined, together with the absence of false-negative results, appears to make 87/312 a very useful reagent for the detection of the enzymes in broth cultures and on agar plates. It can be used qualitatively as a spot-test reagent or quantitatively by measuring changes in its absorption spectrum. In the latter case, it can be used at a minimal level of 10 μg/ml, and the method is more sensitive than other absorption methods because of the greater susceptibility of 87/312 to many β-lactamases. It cannot be used in vivo because of the interfering effect of serum, and care must be taken to exclude the effect of such substances as albumin in media used for growing organisms for test.

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