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# Irreversible Effects of Serum Proteins on Beta-Lactam Antibiotics

CYNTHIA H. O'CALLAGHAN
*Glaxo Research Ltd., Greenford, Middlesex, England*

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The chromogenic cephalosporin nitrocefin (87/312) demonstrates rapid and visible instability to serum from many species. This phenomenon was distinct from serum binding, being significantly slower. Destruction of another cephalosporin, 10485, by serum appeared to account for some anomalous results during investigation into its human pharmacokinetics. Many cephalosporins of very different structures also showed serum instability, unrelated to their degrees of serum binding as measured by plate assay. Extrapolation could not be made from one species to another with regard to either binding or instability. Small changes in the chemical structures of the 3- and 7-substituents of the cephalosporins made profound changes in their susceptibility to serum attack. The decomposition is pH dependent, occurring more slowly at acid pH, and is due to a high-molecular-weight component of serum that resists boiling for several minutes. Isoelectric focusing of serum from several animal species gave various species-specific bands that decomposed nitrocefin. The inactivation of nitrocefin was not entirely parallel with that of 10485 and was inhibited by it. All other β-lactam compounds tested also inhibited the reaction, much greater concentrations usually being necessary when the inhibitor was stable to serum. The complex that causes breakdown of the β-lactam compounds is not necessarily the same as the one causing serum binding. It is postulated that serum may affect most other β-lactam antibiotics in a similar way, although in most cases, this only occurs to a very slight extent.

The binding of chemical substances of small molecular weight to serum proteins by hydrogen bonding modifies their biological activities, sometimes to a considerable extent. Binding to proteins is often considered to be disadvantageous for antibiotics; the greater the degree of binding, the more the antibacterial activity is reduced. The binding shown by clinically available antibiotics is essentially reversible, regardless of the extent to which a compound may be bound. Albumin is usually involved in this binding, and a wide variety of chemical structures are known that bind to it.

Recent work in our laboratories has shown that some penicillins and cephalosporins are bound irreversibly and destructively to serum proteins. Instability to serum has been demonstrated dramatically with the chromogenic cephalosporin nitrocefin (87/312), which rapidly turned a bright cherry red in serum solution (5). At the same time, a new cephalosporin (10485, compound E in Table 4), then undergoing investigations into its human pharmacokinetics in our laboratories, gave unexpectedly poor serum levels and urinary recoveries. These observations led to examination of the stabilities of a range of cephalosporins and other β-lactam compounds in serum from several species, including humans.

## MATERIALS AND METHODS

**Cultures.** The standard laboratory strains used were maintained on Oxoid nutrient agar slopes. Minimal inhibitory concentration determinations were carried out on Casitone agar; pooled human serum was added to the medium in some cases to give a final serum concentration of 5%. The cultures were inoculated with ca. $10^5$ viable organisms and incubated for 18 h at 37°C.

**Microbiological assays.** These were done by the large plate agar diffusion method by using either *Bacillus subtilis* ATCC 6633 in Glaxo Factor B medium (7) or the Oxford strain of *Staphylococcus aureus* in Trypticase soy agar.

**Serum binding.** Either the ultrafiltration method or the plate assay method was used. (i) Ultrafiltration was performed as follows. Serum samples containing added cephalosporin were filtered in an Amicon 8 MC ultrafiltration apparatus. The concentration of cephalosporin in the filtrate was determined by large plate bioassay against the Oxford strain of *S. aureus*. The concentration in the filtrate, calculated as a percentage of the original concentration in the unfiltered sample and subtracted from 100%, gave the percentage bound to serum protein. (ii) Plate assay was performed as follows. Solutions of the test compounds were pre-

pared in either M/20 phosphate buffer (pH 7) or in serum in a series of twofold diminishing concentrations. Both sets of solutions were plated out on large assay plates, and the concentrations in the serum series were estimated in terms of the buffer series. The percentage of discrepancy between the known concentrations and the values obtained experimentally gave an estimate of the degree of serum binding.

**Serum destruction.** Samples of cephalosporin at 200 to 1,000 μg/ml in serum were incubated at 37°C. Samples were removed at intervals up to 6 h and diluted 1:100, and the residual cephalosporin was determined by large plate bioassay against *B. subtilis* or *S. aureus*.

**Isoelectric focusing.** Undiluted serum from several species was focused on thin sheets of polyacrylamide gel containing Ampholine, pH 3.5 to 10 (3). Samples were applied near the anode as drops of liquid on the surface of the gel. Focusing was performed at 200 to 350 V for 48 h. The plates were stained by damping the surface of the gel with nitrocefin (0.5 mg/ml in 0.1 M phosphate buffer, pH 7). After 1 h, pink bands appeared on a yellow background. The plates were photographed by using Kodalith Ortho film with transmitted light and a Wratten green filter.

**Spectrophotometric method for determination of rate of decomposition of nitrocefin by serum.** The rate of breakdown of nitrocefin was determined by measuring the rate of increase of absorbance at 510 nm by using the Unicam SP 800 recording spectrophotometer (Pye-Unicam Ltd., Cambridge, England) with a 1-cm cell. The most convenient concentration was $10^{-4}$ M, i.e., 51.6 μg/ml. For such a solution, the maximal change in optical density observed was from 0.12 to 1.74. Human serum was used at a final dilution of 1:8 in the cell.

In the inhibition studies, the inhibiting compound was present in both the experimental and the reference cells at the same concentration; nitrocefin was in the experimental cell only, with a similar volume of buffer added to the reference cell.

**Human pharmacokinetics.** Adult male volunteers received 1 g of the sodium salt of cephalothin, cefuroxime, or cephalosporin 10485 intramuscularly by injection in 5 to 6 ml of Water for Injection. Blood was withdrawn by venepuncture, and urine was collected at intervals. Antibiotic concentration was measured by large plate bioassay against either *B. subtilis* ATCC 6633 (cephalothin and 10485) or *B. subtilis* NCIB 8993 for cefuroxime (8), with serum or buffer standards as appropriate.

## RESULTS

**Unexpected human pharmacokinetics of cephalosporin 10485.** When 10485 was examined in human subjects, the initial serum levels after one intramuscular injection of 1 g (Table 1) were very high, probably because of its very high serum binding, but then fell very rapidly. Assay problems were also encountered, which appeared to be due to instability of the compound in serum.

Microbiological assay showed that whereas 100% of the given dose of cefuroxime (2) appeared in the urine, only 44% of cephalothin and 25% of 10485 were recovered. Deacetylation, with its concurrent reduction in antibacterial activity, accounted for the poor recovery of cephalothin, and high-pressure liquid chromatography of the urine confirmed that the 3-hydroxymethyl metabolite accounted for all the missing material (4) and that therefore there had been no destruction of the basic cephalosporin structure. The missing 75% of 10485, however, could not be accounted for simply by deacetylation; 10485 was much less susceptible to mammalian esterase attack than cephalothin (Table 2), and no desacetyl metabolite was found in the volunteers. This low recovery of 10485 by both methods of estimation suggested that it had undergone a type of decomposition in the

TABLE 1. *Serum levels of cephalosporin 10485 after 1-g intramuscular injection in human volunteers, compared with cephalothin and cefuroxime*

| Compound | No. of subjects | Avg serum concn in μg/ml at h: | | | | % Urinary recovery | |
|---|---|---|---|---|---|---|---|
| | | 0.5 | 1 | 2 | 4 | Bioassay | Total ultraviolet assay |
| 10485 | 4 | 70 | 54 | 19 | <5.0 | 25.3 | 24.0 |
| Cephalothin | 10 | 10.8 | 6.1 | 2.5 | 0.4 | 44.1 | 100.12 |
| Cefuroxime | 5 | 37.5 | 39.1 | 23.0 | 7.5 | 103.4 | 101.00 |

TABLE 2. *Comparative rates of deacetylation of cephalothin and cephalosporin 10485*

| Compound | Antibiotic remaining in μg/ml at h: | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1 | 1.5 | 2 | 4 |
| Cephalothin | 160 | 90 | 69 | 65 | 64 | 61 |
| Control (no liver) | 255 | 250 | 240 | 253 | 240 | 235 |
| 10485 | 193 | 144 | 134 | 121 | 118 | 106 |
| Control (no liver) | 260 | 255 | 260 | 273 | 245 | 253 |

body that had destroyed the cephalosporin structure.

**Effect of 5% serum on the antibacterial activities of 10485 and nitrocefin.** Both compounds had activity against a range of bacteria, but the minimum inhibitory concentrations were adversely affected in the presence of human serum. The results given in Table 3 show that as little as 5% serum in the test medium produced a profound loss in the activity of nitrocefin, particularly against the strains of *S. aureus*. The activity of 10485 was also considerably reduced in the presence of 5% serum.

**Stability of 10485 and other cephalosporins in human, mouse, and rat sera.** The half-lives of 10485 and cephalosporins with several widely differing 7- and 3-substituents on incubation in serum from several species (Table 4) showed no obvious relationship between the degree of serum binding and the susceptibility of a compound to destruction by that serum. In addition, it was not possible to extrapolate from the stability of a compound in the serum of one species to its stability in the serum from another. Selected groups of cephalosporins that had either the 7-acyl group or the 3-substituent in common (Table 5) showed no obvious structural associations with serum instability.

**Some properties of the serum reaction with nitrocefin.** These are summarized in Table 6.

**(i) Effect of pH on the serum reaction.** The rate at which the colored decomposition product was produced in the presence of serum decreased with decrease in pH, and no color change occurred at pH 5.

TABLE 3. *Antibacterial activity of nitrocefin in the presence of 5% human serum*

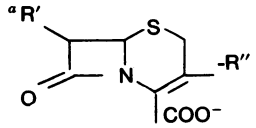
| Organism | MIC[a] in μg/ml in medium | | | |
|---|---|---|---|---|
| | Nitrocefin | | 10485 | |
| | With serum | No serum | With serum | No serum |
| *S. aureus* WH70 | 250 | 4 | 2 | 0.5 |
| 853 | 125 | 1 | 0.2 | 0.025 |
| 1414 | 125 | 0.025 | 0.2 | 0.025 |
| 663 | 31 | 0.025 | 0.2 | 0.025 |
| *Escherichia coli* 851 | >250 | 125 | 16 | 4 |
| *Klebsiella aerogenes* K1⁻ | >250 | 125 | 4 | 1 |
| *Proteus mirabilis* 431 | >250 | 62 | 16 | 4 |

[a] MIC, Minimal inhibitory concentration.

TABLE 4. *Binding and stability of several cephalosporins in sera from humans, mice, and rats*[a]

| | R′ | R″ | Serum from: | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Humans | | Mice | | Rats | |
| | | | % Binding | Half-life (h) | % Binding | Half-life (h) | % Binding | Half-life (h) |
| A. | Phenoxyacetamido | 2-Pyrimidylthiomethyl | 85 | 1 | 64 | 3.5 | 83 | 2.5 |
| B. | Benzylmercapto acetamido | Azidomethyl | 80 | 3 | 80 | >6 | 78 | 5 |
| C. | Phenylglycylamido | Methylene | 33 | 1.5 | 17 | 2 | 38 | >6 |
| D. | 2′-(2-Oxoimidazolidin-1-yl-carboxylamino-2′-(4-hydroxyphenyl)-acetamido | 1:2:3:Triazol-4-ylthiomethyl | 79 | >6 | 12 | >6 | 22 | 2.5 |
| E. | 2(E)-Hydroximino-2-thienylacetamido | Acetoxymethyl | 95 | 1.5 | 38 | >4 | 60 | >4 |
| F. | 2-(Z)-methoximino-2-furyl-acetamido | Carbamoyloxymethyl | 33 | >6 | 20 | >6 | 30 | >6 |
| G. | Thienyl-2-acetamido | 2:4 Dinitro styryl | 100 | <0.5 | ND | <0.5 | ND[b] | <0.5 |

[a]




[b] ND, No meaningful determination possible due to speed of decomposition, but considered to be 100%.

TABLE 5. *Effect of human serum on the stability of cephalosporin analogs with related 7- and 3-substituents*[a]

| | R′ | R″ | % Binding | Half-life (h) |
|---|---|---|---|---|
| E. | 2(E)-Hydroximino thienyl-2-acetamido | Acetoxymethyl | 95 | 1.5 |
| H. | 2(E)-Methoximino thienyl-2-acetamido | Acetoxymethyl | 62 | >6 |
| J. | 2(E)-Hydroximino thienyl-2-acetamido | (5-methyl 1:3:4-thiadiazol-2-yl) thiomethyl | 90 | 1.5 |
| K. | 2(E)-Hydroximino thienyl-2-acetamido | Mercaptobenzthiazol-2-yl-thiomethyl | 100 | >6 |
| D. | 2′-(-2-oxoimidazolidin-1-yl-carboxyl-amino-2′-(4-hydroxyphenyl)-aceta-mido | 1:2:3-triazol-4-yl-thiomethyl | 79 | >6 |
| L. | 2′-(2-oxoimidazolidin-1-yl-carboxyl-amino-2′-phenyl-acetamido | 1-methylpiperidinium-2-yl-thiomethyl | 17 | 4 |

[a]

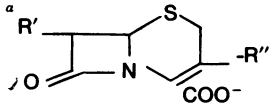



TABLE 6. *Some characteristics of the serum reaction with nitrocefin*

| Treatment | Effect |
|---|---|
| Change in pH | No reaction at pH 5, rate increases as pH rises |
| Ultrafiltration | Retentate active, filtrate inactive |
| Heat | Activity precipitated, but stable to boiling |
| $(NH_4)_2SO_4$ | Activity precipitated at 40% saturation |
| Protein binding | |
| (i) Nitrocefin | Immediate, 100% |
| (ii) Red product | Immediate, 100% |
| Saturation with nitrocefin | ca. 1,500 μg/ml |

**(ii) Ultrafiltration.** The filtrate did not affect nitrocefin, whereas the retentate, containing only compounds with a molecular weight of 10,000 or more, was fully active.

**(iii) Heat.** The clot formed on boiling retained its ability to decompose nitrocefin even after 15 min at 100°C.

**(iv) Ammonium sulfate.** Addition of ammonium sulfate precipitated the active component; the precipitate redissolved with little loss of activity.

**(v) Protein binding.** No nitrocefin was detected in the ultrafiltrate unless the original serum solution contained 1,500 μg/ml or more. The whole procedure took less than 3 min; the retentate did not become noticeably colored at 20°C for at least 5 min, so it was concluded that nitrocefin was bound to serum proteins instantaneously, whereas the decomposition reaction occurred subsequently and more slowly. None of the decomposition product appeared in the filtrate until the serum solution contained 1,500 μg/ml or more.

**Isoelectric focusing.** The possibility of some nonspecific reaction was excluded when clearly defined bands were observed on the isoelectric focusing plates (Fig. 1). Serum from different species gave distinct band patterns in the pH range of albumin. The bands were more clearly observed if the serum used had previously been dialyzed for 24 h against distilled water and the plate had been treated with 4 M phosphate buffer (pH 7) before the nitrocefin was applied.

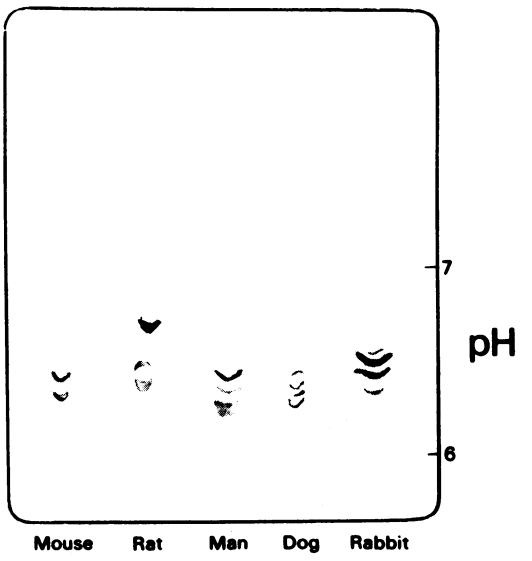



FIG. 1. *Bands produced by isoelectic focusing of serum from various species, followed by treatment of the plates with nitrocefin.*

**Inhibition of the serum decomposition of nitrocefin.** The concentrations at which seven β-lactam compounds in wide clinical use reduced the rate of serum decomposition of nitrocefin by 50% ($I_{50}$) are shown in Table 7. The $I_{50}$s of several experimental compounds with much less serum stability were also determined. All the compounds tested inhibited the serum reaction with nitrocefin, although very high concentrations of the serum-stable compounds were required to reduce the rate of reaction by 50%, whereas the unstable analogs inhibited the reaction at much lower concentrations. Although there was a very large range in $I_{50}$ concentrations from the most effective, 10485 at 20 μg/ml, to the least effective, cephalexin at 20 mg/ml, each compound gave a straight-line, semilogarithmic dose response between 10% and approximately 70% inhibition, depending on the inhibitor used, after which the line flattened markedly. Although 20 μg of 10485 per ml reduced the rate of the serum reaction with nitrocefin by 50%, increase in concentration up to 5 mg/ml did not yield more than 65% inhibition.

There appeared to be a tendency to inverse correlation between the ability of a cephalosporin to inhibit the serum reaction and its stability to incubation with serum (Table 7). 10485 was much more stable in guinea pig serum, and its $I_{50}$, at 290 μg/ml, was more than 10 times greater than in human serum.

TABLE 7. *Inhibition of the serum decomposition of nitrocefin by some penicillins and cephalosporins*

| Inhibitor | $I_{50}$ | | |
|---|---|---|---|
| | mg/ml | M × $10^{-2}$ | Half-life (h) |
| Cephalexin | 20.0 | 5.76 | 24 |
| Cefazolin | 19.8 | 4.16 | 17 |
| Cephaloridine | 8.7 | 2.10 | 15 |
| Cloxacillin | 6.1 | 1.33 | 8 |
| Cephalothin | 2.7 | 0.64 | 4.5 |
| Carbenicillin | 2.3 | 0.53 | NT[a] |
| Benzylpenicillin | 2.1 | 0.60 | 8.5 |
| D[b] | ~12.0 | 1.72 | >6 |
| F | 7.2 | 1.61 | >6 |
| A | 1.6 | 0.36 | 1 |
| B | 0.65 | 0.15 | 3 |
| C[b] | ~0.40 | 0.10 | 1.5 |
| K | 0.26 | 0.05 | >6 |
| J | 0.14 | 0.03 | 1.5 |
| E | 0.02 | 0.004 | 1.5 |

[a] NT, Not tested.

[b] Values approximate due to low solubility. The compounds A through K are identified in Tables 4 and 5.

## DISCUSSION

Serum from different species has been shown here to have differing abilities to destroy cephalosporins, and this may be an unsuspected cause of variations in the pharmacokinetics of these compounds from one animal species to another. Cole et al. (1) showed that penicillins undergo breakdown of the β-lactam ring in the human body; iodometric estimation of the resulting penicilloic acids in the urine showed considerable variation, from 2% for carbenicillin to ca. 20% for benzylpenicillin. They attributed the breakdown to the action of the liver.

The cephalosporins in clinical use tend to give higher urinary recoveries after injection, and it has been assumed that little breakdown of the β-lactam ring would occur in vivo. Such a decomposition would be difficult to detect because cephalosporins in general do not give a stable breakdown product after rupture of the β-lactam ring. The rapid, visible serum reaction with nitrocefin showed that at least one cephalosporin could undergo this kind of degradation; this led to examination of the stability of many cephalosporin compounds to incubation with serum from several species. No relationship between 7- or 3-substituents and serum instability has so far been determined, apart from the highly conjugated 3-substituent in nitrocefin. Closely related compounds could behave very differently, and an unexpectedly wide range of stabilities was observed. The poor stability of 10485 to human serum was thought to be consistent with the very low recovery of total cephalosporin compound in the volunteers and also the reduction of its antibacterial activity in the presence of low concentrations of human serum.

The possibility that nitrocefin was decomposed by bacterial β-lactamases was excluded by several properties such as its stability to heat, its failure to occur below pH 5.5, the pI of the species-specific bands, and its different inhibition spectrum. Cephalosporins and penicillins that inhibit bacterial β-lactamases are stable to those enzymes (6), but there was an inverse correlation between serum stability and ability to inhibit the serum reaction. This was well illustrated by 10485, which was the least stable compound and the most effective inhibitor in human serum. In guinea pig serum, where it was much more stable, it was a much less effective inhibitor.

It seems likely that the complex revealed in the albumin fraction by isoelectric focusing that hydrolyzes the β-lactam ring of nitrocefin can also open the β-lactam ring of the other analogs. However, some decomposition of nitrocefin continued in the presence of concentrations of 10485 that exceed the $I_{50}$ concentration several

hundred-fold, so that at least one component appears to be much more specific for nitrocefin. In addition, it also does not follow that the complex that can destroy some β-lactam compounds is responsible for the more commonly observed phenomenon of serum binding.

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