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Study of the mutual effects of sulphadiazine and ciprofloxacin on their uptakes by *Pseudomonas aeruginosa*

R. MICHAEL E. RICHARDS, RAYMOND G. REID, VIVIENNE E. S. HAMILTON, DOROTHY K. L. XING, *School of Pharmacy, The Robert Gordon University, Aberdeen AB9 1FR, UK*

Abstract—A high-performance liquid chromatography (HPLC) assay was developed for ciprofloxacin and sulphadiazine in Isosensitest broth. Combining the HPLC assay with cell dry-weight determinations indicated that both compounds were able to enhance the uptake of the other by log phase *Pseudomonas aeruginosa* cultured in the presence of the compounds. It is hypothesized that the increased bacterial uptakes are the reason for the enhanced antibacterial activity previously reported for combinations of ciprofloxacin and sulphadiazine.

It has been reported that both *Pseudomonas aeruginosa* and *Staphylococcus aureus* have developed resistance to ciprofloxacin in the clinical situation (Pedersen 1989; Ball 1990; Neu 1991). Combinations of quinolones together with other antimicrobial agents were used (Eliopoulos & Eliopoulos 1989) in order to improve activity against bacteria inadequately inhibited by the fluoroquinolones alone. The results obtained were somewhat variable with addition of activity being the most common effect, although synergism has also been documented. Synergism is the desired effect from antibacterial combinations and since this was demonstrated in-vitro against both *P. aeruginosa* and *S. aureus* with combinations of sulphadiazine and ciprofloxacin (Richards & Xing 1993), the mode of this enhanced activity is worthy of further investigation. Therefore, the object of the present study was to investigate this synergism by developing a single HPLC assay for both ciprofloxacin and sulphadiazine in Isosensitest broth. This would allow determinations to be made of bacterial uptakes from broth containing either antibacterial agent alone or in combination and thus provide evidence of whether the combination produced increased bacterial uptakes.

Materials and methods

Materials. The high-performance liquid chromatography (HPLC) system consisted of an M 6000A pump system (Waters Associates Inc.). Injection was by means of a Rheodyne 7125 valve fitted with a 20 μL fixed volume loop. The 80 mm long, 4·6 mm i.d. column was slurry packed with 5 μm ODS-Hypersil. Detection was at 280 nm using a Waters 440 UV-visible detector connected to a potentiometric recorder (BBC SE120).

Correspondence: R. M. E. Richards, School of Pharmacy, The Robert Gordon University, Aberdeen AB9 1FR, UK.

Pseudomonas aeruginosa NCTC 6750 was the test organism and was from the National Collection of Type Cultures, Colindale, London, UK. Isosensitest broth was obtained from Oxoid Ltd, Basingstoke, UK. Ciprofloxacin and sulphadiazine were obtained from Sigma, Poole, UK. Sodium sulphadiazine was prepared in the laboratory according to Richards et al (1991).

Buffer salt, disodium hydrogen orthophosphate was from Fisons, Loughborough, UK. Tetrabutylammonium bromide (TBA) was from Aldrich Chemical Co. Ltd, Dorset, UK. Acetonitrile was from Rathburn Chemicals Ltd, UK. Water for the HPLC was glass-distilled and further purified by a Millipore Milli-Q System.

Preparation of test sample. Samples of ciprofloxacin (10 μg mL^{-1}) and sulphadiazine (150 μg mL^{-1}) singly and in combination were prepared initially in distilled water and subsequently in Isosensitest broth. A 20 μL aliquot of this preparation was then injected.

Chromatography. The mobile phase system used consisted of 20 mM TBA, 10 mM disodium hydrogen orthophosphate in a 5% v/v acetonitrile/water mixture adjusted to pH 2·5. Detection was at 280 nm and the flow rate was 1·5 mL min^{-1}.

Quantification. The precision of the method was assessed by repeated analysis of Isosensitest samples containing the drugs.

Calibration was determined by adding a range of concentrations of each drug into Isosensitest broth. Peak heights were measured. The correlation coefficient (r^2) for the calibration lines showed good linearity. It was also demonstrated that the assay of ciprofloxacin was not affected by the presence of sulphadiazine and vice versa.

The detection limit of the assay for each of the drugs was determined by successive injections of smaller concentrations of the drug until a signal-to-noise ratio of three was obtained.

Chemical stability. The stability of ciprofloxacin and sulphadiazine in Isosensitest broth was determined by adding 5 μg mL^{-1} ciprofloxacin and 150 μg mL^{-1} sulphadiazine in combination to flasks containing Isosensitest broth. These flasks were incubated at 37°C in a water bath shaking at 100 oscillations min^{-1}. Five flasks were incubated under the conditions described.

Determination of dry cell weights. Dry cell weights were determined as described previously (Richards et al 1991).

Determination of uptake of antibacterial drugs. Two millilitres of an 18-h culture of *P. aeruginosa* was inoculated into 98 mL of prewarmed Isosensitest broth at 37°C in a conical flask and shaken in a water bath at 100 oscillations min^{-1}. After 4 h, 4 mL log phase culture was removed and inoculated into replicate conical flasks containing either of the antibacterial agents singly or both in combination in 96 mL prewarmed Isosensitest broth, prepared as above. Ciprofloxacin was used at a final concentration of 2 $\mu g\ mL^{-1}$ and sulphadiazine at 100, 150 and 200 $\mu g\ mL^{-1}$. These flasks were incubated in the shaking water bath for 4 h at 37°C. The bacterial cultures were centrifuged (6000 *g*, 15 min, 4°C) and the supernatants were removed. The pellets were washed by resuspending in distilled water and recentrifuging. This treatment of the pellets was repeated. The washed pellets were used for dry cell weight determinations and for each culture the concentration of drug in the broth supernatant pooled with the related water washings were determined by HPLC. The supernatant concentration subtracted from the original concentration present in the flasks gave the concentration of drug taken up by the unit mass of cells (Richards et al 1991). All drug uptakes were determined in duplicate.

Results

The separation of ciprofloxacin and sulphadiazine in Isosensitest broth was achieved by a mobile phase system consisting of 20 mM TBA, 10 mM di-sodium hydrogen phosphate in a 5% v/v acetonitrile/water mixture adjusted to pH 2·5 (Fig. 1). Fig. 2 shows the chromatograph of Isosensitest broth alone.

The precision of the method was assessed by repeated analysis of Isosensitest samples containing the drugs. At a detection wavelength of 280 nm the relative standard deviation of ciprofloxacin 5 $\mu g\ mL^{-1}$ was 1·45%, and for sulphadiazine 150 $\mu g\ mL^{-1}$ was 1·28%.

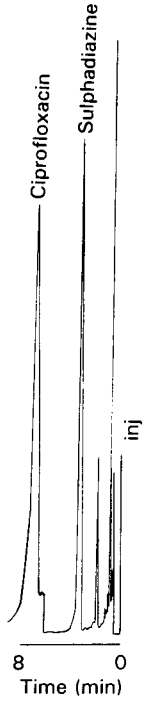

FIG. 1. Chromatogram showing the separation of sulphadiazine and ciprofloxacin in Isosensitest broth. Column: 80 mm long, 4·6 mm i.d. packed with 5 μm ODS-Hypersil. Mobile phase: 5% acetonitrile, 10 mM disodium hydrogen orthophosphate, 20 mM TBA. Sensitivity: 1·28 for sulphadiazine, 0·04 for ciprofloxacin. Detection: 280 nm. Flow rate: 1·5 mL min^{-1}.

FIG. 2. Chromatogram showing the assay of Isosensitest broth. Column: 80 mm long, 4·6 mm i.d. packed with 5 μm ODS-Hypersil. Mobile phase: 5% acetonitrile, 10 mM disodium hydrogen orthophosphate, 20 mM TBA. Sensitivity: 1·28 changing to 0·04. Detection: 280 nm. Flow rate: 1·5 mL min^{-1}.

The linearity of the calibration was assessed using concentrations of 1–9 $\mu g\ mL^{-1}$ ciprofloxacin and 50–300 $\mu g\ mL^{-1}$ sulphadiazine. The correlation coefficient for ciprofloxacin was 0·999 and for sulphadiazine was 0·998. Detection limits during this assay were 0·2 $\mu g\ mL^{-1}$ for ciprofloxacin and 2 $\mu g\ mL^{-1}$ for sulphadiazine. The concentrations of sulphadiazine to be detected were in the range of 100–200 $\mu g\ mL^{-1}$ and the ciprofloxacin range was under 5 $\mu g\ mL^{-1}$.

The uptake by *P. aeruginosa* of sulphadiazine and ciprofloxacin used singly and in combination is given in Table 1. It is seen that the uptake of sulphadiazine was proportionally much greater from the medium plus 200 $\mu g\ mL^{-1}$ sulphadiazine than from the two lower concentrations. This is because the higher

Table 1. Bacterial uptakes of ciprofloxacin and sulphadiazine by log phase *P. aeruginosa* grown in Isosensitest broth for 4 h at 37°C in the presence of the antibacterial agents used singly and in combination.

Antibacterial ($\mu g\ mL^{-1}$)		Antibacterial uptake (μg (mg dry cell wt)$^{-1}$)	
Ciprofloxacin	Sulphadiazine	Ciprofloxacin	Sulphadiazine
2	—	0·027	—
—	100	—	0·305
—	150	—	0·344
—	200	—	0·985
2	100	0·025	1·286
2	150	0·039	1·572
2	200	0·041	2·029

Mean of three duplicate determinations.

concentration is understood to have a much greater effect on the permeability properties of *P. aeruginosa* thus enhancing its own uptake (Richards & Xing 1991; Richards et al 1991, 1993). The sulphadiazine uptake by *P. aeruginosa* is significantly increased when used in combination with ciprofloxacin. The use of 2 μg mL^{-1} ciprofloxacin in combination with 100 μg mL^{-1} sulphadiazine increased the uptake of sulphadiazine by 321% compared with the drug uptake from broth containing 100 μg mL^{-1} sulphadiazine alone.

The uptake of ciprofloxacin also increased at sulphadiazine concentrations of 150 μg mL^{-1} and above. That is, at 150 μg mL^{-1} sulphadiazine, the uptake of ciprofloxacin increased by 44% and at 200 μg mL^{-1} sulphadiazine the uptake of ciprofloxacin increased by 51%.

Discussion

Several HPLC methods have been published for the determination of ciprofloxacin in biological fluids (Walid et al 1987; Chan et al 1989). Sulphadiazine has also been assayed previously using the HPLC method (Taylor et al 1990). No method has been published which determines both antibacterial agents in a biological medium. The method developed during this study shows that ciprofloxacin and sulphadiazine can be separated in Isosensitest broth and that no pretreatment process was required. The quantitative measurements were shown to be reproducible and showed good correlation. Ciprofloxacin and sulphadiazine were also shown to remain stable in Isosensitest broth when incubated under the conditions of growth for *P. aeruginosa*.

Sulphadiazine plus ciprofloxacin has been shown to have a synergistic inhibitory effect against both *P. aeruginosa* and *S. aureus* (Richards & Xing 1993). It was suggested that since sulphadiazine was known to modify cell envelope permeability (Richards & Xing 1991, 1992a, b, 1993; Richards et al 1991, 1993) then it may enhance the uptake of ciprofloxacin by this means. The present study supports this hypothesis and also indicates that both antibacterial agents are able to increase the uptake of the other. Thus it is hypothesized that ciprofloxacin also affects cell envelope permeability and facilitates the uptake of sulphadiazine by *P. aeruginosa* cells. The results presented here indicate that the enhanced antibacterial activity reported for the combinations of ciprofloxacin and sulphadiazine (Richards & Xing 1993) could be the consequence of increased bacterial uptake of both antibacterial agents.

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