Effects of Preservatives, Steroids, and Ethylenediaminetetraacetate on the Antimicrobial Activity of Sulfacetamide

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Abstract □ The effect of EDTA (ethylenediaminetetraacetate), steroids, and preservatives on the antimicrobial activity of 10% sodium sulfacetamide solutions was evaluated in this study by kill rate and minimum inhibitory concentration (MIC) using five representative microorganisms. The results indicate that thimerosal-preserved sulfacetamide solutions containing EDTA are more effective against *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis*, and *Candida albicans* than similar paraben-preserved solutions. Furthermore, the addition of EDTA improves the kill rate, but not the MIC, for the *Pseudomonas*, *Serratia*, and *Candida* species regardless of the preservative. The combination of a steroid with sulfacetamide does not affect its antimicrobial activity.

Keyphrases □ Ophthalmic solutions—sulfacetamide, antimicrobial activity, effects of EDTA, steroids, preservatives □ Sulfacetamide—ophthalmic solutions, antimicrobial activity, effects of EDTA, steroids, preservatives □ Antimicrobial activity—of sulfacetamide in ophthalmic solutions, effects of EDTA, steroids, preservatives

Sodium sulfacetamide is an antimicrobial agent which prevents the growth and division of bacteria by interfering with the uptake of *p*-aminobenzoic acid. *p*-Aminobenzoic acid is required for folic acid synthesis which, in turn, is required for synthesis of purines and the pyrimidine, thymine, the building blocks of DNA. The competitive inhibition of *p*-aminobenzoic acid by sulfacetamide is reduced by some local anesthetics, such as tetracaine, that are derivatives of *p*-aminobenzoic acid (1). It has also been reported that the presence of sodium metabisulfite inhibits the antimicrobial activity of sodium sulfacetamide (2).

Sulfacetamide is the least sensitizing member of the sulfonamide group of antibiotics (3). It has been widely used during the past 25 years to treat blepharitis (4), pustular acne, and seborrheic dermatitis (5), and has also been used prophylactically following cataract surgery (6). It is effective against streptococci (except enterococci), pneumococci, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Clamydia trachomatis*, and some strains of *Haemophilus influenzae*, *Yersinia*, *Nocardia*, and *Actinomyces*. Many strains of *Neisseria* and *Enterobacteriaceae* have acquired resistance to this drug.

Sodium sulfacetamide solutions for ophthalmic use usually contain preservatives. Richards and McBride (2) showed that the inclusion of preservative systems decreases the sterilization time of sulfacetamide solutions from 5 hr to <15 min when challenged with 10^6 colony-forming units/ml of *Pseudomonas aeruginosa* 6750. The preservative systems included either chlorhexidine and phenylethyl alcohol, phenylmercuric nitrate and phenylethyl alcohol, or chlorocresol and phenylethyl alcohol, with or without EDTA (ethylenediaminetetraacetate).

The purpose of the present study was to determine the effects of prednisolone sodium phosphate, EDTA, thimerosal, and methyl- and propylparaben on the *in vitro* antimicrobial activity of solutions containing sodium sulfacetamide. The antimicrobial activities of the solutions tested were determined by kill rate and minimum inhibitory concentration (MIC) techniques using five test microorganisms: *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Candida albicans*, and spores of *Aspergillus fumigatus*.

EXPERIMENTAL

Commercially available 10% sodium sulfacetamide solutions were obtained from pharmaceutical distributors; two experimental formulations were prepared in-house. The ingredients of each solution are listed in Table I. Both standard strains and clinical isolates were used as test microorganisms. The standard strains included *P. aeruginosa* 15442[1], *S. marcescens* 14041[1], *St. epidermidis* 17917[1], *C. albicans* 10231[1], and *A. fumigatus* 10894[1]. The clinical isolates of *P. aeruginosa* were SUH-51278[2] and H-23778[2]. Microbiological growth media included trypticase soy agar[3], trypticase soy broth[3], D/E neutralizing agar[4], and Sabouraud dextrose agar[4]. Other chemicals used in this study included APHA phosphate buffer[3], polysorbate 80[5], and sodium chloride[6].

Microbial cultures were prepared by inoculating 10-ml aliquots of trypticase soy broth with stock bacterial and yeast cultures maintained on either trypticase soy agar or Sabouraud dextrose agar slants and incubated for 18–24 hr at 32–34°. The second 24-hr cultures were harvested by centrifugation, washed three times in phosphate-buffered saline (centrifuged between each wash), and resuspended in sterile distilled water. The suspension of cells was further diluted to give an optical density at 500 nm, corresponding to ~10^6 colony-forming units/ml of test sample. Fungal spores were harvested from Sabouraud dextrose agar plates, washed five times in distilled water (centrifuged between each wash), and diluted to ~10^6 colony-forming units/ml of test sample.

To determine the death rate kinetics, the experiment was initiated by the addition of 0.1 ml of the standardized cultures to 20 ml of each test solution. The microbes were recovered between 1 min and 6 hr, diluted, and cultured in pour plates of D/E medium. The recovery plates were incubated for a maximum of 5 days at 30–35° followed by 5 days at room temperature. The experiments were repeated if recovery times or sample dilutions were inappropriate for the test microbe. The kill rate was expressed as a D-value, the time in minutes required to reduce the number of viable microorganisms by 90% (one log). The D-values were calculated from the best-fitting straight lines determined by linear regression. Thus, a lower D-value indicates a more rapid kill. All recoveries were validated by standard procedures.

MIC values were evaluated using microtiter plates with four overlapping concentrations of each solution, run in duplicate. After incubating the plates for 48 hr at 32–34°, the wells were scored as true positive (turbid), true negative (no turbidity), or plus–minus (very slight turbidity). The MIC was determined by calculating the geometric mean ($\bar{G}$) of the highest concentration of test solution to give a true positive, the lowest concentration to give a true negative, and the two median concentrations to give a plus–minus:

$$\bar{G} = (\text{highest positive value} \times \text{two median plus–minus values} \times \text{lowest negative value})^{1/4}$$

If only one median plus–minus value is used, the cube root of the product is taken; if no plus–minus values are used, the square root of the product is taken.

[1] American Type Culture Collection.
[2] Stanford University Hospital, Palo Alto, Calif.
[3] Baltimore Biological Laboratories, Cockeysville, Md.
[4] Difco Laboratories, Detroit, Mich.
[5] U.S. Biochemical Corp., Cleveland, Ohio.
[6] Fisher Scientific Co., Fair Lawn, N.J.

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Table I—Composition of Sulfacetamide Solutions

Sulfacetamide Solution[a]	Preservative	EDTA[b]	Steroid[c]
A	Thimerosal, 0.01%	—	—
B	Methylparaben, 0.05%; propylparaben, 0.01%	—	—
A′	Thimerosal, 0.01%	0.1%	—
C	Methylparaben, 0.02%; propylparaben, 0.005%	—	0.25%
C′	Methylparaben, 0.02%; propylparaben, 0.005%	0.1%	0.25%

[a] 10% sulfacetamide as active ingredient. [b] The disodium salt. [c] Prednisolone sodium phosphate.

RESULTS

The kill rates of *P. aeruginosa*, *S. marcescens*, *St. epidermidis*, *C. albicans*, and *A. fumigatus* (spores) exposed to two ophthalmic solutions each formulated with 10% sodium sulfacetamide are presented in Table II. The data indicate that the sulfacetamide products are most rapidly bactericidal against *Pseudomonas*, (D = 168 min), slightly bactericidal against *Serratia* (D = 1318 min) and *Candida* (D = 2972 min), and not bactericidal against *Staphylococcus* or fungicidal against spores of *Aspergillus*. The large standard error associated with the D-value for *Serratia* is due to the preservative effect, as seen in Table III. Sulfacetamide solutions preserved with thimerosal are more rapidly bactericidal against *Serratia* than those preserved with parabens (Table III), resulting in lower D-values (faster kill rates). Since ocular anti-infective agents are washed out of the eye by natural tearing, solutions with low D-values would be more effective in controlling infection than those with high D-values.

Table II—Average Kill Rate (D-value) for Two Ophthalmic Solutions[a] Each Formulated with 10% Sodium Sulfacetamide

Microorganism	Number of Determinations	Average D-value[b] for Solutions A and B, min
Pseudomonas aeruginosa (four strains)	17	168 ± 17
Serratia marcescens	4	1318 ± 543
Staphylococcus epidermidis	4	NE[c]
Candida albicans	4	2972 ± 48
Aspergillus fumigatus	6	NE[c]

[a] Both solutions contain 10% sodium sulfacetamide; one was preserved with 0.01% thimerosal (solution A) and one with parabens (solution B). Neither product contains steroids or EDTA. [b] Mean ± *SE*. [c] NE = no significant effect observed during 6 hr of exposure.

Table III—Effect of Preservative Contained in 10% Sodium Sulfacetamide Solutions[a] on the Kill Rate (D-value) of *Serratia marcescens*

Solution	D-Value, min[b] Thimerosal Preservative	D-Value, min[b] Paraben Preservatives
A	436 ± 136	—
B	—	2200 ± 438
A + B	1318 ± 543	

[a] Neither solution contains steroids or EDTA. [b] Mean ± *SE*.

Table IV—MIC Values for Thimerosal- and Paraben-Preserved Sulfacetamide Solutions[a]

Microorganism	Solution A (Thimerosal)	Solution B (Parabens)
Pseudomonas aeruginosa	1.1	1.8
Serratia marcescens	0.4	1.5
Staphylococcus epidermidis	0.2	29.6
Candida albicans	2.0	≥50.0
Aspergillus fumigatus	2.8	6.5

[a] Expressed as %.

Table V—Effect of Steroids on the Kill Rate (D-value) of 10% Sodium Sulfacetamide as Formulated in Two Ophthalmic Solutions[a] Against Several Microorganisms

Microorganism	D-Value, min[b] Solution B (Without Steroid)	D-Value, min[b] Solution C (With Steroid)
Pseudomonas aeruginosa (four strains)	186 ± 28	235 ± 48
Serratia marcescens	2200 ± 438	1218 ± 184
Staphylococcus epidermidis	NE[c]	NE[c]
Candida albicans	2938 ± 56	NE[c]
Aspergillus fumigatus (spores)	1935 ± 136	NE[c]

[a] Both solutions contain 10% sodium sulfacetamide preserved with parabens; neither contain EDTA. [b] Mean ± *SEM*. [c] NE = no significant effect observed during 6 hr of exposure.

Table VI—MIC Values[a] for Steroidal and Nonsteroidal Sodium Sulfacetamide Solutions

Microorganism	Solution B (Without Steroid)	Solution C (With Steroid)
Pseudomonas aeruginosa	1.8	1.5
Serratia marcescens	1.5	1.6
Staphylococcus epidermidis	29.6	41.8
Candida albicans	≥50.0	≥50.0
Aspergillus fumigatus	6.5	6.8

[a] Expressed as percent.

Table VII—Effect of EDTA on the Kill Rate of Sulfacetamide Solutions Preserved with Either Thimerosal or Parabens

Preserved with 0.01% Thimerosal

Microorganism	D-value, min Product A[a]	D-value, min Product A′[b]
Pseudomonas aeruginosa (four strains)	148 ± 19	13 ± 1
Serratia marcescens	436 ± 136	247 ± 49
Staphylococcus epidermidis	1764 ± 306	2848 ± 600
Candida albicans	3006 ± 31	1459 ± 70
Aspergillus fumigatus (spores)	NE[c]	NE[c]

Preserved with Parabens

Microorganism	D-value, min Product C[a]	D-value, min Product C′[b]
Pseudomonas aeruginosa (four strains)	235 ± 48	35 ± 5
Serratia marcescens	1218 ± 184	637 ± 62
Staphylococcus epidermidis	NE[c]	NE[c]
Candida albicans	NE[c]	1612 ± 373
Aspergillus fumigatus (spores)	NE[c]	NE[c]

[a] Without EDTA. [b] With EDTA. [c] NE = no significant effect observed during 6 hr of exposure.

Table VIII—Effect of EDTA on the MIC Values of Sulfacetamide Solutions Preserved With Either Thimerosal or Parabens

Preserved with Thimerosal

Microorganism	Solution A[a]	Solution A′[b]
Pseudomonas aeruginosa	1.1	1.3
Serratia marcescens	0.4	0.4
Staphylococcus epidermidis	0.2	0.1
Candida albicans	2.0	3.1
Aspergillus fumigatus	2.8	0.3

Preserved with Parabens

Microorganism	Product C[a]	Product C′[b]
Pseudomonas aeruginosa	1.5	1.7
Serratia marcescens	1.6	2.0
Staphylococcus epidermidis	41.8	29.8
Candida albicans	≥50.0	≥50.0
Aspergillus fumigatus	6.8	7.4

[a] Without EDTA. [b] With EDTA.

The same anti-infective solutions were compared by MIC values in Table IV. The MIC values represent the bacteriostatic effect of each solution expressed as the percent of product required to inhibit growth; the lower the MIC, the more the test solution can be diluted and still inhibit microbial proliferation. Sulfacetamide solutions preserved with thimerosal have MIC values ranging from 0.2 to 2.8 for the five test microorganisms. The solutions preserved with parabens exhibit slightly higher MIC values for *Pseudomonas*, *Serratia*, and spores of *Aspergillus* (1.5–6.5) and significantly higher MIC values for *Staphylococcus* and *Candida* (19.6 and 5.0, respectively).

The effect of steroids and EDTA on the antimicrobial efficacy of the solutions was evaluated using the kill rate and MIC methods. The kill rates appear to be unaffected by the presence of steroids (Table V). The MIC values agree with this observation (Table VI). However, the addition of 0.1% EDTA to the sulfacetamide solutions significantly reduced the D-value for *Pseudomonas*, *Serratia*, and *Candida* regardless of the preservative (Table VII). This increase in antimicrobial activity, as indicated by smaller D-values, is not reflected by significantly different MIC values (Table VIII).

DISCUSSION

The results of both kill rate and MIC methods used to evaluate ophthalmic anti-infective solutions provide a better understanding of the antimicrobial effects of sulfacetamide and the clinical use of products containing this drug. Both methods indicate greater antimicrobial activity when thimerosal is used as the solution preservative than that seen with parabens: the kill rate for *Serratia* is increased and the MIC values for *Staphylococcus*, *Candida*, and possibly *Aspergillus* are decreased. The results using both methods also indicate that the addition of steroids to sulfacetamide formulations does not affect kill rates or MIC values.

However, only one method could detect the effect of EDTA on the antimicrobial activity of sulfacetamide. The kill rate for *Pseudomonas*, *Serratia*, and possibly *Candida* increased with the addition of EDTA, yet no differences in MIC values were observed. Thus, evaluation of antimicrobial activity using just one technique may not be adequate in determining the efficacy of ocular anti-infective products. MIC values are routinely used to evaluate the microbial sensitivity of parenterally administered antibiotics, yet this method of evaluation may miss interactions of other agents important in ocular therapy. This study shows that sulfacetamide solutions containing EDTA and thimerosal as preservatives are more effective against the organisms tested than sulfacetamide solutions containing paraben preservatives *without* EDTA. The antipseudomonal activity of thimerosal-preserved sulfacetamide solutions is particularly interesting, since they are usually not considered effective against this microorganism.

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High-Performance Liquid Chromatographic Analysis of Diflunisal in Plasma and Urine: Application to Pharmacokinetic Studies in Two Normal Volunteers

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Received May 17, 1982, from the *Department of Clinical Pharmacology, St. Vincent's Hospital, Darlinghurst, New South Wales, 2010, Australia.* Accepted for publication October 19, 1982.

Abstract □ A high-performance liquid chromatographic (HPLC) assay with fluorescence detection has been developed for the determination of diflunisal in plasma and urine. The plasma or urine, containing naproxen as the internal standard, was extracted with ether–hexane (1:1). The samples were analyzed on a microparticulate column, and the compounds were eluted using a mobile phase of 0.05 *M* phosphate buffer (pH 3) and methanol. Plasma samples were analyzed from two healthy male subjects who received a 250- and 750-mg oral dose of diflunisal 3 weeks apart. The data were analyzed according to a two-compartment open model. There was a disproportionate increase in the area under the plasma concentration–time curves (AUC 750 mg/AUC 250 mg was 3.84 for subject A and 4.22 for subject B) and a reduction in plasma clearance after the 750-mg dose of diflunisal. These data suggest that the kinetics of diflunisal may be dose dependent.

Keyphrases □ Diflunisal—high-performance liquid chromatographic analysis, plasma and urine, application to pharmacokinetic studies □ High-performance liquid chromatography—analysis of diflunisal in plasma and urine, application to pharmacokinetic studies □ Pharmacokinetics—diflunisal, high-performance liquid chromatographic analysis, plasma and urine

Diflunisal, 2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid, a salicylic acid derivative with analgesic and anti-inflammatory activity (1, 2), has been assayed in biological fluids by a fluorescence method (3). This assay procedure lacks the specificity of GLC and high-performance liquid chromatography (HPLC) (4). The more tedious GLC method (3) has been superseded by HPLC assays (4–6), but these methods have thus far been relatively insensitive.

The pharmacokinetics of diflunisal have been investigated, but these studies have used either the nonspecific fluorescence assay to measure plasma concentrations (3, 7–9) or the relatively insensitive HPLC procedures (10) which necessitated the administration of high doses (750 mg) of diflunisal. This paper reports the development of a sensitive and specific HPLC assay for quantitating diflunisal in plasma or urine and its application to a preliminary study of the pharmacokinetics of diflunisal in humans.

EXPERIMENTAL

Chemicals and Reagents—Diflunisal[1] and naproxen[2] were the reference and internal standards, respectively. Stock solutions of these

[1] Dolobid; Merck Sharp & Dohme, Sydney, Australia.
[2] Syntex, Sydney, Australia.

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