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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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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compounds were prepared daily by dissolving the drugs in acetone[3]. Chromatographic-grade hexane[4] and methanol[5] were used, while all other reagents were analytical grade. Ether[6] was distilled in glass.

Extraction Procedure—The plasma or urine containing the internal standard was extracted according to the method of Van Loenhout *et al.* (6) with the following modifications: (*a*) to achieve adequate recovery of diflunisal from urine, samples were extracted twice with 5 ml of ether–hexane (1:1), and (*b*) the residue remaining after evaporation of the ether–hexane was resuspended in the HPLC mobile phase.

Clinical Study—Single 250- and 750-mg doses of diflunisal were administered to two healthy male subjects. The doses were not randomized, and the same formulation was used in both studies. Subjects fasted overnight before the drug administration and continued fasting until 4 hr postdose. All doses were administered at 8:00 a.m. and the tablets were swallowed whole with 200 ml of water. Venous blood samples were drawn into potassium oxalate–sodium fluoride[7] tubes before drug administration and at 0.5, 1, 1.5, 2, 2.5, 4, 5, 6, 8, 10, 12, 18, 24, 30, 36, 42, 48, 60, and 72 hr after the drug was given.

Instrumentation—Assays were carried out using a constant-flow high-performance liquid chromatograph[8]. It consisted of a solvent delivery system[9], an autosampler injector[10] with a pneumatic-actuated injection valve fitted with a 20-μl sample loop[11], a microparticulate reverse-phase column[12] (5 μm; 25 cm × 4.6-mm i.d.), a guard column[13], and a spectrophotofluorometer[14] with the excitation monochromotor set at 315 nm and the fluorescence emission at 389 nm (cutoff filter).

Chromatography—The compounds in plasma were eluted with a mobile phase of 64% methanol in 0.05 *M* phosphate buffer (pH 3), prepared from monobasic sodium phosphate with the pH adjusted using sulfuric acid. The column temperature was 50° and the flow rate was 1 ml/min. The same conditions were used to analyze urine samples, but a linear gradient (10 min) from 60% methanol in 0.05 *M* phosphate buffer (pH 3) to 70% methanol in phosphate buffer was necessary to move the diflunisal and naproxen peaks from interfering peaks at the solvent front.

Pharmacokinetic Analysis—The data were analyzed assuming a two-compartment open model with delayed absorption (11) and were fitted to the appropriate triexponential equation using a nonlinear regression analysis program (12). The equation fitted was:

$$C_1 = Ae^{-\alpha(t-t_0)} + Be^{-\beta(t-t_0)} - Ce^{-k_a(t-t_0)}$$

where C_1 is the plasma concentration, t the time after the dose, t_0 the lag time, and k_a the apparent first-order absorption rate constant. A, B, α, and β are constants related to model parameters (11); A and B are also related to the dose (D) and the fraction of the dose that reaches the systemic circulation (f). Initial estimates of these parameters were obtained graphically using the method of residuals (11). The volume of distribution V_d (area), the total body clearance (CL), and area under the curve (AUC) were estimated as described by Wagner (11).

RESULTS AND DISCUSSION

Assay—Reverse-phase HPLC with fluorescence detection was an effective method of quantitating diflunisal in human plasma and urine. Typical chromatograms obtained from blank plasma, blank urine, plasma containing diflunisal and naproxen (internal standard), and urine containing diflunisal and naproxen are shown in Fig. 1. Interference by endogenous substances did not occur from either drug-free plasma or urine (Fig. 1).

The standard curve for diflunisal was linear over the concentration range of 0.05–100 μg/ml in both plasma and urine. The correlation coefficient for the standard curves over this concentration range was >0.999 (n = 4). The coefficient of variation was <5% for all concentrations measured (Table I), and the sensitivity of the method was 50 ng/ml. Under the assay conditions described, acetylsalicylic acid and salicylic acid had elution times of 2.9 and 3.1 min, respectively, and did not interfere with the diflunisal or naproxen peaks.

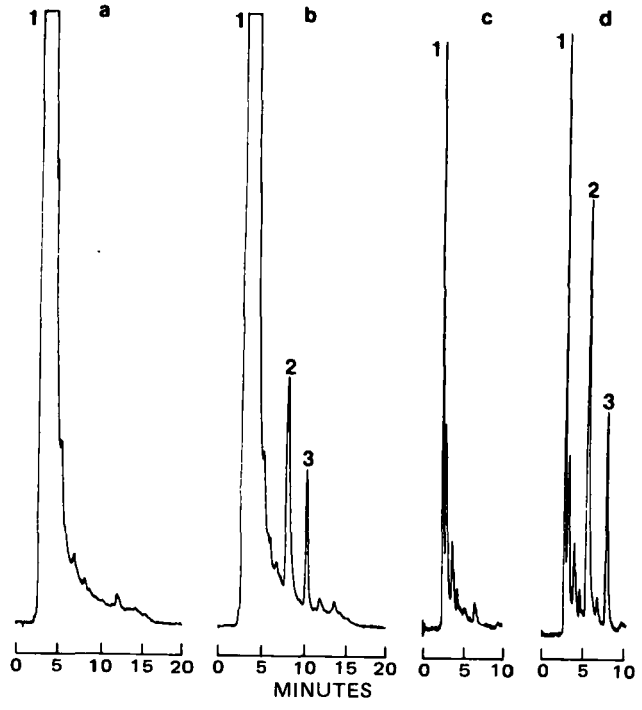

Figure 1—*Chromatograms of blank urine (a), spiked (200 ng/ml) urine (b), blank plasma (c), and spiked (200 ng/ml) plasma (d). Key: (1) solvent front; (2) diflunisal; (3) naproxen (internal standard).*

The method described was sufficiently sensitive to detect plasma concentrations of diflunisal for 80 hr after oral doses of 250 and 750 mg of the drug. The procedure has marked advantages over previous methods in both sensitivity (4–6) and specificity (3). The use of identical extraction and similar chromatographic conditions for the detection of diflunisal in plasma and urine makes the method simple and convenient for pharmacokinetic studies.

Pharmacokinetics—After the 250-mg dose of diflunisal, peak concentrations of 39.6 and 33.9 μg/ml were reached at 3.5 and 2.1 hr, respectively (Fig. 2). The pharmacokinetic data were analyzed according to a two-compartment open model although the elimination phase in subject B (250-mg dose) was not strictly linear. Agreement between the computer-generated curves and the experimental values was good. The various phramacokinetic parameters are shown in Table II. The half-lives of the initial α phase were 1.7 and 0.9 hr, while those of the β phase were 10.8 and 9.8 hr for the two subjects. The total body clearance was 8.6 and

Table I—Recovery and Reproducibility of the Diflunisal Assay [a]

Concentration, μg/ml	CV, %	Recovery, %
	Plasma	
0.050	4.2	97.4
0.075	3.0	96.2
0.100	2.7	97.8
0.150	2.6	97.9
0.200	2.7	97.6
10	4.8	105.0
25	3.2	98.1
50	4.2	94.3
100	2.2	99.9
	Urine	
0.050	2.1	101.0
0.075	2.6	96.8
0.100	2.4	97.9
0.150	3.5	98.9
0.200	2.1	98.4
10	4.0	96.1
25	2.7	103.3
50	1.6	97.2
100	2.4	98.9

[a] Number of estimations = 4.

[3] Hopkin & Williams, Ajax Chemicals, Sydney, Australia.
[4] Unichrom Reagents, Ajax Chemicals, Sydney, Australia.
[5] Waters Associates, Sydney, Australia.
[6] Ajax Chemicals, Sydney, Australia.
[7] Venoject; Kimble-Terumo, Inc., Elkton, Md.
[8] Varian Pty. Ltd., Sydney, Australia.
[9] Model 5000; Varian Pty Ltd., Sydney, Australia.
[10] Model 8055; Varian Pty Ltd., Sydney, Australia.
[11] Model 7126; Rheodyne, Cotati, Calif.
[12] Ultrasphere-ODS; Altex, Berkeley, Calif.
[13] Lichrosorb RP-8 (Cartridge); Brownlee Labs. Inc., Santa Clara, Calif.
[14] Model FS970; Schoeffel Instruments, Westwood, N.J.

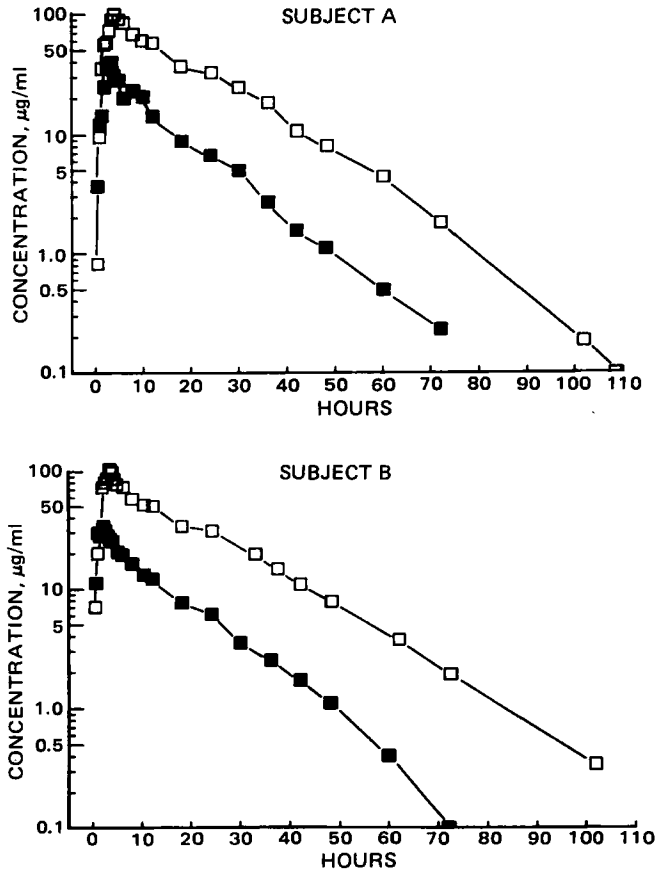

Figure 2—*Diflunisal plasma concentrations in two normal male subjects after a single 250-mg oral dose (■) and a single 750-mg oral dose (□).*

Table II—Plasma Pharmacokinetic Parameters in Two Normal Subjects After a 250-mg Oral Dose

Parameter	Subject A	Subject B
Lag time, hr	0.5	0.4
Peak time, hr	3.5	2.1
k_a, hr^{-1}	0.5	1.7
$t_{1/2,\alpha}$, hr	1.7	0.9
$t_{1/2,\beta}$, hr	10.8	9.8
V_d (area), liters[a]	14.1	8.6
CL, ml/min	8.6	10.0
$AUC_{0-\infty}$, μg·hr/ml	482.2	415.5

[a] Calculation assumes $f = 1$ (7).

10.0 ml of plasma/min. The volume of distribution was 14.1 and 8.6 liters. These data are in good agreement with those previously obtained after a 500-mg oral dose (7). The volume of distribution and clearance of diflunisal are low, but similar to other nonsteroidal anti-inflammatory drugs (13). Pharmacokinetic parameters were not estimated after the 750-mg oral doses of diflunisal in the two subjects because the log plasma concentration–time curves suggested a nonlinear elimination phase.

It has been suggested that diflunisal exhibits dose-dependent kinetics (3, 7, 8). Tocco *et al.* showed that the AUC values for [^{14}C]diflunisal in plasma of healthy volunteers were ~18 times higher after a 500-mg dose when compared with a 50-mg dose of diflunisal (3). Further, these authors reported a shorter elimination half-life (6 hr) after a 50-mg oral dose, compared with an elimination half-life of 10.8 hr after a 500-mg oral dose. However, these data are difficult to interpret because the assays lacked specificity. Dresse *et al.* (8) administered 500 mg of diflunisal twice a day for 8 days to 10 male volunteers and suggested that the elimination of diflunisal might be nonlinear for plasma concentrations >10 μg/ml. A third study, in which a nonspecific fluorescence assay was used, reported that the plasma elimination half-life of diflunisal decreased with declining plasma concentration, again suggesting nonlinear elimination of the drug (7).

Table III—Plasma Pharmacokinetic Parameters in Two Normal Subjects After a 750-mg Oral Dose

Parameter	Subject A	Subject B
Peak time, hr	4.0	3.5
Peak concentration, μg/ml	99.0	99.3
CL, ml/min	6.74	7.13
$AUC_{0-\infty}$, μg·hr/ml	1855	1752
AUC 750 mg/AUC 250 mg	3.84[a]	4.22[b]

[a] This ratio is significantly different from 3.0 ($p < 0.05$), one-sample t test. The one-sample t test is used to find the probability that the mean of a single sample of a population is different than an hypothesized value (15). [b] This ratio is significantly different from 3.0 ($p < 0.01$), one-sample t test.

The pharmacokinetics of diflunisal after 250- and 750-mg oral doses of the drug are shown in Tables II and III. There was a disproportionate increase in the plasma concentration–time AUC (Table III) and a reduction in plasma clearance (Tables II and III) when comparing the 750-mg oral dose of diflunisal with the 250-mg oral dose. Visual inspection of the plasma–concentration time curve for subject A suggests nonlinear elimination of the drug following the 750-mg dose of diflunisal. However, this phenomenon is not apparent in subject B.

The elimination of diflunisal is almost exclusively dependent on glucuronidation of the parent compound to ether glucuronide (64%) and ester glucuronide (20%), which are excreted in the urine (3). Pharmacokinetic studies with sodium salicylate, a compound structurally similar to diflunisal, have demonstrated that humans have a limited capacity for salicyl phenolic glucuronide formation (14), while the salicyl acyl glucuronide formation follows a first-order process (14). Data in normal volunteers suggest that the diflunisal phenolic glucuronide formation may be capacity limited (3). The data obtained from our study suggest that the kinetics of diflunisal may be dose dependent, but further study is needed to confirm this.

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