Liquid Chromatographic Analysis of Sulfaquinoxaline and Its Application to Pharmacokinetic Studies in Rabbits

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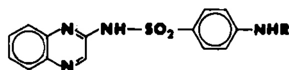
Abstract ☐ A specific, sensitive high-performance liquid chromatographic method is described for sulfaquinoxaline (I) and its major metabolite, the N^4-acetyl metabolite (II), in biological fluids. Detection limits for I and II were 0.25 and 0.50 μg/mL in plasma and 0.10 and 0.20 μg/mL in urine, respectively. The pharmacokinetics of sulfaquinoxaline and its metabolite were studied in New Zealand White rabbits after individual doses of 50 mg/kg of sulfaquinoxaline and its metabolite were administered intravenously. Mean (±*SD*) plasma half-lives were 12.7 (8.0) h for sulfaquinoxaline and 15.4 (3.5) h for the metabolite. After administration of the *N*-acetyl metabolite sulfaquinoxaline appeared in the plasma and urine indicating that deacetylation had occurred. The repercussions of this observation are briefly discussed with respect to two rabbits in which plasma analyses and complete urine collections were made. As sulfaquinoxaline is administered prophylactically to rabbits by some breeders, it is recommended that investigators allow a washout period of about one week after receipt of the animals.

Keyphrases ☐ Sulfaquinoxaline—HPLC, pharmacokinetics, rabbits ☐ Pharmacokinetics—sulfaquinoxaline, rabbits, HPLC

Laboratory animal breeders frequently use sulfaquinoxaline (I) on a chronic basis in feed or water to prevent outbreaks of coccidiosis in rabbits. Measurable quantities of sulfaquinoxaline or its metabolite may therefore be present in the blood or tissues of these animals after delivery to the laboratory. This report examines the hitherto unreported pharmacokinetics of the drug and its major metabolite, the N^4-acetyl metabolite (II), in rabbits, to determine the washout period needed to avoid incorrect conclusions from pharmacokinetics or pharmacodynamic studies.



BACKGROUND

A number of analytical procedures have been used to detect sulfaquinoxaline and related compounds. These include spectrophotometry (1-4), GC (5), TLC and column chromatography (6-10), and high-performance liquid chromatography (HPLC) (11-15). A new HPLC assay has been developed for both sulfaquinoxaline and its major metabolite in order to improve upon the specificity, sensitivity, simplicity, and/or analysis time of available methods, and to explore the pharmacokinetics of the drug and its metabolite in selected species.

Pharmacokinetic studies of sulfaquinoxaline and/or its metabolites have been conducted in a number of species but not, surprisingly, in rabbits. Elimination half-lives for sulfaquinoxaline reportedly range from 6 to 15 h in cattle (16, 17), to ≥1 d in rats (18-20) and dogs (19, 20). This wide range of half-lives obviates a reliable estimate of the washout time required for sulfaquinoxaline or its metabolites in rabbits. This report, therefore, provides information on the pharmacokinetics of sulfaquinoxaline and its N^4-acetyl metabolite in rabbits.

EXPERIMENTAL SECTION

Instrumentation—A high-pressure pump[1], a high-pressure injector[2], a UV detector[3] (252 or 360 nm), and a recorder[4] comprised the chromatographic apparatus. Chromatography was performed on a reverse-phase column[5], using a reverse-phase precolumn[6] to extend column life. The mobile phase, at a flow rate of 1.7 mL/min, consisted of 35 parts methanol[7] and 65 parts 0.2 M KH_2PO_4-Na_2HPO_4 buffer[8] (pH 7). Prior to use, the mobile phase was sonicated for 5 min to remove trapped gas.

Preparation of Standards—An alkaline, aqueous stock solution (pH ~11) was prepared containing 0.2 and 0.4 mg/mL of sulfaquinoxaline[9] and the N^4-acetyl metabolite[9], respectively. Appropriate dilutions with water were prepared to yield standards ranging in concentration from 0.5 to 200 μg/mL for sulfaquinoxaline and 1.0 to 400 μg/mL for the metabolite. At the time of analysis, 50 μL of the standards was added to blank rabbit plasma (100 μL), or 100 μL of the standards was added to blank rabbit urine (500 μL).

Sample Preparation—A fixed plasma volume (100 μL) was used for all samples. Acetone[8] (300 μL) was added slowly, while vortexing, to precipitate proteins. The mixture was centrifuged (1700×*g*) for 4 min, 100 μL of the supernatant was evaporated to dryness at 65°C under a nitrogen stream, and the residue was reconstituted with 200 μL of mobile phase. An appropriate volume (20-100 μL) was injected. Glacial acetic acid-sodium acetate buffer[10] (3 M, pH 6, 500 μL) was added to a fixed volume of urine (500 μL). After mixing, the solution was extracted with methylene chloride[11] (4.0 mL). The organic phase (0.5-1.5 mL) was evaporated to dryness, following which the sample was prepared for chromatography as described above.

Pharmacokinetic Studies in Rabbits—Sulfaquinoxaline or its metabolite (50 mg/kg) was administered as an intravenous bolus dose to male New Zealand White rabbits[12] receiving 150 g of rabbit feed[13] daily and water *ad libitum*. The sulfonamide solution was prepared by dissolving sulfaquinoxaline or its metabolite in a minimum of base and back-titrating with acid to a pH of ~9 and ~10 for the drug or the metabolite, respectively. A portion of the filtered[14] solution was retained for analysis. After dose administration *via* a marginal ear vein, blood was collected from the contralateral vein through a catheter[15]. Harvested plasma from heparinized blood was frozen until analyzed. Complete, quantitative urine collections were accomplished with an indwelling bladder catheter[16] for the first 8 h followed by further recovery from a metabolic cage[17]. The urine samples were carefully mixed, centrifuged, and the supernatant was frozen until analyzed.

Data Analysis—Calibration curves of sulfaquinoxaline or the N^4-acetyl metabolite were based upon absolute peak heights obtained from a fixed HPLC injection volume. Unknown concentrations were obtained by interpolation from these linearized curves. Pharmacokinetic parameters were computed by standard methods (21). Data were analyzed[18] with a nonlinear regression program developed by D'Argenio and Schumitsky (22). The weighting factor throughout was the inverse-square of the observed data. The required number of exponentials was established by the method of Boxenbaum *et al.* (23).

RESULTS AND DISCUSSION

Development of Assay—Figure 1 illustrates chromatograms of sulfaquinoxaline and its metabolite obtained after processing rabbit plasma and urine samples. The drug and its metabolite eluted in a reasonable period of time, were well resolved, and well separated from materials inherently present in biological fluids. A number of compounds, including nine other sulfonamides,

[1] Model 100A; Altex.
[2] Universal Inlet HPLC valve; Valco.
[3] Waters Associates.
[4] Tracor Westronics MT.
[5] Hibar II, C_{18} 10 μm, 250 × 4.6 mm i.d.; E. Merck.
[6] C_{18}, 30-40 μm, 40 × 3.2 mm i.d.; Perisorb.
[7] HPLC grade; Fisher Scientific.
[8] Certified ACS; Fisher Scientific.
[9] Merck Sharp and Dohme.
[10] BDH Analar; J.T. Baker.
[11] Glass distilled; Burdick & Jackson.
[12] Riemans Fur Ranch, Ontario.
[13] Purina.
[14] 0.2 μm; Amicon.
[15] Deseret; Angiocath.
[16] 3mL pediatric; Foley.
[17] Nalgene rabbit cage.
[18] LSI 11/23; Digital Equipment Corp.

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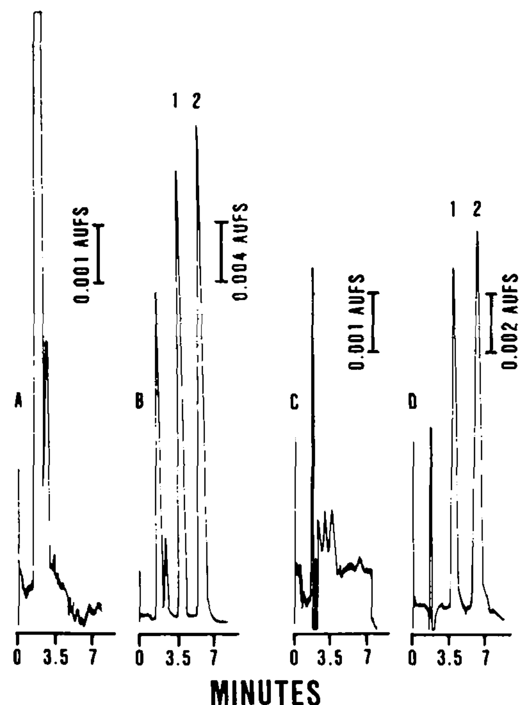

Figure 1—*Chromatograms obtained for sulfaquinoxaline (1) and the* N⁴-*acetyl metabolite (2) in rabbit plasma and urine. The mobile phase consisted of 35 parts methanol and 65 parts 0.2 M, pH 7, phosphate buffer flowing at 1.7 mL/min through an RP_{18} column (250 × 4.6 mm i.d.). Detection was at 252 nm for plasma and 360 nm for urine. Key: (A) blank rabbit plasma; (B) sulfaquinoxaline (52.25 μg/mL) and the* N⁴-*acetyl metabolite (100.5 μg/mL) in blank rabbit plasma; (C) blank rabbit urine; (D) sulfaquinoxaline (39.80 μg/mL) and the* N⁴-*acetyl metabolite (83.40 μg/mL) in blank rabbit urine.*

were tested as potential internal standards. Invariably they eluted too quickly or interfered with the quantitation of I and II which exhibited retention times of ~3.6 and 5.5 min, respectively. Since no suitable internal standard was found, a fixed-volume injection method was employed. The absolute limits of detection, expressed as a measurable peak at least three times the baseline noise, were 2.8 and 5.5 ng for I and II, respectively. Using the aforementioned sample preparation methods, the limits became 0.25 and 0.50 μg/mL in plasma and 0.10 and 0.20 μg/mL in urine, respectively.

The composition of the mobile phase was examined and it was found that decreasing the methanol concentration increased the retention times for both compounds. However, the retention time of II increased at a faster rate than I. Buffer strength had little effect on the range from 0.10 to 0.25 M. The pH of the buffer had a pronounced effect on I and II. Increases in pH >4 led to rapid decreases in times, especially for II. The results for sulfaquinoxaline are in keeping with its reported pK_a of ~6 (11, 19). Surprisingly, pH 7 proved to be a good compromise between short times and well-resolved peaks, even though it exceeded the pK_a.

Table I—The Effect of Urine pH Buffered With 3 M Acetate Buffer on the Recovery of Sulfaquinoxaline (I) and the N^4-Acetyl Metabolite (II)

Compound	Buffer-Urine pH	Relative Compound Peak Height[a]	Relative Interference Peak Height[b]
I	4.49	0.91	0.0275
	4.94	0.95	0.0221
	5.42	0.97	0.0176
	5.95	0.89	0.0097
	6.52	0.85	0.0024
II	4.49	0.97	0.0056
	4.94	1.00	0.0069
	5.42	1.00	0.0080
	5.95	0.88	0.0100
	6.52	0.42	0.0123

[a] Ratio of the peak height of the compound, after adjusting for interference peaks, to the height from a standard prepared in water. [b] Ratio of the interference peak height observed in blank urine to the peak height of the compound corrected for the interfering peak; measured at 252 nm.

Table II—Intra-assay Variability in the Analysis of Plasma or Urine Sulfaquinoxaline (I) and the N^4-Acetyl Metabolite (II) Standards[a]

Compound	Conc., μg/mL		CV, %	
	Plasma	Urine	Plasma	Urine
I	0.2662	0.2130	26.04	8.97
	2.662	1.065	2.89	19.04
	26.62	10.65	1.14	1.83
	106.5	42.60	1.35	2.97[b]
II	0.5175	0.4140	20.60	10.48
	5.175	2.070	7.34	7.03
	51.75	20.70	6.85	1.49
	207.0	82.80	1.41	1.36[b]

[a] n = 5. [b] n = 4.

Acetone, acetonitrile, anhydrous ethanol, methanol, and perchloric acid were examined as plasma protein precipitating agents. Perchloric acid was eliminated because the evaporation step was too time-consuming. Peak heights obtained for the drug and the metabolite after acetone treatment were consistently higher (4-8%) than those seen after treatment with the other agents. Furthermore, acetone provided the finest, most stable precipitate, clearest supernatant after centrifugation, and has been shown to be thorough in removing proteins (24). It was, therefore, the agent of choice.

It was initially anticipated that the plasma assay for sulfaquinoxaline and the N^4-acetyl metabolite could simply be extended to urine analysis. However, it was found that unknown, apparently endogenous components appeared as interfering peaks in the chromatograms. Consequently, various approaches were used with the following observations: (*a*) methylene chloride was chosen as an extracting solvent because solubility tests indicated that the drug and its metabolite were about four times more soluble in this solvent than in acidic water, and this ratio exceeded that observed for ether;. (*b*) because of its high buffering capacity, a strong buffer was needed to acidify the normally alkaline [pH 8-9 (25)] rabbit urine. Phosphate buffer was unsuitable due to solubility problems at concentrations >1 M. Acetate buffer, 3 M, provided the most accurate and predictable buffering of the urine. Table I indicates the degree of recovery of the drug and the metabolite in urine with this buffer; (*c*) a pH of 6 was selected as a compromise between adequate recovery of the sulfonamides and minimal solvent front and interference peak heights. To eliminate the latter, the secondary maximum, 360 nm ($\epsilon = 7.2 \times 10^3$ for the drug and the metabolite), was chosen as the detection wavelength even though 252 nm ($\epsilon = 3.2 \times 10^4$ for the drug and its metabolite), as used for plasma samples, was preferred.

Calibration curves were highly linear ($r > 0.99$) over the concentration ranges in plasma and urine of 0.25-200 μg/mL and 0.10-40 μg/mL, respectively, for sulfaquinoxaline and 0.50-400 μg/mL and 0.20-80 μg/mL, respectively, for the N^4-acetyl metabolite. Intra-assay variability is summarized in Table II and, except for the lowest concentrations, was <10%. Interassay analysis of the calibration curves in plasma provided mean (±*SD*) slopes and intercepts of 0.395 (0.166) and 0.00405 (0.0236), respectively, for sulfaquinoxaline (n = 9) and 0.348 (0.0261) and −0.00385 (0.0138), respectively, for the N^4-acetyl metabolite (n = 4). Such variability, when based on peak-height analysis without an internal standard, reflects changes in column characteristics. However, this presents no difficulty if calibration curves are generated with each set of samples. As only two calibration curves were prepared for urine samples, interassay results are not available.

Of the reported HPLC assays for sulfaquinoxaline (11-15), none describes the simultaneous measurement of the drug and its N^4-acetyl metabolite. Only one of these assays (14) has been developed to measure sulfaquinoxaline in plasma or tissues, and it employs inconvenient extraction methods and yields

Table III—Mean (±*SD*) Pharmacokinetic Parameters Obtained From Plasma Analysis of Sulfaquinoxaline (I) and the N^4-Acetyl Metabolite (II) After Single Intravenous Doses of I and II, Respectively

Variable	I	II
n	11	4
Dose, mg/kg	49.64 (0.76)	49.94 (1.29)
AUC, μg·h/mL[a]	836.1 (363.6) 737.3 (166.6)[b]	2769.9 (326.6)
TBC, (mL/h/kg)[c]	66.47 (19.75) 70.36 (15.78)[b]	18.18 (1.82)
V_1, mL/kg[d]	210.7 (41.85)	140.2 (59.37)
Terminal Constant, h^{-1}	0.0645 (0.0188)	0.0494 (0.0174)
Terminal Half-Life, h	12.67 (7.99)	15.96 (7.40)

[a] Area under the plasma concentration-time curve from time 0 to infinity. [b] Mean (±*SD*) results excluding Rabbit No. 83 (see text). [c] Total body clearance from plasma. [d] Volume of distribution in the central compartment.

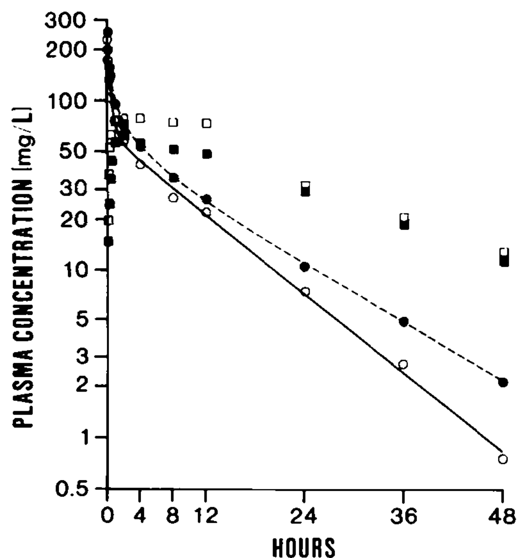

Figure 2—*Plasma sulfaquinoxaline (○) and the* N[4]*-acetyl metabolite (□) observed in two rabbits following intravenous sulfaquinoxaline administration. Key: rabbit 143 (○,□) bolus dose was 49.03 mg/kg [(—) indicates the best fit of the data to a biexponential equation]; rabbit 150 (●,■) bolus dose was 49.51 mg/kg [(- - -) indicates the best fit of the data to a triexponential equation].*

a retention time of 13 min. The new method described herein was simple, reproducible, and had the requisite sensitivity to measure the drug and its metabolite in animal tissues or fluids. Also, interference from potential metabolites was not encountered.

Pharmacokinetic Studies in Rabbits—The intravenous administration of I to rabbits provided multiexponential profiles for the drug and the metabolite in plasma. Table III includes the mean ($\pm SD$) pharmacokinetic parameters for the drug obtained after fitting the data to bi- or triexponential equations. Seven rabbits required three exponentials while four required only two exponentials. Plasma profiles for two of the rabbits are shown in Fig. 2.

One rabbit (No. 83) displayed a total body clearance for the drug that was one-third as great as the mean clearance observed for the remainder of the rabbits. Plasma levels of the drug in this animal were elevated, while plasma levels of the metabolite were reduced. Also, the terminal phase of the metabolite profile was parallel to that of the parent drug. All of the other rabbits displayed nonparallel terminal phases with a mean ($\pm SD$) metabolite half-life of 15.4 (3.5) h. The data would suggest that rabbit 83 is a slow acetylator, although the clearances of the other animals could not be allocated to definable

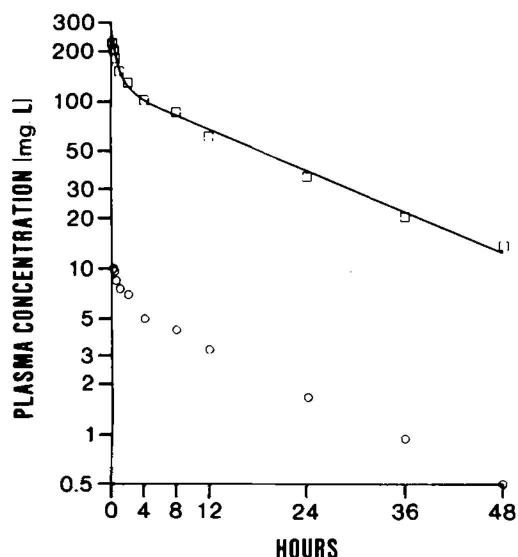

Figure 3—*Plasma sulfaquinoxaline (○) and the* N[4]*-acetyl metabolite (□) observed in rabbit 143 after an intravenous bolus dose (51.16 mg/kg) of the acetylated compound. The solid line represents the best fit of the data to a biexponential equation.*

Table IV—Urinary Excretion Data Observed in Two Rabbits Given Both Sulfaquinoxaline (I) and Its Metabolite (II) on Separate Occasions

Rabbit	Compound Administered	Dose, mg/kg	Amount Excreted, mg/kg[a] I	Amount Excreted, mg/kg[a] II	Renal Clearance, mL/h/kg[b] I	Renal Clearance, mL/h/kg[b] II
179	I	48.96	3.172	13.36	5.978	8.054
	II	49.71	5.343	32.06	61.35	10.85
184	I	49.08	3.035	16.47	5.421	8.477
	II	48.21	3.588	31.83	32.06	13.42

[a] 0-96 h. [b] Amount excreted to 48 h/AUC from 0 to 48 h.

groups. While the phenomenon of fast and slow acetylation has been widely reported and reviewed (26), no known studies have used sulfaquinoxaline as a probe.

Rabbits reportedly lack the ability to deacetylate (27) whereas such metabolism has been observed in other species (27-30). The N^4-acetyl metabolite was, therefore, administered intravenously to 4 rabbits. Unexpectedly, a plasma chromatographic peak was observed with a retention time corresponding to sulfaquinoxaline. Initially it was felt that the presence of sulfaquinoxaline reflected contamination of the metabolite dose by the drug. However, HPLC analysis of the filtered metabolite solution administered to the rabbits confirmed the absence of the drug. Figure 3 illustrates the profiles observed in one of the experiments. Table III also includes the mean ($\pm SD$) pharmacokinetic parameters obtained for the metabolite after its administration to four rabbits. The mean terminal half-life of the metabolite in these experiments agrees well with that observed after sulfaquinoxaline administration. Two animals (Nos. 179 and 184) were studied in greater detail by monitoring both plasma and urine for 96 h after administering the drug and the metabolite on different occasions, two weeks apart. The results in Table IV indicate a sizeable urinary excretion of the drug following administration of the metabolite. The results also demonstrate that urinary recovery of the drug and the metabolite do not account for either administered dose. Perhaps the sulfonamides undergo partial elimination *via* the feces (18) and/or alternate routes of metabolism (17, 19, 31). Finally, the renal clearances of sulfaquinoxaline are notably different when sulfaquinoxaline or the N^4-acetyl metabolite are administered. This anomaly remains to be explored.

From the total body clearance of rabbits 179 and 184 (90.52 and 86.17 mL/h/kg, respectively), one might surmise that the rabbits disposed of the drug in an identical manner and that they might even be identical genetic acetylators. Analysis of the plasma data from these rabbits according to the technique proposed by Wagner *et al.* (32) for prednisone-prednisolone interconversion *in vivo*, provided intriguing results. The method, which permits a calculation of each clearance in the interconversion, pointed to a 40% greater acetylation clearance in rabbit 184 accompanied by a 32% greater deacetylation clearance. The obvious effect of these parallel differences would to be provide an identical total body clearance of the drug in the two rabbits. This preliminary observation has important implications in defining the genetic acetylation characteristics of humans and animals.

The primary purpose in developing an HPLC assay for sulfaquinoxaline and the N^4-acetyl metabolite was to monitor their presence in purchased rabbits. The relatively high single dose of the drug administered in this study required ~4 d to reach plasma levels of the drug and the metabolite, <0.25 and 0.50 μg/mL, respectively. Even though animal breeders undoubtedly use lower chronic daily doses for prophylactic treatment, it would seem prudent for investigators to avoid using rabbits for 1 week after receipt from a supplier.

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