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ACKNOWLEDGMENTS

T. A. Iranloye is grateful to University of Ife for granting him study leave.

Analytical Methods for the Determination of Sulindac and Metabolites in Plasma, Urine, Bile, and Gastric Fluid by Liquid Chromatography Using Ultraviolet Detection

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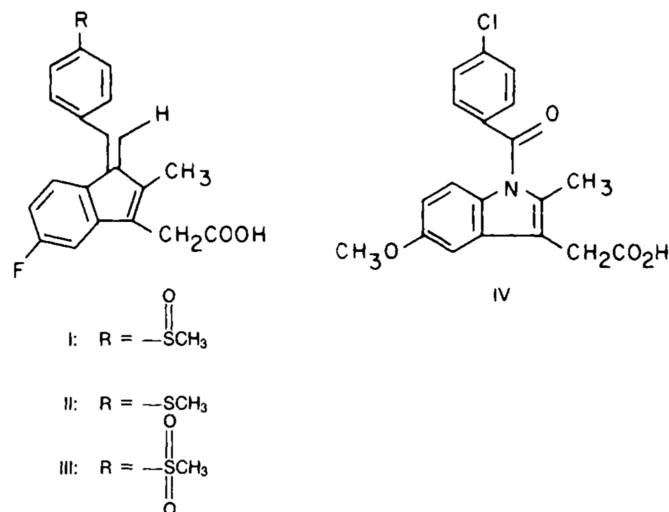
Received March 15, 1983 from the *Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.* Accepted for publication October 17, 1983.

Abstract □ A high-performance liquid chromatographic method using a linear elution gradient has been developed for the analysis of sulindac, sulindac sulfone, and sulindac sulfide in plasma, urine, bile, and gastric fluid. The methodology uses reverse-phase, radial compression chromatography with gradient elution, and UV detection. Sulindac and its metabolites in plasma can be quantitated at 0.25 μg/mL with a mean *CV* of 6.0 ● 2.9%; urine, bile, and gastric fluid (0.5 μg/mL) yield a mean *CV* of 5.5 ± 1.9%.

Keyphrases □ Sulindac—metabolites, liquid chromatography with UV detection, human plasma, urine, bile, and gastric fluid □ Liquid chromatography—sulindac and its metabolites, human plasma, urine, bile and gastric fluid



Sulindac (*cis*-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)-benzylidene]indene-3-acetic acid; I) is an anti-inflammatory drug with analgesic and antipyretic properties. Metabolites include sulindac sulfide (II) the active species (I), and sulindac sulfone (III). Sulindac and its metabolites are detected in plasma, while sulindac, sulindac sulfone, and their respective conjugates are the major constituents excreted in urine (2).

Previous methods of analysis include an isotope dilution radioimmunoassay (3), a computerized mass spectral assay (4), and a high-performance liquid chromatographic assay (HPLC) (5), and appear to be restricted to serum or plasma. A stepwise isocratic HPLC assay has been recently reported (6) for plasma and urine determination; however, it involves tedious extraction procedures and is not free of interfering substances.

Preliminary investigations using the Dusci and Hackett method (5) led to incomplete resolution of sulindac and its metabolites from endogenous substances. Modifying this method with gradient elution afforded an assay that is applicable for measuring total levels of sulindac and its metabolites in plasma, urine, bile, and gastric fluid, and one that appears to be free from interfering substances.

EXPERIMENTAL SECTION

Apparatus—The chromatography was performed on HPLC equipment which included two solvent delivery systems[1], an auto sampler[2], a fixed-wavelength absorbance detector with a 340-nm filter[3], and a solvent programmer[4]. The absorbance responses were recorded by a computing integrator[5] (attenuation 16 mV full scale; chart speed 0.5 cm/min).

Materials—Sulindac[6], sulindac sulfone[6], sulindac sulfide[6], and indomethacin[6] (IV, internal standard) were used as received (7, 8); acetonitrile,

[1] Model 6000A Solvent Delivery Systems; Waters Associates.
[2] WISP 710B auto sampler; Waters Associates.
[3] Model 440 Absorbance Detector; Waters Associates.
[4] Model 660 Solvent Programmer; Waters Associates.
[5] SP4100 Computing Integrator; Spectra Physics.
[6] Merck Sharp & Dohme Research Laboratories. Purity for sulindac and metabolites was determined by HPLC and elemental analysis. Sulindac sulfide: calc. for $C_{20}H_{17}FO_2S$: C, 70.6; H, 5.00. Found: C, 71.0; H, 4.68. Sulindac sulfone: calc. for $C_{20}H_{17}FO_4S$: C, 64.5; H, 4.57. Found: C, 64.3; H, 4.48. Sulindac: calc. for $C_{20}H_{17}FO_3S$: C, 67.4; H, 4.81. Found: C, 67.4; H, 4.72.

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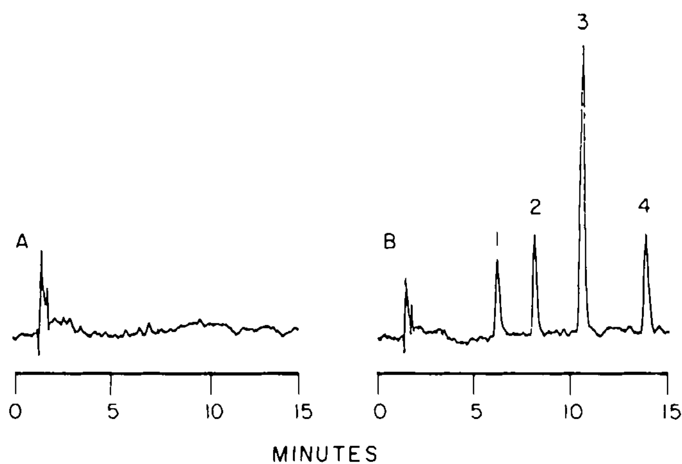


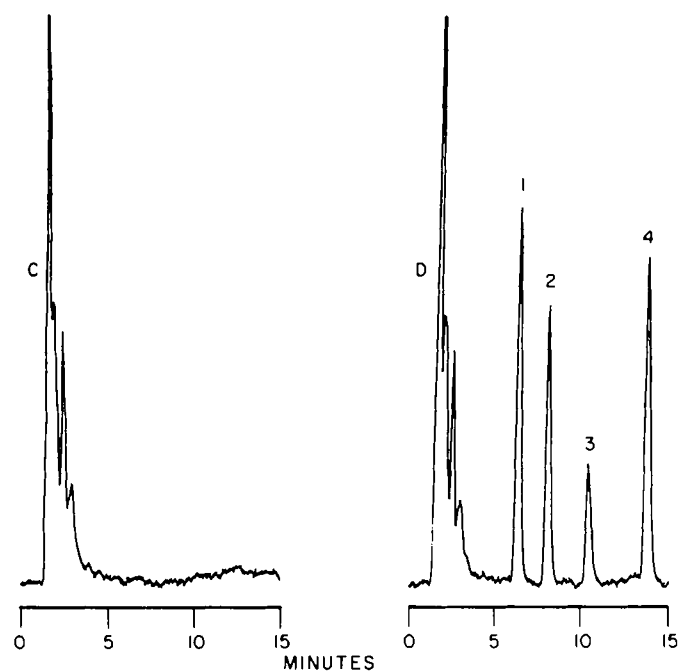


Figure 1—*Chromatograms from a stainless steel C_{18} column. Key: (A) is a plasma control blank; (B) is a plasma sample containing sulindac (1) at 1.5 μg/mL, sulindac sulfone (2) at a 1.7 μg/mL, sulindac sulfide (4)(at 1.2 μg/mL, and indomethacin (3) at 37.5 μg/mL; (C) is a urine blank; (D) is a urine sample containing sulindac at 2.7 μg/mL, sulindac sulfone at 2.0 μg/mL, sulindac sulfide at 2.3 μg/mL, and internal standard at 37.5 μg/mL.*

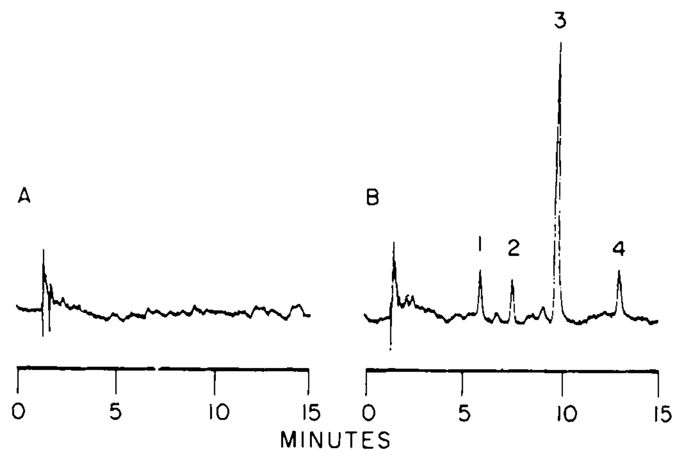
HPLC grade[7], was filtered[8]; and water was purified[9]. Heparinized control plasma was collected and prepared in-house[6].

A C_{18} stainless steel column (30 cm × 3.9 mm i.d., 10 μm)[10], a C_{18} plastic cartridge (10 cm × 8 mm i.d., 10 μm) housed in a radial compression module[11], and a reverse-phase guard column[12] were used.

Prepared Solutions—Standard stock solutions of I, II, and III were prepared weekly by dissolving 2.0 mg of the appropriate compound in 2 mL of absolute ethanol and 8 mL of sodium borate buffer, pH 7.2 (12.4-g boric acid, 10.0-mL 1 M NaOH diluted to 1 L with purified[9] water). Working standards were mixed with appropriate dilutions from the stock solution with the boric acid buffer. Internal standard stock solutions were prepared weekly by dissolving indomethacin in the sodium borate buffer, pH 7.2 (300 μg/mL).

Chromatographic Conditions— The mobile phase gradients for the plasma, urine, bile, and gastric fluid assays were: a potassium phosphate buffer, pH 3.0 (6.8-g KH_2PO_4 in 1000 mL water with the pH adjusted to 3.0 with 85% phosphoric acid) and a mixture of acetonitrile and the phosphate buffer (70:30

[7] Burdick & Jackson Laboratories.
[8] 0.5 μg Zeflor filter; Rainin.
[9] Milli-Q Water Purification System; Millipore Corp.
[10] μ-Bondapak C_{18} column; Waters Associates.
[11] RCM-100 containing a Radial-PAK C18 cartridge; Waters Associates.
[12] Microguard column; Bio-Rad.



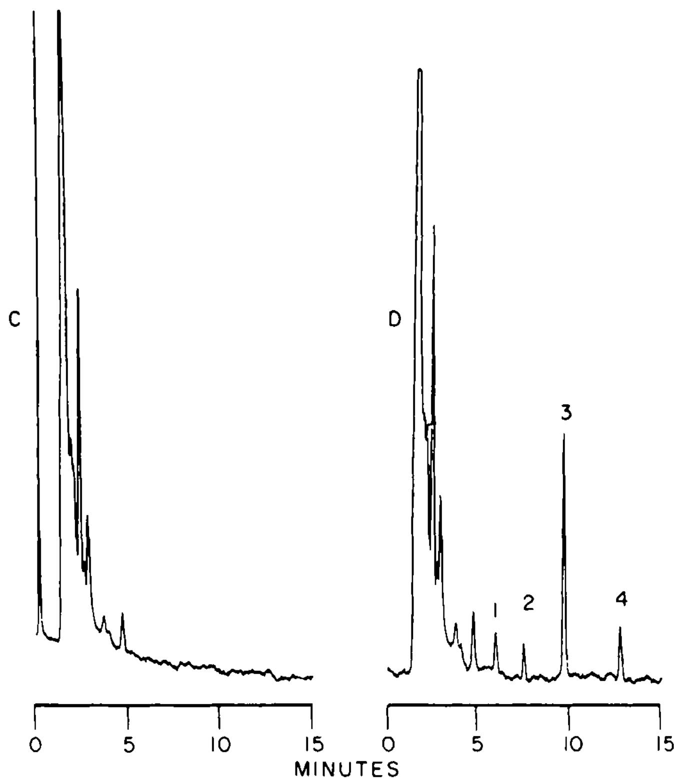


Figure 2—*Chromatograms from a radially compressed C_{18} column. Key: (A) is a control plasma blank; (B) is a plasma standard containing sulindac and its metabolites; (C) is a control urine; (D) is a urine standard. All concentrations of sulindac and its metabolites are at 0.5 μg/mL; the internal standard concentrations are 40.0 μg/mL for plasma and urine standards.*

v/v). Both solutions were filtered (cellulose, 0.5 μm) and kept under a helium atmosphere. During a gradient run the two solutions were mixed at 53:47, respectively, and changed to 7:93 over 10 min (linear gradient time) at 2 mL/min. The final solvent ratio continued to run for 5 min. The radial C_{18} column was reequilibrated for 5 min, with the initial mobile phase conditions, before the next sample run. Due to chromatographic interferences in the bile of certain patients in clinical studies, the gradient conditions for the bile assay required a 15-min gradient time and a 3-min hold.

Plasma Preparation—A 0.3-mL plasma sample was spiked with the internal standard, indomethacin (40.0 μL). One milliliter of acetonitrile was mixed with the plasma aliquot on a vortex mixer (30 s). The tube was then centrifuged (3500 rpm)[13], and the liquid sample was transferred to a tapered tube. The volume was reduced to 0.1 mL with a stream of nitrogen and a warm water bath (50°C) and then analyzed by HPLC (20-μL injection volume).

Urine, Bile, and Gastric Fluid Preparation—A 0.3-mL fluid sample of urine, bile, or gastric fluid was mixed with 0.1 mL of 5 M NaOH. The sample was

[13] International Centrifuge, Size 2, Model K.

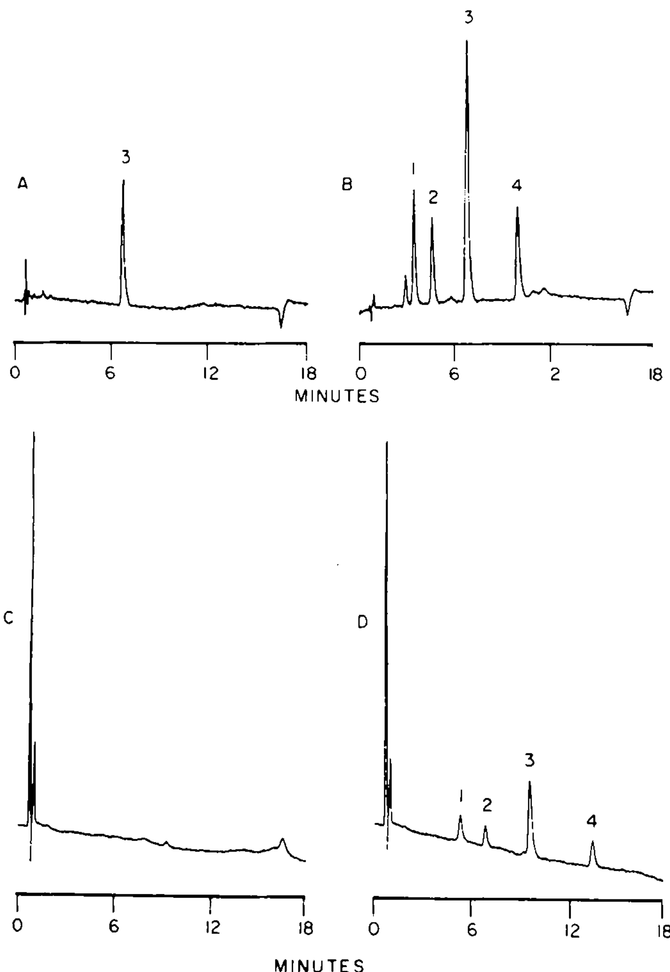


Figure 3—*Chromatograms from a radially compressed C_{18} column. Key: (A) is a control gastric fluid blank containing internal standard; (B) is a gastric fluid standard; (C) is a control bile blank; (D) is a bile standard. All concentrations of sulindac and its metabolites are at 0.5 μg/mL; the internal standard concentrations are 6.0 μg/mL for gastric and 25.0 μg/mL for bile standards.*

incubated at room temperature (25–27°C) for 15 min. The pH was then adjusted with the addition of 0.1 mL of phosphoric acid (28.3%). The internal standard (40 μL for urine, 6 μL for gastric fluid, and 25.0 μL for bile) and 1 mL of acetonitrile (1.5 mL for gastric and intestinal fluids) was mixed with the sample. In all cases, the samples were vortex-mixed (30 s) and centrifuged (3500 rpm)[13]. The organic layer was transferred to a tapered tube, the volume was reduced to 0.1 mL, and then it was analyzed by HPLC (20-μL injection volume for urine, 35-μL for bile, and 40-μL for gastric fluid).

Standard Curves—Plasma standards were prepared by mixing 1 mL of control plasma with the internal standard and appropriate volumes of I, II, and III to achieve concentrations from 0.25 to 10 μg/mL, *e.g.*, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 μg/mL. From these standards, 0.3-mL aliquots were removed and processed as above.

Urine, bile, and gastric fluid standards (1 mL) were prepared similarly but without the internal standard, which is unstable under the basic conditions required for conjugate hydrolysis. The samples were hydrolyzed, pH adjusted, spiked with internal standard, and processed as above.

The standard curves were plotted with peak height ratios *versus* concentrations. A validation curve consisted of the above concentrations with six replicates per concentration (each replicate was prepared separately). A daily standard curve was assayed with the samples; unknown sample concentrations were interpolated from these daily curves.

Recovery Values—Recovery values were measured by comparing the amount of labeled material prepared in a standard sample with the amount detected in acetonitrile after deproteination and/or extraction. Standards of labeled sulindac ([2-^{14}C]acetic acid, 26 μCi/mg)[14], and sulindac sulfone ([^{3}H]methylene, vinyl, 50 μCi/mg), and sulindac sulfide ([^{3}H]methylene, 256 μCi/mg) were prepared in concentrations of 0.25, 1.0, and 10.0 μg/mL (3, 9) and processed. The acetonitrile extracts were measured for volume and counted for radioactivity. The radioactivity measured for the extracts was divided by the radioactivity of the standard solutions.

Table I—Linear Regression Parameters

	Slope	Intercept	r^2	Range, μg/mL
Plasma				
Sulindac	0.3703	−0.0232	0.9999	0.25–10
Sulindac Sulfone	0.3119	0.0326	0.9999	0.25–10
Sulindac Sulfide	0.3517	0.0084	0.9998	0.25–10
Urine				
Sulindac	0.2942	0.0072	0.9996	0.5–10
Sulindac Sulfone	0.2647	−0.0006	0.9996	0.5–10
Sulindac Sulfide	0.2677	−0.0123	0.9980	0.5–10
Bile				
Sulindac	0.0967	0.0345	0.9999	0.5–10
Sulindac Sulfone	0.0955	0.0011	0.9998	0.5–10
Sulindac Sulfide	0.1147	−0.0139	0.9995	0.5–10
Gastric				
Sulindac	1.0202	−0.0880	0.9998	0.5–5.0
Sulindac Sulfone	0.7213	−0.0402	0.9996	0.5–5.0
Sulindac Sulfide	0.8423	−0.1231	0.9950	0.5–2.5

Table II—Precision of the Assay

	CV, %[a]		
	Sulindac	Sulindac Sulfone	Sulindac Sulfide
Plasma Standards			
0.25	8.76	6.34	3.01
1.0	7.76	3.67	6.75
10.0	1.63	1.84	2.69
Urine Standards			
0.5	4.23	9.10	6.01
2.5	5.66	7.56	6.77
10.0	4.22	4.29	6.50
Intestinal Fluid			
0.5	6.4	6.3	2.9
2.5	10.4	7.3	8.4
10.0	11.8	8.1	8.6
Gastric Fluid			
0.5	5.2	6.3[b]	3.2
1.0	5.3	[b]	3.2
2.5	6.5	4.8	5.9
5.0	3.6	5.7	—[c]

[a] n = 6. [b] No replicates run. [c] Standard curve upper limit 2.5 μg/mL.

RESULTS AND DISCUSSION

This assay, a modification of an isocratic, reverse-phase HPLC method for the determination of sulindac in plasma (5), involves the use of a linear elution gradient to resolve sulindac and its metabolites from interfering peaks found in clinical plasma samples and other human fluids, *e.g.*, urine, bile, and gastric fluid. The mobile phase of acetonitrile and potassium phosphate buffer (pH 3.0) was adjusted so that separation from endogenous substances was achieved, the resolution of the sulfone metabolites and sulindac was enhanced, and the elution of the sulfide metabolite was hastened.

Chromatographic separation using a linear elution gradient was achieved, initially, for plasma and urine on a stainless steel C_{18} column[10] at 40:60 acetonitrile–potassium phosphate buffer (2 mL/min) changing to 65:35 over 10 min. Hold time and equilibration delay were set at 5 min. These HPLC conditions gave an assay applicable only to plasma and urine fluids (Fig. 1). Adaptation of the mobile phase conditions to a radially compressed C_{18} cartridge resulted in an assay applicable to plasma, urine, bile, and gastric fluid. This latter assay elutes and resolves sulindac and its metabolites in a chromatographic manner similar to the former assay with the stainless steel column. The chromatograms of plasma, urine, bile, and gastric fluid blanks did not show endogenous substances that would interfere with sulindac, its me-

[14] [^{14}C]Sulindac, (*Z*)-5-fluoro-2-methyl-1-[[4-(methyl-sulfinyl)phenyl] methylene]-1*H*-indene-3-[2-^{14}C]acetic acid, was prepared by Mr. Gregory, J. Gatto, and Miss Jane Chan in the Radiochemical Synthesis Laboratory of MSDRL, Rahway, N.J. under the direction of Dr. Holly E. Mertel. The procedure is unpublished. Labeled carbon was introduced by treating cyano[2-^{14}C]acetic acid (American Radiochemical Co., Sanford, Fla.) with 6-fluoro-2-methyl-1-indone, followed by hydrolysis and decarboxylation steps. The resulting 5-fluoro-2-methyl-1*H*-indene-3-[2-^{14}C]acetic acid was condensed with *p*-methyl-sulfinylbenzaldehyde to furnish the title tracer compound. A description of a related sequence is found in Canadian patent 1,027,967, March 14, 1978, (to John M. Chemerda and Seemon H. Pines).

Table III—Human Urine, Bile, and Gastric Fluid Levels of Sulindac and Metabolites (200 mg im of Sulindac)

Time[a]	Sulindac, µg/mL	Sulindac Sulfone, µg/mL	Sulindac Sulfide, µg/mL
Urine			
−1-0	0	0	0
0-6	34.60	9.15	0
6-24	11.60	14.90	0
Bile			
0-60	0	0	0
60-80	1.31	0.56	0
80-100	2.42	1.55	0
100-120	4.02	2.61	0
120-140	8.73	5.85	0
140-160	8.24	5.50	0
160-180	8.75	5.90	0
180-200	8.42	5.87	0
200-220	9.92	7.69	0
220-240	8.42	7.24	0.52
240-260	7.98	7.11	0.55
260-280	7.67	7.32	0.62
280-300	9.40	8.84	0.80
Gastric Fluid[d]			
0-160	—[c]	0	0
160-180	3.96	3.66	0
180-200	5.59	5.49	0
200-220	4.83	4.96	0
220-240	2.29	2.43	0
240-260	7.92	9.49	0
260-280	7.82	9.62	0
280-300	6.39	8.16	0
300-320	5.13	6.59	0
320-340	1.74	2.47	0

[a] Time period: for urine in hours, for bile and gastric fluid in minutes. [b] 20-min period collections gave 0.0 conc. [c] Only sulindac was found in the 100–120-min interval (1.55 µg/mL). [d] The gastric fluid levels of sulindac and its metabolites are due to the reflux of bile into the stomach.

tabolites, or indomethacin. Sulindac, sulindac sulfone, sulindac sulfide, and indomethacin were completely resolved (Figs. 2 and 3).

Acetonitrile deproteination of plasma (4) provided a method with acceptable reproducibility and linearity. Organic extraction techniques (*e.g.*, diethyl ether) consistently gave coefficients of variation of intraday replicates exceeding 10%.

Standard curves for I, II, and III in plasma were linear over the concentration range of 0.25–10.0 µg/mL; urine and bile gave linear curves over the range of 0.5–10 µg/mL; gastric fluid gave a linear curve over the range of 0.5–5 µg/mL (Table I). Concentrations at 0.1 µg/mL in plasma and in urine could be detected, but poor reproducibility restricted the low concentrations to 0.5 µg/mL. Linearity is lost at the higher concentrations (tested at 20 and 40 µg/mL) due to lower absorbance responses.

The *CV* measured for the plasma assay of the three components gave an average of 4.7 ± 2.7% over the linear concentration range. The urinary, bile, and gastric fluid assays gave average *CV* values of 6.04 ± 1.6%, 7.80 ± 2.5%, and 4.87 ± 1.24%. Representative *CV* are listed in Table II.

Recoveries of I, II, and III from plasma *via* acetonitrile protein precipitation are 39.9 ± 4.2%, 49.8 ± 6.7%, and 86.3 ± 3.7%, respectively. Similarly, representative recovery values for acetonitrile extraction (urine) are 58.2 ± 7.4%, 65.1 ± 9.9%, and 96.7 ± 6.9%. These values are means over three concentrations.

The hydrolysis of urine conjugates for total sulindac and its two metabolites by base catalysis were studied over time. Peak height values for sulindac and its metabolites reached a maximum in the first 15 min and began to decline

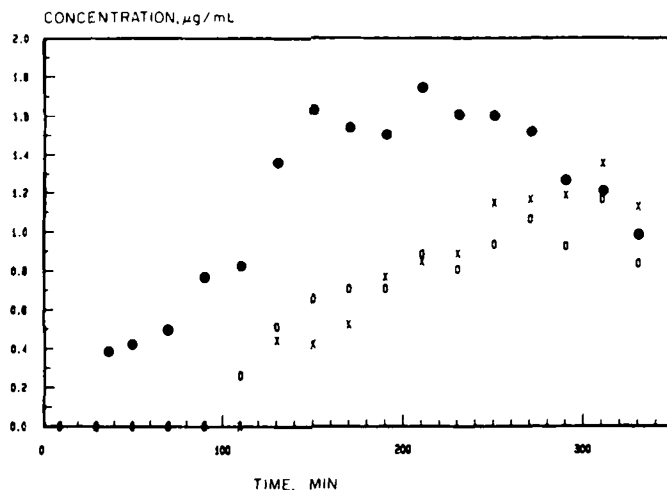


Figure 4—*Plasma levels of sulindac and its two metabolites in one subject administered a single 200-mg im injection of sulindac. Key: (●) sulindac; (O) sulindac sulfone; (X) sulindac sulfide.*

at 60 min. Reaction conditions were set with a 15-min incubation period at room temperature. Similar results were found for bile and gastric fluid.

Concentrations of sulindac and its metabolites in human plasma, urine, bile, and gastric fluid are represented in Fig. 4 and Table III. The data are of one subject who received a single intramuscular injection of sulindac (200 mg).

CONCLUSIONS

Gradient elution with acetonitrile and potassium phosphate buffer over a radially compressed, reverse stationary phase provides the necessary selectivity of the assay for quantitation of sulindac and its metabolites in plasma, urine, bile, and gastric fluid. The advantages of this gradient system over other available isocratic assays (5, 6) are: (*a*) a cleaner chromatogram for the plasma medium, (*b*) a shorter cleanup procedure for plasma and urine, and (*c*) a broader application of the assay to other biological fluids.

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