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## ACKNOWLEDGMENTS AND ADDRESSES

Received November 6, 1975, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907.*
Accepted for publication May 19, 1976.
Presented at the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Atlanta meeting, November 1975.
Supported in part by a David Ross Fellowship, Purdue Research Foundation.
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# Analysis of Sulindac and Metabolites by Combined Isotope Dilution–Radioimmunoassay

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**Abstract** □ Sulindac, a new anti-inflammatory agent, and its sulfone and sulfide metabolites were conjugated to bovine serum albumin by the *N*-hydroxysuccinimide active ester procedure. Antiserum from rabbits immunized with each of these haptens exhibited extensive cross-reactivity, precluding differential analyses of the three species by displacement assay without prior separation. Therefore, an analytical method based on a combination of isotope dilution and radioimmunoassay was devised. A known mixture of the three chemical species, each labeled with tritium, was equilibrated with plasma or urine samples, reisolated chromatographically, and quantitated by binding to an appropriate immunoglobulin. The radiolabeled materials thus served as recovery standards as well as labeled antigens for each displacement assay. Sulindac and each of its metabolites in plasma or urine at concentrations as low as 500 ng/sample were differentially determined by this procedure. However, since an extraction is required, several milliliters of plasma can be used for each sample, thus increasing the actual sensitivity of the assay.

**Keyphrases** □ Sulindac—and metabolites, combined isotope dilution–radioimmunoassay, human plasma and urine □ Radioimmunoassay—combined with isotope dilution, analysis of sulindac and metabolites, human plasma and urine □ Isotope dilution–radioimmunoassay—analysis of sulindac and metabolites, human plasma and urine □ Anti-inflammatory agents—sulindac and metabolites, combined isotope dilution–radioimmunoassay, human plasma and urine

Sulindac, *cis*-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)benzylidenyl]indene-3-acetic acid (I), is a new anti-inflammatory agent (1, 2) currently in clinical trials. Its two principal metabolites, the sulfone (II) and sulfide (III), differ from the parent drug only with respect to the oxidation state of the sulfur moiety (3). Metabolic reduction of sulindac to the sulfide is reversible, but oxidation to the sulfone is irreversible; thus, disposition patterns of this drug are complex. Furthermore, the sulfide metabolite has been proposed as the pharmacologically active species[1]. Thus, any analytical procedure should ideally be capable of distinguishing among these closely related compounds.

GC analysis of sulindac and metabolites was attempted without success[2]. The principal difficulties encountered were the lack of sensitivity for sulfide and the insufficient separation of sulindac and its sulfone metabolite. Another potentially useful method is mass fragmentography, and such an assay for sulindac is in the final stages of development[3].

A third possible method is radioimmunoassay, provided that specific antiserums to each compound are available. Early results suggested that specific antibodies to sulindac and metabolites could not be obtained. One solution to the problem of poor selectivity is to separate the compounds chromatographically prior to radioimmunoassay. This approach has been applied to assays of steroids and prostaglandins (4–6). A slightly different technique utilizing isotope dilution in combination with radioimmunoassay has been devised for the differential determination of sulindac and its metabolites and is the subject of this report.

## EXPERIMENTAL

**Materials**—Bovine serum albumin[4], *N*-hydroxysuccinimide[5], dicyclohexylcarbodiimide[5], neutral charcoal[6], and dextran[7] were used. Dextran-coated charcoal was prepared by suspending 6.25 g of prewashed charcoal and 0.625 g of dextran in 100 ml of 0.05 *M* phosphate buffer, pH 7.5.

**Preparation of Labeled Antigens**—[$^3$*H-Methylene*]*sulindac Sulfide*—A mixture of sulindac sulfide (0.5 g), potassium *tert*-butoxide (0.6 g), and tritiated water (2 ml, 25 Ci) was heated at 90° for 2 hr. Water was then added, and the mixture was acidified. The labeled sulfide that precipitated was collected and dissolved in methanol. The solution was evaporated (several times) to remove labile tritium. Several recrystallizations from benzene afforded pure tritiated sulfide (350 mg), mp 186–187°, specific activity 172 $\mu$Ci/mg.

[$^3$*H-Methylene*]*sulindac*—Sodium metaperiodate (144 mg) in water (2 ml) was added to a solution of tritiated sulindac sulfide (102 mg) in methanol (8 ml) and acetone (2 ml), and the mixture was stirred at 25° for 16 hr. The mixture was concentrated, diluted with water, acidified, and extracted with ethyl acetate. The solid obtained by evaporating the ethyl acetate was recrystallized several times from ethyl acetate to yield 53 mg of pure sulindac, mp 181–183°, specific activity 167 $\mu$Ci/mg.

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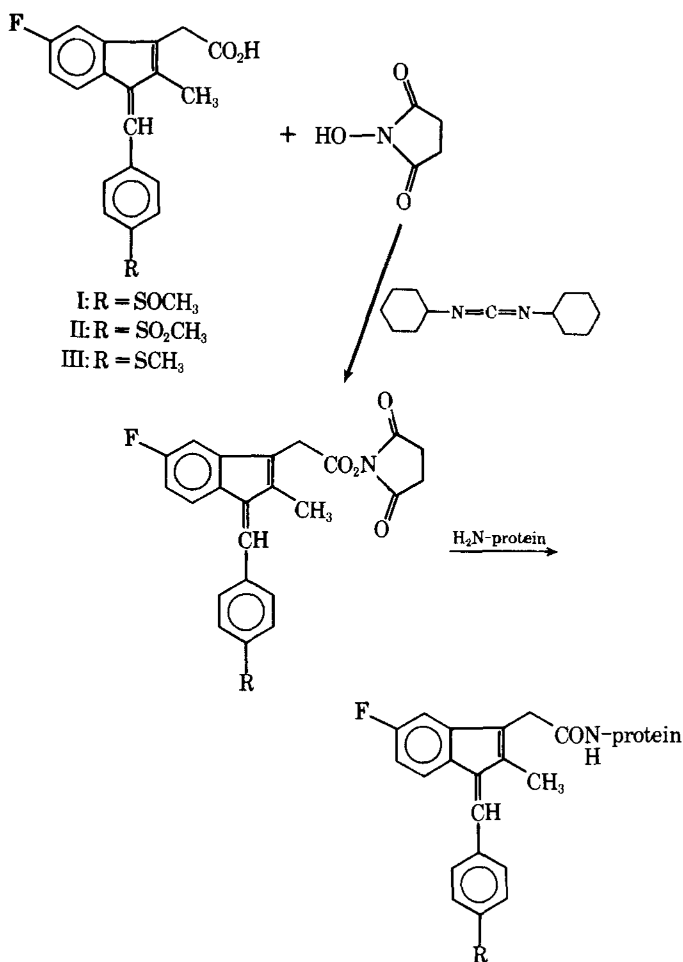


*Scheme I*

[$^3$*H-Methylene, vinyl*]*sulindac Sulfone*—Sulindac sulfone was labeled in the same manner as the sulfide. The crude product was redissolved in 1 *N* sodium hydroxide for 5 min and then precipitated with acid (several times) to remove labile tritium from the sulfonylmethyl and carboxyl groups. Two recrystallizations from acetic acid–water afforded pure tritiated sulfone, mp 200–201°, specific activity 337 $\mu$Ci/mg. The NMR spectrum of the deuterated analog showed partial exchange of the vinyl proton as well as the methylene protons.

**Preparation of Conjugates**—Sulindac and metabolites were each conjugated through the carboxylic acid moiety to bovine serum albumin by the *N*-hydroxysuccinimide active ester procedure (7, 8) (Scheme I). Conversion of the acids to the succinimide esters was verified by the appearance of characteristic imide peaks at 1800, 1780, and 1720 cm$^{-1}$ in the IR spectrum. Each active ester (0.15 mmole) in dioxane (4 ml) was added dropwise to a solution of albumin (340 mg, 0.3 mmole of lysine equivalents) in 0.1 *M* borate buffer, pH 9.0 (30 ml).

The solutions were allowed to stand at room temperature overnight and then were extensively dialyzed against distilled water. Insoluble material was removed by centrifugation, and the solution was lyophilized. The extent of conjugation was 10–30 molecules of sulindac or metabolites per molecule of protein, as estimated by UV absorption (sulindac, $\epsilon$ 325 nm = 1.31 × 10$^4$; sulindac sulfone, $\epsilon$ 327 nm = 1.17 × 10$^4$; and sulindac sulfide, $\epsilon$ 350 nm = 1.72 × 10$^4$).

**Immunization of Animals**—New Zealand White rabbits were given sulindac–albumin conjugates (2–5 mg im) dissolved in saline and emulsified with an equal volume of Freund's complete adjuvant. Booster injections of 1 mg of conjugate were given at monthly intervals in incomplete adjuvant. Bleedings were taken from central ear arteries about 2 weeks after booster injections.

To determine antibody titer, the quantity of tritiated sulindac and metabolites bound to the various antiserums was compared with the binding observed for normal serums. The high degree of sulindac sulfide binding to serum proteins necessitated the purification of the $\gamma$-globulin fraction of antiserum to this compound by precipitation from 18% sodium sulfate (9). Antiserums were used at dilutions that bound 30–50% of the added label in control tubes containing no unlabeled antigen.

Table I—Cross-Reactivity of Sulindac Antiserums

| Immunoglobulin | Unlabeled Antigen, $I_{50}$[a], ng/ml | | |
|---|---|---|---|
| | I | II | III |
| Anti-I or anti-II[b] | 40 | 40 | >1000 |
| Anti-III[c] | 29 | 23 | 33 |

[a] $I_{50}$ = drug concentration resulting in 50% inhibition of binding of labeled antigen. [b] Labeled antigen = $^3$H-I or $^3$H-II (50 ng). [c] Labeled antigen = $^3$H-III (20 ng).

**Combined Isotope Dilution–Radioimmunoassay**—A known mixture of I–III, each labeled with tritium (250 ng, ~1 × 10$^5$ dpm each), was equilibrated with 1-ml plasma or urine samples containing standard or unknown solutions of sulindac and metabolites in the 500–3000-ng/ml range. The aqueous solutions were adjusted to pH 5 by the addition of 0.2 ml of 1 *M* citrate buffer, pH 5, and were extracted with 3 ml of benzene–2-propanol (9:1). The aqueous phase was discarded.

The organic phase was removed and evaporated to dryness. The residue was dissolved in 0.03 ml of ethanol, and approximately one-third was chromatographed on paper[8] with toluene–xylene–2-propanol–dioxane–6% ammonium hydroxide (1:1:3:3:2) as the developing solvent. Areas containing the three chemical species (sulindac, $R_f$ 0.45; sulindac sulfone, $R_f$ 0.54; and sulindac sulfide, $R_f$ 0.71) were excised and eluted with 15 m*M* sodium hydroxide (1 ml). The aqueous solutions were adjusted to pH 7.5 by the addition of 30 m*M* monobasic sodium phosphate (0.5 ml), and one 0.3-ml aliquot was used for determination of recovery.

For the binding assay, a solution consisting of 0.9 ml of the eluate and 0.1 ml of the appropriate immunoglobulin was mixed and allowed to equilibrate at 4° overnight. "Bound" and "free" antigens were separated by addition of 0.2 ml of dextran-coated charcoal. The charcoal suspension was kept at 4° for 10–12 min and then separated by centrifugation; the supernate was counted to determine the fraction of radioactivity bound to the antibody.

Standard curves were plotted as drug concentration (labeled plus unlabeled drug) in the assay tube *versus* percent of total radioactivity bound to antibody. Concentrations of unknowns ($C$, nanograms per milliliter) were calculated from the following equation: $C = (c/r) - 250$, where $c$ and $r$ represent drug concentration as extrapolated from the standard curve and recovery, respectively. The 250 value represents the mass of each radioactive species added to the original sample.

In urine, glucuronide conjugates were hydrolyzed before analysis, thereby providing a measure of the total amount of each species present in free and conjugated forms. Appropriately diluted urine samples (0.75 ml) and 0.25 ml of 1 *N* sodium hydroxide were incubated for 30 min at room temperature and adjusted to pH 5 by the addition of 2 *M* acetic acid (0.17 ml). The mixture of the labeled compounds was added, and the samples were chromatographed and assayed as described.

**Cross-Reactivity**—Conventional radioimmunoassay was used for the estimation of cross-reactivity. This procedure was similar to that already described, except that extraction was necessary only in the case of sulindac sulfide due to binding of this species to plasma proteins. In addition, all chromatography steps were eliminated.

**Comparison of Methods**—Plasma samples containing labeled sulindac and metabolites of low specific activity (8 $\mu$Ci/mg) were prepared to mimic concentrations found in humans following sulindac administration. Each sample was analyzed by isotope dilution–radioimmunoassay as already described and by reverse isotope dilution. In the latter procedure, samples (1 ml) were equilibrated with a known mixture (0.25 $\mu$mole each) of the three compounds and were extracted with benzene–2-propanol (9:1). The organic phase was evaporated to dryness, and the mixture was chromatographed as described. Papers were eluted with ethanol, and drug concentrations were determined spectrophotometrically. The eluates were counted, and the concentration of each component was determined from the specific activities of each species and the known ratio of carrier to radioactivity initially present.

## RESULTS

Antibodies to sulindac and metabolites were detected within 6 weeks after immunization of rabbits. Antibody titers reached plateau values after 6 months and began to fall off after 1 year despite continued boosting. The amount of drug attached to the protein did not appear to

[8] Whatman No. 1.

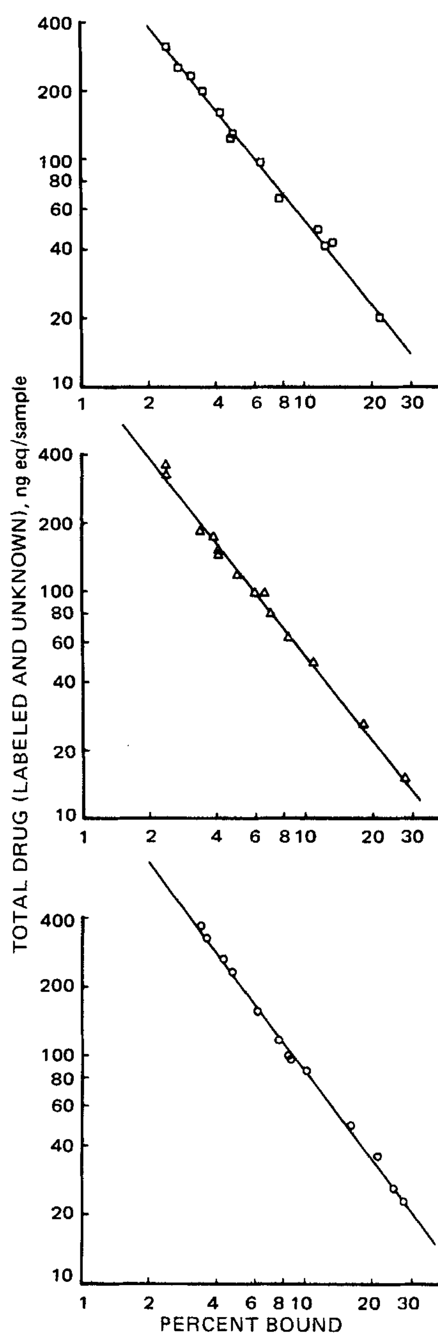


**Figure 1**—*Typical standard displacement curves for determination of sulindac* (○), *sulindac sulfone* (△), *and sulindac sulfide* (□).

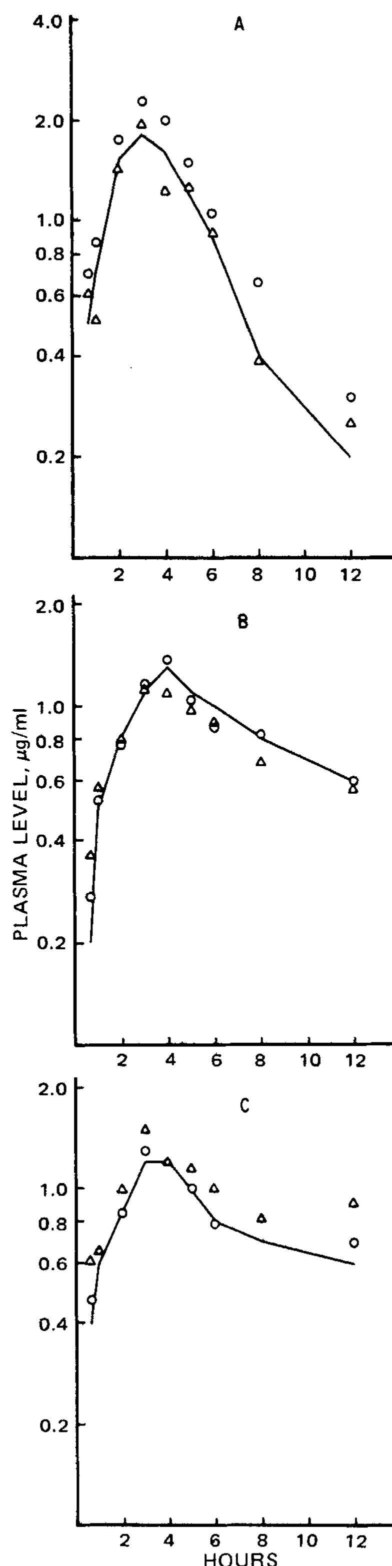


**Figure 2**—*Observed plasma concentrations of sulindac (A), sulindac sulfone (B), and sulindac sulfide (C) determined by isotope dilution–radioimmunoassay* (△) *and reverse isotope dilution* (○). *The line represents theoretical values.*

be critical, since conjugates in which 25–50% of the lysine residues were modified afforded equivalent antibody titers. Final antiserum dilutions of approximately 1:500 in the reaction tube bound 35% of labeled antigen and were used for routine assay.

All antiserums exhibited extensive cross-reactivity (Table I). Antiserums to I and II each possessed identical affinity for I and II. However, neither antibody bound III significantly. The anti-III immunoglobulin was less selective, exhibiting virtually 100% cross-reactivity to all three compounds. Thus, these serums possessed high affinity but low selectivity and were unsuitable for direct, differential determinations of sulindac and metabolites.

The combined isotope dilution–radioimmunoassay method produced the necessary specificity *via* the chromatography step while retaining the sensitivity of the antibody-binding assay. Radioactive recovery following sample extraction, aliquoting, and chromatography ranged from 20 to 70%, and good reproducibility of the assay was obtained within this range. This variability of recovery was probably due to the inability to locate with certainty the three redox species on the paper. Absolute recovery is not critical, since the recovery standard also serves as labeled antigen.

Standard displacement curves are illustrated in Fig. 1. These curves were linear when drawn on a log–log scale and possessed correlation coefficients greater than 0.997. Significant displacement was obtained with approximately 20 ng of unlabeled antigen/tube. Therefore, the effective sensitivity of the assay was 200 ng/sample, assuming a 50% re-

Table II—Assay of Sulindac and Metabolites Added to Normal Plasma

| Standard Concentrations, μg/ml | Experimental Values[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sulindac | | | Sulindac Sulfone | | | Sulindac Sulfide | | |
| | μg/ml | CV | Recovery, % | μg/ml | CV | Recovery, % | μg/ml | CV | Recovery, % |
| 0.1 | 0.07 ± 0.015 | 0.22 | 70 ± 15 | 0.089 ± 0.012 | 0.13 | 89 ± 12 | 0.046 ± 0.023 | 0.50 | 46 ± 23 |
| 0.5 | 0.5 ± 0.042 | 0.08 | 99 ± 8 | 0.55 ± 0.062 | 0.11 | 111 ± 12 | 0.44 ± 0.022 | 0.05 | 89 ± 4 |
| 1.0 | 1.2 ± 0.083 | 0.07 | 115 ± 8 | 1.1 ± 0.051 | 0.05 | 106 ± 5 | 0.89 ± 0.057 | 0.06 | 89 ± 6 |

[a] Means of 10 samples ± *SD*.

covery. However, since an extraction step is employed, sensitivity can be increased by using sample volumes larger than 1 ml with a concomitant increase in the volume of the first organic phase.

Precision and accuracy were demonstrated by analysis of replicate standards in pooled normal plasma (Table II). Coefficients of variance for sulindac and the sulfide at 0.1 μg/ml were unacceptable. However, at concentrations near the midpoint of the standard curves, *i.e.*, 0.5–1.0 μg/ml, coefficients of variance averaged 7% while mean accuracy was 102 ± 7% for all three compounds. Thus, samples should be adjusted to the 0.5–5.0-μg range by dilution or concentration prior to assay.

Results obtained by this method also were validated by comparison with results of the classical reverse isotope dilution assay. The two methods were employed to analyze levels of $^3$H-sulindac and its sulfone and sulfide metabolites in plasma. The specific activity of these samples was much lower than that of the labeled antigen and did not interfere with the combined isotope dilution–radioimmunoassay procedure. Plasma concentrations are shown in Fig. 2. In general, good correlation was seen among values obtained by the two methods.

Since sulfoxides are enantiomeric, the binding of partially resolved biogenic sulindac to the antibody was examined. Biogenic material was purified from the urine of rabbits treated with sulindac sulfide and possessed an $[\alpha]_D$ of +11° compared to the value of +21° determined for chemically resolved compound[9]. Thus, biogenic sulindac was approximately 67% enriched with respect to the dextrorotatory isomer. Standard displacement curves for the biogenic sample and for racemic sulindac were identical.

## DISCUSSION

A common disadvantage of many radioimmunoassay procedures is a lack of specificity. Thus, closely related compounds often cannot be distinguished. For this reason, separation procedures have been utilized in combination with radioimmunoassay in several cases. For example, prostaglandins E, A, and F were separated on silicic acid columns (4), estrogens were analyzed by dextran gel[10] chromatography (5), and dehydroepiandrosterone was purified by paper chromatography (6) prior to radioimmunoassay. In all of these procedures, tracer quantities of radiolabeled drug were added to samples to determine recovery. Then, larger amounts of radioactive drug, usually two to 10 times the tracer amount, were added as labeled antigen for radioimmunoassay. Relatively high specific activities are required to ensure that the recovery standard is indeed added in negligible amounts relative to the material being measured and, therefore, will not interfere with radioimmunoassay.

The described procedure (combined isotope dilution–radioimmunoassay) for sulindac differs from radioimmunoassay in that it is fundamentally an isotope dilution method employing a binding assay for detection. Added radioactive drug serves as both the recovery standard and labeled antigen, and standard curves represent total labeled and unlabeled drug (*i.e.*, specific activity of diluted material) present in the assay tube. Thus, whereas radioimmunoassay is an indirect measurement of unlabeled material causing a certain degree of displacement of labeled antigen from the antibody, combined isotope dilution–radioimmunoassay is a direct measure of the total material bound to the antibody. Therefore, the modified procedure offers the advantages that absolute recovery is not a limiting factor and that standards are treated in the same manner as unknowns, thus avoiding unaccountable procedural changes or losses. In addition, a lower specific activity of the labeled antigens is acceptable and a pipetting step is eliminated.

An apparent disadvantage of this method is that the critical antibody–labeled antigen ratio can vary with recovery, resulting in irreproducible data. This is true if recovery falls below a certain critical value of approximately 15%. However, as indicated by the precision of the assay, this is not generally a problem. Combined isotope dilution–radioimmunoassay requires that labeled antigens be chemically identical to each compound being determined and is not applicable to materials such as peptide hormones that utilize iodine-labeled antigens.

Incorporation of isotope dilution into radioimmunoassay procedures does result in a rather tedious method, but such a combination will, in most cases, provide specificity and sensitivity not otherwise obtainable. The disadvantages, in the case of sulindac, may be eliminated in some instances by determining the parent drug plus sulfone or all three compounds directly by radioimmunoassay using a nonselective antibody such as the anti-III immunoglobulin (Table I). Such an approach might be justified in certain bioavailability or drug interaction studies in which drug absorption is the principal consideration.

Examination of the sulindac metabolic pattern suggested that the sulfoxide might be resolved into one enantiomeric form *in vivo* by repeated reduction to sulfide followed by reoxidation, and this has been shown to be the case. The stereospecificity of antibodies was first demonstrated by Landsteiner and Scheer (10), who found that antibodies produced against one enantiomer of a pair were far less reactive to the second enantiomer. Since the sulindac antibody was produced against a recemic compound, the possibility exists of altered displacement kinetics involving an optically pure or even partially resolved enantiomer of the drug. However, the superimposability of standard curves for biogenic and racemic sulfoxide indicates that partial resolution does not compromise the validity of this assay.

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## ACKNOWLEDGMENTS AND ADDRESSES

Received October 8, 1975, from the *Merck Institute for Therapeutic Research, West Point, PA 19486.*

Accepted for publication May 26, 1976.

Presented in part at the American Society for Pharmacology and Experimental Therapeutics, Montreal, Canada, fall meeting, 1974 [*Pharmacologist*, **16,** 221 (1974)].

The authors thank Dr. J. E. Baer for encouragement and comments and Ms. K. F. Hooke for assistance with animals.

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