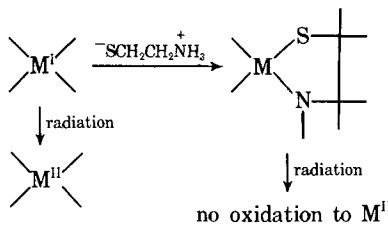

Scheme II

Comparison with Conformational Studies by Other Methods—While the solid-state and solution data on the preferred conformations of the compounds are not identical, they do show a high degree of similarity. For cysteamine in deuterium oxide at physiological pH, the relative population of the *gauche*-conformer is greater (75%) than the *trans*-conformer. X-ray diffraction studies (9), on the other hand, showed that in the solid state the molecule crystallizes only in the *gauche*-form. In crystals, the observed structure is stabilized by intramolecular nitrogen–hydrogen–sulfur bonds.

For thiazolidinecarboxylic acid, X-ray crystallography indicates that the ring has a one-atom pucker (envelope) conformation, with the sulfur atom lying about 0.8 Å ($\delta = 27°$) out of the plane described by the other four atoms. The solution data indicate a slightly higher pucker ($\delta = 45°$) for both thiazolidine and thiazolidinecarboxylic acid. If the bond length and bond angles observed in the solid state are preserved in solution, then the NMR data indicate that both sulfur and nitrogen atoms are puckered relative to the plane described by the three ring carbon atoms. The structure of thiazolidinecarboxylic acid is held in the crystal by several intra- and intermolecular NH···O=C hydrogen bonds. When such hydrogen bonds are replaced by solute–solvent hydrogen bonds in aqueous solutions, the molecular conformation apparently switches to a more relaxed two-atom twisted conformation.

The solution results are in better agreement with those predicted by EHT and CNDO theory. Theoretical results correspond more closely to conformations for isolated molecules and indicate a slight preference for the intramolecularly hydrogen-bonded form of I*b* while almost equal energies are predicted for the G, T, and G′ conformers of I*a* and I*c*. For thiazolidine systems, such calculations indicate that a two-atom twisted conformation (with $\delta = 38°$) is most stable.

Biological Action—Based on the conformer population of cysteamine in solution, it can be argued that the radioprotective action of this molecule arises from a structure in which the essential atoms, sulfur and nitrogen, are placed in a *gauche*-arrangement. In this conformation, the sulfur and nitrogen atoms come in close proximity and their steric repulsion may be offset by intramolecular hydrogen bond NH···S and charge interactions. This type of molecular arrangement allows the possibility of chelation of metal ions involving sulfur and nitrogen atoms (Scheme II) and prevents their oxidation by free radicals formed in the biophase by the radiolysis of water (19). With thiazolidines, the antiradiation action may be due to their *in vivo* hydrolysis (20) to a protective compound, cysteamine, which then undergoes molecular arrangement as shown in Scheme II.

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Radioimmunoassay of Indomethacin in Biological Fluids

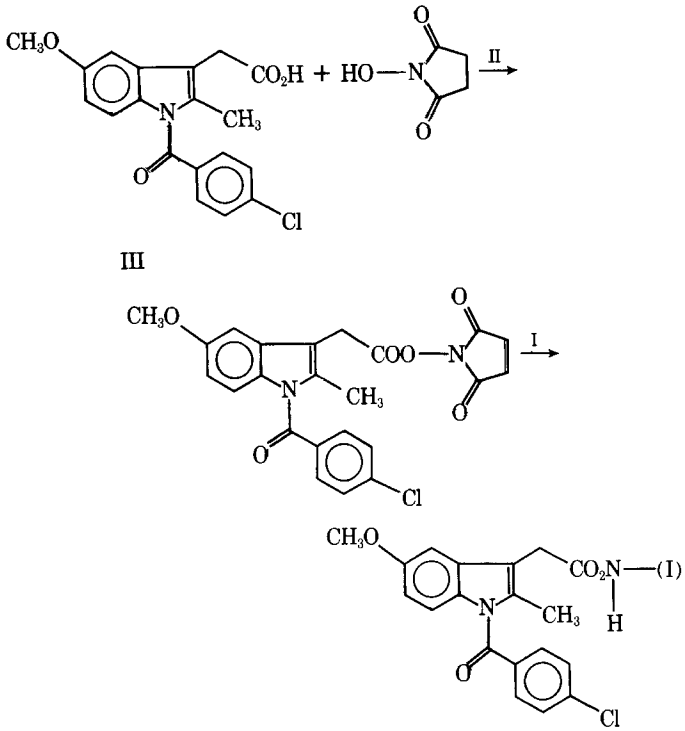
L. E. HARE, C. A. DITZLER, and D. E. DUGGAN [x]

Abstract □ A radioimmunoassay was developed for the determination of indomethacin in biological fluids at concentrations as low as 50 ng/ml. Antibodies were produced in rabbits immunized with a conjugate of bovine serum albumin and indomethacin. This conjugate was prepared by an *N*-hydroxysuccinimide active ester procedure. Antiserums exhibited minimal cross-reactivity with the *O*-desmethyl and deschlorobenzoyl metabolites. However, the glucuronide conjugate was about three times as reactive as indomethacin, thus invalidating direct determinations of indomethacin in urine. This difficulty was circumvented by analyzing urine aliquots before and after conjugate hydrolysis. Concentrations of free and conjugated indomethacin were calculated by difference.

Keyphrases □ Indomethacin—radioimmunoassay, human plasma and urine □ Radioimmunoassay—indomethacin, human plasma and urine □ Anti-inflammatory agents—indomethacin, radioimmunoassay, human plasma and urine

Analyses of the widely used anti-inflammatory agent indomethacin[1] have previously been accomplished by spectrofluorometric (1) or radioisotopic (2) methods. These procedures are useful in certain situations but are subject to limitations. The spectrofluorometric method lacks sensitivity, and the presence of other fluorescent materials,

[1] Indocin, Merck Sharp & Dohme.



Scheme I

such as salicylic acid, causes interference. Reverse isotope dilution offers the advantage of quantitating all metabolites but necessitates administration of radioactive drug.

GC (3), mass fragmentographic (4), and liquid chromatographic (5) techniques recently were developed. Their primary disadvantages are the necessity of careful sample preparation and the relatively slow rate of analysis. This paper reports a radioimmunoassay procedure for the direct analysis of indomethacin in biological fluids requiring no prior isolation.

EXPERIMENTAL

2-^{14}C-Indomethacin[2] (52 μCi/mg), bovine serum albumin[3] (I), *N*-hydroxysuccinimide[4], dicyclohexylcarbodiimide[4] (II), β-glucuronidase[5], neutral charcoal[6], and dextran[7] were used as received. Dextran-coated charcoal was prepared by suspending 6.25 g of previously washed charcoal and 0.625 g of dextran in 100 ml of 0.05 *M* phosphate buffer (pH 7.5).

Preparation of Conjugates—Indomethacin (III) was conjugated through the carboxylic acid moiety to I by an *N*-hydroxysuccinimide active ester procedure (6, 7) (Scheme I). Conversion of indomethacin to the succinimide ester was verified by the appearance of characteristic imide peaks at 1800, 1780, and 1720 cm^{-1} in the IR spectrum. Indomethacin active ester (125 mg, 0.35 mmole) in dioxane (3 ml) was added dropwise to a solution of I (200 mg, 0.18 mmole lysine equivalents) in 0.05 *M* phosphate buffer (pH 7.8) (20 ml).

The solution was allowed to stand at room temperature overnight and was then extensively dialyzed against distilled water. The small amount of insoluble material present was removed by centrifugation, and the solution was lyophilized. The extent of conjugation was 17 molecules of indomethacin/molecule of I as estimated by UV absorption (indomethacin $\epsilon_{320} = 6.3 \times 10^3$). Protein concentration was determined by a microbiuret method (8).

Immunization of Animals—New Zealand White rabbits were injected intramuscularly with indomethacin–I conjugates (5 mg), which had been dissolved in 0.5 ml of 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. The antibody titer was determined from the binding of 2-^{14}C-indomethacin by the antiserums, corrected for binding to normal serum.

Radioimmunoassay of Plasma—Antiserum was used at a dilution that bound 35–50% of the added label in control tubes containing no unlabeled antigen. The assay solution consisted of 0.1 ml of pooled human plasma containing unlabeled indomethacin standards (50–3000 ng/ml) or 0.1 ml of plasma from indomethacin-treated subjects, 0.1 ml of a 1:12 dilution of the antiserum, 20 ng of 2-^{14}C-indomethacin (2.3×10^3 dpm) in 0.1 ml of 0.05 *M* phosphate buffer (pH 7.4), and sufficient buffer to achieve a final volume of 1.0 ml. The solutions were allowed to equilibrate at 4° overnight, and then 0.2 ml of dextran-coated charcoal suspension was added with thorough mixing. The tubes were allowed to stand at 4° for 10–12 min, and the charcoal was separated by centrifugation. Antibody-bound radioactivity contained in the supernate was determined by scintillation counting.

Standard curves were plotted on log-probit paper as unlabeled indomethacin added *versus* percent of control binding ($100 \times B/B_0$, where B = bound antigen in the presence of unknown sample, and B_0 = bound antigen in the absence of unlabeled drug).

Radioimmunoassay of Urine—*Unconjugated Indomethacin*—One aliquot (1 ml) of each appropriately diluted urine sample (5–300 ng/ml) was treated with 0.2 ml of 1 *M* citrate buffer (pH 5.0) and extracted with 3 ml of benzene–2-propanol (9:1). The organic phase was allowed to evaporate, and the residue was dissolved in 0.1 ml of 0.05 *M* phosphate buffer (pH 7.4). Radioimmunoassay was performed as described for plasma.

Total Indomethacin—A second 1-ml aliquot (5–300 ng/ml) was treated with 1 ml (5000 units) of β-glucuronidase in the same buffer as was used for unconjugated indomethacin for 3 hr at 37° to hydrolyze the glucuronide conjugate. Thereafter, this aliquot was treated in an identical manner as the first.

Indomethacin Glucuronide—The numerical difference between results of total and unconjugated procedures represents conjugated indomethacin.

RESULTS

Antibodies to indomethacin could be detected in two of five rabbits 8 weeks after immunization. With continued boosting, antibody titers increased slowly up to about 4 months, at which time a final antiserum dilution of 1:120 bound 35% of added 2-^{14}C-indomethacin in the absence of unlabeled drug. Antiserum was used at this dilution for routine assay.

Standard curves for detecting indomethacin in human plasma were prepared as described under *Experimental*. Significant displacement was seen with 5 ng of added indomethacin, and the curves were linear (r = 0.98) up to about 300 ng. Thus, the useful range of the method is 50–3000 ng/ml plasma. Nonspecific binding to antiserum was less than 5%, indicating that there were no interfering substances in normal plasma. Assay of standards prepared in urine resulted in standard curves that were slightly displaced from plasma curves but had identical slopes. Thus, separate standard curves are required for urine and plasma.

Recovery and reproducibility of the assay were determined by 10 replicate assays of known indomethacin samples in pooled normal plasma:

standard concentration, ng	7	25	100
mean ± *SD* of 10 assays, ng	9.5 ± 0.9	29 ± 1.5	98 ± 7.0
CV, %	9.5	5.2	7.1
recovery, %	135 ± 13	115 ± 6	98 ± 7

Recovery was rather poor at lower indomethacin levels due, in part at least, to errors in sample preparation as well as variations in the assay itself.

Potential interference of metabolites and other substances with the assay was evaluated by determining the concentrations of these compounds necessary to displace 50% of the bound 2-^{14}C-indomethacin from the antibody (Table I). The *O*-desmethyl metabolite (IV) was 12% as reactive as indomethacin, whereas the deschlorobenzoyl metabolite (V) was nonreactive. Salicylate, which is sometimes present in indomethacin plasma samples (following simultaneous aspirin administration), also did not interfere with the assay. However, several ester and amide derivatives of indomethacin were highly cross-reactive. The glycoside de-

[2] Prepared by Dr. R. L. Ellsworth, Merck Sharp & Dohme Research Laboratories.
[3] Nutritional Biochemical Co.
[4] Pierce Chemical Co.
[5] Ketodase, Warner-Chilcott.
[6] Norit A, Amend Drug and Chemical Co.
[7] T-70, Pharmacia Labs.

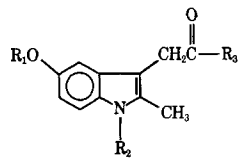
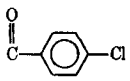
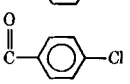
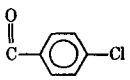
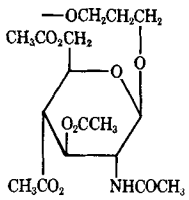
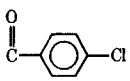
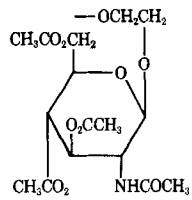


Table I—Cross-Reactivity of Indomethacin–I Antiserum

Compound	R_1	R_2	R_3	Mass Required to Displace 50% of Bound 2-^{14}C-Indomethacin, ng	Relative Cross-Reactivity, %
III	CH_3		OH	35	100
IV	H		OH	296	12
V	CH_3	H	OH	>20,000	<0.2
VI	CH_3			9	390
VII	CH_3			3	1170

rivatives (VI and VII) were several times more reactive than indomethacin. Thus, the glucuronide conjugate of indomethacin, which accounts for at least 50% of the drug-derived material excreted in urine, might be expected to interfere with the determination of urine indomethacin levels.

To explore this possibility, indomethacin and indomethacin glucuronide concentrations were determined in human urine by both radioimmunoassay and reverse isotope dilution (2) (Table II). In this study, 2-^{14}C-indomethacin (25 mg, 1 μCi/mg) was administered intravenously; urine was collected over the 0–2-hr period. The specific activity of the injected indomethacin was <2% of that used in radioimmunoassay and, therefore, did not interfere.

Both methods afforded comparable total indomethacin concentrations following glucuronidase treatment of the samples. Unconjugated indomethacin was determined by extraction of the urine samples with benzene–2-propanol (9:1) prior to analysis. Similar results were obtained by both methods. The slightly high radioimmunoassay value may have been due to 10% interference by IV (2.6 μg equivalent/ml as determined by reverse isotope dilution).

Direct radioimmunoassay or reverse isotope dilution of untreated urine samples should also afford unconjugated indomethacin concentrations if the glucuronide conjugate does not interfere with either method. Values obtained by reverse isotope dilution (3.7 μg/ml) were as expected, but results of the radioimmunoassay analysis were much too high (17.3 μg/ml). Thus, the assay is highly cross-reactive to indomethacin glucuronide. Whether this effect involves perturbation(s) of the indomethacin–antibody or indomethacin–charcoal equilibria or both is not apparent.

The difference between free indomethacin measured by radioimmunoassay after extraction (4.63 μg/ml) and total indomethacin in unextracted samples (17.3 μg/ml) represents the apparent glucuronide level. This value (12.7 μg equivalent/ml) is 3.25 times higher than the true conjugate concentration (3.91 μg equivalent/ml). Radioimmunoassay of indomethacin in unextracted urine samples, therefore, will result in high estimates of indomethacin concentrations. Furthermore, the small but significant cross-reactivity to the desmethyl metabolite (Table I), which is a major constituent of later urine samples (2), precludes the use of direct radioimmunoassay for specific measurement of indomethacin in urine. However, urine concentrations can be determined by analyzing aliquots before and after conjugate hydrolysis as described later.

The utility of the assay for determination of plasma indomethacin concentrations was also demonstrated by a comparison of results obtained by radioimmunoassay and reverse isotope dilution following administration of labeled drug (Fig. 1). Results from the two procedures were in excellent agreement (r = 0.995).

DISCUSSION

The production of antiserums to indomethacin has permitted development of a simple and sensitive method for the determination of this drug in biological fluids. The present sensitivity (50 ng/ml) is adequate for pharmacological levels commonly encountered but could be improved by the use of radiolabeled indomethacin of greater specific activity.

The indomethacin radioimmunoassay is not a totally specific method. The described antiserum is not reactive with V but is about threefold more reactive with indomethacin glucuronide than with indomethacin

Table II—Indomethacin and Indomethacin Glucuronide Concentrations in Urine[a]

Method	Total Indomethacin[b] and Indomethacin Glucuronide	Unconjugated[c] Indomethacin	Indomethacin[d] Glucuronide	Indomethacin[e] and Indomethacin Glucuronide	Indomethacin[f] Glucuronide
Reverse isotope dilution	9.20 ± 0.42	3.70 ± 0.45	5.50	—	—
Radioimmunoassay	8.54 ± 0.47	4.63 ± 0.55	3.91	17.3 ± 1.35	12.0 ± 0.76

[a] Mean ± *SD* of 10 determinations. Values are in micrograms per milliliter. [b] β-Glucuronidase treatment. [c] Reverse isotope dilution data were obtained by direct analysis; radioimmunoassay data were obtained after extraction of free indomethacin. [d] Determined by difference. [e] Direct determination by radioimmunoassay. [f] Radioimmunoassay of aqueous phase from reverse isotope dilution method.

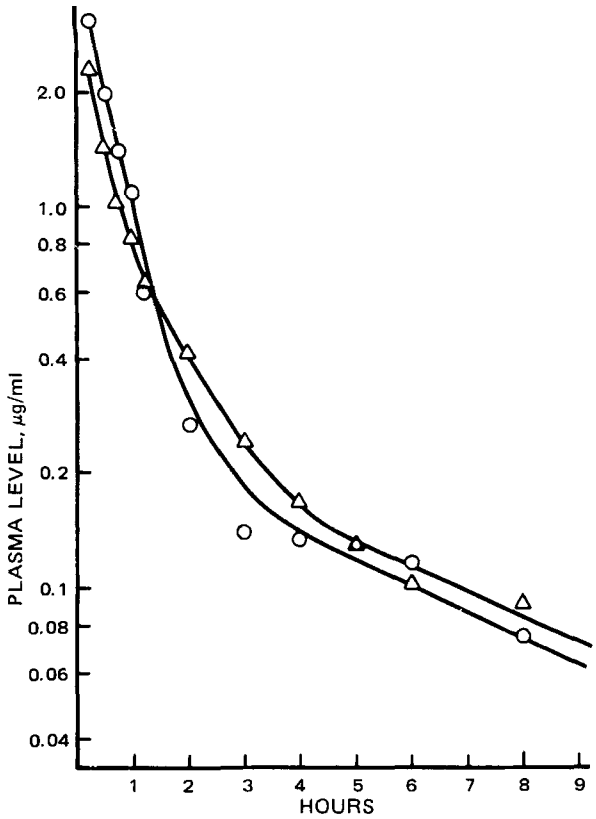


Figure 1—*Human plasma indomethacin concentrations determined by radioimmunoassay (○) and reverse isotope dilution (△). Subjects received 25 mg of 2-^{14}C-indomethacin (1 μCi/mg) intravenously.*

itself. Furthermore, IV exhibits 12% cross-reactivity to the antibody used, and some animals produce serums with 100% cross-reactivity to IV. Even animals with serums of good selectivity produce less selective antibodies over time. Therefore, care must be taken to select appropriate antiserums. Several animals should be immunized, and bleedings should be taken very early.

The cross-reactivity of the antiserum with indomethacin glucuronide is not surprising, since the material used for immunization of rabbits was an acyl conjugate of indomethacin and I. Thus, antibodies were expected to be more reactive to amide and ester derivatives than to the free acid of indomethacin. This supposition was verified by cross-reactivity studies in which glycosyl esters were bound more tightly to the antibody than was free indomethacin and by experiments with human urine samples.

Indomethacin and its conjugate in urine can be estimated accurately by analyzing two sample aliquots as described under *Experimental*. One aliquot is extracted with benzene–2-propanol, thus providing a measure of unconjugated indomethacin. The second aliquot is treated with β-glucuronidase to hydrolyze the conjugate before assay. The value obtained by the latter procedure represents total indomethacin, and the difference between the two values is an estimate of indomethacin glucuronide concentration.

Plasma samples can be analyzed directly without extraction. The small amount of interference (12%) caused by IV would not generally be a problem, since plasma levels of this metabolite only begin to approach indomethacin concentrations about 6 hr after administration of the parent drug, whose plasma half-life is about 2 hr (2). Urinary concentrations of indomethacin are also much greater than concentrations of IV for the first 4 hr. However, IV equals or exceeds indomethacin levels for the 4–48-hr period; IV will, therefore, interfere with the radioimmunoassay procedure outlined for urine.

If differentiation is necessary, a combined isotope dilution–radioimmunoassay method analogous to that described for sulindac (9) could be used. In this case, metabolites are separated chromatographically prior to radioimmunoassay, thus allowing the determination of each compound if suitable antiserums and labeled antigens are available.

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