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METHOD FOR EXTRACTION AND SEPARATION OF DRUGS AND METABOLITES FROM BIOLOGICAL TISSUE*

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Abstract—A method has been developed for the quantitative extraction, partitioning, and separation of indomethacin, its metabolites and other anionic drugs (salicylic acid and phenylbutazone) from biological tissues. Tissues are extracted with chloroform and anhydrous, acidic methanol. When the extract is made biphasic with aqueous acid, conjugated drug species are partitioned to the aqueous, acid phase while protonated and esterified drug species are partitioned to the organic phase. The ionized drugs (indomethacin, its nonconjugated metabolites, and salicylic acid) in this phase are partitioned to an aqueous, basic phase (pH 8·6) and separated by anion-exchange chromatography. Phenylbutazone is extracted at pH 12–13 and esterified drug species remain in the organic phase. The quantitative aspects of this method have been evaluated with biological samples from rats which had received ^{14}C-indomethacin or ^{14}C-salicylic acid *in vivo*.

THE URINARY metabolites of indomethacin and concentration of indomethacin in plasma have been described for several animal species by Harman *et al.*[1] and Hucker *et al.*[2] However, quantitative evaluation of the species differences in metabolism, distribution, and excretion of indomethacin and a study of the effect of other drugs on these parameters[3, 4] required the development of a new method to separate indomethacin, its major metabolites, and other anions from biological tissues. A preliminary report of these findings has been presented.[5]

MATERIALS AND METHODS

Indomethacin and 2-^{14}C-indomethacin were obtained through the courtesy of Merck Sharp & Dohme Research Laboratories, West Point, Penn. Two metabolites of indomethacin, desmethylindomethacin and deschlorobenzoylindomethacin were also donated by Merck Sharp & Dohme Research Laboratories and were recrystallized from aqueous acetone and from absolute ethanol, respectively, before use. Salicylic acid was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisc., and 7-^{14}C-salicylic acid from New England Nuclear Corp., Boston, Mass. Phenylbutazone and ^{14}C-phenylbutazone (uniformly labeled phenyl) were obtained through the courtesy of J. R. Giegy S.A., Basel, Switzerland. Diethylaminoethyl cellulose (Whatman DE-23) was purchased from the Reeve Angel Co., Clifton, N. J.; Tris-(hydroxymethyl)-aminoethane from Sigma Chemical Co., St. Louis, Mo.; and Freon 113 (1,1,2 trichloro-1,2,2 trifluoroethane) from E. I. DuPont de Nemours, Wilmington, Del. All

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other chemicals and solvents were the best commercial reagent grades. Anhydrous HCl-methanol was prepared according to Fieser[6] and diluted to 0·3 N HCl with anhydrous methanol.

Indomethacin was dissolved in absolute ethanol. An aliquot of this preparation was diluted with 0·05 M phosphate buffer (pH 8) to a concentration of 20 mg/ml on the days of the experiments and used within 1 hr of preparation.

Sprague–Dawley rats were obtained from the Blue Spruce Farms, Inc., Altamont, N. Y. Rats from 140 to 175 g were given ^{14}C-indomethacin (8–10,000 dpm/μg) at 10 mg/kg i.v. and were housed in individual No. 110 metabolism chambers (Maryland Plastics, Inc., New York, N. Y.) to collect urine during the drug studies. The technique for preventing coprophagy and collecting feces has been described.[3, 4] All animals had access to food and water.

Blood, collected in tubes containing citrate, was obtained by heart puncture from lightly anesthetized rats (ether). After blood samples were obtained, the anesthetized animals were exsanguinated. Tissues were taken immediately and frozen in liquid nitrogen. Human plasma was obtained through the courtesy of a local hospital.

The animals selected for bile duct cannulation were anesthetized with 360 mg/kg of chloral hydrate U. S. P. (Fisher Scientific Co., Fair Lawn, N. J.) and the bile duct was cannulated with Adams polyethylene tubing P. E. 10 (E. F. Mahady Division, Cambridge, Mass.). These surgically prepared rats were restrained but had access to food and water at all times. Radioactive indomethacin or salicylic acid was injected i.v. into the caudal vein of these animals 3 hr after surgery. Bile was collected at hourly intervals for 6 hr, then every 6 hr for a further 18 hr. Urine collections were made every 3 or 6 hr for 24 hr.

Radioactivity was determined with a Nuclear Chicago Mark I liquid scintillation system having an external standardization component. Organic solvents were removed from samples prior to counting, but aqueous samples were counted directly. The scintillation fluid, TEN,* contained 0·4 g of POPOP,† 20 g of PPO,* and 120 g of naphthalene in a total volume of 4 l. of toluene:ethylene glycol monoethyl ether (2·2:1·8, v/v). Ten or 15 ml of TEN was used with 0·5- or 1-ml aqueous samples respectively.

Quantities of drug and metabolites were determined with a Zeiss PMQ II spectrophotometer. Each compound was measured at its maximal absorption: indomethacin, 265 mμ; deschlorobenzoylindomethacin, 280 mμ; desmethylindomethacin, 265 mμ; phenylbutazone, 264 mμ; and salicylic acid, 297 mμ.

Indomethacin and its two metabolites, desmethylindomethacin and deschlorobenzoylindomethacin, were further identified by their chromatographic behavior on silica-gel thin layers and Whatman DE-23 cellulose columns. The solvent system for thin-layer chromatography with Brinkman precoated, F254, silica-gel plates was acetic acid:chloroform (5:95, v/v).[1] The procedure for precycling and standardizing Whatman DE-23 cellulose was identical to that described in the Whatman brochure. For the equilibration procedure, 0·05 M Tris (pH 8) in 2 M NaCl was used. A 50 per cent slurry of this standardized Whatman DE-23 cellulose in 0·05 M Tris (pH 8) without NaCl or toluene was packed in columns (1·1 × 30 cm) at 2–3 psi and equilibrated

* P. E. Baronowsky, personal communication.

† POPOP is *p*-bis [2-(5-phenyloxazolyl)]benzene and PPO is 2,5-diphenyloxazole. These chemicals were purchased from Pilot Chemicals, Inc., Watertown, Mass.

under the same pressure with about 200 ml of the Tris buffer. (Whatman DE-23, which was stored under toluene, did not resolve indomethacin and its metabolites sufficiently and is not recommended for these compounds.) After loading the sample (ca. 6 ml) on to the column with pressure, the samples were eluted with a nonlinear gradient; the mixing chamber contained 50 ml of 0·05 M Tris buffer (pH 8) and the reservoir contained 250 ml of 0·05 M NaCl in the Tris buffer. The flow rate of the column was approximately 2·5 ml/min. The eluate flowed through a 2·5- or 3-cm flow cell which was monitored at 297 or 265 mμ. The 265-mμ monitor with a 3-cm flow cell was made by Gilson Medical Electronics, Middleton, Wisc. The Zeiss PMQ II spectrophotometer was modified to house a 2·5-cm flow cell and was used to monitor eluates at any desired wavelength.

The general method and techniques which had been developed previously for the extraction and purification of lipids and drugs from tissues were used with some modifications.[7–9] Biological tissue (1 g) was homogenized with 6·5 ml of anhydrous 0·3 N HCl-MeOH and 13·0 ml of $CHCl_3$.[6] This homogenate was made biphasic with 6·5 ml of aqueous 0·1 N HCl, then centrifuged at 1900 *g* for 10 min to separate an acidic, aqueous phase, a solid interphase and an organic phase. The acidic, aqueous phase contained the conjugates of indomethacin and its metabolites; the chloroform phase contained the protonated (nonionized) and esterified compounds. Indomethacin, its metabolites and salicylic acid could be extracted from a 10-ml aliquot of the chloroform phase by adding 10 ml of Freon 113 and partitioning twice with 5 ml of 0·05 M Tris buffer (pH 9) at 4°. Phenylbutazone remained primarily in the nonaqueous phase and was subsequently extracted into 5 ml of 0·1 N NaOH solution. Shaking with basic, aqueous, Freon 113 biphases produced emulsions that could be broken by freezing. Basic, aqueous phases were neutralized to pH 8 prior to chromatography on Whatman DE-23 cellulose.

RESULTS

The extraction method of Hucker *et al.*[2] and the separation techniques of Harman *et al.*[1] were unsatisfactory for the quantitative extraction and separation of indomethacin and its metabolites from tissues. Consequently, the general method and techniques we previously used for the extraction and purification of lipids and drugs from tissues[7–9] were evaluated.

Indomethacin, its metabolites and phenylbutazone partitioned greater than 95 per cent to the organic, chloroform phase in an acidic, biphasic, solvent system. Salicylic acid partitioned greater than 90 per cent to the organic phase of the same system. The presence of plasma (1 or 5 ml) had no effect on the partitioning characteristics of ^{14}C-indomethacin and ^{14}C-phenylbutazone. Approximately 99 per cent of these ^{14}C-labeled drugs partitioned to the organic phase and little or no indomethacin was associated with the solid interphase. Although quantitative u.v. spectroscopy of desmethylindomethacin, deschlorobenzoylindomethacin and salicylic acid in the presence of plasma was difficult because the u.v. absorption of the organic phase from plasma was comparatively high, it was determined semiquantitatively that these three compounds did partition primarily to the chloroform phase. However, a second extraction of the aqueous phase with an equilibrated organic phase was necessary for quantitative recovery of salicylic acid.

The partitioning of these five compounds from a 10-ml aliquot of chloroform–

methanol extract of plasma to various buffered, aqueous phases is summarized in Table 1. Below pH 6, indomethacin, desmethylindomethacin and deschlorobenzoylindomethacin remained in the chloroform phase. At higher pH values, these compounds were extracted into the aqueous phase. The addition of nonpolar, Freon 113 was useful in enhancing the partitioning of indomethacin and desmethylindomethacin into an aqueous phase from an organic phase. Salicylic acid was quantitatively partitioned with 0·05 M Tris buffer (pH 8·6) and Freon 113, but under these conditions

TABLE 1. PARTITION OF INDOMETHACIN, ITS NONCONJUGATED METABOLITES, SALICYLIC ACID AND PHENYLBUTAZONE BETWEEN CHLOROFORM EXTRACT OF PLASMA AND BUFFERED AQUEOUS PHASE

Aqueous type*	Phase pH†	Freon 113 (ml)	Indomethacin	Desmethyl-indomethacin	Deschloro-benzoyl-indomethacin	Salicylic acid	Phenyl-butazone
Citrate	5		0	0	< 5		
Citrate	6		0	0	< 5		
Tris	8		ca. 40	80–90	> 95		
Tris	8	5	60–70	> 95	> 95		
Tris	8	10	59·9 ± 8·1‡,§				
Tris	8·6‖	10	79·2 ± 4·3‡	> 95	> 95	> 95	7·2 ± 0·1‡
Tris	9	10	90·5 ± 7·3‡				
Tris	10	10	94‡				
NaOH	> 12	10					94 ± 2‡

* The molarity of the citrate buffer was 0·1 M, Tris buffer was 0·05 M, and NaOH was 0·1 M.
† The pH was determined prior to making biphasic solvent system.
‡ Radioactive drug. Specific activity of ^{14}C-indomethacin was 14,000 dpm/μg and that of ^{14}C-phenylbutazone was 12,000 dpm/μg.
§ Mean ± S. D. for six to eight determinations.
‖ Partitioning performed at 4° and pH 9·0; the pH of this Tris buffer was 8·6 at room temperature. The others were partitioned at room temperature.

phenylbutazone partitioned minimally to the aqueous phase. However, phenylbutazone partitioned quantitatively to the aqueous phase with 0·1 N NaOH and Freon 113.

Indomethacin and its metabolites are stable under the acidic extraction procedures we used but, as reported by Hucker *et al.*,[2] these drug species are relatively unstable in basic solutions. We found that desmethylindomethacin was also unstable in basic solutions. As shown in Figs. 1 and 2, indomethacin decomposed to deschlorobenzoylindomethacin fairly rapidly at pH 9 and room temperature, but minimally at pH 8 and room temperature. Since hydrolytic processes are temperature-dependent, the increased stability of indomethacin at pH 9 and 4° was expected. Comparable studies with desmethylindomethacin showed rates of decomposition similar to those of a compound which resembled deschlorobenzoylindomethacin in its chromatographic characteristics. Because of the instability of indomethacin and desmethylindomethacin in base, the partitioning step was routinely done in an ice bath and in less than 1 hr.

Since indomethacin partitioned approximately 80 per cent in a single extraction with 5 ml of 0·05 M Tris (pH 9)* in an ice bath (Table 1), a second extraction was performed routinely. An aliquot of the combined extracts was chromatographed on an

* pH 8·6 at room temperature.

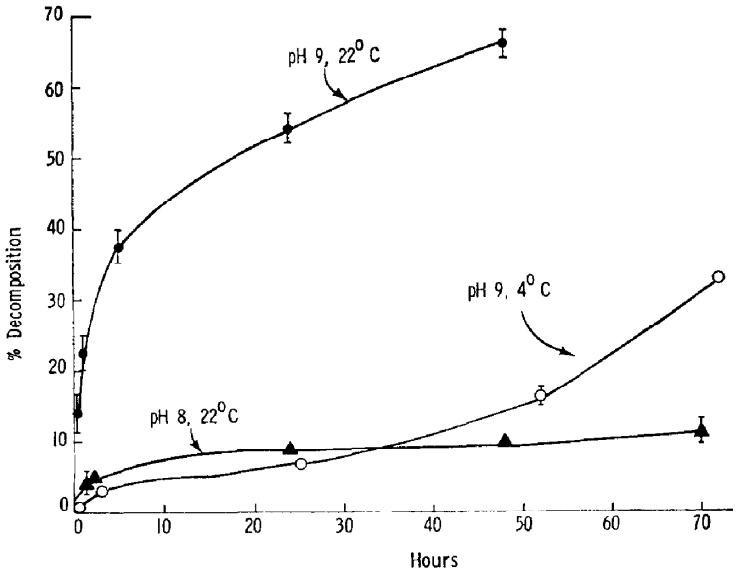

FIG. 1. Stability of indomethacin in basic solutions. Indomethacin in ethanol (40 mg/ml) was diluted to a concentration of 50 μg/ml with 0·05 M Tris buffer, pH 8 or pH 9, at room temperature, or with 0·05 M Tris, pH 9, at ice temperature (4°). At various times aliquots of these solutions were chromatographed on Whatman DE-23 columns (see Methods). The radioactivity from deschlorobenzoylindomethacin and indomethacin was determined by liquid scintillation (see Methods).

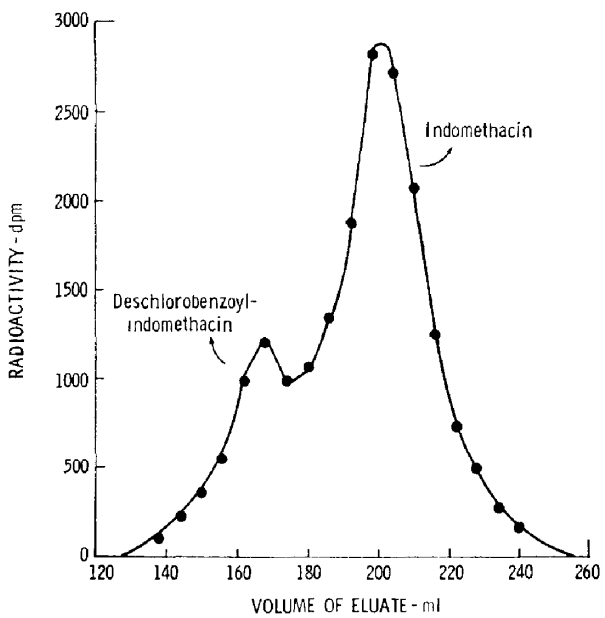

FIG. 2. Decomposition product of indomethacin after standing in Tris buffer pH 8 for 72 hr.

equilibrated DE-23 cellulose column using a nonlinear gradient of 0·05 M Tris buffer at pH 8. Indomethacin and its two metabolites, deschlorobenzoylindomethacin and desmethylindomethacin, were separated as shown in Fig. 3. Typical recoveries of indomethacin and its two metabolites from these columns were about 100 per cent. Equal or better separation of indomethacin and its metabolites was obtained with one-half the elution volume by using Whatman DE-23 at pH 9·6 with a nonlinear gradient of 0·2 M NaCl in 0·2 Tris (pH 9·6). The stability of the eluted indomethacin could be controlled by lowering the pH of the eluate to less than 8 soon after it came

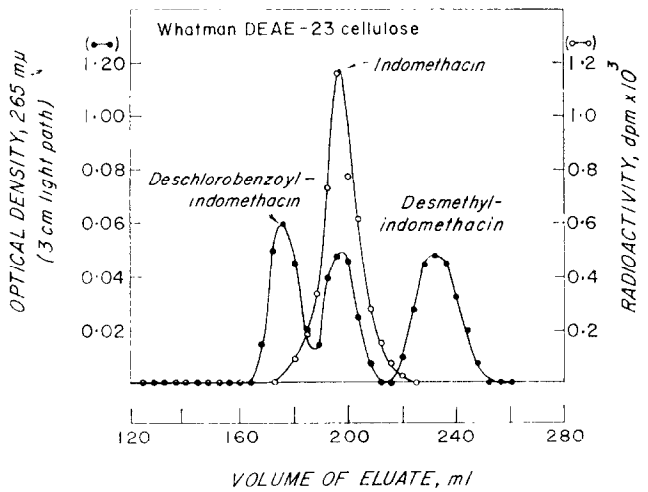

FIG. 3. Chromatogram of extract of human plasma incubated with pure compounds. Approximately 30–50 μg of each compound was added to plasma and incubated for 30 min at room temperature. The extraction, partitioning, and chromatography of the compounds are described in Methods.

out of the column. Other resins (e.g. Dowex-1) were evaluated and discarded because nonspecific adsorption of indomethacin to the polystyrene resins was observed. Similar nonspecific adsorption was seen with the Sephadex-G series.

Phenylbutazone and salicylic acid were also chromatographed on Whatman DE-23 cellulose. As Fig. 4 shows, salicylic acid was co-eluted with deschlorobenzoylindomethacin. The small radioactive area of 160 ml was deschlorobenzoylindomethacin, a decomposition product of the ^{14}C-indomethacin. Both phenylbutazone and salicylic acid from extracts of human and rat plasma were quantitatively (>95 per cent) separated and recovered on DE-23 cellulose columns.

Most of the recovery experiments were done with human plasma. However, recoveries of ^{14}C-indomethacin from rat plasma, rat liver and rat kidney were also quantitative for each extraction, partition, and chromatographic step. In addition, this method has been used in the analysis of indomethacin and its metabolites in tissues from rats that had been given i.v. doses of ^{14}C-indomethacin. The recovery of radioactivity for each separation step is summarized for plasma, kidney, liver, bile, feces, and urine in Table 2. Except for bile, liver, and kidney (basic aqueous phase) and for bile and urine (chloroform phase and DE-23), the recovery of radioactivity

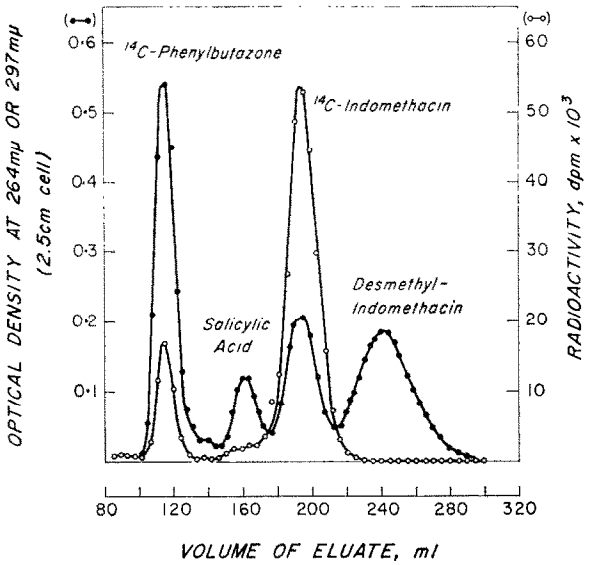

FIG. 4. Chromatogram of ^{14}C-phenylbutazone, salicylic acid, ^{14}C-indomethacin, and desmethylindomethacin on Whatman DE-23 cellulose. Approximately 40 μg of phenylbutazone (ca. 1100 dpm/μg) and 30 μg each of indomethacin (9500 dpm/μg), desmethylindomethacin, and salicylic acid were chromatographed. Recovery of radioactivity from column was about 110 per cent for ^{14}C-phenylbutazone and about 95 per cent for ^{14}C-indomethacin. The eluate corresponding to salicylic acid was monitored at 297 mμ.

TABLE 2. RECOVERY OF RADIOACTIVITY FROM TISSUES OF RATS TREATED WITH ^{14}C-INDOMETHACIN

Biological samples	Per cent recovery of radioactivity at each separation step*		
	$CHCl_3$ phase	Basic aqueous phases	DE-23 column chromatography†
Plasma	98·0 ± 1·8‡	98·1 ± 0·5	100·9 ± 6·3
Kidney	96·7 ± 1·0	90·5 ± 3·9	105·7 ± 9·7
Liver	97·2 ± 1·7	82·4 ± 4·2	103·7 ± 7·9
Bile	68·4 ± 5·2	89·4 ± 6·6	83·4 ± 5·9§
Feces	94·2 ± 2·8	96·0 ± 1·7	103·5 ± 11·3
Urine	63·3 ± 5·9	97·0 ± 4·8	90·8 ± 7·8§

* Rats were treated i.v. with ^{14}C-indomethacin (8–10,000 dpm/μg, 10 mg/kg). Tissues were collected at 20 min, 1, 3, 6 and 24 hr. Feces and urine were collected for 0–6 and 6–24 hr. Bile was obtained from a bile cannulated rat.

† The radioactivity was present as indomethacin (1–60 μg/g), deschlorobenzoylindomethacin (1–30 μg/g), and/or desmethylindomethacin (1–40 μg/g).

‡ Mean ± S. D. for ten to twelve determinations.

§ Represents that recovered as indomethacin, deschlorobenzoylindomethacin and desmethylindomethacin.

in all fractionation steps was greater than 95 per cent over rather wide ranges of indomethacin, desmethylindomethacin, and deschlorobenzoylindomethacin concentrations.

The conjugates of deschlorobenzoylindomethacin and desmethylindomethacin in rat urine[1] which partitioned to the acidic, aqueous phase were extracted with ethyl acetate and characterized by thin-layer chromatography.[1] The remaining radioactivity

in the acidic, chloroform phase from urine was mostly the free drug and metabolites (>90 per cent). The glucuronides (<10 per cent of deschlorobenzoylindomethacin and desmethylindomethacin) partitioned quantitatively to the basic, aqueous phase and were recovered after chromatography on Whatman DE-23 cellulose. The conjugates were eluted at 100–120 ml (deschlorobenzoylindomethacin) and 120–140 ml (desmethylindomethacin). The other ionizable drug species were eluted as shown in Figs. 3 and 4. Similar drug species were also found in bile but in different percentages.

The partitioning characteristics of the radioactivity in chloroform–methanol extracts of liver and kidney between the basic, aqueous and Freon 113 phases were significantly different from those seen for plasma, feces, and urine. Most of the radioactivity from liver and kidney which partitioned to the basic, aqueous phase was quantitatively recovered from DE-23 columns as indomethacin, deschlorobenzoylindomethacin and desmethylindomethacin (Table 2). However, approximately 10–18 per cent of the radioactivity from ^{14}C-indomethacin remained in the nonaqueous, Freon-113 phase; it had previously been partitioned to the acidic, chloroform–methanol phase. These partitioning characteristics indicate that the carboxyl group of the radioactive compound(s) cannot be ionized.

This method was also used in the analysis of ^{14}C-salicylic acid in bile and urine from rats with cannulated bile ducts (Table 3). In the initial aqueous acid:$CHCl_3$–

TABLE 3. RECOVERY OF RADIOACTIVITY FROM URINE AND BILE OF RATS TREATED WITH ^{14}C-SALICYLIC ACID*

Sample	Per cent recovery of radioactivity at each separation step		
	$CHCl_3$ phase†	Basic aqueous phase	DE-23 column chromatography
Urine	49·8 ± 14·1‡	99·9 ± 0·1	93·9 ± 2·2
Bile	54·8 ± 9·9	99·9 ± 0·1	100·6 ± 8·4

* Bile-cannulated rats were treated with ^{14}C-salicylic acid (1000 dpm/μg, 100 mg/kg). Urine and bile were collected; the average excretion for three rats was 57·5% of i.v. dose in urine and 5·6% in bile in 24 hr.

† A major (>95 per cent) radioactive peak from the $CHCl_3$ phase was eluted at 160 ml and was ^{14}C-salicylic acid. The radioactivity in the aqueous acid phase of the $CHCl_3$:MeOH:acid biphase was not identified but presumed to be conjugates.[10]

‡ Mean ± S. D. for eight to ten determinations.

MeOH biphase of urine and bile, about 50 per cent of the radioactivity partitioned to the chloroform phase. The radioactivity in the chloroform phase was extracted quantitatively into base and chromatographed primarily (>95 per cent) as salicylic acid on Whatman DE-23 as a single radioactive peak.

DISCUSSION

The quantitative method for determining indomethacin and its metabolites in biological tissues is outlined in Fig. 5. The volume ratio of organic solvents (chloroform:anhydrous, 0·3 N HCl-methanol; 2:1, v/v) to tissue of 20:1 is important for the initial extraction of tissue. At this solvent–tissue ratio, lipid extraction is optimal[6] and water can be present up to 5 per cent of the volume of homogenate. If extraction of more tissue and/or the addition of aqueous acid exceeds the limit of 5 per cent water,

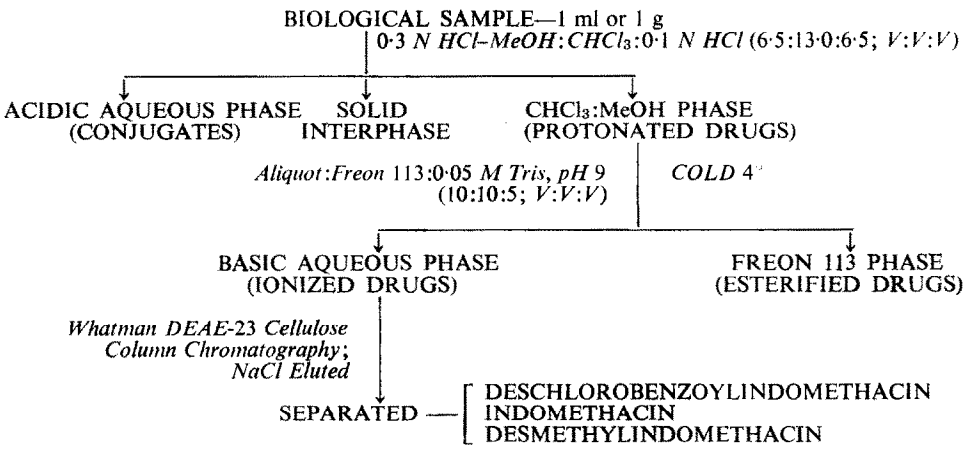

FIG. 5. Extraction, partition, and separation of indomethacin, deschlorobenzoylindomethacin, and desmethylindomethacin and their conjugates.

the tissue will be poorly homogenized (tissue phase will float) and the extraction of lipids and drugs will not be quantitative.

The selective partitioning of drugs from the acidic, chloroform–methanol phase to a basic, aqueous phase is feasible for drugs having widely separated pK_a values (e.g. phenylbutazone and indomethacin), but is not feasible for drugs having similar pK_a values (e.g. indomethacin and deschlorobenzoylindomethacin). However, this partitioning step does separate ionized species from esterified compounds and from tissue lipids. The use of Freon 113 is important because it tends to exclude water (to which drug would partition) from the organic phase without changing the partitioning characteristics of most lipids.[9]

The chromatographic conditions were chosen because indomethacin is unstable above pH 8 at room temperature, and because the molarities (0·05 M) of sodium chloride and of Tris buffer were nearly maximal for the exchange capacity of Whatman DE-23 at pH 8. Higher molarities at pH 8 caused faster elution and incomplete separation of deschlorobenzoylindomethacin and indomethacin. However, the use of Whatman DE-23 at the higher pH values (e.g. 9·6) with an eluent of higher molarity (e.g. 0·2 M Tris buffer, pH 9·6) is possible for indomethacin and its metabolites if chromatography is done in a cold room and/or the eluate is immediately adjusted to pH 8 or lower. For other anions (phenylbutazone) these restrictions do not apply. The chromatographic separation of salicylic acid and phenylbutazone from indomethacin and its metabolites was evaluated and shown to be feasible, with the exception of the separation of deschlorobenzoylindomethacin and salicylic acid. Although the selective separation of metabolites of salicylic acid and phenylbutazone from indomethacin has not yet been studied, it is considered feasible inasmuch as salicylic acid is metabolized primarily to conjugates[10] and phenylbutazone is converted to several hydroxylated metabolites.[11]

The value of this new methodology, in addition to separating indomethacin, its metabolites, and other anionic drugs, was further apparent from our studies *in vivo*. The relative amounts of conjugates and free drugs in urine were immediately apparent,

as was the absence of any significant concentrations of conjugates in extracts of feces and other biological tissues. The partitioning characteristics of the radioactive species from liver and kidney in the basic, aqueous, Freon 113 biphase suggest that an unidentified indomethacin metabolite was also present.

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