ometry. The stomach is neither circular nor does its average diameter approximate that of the small intestine. Consequently, there is no reason to expect that simultaneous excitation of the muscularis mucosa and inhibition of the tunica muscularis would increase intraluminal pressure sufficiently to register with a water-filled intraluminal recording balloon, and the contrary might well be the case if the external effector layers are indeed inhibited.

No acceptable adaptation of the split bowel preparation to the stomach appears practical, but indirect means are available to estimate at least the presence or absence of spontaneous motor activity. Such demonstrations will be necessary to determine whether the observed net inhibition of canine gastric motor activity is due to the relative geometries of coupled effector systems.

With the canine small intestine, paradoxical motor effects were observed by intraluminal pressure measurements, probably because the test agents had opposite effects on the two muscle layers. This is amply illustrated in Fig. 8, which shows the effect of somatostatin on the canine ileum. Clearly, the tunica muscularis is inhibited and the muscularis mucosa is stimulated, while an overall stimulatory effect is seen from an intraluminal balloon in an adjacent intact small bowel segment. These data confirm and explain the excitation mechanism reported by several investigators (1–3) in the somatostatin effect on the motor activity of the canine small intestine. These results also lead to the conclusion that the circular components of the canine small intestine muscularis mucosa possess physiologically significant contractile powers.

The overall significance of these findings is that both the canine and human small intestine constitute pharmacologically separate, but mechanically coupled, muscular systems, either one of which can show excitation when the coupled effects are measured by any system that does not differentiate the identity of the effector system involved. In the future, it will be necessary to evaluate motor effects of test agents on a separate basis. Those that have been reported to produce paradoxical effects should be reevaluated. Obviously, the use of the split bowel technique would not be appropriate for chronic preparations, but these results also suggest a cautious interpretation of the mechanisms from experimental data obtained from either open-ended catheters or intraluminal pressure telemetry capsules.

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High-Performance Liquid Chromatographic Microdetermination of Indoprofen in Human Milk

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Received August 30, 1978, from the **Chemical Sciences Division, Midwest Research Institute, Kansas City, MO 64110,* and ‡*Adria Laboratories, Inc., Columbus, OH 43216.* Accepted for publication March 6, 1979.

Abstract □ A previously reported high-performance liquid chromatographic (HPLC) method for indoprofen determination in physiological fluids was modified and extended to provide quantitative data on drug concentrations in human milk samples at a low nanogram per milliliter level. The reversed-phase HPLC technique was modified to give a better separation of the drug and milk components. To achieve the necessary cleanup for low level determination, the milk samples required protein precipitation, liquid–liquid drug extraction, and concentration. Excellent indoprofen recovery was obtained with this technique; the average recovery from 20 milk samples spiked with various nanogram drug levels was 95%. The analytical technique showed excellent reproducibility; the calibration solutions over 15 days had a relative standard deviation of 3.2%. Results for indoprofen levels in milk and plasma samples from seven subjects who received either a single or multiple oral drug dose are presented.

Keyphrases □ Indoprofen—analysis, high-performance liquid chromatography, human milk □ High-performance liquid chromatography—analysis, indoprofen, human milk □ Anti-inflammatory agents—indoprofen, high-performance liquid chromatographic analysis, human milk □ Milk, human—analysis, indoprofen, high-performance liquid chromatography

Indoprofen, 4-(1,3-dihydro-1-oxo-2*H*-isoindol-2-yl)-α-methylbenzeneacetic acid (I), has been reported to have analgesic activity in animals and humans (1–4). In an earlier report (5), a reversed-phase high performance liquid chromatographic (HPLC) method was presented for the quantitation of drug levels in plasma and urine samples. This method was very simple and had sufficient sensitivity for determination of microgram per milliliter levels. However, in certain cases, such as determinations in later plasma time point samples for pharmacokinetic studies or in physiological fluids such as milk where the drug level is expected to be low, a more sensitive technique is required. Therefore, a sample preparation procedure was developed that provides the necessary cleanup and sensitivity for low level analyses. The method has the necessary precision and accuracy to provide quantitative data for nanogram indoprofen levels in milk samples and may be applicable to other physiological fluids containing low drug levels.

EXPERIMENTAL

Apparatus and Reagents—The liquid chromatograph consisted of a high-pressure pump[1], a loop injector[2], a UV detector[3], and a strip-chart recorder[4]. The chromatographic column was μBondapak C_{18}[5] (300 × 4 mm i.d.).

[1] Model 6000A, Waters Associates, Milford, Mass.
[2] Model U6K, Waters Associates, Milford, Mass.
[3] Model 440, Waters Associates, Milford, Mass.
[4] Model SR-240, Heath/Schlumberger Instruments, Benton Harbor, Mich.
[5] Waters Associates, Milford, Mass.

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Table I—Indoprofen Recovery from Human Milk

Subject	Hours after Administration	Indoprofen Added, ng	Calculated Indoprofen/0.5 ml of Milk, ng		Recovery, %
			Neat	Spiked	
		Part A			
1	Baseline	10	ND[a]	11	110
2	Baseline	25	Tr[b]	33	102
3	Baseline	10	Tr	13	95
4	Baseline	50	20[c]	77[c]	—
5	Baseline	25	ND	25	100
6	Baseline	10	ND	10	100
7	Baseline	25	ND	23	92
				Average ± *SD*	100 ± 6
		Part B			
1	12	25	17	37	88
1	48	25	ND	20	80
2	12	25	96	124	112
2	24	25	Tr	23	92
3	2	200	39	225	94
3	12	25	99	117	96
3	24	25	11	30	83
4	26	25	229	239	94
5	26	50	137	171	91
5	50	50	211	246	94
6	26	50	86	138	102
6	50	50	68	118	100
7	50	50	109	152	96
				Average ± *SD*	94 ± 8
				Overall average	96

[a] ND = not detected. [b] Tr = trace, indoprofen level <10 ng/ml. [c] Subject 4 had an interfering milk component eluting 1 min before indoprofen, thus preventing recovery calculation.

Acetonitrile[6] was of "distilled in glass" purity. The water was first deionized and then distilled to remove contaminants. Hexane[7] and ethyl acetate[7] were of "nanograde" purity.

An indoprofen reference standard[8] was provided, and the internal standard was the pentanoic acid indoprofen homolog[8]. All calculations were made using the internal standard method and the relative weight response (*RWR*) of calibration solutions.

HPLC Conditions—The HPLC conditions used throughout consisted of a μBondapak C_{18} column, 300 × 4 mm i.d., and acetonitrile in 0.175 *M* acetic acid, 35:65 for milk samples and 40:60 for plasma determinations. The flow rate was maintained at 1.0 ml/min, and the temperature was ambient. A 280-nm filter was used in the UV detector since indoprofen has a λ_{max} at 282 nm. Injection volumes varied between 20 and 100 μl, depending on the drug concentration.

Sample Preparation Procedure—Since the indoprofen level in human milk samples was expected to be low, the analytical technique required sensitivity in the low nanogram per milliliter range. Studies were undertaken to define the optimal procedures for extracting the drug from milk and concentrating the extract to provide sufficient sensitivity.

The final method consisted of adding 0.1 ml of 0.1 *N* NaOH to the milk (0.5 ml) to ensure complete ionization of the drug. Then, acetonitrile (2.0 ml) containing the internal standard was added; the sample was mixed and allowed to stand for at least 1 hr at 4° to precipitate the protein. The sample was centrifuged at 2000 rpm for 15 min, and the supernate was transferred to a culture tube. The supernate was extracted with 3 × 3 ml of hexane, which was discarded. The solution was neutralized with 0.1 *N* HCl, and the drug was extracted with 1 × 4 ml, followed by 2 × 2 ml of ethyl acetate.

The extracts were combined and evaporated to dryness on a 50° hot plate under nitrogen stream. The residue was dissolved in 400 μl of the HPLC eluent and filtered through a 0.45-μm[9] filter. An aliquot was injected onto the HPLC column.

For the indoprofen determination in plasma samples, the method reported earlier (5) was used and consisted of precipitating the protein with acetonitrile and filtering the supernate prior to injection.

Samples and Sample Handling—Milk and plasma samples were obtained from seven subjects who received oral indoprofen doses. All subjects were in the early postpartum period. Three subjects received a single 200-mg dose in capsules, and the other four were given 2 × 100 mg of indoprofen every 6 hr for nine doses. A baseline milk sample was obtained from each subject before dosing to determine possible interfering components. Additional samples were obtained at 2.0, 12, 24, and 48 hr for the single-dose subjects and at 2.0, 26, and 50 hr after the initial dose for the multiple-dose subjects. Plasma samples from each subject were obtained at 2.0 hr after the initial drug dose and at 26 and 50 hr for subjects receiving multiple doses. All samples were frozen in dry ice immediately after collection and maintained at −80° until analysis.

Calculations—The calculations were based on an internal standard and the relative weight response of calibration solutions. The average relative weight response of standards was used to calculate indoprofen levels by the following equations:

$$RWR\ \text{std} = \frac{\text{PH I std}}{\text{PH II}} \times \frac{\text{wt II}}{\text{wt I std}} \qquad \text{(Eq. 1)}$$

$$\text{wt I} = \frac{\text{PH I}}{\text{PH II}} \times \frac{\text{wt II}}{RWR\ \text{std}} \qquad \text{(Eq. 2)}$$

where PH I and PH II are the peak heights and wt I and wt II are the weights of the drug and internal standard, respectively.

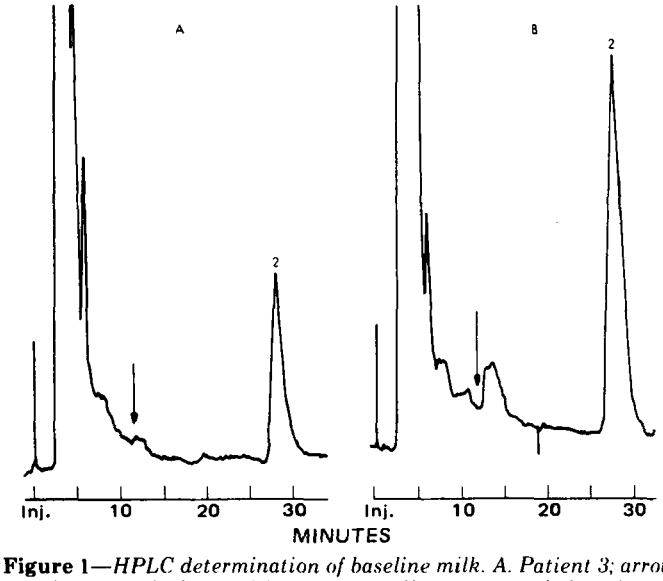

Figure 1—*HPLC determination of baseline milk. A. Patient 3; arrow = indoprofen elution position corresponding to 10.4 ng/ml, and peak 2 = internal standard, 206 ng. B. Patient 7; arrow = indoprofen elution position; and peak 2 = internal standard, 411 ng. Sample preparation and HPLC conditions are given in text.*

[6] Burdick & Jackson Laboratories, Muskegon, Mich.
[7] Mallinckrodt Chemical Works, St. Louis, Mo.
[8] Adria Laboratories, Columbus, Ohio.
[9] Floropore, Millipore Corp., Bedford, Mass.

Table II—Indoprofen in Milk and Plasma

Subject	Nanograms of Indoprofen per Milliliter of Milk, Time from First Dose, hr					Micrograms of Indoprofen per Milliliter of Plasma, Time from First Dose, hr		
	Baseline[a]	2.0	12	24	48	2.0	26	50
1	Tr	681	34	Tr	ND[b]	9.4	—	—
2	ND	Tr	192	Tr	Tr[c]	10.5	—	—
3	Tr	80	198	Tr	Tr	12.4	—	—
	Baseline[a]	2.0	26	50				
4	Tr	60	459	488		3.4	19.4	15.2
5	ND	41	273	422		3.5	5.6	11.3
6	ND	176	172	136		13.5	9.5	12.4
7	ND	Tr	184	219		0.8	6.6	23.8

[a] Calculated baseline level subtracted from other samples to obtain indoprofen level. [b] ND = not detected. [c] Tr = trace, <25 ng/ml.

Table III—Day-to-Day Precision of Relative Weight Response of Reference Standards[a]

Indoprofen Level, ng/ml	Run	Day						
		1	3	4	5	9	11	15
20	1	2.24	2.33	2.38	2.31	—	—	2.31
	2	2.21	—	—	2.42	—	—	—
50	1	2.16	2.22	2.26	2.39	2.18	2.28	2.25
	2	2.23	2.19	—	2.16	2.24	2.26	2.30
100	1	2.14	—	2.18	2.14	2.17	2.18	2.24
	2	2.22	—	2.20	—	2.24	2.24	2.31

[a] Average relative weight response = 2.24, $SD = \pm 0.07$, and $RSD = 3.2\%$.

The recovery of indoprofen added to samples to determine the procedure accuracy was calculated as follows:

$$\%R = \frac{\text{ng I "spiked" sample}}{\text{ng I added} + \text{ng I "unspiked" sample}} \times 100 \quad \text{(Eq. 3)}$$

where $\%R$ is the percent recovery and ng I is the nanograms of indoprofen calculated in the spiked and neat (unspiked) sample.

The analysis precision was determined by analyzing duplicate aliquots from each sample and calculating the standard deviation (SDP) and relative standard deviation ($RSDP$) of pairs:

$$SDP = (\sum |A - B|^2/2 \times P)^{1/2} \quad \text{(Eq. 4)}$$

$$RSDP = SDP/\text{average of all } A \text{ and } B \times 100 \quad \text{(Eq. 5)}$$

where A and B are values of duplicate determinations and P is the number of pairs.

RESULTS AND DISCUSSION

The previously developed HPLC analytical technique had sufficient sensitivity and selectivity to detect and measure nanogram quantities of indoprofen standards. However, prior to physiological fluid analysis at these levels, the sample required concentration and the removal of possible interfering components. This study was initiated to develop the necessary sample cleanup procedure and to determine indoprofen in human milk.

Initial experiments were conducted using cow's milk spiked with varying indoprofen levels. Many different parameters were evaluated, and the steps necessary to provide satisfactory drug recovery and sufficient sample cleanup were defined. The final cleanup procedure is outlined in the *Experimental* section. The indoprofen retention volume was increased by changing the acetonitrile content of the eluent from 40% (for plasma samples) to 35%. This eluent modification separated the drug from some major interfering milk components.

A final evaluation of the method was conducted by analyzing two or more milk samples spiked with 0, 10, 25, 50, 100, and 200 ng of drug/ml. Even with the eluent modification, a minor milk component was detected in the blank samples at the indoprofen retention volume. The level of this compound corresponded to 6.0 ng of indoprofen/ml, and this value was subtracted from the indoprofen levels in the spiked samples prior to recovery calculations. The average recovery for 15 spiked samples of cow's milk was 103% with a standard deviation of ±8.1.

Prior to using the method to determine indoprofen levels in human milk, the sample preparation procedure was further evaluated using the baseline milk samples from the seven subjects (Table I, Part A). Duplicate samples from each subject were prepared, one of which was spiked with 20, 50, or 100 ng of indoprofen/ml. Two analytical runs were made on a number of these samples to ensure technique reproducibility. An interfering milk component was observed in three baseline milk samples (Subjects 2–4) at an elution position near indoprofen. For Subjects 2 and 3, this component represented <10 ng of indoprofen/ml; however, for Subject 4, the peak was equivalent to 20 ng/ml and eluted 1 min before indoprofen. The interfering peak in Subjects 2 and 3 was subtracted from the drug level calculated in the corresponding spiked sample for recovery determination; Subject 4 was not included.

The average recovery for these analyses was 100% with a standard deviation of ±6. Thus, the cleanup technique provided excellent drug recovery and was able to give relatively clean chromatograms for nanogram determination. However, since some samples had interfering components, the method provided quantitative data on samples with drug levels greater than 25 ng/ml. Below that level, indoprofen levels were considered to be semiquantitative and were designated as trace (Tr) if a peak was calculated to be <10 ng/ml and as not detected (ND) if no peak was observed. Figure 1 presents representative chromatograms for two baseline milk samples.

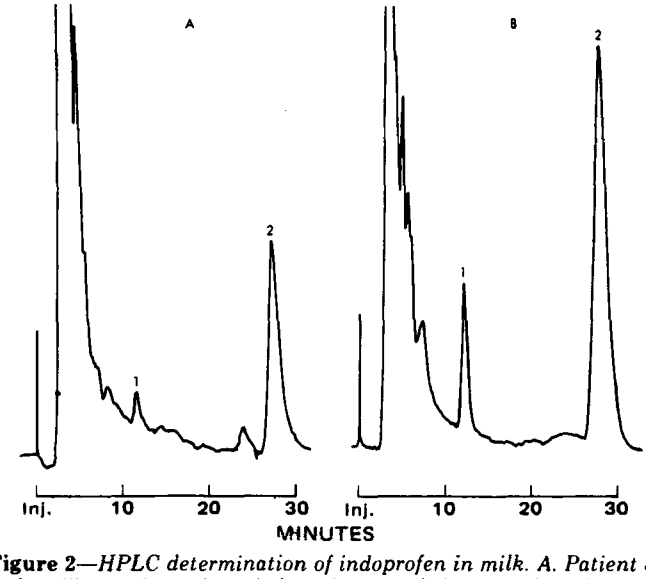

Figure 2—*HPLC determination of indoprofen in milk. A. Patient 3; 24-hr milk sample; peak 1 = indoprofen, 21 ng/ml; and peak 2 = internal standard, 206 ng. B. Patient 6; 24-hr milk sample; peak 1 = indoprofen, 136 ng/ml; and peak 2 = internal standard, 411 ng. Sample preparation and HPLC conditions are given in text.*

The analytical procedure was employed for the determination of indoprofen levels in human milk from seven subjects (Table II). All samples were analyzed in duplicate, and each value is the average of these determinations. The relative standard deviation of pairs was 5.1%. Subjects 1, 3, and 4 had detectable levels of a component in the baseline samples, and these values were subtracted to obtain the corrected indoprofen levels. The impurity level was similar to that observed previously (~10 ng/ml). This interfering compound was possibly present in all samples but was not completely removed in some during the sample preparation. Thirteen recovery samples were analyzed (Table I, Part B). The average recovery was 94% with a standard deviation of ±8. Representative indoprofen chromatograms in milk samples are presented in Fig. 2. For the 20 spiked samples analyzed, the average recovery was 96%.

Plasma samples were obtained at 2.0 hr after administration for the single-dose subjects and at 2.0, 26, and 50 hr after the first dose for multiple-dose subjects. Each plasma sample was analyzed in duplicate, and the averages are given in Table II. The precision of the plasma determinations was excellent, with a relative standard deviation of pairs of 5.1%.

Indoprofen calibration standards were analyzed after every fourth sample to determine the relative weight response for drug level calculations. New standards were prepared for each subject. A comparison of the relative weight responses obtained during milk sample analysis is given in Table III. During the 15-day period, the day-to-day variation in standards was very small, with a relative standard deviation of 3.2% for the relative weight response. Thus, the analytical system had excellent stability and provided quantitative data for extended periods.

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Simple Analogs of the Toxin Callicarpone

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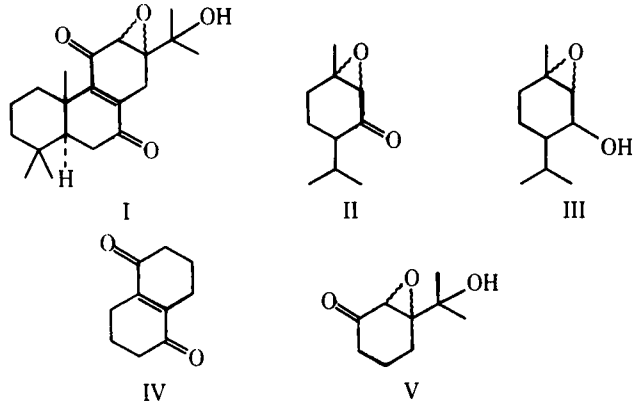
Received June 2, 1978, from the *Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS 66045.* Accepted for publication March 14, 1979. *Present address: Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677.

Abstract □ Callicarpone, a component 10 times as toxic to fish as rotenone, has been isolated from the leaves of *Callicarpa candicans*. It is reasonable to assume that callicarpone will act as an insecticidal agent as does rotenone. Therefore, the structure–activity relationship of callicarpone was examined by synthesizing a series of compounds having certain of its structural features. Those compounds were tested for insecticidal and antimicrobial activities. A study of synthetic analogs elucidated the functional group chemistry of callicarpone so that a synthesis might be undertaken. Piperitone oxide showed ~1/100th the activity of rotenone against *Daphnia magna*. 1-(α-Hydroxyisopropyl)-3-oxocyclohexene oxide showed activity against mycobacterium while 2,3,4,6,7,8-hexahydronaphthalene-1,4-dione showed inhibitory activity against the mycobacterium and two yeasts.

Keyphrases □ Callicarpone—analogs, structure–activity relationships, toxicity, antimicrobial activity □ Insecticides—callicarpone, analogs, structure–activity relationships, toxicity, antimicrobial activity □ Structure–activity relationships—callicarpone analogs, antimicrobial activity

The use of decoctions of various plants as fish poisons has been practiced for many centuries by native tribes in Africa, India, and South America. Usually, the poisonous plants were macerated with water, the decoction was poured into a selected body of water, and the fish that rose to the surface were collected. It was reasonable to assume that these poisonous plants should exhibit toxicity against organisms besides fish, and some of these plants have been found to contain useful insecticides.

A component toxic to fish has been isolated from the leaves of *Callicarpa candicans* (1). These leaves have long been used for stupefying fish by natives of Palau and the Philippine Islands. The active principle was named callicarpone (I). Callicarpone exhibited 10 times stronger toxicity against loach fish (*Misgurnus anguillicaudatus*) than did rotenone. The structure of callicarpone was deduced from spectral and chemical evidence.



Since callicarpone possesses stronger toxicity against fish than does rotenone, it is reasonable to assume that callicarpone also might act as an insecticidal agent. Therefore, a study of the structure–activity relationship of callicarpone was initiated by synthesizing a series of compounds having certain of its structural features and by testing these compounds for their toxicity to *Daphnia magna* (a fresh water crustacean) (2). To determine if these functional groups might show a more general biological activity, the agents also were tested for antimicrobial activity. A study of synthetic analogs provided information about the functional group chemistry of callicarpone so that a synthesis might be undertaken.

Callicarpone possesses some unique structural and

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