momycin was observed. Litmomycin was added to such a mixture at different time intervals during the reaction.

When litmomycin was added at 5 and 10 min after initiation of the polymerization reaction, the enzyme activity continued for about 20 min, comparable to the control. But when the litmomycin was added after 20 min, the enzyme activity stopped instantly, as indicated by the ^{3}H-thymidine monophosphate incorporation (Fig. 3). The results suggest that litmomycin did not inhibit the initial formation of an RNA–DNA hybrid but blocked the subsequent phase of the polymerization processes.

In conclusion, litmomycin inhibits DNA polymerase activity of RNA tumor viruses by interaction with (riboadenylate)$_n$–(deoxythymidylate)$_n$ or adenine–thymine bases and (ribocytidylate)$_n$–(deoxyguanylate)$_n$ or cytosine–guanine bases with a different degree of binding. It may be of interest to investigate the antiviral activity of litmomycin in cell culture.

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ACKNOWLEDGMENTS AND ADDRESSES

Received November 10, 1975, from the *Department of Biomedicinal Chemistry, College of Pharmacy and Pharmacal Sciences, Howard University, Washington, DC 20059*

Accepted for publication March 4, 1976.

The author thanks Dr. H. G. Floss, Purdue University, for a gift sample of litmomycin. The arrangements made by Dr. Jack Gruber and Dr. M. A. Chirigos, National Institutes of Health, for the supply of reverse transcriptase are gratefully acknowledged. The technical help of Mr. Warren W. Schadt in the liquid scintillation counting is appreciated.

GLC Determination of Indoprofen in Plasma

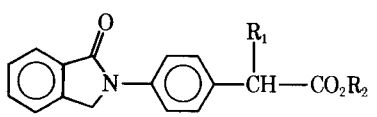
ROBERT V. SMITH ˣ, DAVID W. HUMPHREY, and HENRY ESCALONA-CASTILLO

Abstract □ Implementation of human bioavailability studies with the analgesic indoprofen required a rapid, sensitive, and convenient assay in blood plasma. A procedure based on ether extraction of acidified plasma, derivatization with diazomethane, and GLC analysis using a 3% OV-1 column was sufficiently sensitive for measurement of plasma indoprofen concentrations in the 0.4–16-μg/ml range. An average recovery of 99.0 ± 7.6% (*SD*) was achieved when the pentanoic acid homolog was employed as an internal standard.

Keyphrases □ Indoprofen—GLC analysis, human plasma □ GLC—analysis, indoprofen in human plasma □ Analgesics—indoprofen, GLC analysis in human plasma

Indoprofen, *dl*-2-[4-(1-oxo-2-isoindolinyl)phenyl]propanoic acid (I), possesses promising analgesic and anti-inflammatory properties (1, 2). In humans, I reportedly appears unchanged in plasma (3). Furthermore, enantiomeric enrichment does not seem to be significant in humans (4). Implementation of human bioavailability studies in this laboratory required a rapid, sensitive, and convenient assay of I in blood plasma.

Two GLC methods were reported for the determination of I in plasma (4, 5). Both procedures employ a lengthy series of partition steps. In one (4), a trifluoroethyl ester



I: $R_1 = CH_3$, $R_2 = H$

II: $R_1 = CH_2CH_2CH_3$, $R_2 = H$

III: $R_1 = CH_3$, $R_2 = CH_3$

IV: $R_1 = CH_2CH_2CH_3$, $R_2 = CH_3$

is utilized after preparation with boron trifluoride–trifluoroethanol. The latter reagent is corrosive and difficult to eliminate prior to GLC development. The other method (5) involves preparation and resolution of a diastereomeric pair of amides obtained through reaction of I (as its acid chloride) with *l*-α-methylbenzylamine. While useful in evaluating *in vivo* enantiomeric interconversions, this procedure is not amenable to analyses of large numbers of samples because of its length.

A simplified assay based on a single ether extraction and GLC of I and the internal standard, *dl*-2-[4-(1-oxo-2-isoindolinyl)phenyl]pentanoic acid (II), as their methyl esters (III and IV) was developed and is the subject of this report.

EXPERIMENTAL

Reagents and Materials—Compounds I, II[1], and *N*-methyl-*N*-nitroso-*N'*-nitroguanidine[2] were used as obtained. All other solvents and reagents were analytical reagent grade. GLC columns were prepared with 3% OV-1 on acid-washed silanized Chromosorb W, 100–120 mesh[3].

Reference Methyl Esters—Compound I (1.01 g) was dissolved in 5 ml of methanol; 3 ml of 30% (w/w) boron trifluoride in methanol was added, and the resulting mixture was refluxed for 15 min. The reaction mixture was cooled, reduced to near dryness *in vacuo*, and shaken with 20 ml of water for 5 min. A white precipitate was collected by filtration, and the filtrate was extracted three times with 10 ml of ethyl acetate. The ethyl acetate extracts were combined with the previously collected precipitate, and the total product was crystallized from methanol–acetone (10:1) with a few drops of water to give 1.06 g (100%) of III as white plates, mp 120–122° [lit. (1) mp 124–126°]; mass spectrum, M^+ = 295; GLC, single peak, t_R (solvent front) = 2.87 min.

Compound II (1.00 g) was reacted in the same fashion. Crystallization from methanol–acetone (10:1) with a few drops of water gave 0.99 g (95%) of IV as white plates, mp 125–126°; mass spectrum, M^+ = 323; GLC, single peak, t_R (solvent front) = 4.22 min.

GLC System and Conditions—All chromatography was performed with U-shaped glass columns [0.61 m (2 ft) × 4 mm i.d.] packed with 3% OV-1 on acid-washed silanized Chromosorb W, 100–120 mesh[3]. All newly prepared columns were conditioned at 260° with carrier gas flowing (30 ml/min) for 12 hr. The columns were fitted into a gas chromatograph[4] equipped with dual flame-ionization detectors.

The chromatograph was operated under the following conditions: column and injection port temperature, 255°; detector temperature, 300°; carrier gas (nitrogen) flow, 30 ml/min; hydrogen and air flows, 40 and 300 ml/min, respectively; sensitivity (input attenuation), 10^2 (Mohm); and range, 8 (× 0.01 v). Injections were performed with a 10-μl syringe[5].

Diazomethane Reagent—*N*-Methyl-*N*-nitroso-*N'*-nitroguanidine (296 mg) was reacted with 4 ml of 2 *N* sodium hydroxide in a separator containing 40 ml of ether. The ether solution resulting was sufficient for derivatizing 10 samples and was prepared fresh daily.

Assay Procedure—All glassware was washed in detergent, soaked for 12 hr in alcoholic potassium hydroxide (1000 ml of ethanol added to 120 ml of water and 120 g of potassium hydroxide), thoroughly rinsed with water, silanized by rinsing with 2% trimethylchlorosilane in benzene, and dried at 100° for 15 min.

Preparation of Standards—To six blank plasma samples contained in 60-ml separators, add 2, 4, 6, 10, 12, and 20 μg of I along with 10 μg of II (contained in a total of 2 ml of methanol) and 4 ml of 0.1 *N* hydrochloric acid. Extract and analyze standards in the same manner as described for plasma samples.

Preparation of Samples—Place 2 ml of plasma in a glass-stoppered 60-ml separator and add 10 μg of II (in 2 ml of methanol), 4 ml of 0.1 *N* hydrochloric acid, and 40 ml of ether. Extract by shaking for 5 min and transfer the ether layer to a 50-ml conical flask; evaporate to dryness *in vacuo* at 40° with a rotary evaporator. Add 0.5 ml of methanol and 4 ml of ethereal diazomethane reagent and allow to react at room temperature for 15 min; quench the reaction (loss of yellow color and cessation of effervescence) by dropwise addition of ethyl acetate–acetic acid (10:1).

Reduce the final reaction mixture (~5 ml) in volume (to ~1 ml) *in vacuo* at 40° with a rotary evaporator; reduce the final volume to dryness under a stream of nitrogen at 25° to assure concentration of the residual solids in the tip of the conical flask. Redissolve the residue in 50 μl of ethyl acetate and inject 5 μl into the chromatograph.

Dilute samples that appear to contain greater than 8 μg of I/ml (from the initial GLC development) with an equal volume of ethyl acetate and chromatograph a 5-μl aliquot.

Calculations—The peak heights for III and IV are measured. Peak height ratios are obtained by dividing the peak height of III by the peak height of IV. Calibration curves are prepared by plotting peak height ratios from the standards *versus* the concentration of I in plasma, expressed as micrograms per milliliter. Values for unknown concentrations of I in sample plasma specimens are obtained by interpolation or calculated from the slope of the calibration curve. Once established, the standard curve need only be revalidated on a day-to-day basis with two or three standard samples.

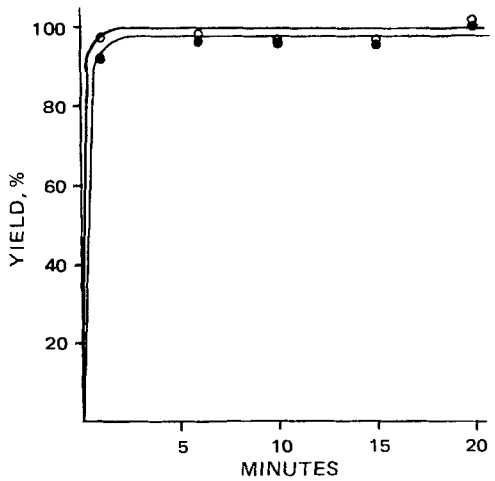


Figure 1—*Diazomethylation of I (●) and II (○).*

RESULTS AND DISCUSSION

Derivatization of I and II—GLC determination of I was initially attempted through use of its trifluoroethyl ester. The latter was reproducibly prepared in good yield through use of the boron trifluoride–trifluoroethanol reagent described previously (4). However, for biological samples containing low microgram amounts of I, it is necessary to eliminate the excess derivatization reagent completely prior to the GLC step, because the broad solvent peak produced by the latter obliterates the drug derivative. Unfortunately, reagent evaporation was not readily accomplished, apparently due to the poor volatility of alcohol–boron trifluoride mixtures[6].

Diazomethylation was studied as an alternative derivatization method[7]. When I and II were reacted with ethereal diazomethane at room temperature in the presence of approximately 10% methanol, essentially quantitative yields of methyl esters were rapidly formed (Fig. 1). Side reactions (6) and reagent polymerization (7) are potential problems with diazomethane. However, if methylation is catalyzed with methanol, these problems are reportedly minimized (7), as was evident in studies with I and II. An impurity appearing at the same retention time as III was observed when *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[8] was employed as a diazomethane-generating reagent. This apparent impurity was probably reagent derived since it did not appear when *N*-methyl-*N*-nitroso-*N'*-nitroguanidine was employed as indicated under *Experimental.*

Assay Selectivity—After confirming the reported high recovery of I and II from acidified aqueous solutions by extraction with ether (4, 5), the potential selectivity afforded by a single liquid–liquid extraction derivatization procedure was studied. Gas chromatograms resulting from this investigation are depicted in Fig. 2 and reveal no interfering peaks at the retention times for III and IV. Essentially identical chromatograms to those in Fig. 2 were produced when derivatized extracts from plasma samples of 10 different individuals were similarly treated.

Accuracy, Precision, and Convenience—Compounds I and II were added at levels of 1–10 μg/ml to 2-ml volumes of blank plasma samples. The spiked specimens were extracted with ether, and the latter extracts were derivatized and chromatographed according to the described procedure.

The peak heights of the resulting esters (III and IV) were compared to calibration curves prepared with authentic samples of III and IV. The absolute recoveries of I and II from plasma were 69.8% (n = 5) and 71.2% (n = 5), respectively. These results indicate that II could serve as a suitable internal standard for the determination of I.

Accordingly, blank plasma samples were spiked with 0.4–8-μg/ml concentrations of I and 5 μg/ml of II and analyzed using a standard curve prepared with triplicate standards equivalent to 0.4, 1.0, 3.0, 5.0, and 8.0 μg/ml of I in plasma (slope = 0.262; y-intercept essentially zero; r = 0.997). The accuracy and precision of these determinations are presented in Table I. The excellent recovery and acceptable reproducibility observed

[1] Adria Laboratories, Wilmington, Del.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Chromosorb W HP, Analabs, North Haven, Conn.
[4] Schimadzu GC-4BM, American Instrument Co., Silver Spring, Md.
[5] Hamilton 701 N.

[6] This was also found to be true when boron trifluoride–methanol was evaluated as a possible derivatization reagent.
[7] *N,O*-Bis(trimethylsilyl)acetamide was tried as a derivatization reagent. However, the trimethylsilyl esters of I and II did not chromatograph well.
[8] Diazald, Aldrich Chemical Co., Milwaukee, Wis.

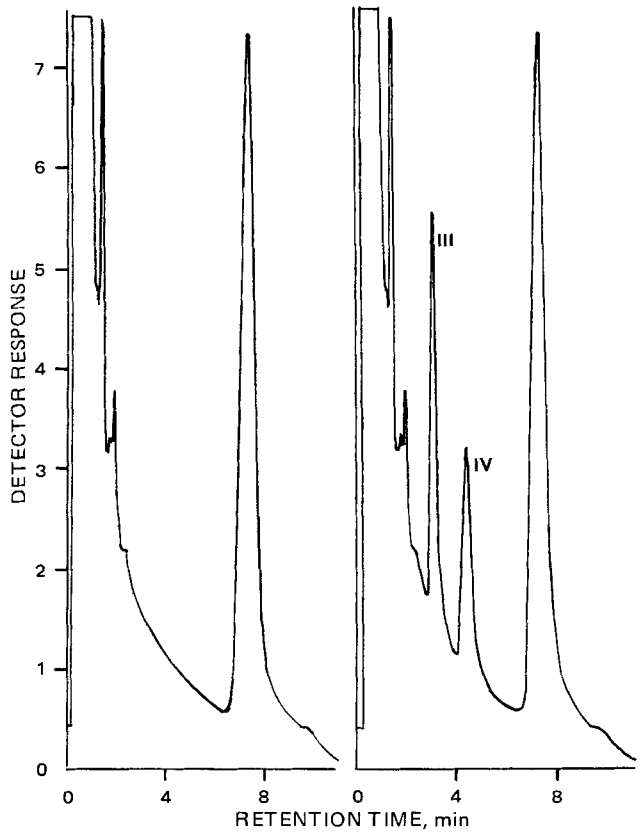


Figure 2—*Gas chromatograms of derivatized human plasma extracts. Key: left, normal plasma specimen; right, plasma specimen containing 6.6 μg of I/ml and 5.0 μg of II/ml; III, indoprofen methyl ester; and IV, internal standard methyl ester.*

provide good evidence for the utility of the developed method for plasma levels of I down to 0.4 μg/ml.

During routine analyses, it was found that a standard curve prepared on Day 1 (as described in *Experimental*) is validated day after day by simple comparison of results from two or three standard samples. In a recent series of determinations, for example, two to five (total = 13) standard samples were analyzed on 4 separate days over a 3-week period. After plotting the data from these determinations, a curve was produced with a slope of 0.269, an r of 0.999, and a y-intercept essentially equal to zero. These data are in excellent agreement with the parameters of the standard curve utilized in evaluating the accuracy and precision of the method and support the utility of the proposed method for routine determinations.

Table I—Accuracy and Precision of GLC Method for I

Concentration of I, μg/ml		
Added	Found	Recovery, %
0.4	0.40	100
0.4	0.41	103
1.0	0.82	82.0
1.0	1.00	100
1.0	1.12	112
3.0	2.65	88.3
3.0	2.99	99.7
3.0	3.08	103
5.0	4.55	91.0
5.0	5.06	101
5.0	5.36	107
8.0	7.55	94.4
8.0	7.73	96.6
8.0	8.16	102
8.0	8.39	105
Mean = 99.0% (n = 15)		
SD = ±7.6%		

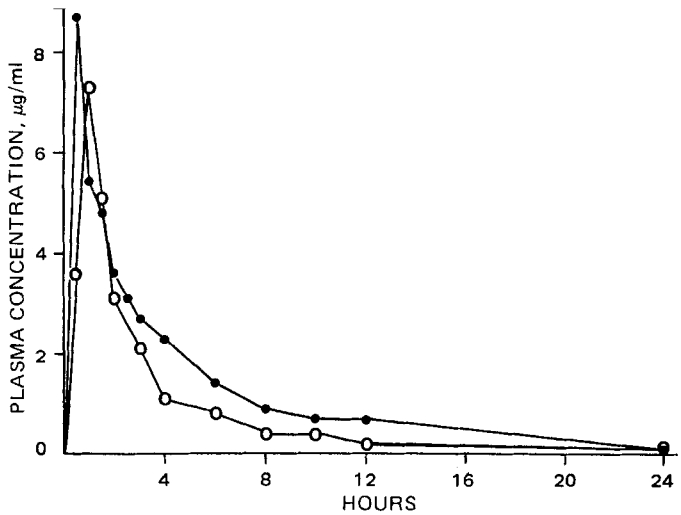


Figure 3—*Individual plasma levels of indoprofen following oral administration of single 100-mg capsules to two subjects as determined by the GLC method. Values less than 0.4 μg/ml are approximations only, since the method was not validated at concentrations below this level.*

The described method was devised as a convenient and more rapid procedure than those reported previously (4, 5). During ordinary usage, the described method permits analysis of 15 samples/one-worker day. This is a marked improvement over the 3–4 hr/sample required to assay I by the previously reported boron trifluoride–trifluoroethanol procedure (4), which is estimated to be the shorter of the two methods described in the literature (4, 5).

Plasma levels obtained in two patients administered single oral doses of 100 mg (capsules) of I were followed with the method described. Results of these analyses are presented in Fig. 3 and nicely parallel the findings published by Tosolini *et al.* (4).

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ACKNOWLEDGMENTS AND ADDRESSES

Received December 22, 1975, from the *Drug Dynamics Institute, College of Pharmacy, University of Texas at Austin, Austin, TX 78712.*

Accepted for publication March 8, 1976.

Supported in part by a grant from Adria Laboratories, Wilmington, Del.

The authors are grateful to Ms. B. Diane Phillips for technical assistance.

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