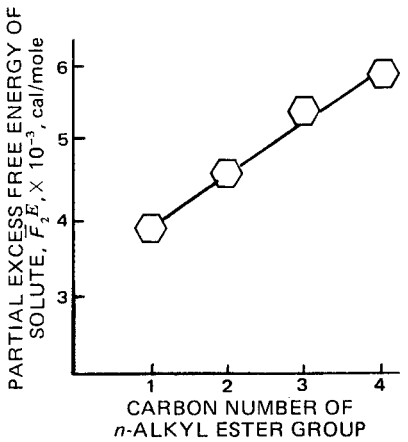

Figure 5—*Plot of the average partial excess free energy for the solute, in calories per mole, as a function of the carbon number of the* n*-alkyl ester group of the parabens.*

free energies increase with an increasing carbon number. The values obtained for these free energy functions, the actual and excess free energies, are substantial in magnitude and imply highly nonideal solution behavior for these parabens in water. This fact was also supported by the rather large activity coefficients determined for these solutes.

Thus, the free energy functions possess a nonlinear relationship with carbon number or molecular size.

An additional thermodynamic parameter, the partial excess free energy of the solute, was investigated. This parameter can be considered to be an overall property of the solution and can also be calculated from these results. The partial free energy, $\overline{F}_2^E$, can be written as:

$$\overline{F}_2^E = RT \ln \gamma_2 \qquad \text{(Eq. 1)}$$

where R is the gas constant, T is the absolute temperature, and γ_2 is the activity coefficient. Previously, it was stated that these activity coefficients were quite large in magnitude and implied highly nonideal behavior. However, when using these values and solving Eq. 1 for the partial free energy, entirely reasonable values of free energy were obtained (Table V). The calculated partial free energy values occurred in regular order with carbon number, and there were regular increases with increasing temperature. This result was expected as the solubility increased with temperature because the energy requirement of the overall system also increased.

Figure 5 shows plots of the partial excess free energy for these solutes as a function of the carbon number of the ester group. In this case, a linear relationship of partial excess free energy with carbon number is evidenced with an approximate slope of about 680 cal/mole/carbon atom.

The expression of the rate of change in partial excess free energy with carbon number allows for a definitive relationship of a thermodynamic parameter and molecular size.

Finally, a spectrum of thermodynamic elements can be easily obtained by studying solubilities at various temperatures once the heat of fusion values have been determined. Studies along these lines will be the subject of future reports.

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Electron-Capture GLC Determination of Ibuprofen in Serum

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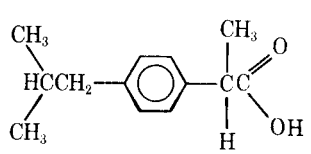
Abstract □ To evaluate drug–protein binding, a sensitive method for the determination of ibuprofen in submilliliter amounts of serum was required. A specific and highly sensitive procedure, based on benzene extraction of the acidified specimen, TLC of the benzene extract residue, formation of the pentafluorobenzyl esters of the materials eluted from the thin-layer chromatogram, and quantification of the pentafluorobenzyl esters by GLC, was developed. Utilizing electron-capture detection, the method is sensitive to 0.1 μg of ibuprofen/0.1 ml of serum. Statistical analyses indicated an average recovery of 97.7% with a standard deviation of ±7.3%. Mass spectrometric analysis, in conjunction with GLC, confirmed the specificity of the method for the intact drug. The procedure was applied successfully to drug absorption and drug–protein binding studies in humans.

Keyphrases □ Ibuprofen—electron-capture GLC analysis in serum, time course of protein binding in humans □ GLC, electron capture—analysis, ibuprofen in serum □ Protein binding—ibuprofen in humans, time course studied using electron-capture GLC analysis in serum □ Anti-inflammatory agents—ibuprofen, electron-capture GLC analysis in serum, time course of protein binding in humans

The pharmacology, toxicology, and biochemistry of ibuprofen[1] [(*RS*)-2-(4-isobutylphenyl)propionic acid] (I), a potent orally active anti-inflammatory agent in animals (1–6) and humans (7–9), have been reported.

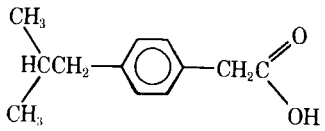
In studies of the absorption, metabolism, and excretion of I, paper chromatographic (10) and GLC (5, 11–14) methods were utilized. All of these procedures required at least 1–5 ml of biological fluid for analysis of intact drug. This quantity constituted a serious limitation in studies where multiple blood specimens were needed for multiple analyses. The need for a sensitive analytical method to measure individual drugs in small amounts of biological fluids was apparent during drug interaction studies with I and aspirin (15).

To determine the time course of drug–protein binding in human volunteers participating in pharmacokinetic studies of I, a simple, specific, and highly sensitive method for the measurement of the intact drug in submilliliter amounts of serum was developed.



I

[1] Motrin, The Upjohn Co., and Brufen, Boots Co.



II

EXPERIMENTAL

Reagents and Materials—Compound I was synthesized[2], as was the internal standard ibufenac[3] (4-isobutylphenylacetic acid) (II). Acetone, benzene, cyclohexane, and methanol, distilled in glass, were used as supplied[4].

Stock solutions of I and II in methanol (60 μg/ml), pentafluorobenzyl bromide[5] in acetone (0.1 ml/100 ml), and aqueous 1 *N* HCl were stored in glass containers. All other chemicals were analytical reagent grade. 3-Cyanopropyl silicone[6] on 100–120-mesh Gas Chrom Q (10% w/w) was used as supplied.

Instrumentation—A two-speed reciprocating shaker[7] was used for shaking samples horizontally. A mixer[8] was used in eluting the zones removed from thin-layer chromatograms. GLC measurements were made with a chromatograph[9] equipped with a ^{63}Ni-electron-capture detector, a freestanding electron-capture linearizer[10], and a −0.1–1.0-mv dual-pen recorder[11]. All gas cylinders used for chromatography [*i.e.*, methane (5% v/v) in argon] were fitted with filters containing molecular sieve 4A.

TLC—All analytical chromatography was conducted on thin layers (250 μm) of silica gel 60 F_{254}[12], ascendingly developed in 10% (v/v) acetic acid in toluene. The separated materials were visualized by irradiation of the plates with a short wavelength (254-nm) UV lamp. Under these conditions, I and II as the free acids had approximately the same R_f values: 0.45 and 0.40, respectively.

GLC Conditions—All chromatography was conducted using U-shaped glass columns (1.83 m × 3 mm i.d.) packed with 10% (w/w) 3-cyanopropyl silicone on 100–120-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 250° for 1 hr with no carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and electron-capture detector were maintained isothermally at 190, 260, and 325°, respectively. The flow rate of the methane (5% v/v) in argon, used as the carrier gas, was maintained at 55 ml/min. Under these conditions, the pentafluorobenzyl esters of I and II had retention times of 9.1 and 13.3 min, respectively (Fig. 1).

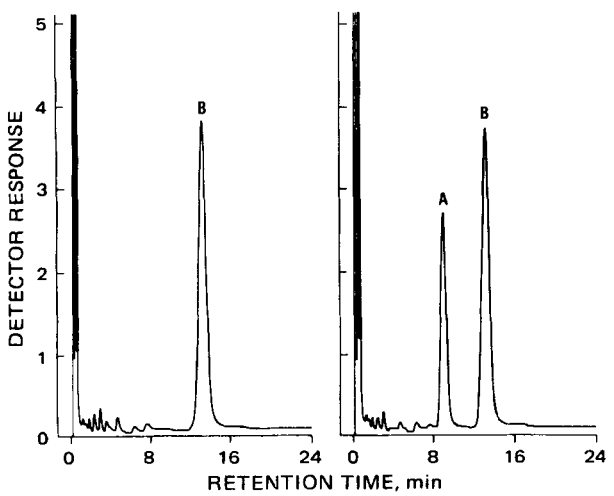


Figure 1—*Gas–liquid chromatograms of human serum extracts. Left: normal serum specimen. Right: serum specimen from subject 1 hr after single-dose oral administration of 400 mg of ibuprofen. Key: A, ibuprofen pentafluorobenzyl ester; and B, ibufenac pentafluorobenzyl ester.*

[2] Research Division, The Upjohn Co., Kalamazoo, Mich.
[3] Research Division, Boots Co., Nottingham, England.
[4] Burdick and Jackson Laboratories, Muskegon, Mich.
[5] Pierce Chemical Co., Rockford, Ill.
[6] Silar 10C, Applied Science Laboratories, State College, Pa.
[7] Eberbach and Sons, Ann Arbor, Mich.
[8] Vortex model K-500, Scientific Industries, Queens Village, N.Y.
[9] Model MT-220, Tracor Inc., Austin, Tex.
[10] Model 114556 B, Tracor Inc., Austin, Tex.
[11] Model MT-22, Westronics Inc., Fort Worth, Tex.
[12] EM Laboratories, Elmsford, N.Y.

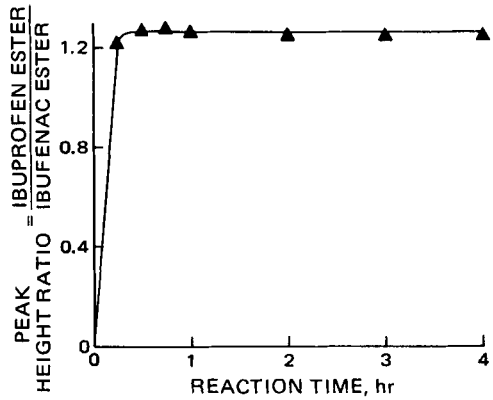


Figure 2—*Effect of reaction time on formation of ibuprofen pentafluorobenzyl ester.*

Protein Binding—For equilibrium dialysis studies, aliquots (0.8 ml) of serum from I-treated subjects were placed in a multicavity dialysis cell[13] on the side opposite to 0.8 ml of aqueous sodium phosphate buffer (0.01 *M*, pH 7.4) containing 0.09% (w/v) sodium chloride (5). The cells were agitated gently for 24 hr at 25°. The I concentration on each side of the dialysis membrane[14] was measured by GLC.

For ultrafiltration experiments, the apparatus (16) and procedure (17) reported previously were utilized. All specimens were centrifuged at 2000 rpm for 3 hr at 25°. The I concentration on each side of the membrane was measured by GLC.

Synthesis of Standard Materials—Place 500 mg of I in a suitable reaction flask. Add 20 ml of acetone, 1.5 ml of undiluted pentafluorobenzyl bromide, and 300 mg of anhydrous potassium carbonate and heat in a water bath at 60° for 5 hr. Evaporate to dryness with a gentle stream of nitrogen. Add 5 ml of water and 10 ml of cyclohexane. Shake for 10 min and allow the phases to separate. Transfer the cyclohexane to a fresh 15-ml centrifuge tube. Repeat the extraction with three additional 10-ml portions of cyclohexane and evaporate the pooled cyclohexane extracts to dryness with nitrogen. Recrystallize from methanol–ether and water.

Synthesize standard material for identification of the II pentafluorobenzyl ester, utilizing the same general reaction conditions as described for I.

Assay—*Preparation of Standards*—Pipet aliquots of the I stock solution, equivalent to 0.5, 1, 2, 3, 4, 5, and 6 μg, into polytef-lined screw-capped centrifuge tubes[15]. Add aliquots of the II stock solution, equivalent to 10 μg, to the same series of centrifuge tubes. Evaporate to dryness with a gentle stream of nitrogen. Add 0.1 ml of control serum to each centrifuge tube and mix well. Prepare an appropriate blank. Extract all standards in the same manner as described for the serum specimens. For analysis of submicrogram amounts of I in aqueous buffer, prepare appropriately diluted standards.

Preparation of Samples—Pipet aliquots of the II stock solution, equivalent to 10 μg, into a series of centrifuge tubes and evaporate to dryness with a gentle stream of nitrogen. Add 0.1 ml of the serum specimens and mix well. Add 0.25 ml of aqueous 1 *N* HCl and 5 ml of benzene and shake horizontally for 10 min. Centrifuge for 10 min at 2000 rpm and aspirate off the aqueous layer. Transfer a 4-ml aliquot of the benzene layer to a fresh 15-ml centrifuge tube and evaporate to dryness with a gentle stream of nitrogen. Wash down the walls of the centrifuge tube with 0.5 ml of chloroform and evaporate to dryness.

Reconstitute the benzene extract residues from the serum standards and samples in 50 μl of chloroform. Together with appropriate standards, spot all of each sample on thin layers of silica gel 60 F_{254} (*vide supra*). Ascendingly develop each plate for a distance of 165 mm, air dry all chromatograms thoroughly at room temperature (24°), and visualize the zones by irradiation with a 254-nm UV lamp. Then scrape the zones corresponding to I and II into screw-capped centrifuge tubes. Add 4 ml of methanol to each tube and gently agitate using the mixer. Centrifuge for 5 min at 2000 rpm. Transfer a 3-ml aliquot of the methanol layer to a fresh screw-capped centrifuge tube and evaporate to dryness with nitrogen.

[13] Chemical Rubber Co., Cleveland, Ohio.
[14] Visking cellophane, Union Carbide Corp., Food Product Division, Chicago, Ill.
[15] Bellco Glass, Vineland, N.J.

Table I—Recovery of Ibuprofen from Human Serum [a]

Added, μg/ml	Found, μg/ml	Recovery, %
0.10	0.083	83.0
0.20	0.215	107.5
0.50	0.549	109.8
5.0	4.75	95.0
10.0	9.54	95.4
20.0	19.21	96.1
30.0	29.21	97.4
40.0	39.62	99.1
50.0	48.50	97.0
60.0	58.18	97.0
	Mean ± *SD*	97.7 ± 7.3

[a] As compared to simple aqueous solution.

Add 25 mg of anhydrous potassium carbonate and a 1-ml aliquot of the pentafluorobenzyl bromide stock solution to each centrifuge tube. Cap tightly and heat in a water bath for 1 hr at 60°. Evaporate to dryness with nitrogen. Add 1 ml of water and 4 ml of cyclohexane. Shake for 10 min, and allow the phases to separate. Transfer 3 ml of the cyclohexane layer to a fresh centrifuge tube and evaporate to dryness. Reconstitute the residues in 0.2 ml of cyclohexane and inject a 2-μl aliquot for analysis into the chromatograph.

Calculations—The peak heights for I and II pentafluorobenzyl esters are measured. Peak height ratios are obtained by dividing the peak height of the I pentafluorobenzyl ester by the peak height of the II pentafluorobenzyl ester. Calibration curves for known concentrations of I in serum are prepared by plotting peak height ratios *versus* I concentrations, expressed as micrograms per milliliter. Values for unknown concentrations of I in serum specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curve.

Drug Administration to Humans—All subjects[16] were between 25 and 28 years; they ranged in body weight from 75.3 to 90.7 kg and in height from 1.70 to 1.83 m. All subjects were fasted for 16 hr prior to drug administration. Each then received a 400-mg dose of I in aqueous solution, and food was withheld for an additional 2 hr.

Blood specimens (10 ml) were withdrawn at predetermined time intervals from 0 to 16 hr after drug administration. The serum was harvested and stored at −18°.

RESULTS AND DISCUSSION

Synthesis and Identification of Pentafluorobenzyl Esters—Methods for the preparation of pentafluorobenzyl esters of carboxylic acids have been reported (18–20). The utility of pentafluorobenzyl derivatives for measuring microgram and submicrogram amounts of selected drugs in biological fluids has been described (21–23). A series of samples, containing known amounts of I and II, was prepared to determine optimal reaction times for formation of the pentafluorobenzyl esters. In studies with I, known amounts of II pentafluorobenzyl ester were added as the internal standard. Similarly, in studies with II, known amounts of I pentafluorobenzyl ester were added as the internal standard. The results indicated that ester formation for both compounds was completed within 30 min (Figs. 2 and 3). A reaction time of 1 hr was selected for convenience. In the presence of water and cyclohexane, the derivatives were stable for at least 96 hr.

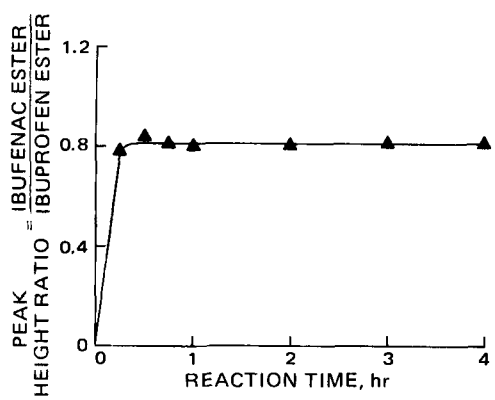


Figure 3—*Effect of reaction time on formation of ibufenac pentafluorobenzyl ester.*

[16] Informed written consent was obtained from each of the three normal human male volunteers prior to participation.

Table II—Recovery of Ibufenac from Human Serum [a]

Added, μg/ml	Found, μg/ml	Recovery, %
5.0	4.44	88.8
10.0	9.32	93.2
20.0	21.56	107.8
30.0	34.11	113.7
40.0	45.49	113.7
50.0	57.31	114.6
60.0	68.20	113.7
	Mean ± *SD*	106.5 ± 10.9

[a] As compared to simple aqueous solution.

Synthesis of standard material showed that the pentafluorobenzyl ester of I was a white crystalline material at room temperature, mp 32–34°; IR (mineral oil): 1745, 1655, 1525, 1510, 1165, 1125, 1055, and 940 cm^{-1}; NMR ($CDCl_3$): δ 0.95 (d, 6H), 1.50 (d, 3H), 1.90 (m, 1H), 2.45 (d, 2H), 3.70 (q, 1H), 5.2 (s, 2H), and 7.0–7.3 (m, 4H) ppm; mass spectrum: *m/e* 386 (M^+), 343 ($M^+ - 43$), 181, 162, 161, 119, 118, and 117.

Anal.—Calc. for $C_{20}H_{19}F_5O_2$: C, 62.17; H, 4.96; F, 25.36. Found: C, 62.44; H, 5.05; F, 24.38.

The pentafluorobenzyl ester of II was isolated as white crystals, mp 48–50°; IR (mineral oil): 1745, 1660, 1525, 1510, 1170, 1050, 935, and 925 cm^{-1}; NMR ($CDCl_3$): δ 0.90 (d, 6H), 1.85 (m, 1H), 2.4 (d, 2H), 3.6 (s, 2H), 5.2 (s, 2H), and 7.0–7.2 (m, 4H) ppm; mass spectrum: *m/e* 372 (M^+), 329 ($M^+ - 43$), 181, 147, 105, and 104.

Anal.—Calc. for $C_{19}H_{17}F_5O_2$: C, 61.29; H, 4.61; F, 25.51. Found: C, 61.35; H, 4.61; F, 26.04.

IR and mass spectrometric analyses, before and after GLC, supported the proposed structures and confirmed that the pentafluorobenzyl esters of I and II chromatographed as the intact molecules.

Assay Sensitivity and Specificity—At an attenuator setting of 32 on the electron-capture linearizer, 7.2 ng of I as its pentafluorobenzyl ester produced a full-scale response. However, under the assay conditions, the lower limit of detection sensitivity for I in extracts of human serum is 0.1

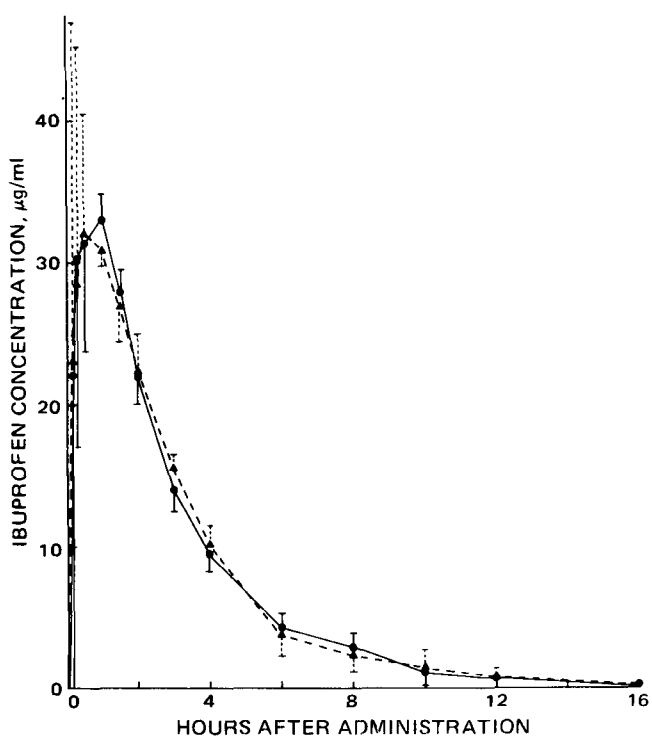


Figure 4—*Average* (±SD) *serum ibuprofen concentration* versus *time in humans* (n = 3) *after single-dose oral administration of 400 mg of drug in aqueous solution. Key:* ●—, *ibuprofen measured as methyl ester; and* ▲– –, *ibuprofen measured as pentafluorobenzyl ester.*

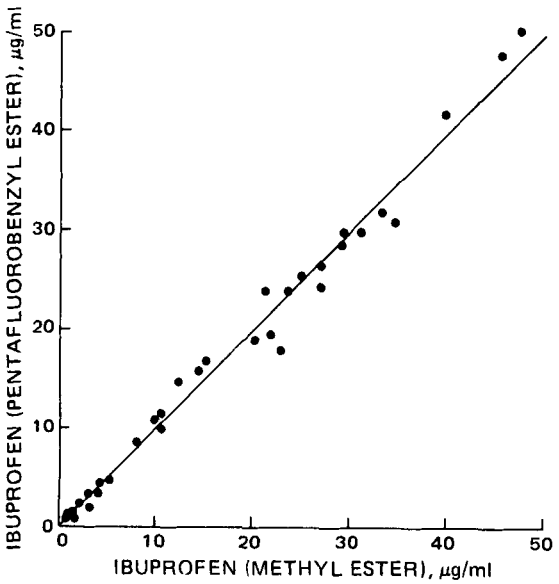


Figure 5—*Correlation of serum ibuprofen concentrations in drug-treated subjects when measured as the methyl ester (abscissa) and pentafluorobenzyl ester (ordinate). Least-squares regression analysis showed a slope of 0.999 and a correlation coefficient of 0.992.*

μg in 0.1–1.0 ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions described, a linear relationship between detector response and concentration is obtained for I over the range of 0–60 μg/ml. Quantification of serum specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay was identical to known I pentafluorobenzyl ester.

Recovery Experiments—Water or serum was added to known amounts of I and II in centrifuge tubes. The samples were mixed thoroughly and extracted with benzene. All extract residues were chromatographed on thin layers of silica gel 60 F_{254}, and the zones corresponding to I and II were eluted. The materials were esterified and analyzed *via* GLC. The results (Tables I and II) indicated that recoveries of I (97.7 ± 7.3%) and II (106.5 ± 10.9%) from serum were essentially quantitative as compared to simple aqueous samples.

Serum Levels of Ibuprofen in Humans—Results from the measurement of serum I concentrations in three normal human volunteers, after single-dose oral drug administration, demonstrated the utility of the method (Fig. 4). A peak mean (±*SD*) level of 31.9 ± 8.8 μg/ml was observed at 0.5 hr after drug administration, indicating rapid drug absorption from the aqueous solution. At 16 hr, serum drug concentrations were below the sensitivity level (*i.e.*, <0.1 μg/0.1 ml), indicating rapid drug disappearance from peripheral circulation. The serum drug disappearance half-life, as estimated from the average serum drug concentrations, was 1.94 hr. Analysis of the same samples, based on GLC of the I methyl ester (11), showed excellent agreement (Fig. 4) as evidenced by a slope of 0.999 and a correlation coefficient of 0.992 (Fig. 5).

In drug–protein binding studies, using equilibrium dialysis or ultrafiltration techniques, I concentrations on each side of the dialysis membrane were measured by GLC of the pentafluorobenzyl esters. The results, in excellent agreement with the *in vitro* studies of Mills *et al.* (5), showed that 99.7 ± 0.2% of the intact drug in peripheral circulation was bound to serum proteins during the 0–12-hr interval after drug administration. The combined results from these investigations showed that GLC of I, as the pentafluorobenzyl ester, could be used for evaluating drug–protein binding, pharmacokinetics, and drug availability from various dosage formulations with submilliliter amounts of human serum.

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