GLC–Mass Spectrometric Procedure with Selected-Ion Monitoring for Determination of Plasma Concentrations of Unlabeled and Labeled Barbital following Simultaneous Oral and Intravenous Administration

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Received September 26, 1979, from the **Department of Pharmacology, University of Montreal, Montreal, Quebec H3C 3J7, Canada*, and the ‡*Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario K1A 0L2, Canada.* Accepted for publication January 7, 1980. §Present address: College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada. †Deceased.

Abstract □ A GLC–mass spectrometric method employing specific-ion monitoring was developed for the determination of plasma concentrations of labeled ($^{15}N_{1,3}$, $^{13}C_2$) and unlabeled barbital following simultaneous intravenous and oral administration. This method proved to be more sensitive and precise than the method employing GLC with flame-ionization detection or GLC with alkali flame-ionization detection. After extraction of [$^{15}N_{1,3}$, $^{13}C_2$]barbital, barbital, and the internal standard, butalbital, from plasma with ether, the organic solvent is evaporated, and the labeled and unlabeled drug as well as the internal standard are converted into their *N,N*-dimethyl derivatives by treatment with diazomethane. The excess reagent is evaporated, and the resulting methyl derivatives are analyzed by GLC–mass spectrometry with selected-ion monitoring. The method is sufficiently sensitive to determine 0.5 μg of the labeled and unlabeled drug/ml with a relative standard deviation of <5%. The application of the method to the determination of the plasma concentration of labeled and unlabeled drug over 6 days following simultaneous oral and intravenous administration of a single dose is demonstrated.

Keyphrases □ Barbital—plasma concentrations of unlabeled and labeled drug following simultaneous oral and intravenous administration, GLC–mass spectrometric analysis □ GLC–mass spectrometry—analysis of plasma concentrations of unlabeled and labeled barbital following simultaneous oral and intravenous administration, specific-ion monitoring □ Pharmacokinetics—barbital, plasma concentrations of unlabeled and labeled drug following simultaneous oral and intravenous administration, GLC–mass spectrometric analysis

Barbiturates have been investigated extensively. GLC procedures employing flame-ionization detection have been preferred for the quantitative determination of barbiturates in biological fluids and tissues (1–5). GLC techniques using newer and more sensitive detectors than flame-ionization detectors, such as alkali flame-ionization detectors and electron-capture detectors, also have been explored (6, 7). Analysis of barbiturates by high-pressure liquid chromatography was reported (8). More recently, barbiturates have been analyzed by rapid and specific radioimmunoassay (9, 10) and by GLC–mass spectrometric procedures (11–13).

To study the effect of congestive heart failure on drug disposition, a procedure capable of simultaneously and separately quantitating the amounts of drug in plasma originating from oral as well as intravenous dosing was required. Simultaneous quantitation of the two fractions in the same plasma sample was essential because congestive heart failure is not easily stabilized and pharmacokinetic parameters may differ in successive studies.

Barbital was chosen as the model drug for this study since it is not metabolized; the determination of pharmacokinetic parameters therefore is simplified. Stable isotopically labeled barbital was given intravenously at the same time that unlabeled barbital was administered orally. A GLC–mass spectrometric method employing a specific-ion monitoring technique was developed for the simultaneous quantitation of barbital given by the two routes.

EXPERIMENTAL

Reagents—Barbital sodium[1], ether[2], hydrochloric acid[3], sodium sulfate[4], methanol[5], and butalbital[6] were used. [$^{15}N_{1,3}$, $^{13}C_2$]Barbital sodium[7] was specified to contain at least 90 atom % of carbon 13 and 95 atom % of nitrogen 15. Diazomethane was generated using a commercially available kit[8]. Plasma was obtained from the Red Cross Blood Bank.

Animals—Male Sprague–Dawley rats were assigned randomly to two groups. The first group (276 ± 2 g, n = 5) was administered 25 mg of barbital sodium/kg iv. The second group (271 ± 7 g, n = 5) was treated similarly with [$^{15}N_{1,3}$, $^{13}C_2$]barbital sodium. Two hours after treatment, rats were sacrificed by decapitation; their blood was collected in a heparinized[9] centrifuge tube (50 IU/ml of 0.9% NaCl solution). Plasma obtained after centrifugation for 20 min was frozen until it was assayed.

Standards—Stock solutions equivalent to 1 mg of the acid form/ml were prepared by dissolving the appropriate amounts of the sodium salts of both labeled and unlabeled barbital in methanol. Appropriate dilutions were prepared as required. Aliquots of the stock solution containing 1 mg/ml of butalbital in methanol were diluted before assay with 0.25 N HCl to obtain the working internal standard solution (10 μg/ml).

Extraction from Plasma—To 1 ml of a human or rat plasma sample (spiked or from dosed animals) in screw-capped[10] culture tubes was added 1 ml of the internal standard solution. The samples were mixed[11] and then extracted with 10 ml of anhydrous ether by mixing[12] for 10 min. After separation by centrifugation[13] for 10 min, the organic phase was transferred with a pipet and desiccated with anhydrous sodium sulfate. After further centrifugation for 10 min, the organic phase was decanted into an evaporating tube[14] and evaporated[15] to dryness at 50° under a dry nitrogen stream. The samples then were frozen until analysis.

Methylation was carried out immediately before the GLC–mass spectrometric analysis to avoid any breakdown and formation of side products. The dried extract was dissolved in 50 μl of methanol by mixing, and 1 ml of ethereal diazomethane was added. The sample was mixed and allowed to react at room temperature for 5 min. The excess diazomethane

[1] Reagent grade, BDH Chemicals, Toronto, Ontario, Canada.
[2] Diethyl ether (anhydrous), Baker Analyzed Reagent, Canlab, Montreal, Quebec, Canada.
[3] Baker Analyzed Reagent, Canlab, Montreal, Quebec, Canada.
[4] Anhydrous, Baker Analyzed Reagent, Canlab, Montreal, Quebec, Canada.
[5] Burdick & Jackson Laboratories, Muskegon, Mich.
[6] Provided by Sandoz Pharmaceuticals, Dorval, Quebec, Canada.
[7] Merck Sharp and Dohme Canada Ltd., Montreal, Quebec, Canada.
[8] Diazald, Aldrich Chemical Co., Milwaukee, Wis.
[9] Heparin sodium, Fisher Scientific Co., Montreal, Quebec, Canada.
[10] Kimak with Teflon liner.
[11] Vortex Genie, Fisher Scientific Co., Montreal, Quebec, Canada.
[12] Eberbach reciprocating shaker, Ann Arbor, Mich.
[13] IEC International Centrifuge, Boston, Mass.
[14] Custom made, Montreal Glass Blowing Registered, Montreal, Quebec, Canada.
[15] Thermolyne Dri Bath, Thermolyne Corp., Dubuque, Iowa.

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Table I—Recovery of Barbital and [$^{15}N_{1,3}$, $^{13}C_2$]Barbital from Plasma Determined by the GLC–Mass Spectrometric Assay

Micrograms Added[a] to 1 ml of Plasma	Mean Micrograms Recovered	Mean Percent Recovery ± SD
Barbital		
0.3	0.302	100.56 ± 5.16
4.0	3.36	84.00 ± 3.56
10.0	8.50	84.97 ± 1.47
Mean		89.84 ± 8.61
[$^{15}N_{1,3}$, $^{13}C_2$]Barbital		
0.5	0.560	112.08 ± 2.50
2.0	1.90	95.21 ± 6.19
10.0	8.98	89.80 ± 2.74
Mean		99.00 ± 10.56

[a] $n = 4$.

was removed by evaporation at 50°, leaving a methanolic solution that was injected in aliquots of 1-2 µl into the gas–liquid chromatograph.

GLC with Flame-Ionization Detection—The gas-liquid chromatograph[16] was equipped with a flame-ionization detector. The column was coiled glass tubing, 1.83 m long × 2 mm i.d., packed with 3% phenylmethylsilicone[17] on acid-washed, dimethylchlorosilane-treated, flux-calcined diatomite support[18] (80–100 mesh). The operating temperatures were: injection port, 250°; column, 225°; and detector, 275°. The carrier gas was nitrogen at a 35-ml/min flow rate. The hydrogen and compressed air flow rates were adjusted to give the maximum response. Pentobarbital[19] (30 µg/ml of plasma) was the internal standard. Flash-heater methylation was carried out with trimethylanilinium hydroxide in methanol[20].

GLC with Alkali Flame-Ionization Detection—The gas-liquid chromatograph[16] was equipped with an alkali flame-ionization detector. The operating temperatures were: injection port, 275°; column, 170°; and detector, 300°. The internal standard was aminopyrine[21] (10 µg/ml of plasma). The column was the same as that described for the method with flame-ionization detection.

GLC-Mass Spectrometry—The gas-liquid chromatograph–mass spectrometer[22] was operated in the electron-impact mode with 70 ev as the ionization beam energy. The source was maintained at 200°. The chromatographic column was a coiled glass tube, 1.22 m long × 2 mm i.d., packed with 3% cyanopropylmethyl phenylmethylsilicone[23] on acid-washed, dimethylchlorosilane-treated, flux-calcined diatomite support[24] (100–120 mesh). The injection port, the column, and the interface temperatures were 250, 150, and 275°, respectively. The carrier gas was helium at a 35-ml/min flow rate.

Reference Samples for Recovery—The acid form of barbital was obtained by successive extractions of an acidified aqueous solution of barbital sodium with ether. The combined extracts were evaporated and dried at 80° overnight. The reference solution of barbital (1 mg/ml) was prepared by dissolving appropriate amounts of the drug in methanol. Different volumes of this solution were added to each evaporating tube to obtain four solutions at each of three concentrations (Table I). The external standard (10 µg of butalbital) and 10 ml of anhydrous ether then were added. The mixture was evaporated to dryness at 55° under a dry nitrogen stream. Methylation was carried out as described, and aliquots (1-2 µl) were injected into the gas-liquid chromatograph–mass spectrometer. The mean of the peak area ratio thus obtained was designated as the 100% value.

For the recovery experiments for barbital sodium, amounts equivalent to the acid form added to plasma (1.0 ml) were extracted with ether as described. Then 10 µg of butalbital was added to the organic extract and the procedure was followed. The recovery of barbital was calculated from the peak ratio thus obtained (mean ± *SD*) compared to the value designated as 100%. The same procedure was followed for the recovery of [$^{15}N_{1,3}$, $^{13}C_2$]barbital sodium.

Calibration Curve—Calibration curves were assembled from the

[16] Model 5830A, Hewlett-Packard, Montreal, Quebec, Canada.
[17] OV-17, Chromatographic Specialties, Brockville, Ontario, Canada.
[18] Gas Chrom Q, Chromatographic Specialties, Brockville, Ontario, Canada.
[19] Abbott Laboratories Ltd., Montreal, Quebec, Canada.
[20] Methelute, Chromatographic Specialties, Brockville, Ontario, Canada.
[21] Casgrain et Charbonneau, Montreal, Quebec, Canada.
[22] Model 5985 data system, Hewlett-Packard, Avondale, Pa.
[23] OV-225, Chromatographic Specialties, Brockville, Ontario, Canada.
[24] Chromosorb W (HP), Chromatographic Specialties, Brockville, Ontario, Canada.

Table II—GLC–Mass Spectrometric Estimation of Barbital Added to Plasma

Barbital[a] Added, µg	n	Mean Peak Area Ratio ± SD	CV, %
0.5	6	0.059 ± 0.003	4.55
2.0	5	0.252 ± 0.010	3.84
4.0	5	0.445 ± 0.019	4.31
6.0	5	0.660 ± 0.037	5.59
10.0	6	1.081 ± 0.038	3.49

[a] Mean *CV* was 4.36% for $y = mx$, where $m = 0.109 \pm 0.002$; $r^2 = 0.998$.

Table III—GLC–Mass Spectrometric Estimation of [$^{15}N_{1,3}$, $^{13}C_2$]Barbital Added to Plasma

[$^{15}N_{1,3}$, $^{13}C_2$]-Barbital[a] Added, µg	n	Mean Peak Area Ratio ± SD	CV, %
0.3	6	0.030 ± 0.001	4.71
1.0	5	0.160 ± 0.004	4.08
2.0	5	0.197 ± 0.008	4.06
3.0	6	0.299 ± 0.010	3.23
6.0	6	0.590 ± 0.015	2.57

[a] Mean *CV* was 3.73% for $y = mx$, where $m = 0.099 \pm 0.001$; $r^2 = 0.999$.

results of spiked control plasma by plotting the peak area ratios *versus* the concentration of the drug (Tables II and III). Peak area ratios were calculated by dividing the peak area under each ion investigated (*e.g.*, *m/e* 169 for barbital and *m/e* 172 for labeled barbital) by the peak area under the ion (*m/e* 196) monitored for the internal standard.

RESULTS AND DISCUSSION

Methylation of barbital with diazomethane gave a major peak (99% by the peak area on the total-ion monitor) when it was analyzed under the described conditions. GLC–mass spectrometric analysis of the eluted compound did not show a molecular ion (*m/e* 212); however, other diagnostic ions at *m/e* 184, 183, 169, 126, and 112 supported identification of the compound as N_1,N_3-dimethylbarbital. Similarly, methylation of [$^{15}N_{1,3}$, $^{13}C_2$]barbital gave a peak with the same retention time (1.1 min) with major ions at *m/e* 187, 186, 172, 127, and 113 (Fig. 1). The ions were consistent with the fragmentation pattern reported (14) for N_1,N_3-dimethylbarbital. The isotopic purity of the labeled compound is demonstrated by the absence of an ion at *m/e* 169 (Fig. 1).

Mass spectral analysis of the methylated internal standard, butalbital, suggested that the N_1,N_3-dimethylated derivative was formed (99% by the peak area). The mass spectrum showed no molecular ion; however, characteristic ions at *m/e* 209, 196, 195, 181, and 74 were consistent with identification of the compound as the N_1,N_3-dimethyl derivative.

The use of diazomethane as a suitable methylating agent for barbiturates was reported previously (15). Dried extracts of samples are stable for periods of months when kept at −20°. However, when diazomethane is added to them, analysis must be completed within 12 hr. In preliminary studies, flash-heater methylation with the safer reagent, trimethylanilinium hydroxide, was tried; however, because of the decomposition of the barbital derivative, it was abandoned. Diazomethane methylation of barbital and bultalbital gave the desired N_1,N_3-dimethyl derivative of the compounds with negligible amounts of by-products (methyl enol ethers).

Single-ion chromatograms obtained after methylation of a reference sample containing [$^{15}N_{1,3}$, $^{13}C_2$]barbital and butalbital in methanol are

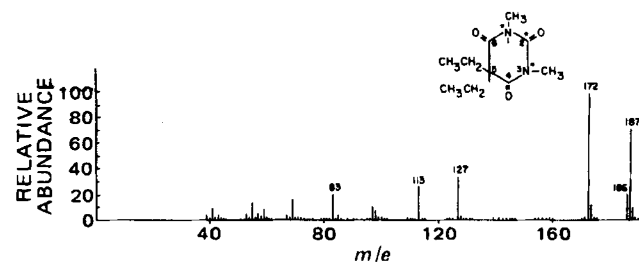

Figure 1—*GLC–mass spectrometry (normalized) of diazomethane-methylated [$^{15}N_{1,3}$, $^{13}C_2$]barbital.*

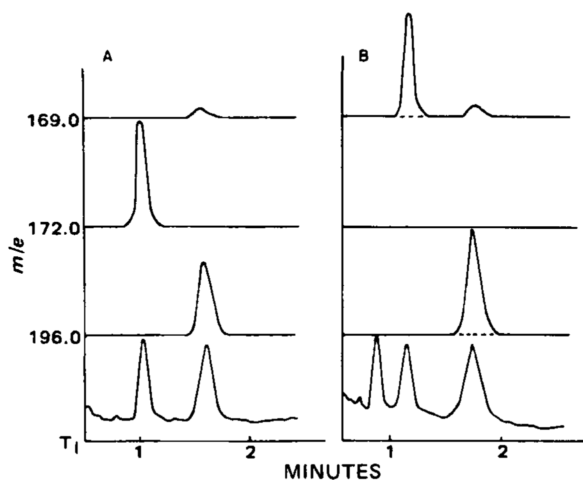

Figure 2—*Typical selective-ion and total-ion chromatograms from the assay of barbital and labeled barbital. Key: A, no plasma; and B, 2-hr plasma sample from a barbital-treated rat. The ions at* m/e *169, 172, and 196 represent barbital, [$^{15}N_{1,3}$, $^{13}C_2$]barbital, and the internal standard, butalbital, respectively.*

shown in Fig. 2*a*. The isotopic purity of the labeled compound is established further by the absence of a signal due to the *m/e* 169 ion (relative abundance <0.2%). Figure 2*b* shows a typical single-ion chromatogram obtained from a 2-hr plasma sample of a barbital-treated rat. The plasma had to be diluted with Red Cross Blood Bank plasma to fit the peak in the linear portion of the detector response curve. To rule out the possibility of any difference between the plasma disposition of barbital and [$^{15}N_{1,3}$, $^{13}C_2$]barbital, two groups of rats were treated with each drug respectively as described under *Experimental*. Comparison of 2-hr plasma concentrations in treated rats is illustrated in Table IV. Results are expressed as the mean for five rats. Results were compared using the Student *t* test, and no statistical difference could be found. Therefore, it was concluded that there was no isotopic effect in the plasma disposition of [$^{15}N_{1,3}$, $^{13}C_2$]barbital sodium in rats.

The overall recoveries of barbital and labeled barbital from plasma are summarized in Table I. Mean percent recoveries of 89 and 99% were considered satisfactory. Recoveries of slightly over 100% sometimes were observed at very low concentrations. This artifact probably is due to background noise.

The accuracy and precision of the GLC–mass spectrometric assay are demonstrated in Tables II and III. Results from at least five determinations of each barbital concentration, ranging from 0.3 to 10 μg/ml of plasma, were obtained as described under *Experimental*. The overall coefficients of variation for barbital and [$^{15}N_{1,3}$, $^{13}C_2$]barbital were 4.36 and 3.73%, respectively. The GLC–mass spectrometric response was linear for both compounds within this concentration range. The detection limit was 1 ng on-column (3× noise). The calibration curves for barbital and labeled barbital were straight lines with an intercept of 0.020 and 0.003, respectively, when they were not forced through the origin. Mean slopes of 0.109 ± 0.002 and 0.099 ± 0.001 for the two compounds were obtained when linear regression without the intercept program was applied.

The sensitivity of the GLC–mass spectrometric detection using single-ion monitoring was compared against GLC with flame-ionization detection and GLC with alkali flame-ionization detection when the same

Table IV—Comparison of 2-hr Plasma Concentrations in Treated Rats

Compound	Plasma Concentration, μg/ml	Mean ± *SD*
[$^{15}N_{1,3}$, $^{13}C_2$]Barbital sodium	25.03	
	24.81	
	24.04	25.13 ± 0.88
	26.47	
	25.29	
Barbital sodium	24.95	
	24.42	
	24.13	24.81 ± 0.61
	25.73	
	24.82	

Table V—Estimation of Barbital Added to Plasma by GLC with Flame-Ionization Detection

Barbital[a] Added, μg	*n*	Mean Peak Area Ratio ± *SD*	*CV*, %
5.0	12	0.13 ± 0.015	11.8
8.0	14	0.227 ± 0.031	13.7
15.0	8	0.414 ± 0.012	2.83
30.0	12	0.765 ± 0.016	2.09
50.0	10	1.309 ± 0.036	2.77

[a] Mean *CV* was 6.64% with $y = mx + b$, where $m = 0.025 \pm 0.012$ and $b = 0.027$; $r^2 = 0.995$.

Table VI—Estimation of Barbital Added to Plasma by GLC with Alkali Flame-Ionization Detection

Barbital[a] Added, μg	*n*	Mean Peak Area Ratio ± *SD*	*CV*, %
1.0	6	0.132 ± 0.024	18.41
5.0	8	0.543 ± 0.036	6.78
10.0	8	1.028 ± 0.059	5.71
20.0	6	2.168 ± 0.087	3.87
30.0	8	3.257 ± 0.212	6.53

[a] Mean *CV* was 8.26% with $y = mx + b$, where $m = 0.107 \pm 0.050$ and $b = 0.008$; $r^2 = 0.993$.

extraction procedure was employed (see *Experimental*). As shown in Tables V and VI, both methods are applicable when plasma barbital concentrations of >5 μg/ml are expected. However, the GLC–mass spectrometric method offers the greatest sensitivity and precision and is more suited to quantitation in the therapeutic range, *e.g.*, <5 μg/ml.

Figure 3*a* shows selective-ion and total-ion chromatograms obtained from control plasma processed as described under *Experimental* but with the internal standard omitted. Most of the endogenous plasma constituents were eluted at 0.86 min. No extraneous peaks that interfered with the selected ions for labeled (*m/e* 172) and unlabeled (*m/e* 169) barbital or butalbital (*m/e* 196) were observed over 20 min (2 min shown). Figure 3*b* shows typical selective-ion and total-ion chromatograms from a 2-hr plasma sample collected from a human volunteer treated with [$^{15}N_{1,3}$, $^{13}C_2$]barbital sodium intravenously and barbital sodium orally at the same time. The signals at *m/e* 169 and 172 represent 7.62 μg of unlabeled barbital/ml and 2.51 μg of labeled barbital/ml, respectively.

Application of the GLC–mass spectrometric procedure to the determination of plasma levels of both labeled and unlabeled barbital is shown in Fig. 4. A dose of 1.5 mg of [$^{15}N_{1,3}$, $^{13}C_2$]barbital sodium/kg was injected intravenously (within 20 sec) into a healthy female volunteer (65 kg); at the same time, she swallowed a capsule containing 4.5 mg of barbital

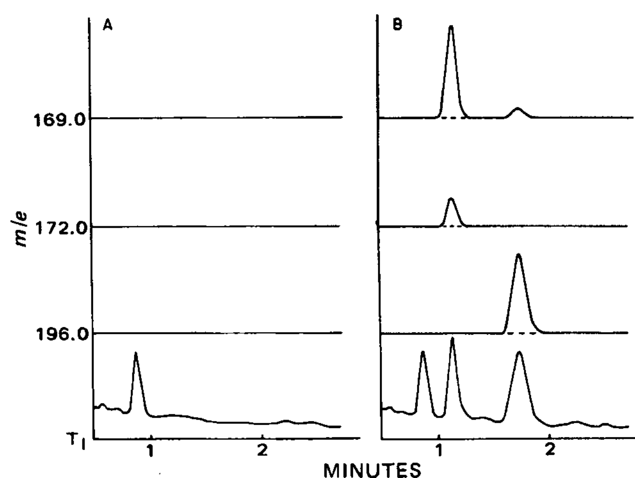

Figure 3—*Typical selective-ion and total-ion chromatograms from the assay of barbital and labeled barbital. Key: A, plasma blank; and B, 2-hr plasma sample from the same volunteer (65 kg) who received [$^{15}N_{1,3}$, $^{13}C_2$]barbital sodium intravenously and barbital sodium orally at the same time. The signals at* m/e *169 and 172 represent 7.62 μg of barbital/ml and 2.51 μg of labeled barbital/ml, respectively. The signal at* m/e *196 is that for the internal standard, butalbital.*

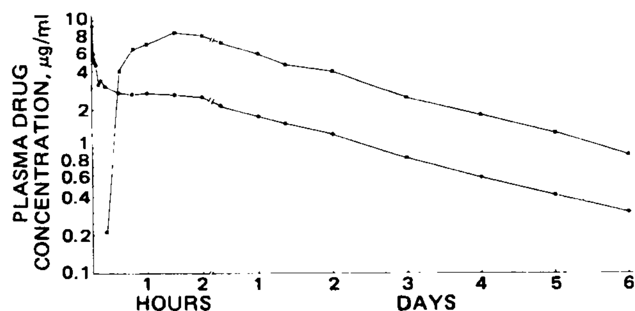

Figure 4—*Barbital (■) and labeled barbital (●) concentrations in the plasma of a female volunteer (65 kg) who swallowed a capsule containing 4.5 mg of barbital sodium/kg and simultaneously received 1.5 mg of* [$^{15}N_{1,3}$, $^{13}C_2$]*barbital sodium/kg iv (within 20 sec).*

sodium/kg. Blood (7 ml) was withdrawn at intervals over 6 days, and aliquots of plasma (1.0 ml) were assayed for barbital. Concentration–time curves of both isotopes in plasma are illustrated in Fig. 4, and the parallelism observed between their elimination phases strongly supports the evidence that both isotopes behave similarly in the organism.

In conclusion, the described GLC–mass spectrometric procedure is sensitive and specific and should be applicable to studies on the effect of congestive heart failure on barbital disposition.

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Permeability of Everted Rat Small Intestine to Lidocaine and Derivatives

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Received June 11, 1979, from the **College of Pharmacy, South Dakota State University, Brookings, SD 57006,* and the *‡Department of Pharmaceutics, University of Houston, Houston, TX 77004.* Accepted for publication January 8, 1980.

Abstract ☐ The transfer rates of lidocaine and five derivatives were measured across the everted rat intestine. No obvious relationship was observed between the first-order rate constants for transfer and the lipophilicity of the compounds as measured by their apparent partition coefficients. The intestinal mucosal epithelium also did not appear to be the rate-limiting barrier for the passage of these agents across the intestinal membrane regardless of the respective clearance of the compound. It is suggested that the ionized form of these agents may be capable of crossing the intestinal barrier at substantial rates, although it is not known if a similar situation exists *in vivo*. The transfer rate is believed to be due to the passage of intact drug and not the metabolite.

Keyphrases ☐ Lidocaine—determination of transfer and clearance rates of parent drug and analogs across everted rat intestinal mucosa, mucosal concentrations and pH, *in vitro* absorption ☐ Absorption—lidocaine and derivatives, determination of transfer rates across everted rat intestinal mucosa, *in vitro* ☐ Permeability—lidocaine and derivatives across everted rat intestinal mucosa, *in vitro* absorption

The *in vitro* everted rat small intestine is a technique that has been utilized as a biological screen for the assessment of drug permeability and may be indicative of *in vivo* absorption problems. Feldman *et al.* (1) found a rank-order agreement between the *in vitro* transfer of prednisolone across the everted rat small intestine in the presence of various substituted propionamides with that reported for *in situ* experiments in the rat. Kaplan and Cotler (2) used the everted rat intestine as a biological screen to assess permeability characteristics of numerous compounds and concluded that this technique, employed along with a dissolution screen, allows evaluation of potential problems in the initial stages of product development.

The nature of the mechanical separation of the intestine from the intact animal imparts inherent advantages and disadvantages to the technique. Separation enables the investigator to control the physical environment of the intestinal preparation and to study some effects of the physicochemical properties of the drugs on the transfer of these agents across the intestinal preparation. However, separation of the intestinal preparation may alter or obliterate some important influencing factors present in the absorption of a compound *in vivo*. Concern has been expressed over viability (2–5), structural integrity (6), and the presence of unnatural absorption barriers.

This study concerned the transfer rates of lidocaine (I) and five experimental derivatives across the everted rat intestine. These compounds were chosen because they represent minor molecular modifications of the parent

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