chromatographed as a single peak with the same retention time as that observed by on-column methylation of phenobarbital; its mass spectrum was consistent with that previously reported (13). Compound I was prepared by the method of Brochmann-Hanssen and Oke (2), and deuterated I was prepared in a similar manner using methyl iodide-d_3[5]. A 1.8 *M* solution of I was obtained from a commercial source[6].

2-Phenylbutyryl chloride was prepared by refluxing 10 g of 2-phenylbutyric acid[7] and 15 g of thionyl chloride for 30 min and then distilling off the excess thionyl chloride. The acid chloride was divided into three portions. Compounds V and VI were prepared by reacting portions of the 2-phenylbutyryl chloride with 20 g of methylamide hydrochloride and 25 g of dimethylamine hydrochloride, respectively, which had been previously adjusted to about pH 12. The reaction mixture was allowed to stand overnight, during which time the crude products separated. The mixture was again made alkaline and extracted with ether. The extract was dried with anhydrous sodium sulfate, and the ether was evaporated. Compound V was obtained as a solid, which was purified by treatment with charcoal[8] in ethanol. Then the ethanol was evaporated, and the compound was recrystallized from hexane, mp 97° [lit. (14) mp 95–96°]. Compound VI was obtained as a viscous liquid, which was purified by repeated treatment with charcoal in ethanol.

Compound VII was prepared by refluxing 5 g of 2-phenylbutyric acid, 10 ml of methanol, and 0.2 ml of concentrated sulfuric acid for 4 hr. Then the cooled reaction mixture was poured into 25 ml of water and saturated with sodium bicarbonate. The alkaline solution was extracted with ether and dried overnight with anhydrous sodium sulfate, the ether was evaporated, and the ester was distilled, bp 225° [lit. (15) bp 228°].

Methods—GLC was performed on a 1.82-m × 3.2-mm o.d. glass column packed with 3% OV-17 on Chromosorb WHP[9]. The carrier gas was helium at a flow rate of 30 ml/min, the injector temperature was 250°, and the oven temperature was either maintained at 220° or programmed from 90 to 260° at 10°/min during the chromatography. A flame-ionization detector was used.

In the appropriate experiments, a portion of the effluent was passed through a heated glass jet separator into a mass spectrometer[10] operating in the electron-impact mode with an ionizing voltage of 70 ev. A mass range of 41–500 was automatically scanned at 2 sec/decade approximately every 5 sec during the elution of peaks. Normalized mass spectra of compounds included all ions within the mass range scanned with intensity greater than 5% of the base peak.

[5] Merck Sharp and Dohme Isotope Division.
[6] Supelco.
[7] Aldrich Chemical Co.
[8] Norit-A, Fisher Scientific Co.
[9] Applied Science Laboratories.
[10] DuPont 21-490 interfaced with a DuPont 21-094 data system.

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Use of Pharmacological Data for Bioavailability and Pharmacokinetic Analyses

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Abstract □ The use of pharmacological responses such as pupil diameter for dosage individualization, bioavailability, and pharmacokinetic analyses is becoming more widespread. Attempts to use pupil diameter to assess morphine bioavailability illuminate the fact that multiple responses, nonlinearities, and the condition of the subject can produce misleading results unless the applicability of the method is confirmed.

Keyphrases □ Pharmacological response data—use in bioavailability and pharmacokinetic analyses, situations producing misleading results determined □ Bioavailability—analyses based on pharmacological response data, situations producing misleading results determined □ Pharmacokinetics—analyses based on pharmacological response data, situations producing misleading results determined □ Pupil diameter—measurements used to assess morphine bioavailability □ Morphine—bioavailability analyzed using pupil diameter measurements

Several recent publications underscored the utility of pharmacological response data for performing drug bioavailability and pharmacokinetic analyses (1–3). The theoretical foundation and limits of applicability of the approach were described in detail (2, 4), and this approach was applied successfully to several systems that would have been difficult or impossible to analyze by conventional means (5, 6). An obvious necessity for using pharmacological data is the existence of a clearly defined, measurable, graded response to the administered drug, but the availability and quantitation of such responses are often cited (7) as limiting factors in applying the method.

The objective of this study is to determine the usefulness of pharmacological methods for analyzing the bioavail-

Table I—Miotic Response of Rabbits to Intravenous Doses of Morphine Sulfate[a]

Rabbit Number	Dose, mg/kg	I_{max}[b]	t_{max}[c], min	Duration of Action $I > 0.1$, min	Total Morphine Plasma Level at End of Effect ($I = 0.1$), μg/ml
1	5	0.58	3	180	2.0
2	5	0.59	1	240	0.35
3	7.5	0.52	1	17	6.5
4	4	0	—	—	6.0[d]
5	6	0	—	—	13.2[d]
6	6	0	—	—	20.0[d]
7	5	0.32	1	120	3.0
8	5	0	—	—	8.0[d]
9	5	0.40	1.5	120	3.6

[a] Using 50 mg/ml in normal saline. [b] Maximum miotic response intensity defined by $(D_0 - D_{t_{max}})/D_0$, where D_0 = baseline pupillary diameter and $D_{t_{max}}$ = diameter at time of maximum miosis. [c] Time of occurrence of maximum miotic response intensity. [d] Measured 30 min after dosing by radioimmunoassay.

ability and pharmacokinetics of narcotics or narcotic antagonists. Narcotics such as morphine and heroin produce pupillary diameter changes that can be quantitated accurately, and the abolition of these changes by narcotic antagonists might also be interpreted as a pharmacological effect and used to assess the bioavailability and absorption characteristics of these agents. The steady-state miotic response to morphine could, for example, be utilized to determine the rate and extent of bioavailability of naloxone from a sustained-release device by analysis of the antagonist's ability to abolish these miotic effects.

Besides a graded pharmacological response, another criterion for using the method is that the rate-limiting process in the production of that response be the drug's ability to gain access to the biophase. All processes must also be entirely reversible, *i.e.*, nonhysteretic and the instantaneous result of a continuous equilibrium between free and receptor-bound drug, and the biokinetic behavior of the drug must be linear. As pointed out previously: "It is not generally sufficient to assume arbitrarily that these conditions are met for any given drug and biological system" (4).

Preliminary results with morphine in rabbits are reported here to underscore the importance of testing these assumptions before using a dose–effect curve to perform pharmacokinetic analyses.

EXPERIMENTAL

Pupillometry—Male New Zealand White rabbits, 3.0–4.0 kg, were fasted overnight and placed in a rabbit restrainer consisting of a rectangular metal box with a neck ring through which the rabbit's head could extend. An IR illuminator provided a constant low level of background light for accentuating the miotic effects of morphine and facilitated drug administration and plasma sampling. All rabbits were acclimated to the experimental setup by putting them through mock experiments for several weeks.

A closed circuit TV camera[1], equipped with an IR light source and a telephoto lens[2], was positioned about 17 cm from the rabbit's right eye and connected to a 22.9-cm television monitor[3]. The near IR light used did not affect pupillary diameter. The camera was set at a fixed focal length and was not altered during the measurements of pupillary diameter. Head movements, which were infrequent, were corrected for by repositioning the camera so that the pupil was returned to perfect focus.

Pupillary diameters were calibrated to the TV screen image by measuring with calipers the TV image size of a series of dots of known diameter located at the position of the rabbit's eye. Pupil diameters were then measured with the calipers on the TV screen and converted to actual diameters using the calibration curve. When a rabbit pupil was not a perfect circle, all measurements were made vertically through the center point of the pupil. Pupil diameters at any given time were measured four times, and the average value was recorded.

Drug Administration—A 23-gauge butterfly infusion set was placed in the marginal vein of one ear. After a brief acclimation, pupillary diameters were measured in the absence of drug to obtain baseline miotic data. Morphine sulfate was then administered in 0.9% NaCl either as a bolus dose or with an infusion pump[4]. Saline solutions buffered to the pH (4.8) of morphine sulfate did not produce a miotic effect when injected into the ear vein as a control.

Plasma Sampling and Radioimmunoassay—Blood samples (100 μl) were collected in heparinized capillary tubes through a small cut in the marginal vein of the opposite ear. The cut was made prior to the experiment and reopened by gentle rubbing to minimize discomfort. Plasma was obtained by centrifugation and was assayed using a commercially available tritiated morphine radioimmunoassay kit[5]. Dialysis studies showed that the procedure measured both free and plasma protein-bound morphine. However, at the high plasma concentrations found in this study during miosis (1–20 μg/ml), a negligible fraction (less than 3%) of the total morphine present was protein bound.

RESULTS

Table I lists the miotic responses of nine rabbits to intravenous doses of morphine sulfate. The intensity of response, I, was taken to be the fractional reduction in pupil diameter at any given time after dosing. Large intrasubject variability was observed, with four rabbits showing substantial responses, four not responding, and one exhibiting a transient response. Plasma morphine levels in nonresponders verified that high plasma levels often did not produce miosis.

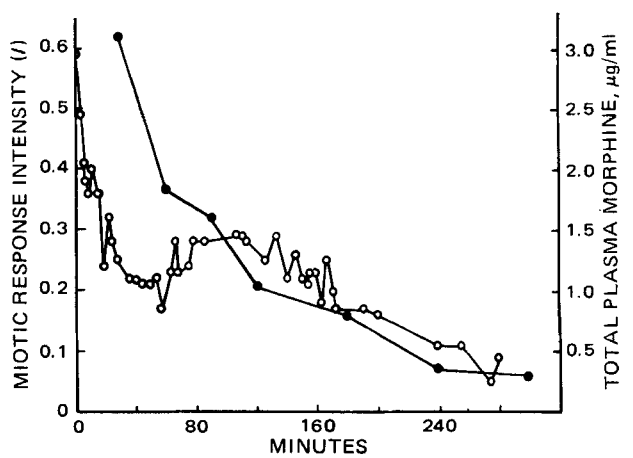

Figure 1—*Typical plot of miotic response intensity (I) as a function of time in a responding rabbit (Rabbit 2) after intravenous administration of a 5-mg/kg dose of morphine sulfate. Each response is the average of four determinations.*

[1] Shibaden model HV40SU, Nucleus Video Co., Indianapolis, Ind.
[2] Wollensack Reptar 7.62 cm f/2.5.
[3] Sony.
[4] Harvard dual syringe, model 2681, Millis, Mass.
[5] Roche Diagnostics, Nutley, N.J.

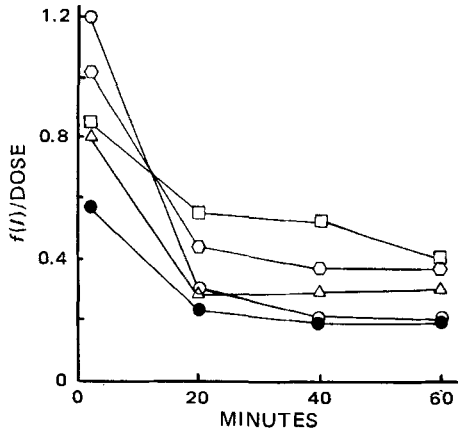

Figure 2—*Congruency test on dose-normalized relative quantities of drug in the biophase, f(I). Each value is the average of four replications on the rabbit that provided the dose–effect data (Fig. 3) used to determine f(I). Doses were 2.0 (□), 3.0 (○), 4.0 (△), 5.0 (○), and 6.0 (●) mg/kg. Curves would be superimposable if system were linear.*

Plots of the time course of I in responding rabbits (Fig. 1) consisted of three phases: an initial rapid decline, a plateau where response was independent of time, and a final slow decline. The relative quantities of drug in the biophase, $f(I)$, at various times were computed from these data (4) and normalized for dose. Plots of dose-normalized values of $f(I)$ *versus* time (Fig. 2) for various intravenous doses are not superimposable as they would be if the system were linear. Therefore, this "congruency test" (4) indicates that the use of a linear compartment model to describe the data is inappropriate. Failure to recognize this nonlinearity can lead to erroneous predictions of expected responses in multiple dose or infusion therapy (see *Discussion*).

When the maximum response intensities (occurring 1–3 min after administration) were plotted as a function of dose, a hyperbolic dose–effect curve was obtained (Fig. 3). The fact that a parallel curve was not obtained when intensities were chosen at times other than t_{max} (such as 30 min) indicated that the response to the drug was hysteretic and not solely the instantaneous result of a continuous equilibrium between free and receptor-bound drug at the site of action ["function of I ratio test" (4)]. Identical challenge doses of morphine were administered to a responding rabbit 1 week after each experiment that contributed to the dose–effect curve (Fig. 3) over several months. No alteration in the miotic response to the challenge dose was observed, indicating that the development of tolerance to morphine did not affect the dose–response relationship.

DISCUSSION

While the rabbit may not be the ideal species for studying the miotic effects of morphine because of the large number of nonresponders, researchers are not totally free to select an appropriate species for many applications; the effective utilization of pharmacological response data ultimately depends on measurements made in a single species, humans.

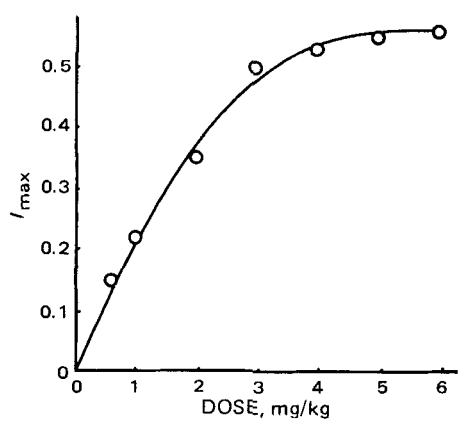

Figure 3—*Intravenous dose–response curve for morphine sulfate in a responding rabbit (Rabbit 2). Response was taken at time of maximum miotic effect (1–3 min), and each response is the average of four determinations. Parallel curves are not obtained using I values obtained at longer times (such as 30 min) after dosing.*

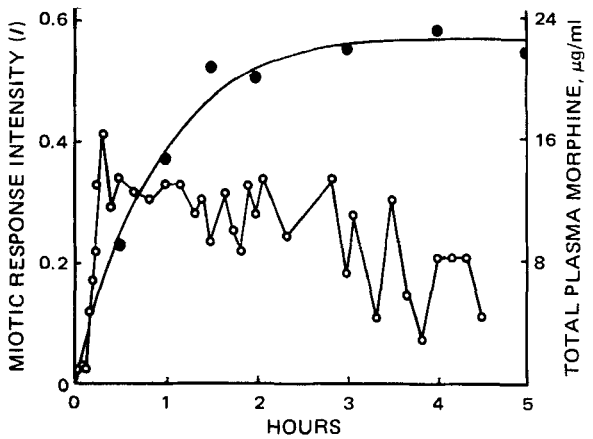

Figure 4—*Miotic response intensity (I) and plasma total morphine levels during an intravenous infusion of morphine sulfate (48 mg/hr) into a responding rabbit (Rabbit 2). Each response is the average of four determinations.*

The presented results are indicative of the potential pitfall one may encounter when a substantial number of nonresponding subjects are encountered for a given drug in a given species. While careful selection of responding subjects or averaging of results over a large number of subjects might prove adequate for some applications such as the determination of bioavailability, dosage individualization using pharmacological responses and attendant calculations specific to a particular subject may not be possible in a substantial fraction of a given subject population.

Even when graded responses are observed in an individual subject, one could easily obtain a dose–effect curve like that shown in Fig. 3, fail to check if the required conditions were met before applying the pharmacological method, and proceed to predict erroneously the outcome of subsequent drug administrations. Figure 4 shows measured plasma total morphine levels during intravenous drug infusion and the miotic response that resulted in a single responding rabbit. This analysis could serve as a model for dosage individualization in a single human or as a check on the validity of using miotic response data to determine the bioavailability from a zero-order sustained-release dosage form.

The responses to the intravenous bolus doses were nonlinear, and one would not expect the miotic response data to be useful in calculating the cumulative amount of drug entering the bloodstream for comparison with what was actually administered. The data confirm that this was indeed the case. The peak miotic responses (Fig. 4) occurred much earlier than peak plasma total morphine levels, and the miotic response to the zero-order infusion never really plateaued but rather continuously diminished with time. Kinukawa (8) also was unable to observe plateau miotic responses during infusions in rabbits.

One must also be aware of factors that may confound the pharmacological response of interest. These factors can render certain pharmacological response baselines so "noisy" that the use of the technique for dosage individualization may be jeopardized. Factors of potential concern include the effects of coadministered drugs in the patient's overall therapy, physiological effects associated with the patient's disease state, and multiple pharmacological responses that are not independent but rather interact. The last factor is illustrated by these data in rabbits. Previous investigators observed a parallelism between morphine miosis and respiratory rate in the rabbit and showed that miosis increased as the carbon dioxide content of plasma rose (9). Thus, observation of the primary pharmacological effect (miosis) appears to be confounded by the depressant effects of the drug. Patients often take several drugs simultaneously, so similar effects in human subjects can be expected to complicate the practical application of pharmacological response methods.

Pharmacological methods of analysis are extremely powerful and hold great potential in systems where they can be quantitated accurately and applied properly. This preliminary study points out that investigators must proceed cautiously in selecting responses to be monitored.

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High-Pressure Liquid Chromatographic Analysis of Drugs in Biological Fluids II: Determination of an Antiarrhythmic Drug, Tocainide, as Its Dansyl Derivative Using a Fluorescence Detector

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Abstract □ A sensitive and specific method is described for the determination of a new primary aliphatic amine antiarrhythmic drug, tocainide. Tocainide, together with an internal standard, is selectively extracted from plasma or blood and reacted with dansyl chloride. The highly fluorescent dansyl derivatives are separated using high-pressure liquid chromatography and measured using a fluorescence detector. The method can measure therapeutic and subtherapeutic concentrations of the drug (0.1–5.0 μg/ml of plasma) with a standard deviation of less than 2%.

Keyphrases □ Tocainide—high-pressure liquid chromatographic analysis, plasma or blood □ High-pressure liquid chromatography—analysis, tocainide, plasma or blood □ Dansyl chloride—derivatizing reagent in high-pressure liquid chromatographic analysis of tocainide □ Antiarrhythmics—tocainide, high-pressure liquid chromatographic analysis, plasma or blood

Within the last 5 years, advances in high-pressure liquid chromatography (HPLC), the commercial availability of reliable HPLC equipment, and the development of efficient columns have resulted in the application of HPLC to many analytical problems. However, the application of HPLC to drug analysis has lagged behind the increase in its general use (1, 2). Many papers reported the chromatographic conditions required to separate drugs (3–7), but only a few described the quantitative application of HPLC to the analysis of drugs in dosage forms (8–11).

A major cause of the failure to apply HPLC to the analysis of drugs in biological fluids has been the lack of suitable detectors. UV absorption detectors have been commonly used in HPLC. The microgram or nanogram per milliliter concentrations of many drugs found in plasma after therapeutic doses have confined the use of HPLC analysis to drugs having high molar absorptivities at 254 or 280 nm, the wavelengths most frequently used in these detectors (12–18).

The derivatization of pesticides with reagents yielding fluorescent derivatives and their subsequent HPLC analysis have been reported (19–22). Many drugs unsuited for analysis with UV absorption or fluorescence detectors have primary or secondary amino, phenolic or alcoholic hydroxyl, or aldehyde and keto functions suitable for the formation of fluorescent derivatives. The ability of HPLC to resolve compounds with small differences in molecular structure permits derivatization prior to injection onto the column while still achieving adequate separation of the drug of interest from other molecules reacting with the reagent.

This paper reports the use of HPLC with a commercially available fluorescence detector to determine blood and plasma concentrations of a new primary aliphatic amine antiarrhythmic drug, tocainide [2-amino-*N*-(2,6-dimethylphenyl)propanamide], after derivatization with dansyl chloride [5-(dimethylamino)-1-naphthalenesulfonyl chloride]. The described techniques should be generally applicable to drugs containing derivatizable functional groups. The use of reagents such as dansyl chloride may result in the increased utilization of HPLC for drug analysis in biological fluids, in a manner similar to that which occurred with electron-capture detection in GLC when halogenated acylating and alkylating reagents became available.

EXPERIMENTAL

Reagents—Ethyl acetate[1], hexane[1], acetone[1], and carbon tetrachloride[1] were used as received. Hexane[2], dichloromethane[2], and methanol[2] were used as chromatographic solvents. Stock solutions of tocainide[3] were prepared in 0.05 *N* HCl over the range of 0.1–10 μg/100 μl. The internal standard [2-amino-*N*-(2,6-dimethylphenyl)butanamide hydrochloride[3] (I)] was prepared in 0.05 *N* HCl at a concentration of 0.675 μg/50 μl.

A solution of dansyl chloride (1 mg/ml) was prepared in acetone and

[1] Nanograde quality, Mallinckrodt, St. Louis, Mo.
[2] SpectrAR quality, Mallinckrodt.
[3] Astra Pharmaceutical Products, Worcester, Mass.