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Methods for *in Vitro* Percutaneous Absorption Studies I. Comparison with *in Vivo* Results

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Methods for ***in Vitro*** Percutaneous Absorption Studies. I. Comparison with ***in Vivo*** Results. **BRONAUGH, R. L., STEWART, R. F.,** CONGDON, E. R., AND GILES, A. L., JR. **(1982).** ***Toxicol. Appl. Pharmacol. 62,*** **474–480.** The percutaneous absorption of selected radiolabeled compounds through female rat skin was determined after their application in a petrolatum vehicle. Absorption was measured during a 5-day period by ***in vivo*** and ***in vitro*** techniques. Benzoic acid. acetylsalicylic acid, urea, and caffeine were selected because of their differing skin permeability rates. Absorption was measured ***in vivo*** from urinary excretion data and in ***vitro*** with excised skin in diffusion cells. When skin absorption was expressed as the percentage of applied dose, values obtained ***in vitro*** were benzoic acid, **49.1;** acetylsalicylic acid, ***29.0*** and urea, **7.2.** Since similar values were obtained ***in vivo,*** permeability measurements of these compounds with excised skin appear reliable. With the ***in vitro*** data, the rate of skin absorption was more accurately stated in the form of a permeability constant. A method is described for the determination of a permeability constant ***in vivo,*** using measurements obtained from blood and **urine** samples.

Skin permeation can be measured in living humans or animals or ***in vitro*** by using excised skin in diffusion cells. Studies with excised skin seem feasible, since passage through the skin is a passive diffusion process and the primary barrier, the stratum corneum, is composed of nonliving tissue.

In several studies the percutaneous absorption obtained by ***in vivo*** and ***in vitro*** procedures has been compared, and results tend to support the contention that reliable measurements can be obtained from ***in vitro*** data (Burch and Winsor, 1944; Ainsworth, 1960 Sekura and Scala, 1972; Franz, 1975, 1978). Wester and Maibach (1977), however, have reported that a more accurate assessment of permeability is obtained by using ***in vivo*** procedures.

The choice of whether to measure skin permeability ***in vivo*** or by ***in vitro*** techniques depends not only upon the availability of liv ing subjects, but also upon the investigator' philosophy about the value of the data ob tained. ***In vivo*** studies include the physic logical effects on an absorbed compound such as metabolism, distribution, and excre tion. However, absorption rate is usuall measured indirectly, e.g., by determining th excretion of a compound from the body. I diffusion cell studies, the percutaneous a t sorption rate can be measured easily an accurately. In addition, human skin per meation data for many toxic compounds ca be obtained only by using ***in vifro*** tech niques.

Before a method was selected for com paring the permeability properties of th skin of certain animals to those of human. it seemed important to examine further th comparability of ***in vivo*** and ***in vitro*** testing

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An important aspect was to apply the compounds in solution in a nonvolatile vehicle so that the concentration was known and therefore permeability constants could be calculated. In some experiments, a comparison was made of permeability data expressed in terms of (i) a permeability constant and (ii) the amount excreted as a percentage of the applied topical dose.

METHODS

Female Osborne–Mendel rats, 3 to 6 months old, were used in all experiments.

[^{14}C]Benzoic acid (specific activity, 25.6 mCi/mmol), [^{14}C]acetylsalicylic acid (ASA; 33.4 mCi/mmol), [^{14}C]urea (53 mCi/mmol), and [^{14}C]caffeine (49.5 mCi/mmol) were obtained from New England Nuclear, Boston, Massachusetts. All compounds were determined by the manufacturer to have a radiochemical purity of greater than 99%.

Petrolatum was the vehicle because of its ability to adhere to the skin during *in vivo* experiments. Solutions of the radioisotopes were prepared by adding the compounds with stirring to the heated, liquefied vehicle. Because of the sensitivity of detection of the radioisotopes, concentrations could be very dilute. The approximate amounts of solute per milligram of petrolatum were benzoic acid, 160 ng; urea, 15 ng; ASA, 250 ng; and caffeine, 130 ng.

Radioactivity in blood, urine, and receptor solutions was measured by counting the samples in a Beckman LS-9000 liquid scintillation spectrometer (Beckman Instruments, Inc., Irvine, Calif.), with Aquasol (New England Nuclear) as the scintillation fluid.

Determination of Percutaneous Absorption as Percentages of the Applied Dose

In vivo experiments. The procedure was based on the method of Feldmann and Maibach (1969) and its application to animal studies by Bartek *et al.* (1972). Absorption was determined from the amount of compound excreted in the urine in 5 days, and the results are expressed as percentages of the applied dose. To correct for the amount that was percutaneously absorbed but not recovered in the urine samples, 1 μCi of the compound was injected into the tail vein of separate animals and the percentage recovery of this dose in the urine was determined.

Rats were prepared as shown in Fig. 1 to prevent removal of the applied compound. Skin on the midback was lightly shaved with electric clippers, with care being taken to prevent damage to the skin (Wester and Maibach, 1975). A nylon ring with an inside area of 2.0 cm^2 was attached securely to the skin with a cyanoacrylate adhesive. The legs of each rat were prevented from scratching the back by wrapping rubber tubing around the body as shown. Fifty milligrams of the vehicle containing the dissolved radiolabeled compound was applied with a spatula to completely cover the skin inside the ring. The wire mesh cover was attached, and the rats were placed in separate metabolism cages for collection of 24-hr urine samples for a 5 day period.

In vitro experiments. Absorption was measured by standard diffusion cell techniques as previously described (Bronaugh *et al.*, 1981). Full-thickness skin (lightly shaved, subcutaneous fat carefully removed) was clamped tightly between two glass chambers. The lower chamber, which had a volume of approximately 2.5 ml, contained normal saline that was continually mixed with a magnetic stirring bar. Thimerosol (0.0196) was added to the saline to retard bacterial growth. The upper chamber was open at the top so that the surface of the skin was exposed to the ambient environment. Petrolatum containing the radiolabeled compound was applied in a thin layer to the surface of the skin (area, 1.13 cm^2) with a spatula. The 30 mg of petrolatum applied resulted in a calculated thickness of the vehicle of 0.2 mm; this amount contained an excess of test compound, resulting in the attainment of a steady-state or linear rate of skin permeation. The temperature of the cells was maintained at 32°C in a water bath. Samples of the receptor solution were withdrawn periodically from a sidearm with a microliter syringe for determination of radioactivity.

Determination of the Permeability Constant of Absorbed Materials

In vitro experiments. Since the permeability constant (k_p) is defined as the steady-state rate of absorption



FIG. 1. Preparation of a rat for an *in vivo* percutaneous absorption study. Rubber tubing prevents the rat from scratching its back. Additional protection of applied compound is afforded by ring and cover glued to the back.

(amount/cm²/hr) divided by the concentration of solute applied to the skin (amount/cm³), a k_p value (cm/hr) was readily obtained by the diffusion cell procedures described above.

In vivo experiments. The determination of a k_p value from *in* vivo data is based on an accurate measurement of absorption rate. We estimated this rate by considering the absorbed compound to accumulate in both the body of the animal and the excreted waste. Blood and urine samples were collected during steady-state absorption while the rats were under pentobarbital anesthesia. Absorption of caffeine occurred rapidly and the entire study was performed while the rats were under anesthesia. In studies of ASA, which was absorbed more slowly than caffeine, rats were anesthetized for sample collection approximately 24 hr after the compound was applied to the back; they were restrained before this time as previously described.

The collection of samples is illustrated in Fig. 2, which shows a rat treated with [^{14}C]caffeine. The bladder was cannulated with PE-50 polyethylene tubing so that urine could be collected as produced. Blood samples were collected from the tail vein in heparinized capillary tubes. The experiments were initiated by applying caffeine in petrolatum to abdominal skin; the surface area (2.0 cm²) was the same as that in experiments on back skin, when rings were glued on for protection of the applied compound in unanesthetized animals. In the *in vitro* caffeine studies, abdominal skin was also used to permit an accurate comparison.

The amount of compound accumulating in the animal at hourly intervals was estimated by multiplying the blood concentrations (calculated from serum radioactivity, dpm/ml) times the apparent volume of distribution (V_d) of the compound in the rat. It was reasoned that the total compound in the body would be approximated by multiplying the volume a compound appears to occupy (V_d) by the concentration of a unit aliquot (blood sample). The amount of compound excreted each hour was calculated directly from the radioactivity in the urine collected by catheter. Because this study was performed only to determine the feasibility of the method. a bile duct cannulation to measure excretion in the feces was not done; caffeine and ASA are excreted predominantly in the urine so the error was not great.[1] The sum of the rate of appearance of compound in the body and in the urine at steady state was used for calculation of the permeability constant. k_p = (rate of body accumulation + rate of excretion)/concentration of solute in vehicle.

The volume of distribution was determined from the equilibrium plasma concentration following a bolus iv injection, using standard procedures (Goldstein *et al* 1974). For the permeability calculations. it was assumed that no significant difference in parenteral metabolite exists after either topical or iv administration. If this condition were not the case, the V_d determined after iv administration might not accurately reflect the distribution of radioactivity after topical administration. For the purpose of estimating total body compounds, this assumption should usually be justified.

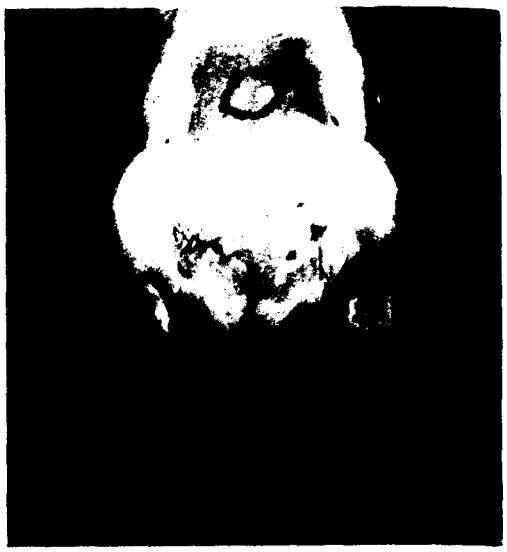

FIG. 2. Rat prepared for sample collection. Bladder is cannulated with PE-50 polyethylene tubing for continuous collection of urine.

RESULTS

Commercially available radiolabeled compounds were selected for the study based on differing octanol–water partition coefficients, which would predict compounds that vary in their skin absorption rates. Benzoic acid, ASA, caffeine, and urea have octanol water partition coefficients of 74, 17, 1, and 0.002, respectively (Leo *et al.*, 1971). For comparative purposes, we selected compounds that had previously been examined in permeability studies by other investigators (Feldmann and Maibach, 1970; Franz, 1975).

The *in vivo* absorption of three of these compounds after topical application is shown in Fig. 3. The values were corrected for in

[1] After iv injection of compound to the rat, recovery in the urine was 78.2% for ASA (see Results) and 74.0% for caffeine (Bartek *et al.*, 1972).

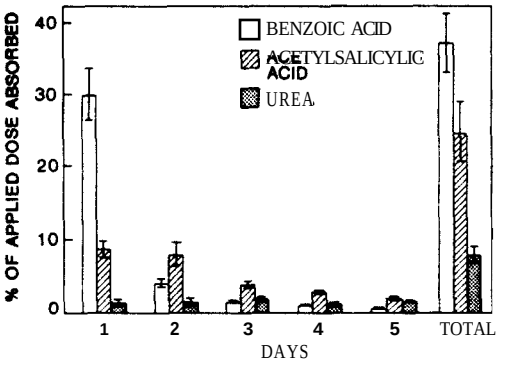

FIG. 3. Percutaneous absorption in the rat *in vivo*. Values are $\bar{x}$ ± SE of five to eight determinations.

complete urinary excretion, using the following percentages for recovery in urine after iv administration: benzoic acid, 75.3; ASA, 78.2; and urea, 70.9. The absorption of these compounds through rat skin in diffusion cells is shown in Fig. 4; results are expressed as percentages of applied dose for comparison with the *in vivo* data. Good agreement was observed between the methods (Figs. 3 and 4), and the compounds were ranked in the same order of increasing permeability: urea, ASA, and benzoic acid. The quantitative agreement was also good, particularly for ASA (Table 1).

As previously stated, *in* vitro measurements can also be expressed in terms of a permeability constant, since the steady-state rate of absorption through skin is accurately

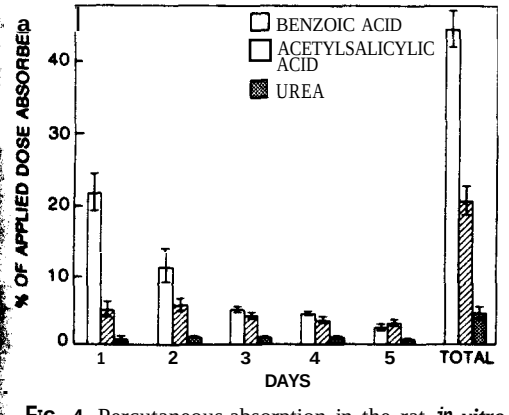

FIG. 4. Percutaneous absorption in the rat *in vitro*. Values are the $\bar{x}$ ± SE of four to eight determinations.

TABLE 1

PERCUTANEOUS ABSORPTION OF ACETYLSALICYLIC ACID IN RATS[a]

Days	Absorption (% of applied dose) *In vivo*	*In* vitro
1	8.5 ± 1.6	8.8 ± 1.2
2	1.9 ± 2.0	8.5 ± 1.2
3	4.0 ± 0.9	4.6 ± 0.5
4	2.8 ± 0.5	4.3 ± 0.4
5	1.9 ± 0.5	2.9 ± 0.1
Total	24.8 ± 4.4	29.0 ± 3.1

[a] Results are expressed as the $\bar{x}$ ± SE of four or five determinations.

measured. The solute concentration in the vehicle must be in large excess so that it remains essentially unchanged during the rate measurement. At low concentrations of solute, the rate of absorption was linearly proportional to concentration. Therefore, the permeability constant is useful for comparison of data since it eliminates experimental variables due to concentration. A comparison of the diffusion cell data calculated in terms of (i) permeability constants and (ii) percentages of the applied dose is shown in Table 2. The rank order of permeability of these compounds was not affected by the method of data expression. However, a greater difference was seen in the relative

TABLE 2

IN VITRO PERMEATION OF RAT SKIN FROM PETROLATUM VEHICLE[a]

Test compound	Permeability constant (cm/hr)	Percentage of applied dose
Urea	1.6×10^{-5} (1)	7.2 (1)
Acetylsalicylic acid	6.5×10^{-5} (4.1)	29.0 (4.0)
Benzoic acid	3.5×10^{-4} (21.9)	49.1 (6.8)

[a] Values are the $\bar{x}$ of four or five determinations. The relative permeability of the compounds with each method of calculation is given in parentheses.

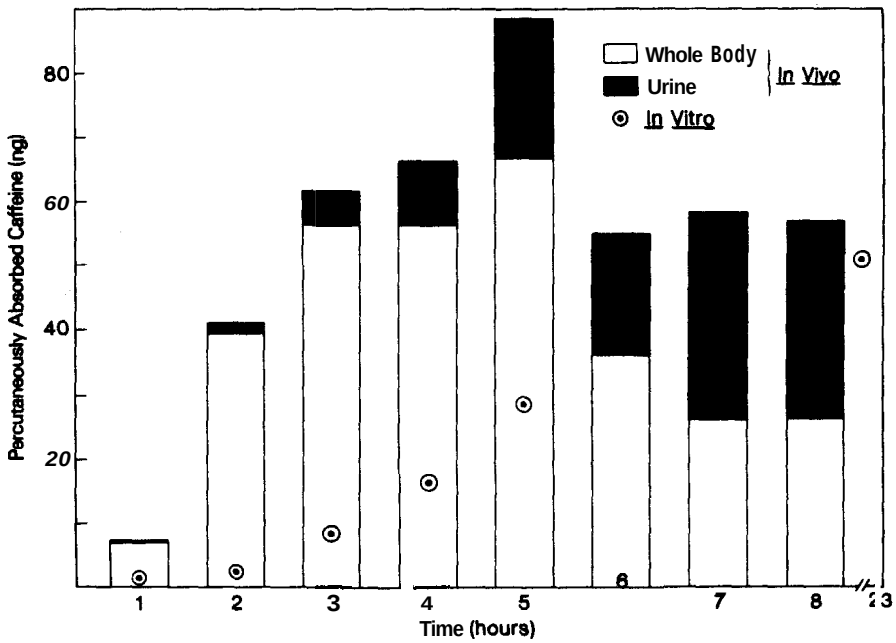

FIG. 5. Percutaneous absorption of ('%]caffeine. *In vivo* values are the average of determinationsin seven animals and are shown by the height of each bar. *In vitro* values a n the average of results from six experiments and are shown by the point of each symbol.

permeability of benzoic acid and ASA when the k_p values were compared.

In vivo permeability constants for caffeine and ASA were determined to ascertain if values obtained by this new procedure were in agreement with those of the established diffusion cell method. The individual measurements for caffeine absorption are shown in Fig. **5**. At **6** hr, the total caffeine absorbed each hour calculated from urine and blood measurements became constant at about **50** ng. Values obtained at similar times from diffusion cell experiments are also shown in Fig. **5**. Although ***in vitro*** measurements were not taken at **6** to **8** hr, the steady-state hourly absorption in these experiments was similar, as shown by the measurement at 23 hr.

The ***in vivo*** rate determinations and a comparison of the ***in vivo*** and ***in vitro*** k_p values for caffeine and ASA are shown in Table 3. **Good** agreement was obtained with the two methods, indicating that the estimation of compound accumulating in the body by the volume of distribution method provides accurate results for these compounds.

DISCUSSION

A comparison of the results of the ***in vivo*** absorption studies (Fig. 3) with the ***in vitro*** studies (Fig. **4**) shows good agreement between the two methods, both from a qualitative and a quantitative standpoint. A similar pattern of absorption on a daily basis was also observed when the data were expressed in terms of the total amount absorbed.

Franz (**1975**) discussed a condition that may result in poor comparison of ***in vivo*** and ***in vitro*** data. In living humans or animals. the process of desquamation results from the turnover of epidermal cells. As the upper layer of the stratum corneum is sloughed off a compound applied to the surface of the skin is also lost. The somewhat smaller absorption occurring in our ***in vivo*** studies may

TABLE 3

IN VIVO VS. *IN VITRO* PERCUTANEOUS ABSORPTION THROUGH RAT SKIN[a]

Test compound	Rate (ng/hr/cm²)		Permeability constant (cm/hr)	
	Body	Urine	*In* vivo	*In* vitro
Caffeine	16.3	27.8	2.1×10^{-4} (7)	3.1×10^{-4} (6)
Acetylsalicylic acid	0	11.4	5.2×10^{-5} (7)	6.5×10^{-5} (5)

[a] Compounds in a petrolatum vehicle were applied to a 2.0-cm' area of skin on the living animals and in diffusion cells. Results are the means of the number of determinations in parentheses.

be due to this effect. The fact that only a small difference was observed is probably due to the method of application of the compounds to the skin. The layer of petrolatum vehicle on the skin undoubtedly retains many of the flakes of stratum corneum that loosen from the skin. In addition, the protective device glued to the animal's back may prevent the loss of additional stratum corneum.

A comparison of the data with those from other studies with human skin is shown in Table 4; the results appear to be similar in spite of some major differences in the studies. Differences in type of skin and vehicle are indicated in the table and, in addition, there were differences in duration of contact of the applied compounds with the skin. In the study of Feldmann and Maibach (1970), the site of application was not protected from rubbing and was washed in 24 hr. In the *in vitro* study of Franz (1975), the percentage absorption was determined after 2 days. Since equivalent results were obtained after 5 days of contact in our study, absorption may occur more slowly from the petrolatum vehicle. We have compared the permeability of excised rat and human skin to these compounds from a petrolatum vehicle (Bronaugh *et al.*, 1982) and found a similarity in the absorption of benzoic acid and ASA, however, urea was absorbed about five times more slowly in human skin than in rat skin.

The methods of calculating diffusion cell data are compared in Table 2. A large difference was observed in the relative amounts of benzoic acid that pentrated skin in the two methods; the difference between benzoic acid and ASA was greater than five-fold when k_p values were calculated and less than two-fold when percentages of applied dose were calculated. This difference is due to the different parameters measured by the two

TABLE 4

COMPARISON OF PERMEATION VALUES WITH THOSE OF OTHER STUDIES

Test compound	Percentage of applied dose			
	In vivo		*In* vitro	
	Rat (Petrolatum)	Human' (Acetone)	Rat (Petrolatum)	Human[b] (Acetone)
Benzoic acid	31.1	42.6	49.1	44.9
Acetylsalicylic acid	24.8	21.8	29.0	40.5
Urea	8.1	6.0	1.2	11.1

[a] Values from Feldmann and Maibach (1970).
[b] Values from Franz (1975).

procedures. The steady-state rate of penetration (after the initial lag time) is represented by the permeability constant and is useful for comparing compounds with regard to rates of absorption. The total amount of compound absorbed can be estimated from this information. On the other hand, the percentage of applied dose absorbed is useful for determining absorption under specific conditions of dose and time. In the example provided in this study, benzoic acid penetrated rapidly during the first day but then slowed down, while the other two compounds continued to penetrate at the same rate during the remaining 4 days. Permeation data expressed as percentages of the applied dose would show smaller differences in absorption than if absorption were considered in terms of penetration rate.

Additional evidence has been presented regarding the good comparability of in *vitro* diffusion cell data with that of *in* vivo measurements. Certain compounds, however, may present problems in an *in* vitro assay; e.g., very lipophilic compounds may have inadequate solubility in the receptor solution (unpublished observation). It seems likely that satisfactory results for percutaneous absorption measurements of most compounds can be routinely obtained with excised skin in diffusion cells. So far, however, only a small number of compounds have actually been studied by both methods.

Although the extra effort required for in *vivo* determination of a k_p value as described in this paper would usually not be warranted, additional compounds should be studied by this method and excretion data from bile cannulation should be included. This procedure may be useful for studies requiring physiological conditions, such as those performed to obtain metabolism or pharmacokinetic data.

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