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Report

In Vitro Skin Absorption and Metabolism of Benzoic Acid, *p*-Aminobenzoic Acid, and Benzocaine in the Hairless Guinea Pig

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The percutaneous absorption and metabolism of three structurally related compounds, benzoic acid, *p*-aminobenzoic acid (PABA), and ethyl aminobenzoate (benzocaine), were determined *in vitro* through hairless guinea pig skin. Benzocaine was also studied in human skin. Absorption of benzocaine was rapid and similar through both viable and nonviable skin. The absorption of the two acidic compounds, benzoic acid and PABA, was greater through nonviable skin. A small portion (6.9%) of absorbed benzoic acid was conjugated with glycine to form hippuric acid. Although *N*-acetylbenzocaine had not been observed as a metabolite of benzocaine when studied by other routes of administration, both PABA and benzocaine were extensively *N*-acetylated during percutaneous absorption. Thus, the metabolism of these compounds should be considered in an accurate assessment of absorption after topical application.

KEY WORDS: percutaneous absorption; metabolism; hairless guinea pig; benzoic acid; PABA; benzocaine.

INTRODUCTION

The potential importance of skin metabolism during percutaneous absorption has recently been demonstrated. Topically applied benzo(*a*)pyrene was significantly metabolized in mouse skin in organ culture (1). Collier *et al.* determined that skin can be kept viable for 24 hr in a flow-through diffusion cell using a HEPES-buffered Hanks' balanced salt solution (HHBSS) as the receptor fluid (2), and small amounts of butylated hydroxytoluene (6%) and the fragrance acetyl ethyl tetramethyl tetralin (2%) were metabolized during percutaneous absorption (3).

However, most *in vitro* studies are currently performed using conditions that do not maintain the viability of skin in diffusion cells. Therefore, it is important to assess the extent of metabolism of different types of compounds during absorption in skin. Of interest, also, is the potential effect of metabolism on rates of percutaneous absorption when results from viable and nonviable skin are compared.

These issues were investigated by using hairless guinea pig skin and a homologous series of compounds susceptible to different metabolic reactions: benzoic acid, *p*-aminobenzoic acid (PABA), and ethyl aminobenzoate (benzocaine). Benzoic acid is excreted almost completely as the glycine conjugate, hippuric acid, after systemic administration (4,5). Acetylation of the primary amino group of PABA, a process that occurs after systemic administration, could also occur after skin absorption (6). Benzocaine, the ethyl ester of PABA, could be acetylated and also hydrolyzed by esterases in skin. The viability of skin was monitored continuously by measuring anaerobic glucose utilization (2). Control experiments were conducted with receptor fluids that produced nonviable skin to distinguish enzymatic and nonenzymatic biotransformations. The absorption and metabolism of benzocaine were also determined in human skin.

MATERIALS AND METHODS

Test compounds were ^{14}C-labeled radioisotopes. [7-^{14}C]Benzoic acid (sp act, 19.3 mCi/mmol; 98% purity) was purchased from New England Nuclear, Boston, MA. *p*-Amino [*carboxy*-^{14}C]benzoic acid (55.6 mCi/mmol, 97% purity) (^{14}C-PABA) was obtained from Amersham Corporation, Arlington, Heights, IL. Radiolabeled benzocaine was synthesized from ^{14}C-PABA by an esterification reaction (7). ^{14}C-PABA was refluxed for 2 hr in ethanol acidified with anhydrous HCl gas. The product was purified to 99% by thin-layer chromatography (TLC) on silica gel plates with a solvent system of hexane:ethyl acetate:acetic acid (250:1000:1). The ^{14}C-benzocaine cochromatographed with unlabeled benzocaine (Sigma Chemical Co., St. Louis, MO). Absorption and metabolism experiments were conducted *in vitro* using flow-through diffusion cells (8). The system was

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sterilized with a 70% ethanol solution to prevent interference from bacterial metabolism. Most studies used female, 3- to 6-month-old hairless guinea pig (Charles River Laboratories, Boston, MA) skin that was prepared with a Padgett dermatome (Padgett Instruments, Kansas City, MO) at a thickness of 200 μm. A surgical specimen of viable human skin obtained from abdominoplasty and dermatomed to 200 μm was used in one study. The integrity of the barrier of this specimen was verified by determining ^{3}H-water absorption (9).

Viability of skin was maintained in the diffusion cells for 24 hr with HHBSS as the receptor fluid (2). Lactate formation from the metabolism of glucose was used as an index of viability and was measured in the collected receptor fluid fractions with a Sigma diagnostic kit (2) (Fig. 1). Control experiments were conducted with skin made nonviable by using distilled water receptor fluid (Fig. 1). The distilled water contained glucose (for viability determinations) and gentamicin sulfate (antibiotic) at the same concentrations as HHBSS. A few experiments were conducted with phosphate-buffered saline with glucose (PBSG) as the receptor fluid. Receptor fluids were sterilized by passage through a 0.2-μm filter (Nalgene Company, Rochester, NY). Some control experiments were conducted by pretreating skin for 4 hr with an HHBSS solution of 0.1 *M* iodoacetamide to inhibit *N*-acetyltransferase and presumably enzymes responsible for glucose metabolism (10).

Compounds were applied in an ethanol vehicle (15 μl/cm^2) to skin at a chemical dose of approximately 2 μg/cm^2. The skin surface was washed at 24 hr with soap and water to remove unabsorbed material. Experiments were continued for another 24 hr to allow additional absorbed material to enter the receptor fluid. Skin specimens were homogenized in HHBSS by a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY). Small aliquots (0.2 ml) of the receptor fluid, which were collected at 6-hr intervals, and skin homogenates were analyzed for radioactivity by a Beckman LS9000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Total recovery of radioactivity (amount absorbed + amount removed in the 24-hr wash) ranged from 85 to 95% of the applied material. Parent compounds and metabolites were extracted from the remaining receptor fluid and skin homogenates with Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA). Benzoic acid and PABA extracts were adjusted to pH 3.5 and filtered through cartridges. Compounds bound to the Sep-Pak cartridges were eluted with 4 ml acetone. Benzocaine and metabolites were extracted by Sep-Pak filtration at both pH 7.4 and pH 3.5.

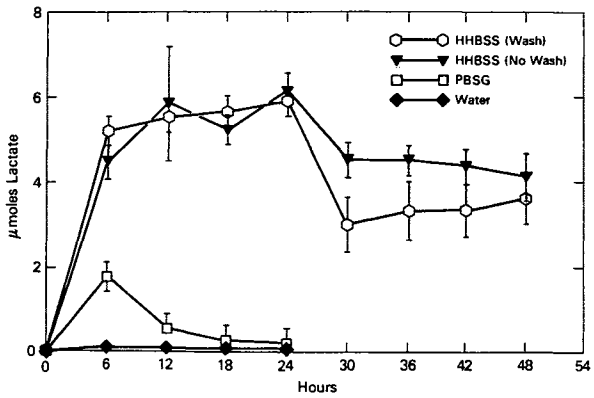

Fig. 1. Anaerobic glucose utilization. Values are the mean ± SE; *N* = 4–8. Lactate formation in the diffusion cell receptor fluids was measured at 6-hr intervals. HHBSS, HEPES-buffered Hanks' balanced salt solution; PBSG, phosphate-buffered saline with glucose.

Extracts containing parent compounds and metabolites were applied to silica gel TLC plates with standards. Plates were developed in an ethyl acetate:ethanol:acetic acid (900:100:1) solvent system. Identification of the compounds and quantitation of radioactivity on the plates were made by a Bio-scan TLC plate scanner (Bioscan Inc., Washington, DC). R_f values of metabolite peaks were compared with those of standards commercially available from Sigma Chemical Co. (hippuric acid, *p*-aminohippuric acid) or synthesized by the Division of Colors and Cosmetics, Food and Drug Administration (FDA), Washington, DC (*p*-acetamidobenzoic acid, *p*-acetamidohippuric acid). *N*-Acetylbenzocaine (acetamidobenzoic acid, ethyl ester) was synthesized from benzocaine and acetic anhydride by a procedure described in the synthesis of acetanilide (7). The following reagents were added to a 250-ml beaker: 50 ml distilled water, 1.8 ml concentrated HCl, 3.1 g benzocaine, and 2.8 g acetic anhydride. The mixture was stirred until all reagents dissolved. The beaker was cooled in an ice bath, and the precipitate that formed was collected by filtration and washed twice with cold distilled water. The structure of the product was verified by gas chromatography/mass spectrometry (GC/MS). The *N*-acetylbenzocaine that formed in skin during an absorption experiment was also identified by GC/MS (Division of Contaminants Chemistry, FDA, Washington, DC).

Absorption data were analyzed with PC SAS/STAT software version 6.03 (SAS Institute, Inc., Cary, NC). A one-way analysis of variance (ANOVA) was performed using a general linear model, followed by a least significant difference test; $P < 0.05$ was considered significant.

RESULTS

The viability of skin was maintained for 24 hr by HHBSS receptor fluid (Fig. 1). Washing the skin at 24 hr further reduced glucose utilization compared with not washing the skin. When PBSG was the receptor fluid, glucose utilization in skin was markedly reduced. Because some glucose was metabolized in the initial 12 hr, PBSG could not be used to create nonviable skin for control experiments. When distilled water was the receptor fluid, however, glucose metabolism was abolished within 1 hr.

Percutaneous absorption of the test compounds is presented as the rate of penetration (pmol/hr) in Fig. 2 and as the percentage of the applied dose absorbed in 48 hr in Table I. Benzocaine penetrated primarily during the first 6 hr, benzoic acid was absorbed primarily in 24 hr, and PABA was absorbed slowly over the 48-hr study (Fig. 2). Maximum absorption rates in HHBSS receptor fluid ranged from a high of about 500 pmol/hr for benzocaine to a low of 57 pmol/hr for PABA. Benzocaine absorption was nearly identical in viable and nonviable skin. For benzoic acid and PABA, a small but significant increase in their total absorption occurred in nonviable skin compared with that in viable skin

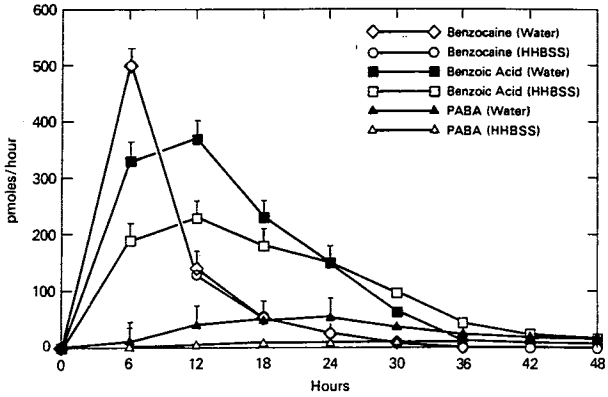

Fig. 2. Percutaneous absorption rates in viable (HHBSS receptor fluid) and nonviable (water receptor fluid) skin. Values are the mean ± SE of four determinations in each of three animals.

(Table I). In contrast to findings with benzoic acid and benzocaine, substantial amounts of absorbed PABA (20.7%, Table I) remained in the skin at the end of the experiments. Human skin absorbed less benzocaine than did hairless guinea pig skin.

A small amount of benzoic acid (equivalent to 6.9% of the receptor fluid radioactivity) was metabolized to hippuric acid during percutaneous absorption (Table II). The identity of the radioactivity at the origin (polar fraction) is unknown. The receptor fluid data in Table III show that topically applied PABA was extensively metabolized to *p*-acetamidobenzoic acid (acetyl-PABA). The substantial radioactivity remaining in skin, however, was primarily unmetabolized PABA. Control experiments verified the enzymatic formation of acetyl-PABA present in the receptor fluid. Compared with that of benzoic acid, no glycine conjugation of PABA was identified.

Absorbed benzocaine was biotransformed primarily to *N*-acetylbenzocaine (Table IV). A small amount of PABA

Table I. Percentage of Applied Dose Absorbed into Human and Hairless Guinea Pig Skin in 48 Hr

Compound	Hairless guinea pig		Human,
	HHBSS	Water	HHBSS
Benzoic acid			
Receptor fluid	47.3 ± 2.3[a]	59.1 ± 5.3	
Skin	2.2 ± 0.7	1.0 ± 0.2	
Total absorbed	49.5 ± 3.0	60.1 ± 5.5*	
PABA			
Receptor fluid	5.0 ± 0.7	18.7 ± 4.8	
Skin	20.7 ± 4.9	14.7 ± 3.2	
Total absorbed	25.7 ± 5.6	33.4 ± 8.0*	
Benzocaine			
Receptor fluid	75.1 ± 2.2	76.0 ± 2.8	48.0 ± 4.1[b]
Skin	1.5 ± 0.2	1.8 ± 0.1	1.5 ± 0.2
Total absorbed	76.6 ± 2.3	77.8 ± 2.9	49.5 ± 4.3

[a] Values are the mean ± SE of four determinations in each of three animals.
[b] Values are from four determinations in one human subject.
* Significantly different from corresponding HHBSS value (ANOVA, $P < 0.05$).

Table II. Benzoic Acid Metabolism in Hairless Guinea Pig Skin[a]

Compound	HHBSS	Water
Receptor fluid		
Benzoic acid	73.8 ± 8.0[b]	92.3 ± 1.7
Hippuric acid	6.9 ± 3.4	0.1 ± 0.1
Polar	11.6 ± 4.1	2.5 ± 1.3
Skin		
Benzoic acid	83.2 ± 2.9	84.2 ± 7.2
Hippuric acid	0.6 ± 0.6	0.0 ± 0.0
Polar	3.8 ± 0.8	8.1 ± 1.7

[a] Percentage of absorbed dose.
[b] Values are the mean ± SE of four determinations in each of three animals.

and acetyl-PABA was also present in the HHBSS receptor fluid. However, these two compounds were also present in equal or greater quantities in the receptor fluid of the nonviable (water receptor fluid) skin. In some experiments, skin was perfused 4 hr before applying the test compound with the enzyme inhibitor iodoacetamide (0.1 *M* in HHBSS). The absorption experiment was then conducted using HHBSS as the receptor fluid. Acetylation of benzocaine and PABA was completely blocked. Substantial acetylation of benzocaine was also observed during skin penetration studies with human skin, but this membrane seemed less active than hairless guinea pig skin in the biotransformation of benzocaine.

DISCUSSION

Different absorption profiles were obtained for benzoic acid and its two derivatives when finite doses were applied to hairless guinea pig skin. When total absorption between viable and nonviable skin was compared, only the two acidic compounds showed significant (although small) differences. The pH changes that occur in degenerating, nonviable skin could possibly explain this finding. When glucose utilization decreases in skin, lactate accumulates and lowers the pH (11). A lower pH would increase the amount of benzoic acid and PABA that are nonionized and would, therefore, enhance percutaneous absorption of those compounds. The magnitude of this effect would, of course, depend on the extent of change in pH after cell degeneration and on the pK_a of the test compounds. The similar absorption of benzocaine through viable or nonviable skin indicates that percutaneous absorption rates for this compound may be determined in the

Table III. PABA Metabolism in Hairless Guinea Pig Skin[a]

Compound	HHBSS	Water
Receptor fluid		
PABA	24.6 ± 8.1[b]	93.0 ± 2.4
Acetyl-PABA	60.7 ± 7.7	1.6 ± 0.4
Skin		
PABA	86.0 ± 7.7	83.9 ± 2.2
Acetyl-PABA	6.7 ± 1.1	6.2 ± 0.6

[a] Percentage of absorbed dose.
[b] Values are the mean ± SE of four determinations in each of three animals.

Table IV. Benzocaine Metabolism in Human and Hairless Guinea Pig Skin[a]

Compound	Hairless guinea pig			Human
	HHBSS	Water	HHBSS + inhibitor[b]	HHBSS
Receptor fluid				
Benzocaine	4.4 ± 1.7[c]	66.8 ± 5.2	89.4 ± 1.1	27.7 ± 12.3
N-Acetylbenzocaine	82.4 ± 1.1	7.2 ± 1.2	0	56.7 ± 12.2
PABA	0.2 ± 0.1	5.4 ± 0.4	6.1 ± 1.1	1.5 ± 0.7
Acetyl-PABA	6.2 ± 0.7	7.7 ± 0.3	0	11.4 ± 2.9
Polar	2.5 ± 1.0	6.0 ± 0.5	2.0 ± 0.1	1.2 ± 0.3
Skin				
Benzocaine	25.8 ± 3.1	30.0 ± 1.4	62.1 ± 2.6	26.4 ± 3.0
N-Acetylbenzocaine	31.7 ± 4.3	20.5 ± 3.3	0	47.7 ± 5.2
PABA	5.5 ± 0.7	12.7 ± 0.6	8.7 ± 3.1	2.3 ± 1.3
Acetyl-PABA	9.7 ± 1.1	8.1 ± 0.9	0	7.3 ± 1.9
Polar	14.6 ± 1.7	16.6 ± 0.8	7.5 ± 1.0	4.7 ± 0.5

[a] Percentage of absorbed dose.
[b] Diffusion cells were pretreated for 4 hr with 0.1 *M* iodoacetamide solution in HHBSS. Only HHBSS was used for absorption/metabolism measurements.
[c] Values are the mean ± SE of four determinations in each of three animals. The inhibitor and human experiments represent four determinations in one subject.

stratum corneum before any biotransformation in the viable epidermis.

Acetylation of primary amines was an important metabolic reaction in skin. For benzocaine, which is susceptible to both *N*-acetylation and ester hydrolysis, 80% of the absorbed material was acetylated in viable skin, while less than 10% of the absorbed ester was hydrolyzed. Possibly, competition between these two biotransformation pathways limits ester hydrolysis to the same level as in nonviable skin. PABA was absorbed much more slowly than benzocaine and was also less extensively *N*-acetylated. At the end of the experiments, acetyl-PABA was found primarily in the receptor fluid, which contained only 20% of the absorbed dose. Much of the absorbed PABA remained unmetabolized and in the skin, as would be expected for an effective sunscreen agent. If the compound had localized in the stratum corneum, PABA would probably not have been exposed to *N*-acetylating enzymes.

In several previous studies of metabolism associated with percutaneous absorption, relatively small amounts of substrate were biotransformed by microsomal enzymes. Only 1.9% of absorbed acetyl ethyl tetramethyl tetralin and 6.6% of absorbed butylated hydroxytoluene were metabolized in fuzzy rat skin (3). In studies comparing absorption and metabolism of benzo(*a*)pyrene and ethoxycoumarin in animal (fuzzy rat, hairless guinea pig, Sencar mouse) and human skin, only a small percentage of the absorbed material was metabolized (12). These findings make the extensive amount of acetylation of PABA and benzocaine in this study seem even more remarkable. Acetylation was carried out by soluble enzymes, which resulted in much greater metabolic activity than in previous studies where the observed metabolism was the result of microsomal enzyme activity (3,12).

Metabolism of topically applied compounds by esterases in skin has been studied previously in diffusion cells under conditions that would not maintain the viability of skin (13–15). Esterases, however, are relatively stable enzymes that do not require pteridine cofactors and can function in nonliving cells such as those of the stratum corneum. Although substantial esterase activity has been observed frequently in these studies, the results should be viewed cautiously. Enzymes may be localized differently in nonviable skin when cells in nonviable skin are disrupted by degradative processes. Additionally, esters may also be subject to metabolism by other enzymes that do not function in nonviable skin.

Control experiments with nonviable skin or enzyme inhibitors are important to rule out nonenzymatic biotransformation reactions and impurities in the test material as being responsible for any metabolite formation. When distilled water was used as the receptor fluid, only small amounts of acetylated PABA and benzocaine were observed in the receptor fluid and skin. These products may have been formed in the short period of time (<1 hr) at the start of the experiment when some metabolic capacity still remained in the skin.

The metabolism of benzoic acid and PABA during skin absorption was similar qualitatively to that previously reported for systemic administration. However, only a small percentage of benzoic acid was converted to hippuric acid in skin, in contrast to almost complete conjugation after a systemic dose to humans and many animal species (4). PABA acetylation following iv infusion in the rabbit (6) was similar (40–80%, depending on dose) to that observed in these studies.

Benzocaine was reported to be extensively metabolized after rectal administration to rats (16). Ayres *et al.* hypothesized that benzocaine was converted to PABA, which was then further metabolized. In another rat study (17), benzocaine was metabolized mainly to PABA, which could be further metabolized to acetyl-PABA, *p*-aminohippuric acid, or acetyl-*p*-aminohippuric acid. However, in the skin of the hairless guinea pig, benzocaine was almost completely acetylated. Only small amounts of PABA and PABA metab-

olites were formed. Thus, the acetylation reaction in hairless guinea pig skin seems to predominate over ester hydrolysis.

Both the absorption and the metabolism of benzocaine were substantial in the skin of the hairless guinea pig and, to a lesser extent, in human skin. This finding is consistent with that of a previous study of benzo(*a*)pyrene and ethoxycoumarin (12). Human skin is generally recognized as less permeable to chemicals than is animal skin; human skin may also be less capable of biotransforming certain topically applied chemicals.

The study of percutaneous absorption is most informative using viable skin preparations. Small differences in absorption between viable and nonviable skin may occur with ionizable compounds. The significant skin metabolism of benzoic acid, PABA, and benzocaine that occurred in this study could be an important consideration when reviewing the effects of any topical pharmaceutical formulation.

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