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The in vivo dermal absorption and metabolism of [4-^{14}C]coumarin by rats and by human volunteers under simulated conditions of use in fragrances

R.A. Ford [a,1], D.R. Hawkins [b], B.C. Mayo [b], A.M. Api [a,*]

[a] Research Institute for Fragrance Materials, Inc., 2 University Plaza, Suite 406, Hackensack, NJ 07601, USA
[b] Huntingdon Life Sciences, Ltd, Huntingdon PE18 6ES, UK

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Abstract

The disposition and metabolic fate of [4-^{14}C]coumarin in a 70% aqueous ethanol solution was studied in male Lister Hooded rats after occluded dermal application and in three male volunteers after an exposure designed to simulate that which may be encountered when using an alcohol-based perfumed product. In both cases, the 6-h exposure was 0.02 mg/cm^2 (rats 0.023 mg/kg and humans 0.77 mg/kg). In both, coumarin was quickly absorbed, distributed and excreted in urine and feces, although fecal excretion of coumarin in humans was only 1% of the applied dose as opposed to 21% in rats. Total absorption was 72% of the applied dose with rats and 60% with humans. Peak plasma radioactivity in both was at 1 h. The mean plasma half-life of coumarin and metabolites was approximately 1.7 h for humans and 5 h for rats. In humans, coumarin was primarily metabolized to and excreted in urine as 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate. Small amounts of unconjugated 7-hydroxycoumarin and *o*-hydroxyphenylacetic acid (*o*-HPAA) were also excreted. In rats, about twenty metabolites were present, but only *o*-HPAA was identified. These studies show the rat is a very poor model for humans and toxicity in the rat cannot be extrapolated to humans.
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Keywords: Skin absorption; Coumarin; Rat; Human

1. Introduction

Coumarin (1,2-benzopyrone; CAS no. 96-64-5) is a white crystalline solid with an odor like new-mown hay (Clark, 1995). Its chemical structure (Fig. 1) consists of an aromatic ring fused to an unsaturated cyclic lactone. It was previously used as a flavoring ingredient (US Food and Drug Administration, 1954); however, it may still be consumed in small amounts as a result of its natural occurrence in roots, bark, stem, leaves and fruits of a variety of plants (Furia and Bellanca, 1975; Leung and Foster, 1996). Coumarin was shown to be effective in the treatment of brucellosis and high protein lymphedema (Egan et al., 1990). It has also been used in the treatment of metastatic renal carcinoma (Marshall et al., 1987; Dexeus et al., 1990). Although the pharmacologic use of coumarin results in doses up to 7000 mg/day (Lake, 1999), its use in fragrances constitutes the principle exposure to coumarin over time.

Coumarin is used as a fragrance ingredient in a wide variety of perfumes, cosmetics and functional products such as soaps and detergents (Opdyke, 1974; Bauer et al., 1990). Alcohol-based products such as perfumes, colognes and eau de toilettes contain the highest levels of fragrance (Streschnak, 1991) and therefore dermal exposure to fragrance ingredients, such as coumarin, is highest in these alcohol-based products. The maximum long-term exposure of humans to coumarin in cosmetic products has been estimated as 9.8 mg/day or 0.16 mg/kg/day (International Fragrance Association, 1998 pers. commun.). The US Food and Drug Administration has estimated the daily exposure to coumarin from its use in fragrances as 1.2 mg/day (Yourick and Bronaugh, 1997).

Substantial in vitro skin absorption of coumarin has been reported using skin samples from human, rat and mouse (Ritschel and Hussain, 1988; Beckley-Kartey et al., 1997; Yourick and Bronaugh, 1997). Percutaneous absorption of coumarin in ethanol solution through unoccluded full-thickness mouse, rat, and human breast

Abbreviations: *o*-HPAA, *o*-hydroxyphenylacetic acid; TLC, thin-layer chromatography

* Corresponding author. Tel.: +1-201-488-5527; fax: +1-201-488-5594.

E-mail address: api@rifm.org (A.M. Api).

[1] Present address: 7 Alwyn Ave, London W4 4PA, UK.

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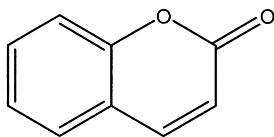

Fig. 1. Structure of coumarin.

skin in vitro was reported to be approximately 50% of the applied dose in 72 h. Occlusion of the skin appeared to increase this figure to approximately 60–75% absorption (Beckley-Kartey et al., 1997). However, in vivo dermal absorption in humans has not been reported and only one study in rats has been reported. Absorption of coumarin after dermal application in an oil/water emulsion on male Sprague–Dawley rats was reported to be only 8.6% after 6 h when applied over a 9.91 cm^2 area, but this increased to approximately 66% when applied over a 30 cm^2 area for 24 h (Ritschel and Hussain, 1988).

The understanding of coumarin's metabolism has been revised over the last several years. In humans, coumarin, after oral ingestion, is primarily metabolized by 7-hydroxylation to 7-hydroxycoumarin that is excreted in the urine mostly as a glucuronide or sulfate conjugate (Shilling et al., 1969; Egan and O'Kennedy, 1992; Rautio et al., 1992). A small portion of coumarin is converted by humans to *o*-hydroxyphenylacetic acid (Shilling et al., 1969; Hadidi et al. 1998; Meineke et al., 1998). In oral studies, a wide species diversity of metabolism is seen with rats, most mouse strains, Syrian hamsters, guinea pigs, ferrets, dogs, marmosets and squirrel monkeys, being poor 7-hydroxylators; rabbits, cats and pigs being intermediate in their ability to 7-hydroxylate, and baboons and humans being very good 7-hydroxylators (Lake, 1999).

The primary metabolite in rats after oral administration is *o*-hydroxyphenylacetic acid (Kaighen and Williams, 1961; Cohen, 1979; Lake et al., 1989; Fentem and Fry, 1993). In vitro studies in primary rat hepatocytes have demonstrated that an important pathway is via the unstable 3,4-epoxide, with rearrangement and loss of carbon dioxide to form *o*-hydroxyphenylacetaldehyde followed by oxidation to *o*-hydroxyphenylacetic acid (Born et al., 1997, 1998b). The major metabolite of coumarin detected in rats in vivo is *o*-hydroxyphenylacetic acid (Kaighen and Williams, 1961; Cohen, 1979), the oxidation product of *o*-hydroxyphenylacetaldehyde. Coumarin-induced toxicity in the rat liver is dependent on the formation of coumarin 3,4-epoxide (Lake et al., 1989; Born et al., 1998b). Extensive first-pass metabolism of coumarin by humans and rats following oral administration has been reported (Ritschel et al., 1979; Ritschel and Hoffman, 1981; Ritschel and Hussain, 1988).

This in vivo dermal study was undertaken to determine the absorption, metabolism and excretion of coumarin by humans under simulated conditions of use in alcohol-based fragranced products.

In order to estimate radiological exposure and hence an acceptable amount of radioactivity to administer in the human study, initial investigations were conducted in rats. Male Lister Hooded (pigmented) rats were used to satisfy the requirements of the Institutional Review Board. This study also enabled a comparison of the rate and extent of dermal absorption and metabolism of coumarin in rats and humans conducted under similar conditions. Another purpose of the rat study was to establish the reliability of using excreted radiolabel as a marker for material absorbed in the human study.

The human study was done under conditions as similar as possible to those that may be encountered when using a typical consumer product. Because the highest concentrations of coumarin are found in alcohol-based products, it was decided to conduct the human simulated exposure study by applying the materials in a 70% alcohol solution. In order to simulate as closely as possible normal exposure conditions, the solutions were applied without occlusion. An exposure time of 6 h was chosen simply because of the practical constraints imposed by conducting such studies using human volunteers.

2. Materials and methods

2.1. Chemicals

Coumarin [4-^{14}C], specific activity 25 mCi/mmol, was purchased from Wizard Laboratories (Davis, CA, USA). Radiochemical purity was found to be greater than 97% by TLC in three solvent systems. Non-radioactive coumarin (purity >99%) was obtained from Rhone-Poulenc Inc. (New Brunswick, NJ, USA). Standard *o*-hydroxyphenylacetic acid, 4-hydroxycoumarin, 7-hydroxycoumarin and *o*-hydroxycinnamic acid were supplied by Aldrich Chemical Co. (Gillingham, Dorset, UK). 3-Hydroxycoumarin and 6,7-dihydroxycoumarin were purchased from Apin Chemicals Ltd (Abingdon, Oxfordshire, UK). Sulphatase (*Aerobacter aerogenes*, Type VI), β-glucuronidase (Type IX-A) and the combined β-glucuronidase/sulphatase (*Helix pomatia*, type H1) were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK). The purity of the sulphatase enzyme was checked by adding saccharo-1,4-lactone (a β-glucuronidase inhibitor) and was shown not to be inhibitory of the sulphatase enzyme.

2.2. Animal studies

Male Lister Hooded (pigmented) rats, 6–8 weeks old (210–220 g) were obtained from Charles River U.K. Ltd (Margate, Kent, UK). Prior to dosing the rats were housed in stainless-steel cages for 5 days. Food (LAD1, Labsure, Manea, UK) and tap water were provided ad lib. throughout the study. A single dose volume (nominal) of

200 μl ^{14}C-coumarin in 70% (v/v) aqueous ethanol (171.1 μCi/mg) was applied evenly to the 9 cm^2 shaved back area with a Hamilton syringe. This dose was designed to be equivalent to the human dose on a dose per unit area (mg/cm^2) basis. The actual mean values for application to the 18 rats were 28 μCi/rat, 0.17 mg/rat, 0.018 mg/cm^2 and 0.77 (range 0.73–0.81) mg/kg body weight. After application, the solvent was allowed to evaporate and the treated area was occluded with aluminium foil. The occlusion was removed at 6 h or at the time of necropsy if earlier, and the residual dose was wiped from the skin with a cotton wool swab moistened with 70% ethanol. The treated area was then re-occluded with clean aluminium foil until necropsy. Those animals that were exposed for 6 h were placed in individual metabolism cages under a 12-h dark/light cycle. Animals exposed to coumarin for less than 6 h were maintained in steel cages and excreta were not collected.

Two animals each were killed by cervical dislocation under halothane/oxygen anesthesia at 0.5, 1, 3, 6, 12, 24, 48, 72 and 120 h after dosing. Tissues and organs including contents of the stomach and intestines were collected and stored at −15°C until analyzed. Whole blood was stored at 4°C. For animals killed at 6 h or later, urine and feces were collected. Urine was collected on dry ice. Feces, up to the time of necropsy or at 24-h intervals, were collected at room temperature. Air from the metabolism cages was collected in a series of two traps containing 2-ethoxyethanol:ethanolamine (3:1, v/v). After removal of the rats for necropsy, the cages were washed and the washings retained for radioactivity measurement. Swabs and dressings were extracted twice with ethanol and aliquots taken for radioactivity measurement. Estimates of half-life of coumarin and metabolites in the body were based on visual inspection of the plots of μg-equivalents per ml of plasma vs time after application.

Portions of urine samples were hydrolyzed with the combined β-glucuronidase:sulphatase (16 h at 37°C) and with hydrochloric acid. Both enzyme and acid hydrolysates were analyzed by TLC and HPLC.

2.3. Human studies

These studies were carried out at the Leicester Clinical Research Centre (LCRC), Leicester, UK and at Huntingdon Life Sciences, Huntingdon, UK. They were conducted with full approval from the LCRC independent ethics committee, by the Administration of Radioactive Substances Advisory Committee of the UK Department of Health, in full compliance with the 1964 Declaration of Helsinki and with full informed consent of the volunteers. Volunteers remained confined to the clinic from the evening before the dosing until after the 120-h samples were taken. The applied dose of coumarin was based on an assumed typical concentration of coumarin in perfumes of 0.2% and a practical area of skin surface of 100 cm^2 for application.

Three male human volunteers, identified as volunteers 1, 2 and 3, were 32, 51 and 47 years old and weighed 78, 76 and 91 kg, respectively. They received a single dermal application of approximately 1 ml of a 0.2% solution of ^{14}C-coumarin in 70% ethanol evenly spread over a 100 cm^2 marked area on the back to provide 22, 24 and 22 μCi; 1.8, 2.0 and 1.8 mg; 0.018, 0.020 and 0.018 mg/cm^2; and 0.023, 0.026 and 0.020 mg/kg body weight for volunteers 1, 2 and 3, respectively. The solvent was allowed to evaporate for 30 min and the treated area was then covered with gauze dressing. 6 h after dermal application, the residual coumarin was wiped off with 70% ethanol. A 2.5×2.5 cm area was then stripped with five successive applications of adhesive tape. The entire treated site was covered with fresh gauze for the duration of the study.

All urine passed was collected at intervals of 0–2, 2–4, 4–6, 6–12, 12–24, 24–48, 48–72, 72–96, and 96–120 hr after application in containers cooled to 4°C. All feces were collected at 24-h intervals for 5 days after application. Blood samples were collected in heparinized tubes immediately before application and at 15 min, 30 min, and 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 48, 72, 96 and 120 h after application of coumarin. Estimates of half-life of coumarin and metabolites in the body were based on visual inspection of the plots of μg-equivalents per ml of plasma vs time after application.

A urine sample collected at 0–2 h from one volunteer was hydrolyzed with β-glucuronidase, sulphatase and β-glucuronidase:sulphatase (20 h at 37°C) and analyzed by HPLC. An aliquot of the urine sample was also hydrolyzed with hydrochloric acid and analyzed using TLC and HPLC.

2.4. Measurement of radioactivity

Radioactivity was measured using either a Philips Automatic Liquid Scintillation Analyzer, model 4700 (Philips NV, Eindhoven, The Netherlands) or an LKB Analyzer model 1219 Rackbeta Spectral (Wallac Oy, Turku, Finland). Liquid samples (urine, plasma, sample extracts and dose measurement solutions for rats and humans; also cage washes for rats) were added directly to vials containing MI-31 scintillation cocktail (Canberra Packard Instrument Co. Ltd, Pangbourne, Berkshire, UK). Sample aliquots (feces and whole blood for rats and humans: adhesive skin strippings for humans; and gastrointestinal tracts with contents, liver, skin and spleen for rats) were combusted in oxygen using an Automatic Sample Oxidizer (Model 306 Mk 2 Tri-Carb, Canberra Packard Instrument Co. Ltd) prior to adding scintillation cocktail. For rats, whole small organs and portions of homogenates of tissues other than liver and spleen were treated with NCS solubilizer at 50–55°C for up to 24 h and added to the scintillation cocktail.

Radiolabeled components on TLC plates were detected by autoradiography using Hyperfilm β-max film (Amersham International plc, Amersham, UK), or were located and measured directly using Berthold TLC Linear Analyzer (Model LB 2832 or 2842). Metabolites were characterized by co-chromatographic comparison using standard reference compounds with different HPLC and TLC systems.

2.5. TLC

TLC was carried out on Merck F254 silica gel plates or Whatman $KC_{18}F$ reverse phase plates. Developing solvent systems included acetonitrile:water (80:20), hexane:ethyl acetate:acetic acid (80:20:1), dichloromethane:hexane (80:20) and chloroform:methanol:acetic acid (90:10:5). Components were characterized by their R_f values (ratio of the distance moved by a component/distance moved by mobile phase).

For establishing chromatographic correspondence of reference compounds with the radioactive metabolites in samples, the reference compounds were co-chromatographed with samples as follows. The reference compounds were *o*-hydroxyphenylacetic acid, 4-hydroxycoumarin, 7-hydroxycoumarin, 2-hydroxycinnamic acid, 3-hydroxycoumarin, 6,7-dihydroxycoumarin and coumarin. A solution of the reference compound was applied to the TLC plate as a 2-cm wide band. The test samples were also applied as a 2-cm wide band, half of which overlapped with the reference compound band. The developing solvent system was chloroform: methanol: acetic acid (90:10:5, by vol.). Co-chromatographic correspondence was assessed by visual inspection of the TLC plate.

2.6. HPLC analysis

HPLC was conducted using two different systems. System 1 included a Spectra-Physics Model SP 8800 pump and a Waters U6K universal injector (Millipore). A spherisorb S5 ODS1 (25 cm×4.6 mm i.d.) column (Hichrom, Reading, UK) was used with outlet linked to a Spectra-Physics Spectrophotometer fitted with a flow-through cell and operating at 254 nm. The eluate was passed through a Ramona D radioactive detector (Raytest Instruments, Sheffield, UK). Appropriate fractions containing the radioactive components were collected. The mobile phase was water containing 0.5% acetic acid in pump A and methanol in pump B. System 2 included a Waters 600E multisolvent delivery system (Millipore (UK) Ltd, Harrow, Middlesex, UK), a Waters 600 gradient controller and a Waters U6K universal injector (Millipore). A Hypersil BDS (25 cm×4.6 mm i.d.) column (Shandon, Runcorn, UK) was used with the outlet linked to a Waters 486 detector fitted with a flow-through cell operating at 254 nm. The eluate was passed through a Reeve Model 9701 radioactive detector with a Reeve Model 9702 precision mixer (Reeve Analytical, Glasgow, UK). Appropriate fractions containing the radioactive components were collected. The mobile phase was water containing 0.5% acetic acid in pump A and methanol in pump B.

3. Results

3.1. Recovery of radioactivity after dermal application under occlusion of ^{14}C-coumarin to male rats

Table 1 shows recovery of radioactivity in urine, feces, air from the metabolism cages, tissues, treated skin, skin washings, gauze and cage washes. Approximately 90% of the absorbed radioactivity was cleared within the first 24 h. A mean of 32.2% of the applied radioactivity was recovered in urine in the first 6 h following dermal administration. By 120 h, 50% of the applied dose was excreted in urine. Excretion of the radioactivity in feces was 7.2% at 12 h rising to 21% after 120 h. Recovery from the air from the metabolism cages was negligible. A total of 65% of the applied dose was absorbed during the 6-h period of contact with the skin under occlusion. At that time, approximately half of the absorbed dose had been excreted and half was recovered from tissues. The total absorption was to 72% after 120 h and at this time only about 1.3% of the dose remained in the tissues.

Peak plasma radioactivity was observed at 1 h after ^{14}C-coumarin application to rats (Fig. 2). Concentrations decreased with an estimated half-life of about 5 h and were not detectable (<0.01 μg/ml) at 48 h. Maximum concentrations of radioactivity, except for the treated skin, were observed in the large intestine, small

Table 1
Fate of ^{14}C-coumarin after dermal application of 1 mg/kg to male rats[a]

	Time (h after dose application)								
Sample	0.5	1	3	6	12	24	48	72	120
Urine	NC[b]	NC	NC	32.2	38.2	44.3	48.2	48.2	49.7
Feces	NC	NC	NC	0.09	7.23	17.5	14.08	18.9	21.2
Air	NC	NC	NC	0.17	0.15	0.07	0.10	0.14	0.08
Tissues	16.2	26.5	25.2	31.9	19.5	6.6	2.4	1.8	1.3
Total absorbed	64.4	65.1	68.5	64.8	69.0	72.3			
Treated skin	13.4	13.8	9.7	5.4	4.0	2.91	3.58	4.09	1.08
Skin washings	47.6	34.1	26.9	13.8	16.1	13.4	14.0	11.2	10.1
Gauze dressing	NC	NC	NC	0.33	0.26	0.16	0.15	0.13	
Cage wash	NC	NC	NC	0.94	1.02	1.73	0.82	0.31	0.34
Total recovery[c]	–	–	–	84.5	86.5	86.8	83.3	84.7	83.9

[a] Results are expressed a percent of coumarin dose and are the mean of two rats at each time point.

[b] NC, sample not collected.

[c] Radioactivity not accounted for was attributed to evaporation of some of the compound (due to its known volatility) immediately after application to the skin.

intestine, stomach, kidney and liver (Fig. 2). Peak levels in the large and small intestines were observed at 6 h and decreased rapidly thereafter. Peak levels in other tissues were observed at 1 h and by 120 h had generally declined to levels below or just above the limits of detection, with the exception of the liver where a mean concentration of 0.05 μg-equivalents/g of tissue was measured. Adrenal glands, fat, thyroid gland and whole blood demonstrated peak values at 1 h in the range of 0.13–0.53 μg-equivalents/g of tissue. Bone marrow, brain, eyes, heart, lymph nodes, muscle, pancreas, testes, thymus, spleen and untreated skin demonstrated peak values in the range of 0.04–0.10 μg-equivalents/g of tissue. The tissues (not shown in Fig. 2) showed decreases in time in a pattern similar to that of plasma. Radioactivity in treated skin decreased from approximately 5% of the applied dose at the end of 6 h of exposure to 1% at the terminal 120-h period.

3.2. Recovery of radioactivity after exposure of ^{14}C-coumarin to human volunteers for 6 h under simulated conditions of use

Peak plasma radioactivity was observed at 0.5–1 h after ^{14}C-coumarin exposure under simulated conditions of use to human volunteers (Fig. 3). The levels decreased to below the limit of detection by 8 h. The mean half-life of radioactivity in plasma after ^{14}C-coumarin exposure was approximately 1.7 h.

Table 2 shows the recovery for both the non-absorbed and absorbed radioactivity for each volunteer. Elimination of radioactivity in the urine was rapid with mean values of 26, 16, 7 and 9% of the applied radioactivity present at 0–2, 2–4, 4–6 and 6–120-h time periods. Total mean urinary excretion of the applied coumarin dose was 59% (Table 2). Excretion of radioactivity in the

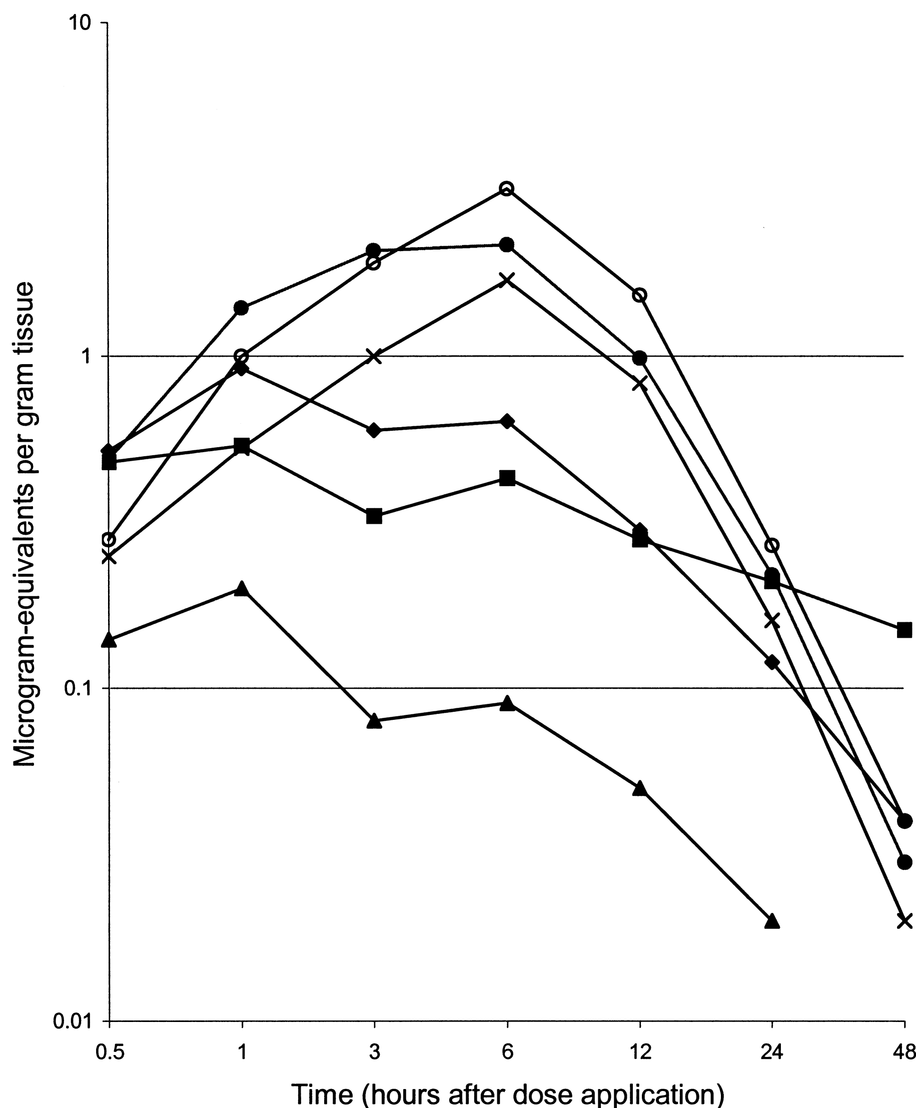

Fig. 2. Concentration of radioactivity in rat tissues after dermal application of 1 mg/kg ^{14}C-coumarin. Measurements at 0.5, 1, 3, 6, 12, 24 and 48 h demonstrate the different compartmentalization for the stomach (×), large intestine (○), small intestine (●), kidney (◆) and liver (■) relative to the plasma (▲) compartment. Radioactivity measurements are the means of two rats at each time point. See the text for description of other tissues.

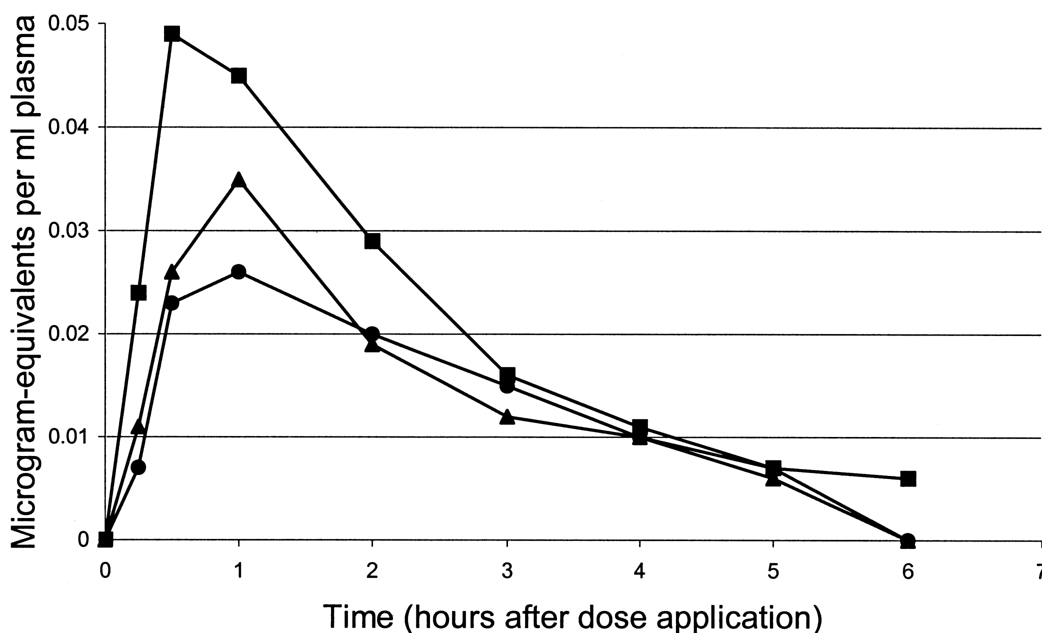

Fig. 3. Concentrations of radioactivity in plasma obtained from male volunteers 1 (▲), 2 (■) and 3 (●) after dermal application of ^{14}C-coumarin to a 100 cm^2 area at an application rate of 0.02 mg/cm^2. The limit of detection was considered to be less than 0.005 μg-equivalents per ml.

feces of human volunteers during the entire study period was only a mean value of 1.1%.

Tape stripping of a 6.25 cm^2 area of the 100 cm^2 dose site immediately after removal of the dose from the surface recovered a mean value of 0.06% of the applied radioactivity. When extrapolated to the entire area of dosing, this demonstrates only about 1% of the applied dose remaining near the surface of the skin at 6 h. After 120 h the gauze dressing contained a mean value of 0.27% of the applied dose.

3.3. Coumarin metabolism in rats and humans after dermal exposure to ^{14}C-coumarin

Fig. 4 shows the radioactive components of rat urine after a 6-h occluded dermal application of ^{14}C-coumarin. The only metabolite (E) identified was *o*-hydroxyphenylacetic acid, which accounted for 10% of the applied dose after 6 h and 23% after 48 h (43% of the total urinary excretion). At least 20 other components in urine were detected but not identified. Components A, D and H each accounted for 5–7% of the applied dose after 48 h; all others were 1% or less. None of the components corresponded to parent coumarin or to 3-, 4- or 7-hydroxycoumarins, 6,7-dihydroxycoumarin or *o*-hydroxycinnamic acid. Treatment of an aliquot of 0–6-h urine with β-glucuronidase/sulphatase or with 6 M HCl at 90°C for 1 h did not significantly change the radioactive profiles. No more than 1–2% of the dose could be accounted for by glucuronide:sulphate conjugates.

The analysis of the radioactive components in the urine of humans exposed dermally to ^{14}C-coumarin revealed five components, as shown in Fig. 5. Three peaks were identified as 7-hydroxycoumarin and its β-glucuronide and sulphate conjugates. A small fourth component was identified as *o*-hydroxyphenylacetic acid and a small fifth component was not identified but appeared to be a glucuronide. Components were identified by treatment of the 0–2-h urine sample of volunteer 1 with β-glucuronidase, sulphatase or β-glucuronidase: sulphatase, as shown in Fig. 5 and quantitated in Table 3. Table 4 shows the mean values for the three volunteers during urinary collection periods through 12 h. Thus,

Table 2
Recovered radioactivity as percent of applied dose after dermal exposure of human volunteers to ^{14}C-coumarin under simulated use conditions[a]

Samples	Volunteer 1	Volunteer 2	Volunteer 3	Mean±SD
6-h Gauze and skin washes[b]	12.7	1.7	4.5	6.3±5.7
6-h Tape strippings[b]	0.1	0.02	0.06	0.06±0.04
6-h Tape strippings ×16[bc]	1.6	0.32	0.96	0.96±0.64
120-h Gauze[b]	0.3	0.2	0.3	0.27±0.06
Total urine (0–120 h)	53.6	61.1	61.2	58.6±4.4
Total feces (0–120 h)	0.9	1.3	1.0	1.07±0.21
Total absorbed	54.5	62.4	62.2	59.7±4.5
Total recovered	67.6	64.3	67.1	66.3±1.8

[a] The radioactivity (μCi), applied as ^{14}C-coumarin, was 21.68, 23.93 and 21.84 for volunteers 1, 2 and 3, respectively, administered topically to an area of 100 cm^2.

[b] Non-absorbed radioactivity.

[c] Value representing total% dose that would have been removed if the total area had been stripped; 16 = 100 cm^2/6.25 cm^2.

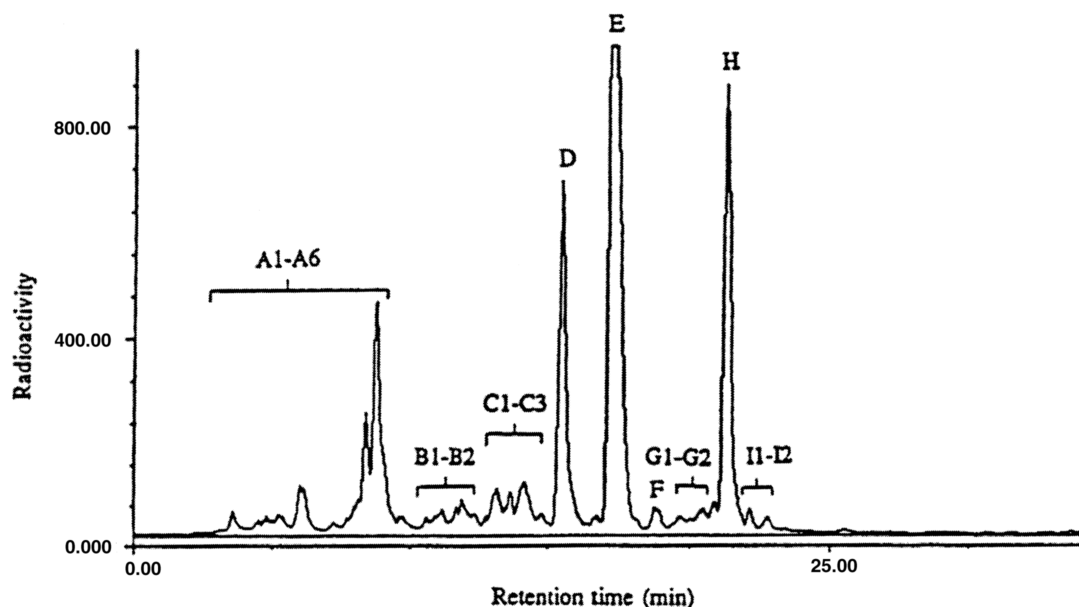

Fig. 4. HPLC radiochromatogram of urine from rats at 0 to 6 h after dermal application of ^{14}C-coumarin. Peaks with letters were quantified, but only peak E (*o*-hydroxyphenylacetic acid) was identified.

after 12 h, approximately 51% of the applied radioactivity was excreted in the urine as 7-hydroxycoumarin, primarily as the glucuronide conjugate. Less than 1% of the applied dose was excreted as *o*-hydroxyphenylacetic acid. Components corresponding to 6,7-dihydroxycoumarin, 3-hydroxycoumarin, *o*-hydroxycinnamic acid, 4-hydroxycoumarin and unchanged coumarin were not detected in urine.

Treatment of the urine sample with 0.6 M or 6 M HCl at 37°C for 20 h resulted in partial hydrolysis of the glucuronide and sulfate metabolites of 7-hydroxycoumarin. Refluxing the urine sample with 6 M HCl for 1 h was effective in completely hydrolyzing these conjugated metabolites with concomitant increases in the amount of 7-hydroxycoumarin.

4. Discussion

Coumarin was rapidly and extensively absorbed in vivo both by rats under occlusion and by humans under simulated conditions of exposure from use in an alcohol-based product. This was demonstrated by the peak plasma levels at 1 hour or less (Figs 2 and 3), the percent of absorbed dose at 120 h after initiation of dosing (about 60% for humans and 71% for rats) and the observation of about 32% of the applied dose in the tissues of rats at the end of the 6-h exposure period. This rapid and extensive absorption following dermal exposure in humans and rats under in vivo conditions is consistent with the reported in vitro data and with studies by the oral and ip routes of administration (Lake, 1999).

There was no evidence for a significant reservoir of coumarin in the skin after dermal application. In rats, only 5% of the applied dose was in the treated area of whole skin after 6 h of occluded contact and this decreased to 1% over the following 5 days. In humans, after 6 h of dermal contact there was no more than 1% of the applied dose in the outer layers of the stratum corneum (five tape strippings) (Table 2). The gauze dressing in contact for the following 5 days showed no more than 0.3% of the applied dose, consistent with some exfoliation of the outer layers of skin.

Beyond the similarities for dermal absorption there were marked differences in elimination pathways between humans and rats. The differences in urinary and fecal excretion are striking. In rats after 120 h, 50% of the applied dose was excreted in the urine and 21% in the feces, while in humans after 120 hr, 59% was excreted in the urine and only 1% in the feces (Tables 1 and 2). Shilling et al. (1969) reported similar results in humans after oral administration of coumarin. This suggests little or no biliary excretion of coumarin metabolites by humans. On the other hand, in rats, the significant fecal excretion and the relatively high concentrations in the liver and large and small intestines (Fig. 2) indicate significant biliary excretion. This is consistent with the results of Williams et al. (1965), who reported biliary excretion of 50% of the dose in 24 h as unidentified ring opened compounds after oral or ip administration of coumarin at 50 mg/kg.

The tissue distribution (Fig. 2) demonstrates the compartmentalization of coumarin for rats. The slower decrease of concentrations in the liver and the delayed peak levels in the large and small intestines are consistent

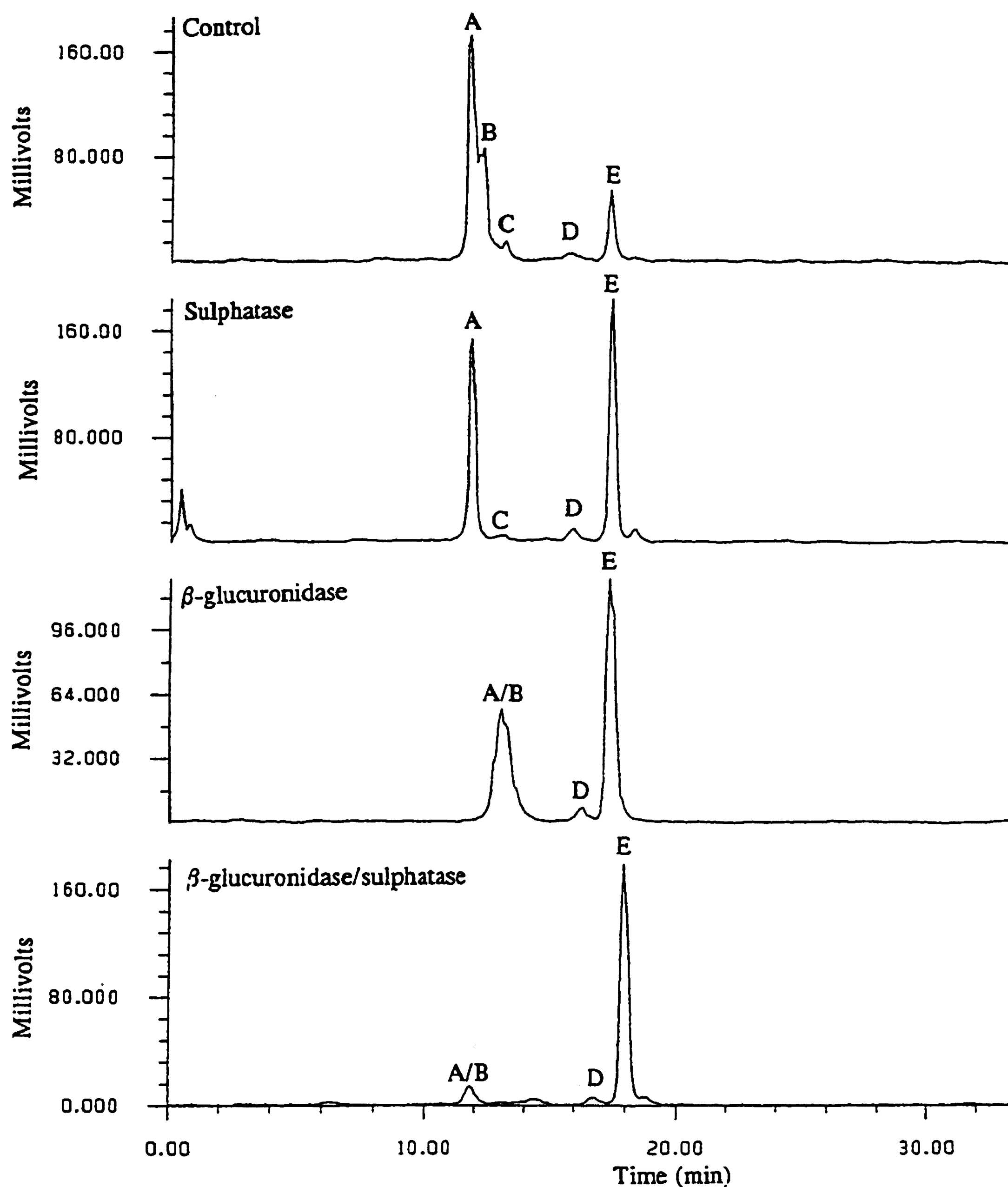

Fig. 5. HPLC radiochromatograms of 0–2 h urine after dermal application of ^{14}C-coumarin to volunteer 1. The top graph shows component metabolites before treatment with enzymes. Metabolites are 7-hydroxycoumarin glucuronide (A), 7-hydroxycoumarin sulphate (B), an unidentified metabolite (C), *o*-hydroxyphenylacetic acid (D) and 7-hydroxycoumarin (E).

with the significant biliary excretion of coumarin by the rat. On the other hand, the similar patterns between the kidney and the plasma are consistent with the significant urinary excretion. The other tissues, not shown in Fig. 1, contained lower concentrations with rates of decrease similar to plasma. The persistence in the liver and the low levels in the lung of rats may be relevant to the observed hepatotoxicity in rats but lack of lung toxicity in rats, in contrast to the lung toxicity reported for mice (Born et al., 1998a; Carlton et al., 1996). Also notable was the longer half-life in plasma of rats (about 5 h) compared with humans (about 1.7 h) following dermal exposure. The value in humans is similar to a report of 1.5–1.8 h after iv administration to humans (Ritschel et al., 1976).

The in vivo metabolism of coumarin after dermal application shows the same differences between humans and rats as found by other routes and recently reviewed by Lake (1999). For humans, the data illustrated in Fig. 5 is entirely consistent with reports by other routes, that is, primary metabolism to 7-hydroxycoumarin and its conjugates with minor metabolism to *o*-hydroxyphenylacetic acid. For rats, the finding of *o*-hydroxyphenylacetic acid as a primary metabolite following

Table 3
Radioactive components as a percent of dose excreted in the 0–2-h urine sample of volunteer 1. Effect of β-glucuronidase, sulphatase and β-glucuronidase:sulphatase[a]

Component	Untreated	Sulphatase	β-Glucuronidase	Combined β-glucuronidase: sulphatase
Unidentified	1.2	1.0	nd	1.2
7-HCG	11.2	9.4	1.3	2.1
7-HCS	6.6	nd	9.2	0.2
o-HPAA	1.1	1.2	0.9	1.0
7-HC	3.6	11.9	13.0	19.3

[a] 7-HCG, 7-hydroxycoumarin glucuronide; 7-HCS, 7-hydroxycoumarin sulfate; *o*-HPAA, *o*-hydroxyphenylacetic acid; 7-HC, 7-hydroxycoumarin; nd, not detected.

Table 4
Proportions of radioactive components excreted in human urine after dermal exposure to ^{14}C-coumarin. Results are the mean of three volunteers and are expressed as percent of dose[a]

	Urine collection period			
Component	0–2 hr	2–4 h	4–6 h	6–12 h
Unidentified	1.70±0.9	0.53±0.29	0.13±0.23	0.23±0.40
7-HCG	16.5±4.9	9.93±4.1	4.87±1.7	5.13±3.2
7-HCS	6.57±2.9	4.47±1.3	1.90±0.6	0.30±0.5
o-HPAA	0.33±0.30	0.33±0.30	0.17±0.29	nd
7-HC	1.17±1.02	0.47±0.45	nd	nd

[a] 7-HCG, 7-hydroxycoumarin glucuronide; 7-HCS, 7-hydroxycoumarin sulfate; *o*-HPAA, *o*-hydroxyphenylacetic acid; 7-HC, 7-hydroxycoumarin; nd, not detected.

dermal application (Fig. 4) was consistent with data for other routes. The more than 20 unidentified components of radioactivity in the urine of rats leaves many unanswered questions about the metabolism of coumarin in rats following dermal application.

In these studies, the coumarin exposure judged to be most relevant to humans was from a 70% alcohol solution because this represents formulations containing the highest levels of fragrance ingredients. While recovery of ^{14}C-coumarin is reported as about 72% in rats and 60% in humans, it is considered that the difference in recovery from percent applied is due to some evaporative loss promoted by the ethanolic solution as has been seen by others (Yourick and Bronaugh, 1997).

Yourick and Bronaugh (1997) reported in vitro absorption with rat skin of 50% after 24 h from ethanol solution, but a higher absorption of 64% after 24 h using human abdominal skin. They further reported (in vitro) absorption from an oil/water emulsion vehicle to be as high as 97% with human abdominal skin and 99% with rat skin. Furthermore, higher in vitro absorption has been reported from human scalp skin than from abdominal skin (Ritschel et al., 1989). In the light of these reports and the 60% absorption from the in vivo study involving skin on the back presented here, it seems prudent for any safety evaluation of coumarin to consider the specific use of the specific product containing coumarin.

In summary, this in vivo study after dermal exposure of rats and humans to coumarin is consistent with the reported similarities and differences between humans and rats treated by other routes of administration. The rat is a very poor model for humans and toxicity in the rat cannot be extrapolated to humans.

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