D&C Red No. 27
[CASRN 13473-26-2]

D&C Red No. 28
[CASRN 18472-87-2]

Prepared October 2000

SUMMARY

D&C Red No. 27 and D&C Red No. 28 are fluorescein-based dyes that were approved in 1982 for use in drugs and cosmetics (except the eye area). Cosmetic retail products containing these dyes are primarily lipsticks and blushers. Dyes physically associated with non-aqueous aluminum or zirconium minerals (lakes) are used in lipsticks, blushers, make-up preparations, hair dyes and colors, rouges and face powders. The toxicological data reviewed by FDA prior to approval of D&C Red No. 27 and D&C Red No. 28 did not include studies of acute phototoxicity or chronic phototoxicity (*i.e.* photocarcinogenesis).

Our current knowledge of the photochemistry and photobiology of D&C Red No. 27 and D&C Red No. 28 raises new concerns about the long-term safety of these colorants. D&C Red No. 28 is now known to be an extremely efficient photodynamic sensitizer whose photo-excitation results in the formation of free radicals and singlet oxygen. These highly reactive species attack cellular components such as lipids, proteins and nucleic acids. Investigators have shown that the genetic damage sensitized by D&C Red No. 28 is mutagenic in bacterial assays. In addition, *in vitro* studies using mammalian cells have shown that D&C Red No. 28 sensitizes photooxidation of guanine bases in cellular DNA (*i.e.* 8-oxo-7,8-dihydroguanine), a lesion known to be mutagenic.

Clinical data and anecdotal reports indicate that fluorescein, and halogenated derivatives of fluorescein, are phototoxic under certain conditions of exposure. Fluorescein is frequently used in ophthalmic examinations and retinal angiography. There have been a number of anecdotal reports of ocular and cutaneous phototoxicity following intravenous administration of fluorescein for this application. Furthermore, halogenated fluorescein dyes are phototoxic if applied topically to scarified skin, but are not phototoxic if applied to intact skin. These results indicate that delivery of the dye to the skin is an important factor to consider in evaluating the potential phototoxicity and photocarcinogenicity of dyes such as D&C Red No. 27 and D&C Red No. 28.

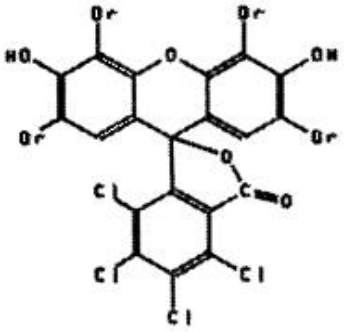
TABLE OF CONTENTS

1.0 BASIS FOR NOMINATION

D&C Red No. 27 and D&C Red No. 28 were selected by the Center for Food Safety and Applied Nutrition for percutaneous absorption and subsequent photocarcinogenicity studies (if percutaneous absorption is significant). Concerns about photocarcinogenicity arise due to the photodynamic activity of these dyes. D&C Red No. 27 and D&C Red No. 28 efficiently photosensitize the formation of reactive oxygen species. These colorants also photosensitize damage to DNA. Structurally related halogenated fluorescein dyes have been found to be photogenotoxic.

2.0 INTRODUCTION

D&C Red No. 27
CASRN 13473-26-2
Color Index No. 45410:1
or 45410:A
RTECS # LM5870000



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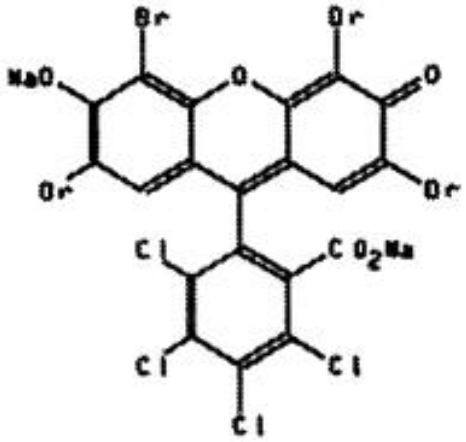
RTECS # LM5900000



2.1 Chemical Identification

The following synonyms are used for D&C Red No. 27:

- 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein or 2',4',5',7'-tetrabromo-4,5,6,7-tetrachloro-3',6'-dihydroxyspiro[isobenzofuran-1(3H), 9'-[9H]xanthen]-3-one
- CI Solvent Red 48
- Acid Phloxine B

The following synonyms are used for D&C Red No. 28:

- Disodium 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein
- Phloxine B
- CI Acid Red 92

2.2 Physical-Chemical Properties of D&C Red No. 27 and D&C Red No. 28

Property	D&C Red No. 27	D&C Red No. 28
Formula[1]	$C_{20}H_4O_5Br_4Cl_4$	$C_{20}H_2O_5Br_4Cl_4Na_2$
Formula weight[1]	785.69	829.65
UV Absorption		
λ_{max} (water)	557 (ethanol)[2]	537[1] (water)
ε	13,480	101,220[1]
Solubility[1]		
Water	insol.	sol.
Glycerol	insol.	sol.
Ethanol	slightly sol.	sol.
Ether	insol.	insol.

[1]Marmion, 1991.
[2]Spectral data obtained in Cosmetic Toxicology Branch/CFSAN using a certified lot of D&C Red No. 27.

D&C Red No. 27 and D&C Red No. 28 are a conjugate acid, base pair. The transition from D&C Red No. 27 to D&C Red No. 28 occurs between pH 3.4 and pH 5.0 (CRC, 1986).

2.3 Photochemical Properties of D&C Red No. 27 and D&C Red No. 28

The photochemical properties of halogenated fluorescein dyes have been extensively studied. These dyes are characteristic photodynamic sensitizers, *i.e.*, photo-excitation of the dye results in the formation of free radicals (type I photodynamic mechanism) or singlet oxygen (type II photodynamic mechanism). Data available suggest that both type I and type II mechanisms are important for D&C Red No. 28. The photochemistry of D&C Red No. 27 has not been described, possibly because of its low solubility in solvents commonly used in photochemical studies and the low absorptivity of this compound.

Type I Photo dynamic Activity:
Photolysis of aqueous D.C. Red No. 28 (10 ppm) using sunlight leads to sequential loss of bromine substituents (Wang *et al.*, 1998). Loss of bromine presumably results in the formation of free radicals, although direct demonstration of free radical formation has not been reported.

Type II Photodynamic Activity:
D&C Red No. 28 has been shown to be one of the most efficient of the halogenated fluorescein dyes for photosensitizing the formation of singlet oxygen, a powerful oxidizing agent. Gandin *et al.* (1983) studied singlet oxygen formation sensitized by a series of halogenated fluorescein dyes. Dyes were dissolved in phosphate buffer (pH 7) and irradiated with visible light restricted to wavelengths greater than 455 nm. Singlet oxygen formation was quantified by measurement of the reaction between singlet oxygen and diphenylisobenzofuran. The quantum yield for singlet oxygen formation sensitized by D&C Red No. 28 was determined to be 0.65, *i.e.*, 65 % of the energy absorbed by D&C Red No. 28 was channeled into the formation of singlet oxygen. Only

rose bengal was found to be a more powerful sensitizer than D&C Red No. 28. Arakane *et al.*, 1996 have obtained similar results using direct measurement of singlet oxygen's near-infrared emission. D&C Red No. 28, dissolved in methanol and irradiated at 514.5 nm with an argon laser, produced singlet oxygen with an efficiency near that of rose bengal.

3.0 PRODUCTION PROCESSES

D&C Red No. 27 is required to contain not less than 90% 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein (Code of Federal Regulations, 2000a). 2',4',5',7'-Tetrabromo-4,5,6,7-tetrachlorofluorescein is manufactured by brominating 4,5,6,7-tetrachlorofluorescein with elemental bromine (Code of Federal Regulations, 2000a). The 4,5,6,7-tetrachlorofluorescein is manufactured by the acid condensation of resorcinol and tetrachlorophthalic acid or its anhydride. The 4,5,6,7-tetrachlorofluorescein is isolated and partially purified prior to bromination.

D&C Red No. 28 is required to contain not less than 85% of the disodium salt of 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein (Code of Federal Regulations, 2000a). The disodium salt of 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein is formed by alkaline hydrolysis of the parent tetrabromotetrachlorofluorecein (Code of Federal Regulations, 2000a).

4.0 REGULATORY STATUS

The US Food, Drug and Cosmetic Act, Chapter VIIB, Section 721, requires pre-market approval for color additives intended for use in foods, drugs, devices and cosmetics. D&C Red No. 27 and D&C Red No. 28 were approved as certifiable color additives for coloring drugs and cosmetics except for the eye area in 1982 (Code of Federal Regulations, 2000a).

The U.S. Environmental Protection Agency has issued an experimental use permit for use of D&C Red No. 28 as a photo-activated pesticide (Federal Register, 1995).

5.0 OCCURRENCE IN COSMETICS

Data available from FDA's Voluntary Cosmetics Registration Program (VCRP), compiled in accordance with Title 21 part 270.4 (d)(1) of the Code of Federal Regulations (Code of Federal Regulations, 2000b), indicate that D&C Red No. 27 is found in 72 cosmetic formulations. Four hundred and seventy one cosmetic products contain D&C Red No. 27 in an aluminum or zirconium lake. Data available from the VCRP indicate that D&C Red 28 is found in 35 cosmetic products and, as an aluminum lake, in an additional 45 cosmetic formulations.

D&C Red No. 27 and D&C Red No. 28 are primarily used in lipsticks and blushers. Lakes containing D&C Red No. 27 and D&C Red No. 28 are used in lipsticks, blushers, makeup preparations, hair dyes and colors, rouges, face powders, bath oils, tablets and salts (Cosmetic, Toiletry, and Fragrance Association, 1999).

6.0 TOXICOLOGICAL STUDIES

6.1 Studies Reviewed for Color Additive Approval

Studies submitted as part of the petition for approval of D&C Red No. 27 and D&C Red No. 28 as color additives in drugs and cosmetics are listed below:

- Mouse Long-Term Feeding Study: (Litton Bionetics In., LBI Study No. 20843) Groups of 60 male and 60 female Charles River CD-1 mice were fed diets containing 0.125, 0.5, and 5.0% D&C Red No. 27 for 18 months. No significant differences in body weights or histopathology were noted in groups receiving D&C Red No. 27.
- 30-Month Chronic Toxicity and Potential Carcinogenicity Study in Rats with *In Utero* and Lifetime Exposure to D&C Red No. 27 in the Diet. (Litton Bionetics, Inc., LBI Project No. 20842) Groups of 60 male and 60 female weanling Charles River CD strain [CRL: COBS CD (SD) BR] rats were fed diets containing 0.25, 1.00, and 2.00% D&C Red No. 27 for eight weeks prior to mating and through mating and gestation, and lactation and weaning of litters. These animals were the parental or F_0 generation in the study. When weanlings (i.e. the F_1 generation) were five weeks old, they were separated from the dams and cycled through the same treatment regimen as the F_0 generation. No significant treatment related effects on body weight, organ weight, fertility, clinical chemistry or histopathology were seen in either the F_0 or F_1 generations.
- Two-year chronic oral toxicity of D&C Red No. 27-Albino Rats. (Industrial Bio-Test Labs, Inc.-Final Report, May 3, 1965) D&C Red No. 27 was fed in the diet to weanling Holtzman (Sprague-Dawley strain) rats at levels of 0.015, 0.25 and 1.0% for 2 years. Test groups consisted of 25 male and 25 female animals and control groups consisted of 65 male and 65 female animals. There were no significant treatment related effects on body weight, organ weight, hematologic determinations and urinanalysis.
- Two-year chronic oral toxicity of D&C Red No. 27-Beagle dogs. (Industrial Bio-Test Labs, Inc.-Final Report, April 15, 1965) D&C Red No. 27 was fed in the diet to pure-bred beagles at levels of 0.015, 0.25 and 1.0%. Each test group consisted of three males and three females while each control group consisted of six males and six females. The study duration was 720 days. There were no significant treatment related effects on blood chemistries, urinanalysis determinations, behavior, body weight, food consumption, or histopathology.
- Dermal study in rabbits (Leberco Laboratories, Assay No. 20737, May 27, 1968) D&C Red 27, in hydrophilic or U.S.P. white ointment was applied to the skin of adult, female rabbits, daily, 5 days/week. Topical treatments included 0.1% and 1.0% D&C Red No. 27. Control groups received white ointment only. Groups of 6 rabbits were assigned to two treatment durations, 28 days or 91 days. For each treatment duration, three rabbits received treatment on intact skin and three rabbits were treated on abraded skin. Body weights and time for abraded skin to heal was not affected by treatment with D&C Red 27. No gross and histopathological changes in the internal organs could be attributed to topical treatment with D&C Red 27.
- Repeated dermal applications in mice (Hazleton Laboratories, Inc., Report No. 444-119, March 31, 1969) D&C Red No. 27, 0.1 ml of a 1% aqueous suspension was applied to the clipped dorsal skin of ICR mice twice weekly for 18 months. There were 50 male and

50 female mice in test and control groups. There were no treatment related effects on survival, or skin lesions.

- Acute oral toxicity-rats. (Industrial Bio-Test Labs, Inc., August 14, 1962) D&C Red No. 27 was administered by stomach tube to groups of 4 Sprague Dawley rats in doses of 4.6, 6.8, 10.2, 15.4 and 23.1 g/kg. The rats were then observed for 14 days. No effects were seen at the lowest dose, sedation and anorexic were observed within one hr of treatment with doses of 6.8 and 10.2 g/kg. Two rats from each of these groups died, the remaining appeared normal after 36 hr. Rats treated with 15.4 and 23.1 g/kg all died within 96 hr. Dye was excreted percutaneously and in urine and feces. The oral LD_{50} was calculated to be 8.4 Å 1.4 g/kg.
- Acute oral toxicity in dogs. (Industrial Bio-Test Labs., Inc., Sept. 15, 1962.) D&C Red 27 was administered in gelatin capsules, at doses of 2.02, 3.04, and 4.56 g/kg to groups of 1 male and 1 female dog. Animals were observed up to 14 days. All dogs vomited from 50% to 90% of the dose, making calculation of the LD_{50} not possible.
- 18-week feeding study in rats. (Hansen, Fitzhugh and Williams, 1958) D&C Red No. 27 was fed at levels of 0, 0.25, 0.5, 1.0 and 2.0% for 18 weeks to groups of 5 male and 5 female weanling rats. There were no significant effects on hematologic determinations, food consumption or organ weights. The only effect was a depression of weight gain in rats receiving 2.0% dietary D&C Red No. 27.
- Three generation reproduction study in rats. (International Research and Development Corp., Feb. 22, 1974). D&C Red 27 was fed in the diet at dosage levels of 0, 5, 50, 150 and 500 mg/kg/day in a multi-generational reproduction study using Charles River CD rats. Each parental test level consisted of 10 males and 20 females. Tests diets were fed to the original parents (*i.e.* F_0 generation) two weeks prior to mating. and was continued throughout the study. No test compound effects were seen on body weight of pups, fertility, litter size and sex composition, viability or survival. Histopathological examination of tissues from generations F_1 and F_2 showed no treatment related effects.
- Metabolism in rats. (Webb, Fonda and Brouwer, 1962). D&C Red No. 27 was administered to groups of 6 Osborne-Mendel rats by stomach tube (0.5 to 500 mg) or intravenously (3 mg/kg). D&C Red No. 27 was found only in the bile, as unchanged dye, after oral administration. An average of 65.6% of the dose was recovered in the bile within 2 hr after intravenous administration.

6.2 Genotoxicity and Photogenotoxicity

A number of bacterial assays for genotoxicity and photogenotoxicity have been reported for halogenated fluorescein dyes including D&C Red 28. Kada *et al.*, 1972, reported using a "rec-Assay" procedure for identifying mutagens and photomutagens. This assay is based on the observation that recombination repair-deficient bacteria (*i.e.*, rec^-) are more sensitive to DNA damaging agents than bacteria capable of recombination repair (*i.e.*, rec^+). If a test compound shows higher inactivation of the rec^- strain than the rec^+ strain, that compound is scored as mutagenic. Treatment of *Bacillus subtilis* rec^- and rec^+ strains with a dye closely related to D&C Red No. 28 (*i.e.* potassium salt instead of sodium salt) followed by UV irradiation resulted in a mutagenic response. These investigators also conducted reverse mutations assays using *Escherichia coli* B/r WP_2 requiring tryptophan. Bacteria were held in broth containing the dye at concentrations between 0 and 45 µg/ml for 20 min. Bacteria were then washed and plated for

identification of revertants. While there was no exposure to UV radiation in this particular study, the dye was mutagenic in the *E. coli* strain.

Yoshikawa *et al.*, 1978, reported that D&C Red 28 was positive in the rec^+/rec^- previously described by Kada *et al.*, 1972. Phloxine B in the presence of radiation from a fluorescent bulb (UV radiation) showed dramatic DNA-damaging effects in this assay system. A second assay for DNA damage was performed by Yoshikawa *et al.*, 1978, where transforming DNA (from *B. subtilis* 45T) was treated with dyes and UV radiation. The treated DNA was added to *B. subtilis* 168 met^- cells. After waiting 30 min for the DNA to be taken up by the *B. subtilis* 168 met^- cells, the cells were plated in minimal-agar and incubated for 48 hr. The number of transformants (met^+) was then counted. Treatment of DNA with phloxine B and UV radiation was found to dramatically reduce the number of transformants indicating the DNA-damaging effects of phloxine B in the presence of UV.

Bockstahler *et al.*, 1990, have demonstrated that halogenated fluorescein dyes (D&C Red No. 21, FD&C Red No. 3, D&C Yellow No. 8) photosensitize damage to normal human skin fibroblasts, resulting in an inability of the cells to support the growth of herpes simplex virus (*i.e.*, the host capacity of human skin fibroblasts). Previous studies have shown that loss of host capacity is closely associated with DNA damage to the host cell. Human skin fibroblasts were incubated in the dark with the dyes in concentrations ranging from 2 µg/ml to 32 µg/ml for 45 to 60 min. Cells were then irradiated with visible light from Sylvania "Cool White" fluorescent bulbs (0 to $6x10^4$ J/m^2). Immediately after irradiation, cells were infected with herpes simplex virus and plaque formation was assessed 3 days later. All the halogenated fluorescein dyes tested were effective in eliciting light-induced reduction of skin fibroblast host capacity for herpes simplex virus. A relationship between the structure of the dye and its efficacy in reducing host capacity was notable. Dyes halogenated with iodine or bromine were more active than the unsubstituted dyes under these test conditions.

Wei *et al.*, 1995, have shown that halogenated fluorescein dyes, including D&C Red No. 28, photosensitize oxidative damage to cellular RNA and DNA and was measured through the formation of 8-oxo-7,8-dihydroguanine (8-oxoG). Human skin fibroblasts were exposed to 20 µM D&C Red. 28 for 2 hr, then irradiated with 3.3 J/cm^2 visible light from filtered Sylvania "Cool White" fluorescent bulbs. Cellular DNA and RNA were isolated immediately after irradiation. Levels of 8-oxoG in RNA were 9.13Å1.26 8-oxoG/10^5 G and 3.59Å1.68 8-oxoG/10^5 dG in DNA. D&C Red No. 28 was the most efficient sensitizer of photooxidation for the three halogenated fluorescein dyes studied.

6.3 *In Vivo* and Clinical Studies

Fluorescein is frequently used in ophthalmology. Fluorescein is topically applied to assess corneal damage or intravenously injected for retinal angiography. Danis and Stephens, 1997, have reported the effect of intravenously injected sodium fluorescein on the sensitivity of patients to sunlight. Intravenous administration of sodium fluorescein for retinal angiography resulted in marked cutaneous erythema, edema, and pain in skin that was exposed to direct sunlight within 1 hr of treatment.

Phototoxic reactions elicited by topically applied halogenated fluoresceins have been reported in an experimental model and clinical tests. Using rabbits, Morikawa *et al.*, 1976, examined the phototoxicity of D&C Red No.22 (structurally similar to D&C Red No. 28 but lacking chlorine substituents) and rose bengal. Dyes were administered intravenously, or topically to intact or abraded abdominal skin. Following administration of the dyes, the abdominal skin was exposed to visible light (fluorescent bulbs) or to sunlight. All animals receiving intravenous injection of dyes showed marked phototoxic reactions. Animals topically treated with dyes on intact skin showed no phototoxic response. In contrast, animals topically treated on abraded skin showed a phototoxic reaction to both visible light and sunlight. In a clinical study, Kaidbey and Kligman (1978) also examined the phototoxicity of D&C Red No. 22 and rose bengal. Fifty microliters of a 1% aqueous solution were applied to 3.2 square cm of intact or scarified skin. Treated sites were occluded for 6 hr and then irradiated (17.5 J/cm^2) with a solar simulator filtered to remove UVB (290 - 320 nm) radiation. Reactions were assessed immediately, and after 24 hr and 48 hr. Topical treatment of intact skin resulted in no phototoxic reaction. However, topical treatment of scarified skin with D&C Red No. 22 and rose bengal produced a marked immediate phototoxic response. This immediate response was characterized by development of “wheals” surrounded by an intense “flare.” By 24 hr and 48 hr, moderate to intense erythema and edema were present in 6/10 subjects treated with D&C Red No. 22, and 5/8 subjects treated with rose bengal.

The above studies illustrate two characteristics of the skin’s responses to fluorescein dyes. One characteristic is the immediate phototoxic response of the skin following irradiation. This immediate, “wheal and flare” response is thought to be typical for phototoxicity elicited by photodynamic sensitizers. The second characteristic is the phototoxicity of fluorescein dyes may be limited by their percutaneous absorption. Only those treatments which increase delivery of dyes into skin (intravenous injection or topical treatment of abraded/scarified skin) result in phototoxicity.

7.0 REQUESTED STUDIES

Since exposure of viable layers of skin to a photosensitizer is a prerequisite for eliciting phototoxicity or photocarcinogenesis, measurement of the percutaneous absorption is requested for both D&C Red No. 27 and D&C Red No. 28 using the animal model selected for phototoxicity and photocarcinogenesis assays. If these colorants are found to penetrate skin, studies of phototoxicity and photocarcinogenesis are requested.

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