Protocol LRC-5A

PRIMARY IRRITATION OF MUCOUS MEMBRANE
THE RABBIT EYE IRRITATION TEST

I. PURPOSE

To evaluate the potential of the test compound to produce injury to human mucous membranes.

II. RATIONALE

Cigarette additives may come in contact with the mucous membranes of the eye, nose, mouth, upper and lower respiratory tract and pulmonary alveoli. Hence, it is appropriate to determine whether there is an irritant potential on these sites. The classical Draize rabbit eye irritation test (1,2,3) has been used to predict the irritant potential of a compound on these mucous membranes in humans. Research indicates that the dosage employed should demonstrate the potential of a compound to produce injury to these membranes and to allow for an estimate of the safety factor for the actual use dose.

III. MATERIALS AND METHODS

A. Animals

New Zealand albino rabbits of either sex and 2-4 kg body weight are used. The animals are housed, according to the Animal Welfare Act (4), in individual cages without bedding in an air conditioned room at 21 $\pm$ 3°C, 50 $\pm$ 10% RH, alternate 12 hours light and 12 hours darkness. They are fed commercial food pellets and water freely. After at least three days of isolation, healthy animals are selected for use. Each animal will be assigned a number unique to it within the population making up the study. Each animal will have its unique number written in its ear with indelible ink. This number will be placed on each individual animal's cage as well as each cage position within the rack. All data records and specimens will have each animal's individual identification number. Body weights will be recorded within two days prior to the initiation of dosing.

Within 24 hours prior to dosing both eyes of each rabbit will be examined by an experienced investigator using a slit lamp biomicroscope. An additional examination of all eyes will be performed under U.V. illumination following application of one drop of 2% sodium fluorescein (U.S.P.) and one minute afterward an isotonic sodium chloride (U.S.P.) rinse. Six rabbits that are free of ocular lesions will be used in this study. Ocular examinations will include observations of the conjunctiva, iris and cornea as well as examination of the lens.

B. Test Description

The test material will be instilled into the lower conjunctival sacs of the right eye; the left eyes will serve as untreated controls. Instillation will be accomplished by pulling the lower lid away from the eyeball to form a cup into which a single dose of 0.1 ml/ or 100 mg (finely ground) of the test material will be placed. The upper and lower lids will be gently held together for approximately one second to ensure distribution of the test material throughout the eyeball and conjunctiva.

C. Scoring

1. Scoring Method: All eyes will be evaluated by an experienced investigator using a slit lamp biomicroscope and irritation will be scored according to the attached scoring and grading system (2) and the illustrated guide for grading eye irritations (5). Any other remarkable findings will also be recorded and included in the final report. Sodium fluorescein staining will be used to evaluate irritation at the 48-hour evaluation and all subsequent examinations.

2. Scoring Interval: Scoring will be performed at 1, 24, 48, 72 and 96 hours and at 7 days. If injury persists, readings shall be made every three days for at least 13 days after dosing or until all signs of reversible toxicity subside. If irritation is still present 13 days after dosing, the project manager will be notified so that additional actions may be taken, if necessary.

3. Grading Scale: Each test material will be classified or rated into a simple, readily comprehended form, according to the attached system described by J. H. Kay and J. C. Calandra (6).

D. Observations

All animals will be observed daily (a.m. and p.m.) for general health, morbidity, or mortality.

E. Unscheduled Deaths and Study Termination

Any animal dying or killed moribund during the test period will be delivered to Pathology and a gross necropsy performed. No tissues will be saved from the gross necropsy. All animals surviving the test will be killed following

the last observation by electrocution. Gross necropsies will not be performed on animals killed at termination.

IV. RECORDS TO BE MAINTAINED

All data generated during the conduct of the study shall be recorded directly, promptly and accurately in ink in bound books with prenumbered pages or on worksheets. All data entries shall be dated on the day of entry and signed or initialed by the person entering the data. Any change in entries for whatever reason (e.g., to correct an error in transposition) will be made in such a manner so as not to obscure the original entry, will indicate the reason for the change and will be dated and signed or initialed at the time of correction. All recorded data will be reviewed, signed, or initialed and dated by a knowledgeable person, other than the person making the entry, to assure adherence to study methods and to verify observations. All raw data will be retained in the Archives of the Research Laboratory for the period set forth in the Good Laboratory Practice Regulations.

V. NON-ROUTINE PROCEDURES TO ASSURE PERSONNEL HEALTH AND SAFETY

Special non-routine procedures, if any, will be indicated in a separate memorandum.

VI. STATISTICAL ANALYSIS

Not applicable.

VII. REPORT

A final report will be issued upon completion of all phases of the study.

VIII. CHANGES OR REVISIONS OF PROTOCOL

All changes or revisions and reasons therefor to this protocol once it is approved, shall be documented, signed by the Study Director, dated and maintained with the original protocol.

The study will be performed in compliance with the Good Laboratory Practice Regulations (7).

IX. DISCUSSION

Results from the study (Appendix Va) should classify the test

substance as to its irritant potential. If the test should indicate that the substance is nonirritating or practically nonirritating, then no further test is needed. However, if the substance should turn out to be minimally irritating, or higher then the substance will be retested at 4000X the actual amount of material used in one cigarette (Appendix Vb). The substance will be rejected if the rating should be greater than practically nonirritating.

REFERENCES

1. Draize, J. H., Woodard, G., and Calvery, H. O., "Methods for the Study of Irritation and Toxicity of Substances Applied Topically to the Skin and Mucous Membranes." J. Pharmacol. Exp. Therap. 82:377, 1944.

2. Draize, J. H., 1959. Dermal Toxicity, In: Association of Food and Drug Officials of the U.S., Austin Texas. Appraisal of the Safety Chemicals in Foods, Drugs, and Cosmetics, pp. 46-59.

3. Acute Eye Irritation Test. In: Testing Standards and Guidelines Work Group of the Interagency Regulatory Liasion Group (I.R.L.G.), U. S. Environmental Protection Agency, Washington, D.C. Draft I.R.L.G. Guidelines for Selected Toxicity Tests, August 1979, pp. 9-38.

4. "Laboratory Animal Welfare Act," Pub. L. 89-544, Pub. L. 91-579, Pub. L. 94-279.

5. Illustrated Guide for Grading Eye Irritations, Digest No. 10239, Consumer Product Safety Commission Directorate for Engineering and Science, Washington, D. C., 20207.

6. Kay, J. H., and Calandra, J. C., "Interpretation of Eye Irritation Tests," J. Cos. Chem. 13: 281, 1962.

7. Fed. Reg. 43 (247), Friday, December 22, 1978, Part II.

Scoring System for Grading Ocular Irritation Studies

BIOMICROSCOPIC	VALUE	DRAIZE SCORING
CORNEA		
Normal cornea	0	No ulceration or opacity
Some loss of transparency with increase of cloudiness, stromal involvement of anterior ½ as evaluated with optical section; underlying structures clearly visible with diffuse illumination, although some cloudiness present.	1	*Scattered or diffuse areas of opacity, details of iris clearly visible.
Slight loss of transparency with increased amount of cloudiness; stroma involvement covers anterior ½ and including involvement of the posterior ½ to the endothelium; including up to entire thickness of stroma underlying structure still clearly visible with diffuse illumination but intensity of cloudiness greater than 1.	2	*+Easily discernible translucent areas of opacity, details of iris slightly obscured.
Entire stroma involvement; with optical section endothelial surface still clearly visible; with diffuse illumination underlying structures barely but still visible so as to allow observer to grade flare, iritis, lenticular changes and light reflex.	3	*+Nacreous areas of opacity, no details of iris visible, size of pupil barely discernible.
Entire stroma involvement; with optical section cannot clearly observe endothelium; with diffuse illumination cannot observe underlying structure with the cloudiness so intense so as to prevent scoring of flare, iritis, lens, light reflex.	4	*+Complete corneal opacity, iris not discernible +Ulceration, absence of a gross patch of corneal epithelium.
IRITIS		
	0	Normal
Minimal injection of secondary vessel but not tertiary, may be uniform but may also only be intense at 1 or 6 o'clock (if confined to 1 and 6 o'clock must have tertiary vessel congestion).	1	*Markedly deepened folds, congestion, swelling, moderate circumcorneal injection (any of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive).

* Grades considered positive for irritation.

\+ Grades considered positive for corrosiveness. In addition, grade 1 opacity evident for any 6 or more days will also be considered as corrosive.

BIOMICROSCOPIC	VALUE	DRAIZE SCORING
IRITIS (Continued)		
Minimal injection of tertiary and minimal to moderate of secondary.	2	+No reaction to light, hemorrhage, gross destruction (any or all of these).
Moderate injection of secondary and tertiary vessels with some slight swelling of muscle fibers of iris (slight rugose appearance confined to only parts of the iris).	3	
Marked injection of secondary and tertiary vessels with marked swelling of muscle fibers giving a rugose appearance; may be accompanied by hemorrhage.	4	
CONJUNCTIVAE (Congestion, Redness)		
Normal, may appear blanched to reddish pink in color, without peri-limbal injection, vessels easily observed.	0	Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris). Vessels normal.
A flushed reddish color predominantly confined to palpebral conjunctivae, may have some peri-limbal injection but only confined to lower (4 to 8 o'clock) peri-limbal region.	1	Some vessels definitely injected.
A bright red color of palpebral conjunctiva with accompanying peri-limbal injection at least 75% around circumference.	2	*Diffuse, crimson red, individual vessels not easily discernible.
A dark, beefy red color with congestion of bulbar conjunctiva and peri-limbal injection and presence *petechia.*	3	*Diffuse beefy red.
CONJUNCTIVAE (Chemosis, Swelling)		
Normal, no swelling	0	No swelling above normal.
Swelling above normal without eversion of lids (can be ascertained by noting that upper and lower lid are positioned as in normal eyes); swelling starts in the lower cul-de-sac near the intercanthus (needs slit lamp examination to observe).	1	Any swelling above normal (includes nictitating membrane).

* Grades considered positive for irritation.
+ Grades considered positive for corrosiveness. In addition, grade 1 opacity evident for any 6 or more days will be considered as corrosive.

BIOMICROSCOPIC	VALUE	DRAIZE SCORING
CONJUNCTIVAE (Chemosis, Swelling) (Continued)		
Swelling which misaligns the normal approximation of lower and upper eyelids, primarily confined to upper lid so that the initial stages of mis-approximation of lids is caused by beginning partial eversion of upper eyelid.	2	*Obvious swelling with partial eversion of lids.
Swelling definite with partial eversion of upper or lower eyelids essentially equivalent.	3	*Swelling with lids about half closed.
Eversion of upper eyelid is pronounced, the eversion of lower and upper eyelids partially close the eyelids so that it is difficult to retract eyelids so as to observe the peri-limbal region.	4	*Swelling with lids more than half closed. +Ulceration or necrosis of palpebral and bulbar conjunctivae or nictitating membrane.
DISCHARGE		
Above normal; not on lids or hairs; ignore small amount inner and outer canthus.	1	Any amount different from normal (does not include small amounts observed in inner canthus of normal animals).
Above normal; on lids and hairs of eyelids.	2	Discharge with moistening of the lids and hairs just adjacent to lids.
Above normal; flowing over lids and wetting hairs on skin.	3	Discharge with moistening of the lids and hairs, and considerable area around the eye.

* Grades considered positive for irritation.
+ Grades considered positive for corrosiveness. In addition, grade 1 opacity evident for any 6 or more days will be considered as corrosive.

Classification *	Maximum** Mean Score	Requirements for Maintenance
Non-irritating (N)	0.0 - 0.5	To maintain this level, the mean total score must be 0 at +24 hours; otherwise raise one level.
Practically Non-irritating (PN)	0.5 - 2.5	To maintain this level, the mean total score must be 0 at +24 hours; otherwise raise one level.
Minimally Irritating (M_1)	2.5 - 15	To maintain this level, the mean total score must be 0 at +48 hours; otherwise raise one level.
Mildly Irritating (M_2)	15 - 25	To maintain this level, the mean total score must be 0 at +96 hours; otherwise raise one level.
Moderately Irritating (M_3)	25 - 50	To maintain this level, the mean total score must be less than 20 at +7 days and the individual rabbit scores at +7 days must be less than 10 in 60% of the rabbits. If the latter is not true, then no rabbit may show a final individual total score over 30; otherwise raise one level.
Severely Irritating (S)	50 - 80	To maintain this level, the mean total score must be less than 40 at +7 days and the individual rabbit scores must be less than 30 in 60% of the rabbits. If the latter is not true, then no rabbit may show a final score over 60; otherwise raise one level.
Extremely Irritating (E)	80 - 100	To maintain this level, the mean total score must be less than 80 at +7 days and the individual rabbit scores must be less than 60 in 60% of the rabbits. If the latter is not true, then no rabbit may show a final total score over 100; otherwise raise one level.
Maximally Irritating (M_x)	100 - 110	To maintain this level, the mean total score must be more than 80 at +7 days and the individual rabbit scores must be more than 60 in 60% of the rabbits. If neither of the above is true; lower one level.

* Kay, J. H. and J. C. Calandra, 1962, Interpretation of Eye Irritation Tests. J. Soc. Cos. Chem. 13:281-289.

** Mean total score for all three tissues occurring within the first 96 hours following instillation for which the incidence of this score plus or minus 5 points is at least 40%.

MAY 26 1981

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Addendum I

DAWSON RESEARCH CORPORATION

Protocol Change Order No. 1

Protocol LRC-5A

Date 5-13-81

Subject Change in the time of the Pre-dose Biomicroscopic Examination

Authorized by Dr. Connie Stone Date 5-13-81 Method Verbal-Phone
(Means of communication)

Authorized to Mr. Charles Burns

Estimated cost of the study will be:

[X] increased [ ] decreased [ ] not affected

Description of change order:

Section III. A. 2nd paragraph - the first sentence is to be changed to read as follows:

> Twenty-four to 30 hours prior to dosing, both eyes of each rabbit will be examined by an experienced investigator using a slit lamp biomicroscope.

The change was made so the protocol more closely conforms with the proposed regulations as stated in the Federal Register, Vol. 44, No. 145, 772.112-24 Primary Eye Irritation Study.

Connie L. Stone 5/19/81

Sponsor Signature

Charles Burns 5/14/81

Study Director Signature

89434213

JUN 26 1981
JUL 10 1981

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Addendum I

DAWSON RESEARCH CORPORATION

Protocol Change Order No. 2

Date 6-10-81

Protocol LRC-5A

Subject Flourescein Stain

Authorized By Dr. Connie Stone Date 6-4-81 Method Verbal-Phone
(Means of communication)

Authorized to Mr. Charles Burns

Estimated cost of the study will be:

[ ] increased [ ] decreased [X] not affected

Description of change order:

Section III. A. 2nd paragraph - the second sentence is to be changed to read as follows:

> An additional examination of all eyes will be performed under U.V. illumination following application of one drop of 2% sodium fluorescein (U.S.P.) and 5 to 10 seconds afterward an isotonic sodium chloride (U.S.P.) rinse.

This change was made to conform with the Dawson Research Corporation standard operating procedures and the recommendation of our consultant ophthalmologist.

Consultant Ophthalmologist

Sponsor Signature

Study Director Signature

8943214

JUL 10 1981

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Addendum I

DAWSON RESEARCH CORPORATION

Protocol Change Order No. 3

Date 6-24-81

Protocol LRC-5A

Subject Compound Disposal

Authorized By Dr. Connie Stone Date 6-23-81 Method Verbal-Phone
(Means of communication)

Authorized to Mr. Charles Burns

Estimated cost of the study will be:

[ ] increased [ ] decreased [X] not affected

Description of change order:

Section III.B - Add the following sentence:

Six months after the completion of each material's test, left-over material is to be disposed of by flushing it down the drain.

This addition was made at the request of Dawson Research Corporation.

Harry J. Munnemeyer
Sponsor Signature

Charles Burns
Study Director Signature

89434215

JUN 26 1981

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Addendum I

DAWSON RESEARCH CORPORATION

Protocol Change Order No. 4

Protocol LRC-5A

Date 6-10-81

Subject Relative Humidity

Authorized By Dr. Connie Stone Date _____ Method This communique (Means of communication)

Authorized to Mr. Charles Burns

Estimated cost of the study will be:

[ ] increased [ ] decreased [X] not affected

Description of change order:

Section III.A - The range for relative humidity will be change to 50% ± 25%.

This change was made so as to maintain the rabbits in a healthier environment more condusive to natural conditions. Since the literature indicates that varing relative humidity is healthier and that only the extreme ranges of relative humidity might be harmful, Dawson Research recommends this change for the good of the animals.

Connie J. Stone

Sponsor Signature

Charles Burns

Study Director Signature

89434216

ADDENDUM I

DAWSON RESEARCH CORPORATION
Primary Irritation of Mucous Membrane
The Rabbit Eye Irritation Test
(Protocol LRC-5A)
DRC 6704-3

ADDENDUM TO THE PROTOCOL

AREAS OF NON-COMPLIANCE WITH THE
GOOD LABORATORY PRACTICE REGULATIONS - 21 CFR 58

58.185 (a)(4), (5)

Data regarding the purity, stability, identity, composition and strength of the test articles are usually the responsibility of the Sponsor and are not included in our final report.

58.35 (1)

At the request of the Sponsor this study does not appear on the master schedule sheet.