TABLE OF CONTENTS

Page Number

LIST OF TABLES ..... **xi**

LIST OF FIGURES ..... **xv**

LIST OF ACRONYMS AND ABBREVIATIONS ..... **xxii**

ACKNOWLEDGEMENTS ..... **xxv**

PREFACE ..... **xxiii**

EXECUTIVE SUMMARY ..... **xxvii**

1.0 Introduction and Rationale for the Proposed Use of *In Vitro* Test Methods to Identify Ocular Corrosives and Severe Irritants **1-1**

1.1 Introduction 1-1

1.1.1 Historical Background of *In Vitro* Ocular Irritation/Corrosion Test Methods and Rationale for Their Development 1-1

1.1.2 Peer Reviews of the HET-CAM Test Method 1-6

1.2 Scientific Basis for the HET-CAM Test Method 1-6

1.2.1 Purpose and Mechanistic Basis of the HET-CAM Test Method 1-6

1.2.2 Similarities and Differences of Modes and Mechanisms of Action Between the HET-CAM Test Method and Human Ocular Irritancy 1-7

1.2.2.1 The Mammalian Eye: Common Anatomy of the Human and Rabbit Eye 1-7

1.2.2.2 Differences Between Human and Rabbit Eyes 1-9

1.2.2.3 The *In Vivo* Rabbit Eye Test Method 1-9

1.2.2.4 The Chorioallantoic Membrane (CAM) 1-13

1.2.2.5 Comparison of the HET-CAM Test Method with the *In Vivo* Rabbit Eye Test Method 1-13

1.2.3 Intended Range of Substances Amenable to the HET-CAM Test Method and/or Limits of the HET-CAM Test Method 1-14

1.3 Regulatory Rationale and Applicability 1-14

1.3.1 Current Regulatory Testing Requirements and ICCVAM Prioritization Criteria 1-14

1.3.2 Intended Uses of the Proposed HET-CAM Test Method 1-16

1.3.3 Similarities and Differences in the Endpoints Measured in the Proposed Test Method and the *In Vivo* Reference Test Method 1-16

1.3.4 Use of Proposed Test Method in Overall Strategy of Hazard for Safety Assessment 1-17

1.4 Validation of the *In Vitro* HET-CAM Test Method 1-17

1.5 Search Strategies and Selection of Citations for the HET-CAM BRD 1-19

2.0 HET-CAM Test Method Protocol Components 2-1
2.1 Overview of How the HET-CAM Test Method is Conducted 2-1
2.2 Description and Rationale for the Test Method Components 2-1
2.2.1 Materials, Equipment, and Supplies Needed 2-2
2.2.1.1 Hen Breed 2-2
2.2.1.2 Criteria for Egg Use 2-2
2.2.1.3 Egg Incubation Conditions (Temperature, Relative Humidity, Egg Rotation During Incubation, and Length of Incubation) 2-2
2.2.1.4 Rotary Saw 2-4
2.2.2 Dose-Selection Procedures, Including the Need for Any Dose Range-Finding Studies or Acute Toxicity Data Prior to Conducting a Study 2-4
2.2.3 Endpoints Measured 2-5
2.2.4 Exposure Parameters 2-5
2.2.4.1 Test Substance Exposure Amount or Volume 2-5
2.2.4.2 Concentration Tested 2-6
2.2.4.3 Application of Test Substance to CAM 2-6
2.2.4.4 Test Substance Exposure Duration 2-7
2.2.5 Known Limits of Use 2-7
2.2.6 Nature of the Response Assessed 2-7
2.2.7 Appropriate Controls and the Basis for their Selection 2-8
2.2.7.1 Negative Controls 2-8
2.2.7.2 Positive Controls 2-8
2.2.7.3 Solvent Controls 2-9
2.2.7.4 Benchmark Substances 2-9
2.2.8 Acceptable Range of Control Responses and the Basis for the Acceptable Ranges 2-10
2.2.8.1 Negative/Solvent Controls 2-10
2.2.8.2 Positive Controls 2-10
2.2.8.3 Benchmark Controls 2-12
2.2.9 Nature of the Data to be Collected and the Methods Used for Data Collection 2-12
2.2.9.1 Time to Development of Observed Endpoints After Exposure to the Test Substance 2-12
2.2.9.2 Lowest Test Substance Concentration Needed to Produce a Minimal Response on the CAM After Exposure to the Test Substance 2-13
2.2.9.3 Severity of Response Observed After Exposure to the Test Substance 2-13
2.2.10 Type of Media in Which Data Are Stored 2-13
2.2.11 Measures of Variability 2-14

2.2.12 Statistical or Non-Statistical Methods Used to Analyze the Resulting Data 2-14
2.2.12.1 Irritation Scores 2-14
2.2.12.2 Q-Score 2-15
2.2.12.3 Mean Detection Time 2-16
2.2.12.4 ITC 2-16
2.2.12.5 S-Score 2-16
2.2.12.6 SIS 2-16
2.2.13 Decision Criteria and the Basis for the Prediction Model Used to Classify a Test Chemical as a Severe Eye Irritant 2-16
2.2.13.1 Irritation Classification Based on IS 2-24
2.2.13.2 Irritation Classification Based on Q-Score 2-18
2.2.13.3 Irritation Classification Based on Mean Coagulation Detection Time 2-18
2.2.13.4 Irritation Classification Based on IS and ITC 2-18
2.2.13.5 Irritation Classification Based on S-Score 2-20
2.2.14 Information and Data that Will Be Included in the Study Report and Availability of Standard Forms for Data Collection and Submission 2-20
2.3 Basis for Selection of the HET-CAM Test Method System 2-22
2.4 Proprietary Components 2-22
2.5 Basis for Number of Replicate and Repeat Experiments 2-22
2.5.1 Sample Replicates 2-22
2.5.2 Experimental Replicates 2-22
2.6 Compliance with Good Laboratory Practices 2-23
2.7 Study Acceptance Criteria 2-23

3.0 Substances Used For Validation of the HET-CAM Test Method 3-1
3.1 Rationale for the Substances Selected for Use 3-1
3.1.1 CEC (1991) 3-1
3.1.2 Gettings et al. (1991, 1994, 1996) 3-1
3.1.3 Bagley et al. (1992) 3-1
3.1.4 Balls et al. (1995) 3-2
3.1.5 Vinardell and Macián (1994) 3-2
3.1.6 Kojima et al. (1995) 3-2
3.1.7 Gilleron et al. (1996, 1997) 3-2
3.1.8 Spielmann et al. (1996) 3-2
3.1.9 Hagino et al. (1999) 3-3
3.2 Rationale for the Number of Substances Tested 3-3
3.3 Chemicals or Products Evaluated 3-3
3.3.1 Substances Evaluated in Reviewed Studies 3-4
3.3.1.1 CEC (1991) 3-4
3.3.1.2 Gettings et al. (1991) 3-5
3.3.1.3 Gettings et al. (1994) 3-6
3.3.1.4 Gettings et al. (1996) 3-6
3.3.1.5 Bagley et al. (1992) 3-6

3.3.1.6 Vinardell and Macián (1994) 3-7
3.3.1.7 Balls et al. (1995)..... 3-7
3.3.1.8 Kojima et al. (1995)..... 3-7
3.3.1.9 Gilleron et al. (1996)..... 3-7
3.3.1.10 Spielmann et al. (1996)..... 3-8
3.3.1.11 Gilleron et al. (1997)..... 3-8
3.3.1.12 Hagino et al. (1999)..... 3-8
3.4 Coding Procedures Used in the Validation Studies..... 3-9
3.4.1 Gettings et al. (1991, 1994, 1996)..... 3-9
3.4.2 Bagley et al. (1992)..... 3-9
3.4.3 Balls et al. (1995)..... 3-9
3.4.4 Spielmann et al. (1996)..... 3-9
3.4.5 Hagino et al. (1999)..... 3-9

4.0 *In Vivo* Reference Data Used for an Assessment of Test Method Accuracy 4-1
4.1 Description of Protocol Used to Generate *In Vivo* Data 4-1
4.1.1 Draize Rabbit Eye Test..... 4-1
4.1.2 Current *In Vivo* Ocular Irritation Test Method Protocols..... 4-2
4.1.3 Current *In Vivo* Ocular Irritancy Classification Systems 4-7
4.2 Detailed Reference Data Used to Assess *In Vitro* Test Method Accuracy..... 4-8
4.3 *In Vivo* Classification Criteria Used for BRD Analysis..... 4-10
4.3.1 GHS Classification Rules Used for BRD Analysis..... 4-11
4.3.2 EPA Classification Rules Used for BRD Analysis..... 4-11
4.3.3 EU Classification Rules Used for BRD Analysis 4-13
4.4 Availability of Original Records for the *In Vivo* Reference Data 4-14
4.5 *In Vivo* Data Quality 4-14
4.6 Availability and Use of Toxicity Information from the Species of Interest..... 4-16
4.7 Information About Accuracy and Reliability of the *In Vivo* Test Method 4-16
4.7.1 Information About the Accuracy of the *In Vivo* Test Method..... 4-16
4.7.2 Information About the Reliability of the *In Vivo* Test Method..... 4-16

5.0 HET-CAM Test Method Data And Results..... 5-1
5.1 Description of the HET-CAM Test Method Protocols Used to Generate Data 5-1
5.2 Availability of Copies of Original Data Used to Evaluate the Accuracy and Reliability..... 5-1
5.3 Description of the Statistical Approaches Used to Evaluate the Resulting Data 5-2
5.3.1 IS 5-3
5.3.1.1 IS Analysis Method 5-3

5.3.1.2 IS Classification Scheme 5-4
5.3.2 Q-Score 5-4
5.3.2.1 Q-Score Analysis Method 5-4
5.3.2.2 Q-Score Classification Scheme 5-4
5.3.3 mtc10 5-5
5.3.3.1 mtc10 Analysis Method 5-5
5.3.3.2 mtc10 Classification Scheme 5-5
5.3.4 IS and ITC 5-5
5.3.4.1 IS and ITC Analysis Method 5-5
5.3.4.2 IS and ITC Classification Scheme 5-5
5.3.5 S-Score 5-6
5.3.5.1 S-Score Analysis Method 5-6
5.3.5.2 S-Score Classification Scheme 5-6
5.4 Summary of Results 5-6
5.4.1 CEC (1991) 5-7
5.4.2 Gettings et al. (1991) 5-7
5.4.3 Gettings et al. (1994) 5-7
5.4.4 Gettings et al. (1996) 5-7
5.4.5 Bagley et al. (1992) 5-8
5.4.6 Vinardell and Macián (1994) 5-8
5.4.7 Balls et al. (1995) 5-8
5.4.8 Kojima et al. (1995) 5-9
5.4.9 Gilleron et al. (1996) 5-9
5.4.10 Spielmann et al. (1996) 5-9
5.4.11 Gilleron et al. (1997) 5-10
5.4.12 Hagino et al. (1999) 5-10
5.5 Use of Coded Chemicals and Compliance with GLP Guidelines 5-10
5.6 Lot-to-lot Consistency of Test Substances 5-10
5.7 Availability of Data for External Audit 5-11

6.0 HET-CAM Test Method Accuracy 6-1
6.1 Accuracy of the HET-CAM Test Method 6-3
6.1.1 GHS Classification System: HET-CAM Test Method Accuracy 6-3
6.1.1.1 Overall Test Method Accuracy 6-4
6.1.1.2 Discordant Results According to the GHS Classification System 6-4
6.1.2 EPA Classification System: HET-CAM Test Method Accuracy 6-12
6.1.2.1 Overall Test Method Accuracy 6-12
6.1.2.2 Discordant Results According to the EPA Classification System 6-16
6.1.3 EU Classification System: HET-CAM Test Method Accuracy 6-20
6.1.3.1 Overall Test Method Accuracy 6-20

6.1.3.2 Discordant Results According to the EU Classification System 6-25
6.2 Accuracy of the HET-CAM Test Method for Identifying Ocular Corrosives and Severe Irritants – Summary of Results 6-30
6.2.1 Discordance Among Chemical Classes 6-30
6.2.1.1 IS(A)-10 and IS(A)-100 Analysis Methods 6-30
6.2.1.2 IS(B)-10 and IS(B)-100 Analysis Methods..... 6-30
6.2.1.3 Q-Score Analysis Method 6-31
6.2.1.4 S-Score Analysis Method..... 6-31
6.2.2 Discordance Among Physical or Chemical Properties of Interest 6-31
6.2.2.1 IS(A)-10 and IS(A)-100 Analysis Methods 6-31
6.2.2.2 IS(B)-10 and IS(B)-100 Analysis Methods..... 6-32
6.2.2.3 Q-Score Analysis Method 6-32
6.2.2.4 S-Score Analysis Method..... 6-32

7.0 HET-CAM Test Method Reliability 7-1
7.1 Selection Rationale for the Substances Used to Evaluate the Reliability of the HET-CAM Test Method 7-1
7.2 Analyses of Repeatability and Reproducibility 7-2
7.2.1 Quantitative Assessment of Intralaboratory Repeatability 7-2
7.2.1.1 Gilleron et al. (1996)..... 7-2
7.2.1.2 Gilleron et al. (1997)..... 7-4
7.2.2 Quantitative Assessment of Intralaboratory Reproducibility..... 7-5
7.2.2.1 Gilleron et al. (1996)..... 7-5
7.2.2.2 Gilleron et al. (1997)..... 7-7
7.2.3 Quantitative and Qualitative Assessments of Assessment of Interlaboratory Reproducibility 7-10
7.2.3.1 Qualitative Analysis of the Interlaboratory Reproducibility of Hazard Classification Category Using the GHS Classification System..... 7-11
7.2.3.2 Qualitative Analysis of the Interlaboratory Reproducibility of Hazard Classification Category Using the EPA Classification System..... 7-15
7.2.3.3 Qualitative Analysis of the Interlaboratory Reproducibility of Hazard Classification Category Using the EU Classification System..... 7-20
7.2.3.4 Common Chemical or Product Classes Among Test Substances with Discordant Interlaboratory Results Based on Qualitative Analyses 7-24

7.2.3.5 Quantitative Analysis of the Interlaboratory Reproducibility of Hazard Classification Category 7-25
7.2.4 Additional Analyses of Interlaboratory Reproducibility 7-35
7.2.4.1 Balls et al. (1995) 7-35
7.2.4.2 Blein et al. (1991) 7-36
7.2.4.3 Doucet et al. (1999) 7-36
7.2.4.4 Spielmann et al. (1991) 7-36
7.2.4.5 Spielmann et al. (1993) 7-36
7.3 Historical Positive and Negative Control Data 7-38
7.3.1 Historical Positive Control Data 7-38
7.3.1.1 Positive Control Studies Using DMF and Imidazole 7-38
7.3.1.2 Positive Control Studies Using SDS and NaOH 7-39
7.3.2 Historical Negative Control Data 7-49
7.4 Summary 7-40

8.0 Test Method Data Quality 8-1
8.1 Adherence to National and International GLP Guidelines 8-1
8.2 Data Quality Audits 8-1
8.3 Impact of Deviations from GLP Guidelines 8-2
8.4 Availability of Laboratory Notebooks or Other Records 8-2
8.5 Need for Data Quality 8-2

9.0 Other Scientific Reports And Reviews 9-1
9.1 Reports in the Peer Reviewed Literature 9-1
9.1.1 Bagley et al. (1992) 9-1
9.1.2 Balls et al. (1995) 9-1
9.1.3 Blein et al. (1991) 9-2
9.1.4 Brantner et al. (2002) 9-3
9.1.5 Brantom et al. (1997) and Steiling et al. (1999) 9-4
9.1.6 Budai et al. (1997) and Budai and Várnagy (2000) 9-5
9.1.7 CEC (1991) 9-5
9.1.8 Dannhardt et al. (1996) 9-5
9.1.9 Demirci et al. (2003) 9-6
9.1.10 Demirci et al. (2004) 9-6
9.1.11 de Silva et al. (1992) 9-6
9.1.12 Djabari et al. (2002) 9-7
9.1.13 Doucet et al. (1999) 9-8
9.1.14 Gettings et al. (1991, 1994, 1996) and Lordo et al. (1999) 9-8
9.1.15 Gilleron et al. (1996) 9-10
9.1.16 Gilleron et al. (1997) 9-11
9.1.17 Hagino et al. (1991) 9-11
9.1.18 Hagino et al. (1993) 9-12
9.1.19 Hagino et al. (1999) and Ohno et al. (1999) 9-12

9.1.20 Kalweit et al. (1987) 9-13
9.1.21 Kalweit et al. (1990) 9-13
9.1.22 Kojima et al. (1995) 9-14
9.1.23 Lawrence et al. (1990) 9-14
9.1.24 Lönnroth et al. (1999) 9-14
9.1.25 Luepke (1985) 9-14
9.1.26 Luepke and Kemper (1986) 9-15
9.1.27 Macián et al. (1996) 9-15
9.1.28 Reinhardt et al. (1987) 9-15
9.1.29 Rougier et al. (1992) 9-16
9.1.30 Schlage et al. (1999) 9-16
9.1.31 Spielmann et al. (1991, 1993, 1996) 9-16
9.1.32 Spielmann et al. (1997) 9-18
9.1.33 Sterzel et al. (1990) 9-20
9.1.34 van Erp et al. (1990) 9-20
9.1.35 Vinardell and García (2000) 9-20
9.1.36 Vinardell and Macián (1994) 9-20
9.1.37 Vives et al. (1997) 9-21
9.1.38 Wilson and Steck (2000) 9-21
9.1.39 Worth and Cronin (2001) 9-21
9.2 Data Received in Response to the ICCVAM *Federal Register* Notice or from Study Authors 9-22

10.0 Animal Welfare Considerations (Refinement, Reduction, and Replacement) 10-1
10.1 How the HET-CAM Test Method Will Refine, Reduce, or Replace Animal Use 10-1
10.2 Requirement for the Use of Animals 10-1

11.0 Practical Considerations 11-1
11.1 Transferability of the HET-CAM Test Method 11-1
11.1.1 Facilities and Major Fixed Equipment Required to Conduct a Study 11-1
11.1.2 General Availability of Other Necessary Equipment and Supplies 11-1
11.2 Training Considerations 11-1
11.2.1 Required Level of Training and Expertise Needed to Conduct the HET-CAM Test Method 11-2
11.2.2 Training Requirements Needed to Demonstrate Proficiency 11-2
11.3 Cost Considerations 11-2
11.4 Time Considerations 11-2

12.0 References 12-1

13.0 Glossary 13-1

Appendix A Protocols for the HET-CAM Test Method A-1
A1 INVITTOX Protocol 47. The HET-CAM Test – Method of Spielmann and Liebsch A-3
A2 Table of HET-CAM Protocols from the Reviewed Literature Used to Assess Ocular Irritancy A-13

Appendix B Chemical and Product Class Information for Substances Tested in the HET-CAM Test Method B-1
B1 Table of Chemical and Product Class Information..... B-3
B2 Table of Formulation Components in Gettings et al. (1991)..... B-19
B3 Table of Formulation Components in Gettings et al. (1994)..... B-25
B4 Table of Formulation Components in Gettings et al. (1996)..... B-35

Appendix C *In Vitro* Data for Substances Tested in the HET-CAM Test Method..... C-1
C1 Test Method Protocols used to Generate *In Vitro* Data C-3
C2 Data Sorted by Reference..... C-15
C3 Data Sorted by Substance for IS(A) Analysis Method C-53
C4 Data Sorted by Substance for IS(A)-10 Analysis Method C-63
C5 Data Sorted by Substance for IS(A)-100 Analysis Method C-69
C6 Data Sorted by Substance for IS(B) Analysis Method..... C-75
C7 Data Sorted by Substance for IS(A)-10 Analysis Method C-89
C8 Data Sorted by Substance for IS(A)-100 Analysis Method C-111
C9 Data Sorted by Substance for Q-Score Analysis Method C-139
C10 Data Sorted by Substance for S-Score Analysis Method... .....C-149

Appendix D *In Vivo* and *In Vitro* Comparison of Ocular Irritancy Classification D-1
D1 Data Sorted by Reference..... D-3
D2 Data Sorted by Substance for IS(A) Analysis Method D-17
D3 Data Sorted by Substance for IS(A)-10 Analysis Method D-23
D4 Data Sorted by Substance for IS(A)-100 Analysis Method D-27
D5 Data Sorted by Substance for IS(B) Analysis Method..... D-31
D6 Data Sorted by Substance for IS(B)-10 Analysis Method D-45
D7 Data Sorted by Substance for IS(B)-100 Analysis Method D-57
D8 Data Sorted by Substance for Q-Score Analysis Method D-75
D9 Data Sorted by Substance for S-Score Analysis Method..... D-81

Appendix E Intralaboratory Repeatability and Intralaboratory Reproducibility Data and Results for Gilleron et al. (1996, 1997) E-1
E1 Individual Egg HET-CAM Scores (Endpoints Evaluated and Overall Irritancy) for Data of Gilleron et al. (1996) E-3
E2 Individual Egg HET-CAM Scores (Endpoints Evaluated and Overall Irritancy) for Data of Gilleron et al. (1997) E-11

E3 Intralaboratory Repeatability Results (Expressed as %CV) for Data of Gilleron et al. (1996).....E-21
E4 Intralaboratory Repeatability Results (Expressed as %CV) for Data of Gilleron et al. (1997).....E-29
E5 Intralaboratory Reproducibility Results (Expressed as %CV) for Data of Gilleron et al. (1996) E-37
E6 Intralaboratory Reproducibility Results (Expressed as %CV) for Data of Gilleron et al. (1997) E-41

Appendix F Interlaboratory Reproducibility Data.....F-1
F1 Individual HET-CAM Test Laboratory Data for CEC (1991).....F-3
F2 Average HET-CAM Scores and Coefficient of Variations for Substances Evaluated in Hagino et al. (1999) and Ohno et al. (1999).....F-13
Appendix G Interlaboratory Correlation Coefficients from the EC/HO Validation Study (Balls et al. 1995) G-1

Appendix H Positive Control Data.....H-1
H1 Individual HET-CAM Egg Data for Dimethylformamide (provided by Drs. Vanparys and van Goethem)..... H-3
H2 Individual HET-CAM Egg Data for Imidazole (provided by Drs. Vanparys and van Goethem) H-9
H3 Individual HET-CAM Egg Data for Sodium Dodecyl Sulfate and Sodium Hydroxide (provided by Drs. Spielmann and Liebsch) H-11

Appendix I Individual HET-CAM Scores for Eggs Treated with 0.9% Sodium Chloride I-1