Doctoral Thesis

Gamma sterilization of chloramphenicol and its eye ointment products

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GAMMA STERILIZATION OF CHLORAMPHENICOL AND ITS EYE OINTMENT PRODUCTS

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of

Doctor of the Natural Sciences

presented by

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Zürich, 2002.
For my son
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CAP</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CAPEO</td>
<td>Chloramphenicol Eye ointment</td>
</tr>
<tr>
<td>CSA</td>
<td>Cetostearyl Alcohol</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode Array Detector</td>
</tr>
<tr>
<td>EMEA</td>
<td>European Agency for the Evaluation of Medicinal Products</td>
</tr>
<tr>
<td>EOB</td>
<td>Eye Ointment Base</td>
</tr>
<tr>
<td>EP</td>
<td>the European Pharmacopoeia</td>
</tr>
<tr>
<td>EtO</td>
<td>Ethylene Oxide</td>
</tr>
<tr>
<td>FDA</td>
<td>the US Food and Drug Administration</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>ISO</td>
<td>International Standards Organization</td>
</tr>
<tr>
<td>kGy</td>
<td>kilo Gray</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectroscopy</td>
</tr>
<tr>
<td>MSD</td>
<td>Mass Spectroscopy Detector</td>
</tr>
<tr>
<td>RT</td>
<td>Retention Time</td>
</tr>
<tr>
<td>R</td>
<td>Correlation Coefficient</td>
</tr>
<tr>
<td>R²</td>
<td>Determination Coefficient</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
</tr>
<tr>
<td>SAL</td>
<td>Sterility Assurance Level</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>USP</td>
<td>the United States Pharmacopoeia</td>
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Summary

Sterilization with γ rays is a cold process and enjoys a reputation as a reliable, environmentally safe and nontoxic technique. Used for sterilization of pharmaceutical products, however, γ rays could induce the degradation of the active ingredient as well as its excipient vehicle and cause the formation of radiolysis products in such quantities that toxicity must be considered. Radiation sterilization of a pharmaceutical product is permissible only when the absence of deleterious effects on the product has been confirmed experimentally.

The main purpose of this work is to determine the feasibility of γ-radiation sterilization for chloramphenicol and its eye ointment from a chemical view. The experiments implemented in four different parts provide us with important data to assess whether or not γ-radiation renders chloramphenicol and its eye ointment risky or unsafe for human.

Part I: Assay and Impurity Test of Irradiated Chloramphenicol Eye Ointment

A sample preparation method was developed to isolate chloramphenicol and its radiolytic products from a petrolatum ointment base. With this method, eye ointment was suspended in n-hexane at 45 °C, and the target compounds were isolated as residues by centrifugation. It was found that keeping the sample solution at 45°C during sample preparation was essential to ensure a satisfactory isolation. Experimental results and statistic analysis confirmed that the method was valid for both assay and impurity tests.

This isolation method is ideal for assay test and also specific for the extraction of unexpected and complex radiolysis products. For assay test, it has advantages over the pretreatment methods described in The United States Pharmacopoeia and British Pharmacopoeia in terms of accuracy, precision, and easy handling.

Part II: Identification and Evaluation of Chloramphenicol Radiolysis Products

In order to assess the safety of the radiolysis products and understand radiolysis pathway, the radiolysis products were studied with simultaneously qualitative and
quantitative analyses. Eight radiolysis products, including two new findings, were identified using HPLC-MS and HPLC-DAD. Carbon-carbon rupture reaction and oxidation reaction were found to be the main radiolysis reactions of chloramphenicol powder.

The qualitative and quantitative results were assessed and compared to the threshold requirements of international regulations for impurities in new drug products in order to evaluate the applicability of \( \gamma \)-sterilization for chloramphenicol products. It is concluded that the toxicity of chloramphenicol radiolysis products produced during \( \gamma \)-sterilization process is negligible, and such radiolysis products are safe for human health from a chemical point of view.

**Part III: Radiolysis Characterization of Chloramphenicol in Powder and Eye ointment**

Radiolysis behaviors of chloramphenicol, in both pure powder state and eye ointment, were investigated systematically. At the reference radiation dose of 25 kGy, the content of CAP decreases by 1.0% in powder state and by 1.2% in eye ointment. The profile of radiolysis products in powder state differs from that in eye ointment state. Hydrophobic radiolysis products of chloramphenicol were observed in eye ointment. Eye ointment base intensified the radiolysis of chloramphenicol during irradiation process.

The microenvironment of chloramphenicol molecule is a key factor governing the radiolysis of chloramphenicol. Oxygen functions as a scavenger and its presence diminishes the radiolysis of chloramphenicol. Residual solvents, in which chloramphenicol is readily soluble, promote radiolytic hydrolysis, while the converses exert no influence on chloramphenicol radiolysis. Inert gas purging or diffusion by exposure to an absorbent can efficiently prevent chloramphenicol powder from radiolysis.

Hydrophobic radiolysis products of chloramphenicol were detected in eye ointment base, however it is difficult to clarify them due to the complexity of eye ointment composition. N-acetyl-L-cysteine can protect chloramphenicol in eye ointment from radiolysis. The use of scavengers or lower irradiation dose could be feasible methods to reduce the radiolysis of chloramphenicol in eye ointment.
Part IV: Radiolysis Characterization of Eye Ointment Base

Gas formation in petrolatum eye ointment products is an unavoidable phenomenon during γ-irradiation. The gas composition was clarified using headspace-gas chromatography-mass spectrometry. Forty-one volatile radiolysis products in irradiated eye ointment base were detected and identified. The main volatile radiolysis products are homologous aliphatic hydrocarbons, which could be divided into groups by their carbon numbers.

The main radiolysis products are assigned to n-alkane and 1-alkene, and 2-methyl-alkane, which are the common radiolysis products of eye ointment base and its ingredient materials (white petrolatum and liquid paraffin, and wool fat). White petrolatum and liquid paraffin have the same radiolysis product profiles, in spite of differences in peak intensity. The profile of wool fat is similar, but with additional acetone as the most prominent radiolysis product. The profile of eye ointment base is a contribution of the radiolysis products of its ingredients.

It is inferred that the rupture of long carbon chains is the main degradation pathway of these materials. There is a close relationship between the mass spectrum of n-alkane and the chromatographic profile of radiolysis products in eye ointment materials. Both are characteristic of abundance of low mass compounds or m/z, having a tendency to just “fall apart”. This confirms that the radiolysis mechanism approximates the degradation mechanism of mass spectrum. The fragment pattern in mass spectrometer can therefore be used as a prediction model for radiolysis product study.

GC method demonstrates that component distribution patterns of eye ointment as well as its ingredients have significant differences before and after γ-irradiation. No condensation reaction was observed. The radiolysis products are present at concentrations ranging from 0.21 to 10.9 ppm under the reference radiation dose of 25 kGy. Both qualitative and quantitative data show that irradiated eye ointment is safe for human use.

The radiolysis behavior of cetostearyl alcohol, another ingredient of eye ointment base, was also studied in its pure state, petrolatum ointment, and chloramphenicol eye ointment, respectively. The radiolysis of cetostearyl alcohol was found to occur in all cases. The degree of radiolysis in each case is directly proportional to the radiation dosage.
Forty-two radiolysis products in irradiated cetostearyl alcohol were identified using gas chromatography-mass spectrometry. These products are assigned to n-alkane, n-aldehyde, and 2-methyl-1-alcohol. None of them exhibits safety threat to pharmaceutical applications. Molecular rupture and oxidation of the hydroxyl group of cetostearyl alcohol are also the main radiolysis reaction pathways. There is evidence that different matrixes have little influence on the radiolysis of cetostearyl alcohol, in contrast to that of chloramphenicol.

In conclusion, this study has achieved the development of a set of effective analytical methods, the clarification of the factors responsible for radiolyses of the target compounds, and the elucidation of the relevant radiolysis pathways. The present work has also established criteria for assessing the feasibility of γ-processing for the sterilization of chloramphenicol and its eye ointment products.

Zusammenfassung


Der Hauptzweck dieser Arbeit ist es, die Eignung der Sterilisierung durch γ-strahlen von Chloramphenicol als Festsubstanz und in Augensalben verarbeitet aus dem Blickwinkel der analytischen Chemie zu bestimmen. Die Experimente, die in vier verschiedene Teile aufgeteilt sind, zeigen analytisch relevante Daten, welche die Entscheidung gestatten, ob γ-strahlen zu Sterilisationszwecken bei Choramphenicol als Festsubstanz und in Augensalben verarbeitet erfolgreich eingesetzt werden kann.
Teil I:

Methoden und Verunreinigungstests für bestrahlte Chloramphenicol Augensalben

Es wurde eine Probenvorbereitungs-Methode entwickelt, welche es ermöglichte die Radiolyseprodukte von Chloramphenicol aus einer Augensalbe abzutrennen, deren Struktur aufzuklären und zu quantifizieren. Die Augensalbe wurde in n-Hexan bei 45° C suspendiert und die gewünschten Verbindungen wurden als Rückstand durch Zentrifugation isoliert. Man fand heraus, dass das Erwärmen der Probe auf 45° C während der Probenvorbereitung eine wichtige Voraussetzung war, um die gewünschte Trennung zu erreichen. Experimentelle Resultate und eine statistische Analyse bestätigen, dass sich die Methode für Gehaltsbestimmung und Verunreinigungstest eignet.


Teil II:

Identifikation und Evaluation der radiolytischen Produkte von Chloramphenicol

Es wurden aufwendige analytische Untersuchungen durchgeführt, um die Radiolyseprodukte zu identifizieren, quantifizieren und die Radiolyseprozesse theoretisch zu interpretieren. Mittels HPLC-MS und HPLC-DAD wurden acht radiolytische Produkte, darunter zwei noch nie beschriebene, identifiziert, in der Struktur aufgeklärt und quantifiziert. Die Hypothese wurde bestätigt, dass Kohlenstoff-Kohlenstoff-Spaltungen und Oxidationen die wichtigsten Reaktionen bei der Bestrahlung von Chloramphenicol als Festsubstanz waren.

Die qualitativen und quantitativen Resultate wurden ausgewertet und verglichen mit den Mindestanforderungen der internationalen Bestimmungen für die Anwendbarkeit von γ-Sterilisierung von Chloramphenicolprodukten. Es war offensichtlich, dass die Toxizität von radiolytischen Chloramphenicolprodukten, welche beim Prozess der γ-
Sterilisierung auftreten, vernachlässigbar ist. Die entstandenen radiolytischen Produkte sind, vom chemischen Blickwinkel aus gesehen, mit sehr hoher Wahrscheinlichkeit für die Gesundheit des Menschen unbedenklich und zeigen bei der Anwendung am Auge keine unerwünschten Nebenwirkungen.

Teil III: Charakterisierung der Radiolyse von Chloramphenicol in Pulver und Salben


Teil IV: Charakterisierung der Radiolyse-Produkte in bestrahlten Salbengrundlagen


Mit der GC-Methode konnte man demonstrieren, dass sowohl das Verteilungsmuster der Komponenten der Augensalbe als auch dasjenige seiner Inhaltsstoffe keine qualitativen Unterschiede aufweisen, vor und nach der Bestrahlung mit γ-Strahlen. Es wurden keine Kondensationsreaktionen beobachtet. Die Konzentrationen der Radiolyseprodukte liegen zwischen 0.2 und 11 ppm bei der
Referenzstrahlendosis von 25 kGy. Sowohl qualitative als auch quantitative Daten zeigen, dass bestrahlte Augensalbe unbedenklich ist für die Anwendung beim Menschen.


Zusammengefasst befasst sich diese Studie mit der Entwicklung von verschiedenen geeigneten Analysemethoden, welche es gestatten die für die Radiolyse verantwortlichen Faktoren und die Charakterisierung der relevanten radiolytisch bedingten Abbaumechanismen von Chloramphenicol und Chloramphenicol-Augensalben zu beurteilen. Diese Arbeit hat ebenfalls Kriterien aufgestellt, für die erfolgreiche Anwendung von γ-strahlen zur Sterilisation von Chloramphenicol und seine Augensalbenprodukte.
Introduction
1.1. GENERAL

D-(threo)-chloramphenicol (CAP) is a broad-spectrum antibiotic that is predominantly active against the pathogenic gram-negative bacteria. It is widely used in medical practice with 164 registered names of pharmaceutical products from 32 countries [1]. Traditionally, sterilization of CAP substance (raw material powder) has to be carried out by the ethylene oxide fumigation method, due to its heat sensitivity [2]. CAP eye ointment is prepared by aseptic process, in which the presterilized CAP powder and petrolatum ointment base are blended, homogenized, and packed.

However, the toxicity issues of ethylene oxide and its degradation products are causing concern worldwide. Regarding ethylene oxide sterilization, the very recent the United States Pharmacopoeia (USP, 2000) [3] points out:

*Among the disadvantages of this sterilizing agent are its highly flammable nature unless mixed with suitable inert gases, its mutagenic properties, and the possibility of toxic residues in treated materials, particularly those containing chloride ions.*

In April 2001, the following recommendation by the European Agency for the Evaluation of Medicinal Products (EMEA) [4] was put into operation:

*In view of the known positive potential of ethylene oxide for genotoxic carcinogenicity, it is recommended that use is acceptable only when pharmaceutically absolutely necessary, and then residual ethylene oxide in the product should be not exceed a limit of 1 ppm. Ethylene oxide sterilization should be used only where safer alternative cannot be used.*

The US Food and Drug Administration (FDA) holds that aseptic manufacture is a process of last resort [5]. If a finished presentation is suitable for terminal sterilization, then this is the method of first choice. Aseptic processing suffers from low degree of sterility assurance, possibility of secondary contamination, and high investment and operation costs. During aseptic operation, the equipment, containers, closures, and other ingredients have to be sterilized by appropriate procedures beforehand.

Therefore, the industrial needs for alternative sterilization technologies of CAP and its products are imminent. This thesis is therefore, dedicated to a systematical
investment of the feasibility of γ sterilization on CAP pharmaceuticals, one of the emerging and most promising means of sterilization.

1.2. SELECTION OF STERILIZATION METHODS

1.2.1. Sterilization for Pharmaceuticals

The Goal of Sterilization

Sterilization process is an indispensable part of pharmaceutical industry. The goal of sterilization is to satisfy the criterion of sterility by complete destruction of all microorganisms on the processed items. Sterility is defined academically as the total absence of viable microorganisms and fundamentally defined by the Sterility Assurance Level (SAL) of a product. The SAL for a given product is expressed as the probability of a non-sterile item in that population. It is not an absolute term, and depends on the usage mode of the product.

For injectable drugs, ophthalmic ointments and eye drops, the SAL must be \( \leq 10^{-6} \). Raw materials must not contain pathogens and Colony Forming Units (CFU) must not exceed the limits proposed in the pharmacopoeias or the in-house standards, if the latter are more stringent.

Sterilization Methods

Currently, the sterilization processes recognized by the European Pharmacopoeia (EP) [6] and the USP [3] are:

- Thermal sterilization (including steam sterilization and dry heat sterilization);
- Ionizing radiation sterilization (including gamma radiation and electron beam radiation);
- Gas sterilization (fumigation by ethylene oxide);
- Filtration;
- Aseptic processing.
Sterilization may be carried out by one of the above methods. Modifications to, or combinations of, these methods may be used provided that the chosen procedure is validated with respect to both its effectiveness and the integrity of the product, including its container or package.

**General Rules for Method Selection**

Every sterilization method has its particular advantages and limitations. The choice of method should take account of any effects it may have on the quality of the product. There are two general rules for sterilization selection:

- Where possible and practicable, thermal sterilization is the method of choice, because they have proven track records in their practicality, product integrity, and economics [7].

- Wherever possible, products intended to be sterile should be preferably terminally sterilized in their final container [6, 8].

Sterilization by saturated steam under pressure is the proven and most economical method of inactivating microorganisms. When requirement for sterility arises for a new medical or pharmaceutical product, steam sterilization should always be given the first consideration. This method is only suitable for water-wettable materials and aqueous formulations, however. For moisture-sensitive products, dry heating is the next method of choice, suiting for non-aqueous liquids or dry powder products, which are damaged by moisture or are impervious to steam. Both steam and dry heating methods are limited to heat-stable items, of course.

**1.2.2. Sterilization for Chloramphenicol Substance**

Medical practice has always included objects that could not withstand high temperatures. This is the case of CAP, which can only be sterilized under a "cold process" – sterilization at room temperature. Typically, "cold processing" includes filtration, ethylene oxide fumigation and ionizing radiation.

Filtration achieves sterility by removing microbes from the product. Certain solution and liquids can be filtered through a sterile filter of pore size 0.22 μm (or less),
or with at least equivalent microorganism retaining properties. It does not rely on chemical reaction to inactive microorganisms, and is suitable for heat sensitive fluids. Filtration is inherently not an option for solid products, and certainly is not feasible method for CAP substance.

Ethylene oxide is an effective bactericide at a temperature as low as 60 °C, and can easily diffuse into the materials to be sterilized. It is a traditional method for the sterilization of CAP substance. Ethylene oxide is a substance which, due to its structure, is counted among the very reactive compounds. This reactivity also includes organic structure within cells and cell nuclei. In this case, alkylation and reactions with DNA, RNA and proteins occur. The DNA damaging active of ethylene oxide provides its effectiveness as a sterilant.

However, the bactericidal effects of ethylene oxide are not peculiar to microorganisms, but all biological systems. Exposure to ethylene oxide induces irreversible chromosomal aberrations and other precancerous changes in the peripheral lymphocytes. In 1985, ethylene oxide was first listed as reasonable anticipated to be a human carcinogen based on limited evidence of its carcinogenicity in humans and sufficient evidence in experimental animals [9].

Cytotoxicity [10], carcinogenicity [11-13] and mutagenicity [14, 15] of ethylene oxide have been demonstrated in vitro and in vivo tests. Ethylene oxide can cause hemolysis of blood [16]. Acute effects of ethylene oxide and its by-products ethylene chlorhydrin (CH₂ClCH₂OH) produced in reaction with chloride ions lead to also symptoms of nausea, dizziness, and signs of mental disturbance and ethylene chlorhydrin may cause kidney and liver degeneration. In 1994 IARC (International Agency for Research on Cancer) concluded that ethylene oxide was carcinogenic to humans [9].

In addition, ethylene oxide may react with some pharmaceutical elements to form hazardous compounds, and can also attach to a molecule, thus changing its chemical identity. Accordingly, the application of ethylene oxide sterilization for pharmaceutical products is recommended only when no other option is available [7]. It is widely expected that this sterilization method will be finally banned from a valid sterilization method in the near future.
Introduction

Gamma processing is, therefore, left as the only cold sterilization method for thermal unstable pharmaceuticals such as CAP substance.

1.2.3. Sterilization for Chloramphenicol Eye Ointment

In addition to its thermal instability, CAP eye ointment products cannot be thermally sterilized also because ointment products in final package will be melted down at high temperatures, although eye ointment base is generally thermal stable. Traditionally eye ointment products are manufactured under aseptic process as shown in Figure 1-1, where ointment base is sterilized by dry heating, CAP powder is sterilized by ethylene oxide fumigation, and containers are sterilized by gamma radiation.

![Diagram of traditional sterilization process of chloramphenicol eye ointment](image)

**FIG. 1-1. Traditional sterilization process of chloramphenicol eye ointment**
In the Federal Register of October 11, 1991, the FDA formally proposed that a rule should be established whereby aseptic processing of sterile pharmaceuticals should only be justifiable on the basis that the product has been proven to be unsuitable for terminal sterilization. This is because the filling stage in an aseptic manufacture is essentially a passive process of contamination control, whereas terminal sterilization is an active process of eliminating contaminants within the sealed presentation. The degree of sterility assurance cannot be predicted for aseptic manufacture in the way that it is predicted for terminal sterilization, and in truth it cannot be expected to be good.

Gamma radiation is obviously the most relevant method of terminal sterilization, although fully validated terminal sterilization can be carried out by steam and dry heating as well. Gamma radiation has the advantage of thorough material penetration,
Introduction

even for high-density products. In addition, a product in heat unstable container such as plastic tubes and soft gelatin capsules can be terminally sterilized by γ radiation.

Consequently, radiation process is considered as the only potential terminal sterilization method for CAP eye ointment products. Manufacture process using radiation sterilization is suggested in Figure 1-2. Comparing with the aseptic operation, the γ processing has the advantages of terminal sterilization, simplified process, high level of sterility assurance and reduced manufacturing cost.

Therefore, it would be of great scientific and economic interests to investigate the feasibility of gamma irradiation sterilization for CAP raw material and its preparation products.

1.3. HISTORICAL REVIEW AND FUTURE PERSPECTIVE

1.3.1. Radiation Sterilization in Pharmaceutical Industry

Since the early 1960s gamma radiation processing has been a recognized method for pharmaceutical industry. Association of the British Pharmaceutical Industry published one of the earliest extensive reviews on the effects of gamma radiation on pharmaceuticals in 1960 [17]. Jefferson introduced radiation sterilization of medical equipment and pharmaceutical products, especially the details of bacterial radiosensitivity in 1964 [18]. An extensive literature review of gamma radiation of pharmaceutical materials was published by Gopal in 1978 [19] and update by Jacobs [20] and Bögl [21] in 1985, covering most of the literature on the radiation effects in pharmaceutical systems until 1984. Jacobs further refreshed the review in 1995 [22]. Bögl et al. published a survey of radiation treatment on alkaloid, morphine derivation, and antibiotics in 1996 [23]. Of special significance is the paper by Bögl et al. in 2000 [24], which comprehensively summarized the influence of radiation on pharmaceuticals.

In contrast to investigation into the gamma sterilization of package materials and medical devices, feasibility studies of the radiation sterilization of pharmaceuticals have not been carried out in a systematic manner. Earlier investigation revealed that about 50% of the solid substance, which are in a dry form of powders, crystals, or lyophilized material, did not show any decomposition upon gamma irradiation. Many of the
reported investigations have been dealt only with a somewhat superficial examination of irradiated products.

Nevertheless, the data reported so far provide useful insights into the overall radiation stability of these products, and often indicate whether more thorough and extensive testing of a product or drug is worthwhile. Two consensuses are achieved. First, drugs in solid state or in non-aqueous matrices are generally more stable than in aqueous form, and the presence of moisture/water usually reduces the radiation stability of a drug. Second, it is not realistic to present a specific testing protocol applicable to all pharmaceuticals; instead each compound should be assessed individually, even though data may be available on other closely related compounds. In any formulation, the stability of an individual component may be altered under irradiation. Generally, damage due to radiolysis can be minimized by the choice of appropriate treatment conditions, including the absence of air, at low temperatures, or incorporation of suitable additives based on understanding of the degradation mechanism.

Currently, ethylene oxide and gamma radiation are two most common techniques for industrial sterilization (Figure 1-3), however sterilization by gamma radiation is increasingly used in preference to the ethylene oxide method. The application of gamma rays to the sterilization of medical devices is now commonplace. Ionizing radiation is used extensively for sterilization of heat-sensitive medical devices and for heat-sensitive pharmaceutical packaging components prior to aseptic processing. Comparatively, sterilization of pharmaceuticals by ionizing radiation is rare, though it is also increasing. Generally speaking, pharmaceuticals are more sensitive to irradiation as compared with medical devices. The slow conversion from ethylene oxide sterilization to gamma radiation and slow development of radiation sterilization of pharmaceuticals are no doubt related to two main reasons:

- Regulations for pharmaceutical manufacture are getting more and more stringent. According to International Conference on Harmonisation Guideline Q3A(R) and Q3B(R), the degradation products in new drug substances and products must be reported with qualitative or quantitative data when their concentrations exceed certain thresholds [25, 26].

- Potential chemical interactions in pharmaceuticals are less well known, although the physical changes that occur with irradiation are well documented. Generally,
the studies of chemical changes and degradation products are very complex and expensive because radiolysis products are trace, unusual, and complicated, furthermore, each compound should be considered individually according to the previous conclusion that there are no universal testing protocols that could be applied to all pharmaceuticals.

It is believed that if the manufacturers are able to switch away from EtO easily, they have already done so. The pursuit to radiation sterilization is being continued for new products. In fact, a number of pharmaceutical companies have irradiated their products, but often for commercial reasons they prefer not to publicize this information. It is foreseeable that economics, statutory regulations and scientific proofs will facilitate continued growth in radiation sterilization.

![Graph showing industrial sterilization market in 1994 and 1999](image)

*FIG. 1-3. Industrial sterilization market in 1994 and 1999 [27]*
DECISION TREE FOR STERILISATION CHOICES FOR NON-AQUEOUS LIQUID, SEMI-SOLID OR DRY POWDER PRODUCTS

Can the product be sterilised by dry heat at 160°C for 120 minutes?

**NO**

Can the product be sterilised by dry heat with an alternative combination of time and temperature to the standard cycle achieving an SAL of $\leq 10^{-6}$?

**NO**

Can the product be sterilised by a method different from dry heat e.g. ionising radiation with an absorbed minimum dose of $\geq 25$ K Gy?

**NO**

Can the product be sterilised using a validated lower irradiation dose (ref ISO 11137)?

**NO**

Can the formulation be filtered through a microbial retentive filter?

**NO**

Use pre-sterilised individual components and aseptic compounding and filling

**YES**

Use filtration and aseptic processing

**YES**

Use sterilisation with an absorbed minimum dose of $\geq 25$ K Gy

**YES**

Use dry heat with alternative combination of time and temperature to the standard cycle achieving an SAL of $\leq 10^{-6}$

**YES**

Use sterilisation at 160 °C for 120 minutes

**YES**

Use sterilisation by validated irradiation dose.

FIG. 1-4. Flowchart of sterilization choices for non-aqueous liquid or dry powder products (recommended by the European Agency for the Evaluation of Medicinal Products, 1999) [7].
As mentioned before, the use of ethylene oxide for sterilization is getting more restricted [4]. The latest recommendation for the selection of sterilization methods by the European Agency for the Evaluation of Medicinal Products is listed in Figure 1-4, in which the following points are noticeable:

- Radiation sterilization method is the first choice for heat-sensitive materials.
- Gas sterilization was excluded as a valid sterilization method.
- Aseptic processing is only recommended as a last choice.

Clearly, γ sterilization is favored for the thermal unstable pharmaceuticals. This regulation change will certainly accelerate applications of radiation sterilization in pharmaceutical industry. No other technology is as easily validated as gamma radiation. No other current technology can provide the same degree of assurance of microbial inactivation, with few side effects on the sterilized product or package.

A better understanding of radiation effects on chemical changes in pharmaceuticals will encourage the regulatory agencies to accept the use of this technology. As happened to medical devices in the 1970s and 1980s, radiation processing of pharmaceuticals will be rapidly growing.

There is a saying [28]: “You will have better products after gamma radiation processing than you would have if you didn’t use the process.”

1.3.2. Previous Studies on Chloramphenicol and its Eye Ointment

Chloramphenicol Powder

Previous reports on gamma sterilization of CAP substance are rather fragmental and contradictory. During the 1960-70s, three groups reported that CAP powder was not subject to decomposition at doses between 25, 50, and 100 kGy [29-31]. Schulte and Henke [32] found that CAP decomposed by 1.5% at 60 kGy. Diding et al. [33] claimed that after being sterilized at 25 kGy from either a 60Co source or a linear electron accelerator, CAP did not meet some of the Nordic Pharmacopoeia quality requirements any more. Altorfer estimated up 1-2% of decomposition at these doses and detected at least five radiolysis products by TLC [34]. It was reported that changes could be observed in physical properties like discoloration, crystallinity and solubility at the
sterilization dose of 15 kGy [35, 36]. The color change decreased in different atmospheres, in the order of air < N₂ < N₂O < H₂. Varshney [37] concluded that CAP in dry powder form can be sterilized by ⁶⁰Co or electron beam at 15 kGy, without significant radiation effects being observed.

**Chloramphenicol Solution**

Irradiation of a 1% aqueous solution at 25 kGy caused noticeable decomposition. Altorfer [34] detected 11 unidentified radiolysis products by TLC. At 25 kGy, the decomposition in a 0.1% aqueous solution was 41% by the measurement of polarography, whereas UV measurement showed only 19% [29, 36]. The same measurement on a 0.05% solution gave the values of 62% (polarographic measurement) and 31% (UV) [29, 36]. Apparently, decomposition is proportional inversely to the concentration of the treated CAP solution. In addition, Schulte and Henke [32] detected 84% decomposition in a 0.25 solution irradiated at 80 kGy.

**Chloramphenicol Ointment**

Among the numerous references to gamma sterilization, there are relatively few studies concerning ointment. In 1967, Hangay et al. reported that hydrocortisone eye ointment containing two active ingredients (hydrocortisone acetate and CAP) was suitable for radiation sterilization since the quantitative determinations and assays of the two active ingredients did not show changes either as the pure substance or in the ointment [38]. The unpublished data from Gopal in 1978 claimed that gamma irradiation at 25 kGy causes principally the formation of p-nitro-benzaldehyde (I) and p-nitro-benzoic acid (II) and a few other unknown products as revealed by TLC and HPLC. The content of I and II was about 0.06% each at 25 kGy and increased to 0.12% at 60 kGy [19]. Gas formation in petrolatum eye ointment products is known to be a phenomenon unavoidable during gamma irradiation.

Furthermore, CAP eye ointment had been approved by regulatory authorities for radiation sterilization and decontamination in UK, Norway and India [19, 39]. Ophthalmic ointments are regarded as one of the most successful dosage forms to which radiation sterilization has been applied [40]. The work by Nash [41] described the
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procedures undertaken by Lederle Laboratories in order to obtain FDA approval for radiosterilized tetracycline preparations. Nevertheless, no reports were available about the details of performing gamma sterilization on CAP eye ointment and chemically validated irradiation dosage. The radiolysis degradation products of CAP in eye ointment had never been examined.

In conclusion, the gamma process causes unacceptable CAP degradation in aqueous solution at the reference dose of 25 kGy. It is not sure whether or not it is feasible to sterilize CAP in powder state with gamma rays at 25 kGy. Gamma sterilization of CAP ointment products has to be studied to determine suitable radiation conditions. In addition, there is still lack in investigation into CAP radiolysis behavior, concerning the following aspects in particular:

- Radiolysis degradation products of CAP in different matrices;
- Factors governing CAP radiolysis and methods preventing CAP from radiolysis;
- Chemical changes in the irradiated CAP ointment base.

1.4. BASICS OF GAMMA RADIATION

1.4.1. History

The first scientific publication on the effects of ionizing radiation on bacteria occurred in 1896 [42]. The pioneering work directed specifically toward evaluating the effectiveness of irradiation as a microbicidal process at an industrial scale was conducted in the 1940s [43]. In the early days of industrial application, it was food industry that excited the greatest interest. However, the first commercial application of the microbicidal effects of radiation was the sterilization of surgical sutures with a Van de Graff accelerator by Ethicon Inc. (USA) in 1956.

The earliest 60Co gamma irradiation plant was opened in Australia in 1959 to eliminate anthrax spores from bales of imported goat hair, followed by the plant at Wantage Research Laboratory by the United Kingdom Atomic Energy Authority, which eventually become allied to the sterilization of medical devices. In 1964, Ethicon Inc. established three commercial gamma sterilization facilities in Peterborough (Ontario),
San Angelo (TX), and Somerville (NJ), respectively. These plants were designed and installed by AECL - commercial products.

Gamma irradiation as sterilization method for pharmaceutical products first appeared in the British Pharmacopoeia of 1963, and in the United States Pharmacopoeia (USP) of 1965 (XVII Edition). Since then the compendia have been periodically revising their recommendations on the radiation treatment. By the end of the 1970’s, gamma sterilization had been adopted as regular sterilization method by large medical manufacturers, such as Baxter International, Becton Dickinson, and Sherwood Medical, etc.

1.4.2. Principle

Irradiation exposes an object to radiant energy. Source of the ionizing energy may be gamma rays from $^{60}\text{Co}$ or $^{137}\text{Cs}$, although the most widely used source is radioactive isotope $^{60}\text{Co}$, which is produced from an isotope of non-radioactive cobalt $^{59}\text{(Co)}$. The refined $^{59}\text{Co}$ slugs or pellets are assembled into rods and placed in a nuclear reactor for a period of 18-24 months. While in the reactor, the $^{59}\text{Co}$ atom absorbs one neutron to become radioactive $^{60}\text{Co}$, and over time the ratio of $^{60}\text{Co}$ to $^{59}\text{Co}$ increases. The radioactivity or "energy level" is measured in curies or Becquerels per gram.

The $^{60}\text{Co}$ atom is naturally unstable because of the extra neutron. It will eventually emit one electron and two successive gamma rays at energies of 1.17 MeV and 1.33 MeV, and decays to non-radioactive nickel-60 ($^{60}\text{Ni}$) (Figure 1-5). This decay is a well-understood natural phenomenon, which is a constant process over time.

The amount of energy absorbed is referred as dose (correctly, absorbed dose). The dose, or amount of radiation absorbed by an item submitted to gamma, is measured in Grays (Gy), which is the ISO (International Standards Organization) unit of dose. 1 Gray is equivalent to the absorption of 1 Joule of energy by 1 kg of material. 1'000 Grays = 1 kilo Gray (1 kGy). In a well-designed irradiation facility, the only variable determining the amount of radiation that is received by a material of any given density is the time that the material has spent within the radiation field.
An atom consists of bound electrons in orbit around a nucleus that contains protons and neutrons. The energy transferred from gamma rays to the atoms of the irradiated material overcomes the electrical attraction binding the electrons, and the orbiting electrons can thus move to higher energy orbits, or be separated from the atoms. This process is known as ionization.

The ionization reactions are the primary mechanisms by which ionizing radiation affects living microorganisms. In the secondary process, the ions either revert to a stable state or to long-lived free radicals through energy exchange with the immediate environment.

During the sterilization process (see Figure 1-6), product is conveyed from the "non-irradiated" area of a segregated warehouse into a shield room. Here it passed around an array of $^{60}$Co rods, from which it receives a uniform dose of radiation. It is then released into the "irradiated" section of the segregated warehouse.
1.4.3. Microbiological Effects

Gamma rays (electromagnetic energy) emitted from $^{60}$Co penetrate deep into materials to destroy microorganisms. Ionization can cause lethal effects on living organisms through two mechanisms: the DNA chain may be ruptured directly through ionization of cellular materials; or the cell may be destroyed by chemical reactions either within the organism or from its immediate surrounding.

In a non-aqueous environment, as is often the case for raw materials, direct effect is the primary mode of lethality. In complex aqueous media, such as biological products and finished pharmaceutical preparations, most changes originate from the recombination of radicals produced by the radiolysis of water. The final result depends on the micro-environment surrounding the irradiated object, such as the presence of water, oxygen, impurities, radical absorbent (e.g. cysteine, certain alcohols and glucose), or antioxidant (vitamins E and C), etc.
Introduction

The changes in the DNA molecules of the micro-organism will either render their division impossible or kill them. This is the main way in which irradiation reduces the microbiological contamination.

The lethal effect of irradiation depends on the total dose absorbed rather than the dose rate. The population of viable organisms decreases exponentially with increased dose. Each species exhibits a specific sensitivity to irradiation.

1.4.4. Features

Cold Process

The highest product temperature reached in industrial-scale gamma irradiators usually ranges from 30 to 40 °C depending on the source strength.

High Efficiency

All common bacteria show very low resistance to irradiation and none can survive the treatment. The strong penetration of gamma radiation also allows any area of the product, its components, or packaging being thoroughly sterilized after treatment. As shown in Figure 1-7a, for a one-sided treatment after 12 cm at density 1 still 50 % of the initial dose is present. By treating the goods from several sides (in Figure 1-7b), penetration and homogeneity can be further improved.

Furthermore, gamma processing is highly reliable due to its simplicity that time is the only variable to control, thereby reducing the possibility of deviation to an absolute minimum. This makes validation an easy task, without the need of using vacuum system, or gas/steam system to monitor, and/or power variables (i.e. voltage or current) to regulate.

No Residuals

Gamma rays emitted from $^{60}$Co, are pure energy with very short wavelengths - similar in many ways to ultraviolet and microwaves. Not only gamma rays are highly effective in killing microorganisms, but also they neither create residues nor have sufficient
energy to effectively change the nuclei or to elicit neutrons of the atoms. The object never comes in contact with any radioactive material during the irradiation process.

Consequently, most of the energy delivered during radiation sterilization simply passes through the object. The amount of energy that does not pass through the object is negligible and is retained as heat. No radioactivity can be created or induced within the product, regardless of the absorbance dose. The irradiation-treated products can be released for sale immediately.

**FIG 1-7 Penetrability of gamma rays (Gamma absorption curves at 25 kGy) [46].**
Terminal Sterilization

The process can be carried out on the final packaged products in their shipping cartons. Multiple packaging layers can be penetrated and treated. Packaging remains intact with gamma processing, and the need for permeable packaging materials is eliminated. Tough, impermeable packaging materials can be used to provide a better, long-term sterile barrier.

### Table 1-1. Comparison of Sterilization Methods [47]

<table>
<thead>
<tr>
<th>Consideration</th>
<th>Gamma Radiation</th>
<th>Electron Beam</th>
<th>Ethylene Oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process type</td>
<td>Batch (simple)</td>
<td>Continuous (simple)</td>
<td>Batch (complex)</td>
</tr>
<tr>
<td>Sterilisation Mechanism</td>
<td>Ionising radiation by gamma rays (deep penetration, low dose rates)</td>
<td>Ionising radiation by high energy electrons- (low penetration, high dose rates)</td>
<td>Gas fumigation - diffusion controlled process</td>
</tr>
<tr>
<td>Product Design</td>
<td>No restriction</td>
<td>Limitations with large components packages</td>
<td>Avoid closed chambers and sealed cavities</td>
</tr>
<tr>
<td>Penetration</td>
<td>Complete penetration</td>
<td>Near-complete penetration</td>
<td>Requires gas-permeable packaging</td>
</tr>
<tr>
<td>Product Packaging</td>
<td>Because of the deep penetration capabilities of gamma radiation, product density and package size are not concerned for most medical devices</td>
<td>Penetration correlates directly to product density and carton size. Densely packaged materials and large cartons may more than one pass beneath the beam</td>
<td>Permeable material or a second sealing process is required. Allowances must be given for expansion due to vacuum and pressure.</td>
</tr>
<tr>
<td>Controlled Parameters</td>
<td>Time</td>
<td>Beam current, width and energy; conveyor speed</td>
<td>Gas concentration, pressure, temperature, relative humidity, time</td>
</tr>
<tr>
<td>Post-Sterilisation Testing</td>
<td>Not required</td>
<td>Not required</td>
<td>Biological indicators must be used to verify sterility assurance level (SAL), unless requirements for parametric release can be met</td>
</tr>
<tr>
<td>Post Sterilisation Quarantine Period</td>
<td>Not required</td>
<td>Not required</td>
<td>Aeration is required to remove toxic residue</td>
</tr>
<tr>
<td>Residues</td>
<td>None</td>
<td>None</td>
<td>Ethylene glycol and ethylene chlorohydrin (carcinogens &amp; mutagens)</td>
</tr>
</tbody>
</table>

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Cost Effective

Gamma processing provides fast, flexible, and cost-effective sterilization. The cost effectiveness is largely attributed to the elimination of sterility tests (due to the FDA’s acceptance of dosimetric release), as well as the elimination of inventory quarantine, and the potential for re-processing.

For comparison, features of ethylene oxide sterilization, gamma radiation sterilization and electron beam radiation sterilization are summarized in Table 1-I.

1.4.5. Choice of Dose

The degree of sterilization (sterility assurance level or SAL) is directly related to the amount of ionizing radiation absorbed by the material. There must be sufficient energy available to kill microorganisms, but not so much that unacceptable material alternations are induced. Then, what is an appropriate sterilizing dose of radiation sterilization? The answer to the question is by no means simple. As with most other sterilization technologies, both process specifications and validated sterility assurance level have to be taken into account.

The first published reference of 25 kGy as an appropriate sterilization dose came from Artandi and Van Winkle in 1957 [48]. They established the minimum killing dose for over 150 species of microorganisms, and chose 25 kGy as the sterilizing dose because it was 40 % the dose necessary to kill the most resistant microorganism and well short of that which would damage the product. By 1965, 25 kGy was quoted [49] as the accepted dose of radiation for sterilizing disposable medical equipment in most countries except Denmark, where 45 kGy was claimed to be necessary. Historically, a radiation dose of 25 kGy minimal has been proven to be safe and effective to sterilize medical goods. Traditionally, this dose has been applied and no further verifications were made.

However, with the introduction of new standards, such as EN 552 [50], ISO 11137 [51], requirements became more stringent. According to International Standards Organization (ISO 11137), sterilization dose is selected as followings:

A knowledge of the number and resistance to radiation of the nature microbial population as it occurs on or in the product shall be obtained and used for
determination of the sterilization dose. The dose shall be capable of achieving the preselected sterility assurance level (SAL).

One of two approaches shall be taken in selecting the sterilization dose:

1) selection of sterilization dose using either bioburden information, or information obtained by incremental dosing

2) selection of a sterilization dose of 25 kGy following substantiation of the appropriateness of this dose.

Lower doses may be acceptable if there is technical need, e.g., with dose-dependent deleterious effects on materials. However, there must be evidence to validate the lower dose, namely, it has been demonstrated satisfactorily that the dose chosen delivers an adequate and reproducible level of lethality when the process is operated routinely within the established tolerances. The procedures and precautions employed are such as to give a SAL of $10^{-6}$.

1.5. CHLORAMPHENICOL AND ITS EYE OINTMENT

1.5.1. Feature of Chloramphenicol

CAP is a nearly perfect antibacterial with one major side effect- causing aplastic anemia and other blood dyscrasias in a small percentage of treated patients. This antibiotic distributes uniformly throughout the body and is effective against a wide range of infectious diseases. Mainly, it exerts bacteriostatic effects on a range of gram-positive and gram-negative organisms, particularly effective against H. influenzae, S. pneumoniae, S. typhi and Neisseria species [52, 53]. It is also active against rickettsiae, chlamydia, and mycoplasma, though not active against protozoans. Originally CAP was isolated from streptomyces venezuelae, and it is now produced synthetically.

CAP is only used when sensitivity tests indicate that it is most active agent and safer drugs not effective. Usually, it used for: 1) typhoid, other salmonella infections; 2) H. influenzae meningitis; 3) Anaerobic abscess; 4) Serious urinary tract infections.
Mechanism of Action

CAP, which is lipid soluble, diffuses through the bacterial cell membrane and reversibly binds to the 50 S subunit of the bacterial ribosomes where transfer of amino acids to growing peptide chains is prevented (perhaps by suppression of peptidyl transferase activity), thus inhibiting peptide bond formation and subsequent protein synthesis.

Chemical Structure

The chemical name of CAP (generic name) is 2,2-dichloro-\(N\)-[(1\(\alpha\),2\(\alpha\))-2-hydroxyl-1-hydroxymethyl-2-(4-nitrophenyl)ethyl]acetamide, \((\text{CAS: } 56-75-7)\). The trade name is Chloromycetin.

As shown in Figure 1-8, CAP contains a nitrobenzene ring, an amide bond, and an alcohol function. The presence of chlorides in biologically produced organic molecules is unusual. The nitrobenzene is relevant because it leads to the formation of aromatic amines, which may be carcinogenic. The amide may be hydrolyzed by some resistant bacteria, leading to inactivation. The alcohol serves as a functional group facilitating the formation of esters that improve water solubility of CAP. CAP base has low water solubility and high lipid (in organic alcohols) solubility.

![Molecular structure of chloramphenicol](image)

FIG. 1-8 Molecular structure of chloramphenicol

Properties

CAP is one of the most chemically stable antibiotics in common use, and shows good stability in the solid dosage forms and is subject to both thermal and photochemical degradation. Degradation rate is sufficiently slow in aqueous suspensions, cream, ointment and non-aqueous solutions. Degradation of CAP in aqueous media is mainly caused by the hydrolytic cleavage of the amide linkage. CAP in aqueous solution is
stable at room temperature in the pH range from 2 to 7, with a maximum stability at pH 6. For example, at 25 °C and pH 6, CAP in aqueous solution has a half-life of almost 3 years [54].

CAP is susceptible to photodegradation in aqueous solution even at room temperature. Under the influence of light and in water, CAP undergoes oxidation, reduction, and condensation reactions [55, 56]. Therefore, reasonably precautions are required to protect CAP and its preparations from excessive exposure to light or moisture in order to prevent significant decomposition over an extended period.

1.5.2. Chloramphenicol Eye Ointment

CAP eye ointment is a sterile preparation, manufacturing by incorporation of very finely powdered CAP into a petrolatum base. The base is designed to have a melting point close to human body temperature and can be used as anhydrous medium for the delivery of moisture-sensitive drugs. Each gram of the CAP eye ointment contains 10 mg of CAP and contains no preservatives. All the material used for eye ointments must be non-irritant and chemically inert. Wool fat (as emulsifying agent), cetostearyl alcohols (as emollient, emulsifying agent), and liquid paraffin (as consistency and fluidity regulator) are used as essential ingredients in the petrolatum base. Traditionally, CAP eye ointment is manufactured by mixing sterile ingredients and filling into container under aseptic conditions (Figure 1-1).

1.6. AIM AND SCOPE OF THE PRESENT WORK

1.6.1. Aim

A successful gamma radiation process encompasses three basic elements: product qualification, equipment qualification and process qualification [57]. All three combine to provide a validated sterilization processing. Amongst the three qualifications, the product qualification is of primary importance. In other words, the first consideration in selecting the radiation sterilization for a pharmaceutical substance should be given to the effects of radiation on the stability of the products. Radiation sterilization is
permissible only when the absence of deleterious effects on the product has been confirmed experimentally.

A main test for product qualification is to examine whether there are physical and chemical changes within the materials after receiving a sterilizing dose, and to determine the magnitude of the changes if any. Beyond a certain dose, irradiation could induce the formation of compounds (radiolysis products) in such quantities that toxicity must be considered. The significance of such products should be assessed in order to elucidate suitability of adopting gamma processing.

This thesis focuses primarily on the chemical changes of CAP eye ointment products induced by γ irradiation and on validation of the sterilization method. Efforts have been carried out to answer two fundamental questions:

1) Will the material/product be chemical stable to withstand any radiation dose?

2) Are there any chemical changes to impact the safety of the products?

1.6.2. Scope

An outline of the validation steps is shown in Figure 1-9. The validation process has the following essential features:

- Studying the radiation chemistry of the product with assay test in order to evaluate what radiation dose that material can withstand;

- Examining degradation products and identifying the types of degradation products in order to understand the possible mechanisms of radiation damage, and in the meantime evaluating the magnitude of the degradation;

- Quantifying degradation products if any and evaluating the toxicological risks of such products;

- Determining the mass balance to confirm no omission of degradation products;

- Assessing the significance of any observed changes;

- Developing strategies to minimize the radiation damage.
Introduction

γ Rays Sterilization

Degradation??

Assay test

Degradation No degradation

Quantifying radiolysis products

Acceptable amount Unacceptable amount

Identification

Assessing toxicity

No toxicity

Toxicity

Strategies against radiolysis

FIG. 1-9. Outline of the validation steps of sterilization of a pharmaceutical product
1.7. REFERENCES


Introduction


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Chapter 2

Assay and Impurity Test of Irradiated
Chloramphenicol Eye Ointment

A sample preparation method was developed to isolate chloramphenicol and its radiolytic products from an oily ointment base. The isolation method suspended the eye ointment in n-hexane at 45 °C, and isolated the target compounds as residue by centrifugation. It was found that the main requirement to ensure a satisfactory isolation was keeping the sample solution at 45°C during sample preparation. Linearity, precision, accuracy and suitability of the method were confirmed valid for both assay and impurity tests.

This isolation method was ideal for assay, unique for extraction of unexpected and complex radiolysis products, and had a number of advantages compared to the pretreatment methods described in The United States Pharmacopoeia and British Pharmacopoeia, in terms of accuracy, precision, and easy handling.

The effect of γ-irradiation on chloramphenicol eye ointment was studied by HPLC-DAD, after applying the developed sample preparation method. The present assay and impurity test methods with HPLC-DAD were confirmed to be suitable for irradiated chloramphenicol in eye ointment.

Published in Pharm. Biomedical Analysis 2001, 24, 667-674 [1]
2.1. GENERAL

Chloramphenicol (CAP) was initially determined by microbiological assay [2], but the elucidation of its structure has led to the use of a wide variety of chemical and physicochemical assay methods including argentometric titration, colourimetry [4], thin layer chromatography [5], UV spectroscopy [6], polarography [7, 8], gas chromatography [9-11] and the high-performance liquid chromatographic (HPLC) [12-17]. HPLC for the assay of CAP is superior to other conventional methods in speed, precision, specific and ease of performance.

Reliable determination of the influence of γ-irradiation on chloramphenicol in eye ointment depends critically on proper isolation of CAP and its possible degradation products from the ointment base. Liquid-liquid extraction, solid-phase extraction and centrifugation are generally applied for separating chloramphenicol from matrixes. Being typical traditional isolation methods relative to on chloramphenicol eye ointment (CAPEO), the methods of The United States Pharmacopoeia (USP) [18] and British Pharmacopoeia (BP) [19] employ liquid-liquid extraction using methanol and water as extraction agents. Attia et al [20] presented an extraction method to deal with the effect of ointment bases and temperatures on the stability of chlortetracycline hydrochloride and chloramphenicol in eye ointments. Kim et al [21] used graphitized carbon black as solid-phase to extract CAP from biological samples.

However, liquid-liquid extraction and solid-phase extraction are generally designed for assay of general chloramphenicol products only, and may not be applied directly to investigation of radiolysis products because it could not ensure an exhausted extraction of the unusual, complex, and trace radiolysis products. Separating CAP impurities from petrolatum ointment has been little studied in past.

Centrifugation, dissolving eye ointment in hydrophobic solvent and then separating the ointment part by centrifugation, can keep all the hydrophilic parts remained and ensure exhausted extraction. Although centrifugation is generally used as non-quantitative separation method, the method was used for qualitative separating neomycin from petrolatum based ointment for assay test [22].

The aim of present work was, therefore, to explore the possibility to isolate and CAP and its radiolysis products from CAPEO and develop rapid and reliable method to
determine the chemical changes of chloramphenicol eye ointment after $\gamma$-irradiation. In addition, suitability of traditional analysis methods on irradiated chloramphenicol products has yet to be confirmed.

2.2. EXPERIMENTAL

2.2.1. Material and Reagents

Chloramphenicol eye ointment, chloramphenicol powder, and eye ointment base (EOB, containing no active ingredient) were offered by Ciba Vision AG (Switzerland). All chemicals used in the present study were of reagent-grade or better. Methanol and acetonitrile were of HPLC grade solvent. The samples were irradiated in aluminium collapsible tubes by Cobalt-60 source to 25 or 50 kGy, respectively, in a radiation sterilisation plant of Studer AG (Switzerland). Details of the samples in this study were summarised in Table 2-1.

<table>
<thead>
<tr>
<th>Table 2-1. Sample Description and Their Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye Ointment Base</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Non-irradiated</td>
</tr>
<tr>
<td>Irradiated at 25 kGy</td>
</tr>
<tr>
<td>Irradiated at 50 kGy</td>
</tr>
<tr>
<td>Spiked Samples*</td>
</tr>
</tbody>
</table>

* Eye ointment base spiked with 10 mg CAP-0 and CAP-50, respectively.

2.2.2. Instruments and Operation Conditions

The HPLC experiments were carried out on a Merck Hitachi La Chrom liquid chromatograph equipped with an L-7100 pump, an L-7450 diode array detector, an L-7200 automatic injector, and a D-7000 interface. The operation conditions were summarized in Table 2-II. Impurity test by HPLC was carried out according to the work of Altorfer et al [23]. To minimize hydrolysis, all samples were analyzed within 8 hours after preparation.
Assay and Impurity Test of Chloramphenicol Eye Ointment

**TABLE 2-II. HPLC EXPERIMENTAL CONDITIONS FOR ASSAY AND IMPURITY ANALYSIS**

<table>
<thead>
<tr>
<th></th>
<th>Assay Test</th>
<th>Impurity Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>stainless steel, 125×4mm ID</td>
<td>stainless steel, 250×4mm ID</td>
</tr>
<tr>
<td>Stationary Phase</td>
<td>LiChrospher RP 18, 5μm</td>
<td>LiChrospher 60 RP select B, 5μm</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>water:methanol:glacial acid (55:45:0.1) (a), 1.000 mL.min(^{-1})</td>
<td>gradient: acetonitrile/phosphate buffer (20 mM, pH 2.5), 1.000 mL.min(^{-1})</td>
</tr>
<tr>
<td>Detector Wavelength</td>
<td>280 nm</td>
<td>278 nm</td>
</tr>
<tr>
<td>Sampling Size</td>
<td>10.0 μL</td>
<td>20.0 μL</td>
</tr>
</tbody>
</table>

\(^a\) \(v/v/v\)

Gas chromatograph analysis was carried out on a Varian Star 3400 CX instrument equipped with flame ionization detector. Capillary column: Rtx-5 (crossbond® 5% diphenyl-95% dimethyl polysiloxane, BGB Analytik AG, 30m, 0.32 mm ID, 0.5μm), 50 °C (hold 1 min) to 200 °C at 5 °C.min\(^{-1}\).

**2.2.3. Sample Preparation Procedures**

Samples of non-irradiated/irradiated CAP powder were prepared according to the procedures described in Table 2-III. For CAPEO samples, chloramphenicol and its degradation product were first isolated as dry powder and then prepared with the same procedures as that for CAP powder.

**TABLE 2-III. SAMPLE PREPARATION FOR THE HPLC ANALYSIS**

<table>
<thead>
<tr>
<th></th>
<th>Assay Test</th>
<th>Impurity Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Amount 1</td>
<td>10 mg CAP or equivalent</td>
<td>10 mg CAP or equivalent</td>
</tr>
<tr>
<td>Dilution 1</td>
<td>50 mL, methanol</td>
<td>2 mL, mobile phase</td>
</tr>
<tr>
<td>Initial Amount 2</td>
<td>10 mL of Dilute 1</td>
<td>none</td>
</tr>
<tr>
<td>Dilution 2</td>
<td>50 mL, mobile phase</td>
<td>none</td>
</tr>
</tbody>
</table>

The isolation was carried out as following: equivalent to 10 mg CAP of CAPEO was accurately weighed into a 15-mL glass centrifuge tube. After adding 10-mL n-hexane, the sample was placed in water bath at 45°C for ca. 5 min and agitated until it
was dissolved well. The sample was then centrifuged at 3500 rpm-min\(^{-1}\) for 2 min, and the supernatant liquid was discarded. This procedure was repeated three times. The analysis was carried put with the residues.

2.3. RESULTS AND DISCUSSION

2.3.1. Justification of the Method

With n-hexane as the extraction medium, the present isolation method separated successfully the eye ointment into hydrophilic and hydrophobic portions. It covered the whole hydrophilic part of CAP and its radiolysis products. CAP contained strong polar groups like intro, hydroxyl and dichloro etc., which were very active during gamma processing, therefore the radiolysis products of CAP were normally unexpected and complex. In this case, liquid-liquid extraction or solid phase extraction could not ensure the exhaustive extraction.

Leaving the n-hexane insoluble portion as dry residues, the method assured more freedom to choose solvent or solution concentration to dissolve those compounds for further analyses. This suited extremely well for the cases of analysis of radiolytic products, which were often unusual, complex and trace. This was in contrast to the methods of USP and BP, by which CAP and its degradation products would be extracted into a dilution solution of methanol or water.

The USP employed methanol as the extraction medium to separate CAP from the ointment base. It was found that white precipitates were produced in the resulting solution, which not only interfered with experimental operations of assay, but also resulted in impurity test to fail.

In addition, because CAP and its degradation products were isolated as dry powder, the present isolation made it easy to introduce other techniques (i.e. IR, TLC, NMR, LC-MS, UV etc.) for investigation of assay and radiolysis products in the ointment preparations. Finally, the manipulation of this method was very simple with only three times of centrifugation and reduced solvent consumption as well.
Assay and Impurity Test of Chloramphenicol Eye Ointment

Linearity

Typical chromatogram of radiolytic products by the impurity test was showed in Figure 2-1. Seven main impurity peaks were selected to study the impurities (identification of these peaks will be reported in our further work). Peak areas were used for quantitative calculation.

![Typical chromatograms of impurity test. CAPEO-0 and CAPEO-50 represent chloramphenicol eye ointment non-irradiated and irradiated at 50 kGy, EOB-50 represents eye ointment base (without active ingredient) irradiated at 50 kGy.](image)

In order to elicit the linearity of the present method, six levels over the range of 80-130% and 80-120% of the target concentration were used for assay test and impurity test, respectively. It was found that the peak areas were linearly related to the concentration over the given ranges in both cases. Least-squares regression analysis and statistical evaluation in Table 2-IV showed excellent linear behavior for assay and impurity test, as all the correlation coefficients (R) are more than 0.99.
Chapter 2

Precision

Precision of the isolation method was examined for assay test and impurity test, respectively. In the assay test, ointment samples including CAPEO-0, EOB+CAP-0, and CAPEO-50 were respectively isolated and analyzed with six replicates. The relative standard deviation (RSD) of the final analysis results (Figure 2-2), including the errors of the isolation and the HPLC procedures, fell well into the 95% confidence interval of the RSD of the HPLC determination alone (0.59 - 2.3) [24], which were measured using chloramphenicol reference solution (excluding isolation procedure). The results indicated that experimental errors from the isolation procedure were within that from HPLC procedure, confirming the validity of sample preparation for assay test.

<table>
<thead>
<tr>
<th>No.</th>
<th>Trendline Equation(^a)</th>
<th>(R^2)</th>
<th>Slope</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(y = 29x)</td>
<td>0.991</td>
<td>2.04</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(y = 225x)</td>
<td>0.997</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(y = 94x)</td>
<td>0.993</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(y = 129x)</td>
<td>0.993</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(y = 73x)</td>
<td>0.997</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(y = 14x)</td>
<td>0.994</td>
<td>4.37</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(y = 17x)</td>
<td>0.991</td>
<td>3.37</td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td>(y = 1298x)</td>
<td>0.9993</td>
<td>0.70</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\): set intercept = 0

For impurity test, precision was determined by the sample (EOB+CAP-50) that was prepared by spiking CAP-50 into eye ointment base (EOB). Similarly, the RSD of EOB+CAP-50 included the errors of both the isolation and the HPLC procedures, while the RSD of CAP-50, going through only HPLC analysis, represented the precision of the HPLC analysis procedure only. Table 2-V showed that although RSD of each analyte was different between CAP-50 and EOB+CAP-50, values of \(F_{cal}\), the experimental values of \(F\)-test between the two groups, were all less than the critical value of \(F_{0.05, 5, 5}=5.05\). It suggested that the differences of precision between the two groups were
Assay and Impurity Test of Chloramphenicol Eye Ointment

negligible and that the isolation procedure did not contribute significantly to the experimental errors. Therefore, the precision of isolation method for impurity test was, at least, within that of the HPLC analysis.

![Graph showing precision of assay test](image)

FIG. 2-2. Precision of assay test described by relative standard deviation (RSD). CAPEO-0 and CAPEO-50 represented chloramphenicol eye ointment non-irradiated and irradiated at 50 kGy, BOE+CAP-0 represented eye ointment base spiked with non-irradiated chloramphenicol powder.

<table>
<thead>
<tr>
<th>No.</th>
<th>RTa (min)</th>
<th>CAP-50 Responseb SD</th>
<th>RSD%</th>
<th>EOB + CAP-50 Responseb SD</th>
<th>RSD%</th>
<th>Ftest cal</th>
<th>Recovery %</th>
<th>ttest cal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.1</td>
<td>25452 396 1.56</td>
<td></td>
<td>25880 610 2.36</td>
<td></td>
<td>2.37</td>
<td>101.7</td>
<td>1.44</td>
</tr>
<tr>
<td>2</td>
<td>7.2</td>
<td>16203 303 1.87</td>
<td></td>
<td>16404 149 0.91</td>
<td></td>
<td>4.15</td>
<td>101.2</td>
<td>1.46</td>
</tr>
<tr>
<td>3</td>
<td>13.2</td>
<td>8348 131 1.57</td>
<td></td>
<td>8385 96 1.14</td>
<td></td>
<td>1.85</td>
<td>100.4</td>
<td>1.57</td>
</tr>
<tr>
<td>4</td>
<td>14.1</td>
<td>25630 492 1.92</td>
<td></td>
<td>25162 286 1.14</td>
<td></td>
<td>2.95</td>
<td>98.2</td>
<td>2.02</td>
</tr>
<tr>
<td>5</td>
<td>15.2</td>
<td>7279 148 2.03</td>
<td></td>
<td>7345 106 1.44</td>
<td></td>
<td>1.93</td>
<td>100.9</td>
<td>0.89</td>
</tr>
<tr>
<td>6</td>
<td>17.2</td>
<td>3277 55 1.68</td>
<td></td>
<td>3256 67 2.06</td>
<td></td>
<td>1.52</td>
<td>99.4</td>
<td>0.60</td>
</tr>
<tr>
<td>7</td>
<td>23.0</td>
<td>1929 103 5.34</td>
<td></td>
<td>1956 65 3.32</td>
<td></td>
<td>2.50</td>
<td>101.4</td>
<td>0.54</td>
</tr>
</tbody>
</table>

aRetention Time; bMean response of the impurity peaks from six replicates
Chapter 2

Accuracy

For assay test, the accuracy of the method was evaluated by recovery and t-test from six replicates of spiked samples (EOB+CAP-0) at target concentration (Table 2-VI). The recovery of CAP from spiked sample was 99.2%. Furthermore, the experiment value of t-test ($t_{cal}$) between CAP-0 and EOB+CAP-0 was 1.21, less than the critical value of $t_{0.05/2, 10} = 2.23$, indicating that there were no differences of analytical accuracy between EOB+CAP-0 and CAP-0 by the present method.

### Table 2-VI. Recovery of the Assay Test (N = 6)

<table>
<thead>
<tr>
<th>Method</th>
<th>CAP-0</th>
<th>EOB + CAP-0</th>
<th>Recovery</th>
<th>$t$ test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Response SD</td>
<td>Response SD</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>342698 4412 1.29</td>
<td>340101 2839 0.83</td>
<td>99.2</td>
<td>1.21</td>
</tr>
<tr>
<td>USP</td>
<td>344870 3876 1.12</td>
<td>313394 3949 1.26</td>
<td>90.9</td>
<td>13.93</td>
</tr>
</tbody>
</table>

*: response of chloramphenicol from six replicates

In contrast, the recovery was 90.9 % and $t_{cal}$ equaled 13.9 by the method of USP (Table 2-VI), which was far greater than the critical value. The USP method certainly gave different measured contents of CAP between CAP-0 solution and the spiked sample solution. It significantly undervalued the measured CAP content in the eye ointment, possibly due to the presence of white precipitates. However, proper analysis resulted by the USP method from different calibration curves could not be ruled out.

The results of $t$-test and recovery in Table 2-V demonstrated that the current method was also accurate for impurity test. The $t$-test was performed to measure the closeness of analytical agreement between CAP-50 (going through only the HPLC procedure) and spiking sample EOB+CAP-50 (going through both the isolation and the HPLC procedures). Every experimental value of t-test ($t_{cal}$) was less than critical value $t_{0.05/2, 10} = 2.23$, indicating that there were no significant differences in the measured impurity contents between the two groups. Thus, each impurity was isolated and analyzed accurately.
2.3.2. Characterization of the Isolation Process

Necessity and Validation of Heating

It was found that some components of the eye ointment base could not be fully dissolved in both hydrophilic and hydrophobic solvents without heating. The insoluble residues left in the final solution not only needed to be filtered, but might also cause residue encapsulation or adsorption of the target compounds, which resulted in poor recoveries. Heating the n-hexane suspension at 45 °C made the residues easily dissolved, and improved the recoveries successfully (Figure 2-3).

![Graph showing recovery comparison with and without heating](image)

**FIG. 2-3. Necessity of heating during sample preparation. The sample was treated with heating in 45 °C water bath and without heating at room temperature.**

However, heating treatment rose immediately the question whether or not chloramphenicol was still stable, as it was subject to both thermal and photochemical degradation [25]. In order to check the validation of this treatment, the spiked samples (EOB+CAP-0) were dissolved in 10 mL n-hexane, and heated in water bath at 45°C for different time intervals, then following the same sample preparation procedures.

Figure 2-4 showed that no new compound was formed even after 7 hours of heating treatment, and quantities of the original CAP and impurities had no visible variation as
well. It could be concluded that chloramphenicol kept its thermal stability at 45°C, and the present heating treatment was valid.

![Retention Time (min) vs Absorbance at 278 nm](image)

**FIG. 2-4. Evaluation of thermal stability of CAP at 45 °C**

**Precipitates during Sample Preparation in USP**

Methanol extraction was employed to extract CAP for assay test in USP. Severe white precipitates were formed in the final solution when the sample was suspended to the mobile phase of HPLC. To identify the precipitates, eye ointment base was dissolved and extracted according to the sample preparation procedures of USP. The extract solution was analyzed by gas chromatography.

Figure 2-5 showed that the extract solution included mainly 1-dodecanol, 1-tetracanol, 1-hexadecanol and 1-octadecanol (identification of the other smaller peaks will be reported in Chapter 6). Those compounds were extracted together with CAP and its radiolytic degradation products by the USP method, as they were soluble in methanol. However, they were insoluble in the mobile phase of HPLC for assay test of USP (the mixture solution of water, methanol and glacial acid), and presented as white precipitate. The mixture of 1-hexadecanol and 1-octadecanol was the well-known
ingredient of eye ointment base and functioned as emollient and emulsifying. In the present isolation method, these compounds were soluble in n-hexane and thus were extracted into hydrophobic part.

\[ \text{Retention Time (min)} \]

**FIG. 2-5. Gas chromatogram of the methanol extracts of petrolatum eye ointment base.**

### 2.3.3. Determination of Irradiated CAP

According to the report by Hangay et al. [26], irradiated CAP did not show measurable changes either in pure powder state, or in eye ointment after irradiation of 50 kGy dose. The present result, determined by HPLC, showed in contrast that CAP in eye ointment degraded significantly after irradiation (Figure 2-1). It was noted that UV-spectroscopy method was employed by Hangay et al., and the radiolytic degradation products were not identified in their studies. The influence of impurities on the assay test results was therefore, not clarified.

The three dimensional chromatogram (Figure 2-6) from HPLC diode array detector in the present study illustrated that impurities from CAPEO-50 also contributed to the UV absorbance almost at the same wavelength of maximum absorbance of CAP. Positive experimental errors were thus unavoidable.
The argument was further demonstrated when the assay test results were compared. The UV-spectroscopy method according to BP gave a positive error compared to that of the HPLC method in Table 2-VII. Therefore, the UV-spectroscopy method was unsuitable for assay determination of irradiated chloramphenicol products.

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPLC method</th>
<th>UV-spectroscopya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RSD (%)</td>
<td>Content (%)</td>
</tr>
<tr>
<td>CAPEO-0</td>
<td>1.06</td>
<td>100</td>
</tr>
<tr>
<td>CAPEO-50</td>
<td>1.21</td>
<td>88.9</td>
</tr>
</tbody>
</table>

a: according to the method of the British Pharmacopoeia for assay test (at 278 nm)

FIG. 2-6. Three dimensional HPLC chromatogram of chloramphenicol and impurities by diode array detector.
2.4. CONCLUSION

The present methods of isolation and determination of assay and impurity in CAP eye ointment were accurate, precise and reliable, and keeping the sample solution at 45°C during sample preparation was key to ensure a satisfactory isolation. It described for the first time a method to determine impurities in irradiated eye ointment products of chloramphenicol. In addition to simplified manipulation and low solvent consumption, the method isolated CAP and the impurities as dry residues, which ensured more flexibility for further determination.

The sample preparation methods of USP and BP were certainly not suitable for impurity determination of CAP eye ointment products, due to unsure exhausted extraction and the lean concentration in the resulting solution. Furthermore, methanolic extraction of ointment products by USP was involved in problems with precipitates, which encapsulated the target compounds and undermined experimental results. The UV spectroscopy method in BP certainly was not able to exclude the absorbance contributions from the CAP degradation products, which resulted in positive errors in the assay test of irradiated chloramphenicol eye ointment products. HPLC was clearly a better choice for the determination of assay and impurities of irradiated chloramphenicol eye ointment.
2.5. REFERENCE


Assay and Impurity Test of Chloramphenicol Eye Ointment


Identification and Evaluation of Chloramphenicol Radiolysis Products

The radiolysis products of chloramphenicol under γ-radiation sterilization were investigated systematically. Eight main radiolysis products were identified and quantified by HPLC-MS and HPLC-DAD, including two compounds never before reported. The minor radiolysis products were quantified, which showed that they were at concentration levels < threshold for identification. Carbon-carbon rupture reaction and oxidation reaction were proposed as the main radiolysis reactions of chloramphenicol powder. The applicability of γ-sterilization for chloramphenicol products was evaluated with qualitative and quantitative data that were compared to the threshold requirements of international regulations for identification. It was concluded that toxicities of the radiolysis products of chloramphenicol produced by γ-radiation sterilization could be neglected, the radiolysis products were safe for human health from the chemical view.
3.1. GENERAL

Generally, chloramphenicol (CAP) is chemically stable in solid dosage forms, is subjected to thermal and photochemical degradations [1]. CAP in dry state is more stable on γ-irradiation than in aqueous media. Fleurette et al. reported no loss in antimicrobial activity of CAP powder at irradiation dosage of 50 kGy [2]. Although changes could be observed in physical properties like discoloration, crystallinity and solubility, CAP in solid dry powder form can be radiation sterilized using Co-60 or electron beam at lower radiation dose of 15 kGy, at which insignificant effects have been observed and high chemical purity of irradiated CAP was retained at sterilization dose of 15 kGy [3].

It is therefore of both scientific and industrial interest to explore the chemical changes of chloramphenicol at the reference dose of 25 kGy. A feasibility study is required to ensure that the radiation treatment will not change chloramphenicol to become unsafe or unsuitable for medical use. Irradiated CAP should not be consider as a new drug, if its physicochemical integrity is established using sensitive analytical techniques by experts in the field [4]. To fulfill this requirement, the radiolytic products, if any, have to be identified and the toxicity of those degradation products has to be evaluated. Furthermore, the mechanisms of these reactions are interesting to be explored in order to find possible ways to eliminate irradiation degradation.

Previous investigations only gave fragmentary information of the radiation degradation products of chloramphenicol, and there is no report of simultaneously qualitative and quantitative data. Altorfer reported that there were at least five radiolysis products of chloramphenicol in the solid state and eleven products in 1% aqueous solution in 1974 [5], and separated the main radiolysis products of irradiated chloramphenicol by HPLC in 1997 [6], but without giving identification data. Varshney and Patel [3, 7] irradiated CAP using Co-60 radiation and electron beam at graded radiation doses up to 100 kGy and identified the four degradation products: 4-nitrobenzaldehyde, 4-nitro-benzoic acid, 4-nitrosobenzoic acid and HCl.

Zeegers et al. [8] distinguished 15 radiolysis products of irradiated chloramphenicol using GC-MS and semi-preparative HPLC, but provided no quantitative data. Their GC-MS methods involve derivation techniques, which make it difficult to continue to
perform quantitative analysis. In addition, their HPLC method separated only nine impurities and showed no identification data.

The consensus is that both qualitative and quantitative data for radiolysis products are essential for assessing the feasibility of gamma sterilization for pharmaceutical products, even for such a highly radiation-resistant substance as chloramphenicol. Although it was reported that photochemical degradation of chloramphenicol involved oxidation, reduction and condensation reactions [9, 10], no report involved radiolysis pathway of chloramphenicol.

The aim of the present study is therefore, to systematically identify and quantify the radiolytic degradation products of irradiated chloramphenicol and to achieve qualitative and quantitative analysis simultaneously for the radiolysis products of chloramphenicol. By the knowledge of the identification and quantification, the degradation pathway can be elucidated. This work enables qualitative evaluation of the chemical toxicity of chloramphenicol radiolysis products and gives integrated scientific proof of the feasibility of gamma sterilization for chloramphenicol products.

3.2. EXPERIMENTAL

3.2.1. Sample Preparation

Chloramphenicol powder sample was offered by CIBA Vision Ltd. (Switzerland). The powder was packed in conical glass bottles and irradiated by $^{60}$Co with 25 kGy (CAP-25) and 50 kGy (CAP-50), respectively, in a radiation sterilization plant of Studer AG (Switzerland). CAP-0 represents non-irradiated CAP powder.

3.2.2. HPLC-DAD

The HPLC experiments were carried out using a Merck Hitachi La Chrom liquid chromatograph equipped with a L-7100 pump, a L-7450 diode array detector (DAD), a L-7200 automatic injector, and a D-7000 interface; and managed by a Merck-Hitachi Model D-7000 Chromatography Data System. About 10 mg of accurately weighed CAP
sample was dissolved in a sample tube containing 2.0 mL mobile phase, and 20 μL was injected.

A 250 × 4 mm I.D. column packed with LiChrospher 60 RP select B (particle size 5 μm, Merck) was used for the analysis. The mobile phase was a gradient of 20 mM phosphate buffer (pH 2.5) and acetonitrile (described in Table 3-I) at a flow rate of 1.00 mL·min⁻¹. Chromatograms were recorded at 278 nm, and UV spectra were recorded in the range 200-450 nm [11].

<table>
<thead>
<tr>
<th>HPLC method, time (min)</th>
<th>Buffer (%)</th>
<th>MeCN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>HPLC-MSD method</td>
<td>H₂O (%)</td>
<td>MeCN (%)</td>
</tr>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

### 3.2.3. HPLC-MSD

The HPLC experiments were carried out on a Waters liquid chromatograph equipped with a quaternary Waters 626 LC pump, a Waters 600S controller with Millennium chromatography manager 2010, and a Rheodyne manual injector 7725i. A Waters symmetry C18 column (3.5 μm, 2.1 x150 mm) was used. The mobile phase was a MeCN-H₂O gradient (described in Table 3-I) at a flow rate of 200 μL·min⁻¹.

### ESI-MS

A triple stage quadrupole instrument (Finnigan TSQ 700) was equipped with a combined Finnigan Atmospheric Pressure Ion (API) source, and a quadrupole ion-trap instrument (Esquire-LC, Bruker-Franzen Analytik GmbH) was equipped with a combined Hewlett-Packard API source.
Finnigan TSQ 700

The capillary was held at 200°C, sheath gas pressure at 40psi. Argon was used as the collision gas for MS-MS experiments at a pressure of 1.8 to 2.5mTorr. The collision voltage varied between -9 and -35eV.

Bruker Esquire-LC

Drying gas at 250°C and 3.8 L-min\(^{-1}\), nebulizing gas at 21psi, capillary voltage at 4300V, end plate at 3800V, capillary exit at 75V, and Skimmer 1 at 25V. For MS-MS spectra the fragmentation amplitude was varied between 0.5 and 1.1 V.

3.2.4. Headspace-GC-MS

Headspace-GC-MS analysis was carried out on a Varian 3400cx Saturn 4D/GC-MS-MS equipped with headspace autosampler as in our former work [12]. A column of Rtx\(^\circledast\)-624 (30m×0.32 I.D., 1.8 \(\mu\)m) was used for analysis under the conditions: 45°C (hold 5 min) to 95°C at 2°C-min\(^{-1}\) (hold 25 min), helium carrier gas (5.0 grade), flow rate 1.1 mL-min\(^{-1}\).

Mass spectra were obtained at electron impacts of 70eV and chemical ionization (with methane reagent gas) at 15eV; chromatograms were recorded by monitoring the total ion current in the range 30-400u.; transfer line and ion-trap manifold were maintained at 220°C and 170°C, respectively.

3.3. RESULTS AND DISCUSSION

3.3.1. Identification of Degradation Products and Degradation Pathway

Figure 3-1 shows a typical HPLC chromatogram of the impurity profile of irradiated chloramphenicol powder. By comparing the chromatograms of non-irradiated (CAP-0) and irradiated (CAP-50) chloramphenicol samples, it was clear that degradation occurred during gamma processing.
FIG. 3-1. Determination of degradation profiles of γ-irradiated chloramphenicol (CAP-50) and non-irradiated chloramphenicol (CAP-0) by HPLC-DAD at 278 nm. Peak identification: 1 = 2-amino-1-(4-nitrophenyl)-1,3-propanediol, 2 = 2-formamide-1-(4-nitrophenyl)-1,3-propanediol, 3 = 2-(2-chloroacetamide)-1-(4-nitrophenyl)-1,3-propanediol, 4 = 4-nitrobenzoic acid, 5 = 2-(2,2-dichloroacetamide)-1-(4-nitrophenyl)-1-ethanol, 6 = 4-nitrobenzaldehyde, 7 = 2-(2,2-dichloroacetamide)-3-hydroxy-4-nitropropiophenone, 8 = 2-(2,2-dichloroacetamide)-4-nitro-acetophenone, and U1-U8 = unknown.

By comparing the HPLC chromatograms, UV and MS spectra with those of reference compounds, peak 1 was assigned to 2-amino-1-(4-nitrophenyl)-1,3-propanediol, a hydrolysis product of the amide group of chloramphenicol (a typical reaction in the photochemical degradation of CAP [9]). Peaks 4 and 6 were assigned to 4-nitrobenzoic acid and 4-nitrobenzaldehyde, respectively. Peak 6 gives a lower response factor in the current method because of peak distortion, and this makes it difficult to judge the absence of peak 6 using the current HPLC-DAD method. The
identification and quantitative determination of peaks 4 and 6 in irradiated chloramphenicol were confirmed using thin-layer chromatography [6].

### Table 3-II. Mass Fragment Information from HPLC-MSD Analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>$t_R$ (HPLC)</th>
<th>MW $^a$</th>
<th>Fragmentation information</th>
<th>$m/z$, 100%</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP</td>
<td>11.36</td>
<td>322</td>
<td>[M-H$_2$O]$^+$, 2 Cl</td>
<td>[M+H]$^+$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.29</td>
<td>212</td>
<td>[M-H$_2$O]$^+$, without Cl</td>
<td>[M+H]$^+$</td>
<td>[7, 8]</td>
</tr>
<tr>
<td>2</td>
<td>4.75</td>
<td>240</td>
<td>[M-H$_2$O]$^+$, without Cl</td>
<td>[M+H]$^+$</td>
<td>New finding</td>
</tr>
<tr>
<td>3</td>
<td>7.89</td>
<td>288</td>
<td>[M-H$_2$O]$^+$, 1 Cl</td>
<td>[M+H]$^+$</td>
<td>[6]</td>
</tr>
<tr>
<td>4</td>
<td>12.39</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[5, 6]</td>
</tr>
<tr>
<td>5</td>
<td>14.09</td>
<td>292</td>
<td>[M-H$_2$O]$^+$, 2 Cl</td>
<td>[M-H$_2$O]$^+$</td>
<td>[6]</td>
</tr>
<tr>
<td>6</td>
<td>14.59</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[5, 6]</td>
</tr>
<tr>
<td>7</td>
<td>14.95</td>
<td>320</td>
<td>[M-H$_2$O]$^+$, 2 Cl</td>
<td>[M+H]$^+$</td>
<td>New finding</td>
</tr>
<tr>
<td>8</td>
<td>18.14</td>
<td>290</td>
<td>2 Cl, without [M-H$_2$O]$^+$</td>
<td>[M+H]$^+$</td>
<td>[6]</td>
</tr>
</tbody>
</table>

$^a$ molecular weight

Peaks 2-3, 5, 7 and 8 were identified using HPLC-MSD analysis and assisted by HPLC-DAD absorption spectra (Figure 3-2). In mass spectra, the isotope pattern provided information on the numbers of chlorines in the target compounds, and the species [M-H$_2$O]$^+$ evidenced the presence of at least one hydroxyl group. Molecular weights were calculated using the corresponding [M+H]$^+$ species. As an example, the MS spectrum of peak 5 was characterized by the [M-H$_2$O]$^+$ peak at $m/z$ 275 (100%) and the [M+H]$^+$ peak at $m/z$ 293 (40%). Information for each compound is summarized in Table 3-II. From both the molecular structure of chloramphenicol and MS results, the chemical structures of the above radiolysis products have been determined and listed in Table 3-III.

The wavelengths of the absorbance maximum of each radiolysis product ($\lambda_{max}$) in the present HPLC mobile phase, a mixture of acetonitrile and 20 mM phosphate buffer (pH 2.5), are listed in Table 3-III. Chloramphenicol and 4-nitrobenzaldehyde were used as references for the molecular structure comparison. It was found that UV spectra of the identified radiolysis products perfectly overlapped with those of relevant reference
Identification of Evaluation of Chloramphenicol Radiolysis Products

Compounds (Figure 3-2). UV spectra of peaks 2 and 3 showed the typical shape of the chloramphenicol UV spectrum ($\lambda_{\text{max}}$ 278.2 nm), indicating that peaks 2 and 3 have a molecular structure similar to chloramphenicol. The UV spectra of peaks 7 and 8 coincided with the UV spectrum of 4-nitrobenzaldehyde ($\lambda_{\text{max}}$ 266.6 nm), verifying the presence of a carbonyl group in these peaks. The nitrobenzene ring conjugating with the carbonyl group gives an UV absorbance of shorter wavelength of ($\lambda_{\text{max}}$ 266.6 nm) than without conjugation ($\lambda_{\text{max}}$ 278.2 nm), as in the case of chloramphenicol.

The presence of the compounds corresponding to peaks 3, 5, and 7 in the radiolysis products of chloramphenicol have been reported by Zeegers et al. [8] using different identification methods, however, quantitative data were not available. Peaks 2 and 8 as the radiolysis products of chloramphenicol are new findings from the current investigation.

### Table 3-III. Structure and Measured UV $\lambda_{\text{max}}$ (Maximum Absorption) of Radiolysis Degradation Products in Irradiated Chloramphenicol.

<table>
<thead>
<tr>
<th>No.</th>
<th>$\lambda_{\text{max}}$, nm</th>
<th>Compound Structure</th>
<th>No.</th>
<th>$\lambda_{\text{max}}$, nm</th>
<th>Compound Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP</td>
<td>278.2</td>
<td>$\text{O}_2\text{N}^-\text{V-CH-CH}_2\text{Cl}$</td>
<td>5</td>
<td>273.3</td>
<td>$\text{O}_2\text{N}^-\text{V-CH-CH}_2\text{Cl}$</td>
</tr>
<tr>
<td>1</td>
<td>271.0</td>
<td>$\text{O}_2\text{N}^-\text{V-CH-CH}_2\text{NH}_2$</td>
<td>6</td>
<td>266.6</td>
<td>$\text{O}_2\text{N}^-\text{V-CH}_2$</td>
</tr>
<tr>
<td>2</td>
<td>278.2</td>
<td>$\text{O}_2\text{N}^-\text{V-CH-CH}_2\text{NH-C-H}$</td>
<td>7</td>
<td>266.6</td>
<td>$\text{O}_2\text{N}^-\text{V-CH-CH}_2\text{Cl}$</td>
</tr>
<tr>
<td>3</td>
<td>278.2</td>
<td>$\text{O}_2\text{N}^-\text{V-CH-CH}_2\text{NH-C-Cl}$</td>
<td>8</td>
<td>266.6</td>
<td>$\text{O}_2\text{N}^-\text{V-CH-CH}_2\text{Cl}$</td>
</tr>
<tr>
<td>4</td>
<td>264.5</td>
<td>$\text{O}_2\text{N}^-\text{V-O-H}$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Reference compound*
FIG. 3-2. HPLC-MS and HPLC-UV spectra of peaks 2, 3, 5, 7, and 8.
As shown in Figure 3-3, the identification results suggest that two kinds of reactions occur in chloramphenicol during gamma processing. One is bond rupture, and the other is oxidation. In the case of bond rupture, there are two driving forces that promote the rupture. First, the energy transferred from gamma rays to atoms of the
irradiated material overcomes the electrical attraction binding the electrons to the nucleus. The orbiting electrons thus excite and move to higher energy levels. Some bound electrons are separated from the atom or molecule. This process of ionization is the primary mechanism of bond rupture. The degradation process is controlled by irradiation energy, i.e. the irradiation dosage. In this way, scavengers could eliminate only the formation of chloramphenicol molecule radicals, while playing no role in bond ruptures.

Second, because moisture unavoidably exists in the chloramphenicol powder, γ-irradiation will rupture water molecule to form H* and HO2* radicals through e_{elv}, when the radiation energy is absorbed by the target substance [13]. These radicals could react with chloramphenicol and results in bond cleavage or replacement of functional groups. Using protection techniques, such as scavenging, these degradation reactions could be eliminated.

![Chromatograms of volatile radiolysis products by headspace-GC-MS.](image)

**FIG. 3-4. Chromatograms of volatile radiolysis products by headspace-GC-MS.**
Identification of Evaluation of Chloramphenicol Radiolysis Products

Figure 3-3 shows that 2-formamide-1-(4-nitrophenyl)-1,3-propanediol (peak 2) was formed through carbon-carbon rupture and by eliminating the dichloromethyl group. Headspace-GC-MS analysis showed that dichloromethane was detected in the irradiated chloramphenicol sample, while it was not present in the original sample (Figure 3-4). The concentration of dichloromethane was relevant to that of peak 2 in the irradiated CAP sample. This strongly suggests the formation pathway of 2-formamide-1-(4-nitrophenyl)-1,3-propanediol (peak 2).

Similarly, 2-(2-chloroacetamido)-1-(4-nitrophenyl)-1,3-propanediol (peak 3) forms through rupture of the carbon-chlorine bond, or replacement of a chlorine atom. 2-(2,2-Dichloroacetamido)-1-(4-nitrophenyl)-1-ethanol (peak 5) involves carbon-carbon bond rupture, and 2-amino-1-(4-nitrophenyl)-1,3-propanediol (peak 1) involves nitrogen-carbon bond rupture.

The presence of moisture also unavoidably causes the formation of radical oxidants *OH, HO₂* and peroxide in chloramphenicol powder due to water radiolysis during gamma processing [13]. These oxidant radicals result in oxidation reaction of chloramphenicol. The identification result shows that the oxidation reaction occurs and the hydroxyl group is oxidized to a carbonyl group as shown in Figure 3-3.

2-(2,2-dichloroacetamido)-3-hydroxy-4-nitropropiophenone (peak 7) is the oxidation product of the chloramphenicol hydroxyl group, and 2-(2,2-Dichloroacetamido)-4-nitro-acetophenone (peak 8) is resulted from combination of an oxidation and carbon-carbon rupture. It appears that the concentration of peak 8 is relatively lower than that of peak 7 (Figure 3-1). This observation supports the conclusion that peak 8 is the product of two-step reactions (carbon-carbon rupture then oxidation, or *vice versa*), while peak 7 is a one-step reaction product. In addition, peaks 4 and 6, 4-nitrobenzoic acid and 4-nitrobenzaldehyde could be also the products of two-step reactions (carbon-carbon rupture then oxidation, or *vice versa*) from chloramphenicol and all of the other radiolysis products.

3.3.2. Evaluation of Radiolysis Products

To confirm that, as appropriate, γ-sterilization does not result in unacceptable radiolysis products in the irradiated chloramphenicol, the toxicity of the radiolysis
products must be estimated. Table 3-III shows that molecular structures of those degradation products resemble chloramphenicol. Chloramphenicol contains a nitrobenzene ring, an amide bond, and an alcohol function. The presence of chloride in biologically produced organic molecules is unusual. Nitrobenzene is the main functional group of chloramphenicol and the nitrobenzene is relevant because it leads to the formation of aromatic amines that may be carcinogenic. The hydrolysis of amide leads to inactivation. The alcohol serves as a functional group facilitating the formation of esters that improve the water solubility of chloramphenicol [14].

Health hazard data [15] shows that 4-nitrobenzoic acid and 4-nitrobenzaldehyde are safe to human health at such trace levels. Accordingly, it could be proposed that the molecular structures of these radiolysis products in Table 3-III contain no toxic functional groups, and it could be expected that the toxicities of those radiolysis products identified were similar to that of chloramphenicol. Cytotoxic tests performed previously show that no toxic effects induced by irradiated chloramphenicol were detected. This strongly supports this conclusion.

It is noted that in irradiated chloramphenicol samples there are still some minor impurities (labeled U1-U8) that have not yet been identified (Figure 3-1). Further efforts to identify those impurities were difficult to interpret with confidence due to their low concentration. According to the requirements of EMEA [16], degradation products should be identified or reported when present at levels > the required thresholds, which depend on the maximum daily dose. Since the maximum daily dose of chloramphenicol is 3.0g [17], accordingly, the threshold for identification of its degradation products is 0.1%, and for reporting, 0.05% [16].

Quantitative analysis of the impurities in irradiated chloramphenicol was carried out in order to clarify the necessity for further identification. Non-irradiated chloramphenicol was used to prepare the calibration curves. The results are summarized in Table 3-IV. They show that at an irradiation dose of 25 kGy, all of the unidentified impurities are present at levels of not more than the threshold, except peak U6. However, by comparing the impurity profiles in Figure 3-1, it is noted that peak U6 is not a radiolysis product, but an original impurity in the drug substance. The concentration of peak U6 was not augmented after γ-irradiation.
Identification of Evaluation of Chloramphenicol Radiolysis Products

TABLE 3-IV. QUANTITATIVE ANALYSIS OF IMPURITIES IN IRRADIATED AND NON-IRRADIATED CHLORAMPHENICOL.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Identified Peaks, % (w/w)(^a)</th>
<th>Unidentified Peaks, % (w/w)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.023</td>
<td>0.080</td>
</tr>
<tr>
<td>2</td>
<td>0.0002</td>
<td>0.061</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>0.098</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>0.046</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>0.058</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>0.166</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>0.015</td>
</tr>
</tbody>
</table>

\(^a\): weight/weight, pure non-irradiated chloramphenicol used as reference to prepare calibration curve for quantitative determination of radiolysis products because the references for these radiolysis products are unavailable.

Peak U8 was also present in the non-irradiated chloramphenicol samples as an impurity. Unlike peak U6, the concentration of peak U8 increased slightly after \(\gamma\)-irradiation, but was still below the threshold value after \(\gamma\) irradiation at 25 kGy. No further identification is therefore, necessary. It is clear that concentrations of all the unidentified radiolysis products are not greater than the thresholds of identification and reporting. Their influences on the quality of irradiated CAP can be neglected.

Chloramphenicol contains a nitrobenzene ring, an amide bond, and an alcohol functional group. Reduction of the nitrobenzene ring could lead to the formation of aromatic amines, which may be carcinogenic. To examine further the possible formation of aromatic amines in the degradation products, 4-aminobenzoic acid, 4-aminobenzaldehyde, and 4-aminophenol were spiked into the irradiated chloramphenicol and examined by HPLC-DAD. Retention times of the three compounds were carefully compared with those of all radiolysis products, respectively. UV spectra and peak purity checks were carried out as well. Signs of the existence of these aromatic amines in the radiolysis products were not in evidence. UV spectra of the three compounds were also compared with all the radiolysis products that had different...
retention times from the three, and no coincidence was found. This suggests that the presence of similar aromatic amines in irradiated chloramphenicol samples was unlikely.

3.4. CONCLUSION

The present work makes it possible to evaluate the radiolysis of chloramphenicol products simultaneously with qualitative and quantitative data. Accordingly, it was found that none of the major radiolysis products in irradiated chloramphenicol should be unacceptable and threaten the product safety, as required by The European Agency for the Evaluation of Medicinal Products (EMEA). Quantitative analysis also shows that all unidentified radiolysis products are present at levels not more than the thresholds of identification and reporting as required by EMEA. In conclusion, gamma radiation treatment does not cause chloramphenicol to become risky or unsuitable for medical use.
Identification of Evaluation of Chloramphenicol Radiolysis Products

3.5. REFERENCES


[14] Coppoc, G. L. Purdue Research Foundation 1996,  

[15] Material Safety Data Sheets (MSDS),  
http://chem-courses.ucsd.edu/CoursePages/Uglabs/MSDS/.


Radiolysis Characterization of Chloramphenicol in Powder and in Eye Ointment

The effects of γ radiation sterilization on chloramphenicol, in both pure powder state and petrolatum eye ointment, were investigated with HPLC. The content of chloramphenicol decreased by 1.0% in powder state and by 1.2% in eye ointment at the reference radiation dose of 25 kGy. The profile of chloramphenicol radiolysis products in powder state differed from that in eye ointment.

It was found that microenvironment of chloramphenicol molecule was a key factor governing the radiolysis of chloramphenicol in powder state. Solvent residues in chloramphenicol powder could change the radiolysis behavior of chloramphenicol. The solvents, in which chloramphenicol was readily soluble, promoted radiolytic hydrolysis of chloramphenicol, but the converses did not. Inert gas purging or diffusion by exposing in absorbent was efficient method to prevent chloramphenicol powder from radiolysis.

The influence of the presence of oxygen was explored. Oxygen played a role of scavenger and diminishes radiolysis of chloramphenicol. It was found that N-acetyl-L-cysteine could protection chloramphenicol in eye ointment from radiolysis. Hydrophobic radiolysis products of chloramphenicol were observed in eye ointment part. Using scavengers and lower irradiation could be strategies to resist radiolyses of chloramphenicol in petrolatum eye ointment.
4.1. GENERAL

Due to the poor thermal stability [1], D-(threo)-chloramphenicol (CAP) powder has currently to be sterilized by the ethylene oxide method, and CAP eye ointment (CAPEO) is manufactured under aseptic conditions. However, the ethylene oxide method is involved in the problems of toxic residues [2, 3], and aseptic process is involved in possibility of second contamination as well as high cast of invest and maintenance. Accordingly, there are great scientific and industrial interests to convert the current sterilization methods to γ processing. The key factor for a successful γ sterilization is to ensure that the radiation treatment does not cause the degradation and the formation of toxic compounds in the target products.

The existing reports about gamma sterilization on CAP powder are rather fragmental and conflicting. In the 60s-70s, three group of researchers reported that powder CAP did not decompose when exposed at irradiation doses of 25, 50, and 100 kGy [4-6]. Schulte and Henke found a decomposition of 1.5% CAP at 60 kGy [7]. Diding et al. concluded that after 25 kGy irradiation from either a 60Co source or a linear electron accelerator, CAP did not meet some of the Nordic Pharmacopoeia quality requirements [8]. Altorfer estimated decomposition at these doses at up to 1-2% and systematically reported different methods for the assay and impurity-tests of CAP powder [9, 10]. Varshney et al. concluded that CAP in dry powder form can be sterilized by 60Co or electron beam at 15 kGy [11]. However, it was reported that changes could be observed in physical properties like discoloration, crystallinity and solubility at sterilization dose of 15 kGy [12, 13]. The color change decreased in different atmospheres, in the order air < N2 < N2O < H2.

The previous investigation concluded that there are no universal testing protocols that would be applied to all pharmaceuticals, but rather each compound should be considered individually. Therefore, the stability of CAP in eye ointment may be different from that of CAP in powder state during gamma irradiation. Among the numerous references to gamma sterilization, there are relatively few studies concerning ointment. Hangay et al. reported in 1967 that hydrocortisone eye ointment containing two active ingredients (hydrocortisone acetate and CAP) was suited for radiation sterilization and the quantitative determinations and assays of the two active ingredients
did not show changes either as the pure substance or in the ointment [14]. The unpublished data from Gopal in 1978 claimed that gamma irradiation at 25 kGy causes principally the formation of p-nitro-benzaldehyde (I) and p-nitro-benzoic acid (II) and a few other unknown species as revealed by TLC and HPLC. The content of I and II is about 0.06% each at 25 kGy and increases to 0.12% at 60 kGy [15]. Furthermore, CAP eye ointment had been approved by regulatory authorities for radiation sterilization and decontamination in UK, Norway and India [15, 16]. Nevertheless, no reports released the details of performing gamma sterilization on CAP eye ointment and chemically validated irradiation dosage. The radiolysis degradation products of CAP in eye ointment had never been examined. The factors governing CAP radiolysis and methods protecting CAP from radiolysis had rarely explored.

The aims of present work are 1) to clarify the radiolysis degree of CAP both in powder state and eye ointment state, 2) to examine the radiolysis products of CAP in eye ointment, and 3) to explore protection techniques for eliminating possible radiolysis.

4.2. EXPERIMENTAL

Experimental conditions of assay test, impurity analysis and sample preparation were described in Chapter 2.

4.3. RESULTS AND DISCUSSION

4.3.1. Assay Test

The effect of $\gamma$-irradiation on the CAP content was determined by HPLC (Figure 4-1), which shows that $\gamma$-irradiation treatment induces CAP degradation both in eye ointment and in powder state. Although the degree of degradation in both cases is proportional to the radiation dose, the degree in powder state increases high linearly with irradiation dosage (the correlation coefficient is 0.9997), while the degree shows nonlinear relationship with the dosage in the case of CAP eye ointment. Thus,
differences of degradation degree between CAP and CAPEO at 25 kGy and below are negligible, and CAP content in CAPEO decreased much more significantly than that in powder state at 50 kGy. This indicates that the oily ointment matrix plays a role in the γ induced degradation and CAP molecule may undergo different degradation pathways in powder state and eye ointment during γ processing.

**FIG. 4-1.** Effect of γ-irradiation on chloramphenicol (CAP) content determined by HPLC assay test (n = 6 at each point). The non-irradiated samples were set as 100% and the CAP eye ointment and CAP powder samples were packed respectively in conical glass flasks for irradiation.
At the standard dose of 25 kGy [17], CAP degraded by more than 1% both in powder and in eye ointment states. Although it was reported that radiolytical degradation of CAP did not affect its biological activity [14], the degradation fragments are not always harmless and may influence the secondary effects by increasing toxic or immunologic side effects. According to regulation of Intentional Conference of Harmonization, radiolysis products have to be qualified down to a threshold of 0.1% [18]. Therefore, a loss of more than 1.0 % of the active principle is not acceptable without knowing the toxicity of the radiolytic products and the degradation products must be explored. In addition, it is also of great interesting to further explore the difference with impurity test in order to well understand degradation pathway and to find possible ways to eliminate the irradiation degradation.

4.3.2. Impurity Test

Typical HPLC chromatograms of impurity profiles of CAP powder and CAP eye ointment are shown in Figure 4-2, in which the radiolysis products in CAP powder were assigned according to our previous work [19]. The previous work concluded that none of radiolysis products in irradiated CAP powder may be unacceptable and threatens the product safety. By comparing the impurity profile of irradiated CAP powder and irradiation CAP eye ointment, it is noted that peak 1 is a radiolysis product in CAP powder but a trace original impurity in CAPEO. Peak 1, assigned as 2-amino-1-(4-nitrophenyl)-1,3-propanediol, is a typical hydrolysis product of CAP in thermal and photochemical processing [20, 21].

It is also observed that the relative intensities of other radiolysis product peaks differs significantly between CAP powder and CAP eye ointment samples in Figure 4-3. By means of simultaneously qualifying and quantifying determination, it was possible to quantitatively compare the influence of irradiation conditions (CAP status, matrices) on the degradation products (Table 4-1). Peak 1, the hydrolysis product, and Peak 2 almost disappeared, and the amounts of Peaks 4, 6, 7 and 9 were obviously reduced in CAPEO, while the amounts of radical reduction products Peaks 3 and 5 are higher in CAPEO than those in CAP powder. This impurity test result also indicates that the petrolatum ointment base play a role during CAP molecular radiolysis and the amounts of the radiolysis products varies with the changes of CAP micro-surrounding, especially, the radiolysis product of peak 1, which can be eliminated out by changing the surrounding of CAP.
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1. 2-amino-1-(4-nitrophenyl)-1,3-propanediol
2. 2-formamide-1-(4-nitrophenyl)-1,3-propanediol
3. 2-(2-chloroacetamido)-1-(4-nitrophenyl)-1,3-propanediol
4. 4-nitrobenzoic acid
5. 1-(4-nitrophenyl)-2-(2,2-dichloroacetamido)-1-ethanol
6. 4-nitrobenzaldehyde
7. 2-(2,2-dichloroacetamido)-3-hydroxy-4-nitropropiophenone
8. Unknown
9. 2-(2,2-dichloroacetamido)-4-nitroacetophenone
10. Unknown

FIG. 4-2. Comparison of the radiolysis products of chloramphenicol in powder and in eye ointment, irradiated at 50 kGy.

CAP and CAPEO mean chloramphenicol powder and its eye ointment, respectively.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>In CAP-25</th>
<th>In CAPEO-25</th>
<th>CAP-25/CAPEO-25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>166565</td>
<td>2970</td>
<td>56.1</td>
</tr>
<tr>
<td>Peak 2</td>
<td>29905</td>
<td>4099</td>
<td>7.2</td>
</tr>
<tr>
<td>Molecular rupture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 3</td>
<td>76900</td>
<td>112112</td>
<td>0.68</td>
</tr>
<tr>
<td>Peak 5</td>
<td>48894</td>
<td>67004</td>
<td>0.72</td>
</tr>
<tr>
<td>Oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 4</td>
<td>61564</td>
<td>47728</td>
<td>1.3</td>
</tr>
<tr>
<td>Peak 6</td>
<td>8029</td>
<td>6982</td>
<td>1.2</td>
</tr>
<tr>
<td>Peak 7</td>
<td>89054</td>
<td>52130</td>
<td>1.7</td>
</tr>
<tr>
<td>Peak 9</td>
<td>14750</td>
<td>7032</td>
<td>2.1</td>
</tr>
</tbody>
</table>

^a,b: chloramphenicol powder and chloramphenicol eye ointment irradiated at 25 kGy, respectively

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CAP molecules in CAPEO are likely to be surrounded by the ointment base, instead of the air surrounding conditions in the powder state. The compounds of Peaks 4, 6, 7 and 9 are radiolysis products from oxidation reaction, and Peaks 3 and 5 from CAP molecular rupture or reduction reaction [19]. The data in Table 4-1 suggest that the elimination of the air atmosphere in CAPEO samples has certainly reduced the radiolytical hydrolysis and oxidation reactions, while air free condition in CAP powder reduces molecular rupture during γ-irradiation.

As the well elucidation of the radiolysis behavior of water molecule and oxygen have been well elucidated [9, 22-24], whenever moisture presence, the following ionization of water molecule could occur:

\[ H_2O \rightarrow H_2O^+ + e^- \]

\[ H_2O^+ + e^- \rightarrow H^+ + \cdot OH \]

\[ H_2O + H_2O^+ \rightarrow H_3O^+ + \cdot OH \]

\[ 2\cdot OH \rightarrow H_2O_2 \]

\[ H^+ + H_2O_2 \rightarrow H_2O + \cdot OH \]

\[ \cdot OH + H_2O_2 \rightarrow H_2O + HO_2^* \]

In these reactions, oxidant species (HO*, H₂O₂, HO₂*) are produced, they will unavoidably promote the formation of oxidation products during γ process.

Oxygen may play double roles during γ process. It can produce oxidants, and adsorb the radicals as a proven electron scavenger, which can inhibit the formation of radiolysis products from molecular rupture during γ processing. The chemistry of oxygen during γ can be expressed as following [25]:

\[ O_2 + e^- + M \rightarrow O_2 + M \] (1)

\[ O_2^* \rightarrow 2O \] (2)

\[ O_2 + O_2^* \rightarrow O + O_3 \] (3)

\[ O + O_3 \rightarrow 2O_2 \] (4)

In reaction (1), oxygen acts as scavenger of electron (M is a third body), and in reactions (2)-(4), it acts as precursor of oxidants. However, the energy deposited by the
Radiolysis Characterization of Chloramphenicol in Powder and in Eye Ointment

gamma ray to a component of a mixture, to a first approximation, is proportional to the fraction of molecules of each compound. Oxygen concentration is much less than the CAP or the ointment, thus very little energy is deposited in the oxygen, and it is not a major source of oxidants. Its main effect will be as a radical scavenger. Reactions 2-4 are of no significance in this system.

In the manufacture process, eye ointment base is pretreated at 120°C for two hours under vacuum for sterilization and homogenization. Then the base is blended with CAP powder immediately after cooling under vacuum. Thus the moisture amount in petrolatum eye ointment base is less than that in powder state although the presence of moisture is always unavoidable in eye ointment and CAP powder. The presence of oxygen molecules and moisture in the neighborhood of a CAP molecule is less possible in CAPEO than in CAP powder. Consequently, the amounts of rupture products of Peak 3 and 5 in CAP powder are lower than those in CAPEO, while the amounts of oxidation products (peaks 4, 6, 7, and 9) in CAP powder is higher than those in eye ointment system (as shown in Table 4-I).

4.3.3. Strategies against Radiolysis of Chloramphenicol Powder

Both assay test and impurity test results demonstrate that the microenvironment surrounding CAP molecules is a key factor for the CAP radiolysis and the amount of radiolysis products, especially the compound of peak 1, depends on CAP microenvironment. This suggests a way to diminish the CAP radiolysis by changing the CAP microenvironment. Accordingly, CAP powder samples were pretreated by He and N₂ purging for 48 hours, as well as by drying in exsiccator (containing silica gel) for 48 hours, respectively. All the pretreated samples were then irradiated and analyzed at identical conditions for comparison.

The results show that these pretreatment methods effectively reduce the radiolysis of CAP powder from 2.1% to ca. 0.8% at 50 kGy, and there is no significant difference amongst helium purging, nitrogen purging, and drying in exsiccator (Table 4-II). Impurity profiles show that peak 1, 2-amino-1-(4-nitrophenyl)-1,3-propanediol, was dramatically eliminated by all the three the pretreatment, and the case of helium purging is given as an example in Figure 4-3. Both the assay and impurity determinations
demonstrate that purging and drying treatments are efficient methods to relieve CAP radiolysis in powder state.

**TABLE 4-II. INFLUENCE OF INERT GASES PURGING AND DRYING IN EXSICCATOR ON CHLORAMPHENICOL CONTENT IN CAP POWDER AFTER γ IRRADIATION**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CAP content a, %</th>
<th>RSD%, n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment, 0 kGy</td>
<td>100</td>
<td>0.61</td>
</tr>
<tr>
<td>No pretreatment, 50 kGy</td>
<td>97.9</td>
<td>0.88</td>
</tr>
<tr>
<td>He purge, 48 hours, 50 kGy</td>
<td>99.1</td>
<td>0.50</td>
</tr>
<tr>
<td>N2 purge, 48 hours; 50 kGy</td>
<td>99.2</td>
<td>0.69</td>
</tr>
<tr>
<td>In silica gel exsiccator, 48 hours; 50 kGy</td>
<td>99.3</td>
<td>0.53</td>
</tr>
</tbody>
</table>

a: results by HPLC assay test.

**FIG. 4-3. Effect of helium purging on γ induced degradation of chloramphenicol powder.**

CAP and CAP-He mean chloramphenicol powder and chloramphenicol powder purged by helium for 48 hours, respectively.
Water molecular is very active during gamma processing and aqueous solution of CAP is unstable. Rhee et al. reported that CAP in solution was 90 percent inactivated even at a 15 kGy dose [6]. The moisture might play a role in the radiolysis of CAP, and might be the thing that causes CAP radiolysis. The moisture could be driven by the purging or drying treatment. Hence, about 1000 ppm (w/w) water was spiked into the helium purged CAP powder before γ-irradiation in order to explore the role of water molecular. It was found in surprise the spiked water did not promote the formation of Peak 1 (Figure 4-4). This means that moisture might not be the governing factor of radiolytical hydrolysis of CAP powder. Purging and drying pretreatments may drive off not only moisture, but also other solvent residues. Thus, more solvents that possibly existed in CAP as residues were spiked into the dry-pretreated CAP powder at the level of 1000 ppm, respectively, and then these samples were irradiated at 50 kGy.

FIG.4-4. Influence of typical solvent residues on chloramphenicol radiolysis.

CAP-MeOH-50, CAP-Water-50, CAP-CHCl3-50 mean the chloramphenicol powder spiked respectively with methanol, water, and chloroform after helium purging and irradiated at 50 kGy, and CAP-He-50 means chloramphenicol powder treated by helium purging and 50 kGy irradiation.
It is of interesting to find that presence of solvents, such as chloroform, toluene, dichloromethane, and benzene do not influence the radiolytical hydrolysis of CAP. In contrast, methanol, ethanol, isopropanol, acetonitrile and 1,4-dioxane strongly enhance the formation of the hydrolysis product, Peak 1 (examples are shown in Figure 4-3 & 4-4). It is noted that CAP is well dissoluble in the former solvent group, and has poor solubility in later group. The solubilities of CAP in these solvents are summarized in Table 4-III.

Table 4-III shows a clear relationship between solubility and the formation of Peak 1. It is also noted that the solvents inducing radiolysis are miscible with water, while those solvents without inducing function are nonmiscible with water. Alcohols, dioxin and acetonitrile, having inducing function, are quite different from each other in chemical nature and molecular structure. It is unlikely to that the solvent molecule itself or its fragment directly attack the CAP molecule during γ-irradiation. Further experiment shows that serious radiolytical hydrolysis will happen when the amount of spiked water reaches to 1%. A possible explanation could be that water molecule could be rather difficult to approach CAP molecule without a proper medium due to its poor solubility for CAP. The solvents having inducing function could play a medium role for the reactions between water and CAP. Thus, the solubility of residual solvents is one of important factors for the radiolysis of CAP.

**Table 4-III. Solubility of chloramphenicol in some solvents possibly existed in pharmaceuticals [26].**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility, mg/mL, 28°C</th>
<th>Peak 1 inducing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.4</td>
<td>No</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.145</td>
<td>No</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.26</td>
<td>No</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.95</td>
<td>No</td>
</tr>
<tr>
<td>Ethylene chloride</td>
<td>2.3</td>
<td>No</td>
</tr>
<tr>
<td>Methanol</td>
<td>&gt; 20</td>
<td>Yes</td>
</tr>
<tr>
<td>Ethanol</td>
<td>&gt; 20</td>
<td>Yes</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>&gt; 20</td>
<td>Yes</td>
</tr>
<tr>
<td>Dioxane</td>
<td>&gt; 20</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Radiolysis Characterization of Chloramphenicol in Powder and in Eye Ointment

Acetonitrile > 20 Yes

It is also a good explanation for the conflicting reports on CAP powder radiolysis in literature. The microenvironment of CAP powder from different manufacturers could be different, the presence or absence of the solvents with inducing function can result in different radiolysis behavior. The drying in present work functions actually as exposing to get rid of both solvent residuals and moisture. The inert gases purging and drying are practical methods to clean the environment of CAP, and to protect CAP powder from radiolysis.

4.3.4. Radiolysis of Chloramphenicol in Eye Ointment

The quantitative determination of the radiolysis products had to be carried out semi-quantitatively, due to the lack of relative reference compounds. The non-irradiated CAP was used as reference compounds for all the radiolysis products to prepare calibration curves. The semi-quantitative result is shown in Table 4-IV.

It is noted that the assay test results do not match the impurity test results, especially in the case of CAPEO at higher irradiation dose (50 kGy). Assay tests show that the degradation amount in CAP powder is higher than that in CAPEO, while the sum of all the impurities in the identical samples by impurity test are reverse. This strongly suggests that some of the radiolysis products in CAPEO have escaped from the present HPLC measurement, or from the extraction process during the sample preparation.

Table 4-IV. HPLC Quantitative results of assay and impurity tests after γ sterilization of CAP powder and CAP eye ointment.

<table>
<thead>
<tr>
<th>Assay test</th>
<th>Sum of impurities a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assay test</td>
</tr>
<tr>
<td></td>
<td>CAP</td>
</tr>
<tr>
<td>15 kGy</td>
<td>99.4%</td>
</tr>
<tr>
<td>25 kGy</td>
<td>99.0%</td>
</tr>
<tr>
<td>50 kGy</td>
<td>97.9%</td>
</tr>
</tbody>
</table>

a: response factors of all impurities were assumed the same as chloramphenicol.
As the part from escaped measurement, the radiolysis products in CAPEO could be:

— Too low concentrations to be detected;
— No absorbance at the HPLC detector (UV, 278nm);
— Volatile compounds;
— Retained at the top of HPLC column;
— Hydrophobic compounds and left in petrolatum eye ointment base part.

An assay test, using UV spectroscopy method at the measurement wavelength (278nm), was carried in order to examine the low concentration radiolysis products. In this measurement, all impurities having absorbance at 278 nm are taken into account of assay result, no matter what concentration levels. The value of this assay test for irradiated CAPEO is 92.4% at 50 kGy (RSD% = 0.05, n= 6) and it is higher than that using HPLC method in Table 4-IV. Nevertheless, there is still disagreement between this assay test and impurity test in Table 4-IV.

Further examination was carried out with thin-layer chromatography (TLC). As shown in Figure 4-5, no additional sizeable peaks were found in the chromatogram of CAPEO compared to the chromatogram of CAP powder. It proves that no new degradation products have stocked on the HPLC column during the impurity test. Using universal spray reagents (iodine vapour, vanillin-sulphuric acid, and sulphuric acid) had not reviewed any new compounds either, suggesting that no UV insensitive compounds had escaped detection. Examination with diode array detector over 200-400 nm in the HPLC impurity test detected no new radiolysis products as well.

Volatile radiolysis products may be formed during gamma processing because the radiation energy could be strong enough to break CAP molecule into smaller pieces, possibly volatile compounds. However, the volatile radiolysis profile of irradiated CAPEO determined using Headspace-GC-MS is exactly the same as that irradiated eye ointment base (without CAP), even at very high dose of 100 kGy (in our unpublished data).

These examination results indicate that some of radiolysis products of CAPEO have escaped from the sample preparation process. By the molecular structure of CAP, there is little possibility that CAP forms non-volatile hydrophobic radiolysis products by molecular rupture. Therefore, the only possibility is that CAP molecules or its radicals react with certain radicals from ointment base ingredients during γ-irradiation. By this
Radiolysis Characterization of Chloramphenicol in Powder and in Eye Ointment

way, radiolysis products of CAP become hydrophobic and remain in the hydrophobic medium.

FIG. 4-5. TLC chromatograms of irradiated chloramphenicol in powder (CAP-50) and in eye ointment (CAPEO-50) at 50 kGy. Stationary phase: silica gel 60 F254. Mobile phase: chloroform/methanol/glacial acetic acid = 97:14:7 (v/v/v); Detector wavelength: 278 nm. Peak 1 = 2-amino-1-(4-nitrophenyl)-1,3-propanediol; Peak 2 = 4-nitrobenzaldehyde.

It was observed that after γ-irradiation at 50 kGy, light yellow color of CAPEO became dark yellow, while the color of eye ointment base did not change. The examination of the hydrophobic part was carried out by UV spectroscopy method. The UV spectra of the eye ointment extraction (hydrophobic residues) in Figure 4-6 show that the absorbance of irradiated CAPEO differs from that of non-irradiated CAPEO and
irradiated eye ointment base (Figure 4-6). This indicates the presence of radiolysis products concerning CAP in eye ointment part. The details of this finding could be further investigated using suitable GC and HPLC methods.

**FIG. 4-6.** Influence of γ-irradiation on hydrophobic part of chloramphenicol eye ointment (CAPEO) and eye ointment base (EOB, containing no chloramphenicol), irradiated at 50 kGy.
4.3.5. Strategies against Radiolysis of Chloramphenicol Eye Ointment

**Radical scavenger**

Because CAPEO degraded more than 1% under the standard γ irradiation dose of 25 kGy and hydrophobic part of its radiolysis products is unknown, the gamma sterilization on CAPEO can not be performed without protection. The effects of radical scavengers were therefore explored to determine ways to prevent excessive radical radiolysis of CAPEO. The selected scavengers are preferably simple chemical substances, which are more sensitive to the direct influence of irradiation, or react faster with radicals/electrons than CAP molecule [27].

Vitamin E, N-acetyl-L-cysteine, β-carotin, and glutathione were thus tested as radical scavengers in the present study. They were blended well with CAPEO before irradiation. Assay test by HPLC method shows that N-acetyl-L-cysteine exhibits excellence protection function (Table 4-V). CAP radiolysis in CAPEO was reduced from 90.5% to 95.2% when CAPEO sample was vigorously stirred only in air, again, indicating the scavenging effect of oxygen on CAP molecule. Because all the sample were prepared in air, vitamin E showed no protection function more, glutathione and β-carotin showed only weak protection function. The scavenger function of N-acetyl-L-cysteine could be a synergy with oxygen.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chloramphenicol content, %</th>
<th>RSD%, n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPEO, 0 kGy</td>
<td>100</td>
<td>0.87</td>
</tr>
<tr>
<td>CAPEO, 50 kGy</td>
<td>90.5</td>
<td>0.64</td>
</tr>
<tr>
<td>CAPEO-air, 50 kGy</td>
<td>95.2</td>
<td>1.05</td>
</tr>
<tr>
<td>CAPEO-vitamin E, 50 kGy</td>
<td>95.1</td>
<td>2.57</td>
</tr>
<tr>
<td>CAPEO-glutathione, 50 kGy</td>
<td>97.0</td>
<td>0.42</td>
</tr>
<tr>
<td>CAPEO-β-carotin, 50 kGy</td>
<td>97.8</td>
<td>0.74</td>
</tr>
<tr>
<td>CAPEO-N-acetyl-L-cysteine, 50 kGy</td>
<td>99.6</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*a: chloramphenicol eye ointment (CAPEO) were blended with 0.2 % of each scavenger by hand in air, respectively, and CAPEO-air was prepared by stirring CAPEO in air only.
Chapter 4

Table 4-VI. Comparisons of chloramphenicol content in CAPEO after γ-irradiation in different packages.

<table>
<thead>
<tr>
<th></th>
<th>in aluminum tube</th>
<th>in conical glass flask</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assay</td>
<td>RSD%</td>
</tr>
<tr>
<td>25 kGy</td>
<td>96.7%</td>
<td>1.32</td>
</tr>
<tr>
<td>50 kGy</td>
<td>89.7%</td>
<td>0.87</td>
</tr>
</tbody>
</table>

It is also worthwhile to mention that the radiolysis degree of manufacturer packed CAPEO (in aluminum tube) was higher than the lab packed CAPEO (in conical glass flask) at both 25 and 50 kGy (Table 4-VI). The manufacturer packed CAPEO was prepared and packed to its final container (in aluminum tube) under vacuum condition. The lab packed CAPEO was packed under atmospherically conditions in glass flask. The vacuum operation of manufacturer packed CAPEO obviously diminishes oxygen residues in the CAPEO product. The absence of oxygen results in higher degree of CAP radiolysis.

However, oxygen is unacceptable as scavenger for CAPEO, because it will cause the oxidation reaction of petrolatum eye ointment base. This severely harms the quality of petrolatum ointment products. Therefore, N-acetyl-L-cysteine could be only a good candidate as scavenger. In absence of oxygen, the function of N-acetyl-L-cysteine should be further investigated. Furthermore, it is necessary to clarify the radiolysis behavior and radiolysis products of N-acetyl-L-cysteine.

Lower Radiation Dose

The above shows difficulties to find a proper scavenger. In addition, it is noticed that radiation treatment at 25 kGy results in two other drawbacks as well. One is formation of gas radiolysis products from the petrolatum ointment base, which may cause some swelling of the tube and result in ointment leaks as well as spoil the package. The other is the irradiation treatment produces unpleasant odor from petrolatum ointment base. N-acetyl-L-cysteine shows no relief for the two handicaps.

According to the sterilization selection guideline from The European Agency for The Evaluation of Medicinal Products (EMEA) [28], products could be sterilized using a
validated lower irradiation dose when the product can not withstand the irradiation of 25 kGy, if the Sterility Assurance Level (SAL) of the ophthalmic product can be ensure to be \( \leq 10^{-6} \). In the case of sterilization of CAPEO, lower radiation dose tactics could therefore be considered.

The key for the use of lower radiation dose is to have a relative lower bioburden in the object product before sterilization. For this reason, petrolatum base can be pre-sterilized using dry heating sterilization during homogenizing of base ingredients. The mixture of CAP powder and the pre-sterilized base will have a low bioburden, which may require a sterilizing dose of 10-15 only. The present investigation confirmed that the influence of gamma irradiation at 15 kGy degradation of CAP in petrolatum ointment base was insignificant, the content loss of CAP was 0.3 % (Table 4-IV). The gas formation and the change of the odor are negligible as well.

4.4. CONCLUSION

Gamma irradiation results in degradation of CAP both in powder state and in petrolatum eye ointment, and chloramphenicol degrades more severely in ointment state than that in pure powder state. Because the radiolysis degree is more than 1% and hydrophobic part of radiolysis products in chloramphenicol eye ointment is unknown, \( \gamma \) sterilization may not be directly applied to chloramphenicol eye ointment product under the standard irradiation dose of 25 kGy.

Microenvironments of chloramphenicol molecule during \( \gamma \) processing play a key role in the chloramphenicol radiolysis in both powder state and ointment state. Comparing with the case of powder state, the radiolysis hydrolysis product, 2-amino-1-(4-nitrophenyl)-1,3-propanediol, almost disappears in chloramphenicol eye ointment, and the amounts of oxidation products are lower, while the amounts of reduction products are higher.

Solvent residues that have good solubility for chloramphenicol, such as methanol, acetonitrile and 1,4-dioxane, induce hydrolysis reaction of chloramphenicol in \( \gamma \) process. By means of inert gas purging or diffusion by exposing in absorbent, radiolysis of chloramphenicol in powder can be obviously reduced. Presence of oxygen
significantly eliminates chloramphenicol radiolysis in both powder state and ointment state.

N-acetyl-L-cysteine could be a potential radical scavenger against radiolysis in chloramphenicol eye ointment. Using a lower irradiation dose, such as 15 kGy, is a practical method to perform γ sterilization of chloramphenicol eye ointment.
Radiolysis Characterization of Chloramphenicol in Powder and in Eye Ointment

4.5. REFERENCES


[18] The International Conference of Harmonization, “Impurities in new drug products”. ICH topic Q3B.


Radiolysis Characterization of Eye Ointment Base (I)

The effects of gamma irradiation on petrolatum eye ointment base as well as its ingredients (white petrolatum, liquid paraffin, and wool fat) were studied at different irradiation doses. Fort-one volatile radiolysis products were recognized and identified by a combine system of headspace-gas chromatography-mass spectrometry.

The main radiolysis products were assigned to n-alkane and 1-alkene, and 2-methyl-alkane, which were the common radiolysis products of eye ointment base and its ingredient materials. White petrolatum and liquid paraffin had the same radiolysis product profiles, in spite of differences in peak intensity. The profile of wool fat was similar, but with additional acetone as the most prominent radiolysis product. The profile of eye ointment was a contribution of the radiolysis products of its ingredients. GC method demonstrated that component distribution pattern of eye ointment as well as its individual ingredients showed no differences following irradiation, as compared to prior to γ-irradiation.

It was found that the hydrocarbon radiolysis products exhibited a “fall apart” profile, which was similar to the mass spectrum profile of n-alkane, indicating that hydrocarbons degrade by the similar pathway both in mass spectrometer and gamma radiation process. Influence of gamma treatment on eye ointment base was quantitatively determined at 15, 25 and 50 kGy. The radiolysis products presented at the concentration levels over a range of 0.21-10.9 ppm, at the reference radiation dose of 25 kGy. The concentration of the radiolysis products rose with increasing doses linearly. Both the qualitative and quantitative data showed that irradiated eye ointment was safe for human use.
5.1. GENERAL

Ophthalmic ointment products are among the most successful dosage forms to which radiation sterilization has been applied, mainly due to three factors. First, the most effective thermal sterilization cannot be applied to ointment products because of their low melting point. Second, the aseptic process, a traditional sterilization method for eye ointment products, involves the possibility of secondary contamination and excessive costs. Third, the intrinsic nature of strong penetration of gamma rays enables gamma sterilization to be the only method for terminal sterilization of eye ointment products.

Eye ointments are normally petrolatum-based and designed to have a melting point close to the human body temperature. The petrolatum base is used as an anhydrous medium to deliver moisture-sensitive drugs, like chloramphenicol. The base adopted as the excipient of chloramphenicol eye ointment consists of petrolatum, liquid paraffin, and wool fat. White petrolatum is a purified mixture of semisolid saturated hydrocarbons having the general formula $C_nH_{2n+2}$. Although some cyclic alkanes ($C_nH_{2n}$) and aromatics with paraffin side chains may also be present, the hydrocarbons consist mainly of branched and unbranched chains. Liquid paraffin (mineral oil) is a mixture of refined saturated hydrocarbons from petroleum. Wool fat (lanolin) is a wax-like substance obtained from sheep wool, which has been cleaned, decolorized, and deodorized. Wool fat is mixtures of esters with the following structural formula of the main components:

\[ R-O-CO-R' \]

where $R$ is a fatty alcohol rest and $R'$ is a fatty acid rest.

These ingredients of petrolatum ointment base are mainly used in topical pharmaceutical formulations and are generally considered to be nonirritant and nontoxic materials. Therefore, it is important to clarify whether or not the radiation sterilization process provokes the formation of unsafe radiolysis products for their use in pharmaceuticals.

Earliest investigation of radiated white petrolatum and liquid paraffin were reported during 1960-1963 [1-3]. However, a few cases concerned ointments or ointment ingredients, these limited studies were focused on the changes in pH value, physical properties and active ingredients upon gamma irradiation [4-11]. There has been no report available on whether and how gamma irradiation causes chemical component changes in ointment base as well as its relevant ingredients although the information
about potential decomposition products is required for the validation of gamma radiation sterilization.

Gas formation in petrolatum eye ointment products is a phenomenon unavoidable during gamma irradiation. Thus, the gas composition needs to be clarified and untoward activity or carcinogenic potential of these gases for human health must be assessed. Nevertheless, there has been no report concerning the composition of these gases, though the existence of hydrogen carbon dioxide, and methane in the gases was reported [11].

The present work pursued the chemical impact of gamma radiation on eye ointment base as well as its ingredients (white petrolatum, liquid paraffin and wool fat). The gases and organic volatile impurities produced from these materials during irradiation were clarified, both with qualitatively and quantitatively. The research findings enable the elucidation of radiolysis mechanism and the assessment of the toxic potential of the radiolysis products for human health.

5.2. EXPERIMENTAL

5.2.1. Reagent and Materials

Eye ointment base (EOB, a petrolatum oily base without active ingredient), white petrolatum, liquid paraffin, and wool fat were supplied by CIBA Vision AG (Hettlingen Switzerland). Reference compounds, solvents and reagents were of analytical reagent grade or better from Fluka Chemie GmbH (Buchs SG1, Switzerland).

Samples were irradiated with gamma rays from $^{60}$Co at 15, 25, 50, and 100 kGy respectively, in a radiation sterilization plant, Studer Draht-und Kabelwerk AG (Däniken, Switzerland). 500 mg of each ointment sample was weighed in a headspace vial and capped air-tightly for the chromatographic procedure.

5.2.2. Apparatus

Operation conditions for gas chromatography (Varian 3400cx, Sugar Land, Texas, in the USA) and headspace-gas chromatography-mass spectrometry (Varian Headspace Autosampler-Tekmar 7000, Varian 3400cx, Saturn 4D/GC/MS/MS, Sugar Land, Texas,
Radiolysis Characterization of Eye Ointment Base (I)

in the USA) are summarized in Table 5-I. Conditions for the Headspace Autosampler are shown in Table 5-II. Mass spectra were obtained at the electron impact of 70eV and chemical ionization (with methane as reagent gas) at 15eV. Chromatograms were recorded by monitoring the total ion current in the range of 30-400 amu. Transfer line and ion trap manifold were maintained at 220 °C and 170°C, respectively.

### TABLE 5-I. EXPERIMENTAL CONDITIONS FOR GC AND HEADSPACE-GC-MS ANALYSES.

<table>
<thead>
<tr>
<th></th>
<th>GC analysis</th>
<th>Headspace-GC-MS analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td>BGB-Silaren, 10m×0.32 ID, 0.12 μm</td>
<td>Rtx®-624, 30m×0.32 I.D., 1.8 μm</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>50°C (2 min) to 180°C at 30°C min⁻¹ to 280°C at 5°C min⁻¹ (25 min)</td>
<td>45°C (5 min) to 95°C at 2°C min⁻¹ (25 min) to 125°C at 6°C min⁻¹ (15 min)</td>
</tr>
<tr>
<td><strong>Carrier gas</strong></td>
<td>He (5.0 grade), 2.7 mL min⁻¹</td>
<td>55 kPa, He (5.0 grade), 1.1 mL min⁻¹</td>
</tr>
<tr>
<td><strong>Detector</strong></td>
<td>FID (350°C)</td>
<td>MSD</td>
</tr>
<tr>
<td><strong>Injection</strong></td>
<td>SPI, 50°C (0.5 min) to 280°C at 200°C min⁻¹ (30 min), 1.0 μL</td>
<td>Headspace autosampler (see Table 5-II)</td>
</tr>
<tr>
<td><strong>Sample</strong></td>
<td>4 mg mL⁻¹ in cyclohexane</td>
<td>500 mg original sample per vial</td>
</tr>
</tbody>
</table>

### TABLE 5-II. OPERATION CONDITIONS OF THE HEADSPACE AUTOSAMPLER

*Varian Headspace Autosampler-Tekmar 7000*

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platen temperature</strong></td>
<td>80°C</td>
<td>Loop fill time</td>
</tr>
<tr>
<td><strong>Sample equilibrium time</strong></td>
<td>50 min</td>
<td>Loop equilibrium time</td>
</tr>
<tr>
<td><strong>Mixing time</strong></td>
<td>10 min</td>
<td>Inject time</td>
</tr>
<tr>
<td><strong>Stabilising time</strong></td>
<td>2 min</td>
<td>Valve temperature</td>
</tr>
<tr>
<td><strong>Vial pressure</strong></td>
<td>8 psi</td>
<td>Transfer line temperature</td>
</tr>
<tr>
<td><strong>Pressurising time</strong></td>
<td>1.5 min</td>
<td>Loop volume</td>
</tr>
<tr>
<td><strong>Pressurising equilibrium</strong></td>
<td>0.20 min</td>
<td>Vial size</td>
</tr>
</tbody>
</table>

The compounds identification was carried out by matching mass spectra with those recorded in the NIST (National Institute of Standards and Technology) mass spectral
search program as well as their molecular weights (obtained from chemical ionization process). Identification results were confirmed by the injection of authentic substance as reference, wherever possible (total 27 compounds), and by the regular cycle of retention time of the homologues.

5.3. RESULTS AND DISCUSSION

5.3.1. Degradation Profiles of Eye Ointment Base and Identification

The most obvious variation of irradiated eye ointment is manifested in the production of bubbles after irradiation, even at 15 kGy. This indicates the formation of volatile radiolysis products. HS-GC-MS was therefore employed to analyze these volatile products. A typical total ion chromatogram of an eye ointment base (EOB) sample irradiated at 50 kGy, along with the chromatogram of non-irradiated eye ointment base, is presented in Figure 5-1. Forty-one volatile compounds were detected and identified in the irradiated EOB, while only four small peaks were found in the non-irradiated sample. The size of the four peaks found in the non-irradiated EOB increases after irradiation, evidencing that all volatile compounds detected in the irradiated EOB are radiolysis products.

Hydrocarbons are the dominant radiolysis products, including 14 alkanes, 18 alkene, and 5 cycloparaffin. Alcoholate and ketone were also observed in radiolysis products. Because some alkene compounds have very similar mass spectra, the chemical structures of six alkenes (peaks 4, 11, 12, 22, 33, 34) cannot be assigned with confidence. The main radiolysis products are n-alkanes (peaks 1, 3, 9, 20, 31, and 41), except peak 13 (Figure 5-1). Hydrocarbon compounds in radiolysis products can be divided approximately into six groups sorted by their carbon numbers, i.e. hydrocarbon compounds in the same group have the same carbon numbers. Each n-alkane can be a mark of the group to which it belongs. As Table 5-III shows, the straight chain and branch chain hydrocarbons of radiolysis products appear in a regular order. Whenever the carbon number allows, certain homologues exist in each group.
Radiolysis Characterization of Eye Ointment Base (I)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-Propane</td>
</tr>
<tr>
<td>2</td>
<td>Isobutane</td>
</tr>
<tr>
<td>3</td>
<td>n-Butane</td>
</tr>
<tr>
<td>4</td>
<td>Alkene, C₄H₈</td>
</tr>
<tr>
<td>5</td>
<td>2-Butene</td>
</tr>
<tr>
<td>6</td>
<td>3-Methyl-1-butene</td>
</tr>
<tr>
<td>7</td>
<td>3-Methyl-1-pentene</td>
</tr>
<tr>
<td>8</td>
<td>1-Pentene</td>
</tr>
<tr>
<td>9</td>
<td>n-Pentane</td>
</tr>
<tr>
<td>10</td>
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<td>Alkene, C₅H₁₀</td>
</tr>
<tr>
<td>12</td>
<td>Alkene, C₅H₁₀</td>
</tr>
<tr>
<td>13</td>
<td>Acetone</td>
</tr>
<tr>
<td>14</td>
<td>2-Propanol</td>
</tr>
<tr>
<td>15</td>
<td>4-Methyl-1-pentene,</td>
</tr>
<tr>
<td>16</td>
<td>2-Methyl pentane</td>
</tr>
<tr>
<td>17</td>
<td>Cyclopetane</td>
</tr>
<tr>
<td>18</td>
<td>1-Hexene</td>
</tr>
<tr>
<td>19</td>
<td>2-Hexene</td>
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<tr>
<td>20</td>
<td>n-Hexane</td>
</tr>
<tr>
<td>21</td>
<td>3-Methyl pentane</td>
</tr>
<tr>
<td>22</td>
<td>Alkene, C₆H₁₂</td>
</tr>
<tr>
<td>23</td>
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</tr>
<tr>
<td>24</td>
<td>Methylcyclopentane</td>
</tr>
<tr>
<td>25</td>
<td>2-Butanone</td>
</tr>
<tr>
<td>26</td>
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</tr>
<tr>
<td>27</td>
<td>2-Methyl hexane</td>
</tr>
<tr>
<td>28</td>
<td>Cyclohexane</td>
</tr>
<tr>
<td>29</td>
<td>3-Methyl hexane</td>
</tr>
<tr>
<td>30</td>
<td>1-Heptene</td>
</tr>
<tr>
<td>31</td>
<td>n-Heptane</td>
</tr>
<tr>
<td>32</td>
<td>2-Heptene</td>
</tr>
<tr>
<td>33</td>
<td>Alkene, C₇H₁₄</td>
</tr>
<tr>
<td>34</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>39</td>
<td>3-Methyl heptane</td>
</tr>
<tr>
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<td>1-Octene</td>
</tr>
<tr>
<td>41</td>
<td>n-Octane</td>
</tr>
</tbody>
</table>

FIG. 5-1. Identification of the volatile radiolysis products of irradiated eye ointment base (EOB) by Headspace-GC-MS (EOB-50: irradiated at 50 kGy, EOB-0: non-irradiated eye ointment base, n = carbon number)
TABLE 5-III. MOLECULE STRUCTURES AND THE ORDER OF THE STRAIGHT AND BRANCH HYDROCARBONS OF THE RADIOLYSIS PRODUCTS IN SIX GROUPS.

<table>
<thead>
<tr>
<th>group n = 3</th>
<th>group n = 4</th>
<th>group n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>563 ( \text{H}_2\text{C} = \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>—</td>
<td>436 ( \text{H}_3\text{C} - \text{C} - \text{CH}_3 )</td>
<td>589 ( \text{H}_3\text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>639 ( \text{H}_2\text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>396 ( \text{H}_2\text{C} - \text{C} - \text{CH}_3 )</td>
<td>469 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>650 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>—</td>
<td>511 ( \text{H}_3\text{C} = \text{C} - \text{C} - \text{CH}_3 )</td>
<td>686 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>group n = 6</td>
<td>group n = 7</td>
<td>group n = 8</td>
</tr>
<tr>
<td>859 ( \text{H}_2\text{C} = \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>1356 ( \text{H}_2\text{C} = \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>2125 ( \text{H}_2\text{C} = \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>883 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>1420 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>2153 ( \text{H}_2\text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>953 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>1486 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>2223 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>1009 ( \text{H}_2\text{C} = \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>1623 ( \text{H}_2\text{C} = \text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>2400 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>1028 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>1650 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>2430 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
</tr>
<tr>
<td>1063 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>1668 ( \text{H}_3\text{C} - \text{C} - \text{C} - \text{C} - \text{C} - \text{CH}_3 )</td>
<td>—</td>
</tr>
</tbody>
</table>

\( n = \) carbon numbers, the numbers are scan numbers (the same as in Figure 1)
5.3.2. Degradation Profiles of the Ingredients of Eye Ointment Base

The production of bubbles was also observed in white petrolatum and wool fat, but not in the liquid paraffin. However, volatile radiolysis compounds were detected in all the three materials using HS-GC-MS after irradiation (Figure 5-2), while these volatile compounds were not found in the relevant non-irradiated samples. In addition, the concentration of these volatile radiolysis products in all samples was directly proportional to the irradiation dosage (unpublished data).

Volatile radiolysis products in liquid paraffin and white petrolatum are the same (Figure 5-2, in B and C), but the radiolysis product profile of wool fat looks different from those of liquid paraffin and white petrolatum. In irradiated wool fat, straight chain alkanes (peaks 1, 3, 9, 20, 31, and 41) exist only in smaller amount (Figure 5-2, in A). Acetone (peak 13) is the most predominant radiolysis product in irradiated wool fat, whereas it is much less in irradiated liquid paraffin and almost disappears in irradiated white petrolatum. It is also true in the cases of 2-propanol (peak 14) and 2-butanone (peak 25). Cycloparaffin products (peaks 17, 24, 28 and 35) disappear in irradiated wool fat. Although there are two peaks at the same retention time of peaks 24 and 28 in chromatogram of irradiated wool fat, their mass spectra prove that they are different from the radiolysis products in irradiated white petrolatum or liquid paraffin.

Nevertheless, white petrolatum, liquid paraffin and wool fat produce the same straight chain and branch chain hydrocarbons as radiolysis products, though their relative intensities are different.

All volatile radiolysis products that appear in white petrolatum, liquid paraffin and wool fat are present in irradiated EOB (Figure 5-2, in D). Cycloparaffins are the unique radiolysis products of white petrolatum and liquid paraffin, and acetone, 2-propanol and 2-butanone are the only radiolysis products of wool fat. However, all of these compounds appear in irradiated EOB. This means that acetone, 2-propanol and 2-butanone in irradiated EOB originate from wool fat, while cycloparaffins come from white petrolatum and liquid paraffin.
FIG. 5-2. Comparison of volatile radiolysis products at 50 kGy by Headspace-GC-MS: irradiated wool fat (A), liquid paraffin (B), white petrolatum (C), and eye ointment base (D).
5.3.3. Component Distribution Pattern

The distribution pattern of hydrocarbons was identifying by means of GC method (Figure 5-3). The components of EOB, liquid paraffin and white petrolatum are so

FIG. 5-3. Profiles by gas chromatograms, A) irradiated and non-irradiated eye ointment base (peak 1 = cetyl alcohol, peak 2 = stearyl alcohol, they are also the ingredients of the present eye ointment base); B) irradiated and non-irradiated liquid paraffin; C) irradiated and non-irradiated petrolatum.
complex that the GC method with a direct injection does not achieve a complete separation even with a capillary column. However, the slow and drifted baseline suggests the presence of isoparaffins, cycloparaffins, etc., and the normal alkanes show appreciable peaks [12]. The gas chromatograms of non-irradiated and irradiated samples overlap perfectly in each case, even at very high irradiation dose of 100 kGy (Figure 5-3). Although the GC method could not provide an enough separation capability, it hints that the amounts of the main composition of eye ointment base changes little after irradiation.

Furthermore, normal alkanes in the GC chromatograms provide information on the distribution of carbon numbers. It was reported that for liquid or solid hydrocarbons, an important consequence of radiation effect was the formation of radiolysis products of higher weight molecules [13], and under prolonged irradiation, a sufficient amount of the molecule became linked together to form a gel extending throughout the system [14]. Present GC results show that the distribution patterns of n-alkanes remain unchanged after gamma processing and no peaks appear at the end part of the chromatogram. This provides strong evidences that there are no detectable products of condensation reaction in the petrolatum eye ointment.

In conclusion, the GC results demonstrate that there are no significantly chemical changes in the main composition of the petrolatum ointment base as well as its individual ingredients after gamma irradiation. The distribution of hydrocarbons in eye ointment base does not change significantly after irradiation although volatile radiolysis products are produced during irradiation.

5.3.4. Degradation Pathways

**Long Carbon Chain Degradation**

Paraffin differs from petrolatum in their carbon chains. Wool fat differs from paraffin and petrolatum in molecular structure. EOB differs from paraffin and petrolatum in composition. However, headspace chromatograms (Figure 5-2) show that the radiolysis products of these different materials include the same straight chain and branch chain hydrocarbons. This indicates that the radiolysis products of white petrolatum, liquid paraffin, and wool fat are probably produced by the similar degradation pathway, possibly the same functional group in their molecules degrades in all these materials.
What all these materials have in common is the long carbon chains in their molecular structure. Since the C-C bonds in large straight molecules are of similar strength, and can be broken equally under irradiation [14], the initial ionization can occur virtually anywhere along the chain. Thus, the radiolysis products are independent of the original chain structure and its length after the initial ionization. Accordingly, petrolatum and liquid paraffin give the same degradation products.

In the case of wool fat, as long as the carbonyl and long straight chain radicals are formed by bond rupture reactions, further bond scission of the C-C chains will proceed by the same pathways as in white petrolatum or liquid paraffin. Eventually wool fat has the radiolysis products similar to those of liquid paraffin and white petrolatum.

As unique radiolysis products of wool fat, on the other hands, the appearance of acetone, 2-propanol and 2-butanone reflects the chemical feature of wool fat. The presence of cycloparaffins in irradiated white petrolatum and liquid paraffin could be due to the existence of cycloparaffin groups in molecular structures of the original samples.

The radiolysis product profile of EOB shows that the radiolysis products of EOB are a total contribution of the radiolysis products of white petrolatum, liquid paraffin and wool fat. This indicates that the matrix changes have no influence upon the radiolysis pathway of these materials.

*Mass Spectrum Degradation Model*

The radiolysis products of irradiated EOB exhibit an abundance of low mass hydrocarbons, which looks as if the radiolysis products have a tendency to just “fall apart”. The radiolysis product profile of irradiated EOB has the following characteristics:

- Low mass ion series;
- Characteristic peaks of n-alkanes;
- Independent on the chain length

These are highly characteristic of mass spectra of long carbon chain n-alkane as shown in Figure 5-4, i.e., the radiolysis product profile of irradiated EOB detected by headspace-GC is quite similar to a typical mass spectrum of n-octadecane (an example of long carbon chain n-alkane). The characteristic ions in mass spectrum are \([(C_{n}H_{2n+2})-]^{+}\). 

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1)\textsuperscript{+} and [(C\textsubscript{n}H\textsubscript{2n})-1]\textsuperscript{+}, and the characteristic peaks in irradiated EOB profile are C\textsubscript{n}H\textsubscript{2n+2} and C\textsubscript{2}H\textsubscript{2n}. The series of intensive ions at m/z 43, 57, 71, 85, 99 and so forth is highly characteristic of saturated aliphatic hydrocarbons of n-propane (44), n-butane (58), n-pentane (72), n-hexane (86), n-heptane (100), etc. This indicates that alkane chain degrades by a similar pathway both in mass spectrometer and radiolysis process.

It has been reported that secondary and especially tertiary C-C bonds are particularly liable to fission \[13\]. As a result, the proportion of fragmentation products decreases in the order of «-alkane > iso-alkane >> neo-alkane. Accordingly, n-alkanes and their ions become the main radiolysis products and characteristic ions in mass spectrum, respectively.

In mass spectrometer, the rupture of C-C bond tends to continue from the chain end and results in secondary or further fragmentation in the following way:

\[
\text{R}^1\text{-CH}_2\text{-CH}_2\text{-CH}_2^+ \rightarrow \text{R}^1\text{-CH}_2^+ + \text{CH}_2=\text{CH}_2
\]

The driving force for this fragmentation is the formation of the new \(\pi\)-bond in the extruded molecule of ethylene, which lowers the activation energy toward the secondary fragmentation of high mass ions \[15\]. As a result, intensive peaks of low mass were observed. Similarly, the main radiolysis products of EOB are low mass compounds as well. This result suggests a possible approach for studying the molecular rupture mechanisms during gamma process, based on the molecule rupture pattern in mass spectrum.

Generally, the radiolysis products are a trace, unusual and complex mixture, which is difficult to clarify every degradation product. In studying the radiolysis products of a pharmaceutical product, several analytical techniques have to be employed to avoid escape of detection and mis-identification. Even so, it is still difficult to reach the integrity of analyses due to the complexity. Furthermore, the confirmation of identification results is usually difficult, because reference compounds are often lack.

However, fragments in mass spectrometer can be detected with confidence and without any escape. Similarity of both mass spectrum pattern and radiolysis product profile enables the theoretical prediction of the radiolysis products by means of mass spectra. Thus, a rupture model of radiolysis can be created or confirmed without a great deal of laborious work.
FIG. 5-4. Relationship between n-alkane degradations in MS process and gamma radiation process: in A) the numbers represent ion fragment m/z of [M-1]+; in B) the numbers represent molecular weight of radiolysis products, n-Propane (44), n-Butane (58), 1-Pentene (70), n-Pentane (72), 1-Hexene (84), n-Hexane (86), 1-Heptene (98), n-Heptane (100), 1-Octene (112), n-Octane (114), 1-Nonene (126), and n-Nonane (128).
5.3.5. Quantitative Analysis and Evaluation

In order to achieve accurate quantitative analysis, the matrix effect of EOB should be considered because a volatile organic compound (VOC) in different matrixes would result in different concentrations in headspace [16]. For the preparation of calibration curves, reference compounds were dissolved in 1,3-Dimethyl-2-imidazolidinone (DMI), then spiked in a small volume (10 μL into EOB-0, which contained no detectable VOC compounds under the headspace conditions. Thus, the matrix of standard samples is created to be similar to that of eye ointment samples.

The quantitative determination of radiolysis products of the eye ointment base is summarized in Table 5-IV. The concentrations of all the radiolysis products of EOB increase linearly with increasing irradiation doses. Most of the correlation coefficients (r) of linear regression between concentrations of the radiolysis products and the irradiation doses are higher than 0.997 (r_{0.05}, the critical value at 95% confidence interval), especially for the main radiolysis products.

The main radiolysis products of the EOB irradiated at 25 kGy (Table 5-IV) exist at the levels of about 10 ppm. An immediate question is whether or not these radiolysis products will cause unacceptable toxicity at such a concentration level.

All the radiolysis products of EOB detected in the present work are volatile organic compounds. They can be regarded as volatile residual solvents in pharmaceuticals. According to the Impurities Guideline for Residual Solvents developed by International Conference on Harmonisation (ICH) [17], safety risks of residual solvents to human health are placed into three classes:

- Class 1: solvents to be avoided;
- Class 2: solvents to be limited;
- Class 3: solvents with low toxic potential.

Thus, pharmaceuticals should not contain class 1 residual solvents, and classes 2 & 3 residual solvents in pharmaceuticals should be controlled at the levels lower than those required in the guideline. Some of the relevant requirements in the guideline, which are involved in the present work, are excerpted in Table 5-V.

The present identification result shows that none of radiolysis products from EOB is listed in Class 1, while nine of them are listed in Classes 2 and 3. Comparison of Table 5-IV and Table 5-V shows that concentrations of the nine radiolysis compounds
Radiolysis Characterization of Eye Ointment Base (I)

at 25 kGy (the reference dose), or even at a higher dose of 50 kGy, are lower than their relevant thresholds in ICH guideline. Accordingly, it is concluded that the nine radiolysis products are at safety levels for human health.

Table 5-IV. Quantitative Analysis of the Radiolysis Products of Irradiated Eye Ointment Base.

<table>
<thead>
<tr>
<th>No.</th>
<th>Scan Concentration, ppm, (RSD%)</th>
<th>r</th>
<th>No.</th>
<th>Scan Concentration, ppm, (RSD%)</th>
<th>r</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>50 kGy</td>
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<td>15 kGy</td>
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<td>50 kGy</td>
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<tr>
<td>1</td>
<td>396</td>
<td>26.4 (2.3)</td>
<td>15.5 (1.3)</td>
<td>7.0 (2.4)</td>
<td>0.985</td>
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<tr>
<td>2</td>
<td>436</td>
<td>5.1 (6.8)</td>
<td>2.5 (0.6)</td>
<td>1.6 (3.1)</td>
<td>0.999</td>
</tr>
<tr>
<td>3</td>
<td>469</td>
<td>40.3 (2.9)</td>
<td>21.6 (1.6)</td>
<td>13.3 (2.0)</td>
<td>0.999</td>
</tr>
<tr>
<td>4</td>
<td>491</td>
<td>3.9 (3.4)</td>
<td>2.0 (3.6)</td>
<td>1.3 (3.3)</td>
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</tr>
<tr>
<td>5</td>
<td>511</td>
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</tr>
<tr>
<td>6</td>
<td>563</td>
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<td>1.2 (1.6)</td>
<td>0.7 (5.3)</td>
<td>0.997</td>
</tr>
<tr>
<td>7</td>
<td>589</td>
<td>7.7 (1.9)</td>
<td>3.9 (4.9)</td>
<td>2.3 (4.5)</td>
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</tr>
<tr>
<td>8</td>
<td>639</td>
<td>7.2 (4.5)</td>
<td>3.8 (1.6)</td>
<td>2.2 (6.3)</td>
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<tr>
<td>9</td>
<td>650</td>
<td>21.5 (1.7)</td>
<td>10.9 (4.7)</td>
<td>6.6 (3.2)</td>
<td>1.000</td>
</tr>
<tr>
<td>10</td>
<td>686</td>
<td>2.2 (6.2)</td>
<td>1.1 (16.4)</td>
<td>0.6 (11.2)</td>
<td>0.999</td>
</tr>
<tr>
<td>11</td>
<td>713</td>
<td>1.05 (14.7)</td>
<td>0.55 (5.2)</td>
<td>0.48 (10.2)</td>
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</tr>
<tr>
<td>12</td>
<td>728</td>
<td>0.90 (0.5)</td>
<td>0.54 (3.0)</td>
<td>0.49 (5.1)</td>
<td>0.984</td>
</tr>
<tr>
<td>13</td>
<td>795</td>
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<td>8.2 (0.2)</td>
<td>6.3 (2.1)</td>
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</tr>
<tr>
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<td>1.1 (4.5)</td>
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</tr>
<tr>
<td>15</td>
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<td>1.6 (5.3)</td>
<td>1.3 (6.8)</td>
<td>0.992</td>
</tr>
<tr>
<td>16</td>
<td>883</td>
<td>10.2 (1.0)</td>
<td>5.2 (3.2)</td>
<td>3.7 (2.1)</td>
<td>0.998</td>
</tr>
<tr>
<td>17</td>
<td>904</td>
<td>2.3 (4.2)</td>
<td>1.2 (6.3)</td>
<td>0.5 (8.3)</td>
<td>0.994</td>
</tr>
<tr>
<td>18</td>
<td>953</td>
<td>3.2 (5.5)</td>
<td>1.6 (9.8)</td>
<td>1.0 (5.5)</td>
<td>1.000</td>
</tr>
<tr>
<td>19</td>
<td>1009</td>
<td>8.3 (1.2)</td>
<td>4.3 (1.5)</td>
<td>2.6 (1.8)</td>
<td>0.999</td>
</tr>
<tr>
<td>20</td>
<td>1028</td>
<td>20.3 (2.8)</td>
<td>10.3 (2.1)</td>
<td>6.9 (2.0)</td>
<td>0.999</td>
</tr>
<tr>
<td>21</td>
<td>1063</td>
<td>0.76 (8.0)</td>
<td>0.34 (10.5)</td>
<td>0.28 (8.7)</td>
<td>0.986</td>
</tr>
</tbody>
</table>

\[a\]: peak number in Figure 5-1. \[b\]: \( r_{0.05} = 0.997. \[c\]: the data in parentheses present relative standard deviation, \( n = 3. \)
The rest of other 32 radiolysis products that are not listed in the ICH guideline are hydrocarbon compounds, belonging to a lower toxicological class. Furthermore, their concentrations are much lower than the safety thresholds for classes 2 & 3. Even in Class 1, the concentration thresholds are 2 ppm and 5 ppm for the very toxic compounds, benzene (carcinogen) and 1,2-dichloromethane (toxic and environmental hazard), respectively [17]. In comparison with this, the highest concentration of the 32 radiolysis products is less than 5 ppm at the reference radiation dose of 25 kGy.

Therefore, it is concluded that all these volatile radiolysis products have a low toxic potential, and are at the safety levels acceptable for human use at the reference radiation dose of 25 kGy.

**Table 5-V. Safety limits of residual solvents in pharmaceuticals for human use by International Conference on Harmonisation (ICH) guideline.**

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Name</th>
<th>Class</th>
<th>PDE (^a) (mg/day)</th>
<th>Concentration limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>n-Pentane</td>
<td>Class 3</td>
<td>50</td>
<td>5000 ppm</td>
</tr>
<tr>
<td>13</td>
<td>Acetone</td>
<td>Class 3</td>
<td>50</td>
<td>5000 ppm</td>
</tr>
<tr>
<td>14</td>
<td>2-Propanol</td>
<td>Class 3</td>
<td>50</td>
<td>5000 ppm</td>
</tr>
<tr>
<td>20</td>
<td>n-Hexane</td>
<td>Class 2</td>
<td>2.9</td>
<td>290 ppm</td>
</tr>
<tr>
<td>23</td>
<td>1-Propanol</td>
<td>Class 3</td>
<td>50</td>
<td>5000 ppm</td>
</tr>
<tr>
<td>25</td>
<td>2-Butanone</td>
<td>Class 3</td>
<td>50</td>
<td>5000 ppm</td>
</tr>
<tr>
<td>28</td>
<td>Cyclohexane</td>
<td>Class 2</td>
<td>38.8</td>
<td>3880 ppm</td>
</tr>
<tr>
<td>31</td>
<td>n-Heptane</td>
<td>Class 3</td>
<td>50</td>
<td>5000 ppm</td>
</tr>
<tr>
<td>35</td>
<td>Methylcyclohexane</td>
<td>Class 2</td>
<td>11.8</td>
<td>1180 ppm</td>
</tr>
</tbody>
</table>

\(^a\) permitted daily exposure.

5.4. CONCLUSION

Headspace-GC is an appropriate technique to determine trace volatile radiolysis products in irradiated eye ointment. Forty-one radiolysis products, 14 alkanes, 18 alkenes, 5 cycloparaffin, and 4 others, are found. Acetone, 2-propanol and 2-butano
are the unique radiolysis products of wool fat, and cycloparaffins are the unique radiolysis products of white petrolatum and liquid paraffin. The common radiolysis products of the three materials are straight chain and branch chain hydrocarbon, which appear regularly as homologues in six groups. The radiolysis products of EOB are a total contribution of the radiolysis products of its ingredients (white petrolatum, liquid paraffin and wool fat).

Ruptures of the long carbon chains are the main degradation pathways of eye ointment base as well as its ingredients. Variation in molecular structure and matrix composition has little influence on the radiolysis behavior of these materials. The radiolysis product profile of irradiated EOB determined by headspace-GC is quite similar to a typical mass spectrum of long carbon chain n-alkane, suggesting that the radiolysis mechanism approximates the degradation mechanism in mass spectrometer. The fragment pattern of n-alkane in mass spectrometer can be used as a theoretical prediction for the radiolysis product study.

Gamma radiation treatment imposes no major influence on the composition of petrolatum eye ointment, and no significant condensation reaction can be observed. Both qualitative and quantitative data demonstrate that the volatile radiolysis products in irradiated petrolatum eye ointment base are safe for human health at the standard irradiation dose of 25 kGy. Thus, it is feasible to perform gamma sterilization on petrolatum eye ointment, white petrolatum, liquid paraffin and wool fat.
Chapter 5

5.5. REFERENCE


Radiolysis Characterization of Eye Ointment Base (I)


[17] ICH Steering Committee: Impurities guideline for residual solvents, Switzerland, July 1997, Q3C.
Radiolysis Characterization of Eye Ointment Base (II)
— Cetostearyl Alcohol

A set of methods for sample preparation, assay test and impurity test were established. Effects of γ-irradiation on cetostearyl alcohol in pure state, ointment base as well as in chloramphenicol eye ointment were determined at doses of 25 and 50 kGy. The radiolysis of cetostearyl alcohol was found to occur in all cases. The degree of radiolysis was directly proportional to the dose of radiation in each case.

Forty-two impurities and radiolysis products were identified by means of gas chromatography-mass spectrometry. The radiolysis products were assigned to be n-alkane, n-aldehyde and 2-methyl-1-alcohol. The degradation pathways of cetostearyl alcohol were elucidated accordingly.

Differences in the radiolysis behavior of cetostearyl alcohol in pure state, eye ointment base and chloramphenicol eye ointment were studied by assay and impurity analyses. It was found that the influence of eye ointment matrixes was modest and chloramphenicol molecule exhibits slight scavenger function for cetostearyl. The safety risk of the radiolysis products was assessed with qualitative and quantitative data. It was concluded that the radiolysis products of cetostearyl alcohol in chloramphenicol eye ointment did not cause safety concerns for human use.
6.1. GENERAL

Cetostearyl alcohol (CSA) is a mixture of solid aliphatic alcohols, consisting mainly of cetyl alcohol (1-hexadecanol, C\textsubscript{16}H\textsubscript{34}O) and stearyl alcohol (1-octadecanol, C\textsubscript{18}H\textsubscript{38}O). It contains not less than 40.0% of stearyl alcohol and not less 90.0% of both alcohol substances [1, 2]. CSA is a white or cream-colored unctuous mass, or almost white flakes or granules, with a faint characteristic odor and a bland taste, obtained by the reduction of appropriate fatty acids, or from sperm oils. It melts to clear colorless or pale yellow liquid, free from cloudiness or suspended matter. Solidifying point is 45 - 53 °C. It is insoluble in water, soluble in ether, with a lower solubility in alcohol and light mineral oil.

CSA are similar to cetyl alcohol in their properties. As one ingredient of chloramphenicol eye ointment, CSA functions as an emollient and emulsifying agent, which improves the emollient properties of petrolatum ointments and imparts emollient properties without being greasy. This chemical can also produce oil-in-water emulsions with suitable hydrophilic substances (sulfated acids in Emulsifying Wax, PEG monocetyl ether in "Cetomacrogol Emulsifying Wax"). Therefore, CSA is a material widely used as a component of cream, ointment and emulsifying waxes in the pharmaceutical industry.

Although cetyl alcohol and stearyl alcohol are definitely safe for the pharmaceutical use, the trace impurities of CSA could cause contact hypersensitivity and changes in chemical components of CSA would pose impact on its function [3-5]. Thus, the radiolysis behavior of CSA in pure state and in ointment base has to be investigated to clarify whether or not CAS following γ irradiation are still safe for pharmaceutical use. Knowing qualitative and quantitative chemical changes is a must to validate gamma sterilization process on CSA and ointment.

Previous studies were focused on the physical changes, especially changes in pH value. Hangay reported that no significant changes were found in irradiated CSA at a dose ≤ 50 kGy, the pH value of CSA changed from 7.0 (non-irradiated) to 6.7 (irradiated at dose of 50 kGy), and melt point changed 1°C [6, 7]. It was also reported that a small change of pH value occurred in irradiated CSA, from pH 6.5 (non-irradiated) to pH 5.2
Chapter 6

(25 kGy) and pH 4.14 (100 kGy) [8]. Mace reported the composition of cetostearyl alcohol [9], but no literature is available either on the radiolysis degree of CSA and its radiolysis products, or on the relevant analysis methods.

In the present work, a set of methods for sample preparation, assay test and impurity test were established. The influence of γ-irradiation treatment on CSA was quantitatively studied at different doses in pure state, eye ointment base and chloramphenicol eye ointment. CSA radiolytic products were identified using gas chromatography-mass spectrometry (GC-MS) accordingly, and the safety risk of radiolysis products was assessed.

6.2. EXPERIMENTAL

6.2.1. Reagent and Materials

Cetostearyl alcohol (CSA) is of research grade from SERVA (Germany). Eye ointment base (EOB), a petrolatum oily base (without active ingredient), and chloramphenicol eye ointment (CAPEO) were supplied by CIBA Vision AG (Switzerland). Solvents and reagents are of analytical reagent grade or better.

Samples were irradiated with $^{60}$Co gamma rays at 25 and 50 kGy respectively in a radiation sterilization plant of Studer AG (Switzerland).

6.2.2. Apparatus

Instrumental conditions are shown in Table 6-1. Chromatograms were recorded by monitoring the total ion current in the range of 30-400 u. Transfer line and ion trap manifold were maintained at 220°C and 170°C respectively. Mass spectra were obtained by electron impact (EI) at 70 eV and molecular weights were obtained by chemical ionization (CI, with methane reagent gas) at 15 eV.

Identification analyses were performed by matching their mass spectra with those recorded in the NIST (National Institute of Standards and Technology) instrument library, and referring to their molecular weights. Confirmation was performed using
authentic reference, retention time order and two analytical columns with different polarities.

Headspace-GC-MS was carried out under the conditions described in reference [10], but at a headspace platen temperature of 110°C with the column temperature program being 100°C (hold 5 min) to 200°C at 5°C min⁻¹ (hold 55 min).

<table>
<thead>
<tr>
<th>Table 6-I. Experimental Conditions for Assay and Impurity Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay Test</strong></td>
</tr>
<tr>
<td>Instrument</td>
</tr>
<tr>
<td>Column</td>
</tr>
<tr>
<td>Temperature</td>
</tr>
<tr>
<td>Carrier gas</td>
</tr>
<tr>
<td>Detector</td>
</tr>
<tr>
<td>Injection</td>
</tr>
<tr>
<td>Sample</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

### 6.2.3. Sample Preparation

**Cetostearyl alcohol**

CSA and 1-heptadecanol (Internal Standard) were prepared in n-hexane for assay test. In order to reach better dissolution at high concentrations, CSA was prepared in cyclohexane for impurity test.

**Eye ointment base**

According to Table 6-II, EOB was accurately weighed and dissolved in n-hexane with 45°C heating in a water bath and vortexing. In order to remove other ingredients
from the solution, methanol was added, followed by vigorously shaking, cooling down in ice bath for ca. 10 min, then filtering. The filtrate was used for gas chromatography injection of assay test. For impurity test, the whole filtrate was further treated by vacuum evaporation, and then the dry residues was dissolved in cyclohexane.

*Chloramphenicol eye ointment*

An equivalent amount of CAPEO was accurately weighed into a 15-mL glass centrifuge tube, followed by adding 10 mL internal standard solution (for assay test) or n-hexane (for impurity test). The sample was heated in a 45°C water bath for 5 min with stirring twice for 20 s, then centrifugated at 3000 rpm min⁻¹ for 2 min. The supernatant liquid was quantitatively collected together with washings. Then the above-described procedure for EOB was followed.

**TABLE 6-II. CETOSTEARYL ALCOHOL EXTRACTION FROM OINTMENT BASE FOR ASSAY AND IMPURITY TESTS.**

<table>
<thead>
<tr>
<th></th>
<th>Assay Test</th>
<th>Impurity Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Amount</td>
<td>Equivalent 25 mg CSA</td>
<td>Equivalent 100 mg CSA</td>
</tr>
<tr>
<td>Dilution 1, n-hexane</td>
<td>in 25 mL volumetric flask</td>
<td>30 mL, in 250 mL conical flask</td>
</tr>
<tr>
<td>Dilution 2, methanol</td>
<td>10 mL of Dilution 1, in 50 mL</td>
<td>Add 75 mL, shake, cool to 0°C</td>
</tr>
<tr>
<td>Filtering &amp; washing</td>
<td>None</td>
<td>10 mL methanol, twice</td>
</tr>
<tr>
<td>Evaporation</td>
<td>None</td>
<td>Whole filtrate, to dry</td>
</tr>
<tr>
<td>Dilution 3</td>
<td>None</td>
<td>2 mL cyclohexane</td>
</tr>
</tbody>
</table>

*: n-hexane solution of internal standard should be used for assay test.

6.3. RESULTS AND DISCUSSION

6.3.1. Assay Method Suitability

To determine the degree of radiolysis accurately, a valid assay test (a quantitative analysis of main content) has to be established first. A typical chromatogram of the extraction from CAPEO is presented in Figure 6-1, which indicates that good resolution
Radiolysis Characterization of Eye Ointment Base (II): Cetostearyl Alcohol

is fulfilled among the cetyl alcohol, stearyl alcohol, internal standard compound, and trace impurities.

Six replicate injections of a CSA solution were performed to determine the GC method precision. In order to determine the precision of sample preparation method, six independent CAPEO samples were prepared according to the described sample preparation procedures, and then each sample was analyzed by the GC method with three successive injections. Actually, sample preparation method covers the errors from GC determination and sample preparation procedure. These results were summarized in Table 6-III. Both GC and sample preparation methods have a precision well below 1.5%, the criteria of The United States Pharmacopoeia for CSA assay [2].

![GC chromatogram of assay test, internal standard = 1-heptadecanol. Sample: extraction from chloramphenicol eye ointment](image)

**FIG. 6-1.** GC chromatogram of assay test, internal standard = 1-heptadecanol.
Sample: extraction from chloramphenicol eye ointment

The linearity of peak area responses versus concentrations was studied for the sample preparation method at five levels over a range of 80-120% of the nominal sample active concentration. CAPEO was used and four replicates were performed at each level. Good linearities for both cetyl alcohol and stearyl alcohol over the range were attested by least squares regression and statistical analysis (Figure 6-2).
TABLE 6-III. PRECISION OF GC METHOD AND SAMPLE PREPARATION METHOD FOR CSA ASSAY TEST.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>GC method</th>
<th>Sample preparation method</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>RSD%</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>1.013 0.005 0.48</td>
<td>0.982 0.0031 0.31</td>
<td>96.9</td>
</tr>
<tr>
<td>Stearyl alcohol</td>
<td>1.083 0.005 0.47</td>
<td>1.054 0.0031 0.30</td>
<td>97.3</td>
</tr>
</tbody>
</table>

a mean of GC relative responses versus internal standard

The recovery of 96.9% and 97.3% was obtained for pure target compounds and the compounds in eye ointment, respectively (Table 6-III). Although a recovery of 100% did not reach by this assay method, the excellent precision can ensure accurate determination after relevant calibration.

These results demonstrate that the sample preparation procedure and the GC processing are suitable for CSA quality control and their stability studies.

FIG. 6-2. Linearity of assay test for cetyl alcohol and stearyl alcohol. Samples: extractions from chloramphenicol eye ointment, n= 4 at each point.
6.3.2. Cetostearyl Alcohol Degradation Degree in Different Matrices

Radiolytic behaviors of components in a formulated product may be different from those occurring when these components are exposed to irradiation individually. In order to clarify the radiolysis of CSA in different matrices, investigations were carried out in pure state, EOB, and CAPEO.

Although following irradiation at doses of 25 and 50 kGy CSA in pure state undergoes no changes in color and appearance, the results of assay test show that γ irradiation leads to content losses of both cetyl alcohol and stearyl alcohol in all studied cases, i.e. pure state, EOB, and CAPEO (Figure 6-3). The losses are directly proportional to the irradiation doses. This is different from the previous report that cetyl alcohol remains unchanged at 25 kGy [11].

**FIG. 6-3.** Gamma Radiation effect on CSA content (assay test) in different matrices: pure cetostearyl alcohol (Pure), eye ointment base without chloramphenicol (EOB), chloramphenicol eye ointment (CAPEO), n = 6.
Table 6-IV. **Statistic Analysis of t-test between different groups at 50 kGy.**

<table>
<thead>
<tr>
<th></th>
<th>Pure state</th>
<th>EOB</th>
<th>CAPEO</th>
<th>t-test ($t_{0.05,5} = 2.57$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>97.10</td>
<td>0.954</td>
<td>96.43</td>
<td>0.588</td>
</tr>
<tr>
<td>Stearyl alcohol</td>
<td>96.52</td>
<td>0.383</td>
<td>97.34</td>
<td>0.755</td>
</tr>
</tbody>
</table>

Note: EOB = eye ointment base, CAPEO = chloramphenicol eye ointment and SD = standard deviation.

As Figure 6-3 shows, the influence of surrounding media on CSA content is modest during γ processing and the degree of degradation is somehow different in pure state, CAPEO and EOB. The degradation degrees of both cetyl alcohol and stearyl alcohol in CAPEO were slightly lower than those in both pure state and EOB (without active ingredient chloramphenicol). This suggests that chloramphenicol molecule functions to a certain extent as a scavenger for CSA during γ processing.

Statistic analysis of t-test at 50 kGy shows that differences between pure state and EOB are insignificant because the calculated t-values are less than the critical value of $t_{0.05,5} = 2.57$ (Table 6-IV). However, a slight scavenger function is clearly detected in the case of cetyl alcohol, because the t-value calculated from comparison between EOB and CAPEO is higher than 2.57. The chloramphenicol molecule might somewhat alleviate the radiolysis of cetostearyl alcohol, or its exciting energy could be lower than those of cetyl alcohol and stearyl alcohol [12, 13].

It is noticed that degradation degrees of cetyl alcohol and stearyl alcohol in CAPEO at the reference dose of 25 kGy are 1.10% and 1.55%, respectively, more than the criteria acceptable for a pharmaceutical product [14]. Therefore, the radiolysis products must be further clarified.

### 6.3.3. Identification of Radiolysis Products

Identification of impurities and radiolytic products in irradiated CSA was carried out using GC-MS. Typical total ion current chromatograms describes impurity profile of a CSA sample irradiated at 50 kGy in Figure 6-4.
FIG. 6-4. Total ion current chromatograms of GC-MS of irradiated cetostearyl alcohol at 50 kGy (A: using a nonpolar column of Rtx-5MS®, B: using a mid-polar column of Rtx-624®)
The impurity profile was clarified with two capillary columns. The chromatogram A is the identification result fulfilled using a non-polar column of Rtx-5®. In order to verify the identification result and especially to improve the resolution to the alcohol compounds, the same experiment was performed with a mid-polar column of Rtx-624, as shown in chromatogram B. The assigned compounds were found in both cases and gave the same mass spectra. With column Rtx-624, all peaks of “C” series in chromatogram A are separated into two peaks and assigned to the compounds of series of “Ca” and “Cb”. The presence of compounds of A10 and A12 in chromatogram A is further confirmed in chromatogram B.

Identification results show that the original impurities and radiolytic products in the irradiated CSA appear in a regular order of homologues, which can be sorted into six series of “A”, “B”, “Ca”, “Cb”, “D”, and “E”. Every homologue group gives similar mass spectra, but different molecule weights, which were determined by GC-MS-CI analyses. Based on the mass spectra and the molecular weights, 42 compounds in irradiated CSA were identified (Table 6-V). The series of “A” is assigned as n-alkane, “B” n-alkan-1-ol, “Ca” n-alkan-2-ol, “Cb” n-alkan-3-ol, “D” n-aldehyde, and “E” 2-methyl-1-alcohol, respectively.

By comparing the chromatograms of the non-irradiated and irradiated CSA in Figure 6-5, the radiolytic products are distinguished from the original impurities (the results are summarized in Table 6-V). Compounds A6, A7, A8, D5, D7, E3 and E4 (pentadecane, hexadecane, heptadecane, hexadecanal, octadecanal, 2-methyl-1-hexadecanol and 2-methyl-1-octadecanol) were main radiolytic products, which appear or increase dramatically after irradiation and proportionally to irradiation doses.

Compounds of series D are not unique radiolytic products. They exist in non-irradiated CSA but their amounts increase after irradiation. They could be produced by spontaneous oxidation under air and gamma irradiation. Compounds of series E are produced mainly from γ radiation processing, and they exist at an almost unrecognizable level before irradiation. In contrast, compounds of series C and Cb are definitely non-radiolysis products, and they exist in non-irradiated CSA and remain unchanged after irradiation. The case of alkane compounds is special. Compounds of A1-A8 are unique radiolytic products and they are present only in irradiated CSA. Octadecane (A9)
appears as both radiolytic product and original impurity. Compounds, such as nonadecane (A10), eicosane (A11) and henicosane (A12), are difficult to recognize with column Rtx-5®. However, it can be clearly observed that they are not changed after irradiation in the chromatograms using the mid-polar column of Rtx-624® (Figure 6-6), demonstrating that they are original impurities.

Chromatogram impurity profiles (at 0, 25 and 50 kGy) shown in Figure 6-5 also demonstrate that the amount of all radiolysis products increase proportionally with the irradiation dosage.

**TABLE 6-V. RADIOLYTIC PRODUCTS AND ORIGINAL IMPURITIES OF CETOSTEARYL ALCOHOL**

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Radiolytic products a</th>
<th>No.</th>
<th>Compounds</th>
<th>Radiolytic products</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Decane</td>
<td>+</td>
<td>C2a</td>
<td>3-Pentadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A2</td>
<td>Undecane</td>
<td>+</td>
<td>C2b</td>
<td>2-Pentadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A3</td>
<td>Dodecane</td>
<td>+</td>
<td>C3a</td>
<td>3-Hexadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A4</td>
<td>Tridecane</td>
<td>+</td>
<td>C3b</td>
<td>2-Hexadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A5</td>
<td>Tetradecane</td>
<td>+</td>
<td>C4a</td>
<td>3-Heptadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A6</td>
<td>Pentadecane</td>
<td>+*</td>
<td>C4b</td>
<td>2-Heptadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A7</td>
<td>Hexadecane</td>
<td>+*</td>
<td>C5a</td>
<td>3-Octadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A8</td>
<td>Heptadecane</td>
<td>+*</td>
<td>C5b</td>
<td>2-Octadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A9</td>
<td>Octadecane</td>
<td>+*, Δ</td>
<td>C6a</td>
<td>3-Nonadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A10</td>
<td>Nonadecane</td>
<td>Δ</td>
<td>C6b</td>
<td>2-Nonadecanol</td>
<td>Δ</td>
</tr>
<tr>
<td>A11</td>
<td>Eicosane</td>
<td>Δ</td>
<td>D1</td>
<td>Dodecanal</td>
<td>+, ?Δ</td>
</tr>
<tr>
<td>A12</td>
<td>Henicosane</td>
<td>Δ</td>
<td>D2</td>
<td>Tridecanal</td>
<td>+, ?Δ</td>
</tr>
<tr>
<td>B1</td>
<td>1-Dodecanol</td>
<td>Δ</td>
<td>D3</td>
<td>Tetradecanal</td>
<td>+, ?Δ</td>
</tr>
<tr>
<td>B2</td>
<td>1-Tridecanol</td>
<td>Δ</td>
<td>D4</td>
<td>Pentadecanal</td>
<td>+, ?Δ</td>
</tr>
<tr>
<td>B3</td>
<td>1-Tetradecanol</td>
<td>Δ</td>
<td>D5</td>
<td>Hexadecanal</td>
<td>+*, Δ</td>
</tr>
<tr>
<td>B4</td>
<td>1-Pentadecanol</td>
<td>+, Δ</td>
<td>D6</td>
<td>Heptadecanal</td>
<td>+, Δ</td>
</tr>
<tr>
<td>B5</td>
<td>1-Hexadecanol</td>
<td>Δ</td>
<td>D7</td>
<td>Octadecanal</td>
<td>+*, Δ</td>
</tr>
<tr>
<td>B6</td>
<td>1-Heptadecanol</td>
<td>+, Δ</td>
<td>E1</td>
<td>2-Methyl-1-dodecanol</td>
<td>+</td>
</tr>
<tr>
<td>B7</td>
<td>1-Octadecanol</td>
<td>Δ</td>
<td>E2</td>
<td>2-Methyl-1-tetradecanol</td>
<td>+</td>
</tr>
<tr>
<td>C1a</td>
<td>3-Tetradecanol</td>
<td>Δ</td>
<td>E3</td>
<td>2-Methyl-1-hexadecanol</td>
<td>+*</td>
</tr>
<tr>
<td>C1b</td>
<td>2-Tetradecanol</td>
<td>Δ</td>
<td>E4</td>
<td>2-Methyl-1-octadecanol</td>
<td>+*</td>
</tr>
</tbody>
</table>

a + = radiolytic product; +* = main radiolytic product; Δ = original impurity, ?Δ = possible original impurity.
FIG. 6-5. Comparison of GC chromatogram between irradiated and non-irradiated cetostearyl alcohol (using a nonpolar column of Rtx-5)
FIG. 6-6. Comparison of GC chromatogram between irradiated and non-irradiated cetostearyl alcohol (using a mid-polar column of Rtx-624®)
6.3.4. Radiolysis Pathway

As described above, whether alkane compounds are radiolysis products or not depends on the number of their carbon chain. When their carbon chain numbers are $\leq 18$, they are radiolysis products, and otherwise they are non-radiolytic products. Figure 6-5 shows that peaks of D5 (hexadecanal) and D7 (octadecanal) are approximately identical and they are two highest peaks amongst their homologues, while the peak of D6 (heptadecanal) is much smaller in comparison to D5 and D7. It is also true for A6 (Pentadecane) and A8 (Heptadecane), A7 (Hexadecane) and A9 (Octadecane), as well as E3 and E4.

In the irradiated CSA system, cetyl alcohol (hexadecanol) and stearyl alcohol (octadecanol) are the main components at approximately identical concentration levels, while the concentration of heptadecanol is much lower comparatively. During the irradiation processing, the energy deposited by the gamma rays to a component of a mixture, to a first approximation, is proportional to the fraction of the molecules of each compound. Therefore, it could be inferred that D5, A6, A7 and E3 are the radiolysis products of cetyl alcohol, D7, A8, A9 and E4 are the products of stearyl alcohol, and D6 is the product of 1-heptadecanol.

![Graph showing total ion chromatograms of Headspace-GC-MS of cetostearyl alcohol non-irradiated (CSA-0) and irradiated at 50 kGy (CSA-50)]

FIG. 6-7. Total ion chromatograms of Headspace-GC-MS of cetostearyl alcohol non-irradiated (CSA-0) and irradiated at 50 kGy (CSA-50)
In addition, Headspace-GC-MS analysis reveals a profile of volatile radiolysis products of irradiated CSA (Figure 6-7), showing the presence of lower molecular weight n-alkene compounds. This indicates the occurrence of degradation of long carbon chain radicals.

Accordingly, the main radiolysis pathways in irradiated cetyl alcohol can be elucidated as shown in Figure 6-8, which are similar to the radiolysis pathways of stearyl alcohol, but \( n = 18 \). The principal effect of irradiation on the straight long chain alcohols, cetyl alcohol and stearyl alcohol, is cleavage of \( \alpha \)-carbon-carbon bonds, resulting in the formation of n-alkanes, pentadecane and heptadecane. Both are formed in the greatest yield amongst radiolysis products of CSA, indicating that \( \alpha \)-carbon-
carbon bonds are the most readily broken bonds in the alcohol with long chain. The octadecane and hexadecane are also main radiolysis products. This shows that scission of the carbon-oxygen bond can occur. All the long carbon chain n-alkanes or radicals may be further fragmented, producing alkane ($C_{n-2}$, $C_{n-3}$, $C_{n-4}$, $C_{n-5}$, etc., $n =$ carbon atom of cetyl alcohol or stearyl alcohol). All these reactions belong to radiolytical reduction.

Another important effect is loss of the hydrogen atom from the hydroxyl group, resulting in the formation of aldehydes. In essence, this reaction is an oxidation process. The formation of E3 and E4 could involve rupture of carbon-carbon bond or carbon-oxygen bond and further reaction of hydrocarbon radicals, but the details should be further studied.

6.3.5. Radiolytic Products in Different Matrices and Safety Assess

The impurity analysis of CSA was also carried out for irradiated EOB and CAPEO because the radiolytical behavior of the same compounds could be different in different matrix. Chromatogram impurity profiles of CSA in irradiated CAPEO, irradiated EOB and non-irradiated EOB were compared in Figure 6-9. This experiment clearly illustrates that γ irradiation results in degradation of CSA in CAPEO and EOB. Similar radiolysis product profiles were found in both cases. The peaks of most radiolysis products in irradiated EOB are slight higher than those in irradiated CAPEO at an identical condition. This result is in agreement with the assay result in Figure 6-3, i.e. radiolysis degree in CAPEO is slight lower than that in eye ointment (without chloramphenicol). This shows again the scavenger function of chloramphenicol molecule.

Radiolysis products that exist in pure CSA appear in irradiated CAPEO and irradiated EOB, and no large amounts of new radiolysis products are found. Although there are three new small peaks near the peaks A5, A7 and A9 in irradiated CAPEO sample, the chromatogram of non-irradiated CAPEO shows that they are original impurities rather than radiolysis products (from our unpublished data).
FIG. 6-9. Impurity chromatogram profiles of irradiated chloramphenicol eye ointment (CAPEO-50) and eye ointment base (EOB-50) as well as non-irradiated eye ointment (EOB-0), using Rtx-5® column.
This strongly suggests that the CSA in pure state, EOB and CAPEO degrades in a similar way. By combining assay and impurity tests, it can be concluded that the influence of EOB on radiolysis of CSA is negligible.

Amongst the radiolysis products, alkane series are definitely the compounds safe for human use. 2-mthyl-1-hexadecanol and 2-mthyl-1-octadecanol are the compounds of low toxicity. Although low molecular weight aldehydes, e.g., formaldehyde and acetaldehyde, have sharp, unpleasant odors, the higher molecular weight aldehydes have pleasant, often fragrant odors, which are found in the essential oils of certain plants. Pentadecanal, hexadecanal, and heptadecanal were reported to be found in the essential oil of citrus limon, a traditional medicinal plant [15]. Hexadecanal is listed as a register of flavoring substance used in or on foodstuffs [16]. Therefore, these higher molecular weight aldehydes are low toxic compounds.

Furthermore, CAPEO contains about 2.5% of CSA. As shown in Figure 6-3, the radiolytical amounts of cetyl alcohol and stearyl alcohol in CAPEO are 1.10% and 1.55% at 25 kGy, respectively. This means the absolute percentage of radiolysis products from CSA in CAPEO will not be more than 0.066%, which is a safe level for low toxic impurities in pharmaceutical products [17, 18]. Conclusively, the radiolysis products of CSA impose no unsafety effect on CAPEO at the reference radiation dose of 25 kGy.

6.4. CONCLUSION

The present assay test method is accurate and suitable, and the impurity test method is specific for the determination of radiolysis products. Gamma sterilization processing causes the radiolysis of cetostearyl alcohol in pure state, eye ointment base, and chloramphenicol eye ointment. The influence of eye ointment composition on the radiolysis of cetostearyl alcohol is modest.

Radiolysis products can be sorted into three series, i.e. n-alkane, n-aldehyde, 2-methyl-1-alcohol. Oxidation of hydroxyl group and cleavages of α-carbon-carbon bonds and carbon-oxygen bond are the main radiolytical reaction. Both assay and impurity test
show that chloramphenicol molecule has a very slight scavenger function for cetostearyl alcohol. The present data show that all the radiolysis products belong to low toxicity compounds and they are at safe concentration levels for pharmaceutical products. Thus, gamma process is feasible for sterilization of pure cetostearyl alcohol and the radiolysis products of cetostearyl alcohol in chloramphenicol eye ointment result in no safety risk for human use.
6.5. REFERENCES


Curriculum Vitae

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