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Stability studies of intravenous cyclosporine preparations stored in non-PVC containers

par

Mengqing Li

Faculté de pharmacie

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Ce mémoire intitulé :

Stability studies of intravenous cyclosporine preparations stored in non-PVC containers

Présentée par :

Mengqing Li

a été évalué par un jury composé des personnes suivantes :

Prof. Dr. Jeanne Leblond-Chain, président-rapporteur

Prof. Dr. Grégoire Leclair, directeur de recherche

Prof. Dr. Suzanne Giasson, membre du jury

Résumé

Dans cette étude, la stabilité de préparations intraveineuses de cyclosporine (0.2 et 2.5 mg/mL dans NaCl 0.9% ou dextrose 5%) entreposées dans des seringues de polypropylène, des sacs de polypropylène-polyoléfine et des sacs de vinyle acétate d'éthylène a été évaluée. Une méthode HPLC indicatrice de la stabilité à base de méthanol a été développée et validée suite a des études de dégradation forcée. Les solutions évaluées ont été préparées de façon aseptique, puis entreposées à 25°C. La stabilité chimique a été évaluée par HPLC et la stabilité physique a été évaluée par inspection visuelle et aussi par diffusion dynamique de la lumière (DLS). Tous les échantillons sont demeurés stables chimiquement et physiquement dans des sacs de polypropylène-polyoléfine (>98% de cyclosporine récupérée après 14 jours). Lorsqu'entreposés dans des seringues de polypropylène, des contaminants ont été extraits des composantes de la seringue. Toutefois, aucune contamination n'a été observée après 10 min de contact entre la préparation de cyclosporine non-diluée et ces mêmes seringues. Les préparations de 2.5 mg/mL entreposées dans des sacs de vinyle acétate d'éthylène sont demeurés stables chimiquement et physiquement (>98% de cyclosporine récupérée après 14 jours). Toutefois, une adsorption significative a été observée avec les échantillons 0.2 mg/mL entreposés dans des sacs de vinyle acétate d'éthylène (<90% de cyclosporine récupéré après 14 jours). Une étude cinétique a démontré une bonne corrélation linéaire entre la quantité adsorbée et la racine carrée du temps de contact ($r^2 > 0.97$). Un nouveou modèle de diffusion a été établi. En conclusion, les sacs de polypropylène-polyoléfine sont le meilleur choix; les seringues de polypropylène présentent un risque de contamination, mais sont acceptables pour un transfert rapide. Les sacs de vinyle acétate d'éthylène ne peuvent être recommandés à cause d'un problème d'adsorption.

Mots-clés : Cyclosporine, stabilité, seringue, sac pour perfusion, dégradation forcée, adsorption

Abstract

In the present study, the stability of intravenous cyclosporine preparations (0.2 and 2.5 mg/mL in 0.9% sodium chloride injection or 5% dextrose injection) stored in polypropylene (PP) syringes, polypropylene–polyolefin (PP-PO) bags and ethylene vinyl acetate (EVA) bags was evaluated. A methanol-based high-performance liquid chromatography (HPLC) method was developed and validated to be stability-indicating by stress degradation tests. The test solutions were aseptically prepared and stored at 25 °C. Chemical stability was evaluated by HPLC assay. Physical stability was assessed by visual inspection and a dynamic light scattering (DLS) method. All samples were chemically stable (> 98% of recovered cyclosporine) and physically stable when stored in polypropylene–polyolefin bags for 14 days. When stored in polypropylene syringes, some impurities were leached. However, no leaching was detected when the syringes were exposed to undiluted intravenous cyclosporine for 10 minutes. The preparations of 2.5 mg/mL were chemically and physically stable as stored in ethylene vinyl acetate bags for a period of 14 days (> 98% of recovered cyclosporine), while significant cyclosporine adsorption occurred on the samples of 0.2 mg/mL (< 90 % of recovered cyclosporine) after 14 days. Kinetic study showed that good linear correlations were achieved by plotting the adsorption amount versus square root of contact time ($r^2 > 0.97$). A novel diffusion model was established and successfully predicted long-term drug stability. In conclusion, polypropylene–polyolefin bags were the best choice; syringes were inferior because of leachables. However, they were safe for preparation and transferring undiluted intravenous cyclosporine. Ethylene vinyl acetate bags cannot be recommended due to cyclosporine adsorption.

Keywords : Cyclosporine, stability, syringe, infusion bag, stress degradation, adsorption

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List of abbreviations

I.V.	Intravenous
Abu	2-Aminobutyric acid
API	Active pharmaceutical ingredient
BCS	Biopharmaceutics classification system
Cya	Cyclosporine
D5W	5% dextrose injection
DEHP	Bis (2-ethylhexyl) phthalate
DLS	Dynamic light scattering
ELSD	Evaporative light scattering detector
EVA	Ethylene vinyl acetate
FDA	U.S. Food and Drug administration
GC	Gas chromatography
HPLC	High-performance liquid chromatography
ICH	International Conference on Harmonisation
LC	Liquid chromatography
LD	Laser diffraction
LOD	Limit of detection
LOQ	Limit of quantification
MeBmt	(4R)-4-[(E)-2-butenyl]-4, N-dimethyl-threonine
MeGly	N-Methylglycine
MeLeu	N-Methylleucine
MeVal	N-Methylvaline

MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NP-HPLC	Normal-phase chromatography
NS	0.9% sodium chloride injection
PDA	Photodiode array
PDI	Polydispersity index
PE	Polyethylene
PEEK	Polyetheretherketone polymer
P-gp	P-glycoprotein
PP	Polypropylene
PP-PO	Polypropylene–polyolefin
PVC	Polyvinyl chloride
RI	Refractive index
RP-HPLC	Reversed-phase chromatography
RSD	Relative standard deviation
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
USP	U.S Pharmacopeia
USP-NF	The United States Pharmacopeia and the National Formulary
UV/VIS	Ultraviolet-visible
WHO	World Health Organization

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Chapter one

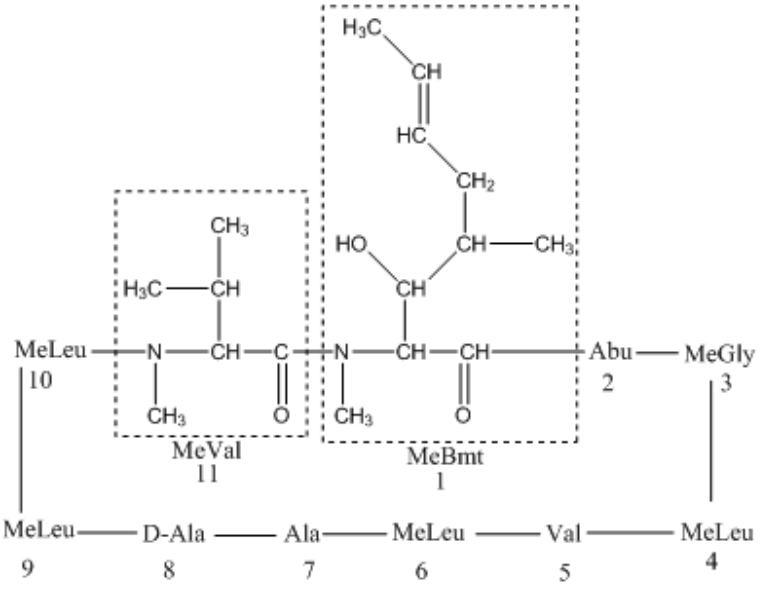
1 Introduction: cyclosporine formulations, chemistry and quality control

1.1 Cyclosporine chemistry and formulation

Cyclosporine (Cya) is a potent immunosuppressive agent in organ transplantation to avoid rejection, as well as for the treatment of various auto-immune diseases. Commercial products include oral and intravenous formulations {Novartis, 2011a #1007}. The stability and compatibility with containers are basically related to its physicochemical nature and formulation composition. The following introduction will focus on the properties of Cya drug substance and drug products.

1.1.1 Nomenclature, structure and property of Cya

Cya (CAS 79217-60-0) or Cya A is a cyclic undecapeptide peptide (formula: $C_{62}H_{11}N_{11}O_{12}$; molecular weight: 1202.6 g/mol). The synonyms such as cylosporin, Cyclosporine, ciclosporin are also used in different countries {Hossan, 1987 #1008}.



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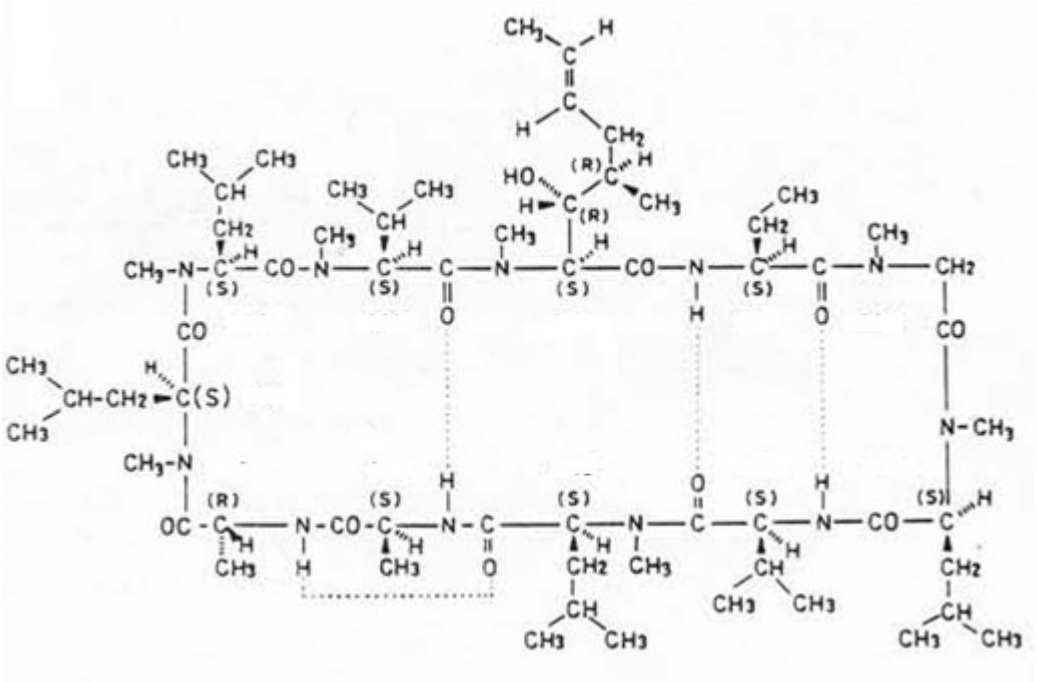
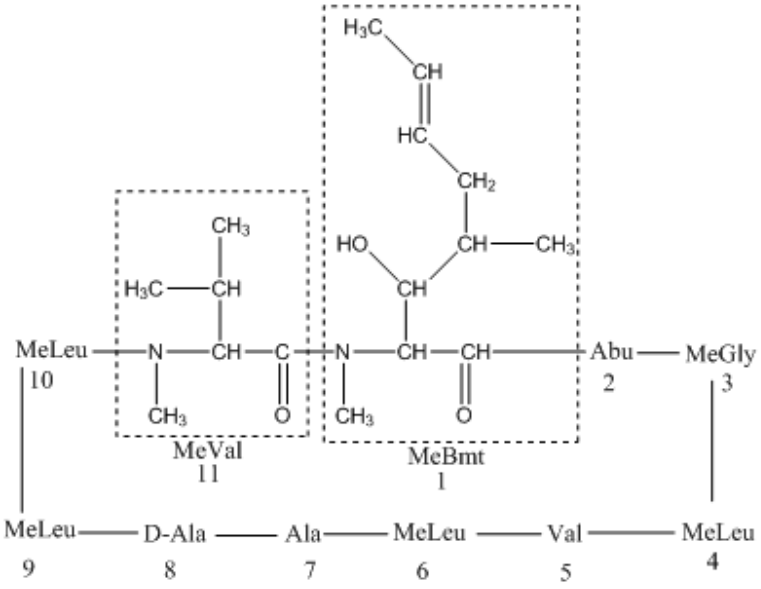


B Modified from {Wenger, 1985 #1001}

Figure 1.1 Structure of cyclosporine, it is composed of 8 natural amino acid residues with L-configuration and 3 non naturals. The achiral amino acid sarcosine (Sar or MeGly) is present at position 3 and D- Alanine (Ala) can be found at position 8. Importantly, an unusual amino acid (4R)-4-[(E)-2-butenyl]-4, N-dimethyl-threonine (MeBmt) at position 1 is required for its biological activity {Hossan, 1987 #1008}. The conformation of Cya has been extensively investigated by X-ray diffraction and NMR techniques. Because of intra-molecular hydrogen bonds, Cya has a rigid conformation either in solid crystal state or in solution {Hossan, 1987 #1100}. Furthermore, the side chain of MeBmt stands out and is primarily responsible for the immunosuppressive activity{Kessler, 1985 #1009}. Likewise, the amino acid residues Abu, MeGly, MeLeu, MeLeu, MeVal at position 2, 3, 4, 10, and 11 are also related to the biological activity {Quesniaux, 1986 #1010}.



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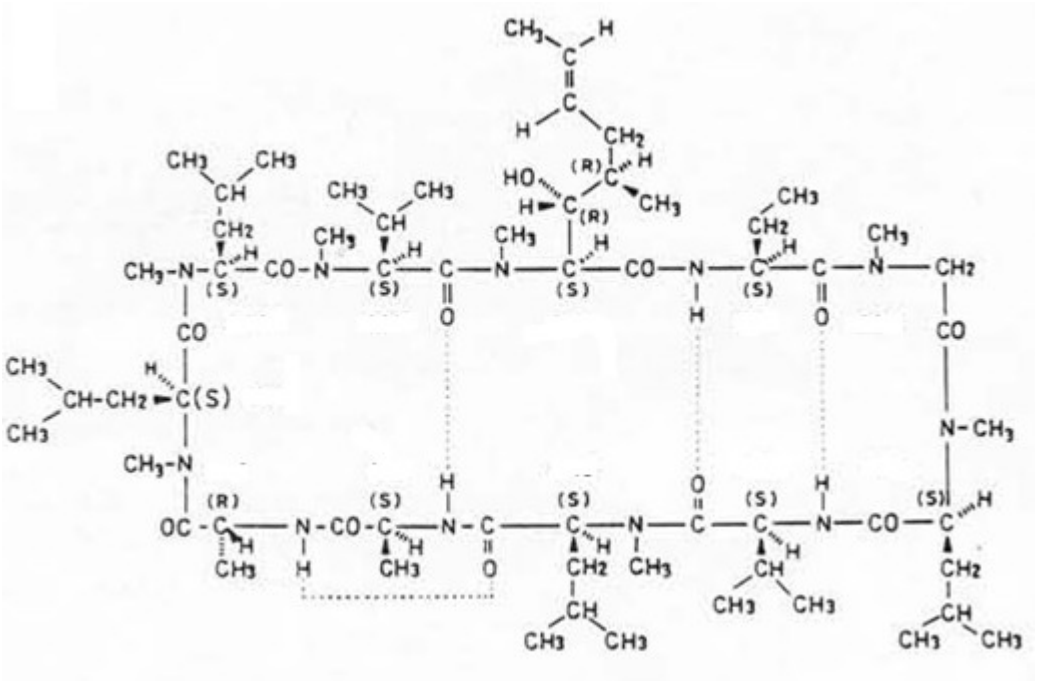


B Modified from {Wenger, 1985 #1001}

Figure 1.1 Structure of cyclosporine

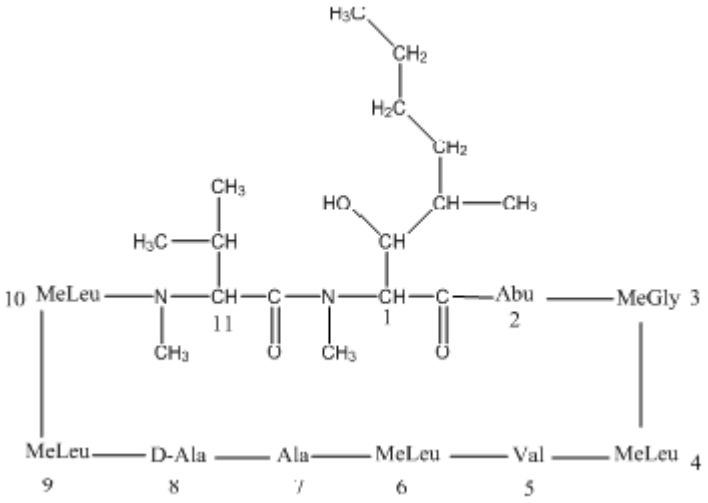


Figure 1.2 Structure of dihydrocyclosporine

Due to the cyclic structure and inherent intra-molecular hydrogen bonds (Figure 1.1 B), cyclosporine is very stable under normal conditions. However, degradation occurs under stress conditions. Several degradation pathways have been reported for the formulations under shelf life conditions {Bonifacio, 2009 #1011}: dehydration on olefinic bond and even loss of the MeBmt side chain; chain opening and oligopeptide formation, and N-O peptidyl shift to form isocyclosporine {Oliyai, 1994 #1012}. Another known degradant is dihydrocyclosporine (Figure 1.2). In the stability study of cyclosporine oral solution, it has been found that isocyclosporine (Figure 1.3) could be produced in acid medium {Kumar, 2001 #1017}.

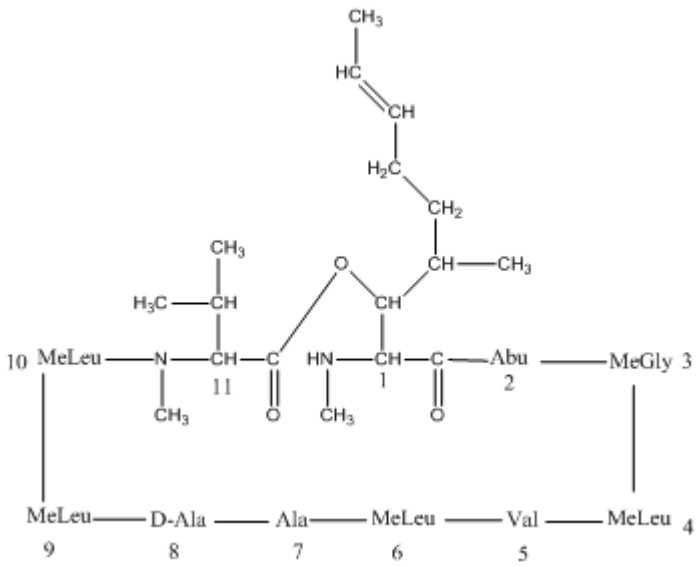


Figure 1.3 Structure of isocyclosporine

Considering the presence of hydrophobic amino acid residues and its neutral nature, cyclosporine is highly hydrophobic. It is poorly soluble in water and slightly soluble in n-hexane, soluble in acetone, ether and chloroform. White prismatic crystal (melting point: 148-151^{o}C) can be obtained from saturated acetone solutions {Hossan, 1987 #1008}. Cyclosporine displays high intestine permeability {Amidon, 2003 #1101} and poor aqueous solubility (0.04 mg/mL at 25°C), as a result, it is classified as BCS II drug substance according to biopharmaceutical classification system (BCS) {Ismailos, 1991 #1018;Augustijns, 2000 #1019}.

1.1.2 History and therapeutic applications of Cya

The discovery of Cya was a cutting-edge revolution in immunopharmacology.

Since 1961, a combination of azathioprine and corticosteroids had been the predominant immunosuppressant in organ transplantation. However, its application is limited due to the main side effects such as depression of the bone marrow and increased susceptibility to infections. Corticosteroid has its drawback in clinic applications as well. It inhibits T lymphocytes and is used as an anti-inflammatory agent, but the action is non-

selective, inevitably generating adverse side effects such as diabetes, avascular necrosis of bones and increased tendency to infections {Upton, 2001 #1020} .

Cya turns out to be a better option due to its stronger immunosuppressive activity and much lower toxicity. It is the first drug that exhibits selective immunoregulation of T cells without excessive toxicity. However, there is still a therapy limitation originating from the nephrotoxicity, hepatotoxicity and incomplete control of chronic rejection {Naesens, 2009 #1175;Erdem, 2011 #1072}. Many efforts have been made to reduce the toxicity by developing novel formulations {Muller, 2006 #1179;Czogalla, 2009 #1099;Muller, 2006 #1179}.

Cya was initially obtained from the fungus Tolypocladium inflatum, an extract of soil sample in Norway {Dreyfuss, 1976 #1103}. Several metabolites of this fungus, including Cya and other analogues (B, C, D, G and H), have been isolated. The main component Cya A, now named as Cya, was found to have strong immunosuppressive activity. In the following years, it was successfully utilized in preventing organ rejection in kidney transplant and liver transplant {Calne, 1978 #1104;Starzl, 1981 #1105}. In 1983, it was approved as an immunosuppressant to prevent graft rejection in transplantation by the U.S. Food and Drug administration (FDA) {Upton, 2001 #1020}. Currently, it has also been used in most autoimmune diseases, such as psoriasis, severe atopic dermatitis, pyoderma gangrenous, chronic autoimmune urticaria, and rheumatoid arthritis. Additionally, Cya has been formulated as an ophthalmic eye drop emulsion and widely applied for dry eye treatment {Salib, 2006 #1177}. Its application for cardiac disease treatment has become another focus of interest {Mott, 2004 #1180}.

Cya as well as its analogs Cya B, C, D, G and H were extracted from fungal metabolites followed by chromatography separation {Ruegger, 1976 #1109;Dreyfuss, 1976 #1103;Ruegger, 1976 #1109}. The total synthesis was first reported in 1984 {Wenger, 1984 #1110}. The successful synthesis of Cya and its analogues provided good understanding of the structure-activity relationship {Wenger, 1985 #1001}. However, none of the analogues showed greater potency than Cya itself. Among them, Cya G, in which norvaline at the 2

position replaces alpha-aminobutyric acid (Abu) in Cya, appears to be an efficacious immunosuppressant and is less nephrotoxic than Cya {Henry, 1995 #1112;Jeffery, 1991 #1111}.

1.1.3 Cya formulation

Table 1.1 Main commercial products of cycolsporine

Product	Company	Main excipient
Sandimmune® Soft Gelatin Capsule	Novartis	Corn oil
Sandimmune® Oral Solution	Novartis	Olive oil
Sandimmune® Injection	Novartis	Cremophor® EL
Neoral® Soft Gelatin Capsule	Novartis	Corn oil-mono-di-triglycerides Cremophor® RH 40 DL-α-tocopherol USP
Neoral® Oral Solution	Novartis	Corn oil-mono-di-triglycerides, Cremophor® RH 40 DL-α -tocopherol USP Propylene glycol USP
Restasis®	Allergan, Inc.	Glycerin; castor oil, Polysorbate 80, Carbomer copolymer type A

Due to poor solubility in water, Cya is generally formulated as suspensions or emulsions for oral and parental administration. The main commercial products in the market are listed in Table 1.1.

The first marketed product under the trade name Sandimmune® was developed by Sandoz, now Novartis {Novartis, 2011 #1007}. The oral products include Sandimmune® Soft Gelatin Capsules and Sandimmune® Oral Solution. They consist of Cya, alcohol and oil and are in the form of oil-in-water emulsions. Such formulation feature leads to erratic absorption depending on food intake, fatty content and gastrointestinal tract secretion. The bioavailability variability between patients and within an individual patient over time gives rise to particular concerns of potential toxicity and dosage monitoring {Yocum, 2000 #1114}. To overcome the drawback of emulsion formulation, a modified formulation in form of microemulsion has been developed and marketed as brand name Sandimmun Neoral®. The microemulsion can be formed immediately when the formulation contacts with aqueous environment. This feature enables fast drug release in the gastrointestinal tract and the absorption becomes less influenced by concomitant food intake and diurnal rhythm. Thus the intra-patient and inter-patient variability of all pharmacokinetic parameters can be reduced to some extent {Kovarik, 1994 #1115;Novartis, 2011b #1116}.

Cya demonstrates considerable P-glycoprotein (P-gp) efflux in intestinal absorption, which is another aspect for poor and variable bioavailability {Fricker, 1996 #1117} . The bioavailability of Sandimmun Neoral® may be in the range from 10% to 60%. Because Cya has a narrow therapeutic window index {Armstrong, 2001 #1118;Shaw, 1999 #1119}, the microemulsion Cya exhibits a pronounced initial plasma peak, which is unfavourable considering its therapeutic index. In the last decade, novel formulations have been developed to reduce toxicity by designing controlled release delivery system, such as solid lipid nanoparticles and micelle delivery systems {Muller, 2006 #1179;Aliabadi, 2006 #1120}.

Intravenous Cya (Sandimmune® Injection) has been developed in the form of microemulsion, using polyoxyethylated castor oil as emulsifier {Novartis, 2011 #1116}.

There are already generic Cya products available in the market, including Cicloral (Sandoz/Hexal), Gengraf (Abbott), Deximune (Dexcel Pharma Ltd) and Sangcya (SangStat). A topical formulation for treating keratoconjunctivitis sicca has been marketed

under the trade name Restasis by Allergan, Inc. {Lallemand, 2003 #1122;Allergan, 2010 #1181}. Inhaled Cya formulations are currently under clinical development, including a solution in propylene glycol and liposome dispersions {Groves, 2010 #1124;Iacono, 2006 #1182}.

1.1.4 Pharmacokinetics of Cya

The absorption, distribution, metabolism and excretion of Cya have been extensively documented. The absorption from the gastrointestinal tract is variable due to the formulation features and the nature of Cya. After administration of oral Sandimmune, blood and plasma concentrations reach maximum 1.0 ng/mL and 2.7-1.4 ng/mL (C_{max}) at about 3.5 h (T_{max}), respectively, while for modified oral Neoral, C_{max} is 59% higher than traditional Sandimmune and achieved at about T_{max} 2.5 h {Novartis, 2011 #1116}.The bioavailability is 29 % higher than oral Sandimmune. The distribution of cyclosporine exhibits multicompartment behaviour, it accumulates in liver, pancreas, lungs, kidneys, adrenal glands, spleen and lymph nodes {Novartis, 2011 #1116}.

Cya is mainly metabolized by cytochrome P-450 enzymes. Even though the drug has a complex structure, the identified metabolites are limited and have the intact cyclic oligopeptide structure of the parent drug. The major route of elimination is biliary, and only 6% of the dose is excreted in the urine through the bile {Novartis, 2011 #1116}.

1.1.5 Administration of intravenous Cya

Cya Sandimmune® I.V. (Novartis) is an intravenous preparation of Cya. The formulation is shown in Table 1.2.

Table 1.2 Sandimmune® I.V. formulation

Formulation	Cya	Ethanol (94% w/w)	Polyoxyethylated castor oil (Cremophor EL)
Sandimmune® I.V 1 mL	50 mg	278 mg	650 mg

Based on the product monograph, the injection solution must be diluted with 0.9% sodium chloride injection (NS) or 5% dextrose injection (D5W) before adminstration {Novartis, 2011 #1116}.

1.2 Infusion container

Large volume parenterals can be packaged in flexible bags, glass bottles or syringes when administrated to patient by infusion. As well known, liquid-based dosage is more likely to interact with the packaging materials than solid forms. For parenterals, contaminants, which may come from the packaging materials or due to the protection failure of packaging, can rapidly and completely be introduced into the blood circulation. Thus, as required in FDA guideline, injectable drug products represent one of the highest risk drug products considering the likelihood of interaction between packaging and injections, and more information should be provided regarding safety and compatibility of packaging system {FDA, 1999 #1128}.

1.2.1 Safety evaluation of packaging containers

Infusion containers both bottles and bags can be defined as container closure systems. According to the FDA guidance documents, four attributes: protection, compatibility, safety and performance/drug delivery are required to meet the related regulation {FDA, 1999 #1128;Albert, 2004 #1183}.

Containers required to protect drug products from light should be subject to light-transmission test and meet the specification regulated in USP 34 <661> containers {USP34-NF29, 2011b #1004}. Infusion bags should be also evaluated in terms of integrity.

The compatibility of containers should be sufficient to maintain the quality of the drug formulations. Herein leaching and extraction tests have to be carried out to evaluate potential chemicals extracted from containers. Leachable can be inorganic or organic, depending on the type of materials used and manufacturing processes. A number of analytical techniques can be employed to detect and quantify leached impurities, including liquid chromatography (LC), gas chromatography (GC), mass spectrometry (MS),

inductively coupled plasma (ICP) spectroscopy {Yu, 2000 #1130}. In addition, other changes like pH shifts, precipitate, and discoloration should be inspected, because these changes may lead to drug degradation and pose hazardous issues during administration.

With respect of safety evaluation, container materials should be verified of no harmful to human health. It is imperative to have a good knowledge of the material composition (*e.g.* production process, solvents used, potential impurities, polymer additives), as well as information of drug substance and excipients, thus having a good understanding of potential leachables and possible interaction of these materials with drug formulations.

Finally, performance should be evaluated on the functionality and appropriate drug delivery. The point is on improvement of patient compliance, minimum waste, or ease of use. Good performance should ensure to deliver the right amount or rate of drug substance. For instance, the container should have low water vapour transmission to ensure minimum water loss, thus keeping drug concentration consistent {Paula Youngberg webb, 2009 #1185}.

1.2.2 Infusion container types

Infusion containers can be bottle or flexible bags. They are used to prepare or store fluid mixtures to be administered to a patient through an intravascular administration set. The material used can be glass or plastic.

1.2.2.1 Glass bottles

In earlier years, glass bottles were the standard containers for intravenous administration, which are gradually replaced by plastic containers or bags due to cost and delivery facilitation. However, in the case of special intravenous liquids, glass bottles are still a good choice due to the inert chemical reactivity. Glass bottle has various advantages. First of all, it is transparent, which is particularly important to inspect particles or aggregates when preparing injection or during administration. Besides, glass bottle is known as gas-proof and chemically inert to most drugs and excipients.

However, glass container has some disadvantages. It is breakable and the delivery is costly. In addition, it must be vented and the administration requires careful monitoring to prevent any possible air embolism at the end of the infusion. Also it does not support a pressure infusion by using pressure cuff. Drug sorption is still a concern, especially in the case of administration of proteins and peptides nutrient {Mizutani, 1984 #1135}. In addition, the possible inorganic leachable such as metal ions may affect the stability of metal sensitive infusion fluids {Dean, 2007 #1136}. Therefore, it is still necessary to evaluate the compatibility of glass material with infusion liquid before use {Sacha, 2010 #1134}.

1.2.2.2 Plastic containers

Currently, flexible plastic bags are predominant infusion containers in the market. Compared to traditional glass bottles, plastic infusion bags have lower weight, lower cost and several application advantages. Firstly, they are more user-friendly, safer, more healthy and more convenient than glass bottles. The flexibility enables the bag to shrink during administration and no air interchange is needed. Thus, air embolism can be avoided during infusion administration. In addition, it is easier to mix the contents when admixtures are required. More importantly, flexibility makes pressure infusion possible. However, barrier and sealing property, transparency and chemical inert property are usually inferior to glass bottles. As the storage time and temperature increase, a high gas and water permeability may cause substantial water evaporation or gas permeation, which will produce inappropriate administration. Therefore, numerous specific tests are required to guarantee the safety of plastic containers.

The major types of materials used for parenteral plastic packaging include polyvinylchloride (PVC), polyethylene (PE), polypropylene (PP), ethylene vinyl acetate (EVA) {Sacha, 2010 #1134}.

PVC bags were the first flexible containers used in intravenous administration and have dominated the market for many years. The polymer PVC is brittle and very hard, thus high amount of phthalate DEHP (bis(2-ethylhexyl) phthalate) has to be added to enhance

flexibility. However, the leaching of DEHP to products has been proven to be a hazardous issue. The Environmental Protection Agency (EPA) has classified DEHP as a probable human carcinogen {EPA, 2000 #1137}. The FDA also issued a public health notification regarding the use of DEHP {FDA, 2011 #1138}. Furthermore, drug sorption on PVC containers occurs. {Martens, 1990 #1139;Noh, 2009 #1140}. Up to date, DEHP/PVC containers have been banned or restricted in a number of countries (*e.g.* Germany, Sweden, France, Canada, Spain, South Korea and the Czech Republic). On the other hand, there is environmental issue arising from PVC disposability. It produces hydrochloric acids waste and dioxin when incinerated {Sittig, 2008 #1186}.

Table 1.2 Comparative properties of plastic bags

Modified from {Sacha, 2010 #1134}

Property	PVC	LDPE [a]	HDPE [b]	PP	EVA
Compatibility with drug products	Poor	Good	Good	Good	Fair
Protection from moisture evaporation	Very poor	Good	Excellent	Good	Very poor
Transparency characteristics	Good	Fair	Poor	Fair	Fair
Collapsibility characteristics	Excellent	Poor	Poor	Poor	Good
Disposability	Poor	Good	Good	Good	Fair
Heat sterilization	Fair	Poor	Good	Excellent	Very poor

[a] LDPE means low density polyethylene.

[b] HDPE means high density polyethylene.

Most companies instead are using DEHP-free non-PVC materials. Basically the alternative materials are biologically inert, plasticizer-free, clear, flexible, non-toxic and biodegradable.

Generally speaking, the drug stability should be highlighted regarding of container materials and drug formulations. The properties of plastic bags are illustrated in Table 1.2 {Sacha, 2010 #1134}.

Plastic syringe is also used for small dose intravenous administration to paediatric patients. The materials typically used are polycarbonate, polyethylene and polypropylene {Anes J, 1992 #1142}. In most cases, these syringes have high compatibility with drug formulations. However, some potential problems may arise from the silicone lubricant, which are incompatible with some drug formulations, for example, silicone may cause protein aggregation due to hydrophobic interaction {Jones, 2005 #1143}. In addition, some impurities may be leached from uncoated rubber plunger. It has been reported that Eprex® formulation leached some impurities from uncoated rubber of syringe plunger, causing the incidence of pure red cell aplasia in chronic kidney disease patients. The investigation showed that surfactant polysorbate 80 in the formulation was responsible for the product-rubber interaction {Yu, 2010 #1144;Boven, 2005 #1187}.

In conclusion, plastic bags are popularly used due to flexibility and delivery convenience. Currently, the standards for plastic containers are less addressed than glass ones. Extra tests and efforts are required regarding the interaction between drug product and container materials. Practically, application of infusion containers are in dilemma and the compatibility is highlighted only when the problems are encountered clinically. Currently, the non-PVC containers have been used for the administration of Cya intravenous injection. However, their compatibility has rarely been investigated.

1.3 Drug stability study

According to the U.S. Pharmacopeia (USP), drug stability is defined as the extent to which a drug product retains the same properties and characteristics within specified limits

throughout its period of storage and use (*i.e.*, its shelf-life) {USP34-NF29, 2011a #1002}. The stability of intravenous injections is affected by the interaction of formulation and container materials:

- The adsorption of drug substance to the surface will lead to drug loss.
- The leachables from the surface materials to the injection fluid may cause toxicity, drug degradation, induce aggregation or precipitation during storage and administration.
- Gas permeation may cause degradation or other incompatibility.
- Moisture evaporation leads to inconsistent drug concentration.

In addition, microbial stability should be examined to avoid contamination. Normally, validated aseptic procedure can prevent from microbiological contamination. Therefore, for most parenteral products of small molecular drug, stability studies mainly focus on the alteration of physical and chemical properties, as well as therapeutic property.

Drug adsorption is essentially related to the drug property and the container composition. The sorption can be caused by electrostatic or hydrophobic interaction. For example, the basic drugs can be ionized in acidic medium and tend to be adsorbed on negatively charged polystyrene surface, the polar molecules are prone to be adsorbed on polar surfaces. The adsorption of lipophilic drugs are usually dominated by hydrophobic interactions. The partition of drug in liquid medium or solid material is a determinant aspect. Hydrophobic drugs with higher log P have tendency to adsorption on plastic surfaces (Palmgren, Monkkonen et al. 2006).

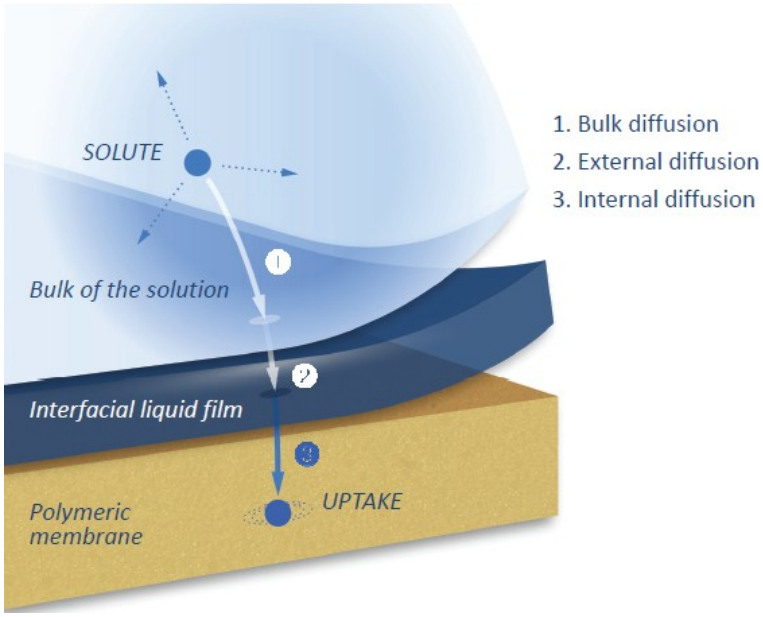


Figure 1.4 Adsorption process

As shown in Figure 1.4, the adsorption process includes bulk diffusion, interfacial diffusion and uptake. External diffusion refers to the diffusion across the liquid film to the solid surface; internal diffusion refers to the liquid contained in the pores of along the pore walls; uptake refers to adsorption to active site or mass action {Qiu, 2009 #484}. For physical adsorption, the adsorption or uptake to active site is very rapid and the adsorption rate is always controlled by external diffusion or internal diffusion rate. The adsorption kinetics of drug on packaging materials is seldom studied. Good understanding of adsorption mechanism may be expected to evaluate the performance of packaging containers regarding drug loss.

1.3.1 Stability-indicating methods

Evaluation of pharmaceutical product stability has been addressed in regulations in U.S. Food and Drug Administration (FDA), U.S Pharmacopeia (USP), World Health Organization (WHO), International Conference on Harmonisation (ICH), and other

agencies. These regulations require that the methods for stability evaluation should be validated to be stability-indicating.

According to FDA guidelines, a stability-indicating method is defined as a validated analytical procedure that could accurately and precisely measure active pharmaceutical ingredient (API) in drug substance or drug products, without interference with degradation products, process impurities or other potential impurities {FDA, 2000 #1146}. It is recommended that all assay procedures for stability studies be stability-indicating {FDA, 2000 #1146;Swartz, 2005 #1147}, in which it can detect the changes of drug substance properties and measure the active ingredient accurately {Velagaleti, 2007 #1148}.

Stability-indicating methods can be specific or selective {Bakshi, 2002 #1149}. The former refers to a method that is able to measure unequivocally the drug in the presence of all degradation products. The later refers to measuring drug unequivocally, as well as all degradation products and impurities. Even though selective stability indicating method is time-consuming and costly, it is required when potential safety concerns arise from degradation product or impurity. In case of old drugs or drug products, if the degradation behaviour is well understood, specific stability-indicating method is sufficient for the routine stability evaluation.

In order to develop a stability-indicating method, stress degradation (also called forced degradation) is required to produce potential degradation products. It can be undertaken under acid and basic hydrolysis, higher temperature above the accelerated storage temperature, humidity, photolysis and oxidation. The drug structure dictates its degradation behaviour. Compounds like amides, esters, lactams, lactones, etc. are susceptible to hydrolysis under acid and base conditions {Velagaleti, 2007 #1148}. Thiols and thioethers undergo oxidation even under mild conditions. Compounds with carbonyl, nitroaromatic, N-O, double C=C bond, suplfides, alkenes, polyenes, and phenols may degrade under light exposure. Oxidation degradation might be catalyzed by trace quantities of metal ions. In addition, Environmental changes such as temperature, pH value, vehicles

and impurities released from packaging materials may influence the degradation {Hovorka, 2001 #1150}.

Current guidance documents do not regulate how stress test should be carried out. Most practices are performed on a sound scientific basis. Stress degradation conditions are optimized depending on the chemical nature of the active ingredient and the type of dosage forms. Successful stress degradation should yield representative degradants and control appropriate rate in order to meet the requirement of stability-indicating demonstration. Importantly, stress degradation may be different from that under shelf-life conditions, thus, mild condition is preferred to yield true degradation products.

In some cases, stress test should be performed both on API and drug products in order to understand the influence of excipients on stability. Generally, degradation should not exceed 5-15% of API. Extensive degradation may lead to limited relevant products and complicated degradation patterns {Klick S., 2005 #1151}. Stability data should be evaluated with physical, chemical, and microbiological properties. The US FDA guidance defines significant changes for drug or drug products as following {FDA, 2003 #1152}:

- Change of 5% in assay during the period of storage.
- Degradants exceeding specifications.
- Physical attributes such as color, phase separation, pH and dissolution failing to meet specifications.

Real time stability tests vary with dosage forms. As for parenterals, various aspects should be considered, including color, clarity for solutions, particulate matter, pH, sterility, and endotoxines. For instance, stability test of suspensions or emulsions for injection should include inspection of phase separation, particle size and size distribution.

1.3.2 Techniques employed in stability-indicating methods

The traditional titrametric, spectrophotometric techniques have been employed in stability tests in pharmacopoeia monographs. However, most of them are lacking of specificity or selectivity (Abdel-Fattah, El-Sherif et al. 2010), separation from related

substances and impurity identification are usually required. So far, chromatographic methods have become predominant in stability assessment {Abdel-Fattah, 2010 #1188}, including thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), HPLC and capillary electrophoresis (CE), etc. TLC is effective in identifying degradation products and impurities. However, the variability and non-quantitative nature in TLC limit its application. Instead, a novel technique of high performance thin layer chromatography (HPTLC) is more accurate and reliable and appears powerful in quantitative drug stability tests {Fakhari, 2008 #1154;Abdel-Fattah, 2010 #1188;Rao, 2011 #1155;Kumar, 2001 #1017;Rao, 2011 #1155}. GC can be used as stability-indicating technique, however, its application is not versatile and restricted when the drugs are not volatile or thermally unstable. Up to now, there are few reports on the use of GC for stability evaluation. Instead, HPLC has been extensively employed due to the attributes of high resolution, sensitivity and specificity. Non-volatile, thermally unstable or polar/ionic compounds can be analyzed on HPLC by rational alteration of stationary phase, mobile phase or even detector. Based on separation mechanism, HPLC methods are classified as normal-phase chromatography (NP-HPLC: using polar stationary phase and a non-polar, non-aqueous mobile phase), and reversed-phase chromatography (RP-HPLC: using non-polar stationary phase and an aqueous, moderately polar mobile phase). Ion-exchange chromatography is also utilized to assay ionic substance. Furthermore, the multiple choices of detectors facilitate versatile applications. The detectors include ultraviolet-visible (UV/VIS), photodiode array (PDA) detector, refractive index (RI) detector, fluorescence detector, evaporative light scattering detector (ELSD), electrochemical detector, and conductivity detector. PDA collects spectra across a range of wavelengths at each retention time, making it more powerful for specificity evaluation and impurity identification.

The application of hyphenated techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-nuclear magnetic resonance (LC-NMR) and capillary electrophoresis-mass spectrometry (CE-MS) are powerful to identify known and unknown degradation

products or impurities {Lee, 1999 #1156;Belal, 2009 #1189;Singh, 2010 #1190;Lee, 1999 #1156}. Capillary electrophoresis (CE) is the latest technique applied in stability study, it has the advantage of high sensitivity, resolution and high efficiencies with minimal peak dispersion {Visky, 2005 #1191;Singh, 2010 #1190}.

Among the stability-indicating methods, HPLC is cost-effective, high throughput and almost universal. The combination with mass spectrometry and PDA facilitates method development in stability study.

1.3.3 Physical stability

Physical stability is closely related to dosage forms. Any changes such as phase separation, particulate, precipitates and discoloration may cause degradation or increase potential risk. Regarding emulsion, liposome and suspension formulations for intravenous administration, particle size and particle size distribution are the major stability parameters {Wong, 2008 #429}.

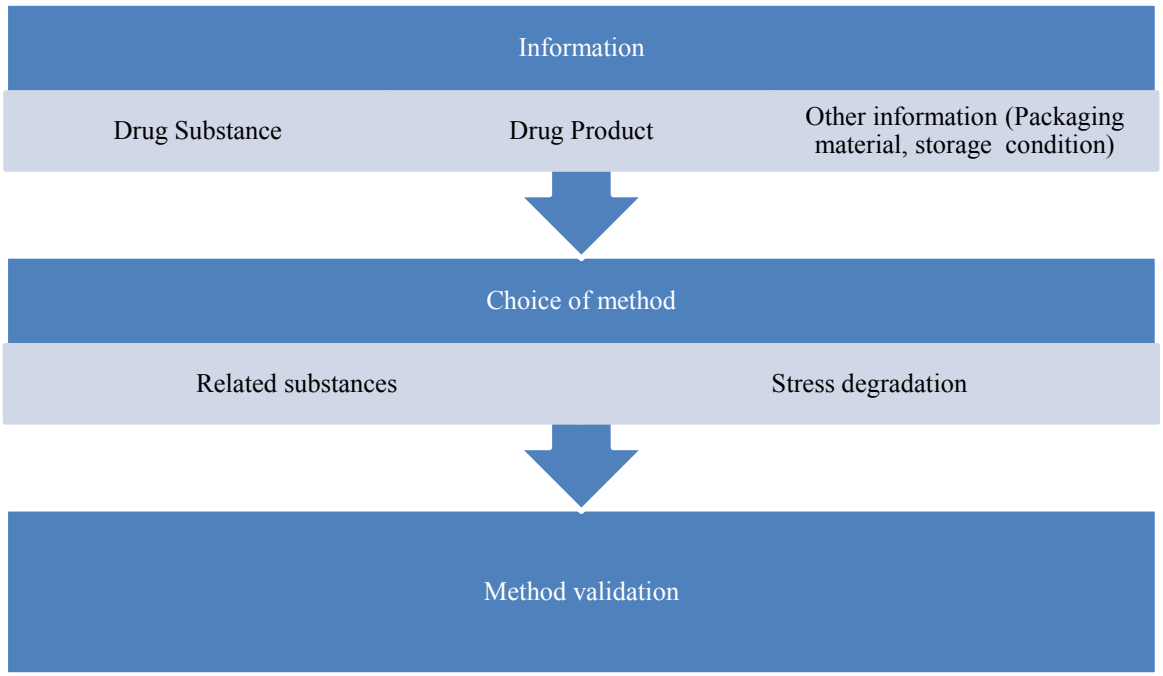
Particle size can be measured by microscopy, coulter counter, dynamitic light scattering (DLS), or laser diffraction (LD) {Wu, 2011 #1160}. The application of each technique should take account of accuracy, precision and particle size range. Thus, accuracy, precision and sample preparation procedure should be validated. Dynamic light scattering (DLS) is a powerful technique to measure particles in the range of 0.3 nm to 10 μm and the measurement is precise in the range of 10 nm to 1000 nm {Malvern, 2011 #1202}. This technique is suitable for micelle systems such as emulsions, lipsomes and nanosuspensions. However, its application is limited to liquid formulations and cannot be applied to dry powder. Polydispersity index (PDI) demonstrates the particle size distribution, values below 0.1 indicate that particles are homogeneous, values of 0.1 to 0.25 show a narrow size distribution, while values above 0.5 mean a wide size distribution and usually unacceptable in drug delivery systems {Patravale, 2004 #1161} .

In terms of parenteral emulsion, the British Pharmacopoeia requires that the diameter of fat globules should not exceed 5μm {Wong, 2008 #429}. The USP Pharmacopeial

Forum has established the requirement and measurement methods. By using DLS method, the proposed intensity-weighted mean droplet diameter must be less than 500 nm, volume-weighted percentage (based on light obscuration) of fat greater than 5μm should not exceed 0.05% {Driscoll, 2006 #1192}. In the previous study, the physical stability of parenteral cyclosporine was generally evaluated visually for clarity, precipitate and color change. The use of particle size analysis will be more precise to detect the physical change during storage. For example, the compatibility of cyclosporine injection with fat emulsion injection has been studied by using DLS to measure emulsion size {Jacobson, 1993 #1174}.

1.3.4 Development and validation HPLC stability-indicating method

Typically, most stability-indicating methods are using HPLC. Method development and validation are mostly determined by intended purpose. For instance, selective stability method requires good resolution of API and each relevant impurity, while in specific method, it is unnecessary to separate each impurity. The general procedure (Figure is described as below {Bakshi, 2002 #1149}:



1.3.4.1 Get Information on drug, drug products, and potential degradation routes

The knowledge of physicochemical properties of drug substance, excipients, and packaging materials is a good starting point for the stability-indicating method development. Degradation routes rely mostly on the structure of compound and preparation vehicles.

In new drug product development, degradation chemistry can be easily postulated from the existing related drugs, since the degradation behaviour of most new drugs are similar to their congeners. For example, penicillin in clinical practice has the same degradation behaviour as at the beta-lactam moiety {Bakshi, 2002 #1149}. Thus, degradation mechanisms of some old drugs are important assets in stability studies of new drugs. The properties such as pKa, logP, solubility, spectroscopy behaviour, drug degradants and potential impurities should be considered for the choice of method and method parameters. For example, the determination of stationary phase, mobile phase, sample extraction and preparation, and detector choice are all based on these properties of drugs, excipients, and degradants.

1.3.4.2 Set up HPLC method and conduct stress degradation

A short cut can be taken benefiting from existing methods. In the new drug product development activity, the existing method should be revalidated to be suitable in actual analysis, for the excipients and vehicles may influence or even alter the degradation pathways. In most cases, some modifications are necessary.

Stress degradation should be designed based on the individual drug as well as drug products. The stress conditions are optimized based on trial and error. Normally, stress test is performed only on drug substance, because in most cases, it can provide sufficient potential degradants possibly present within the storage interval. However, in order to investigate the influence of excipients on degradation behaviour, drug products should also be subjected to stress degradation {Grimm, 1998 #1163}. Cyclosporine is a stable cyclic peptide; high temperature is usually required to get related degradants. In Kumar's study, the acid and base stress degradations for oral cyclosporine formulation were conducted at

80^{o}C. The results showed that cyclosporine content did not change under base degradation, however, some degradant were generated from acid degradation {Kumar, 2001 #1017}.

1.3.4.3 Validation of stability-indicating method

To validate a stability-indicating method, it is imperative to determine the validation parameters. For drug substance assays, detection limit is not critical. However, for impurities or degradants evaluation, low detection limits and quantitative limits should be determined.

1.4 HPLC method validation

Validation of an analytical method is to establish the process through the laboratory studies, in which the performance characteristics should meet the requirement for its intended use {Yuwono, 2005 #1164}. Reliability and reproducibility should be adequate for intended application.

The fundamental attributes of validation include accuracy, precision, selectivity/specificity, detection limit, linearity and range. Validation can be classified as fully validated level and single-laboratory level. Generally, it is not always necessary to validate all parameters. The extent of validation depends on the intended use according to the corresponding regulations in FDA, ICH and USP Documents (Table 1.3).

Table 1.3 Parameters required for analytical method validation

Reproduced from {Shabir, 2003 #1003;Yuwono, 2005 #1164;USP, 2004 #1165;Shabir, 2003 #1003}

Analytical Type		Assay	Impurity Quantitative testing	Impurity Limit	Performance characteristics	Identification
Accuracy	USP	Yes	Yes	*	*	No
	ICH	Yes	Yes	No	N/A	No
Precision	USP	Yes	Yes	No	Yes	No
	ICH	N/A	N/A	N/A	N/A	N/A
Repeatability	USP	N/A	N/A	N/A	N/A	N/A
	ICH	Yes	Yes	No	N/A	No
Intermediate precision	USP	N/A	N/A	N/A	N/A	N/A
	ICH	Yes	Yes	No	N/A	No
Specificity	USP	Yes	Yes	Yes	*	Yes
	ICH	Yes	Yes	Yes	N/A	Yes
LOD	USP	No	No	Yes	*	No
	ICH	No	No	No	N/A	No
LOQ	USP	No	Yes	No	*	No
	ICH	No	Yes	No	N/A	No
Linearity	USP	Yes	Yes	No	*	No
	ICH	Yes	Yes	No	N/A	No
Range	USP	Yes	Yes	*	*	No
	ICH	Yes	Yes	No	N/A	No

"*" May be required, depending on the specific test; N/A: not available.

Before validation, system suitability should be verified to ensure that chromatographic system is appropriate for the intended analysis. In general, acceptable parameters should be in a definite range. For instance, during the suitability test, standards and assay samples should be stable within 2% deviation for 24 h {Shabir, 2003 #1003}. In most cases, change in retention time will impact on peak shape and thus lower result accuracy, so deviation of retention time should be as low as possible and the injection repeatability should be lower than 1%.

Tailing factor TF stands for peak symmetry (Figure 1.5), it is calculated as:

$$TF = \frac{W_{0.05}}{2f}$$

where $W_{0.05}$ is the width of the peak at 5% of height and f is the distance from the leading edge to peak maximum at a point 5% of the peak height {USP, 2011 #1004}. Figure 1.6 represents a separation of two substances with respective retention times of t_{R1} and t_{R2}, and widths of W1 and W_2, $W_{h/2}$ is the width at half-height. t_M corresponds to the solvent front, implying the time required for unretained solvent elution. The resolution of two components in a mixture is calculated:

$$Rs = 2 \times (t_{R2} - t_{R1}) / (W_1 + W_2)$$

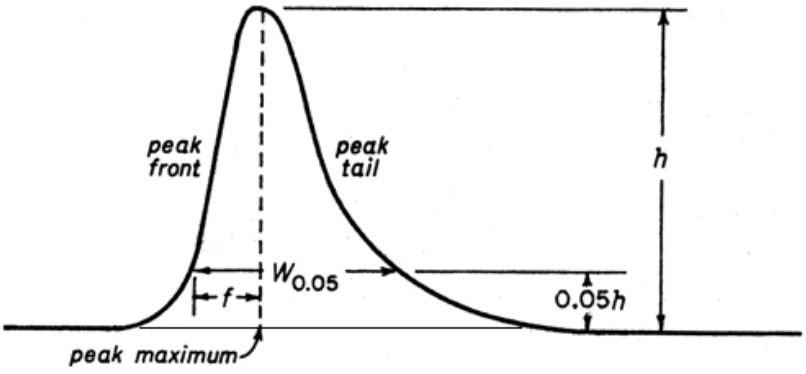


Figure 1.5 Tailing factor illustration of HPLC

Modified from {USP, 2011 #1004}

Number of Theoretical Plates (N) indicates column efficiency. It is usually calculated as {USP, 2011 #1004}:

$$N = 16 \times (t_R / W)^2$$

Where t_R ,W is retention time and peak width of analyte, respectively.

The capacity factor (k), also known as retention factor, is defined as the ratio of substance mass dispersed in stationary phase (m_{sp}) to that in mobile phase (m_{mp}):

$$k = \frac{m_{sp}}{m_{mp}}$$

It is determined as (Figure 1.6):

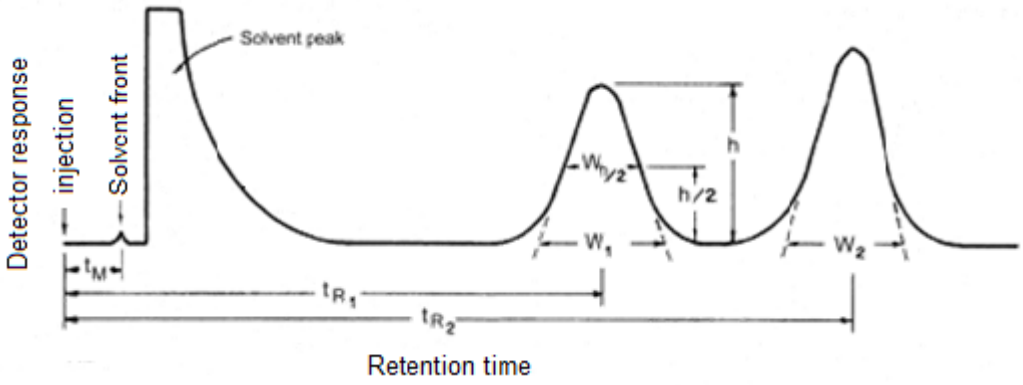


Figure 1.6 Chromatographic separation of two substances

Modified from {USP, 2011 #1004}

$$k = \frac{t_R - t_M}{t_M}$$

The recommended parameters for the suitability test are {Shabir, 2003 #1003}:

- Relative standard deviation (RSD) of repeated injections ≤ 1% (n=5).
- Tailing factor ≤ 2.
- Resolution ≥2.
- Theoretical plate ≥2000.
- Capacity factor ≥2.

Validation parameters are determined by the analytical purpose. For example, according to the regulation of USP and ICH, the quantitative assay of active ingredient requires validation of accuracy, precision, specificity, linearity, range. For the identification of impurities, the validation of accuracy, the limit of detection (LOD) and quantity (LOQ), repeatability, interim precision, linearity and range can be omitted.

1.4.1 Selectivity and specificity

Selectivity and specificity are often interchangeably used. Specificity of analytical method refers to the ability to differentiate and quantify the analyte from other components such as degradation products, metabolites, impurities and matrix. Chromatography techniques require no interference between the analyte and other components. Normally, resolution of the analyte from other components should be more than 1.5-2 to ensure accuracy {Yuwono, 2005 #1164}.

However, compounds of similar structure are likely to coelute. In order to verify coeluted substances, peak purity should be verified. By using a HPLC system coupled with photo diode array (PDA) detector, UV-Vis spectrum at different wavelength can be recorded. Peak purity can be confirmed by comparing spectra from the upslope, apex and downslope, or the whole spectrum simulation {Papadoyannis, 2004 #1166}.

Different terms are used to indicate peak purity. For example, match factor (MF) is used on Agilent system; similarity index is used on Shimadzu/LCsolution system. The principle is described in Figure 1.6 {Papadoyannis, 2004 #1166}. Let's assume that chromatogram A and B are response signals recorded at different wavelengths, in case of the pure peak, the ratio of the signals A and B (called ratiogram) will be constant and the ratiogram will be rectangular. For the first impure peak of coeluting analyses, due to different absorbance spectra and a slight difference in retention time, a significant distortion of rectangular form will take place. This is the basic principle of peak purity determination.

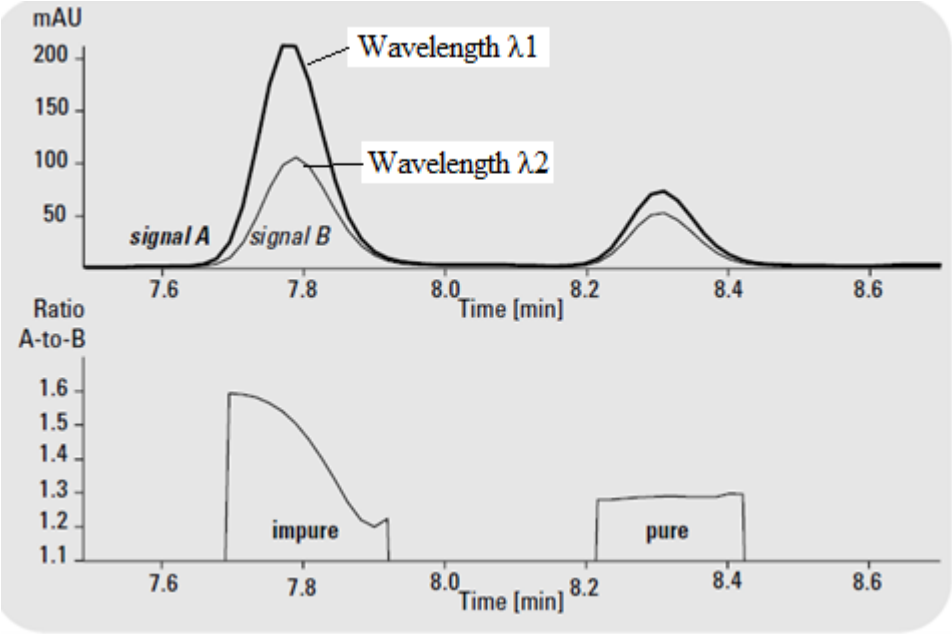


Figure 1.7 Signal ratiograms for impure and pure peaks at wavelength λ_1 and λ_2.

Modified from {Papadoyannis, 2004 #1166}

Based on this principle, impurity as low as 0.5% of the analyte can be detected. For instance, on Shimadzu/LCsolution system, if purity index is below 0.89, it is regarded as not pure, values of 0.90-0.95 indicate that the peak is contaminated. On HPLC without PDA detector, peak purity can be estimated by calculating tailing factor at different wavelengths. Peak purity can be assumed to be acceptable if tailing factors are identical at different wavelengths. However, when the impurity has very similar profile with analyte or has much lower concentration, other techniques LC-MS, GC-MS, LC-FTIR, LC-NMR should be used to verify the peak purity {Velagaleti, 2007 #1148}.

If isolated impurities are available, the specificity can be determined simply by comparing the standard with the results from the samples consisting impurities, degradation products, and placebo ingredients. Normally, it is difficult to obtain and isolate all potential impurities and degradation products. Thus stress degradation is necessary to demonstrate stability-indicating power. Specificity can be evaluated by comparing chromatograms of

original products and degraded samples. Mostly, degradants and impurities do not need to be separated from each other, but need to be adequately resolved from the drug substance peak.

1.4.2 Accuracy

Accuracy of an analytical method is defined as the extent by which the value measured deviates from the true value {Yuwono, 2005 #1164}. There are several approaches to estimate accuracy. The first one is to compare the value obtained in a new method with a value known to be accurate. The second method is to add known concentrations to the sample and then compare the results with the true value. The third method is based on the calculation of recovery of standard or known analyte spiked into blank matrices or products. For the samples spiked into blank matrices, it is recommended that 5 analyte concentrations, covering the range 75% to 125% or 80%-120% of the target concentration. For the standard addition methods, the spiking concentration is in the range of 50-150% of the target concentration. Accuracy is assessed as percent recovery. The acceptance criterion is set depending on the analyte concentrations, recovery rate 98%-102% or 95%-105% can be acceptable for drug preparations. In biological analysis, recovery of 90% -110% can be acceptable {Velagaleti, 2007 #1148}.

1.4.3 Precision

Precision of an analytical method expresses the closeness of agreement between several results. It is usually evaluated at three categories: repeatability, intermediate precision and reproducibility. Repeatability is the intraday precision. It is determined by one analyst using one equipment in one laboratory. According to ICH guidelines, nine determinations covering the specified range (for example, three concentrations 80%, 100%, 120% of the target concentration, each sample is injected in triplicate) or six determinations at 100% of the target concentrations are required. Intermediate precision is evaluated based on the interday variation over a number of days or weeks, at least six determinations of three concentrations at three different times. Reproducibility is performed to assess the

precision between laboratories. These characteristics are essential for method transfer between laboratories.

The criterion of relative standard deviation (RSD) depends on the nature of analysis study. For a finished drug product assay and content uniformity, RSD should be below 2% (intraday precision for 6 injections. n = 6) or 3% (intermediate precision for at least 6 measurements. n ≥ 6). For the dissolution test, the acceptance criterion of repeatability is set as ≤ 3%, for bioanalytical study, it should not be more than 15% {Brodie, 2002 #1167;Yuwono, 2005 #1164}.

1.4.4 Limit of detection (LOD) and quantification (LOQ)

LOD is defined as the lowest quantity at which the substance can be detected under the analytical condition. At LOD, analyte can be identified but not be quantitatively assayed. LOQ is the lowest quantity that can be determined with acceptable accuracy and precision. In chromatography, The LOD can be determined by the signal-to-noise ratio about 2:1 to 3:1. LOQ is estimated with the ratio of 10:1. The alternative estimation is based on the calibration standards. LOD and LOQ are determined as follows {Chan, 2010 #1168}:

$$LOD = 3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where σ is standard deviation of intercept, S is the slope of the calibration curve. In some case, this method need to be verified by measuring several samples near LOD or LOQ. Spectra comparison of sample, placebo and matrix is needed to eliminate noise. LOQ can also be obtained by diluting known concentration of analytes until RSD of multiple injections is larger than 2% {Yuwono, 2005 #1164}.

1.4.5 Linearity

Linearity of a method is defined as that the response directly obtained or after mathematical transformation is proportional to the concentration of the analyte. Either peak area or peak height can be used for calibration curve calculation, depending on the error

produced. Usually, the use of peak area is preferred to height, for peak area is directly related to concentration, while peak height varies depending on the retention time and overloading analyses {Yuwono, 2005 #1164}. Linearity can be determined by measuring the signal of a series of concentrations. According to ICH, at least five concentrations over the entire working range are required. Standard samples can be prepared by weighing different amounts of standards or diluting standard stock solutions. The latter approach is popular and suitable in case of low concentration preparation, in which weighing error would be much higher. Linearity is produced by calculating the regression line using mathematical methods such as least mean squares. Linearity is confirmed by determination coefficient of linear regression. In most cases, more than 0.997 is acceptable {Chan, 2010 #1168}. Otherwise, further statistic analysis is required {Yuwono, 2005 #1164}. Based on ICH regulations, the slope, residual sum of squares, and intercept should be reported {ICH, 2005`, Nov. #1169}.

1.4.6 Range

In USP 34, the analytical method range refers to the interval between upper and lower levels of the analyse that has been demonstrated to have an acceptable level of precision, accuracy, and linearity. Normally, for the assay of drugs or drug products, the range should cover 80% to 120% of target concentrations. For content uniformity, it should be between 70-130%. While for controlled release products, the range should cover 0-110% of the label claim {Yuwono, 2005 #1164;Chan, 2010 #1168}.

1.4.7 Robustness and ruggedness

Robustness of an analytical method is defined as the ability to remain unaffected by small, but deliberate variations in method parameters. For HPLC method development, the parameters required to be tested include flow rate, column temperature, column origin, injection volume, mobile phase composition, the pH of buffer and detection wavelength {Ermer, 2001 #1170}. Ruggedness is determined by following the same procedure by different analysts in different laboratories under slightly different conditions.

In conclusion, validation of HPLC method ensures that the characteristics meet the regulated guidelines for intended analysis. So the validation should be carried out before the routine analysis. The requirement of validation parameters and level depends on the intended purpose and choice of method.

1.5 Cyclosporine stability-indicating methods and stability study

Several analytical methods for cyclosporine have been reported. In brief, these methods include reverse phase HPLC employing acetronitrile- or tetrahydrofuran-based mobile phases. The United States Pharmacopeia (USP) method employed reverse phase column C18 as stationary phase, and acetonitrile based mixture as mobile phase {USP34-NF29, 2011c #1005}. A similar method has been developed for oral cyclosporine stability evaluation, in which Nuclosil RP-2 column was used as stationary phase, a mixture of acetonitrile, water and phosphoric acid was used as mobile phase. This method was demonstrated to be stability-indicating by stress degradation tests {Kumar, 2001 #1017}. Another method using mobile phase of 68% (v/v) acetonitrile in water has also been reported for the compatibility of plastic syringe with oral cyclosporine {Ptachcinski, 1986 #1171}.

In another report, Bonifacioa *et al* developed an HPLC method using Lichrospher RP-18 column and a mixture of tetrahydrofuran (THF) and aqueous phosphoric acid (0.05M in water) (44:56) as mobile phase {Bonifacio, 2009 #1011}. The main degradants and related substances including ID-005-95, cyclosporine H, isocyclosporin H, and potential impurities isocyclosporin A, Cyclosporin B and Cyclosporin G in oral formulations, could be resolved and determined simultaneously. This method can provide more stability information than other reported methods. It accurately measured cyclosporine concentration in oral dosage. However, high proportion of THF in mobile phase may attack polyetheretherketone polymer (PEEK) tubing employed in most modern HPLC systems, thus the application is restricted only to the system compatible with THF solvents {Clayton McNeff, 2011 #1006}.

Approximately 70% of world's supply of acetonitrile was consumed in pharmaceutical industry as the solvent. The prices of HPLC-grade acetonitrile has been increasing rapidly because of worldwide shortage. Laboratories are under pressure to re-develop HPLC methods by use alternate solvents such as methanol or long chain alcohols {Ankur M. Desai , 2011 #1195;Ankur M. Desai , 2011 #1195}. From an environmental perspective, methanol is preferable solvent than the use of acetonitrile and tetrahydrofuran {Christian C, 2007 #1196}. Thus, in this study, methanol-based HPLC method will be developed and validated.

The compatibility of various containers with cyclosporine has been studied. The study on stability of oral cyclosporine liquid (Sandimmune® Oral, Sandoz) stored in syringes showed that cyclosporine remained stable for 28 days at 25°C. there is no stability data of intravenous cyclosporine in syringe. Stability of intravenous cyclosporine (Sandimmune® I.V., Sandoz) in PVC tubes and containers has been extensively studied. A significant amount of drug can be lost due to sorption on PVC surface. The extent of drug loss depended on the concentration, the vehicle and the container {Ptachcinski, 1986 #1127;Shea, 1989 #1173}. In addition, phthalate is leached from PVC containers due to the existence of polyoxyethyleted castor oil in formulation.

1.6 Hypothesis of the thesis

A stability-indicating HPLC methods have been reported using acetonitrile or tetrahydrofuran (THF) mobile phases. THF based mobile phase (THF 44%) is incompatible with PEEK tubing in some HPLC systems. Also, acetonitrile and tetrahydrofuran are more costly and environmental unfavourable. Therefore, there is a need to develop a methanol-water HPLC stability indicating method.

Cyclosporine has a narrow therapeutic window index {Shaw, 1999 #1119;Armstrong, 2001 #1118}, the administration requires consistent concentrations during infusion, thus stability study and compatibility in infusion containers are necessary. Glass containers have been superseded by plastic bags. PVC bags are restricted due to DEHP leaching. Recently, non-PVC containers are alternative for intravenous cyclosporine preparations. Plastic

syringes are commonly used for paediatric patients. Plastic syringes usually consist of rubber plunger. Up to now, no stability data on these non-PVC containers are available in literature, the study on compatibility of these containers with intravenous cyclosporine is important to ensure safety administration.

1.7 Objectives of the thesis

This study is to develop a cost-effective and environmental favourable HPLC method for stability study and routine assays of intravenous cyclosporine. The specific aims of this project are:

- Evaluate the stability of intravenous cyclosporine preparations stored in polypropylene-polyolefin (PP-PO) bags (Baxter Corp., Mississauga, Ontario, Canada).
- Evaluate the stability of intravenous cyclosporine preparations stored in polypropylene (PP) syringes (60 mL, Luer-Lok, Becton-Dickinson, Mississauga, Ontario, Canada).
- Evaluate the suitability of polypropylene (PP) syringes (1 mL, 5 mL Luer-Lok, Becton-Dickinson, Mississauga, Ontario, Canada) for sampling and transferring intravenous cyclosporine.
- Assess the stability of intravenous cyclosporine preparations stored in ethylene vinyl acetate (EVA) bags (250 mL, Baxa).

1.8 Reference

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Chapter two

2 Development and validation of a stability-indicating HPLC method for intravenous cyclosporine assay

2.1 Introduction

Several analytical and bioanalytical method for cyclosporine assays have been developed (cf 1.5 in Chapter one). Acetonitrile-based HPLC methods are generally recommended in USP. {USP34-NF29, 2011c #1005}. A tetrahydrofuran-based HPLC method can simultaneously quantify cyclosporine and possible impurities in oral formulations {Bonifacio, 2009 #1011}. In this section, a methanol-based HPLC is developed for routine assay in hospital and stability study of intravenous cyclosporine.

Based on the properties of cyclosporine and the nature of intravenous formulation, stress degradation of cyclosporine and injection products will be performed to study the stability-indicating properties of the HPLC method. Furthermore, suitability of the chromatographic system including repeatability and column efficiency will be evaluated according to ICH and USP guidelines. Performance parameters such as specificity, accuracy, precision, linearity, detection limit and robustness will also be validated.

2.2 Materials and method

2.2.1 Materials

All reagents used in the experiments were analytical grade. The deionised water (18.2 MΩ) used for HPLC and sample preparation was generated with a Millipore Milli-Q system (Bedford, MA). Methanol (HPLC grade) was purchased from JT Baker (Phillipsburg, NJ). Phosphoric acid (A.C.S., 85%) and hydrogen chloride (A.C.S., 37%) were supplied by Fisher Scientific Canada. Triethylamine (A.C.S.) was obtained from Laboratoire MAT Inc. Nylon membrane filter (Nylaflo^{TM} 0.45 μm) for mobile phase filtration and syringe filter (Supor ® (PES 0.2 μm) was obtained from Pall, Life Sciences. Cyclosporine (98%, lot H890) was purchased from AK Scientific Inc. and used as reference

standard. Sandimmune® I.V. (50 mg/mL, lot S0034, exp FEB2012 and lot S0040, exp MAR2013) were obtained from Novartis, Canada. Dextrose injection 5% (D5W, Lot W9H18B0) and sodium chloride injection 0.9% (NS, Lot W9I14M0) were supplied by Baxter Corporation, Canada.

2.2.2 HPLC system

A Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan) consisting of a vacuum degasser (DGU-20), an autosampler (SIL-20AC), a quaternary-flow solvent-delivery system (LC-20AD), a column oven (CTO-20AC), a diode-array detector (SPD-M20A) and the LC Solution software (version 1.21) was used. Separation was performed on a column C18 (Zorbax, SB-C18, 4.6×250 mm) coupled with security guard cartridge (Phenomenex, C18, ODS, 3.0×4 mm). The isocratic mobile phase consisted of a mixture of HPLC-grade methanol and water (86:14, V/V) at a flow rate of 1mL/min. Column temperature was controlled at 50°C. Quantification was performed by integration of the area at a detection wavelength of 203 nm.

2.2.3 Sample preparation and HPLC assay

2.2.3.1 Preparation of standards

The stock standard solution was prepared by dissolving cyclosporine powder (about 40 mg) with methanol in a 10 mL volumetric flask. Seven calibration standard solutions were prepared by diluting with methanol to obtain concentrations in the range of 0.05 to 0.40 mg/mL. Standards were assayed by HPLC with injection volumes of 20 µL. Another calibration curve was similarly prepared using seven solutions of concentrations varying between 0.50 and 4.0 mg/mL, and assayed on HPLC with injection volumes of 2 µL.

2.2.3.2 Preparation of samples for precision evaluation

Intravenous cyclosporine injections (0.2 and 2.5 mg/mL) were aseptically prepared by diluting Sandimmune® I.V. 50 mg/mL with 5% dextrose or 0.9% sodium chloride. The samples were filled in glass vials and stored in refrigerator (2-8 ^{o}C) as controls. The samples of 0.2 mg/mL and 2.5 mg/mL were injected with volumes of 20 µL and 2 µL,

respectively. Each sample was injected in duplicate. Intraday precision was evaluated by calculating relative deviation of 5 injections. Interday precision was evaluated based on the results at 5 time points during 1 week.

2.2.3.3 Preparation of samples for accuracy evaluation

Accuracy was evaluated by spiking standard cyclosporine with Sandimmune® I.V. Intravenous cyclosporine injection was prepared by diluting Sandimmune® I.V. with methanol to the concentration 0.207 mg/mL. A series of spiked solutions were prepared in triplicate by mixing them with standard solution (0.181 mg/mL) at the ratio (V/V) of 75:25, 50:50, 25:75. Each sample was assayed in triplicate with injection volume 20 µL.

2.2.3.4 Generation of degradation products

A degradation sample was prepared by adding about 13 mg (98%) of cyclosporine powder to hydrochloric acid (12.5 mL, 1 N). After refluxing at 80°C for 4 h, the solution was neutralized with sodium hydroxide (1 N) to pH 6.8, diluted with methanol to about 0.25 mg/mL and filtered with 0.2 µm membrane. The final sample was assayed by HPLC with injection volume of 20 µL {Kumar, 2001 #1017}. Similarly, stress degradation testing was conducted on Sandimmune® I.V. (Novartis) to rule out interference with the excipients. The test sample was prepared by adding Sandimmune® I.V. solution (0.25 mL) to hydrochloric acid (12.5 mL, 1 N) followed by the same procedure as described above. In order to investigate the influence of temperature on degradation and to obtain stability related degradation products, 13.00 mg of standard cyclosporine was mixed with hydrochloric acid (2 mL, 1 N) and kept at room temperature for 5 days without protection from light and similarly assayed.

2.3 Results and discussion

2.3.1 Method development

The current RP-HPLC method used a C18 column as stationary phase. The mobile phase composition was optimized to achieve an adequate resolution of cyclosporine from the impurity peak. The ratio of methanol and water is determined as 86:14 (V/V). It has

been reported that on acetonitrie-water based HPLC, chromatogram of cyclosporine was significantly influenced by column temperature {Nishikawa, 1994 #16016}. The peak became broadening and tailing as temperature decreased from 60 ^{o}C to 20 ^{o}C. Such behaviour was accounted for as the inter-conversion between multiple conformations. Higher temperature facilitated one conformation and a faster equilibrium, thus good performance was achieved with higher separation efficiency. Considering the boiling point of methanol, the column temperature was controlled at 50 ^{o}C. Cyclosporine has UV absorption at 190-210 nm **{Hossan, 1987 #1100}**, based on the absorption profile detected by PDA, the integration area was performed at 203 nm.

2.3.2 Validation of the HPLC method

2.3.2.1 System suitability

System suitability was evaluated by injecting standard solutions. The solution of Sandimmune in D5W was injected to estimate the resolution from the excipient in intravenous dosage. The suitability parameters are shown in Table 2.1. The USP method requires that the number of theoretical plates should not be less than 700 (USP34-NF29 2011). The lower column efficiency can be attributed to broadening cyclosporine peak, which turns out to be a challenge in RP-HPLC assay (Nishikawa, Hasumi et al. 1994). Even though the ICH recommends using columns with theoretical plates N > 2000 in HPLC analysis, in some cases, this criterion is validated if the precision and accuracy can meet the requirement (Épshtein 2004).

An important suitability parameter was resolution. By comparing chromatogram of intravenous cyclosporine to standard, new peaks were observed resulting from Cremophor EL. However, they did not interfere with cyclosporine (Figure 2.1). The resolution was 3.92 from cyclosporine. Tailing factor indicates peak asymmetry. It is critical to the accuracy of area integration, since the determination of end time becomes difficult with increasing tailing factor. Both ICH and USP specifications require that the tailing factor be less than 2. In the current method, the tailing factor was 1.052 and ensured accurate results in stability study {Kumar, 2001 #1017;Shabir, 2003 #1003}.

Table 2.1 HPLC System suitability parameters

	RSD of retention time %	RSD of peak area	Number of theoretical plates	Tailing factor	Resolution
Current HPLC method	0.99%	0.37%	1309 ± 10	1.052 ± 0.004	3.92
ICH specification	< 1 %	< 2 %	> 2000	< 2	> 1
USP specification	< 2 %	< 2 %	> 700[a]	< 2	> 1

[a] Theoretical plate number required for cyclosporine assay

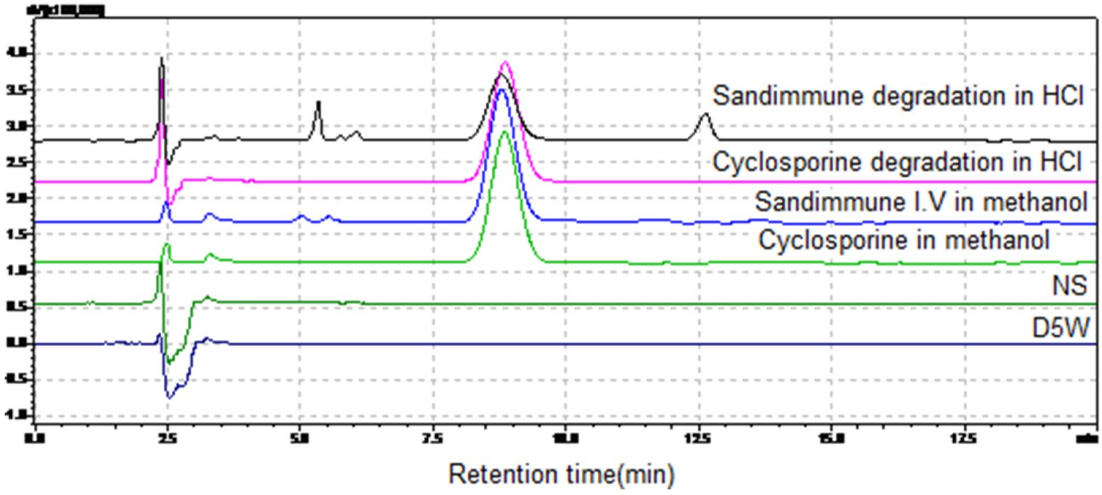

Figure 2.1 Overlay of chromatograms of D5W injection, NS injection, cyclosporine, Sandimmune I.V, Cyclosporine after degradation (1 N HCl at 80°C for 4 h) and Sandimmune I.V. after degradation (1 N HCl at 80°C for 4 h).

2.3.2.2 Specificity

In order to validate the specificity of the method, cyclosporine should be resolved from possible degradants or other potential impurities. Based on the results of stress degradation tests under acid, alkali, hydrogen peroxide, heat or light exposure {Kumar, 2001 #1017}, the main degradation products, such as isocyclosporine, may be produced after acidic treatment. Thus, in this study, the stress degradation was only performed by subjecting cyclosporine or Sandimmune® I.V. to HCl at 80°C for 4 h. The percent of degradation of standard cyclosporine was about 8.9% based on HPLC assays, indicating it was sufficient to provide related degradants. The overlay of chromatograms is shown in Figure 2.1. No interference occurred with cyclosporine after acidic degradation. The cyclosporine peak (retention time 8.9 min) purity was determined by PDA detector in the range of 190 nm to 600 nm, the purity index value is higher than 0.999, showing no significant impurity coeluted with cyclosporine. By comparing the chromatograms profiles of cyclosporine before and after degradation, no significant change was observed. The possible degradation product isocyclosporine is ionisable, thus it might elute much faster than cyclosporine. Considering the oligopeptide structure of cyclosporine, and the storage condition at room temperature, degradation was also conducted at room temperature for 5 days, cyclosporine peak purity was 0.999, showing that there was still no peak interference with cyclosporine.

Due to PDA limitations for detecting coeluting substances with very low concentrations or very similar spectra {Papadoyannis, 2004 #1166}, mobile phases with different pH values were used to verify if any degradants coeluted with cyclosporine. Alteration of pH in mobile phase is possible to discriminate the substances of similar structures. Three types of mobile phases: a mixture of methanol and water (86:14, V/V), a mixture of methanol, water and phosphoric acid (86:14:0.1, V/V, pH 3.1) and a mixture of methanol, water and triethylamine (86:14:0.1, V/V, pH 9.25) were delivered in another HPLC system (Shimadzu HPLC system: consisting of LC-20AT pump, DGU-20 degasser, SIL-20A HT autosampler, SPD-20A UV detector, CTO-20AC column oven and Shimadzu Lcsolution software). The retention time of cyclosporine was 12.5min, 12.8min, 20.3min,

respectively when using different mobile phases (Figure 2.2, 2.3, 2.4). No interfering peak occurred with cyclosporine. In conclusion, both PDA purity determination and HPLC chromatograms achieved by using different mobile phases confirmed no compound coeluted with cyclosporine.

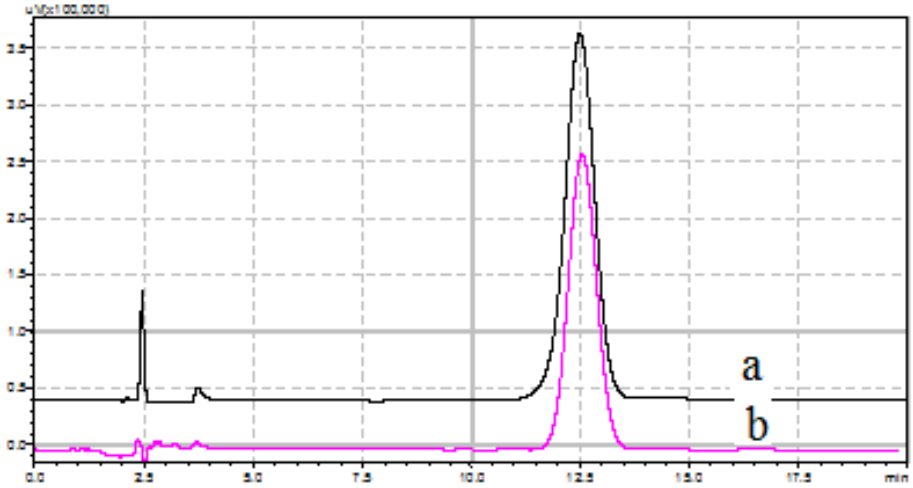

Figure 2.2 Chromatograms of standard cyclosporine (a) and degraded sample (b)

Mobile phase: methanol:water 86:14 (V/V).

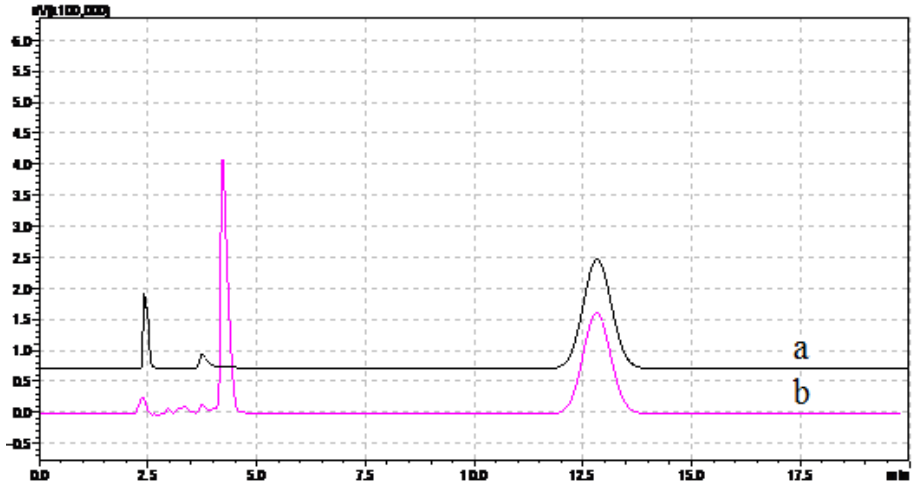

Figure 2.3 Chromatograms of standard cyclosporine (a) and degraded sample (b)

Mobile phase: methanol: water: phosphoric acid 86:14:0.1 (V/V, pH 3.1).

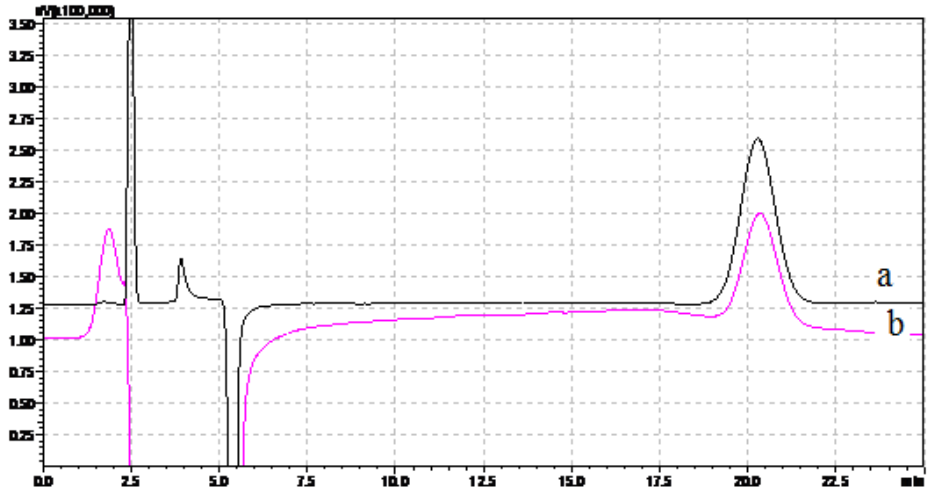

Figure 2.4 Chromatograms of standard cyclosporine (a) and degraded sample (b)

Mobile phase: methanol: water : triethylamine = 60:10:0.07 (V/V, pH 9.25).

Regarding stress degradation of intravenous cyclosporine, some new peaks with retention times of 5.3 min and 12.7 min appeared (Figure 2.1). However, those peaks did not interfere with cyclosporine peak. It was assumed that these new peaks were generated from degradation of Cremophor EL present in the formulation.

Based on the above discussion, the current methanol-based HPLC method was demonstrated stability-indicating.

2.3.2.3 Linearity and range

Calibration standard were prepared in the range of 0.05 mg/mL to 0.40 mg/mL. Peak area of each standard (injection volume of 20 µL) versus concentration was plotted. Good linearity was obtained with regression coefficient 0.99996. As the target concentrations was 0.2 mg/mL and 2.5 mg/mL in the current stability study, to eliminate the measurement error caused by diluting, another calibration curve was prepared in the range of 0.50-4.0 mg/mL with injection volume of 2 µL. As shown in Table 2.2, the linear regression coefficient was 0.99999 (Figure 2.5).

Table 2.2 Linearity regression results

Calibration range CyA mg/mL	Injection volume (µL)	Slope (Au mL/mg)	Intercept (Au)	R^2
0.05 - 0.40	20	$(5.09\pm0.014)\times10^7$	$(1.02\pm0.25)\times10^5$	0.99996
0.50 - 4.0	2	$(5.02\pm0.008)\times10^5$	$(1.69\pm0.91)\times10^4$	0.99999

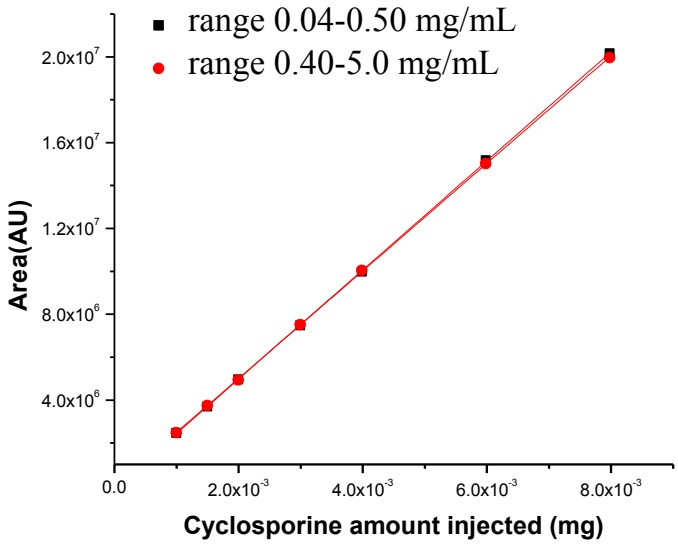

Figure 2.5 Standard curve of cyclosporine: peak area versus amount of cyclosporine injected.

The area was plotted against the amount of cyclosporine injected. As shown in Figure 2.5, the linearity of the two standard curves was similar to each other, illustrating quantification of concentrated sample 2.5 mg/mL can be performed by simply reducing injection volume.

The linear range of calibration curves covered 80% to 120% of the target concentrations 0.2 mg/mL and 2.5 mg/mL, satisfying the USP and ICH regulations.

2.3.2.4 Detection Limit

Based on the calibration curve, the limit of detection (LOD) and quantification (LOQ) were calculated as {Chan, 2010 #1168}:

$$LOD = 3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where σ is standard deviation of intercept, S is the slope of the calibration curve (Table 2.2). The calculated LOD and LOQ were 1.47×10^{-3} mg/mL and 4.91×10^{-3} mg/mL, respectively. As the current study did not require the quantification of impurities, no extra experiment was performed involving the impurities or related substances.

2.3.2.5 Precision

Table 2.3 Intraday and interday precision [a]

Sample	Cya concentration mg/mL	solvent	Intraday RSD	Interday RSD
Standard	0.247	methanol	0.32%(n=5)	0.33% (n=6)
Sandimmune I.V.	0.2	D5W		0.12% (n=5)
Sandimmune I.V.	0.2	NS		0.36% (n=5)
Sandimmune I.V.	2.5	D5W		0.57% (n=5)
Sandimmune I.V.	2.5	NS		0.12% (n=5)

a All samples were stored in glass vials and refrigerated (2-8 ^{o}C).

Precision in this method was evaluated with respect to intraday and interday repeatability. It was expressed as percent of relative standard deviation (RSD). The intraday repeatability was calculated from five replicate injections of standard cyclosporine solution

0.247 mg/mL. As shown in Table 2.3, the RSD was 0.32%, meeting the USP and ICH specification (< 2%). Interday precision was evaluated on the solutions relevant to stability test, including standard solution, the intravenous cyclosporine preparations at 0.2 mg/mL and 2.5 mg/mL concentration levels. All the RSD values were below 1%, illustrating good reproducibility of this method.

2.3.2.6 Accuracy

The accuracy of the method was assessed by standard addition. In order to verify if the possible uncertainty of product composition affects the quantification, a placebo (matrix) was spiked with known quantities of cyclosporine {Épshtein, 2004 #16019}. The placebo for intravenous cyclosporine injection consisted of all the excipients in Sandimmune I.V. and the diluents D5W or NS injections. In our previous studies on intravenous cyclosporine assay, it was found that the diluents: methanol, NS and D5W did not influence the quantification of Sandimmune I.V. In the specificity study (Figure 2.1), there was good baseline and no peak was observed in chromatograms of NS and D5W. Thus, these diluents have no impact on cyclosporine determination.

Table 2.4 Accuracy results [a]

Spiking Sample	Volume fraction of Sandimmune solution added (0.207 mg/mL in methanol)	Volume fraction of standard added (0.181 mg/mL in methanol)	Recovery % [a]
1	75%	25%	99.80±1.04
2	50%	50%	99.92±0.55
3	25%	75%	100.1±0.28

a. Average of 9 determinations. (Each sample was prepared in triplicate and assayed with three injections).

Furthermore, the low solubility of cyclosporine in D5W or NS did not allow the preparation of cyclosporine solutions in these vehicles. Therefore, the spiking solutions were prepared by dissolving both cyclosporine powder and intravenous cyclosporine

(Sandimmune I.V.) in methanol instead of injection diluents, and then mixed at three concentration levels in the linear range. The recovered standard cyclosporine was calculated to represent the accuracy:

$$Recovery\ \% = \frac{The\ amount\ of\ standard\ calculated}{The\ amount\ of\ standard\ added} \times 100\%$$

Assuming that the concentrations of Sandimmune and standard are C_1 and C_2, respectively, the measured concentration of spiking mixture is C, the volume fractions of Sandimmune and standard are F_1 and F_2, respectively, the recovery rate can be calculated as:

$$Recovery\ \% = \frac{C - C_1\ \times F_1}{C_2 \times F_2} \times 100\%$$

Generally, the acceptance criterion for recovery data is 98-102% for drug preparations {Velagaleti, 2007 #1148}, in the current method, the recovery rate was between 99.80% to 100.1% (Table 2.4), indicating good accuracy can be obtained in this method.

2.3.2.7 Robustness

The parameters for robustness assessment should include flow rate, column temperature, mobile phase composition, buffer pH, column supplier and detection wavelength {Ermer, 2001 #1170}. Considering that the method used a reverse phase column and isocratic mobile phase of methanol and water, robustness was assessed under various conditions, including temperature at three different temperatures (45 ^{o} C, 50 ^{o} C, 55 ^{o} C), flow rate at 0.8 mL/min, 1.0 mL/min, 1.2 mL/min. The effect of mobile phase composition was also investigated. The ratio of methanol to water varied slightly from 84: 16 to 88:12 (V/V). The robustness results are illustrated in **Error! Reference source not found.**. The system suitability was primarily indicated by RSD of retention time (RT) and peak area, as well as the resolution values. At each condition, RSD % is less than 1%; the resolution is more than 2, confirming that the system was robust and could applied to the stability assay

if a minor change in instrument parameters. However, the results indicated that retention time was significantly influenced by mobile phase composition and column temperature. Low temperature caused peak broadening and significantly affected the separation efficiency; the number of theoretical plates decreased from 1242 to 824 when performing at low temperature. It means that the temperature and mobile phase composition should be highlighted to ensure good reproducibility when transferring method.

Table 2.5 Robustness with variation of flow rate, column temperature and mobile phase [a]

Parameter	Variable parameter	RT min	RT RSD%	Peak area RSD%	Tailing factor	Theoritical plates number	Resolution
	Normal[b]	9.21±0.005	0.06	0.15	1.155±0.003	1242±5	3.75±0.16
Flow rate (mL/min)	0.9	10.26±0.003	0.03	0.27	1.158±0.002	1360±7	3.78±0.08
	1.1	8.30±0.002	0.02	0.19	1.157±0.002	1163±2	3.04±0.54
Temperature (^{o}C)	45	9.84±0.005	0.05	0.34	1.130±0.004	824±7	3.58±0.33
	55	8.60±0.003	0.04	0.19	1.194±0.002	1796±2	3.87±0.12
Mobile phase methanol:water (V/V)	84:16	12.13±0.005	0.04	0.08	1.131±0.004	1273±14	3.98±0.71
	88:12	7.33±0.006	0.08	0.53	1.183±0.003	1283±8	3.15±0.13

a. RSD% is calculated based on the results of triplicate injections.
b. Normal conditions are under 50^{o} C, 1.0 mL/min, methanol: water 86:14 (V/V).

2.4 Conclusion

Based on the discussion above, a new HPLC method, using C18 column and methanol-water as mobile phase, was successfully developed. The validation parameters indicated it was stability-indicating and could be applied to the stability study of intravenous cyclosporine.

2.5 Reference

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Chapter three

3 Research paper: stability of cyclosporine solutions stored in polypropylene–polyolefin bags and polypropylene syringes

Mengqing Li[1], Jean-Marc Forest[2], Christian Coursol[3], and Grégoire Leclair[1]

[1]Factulty of Pharmacy, Université de Montréal, Montreal, Quebec, Canada, H3C 3J7

[2]B.Pharm. M.Sc., is Pharmacist, Ste-Justine Hospital, Montreal,Quebec, Canada, H3T 1C5

[3]B.Pharm., M.Sc., is Pharmacist, Royal Victoria Hospital,

McGill University Health Center, Montreal, Quebec, Canada, H3A 1A1

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3.1 Abstract

Purpose: the stability of cyclosporine diluted to 0.2 or 2.5 mg/mL with 0.9% sodium chloride injection or 5% dextrose injection and stored in polypropylene-polyolefin containers or polypropylene syringes was evaluated. Methods: intravenous cyclosporine solutions (0.2 and 2.5 mg/mL) were aseptically prepared and transferred to 250-mL polypropylene-polyolefin bags or 60-mL polypropylene syringes. Chemical stability was measured using a stability-indicating high-performance liquid chromatography (HPLC) assay. Physical stability was assessed by visual inspection and a dynamic light scattering (DLS) method. Results: after 14 days, HPLC assay showed that the samples of I.V. cyclosporine stored in polypropylene–polyolefin bags remained chemically stable (> 98%

of initial amount remaining); the physical stability of the samples was confirmed by DLS and visual inspection. The samples stored in polypropylene syringes were found to contain an impurity (attributed to leaching of a syringe component by the solution) that could be detected by HPLC after 1 day; on further investigation, no leaching was detected when the syringes were exposed to undiluted I.V. cyclosporine 50 mg/mL for 10 minutes. Conclusion: samples of I.V. cyclosporine solutions of 0.2 and 2.5 mg/mL diluted in 0.9% sodium chloride injection or 5% dextrose injection and stored at 25 °C in polypropylene-polyolefin bags were physically and chemically stable for at least 14 days. When stored in polypropylene syringes, the samples were contaminated by an impurity within 1 day; however, the short-term (i.e., ≤10 minutes) use of the syringes for the preparation and transfer of I.V. cyclosporine solution is considered safe.

Index terms: Chromatography, liquid; Concentration; Containers; Contamination; Cyclosporine; Dextrose; Diluents; Immunosuppressive agents; Injections; Polyolefin; Polypropylene; Sodium chloride; Stability; Storage; Syringes

3.2 Introduction

Cyclosporine is a potent immunosuppressive agent with a narrow therapeutic range. It strongly suppresses cell-mediated immunity and is used to prolong the survival of allogenic transplants and for the treatment of various autoimmune diseases {Novartis, 2011 #1113}.

Parenteral preparations contain cyclosporine (50 mg/mL), alcohol (278 mg/mL), and polyoxyethylated castor oil (650 mg/mL). Once diluted, I.V. preparations produce a dispersed system of cyclosporine in polyoxylated castor oil micelles. According to the manufacturer's product monograph, this preparation must be diluted with 0.9% sodium chloride injection or 5% dextrose injection at a ratio of 1:20 to 1:100 immediately before administration {Novartis, 2011 #1113}. Phthalate stripping by the polyoxyethylated castor oil from plasticized polyvinyl chloride (PVC) containers is a known problem; therefore, these containers cannot be recommended, and glass containers must be used whenever possible. Furthermore, containers and stoppers should be free of silicone oil and fatty substances {Novartis, 2011 #1113}.

Because the 250-mL glass containers of 5% dextrose have been discontinued, other alternatives for preparation of I.V. cyclosporine are required. A literature search found no useful data on the stability of I.V. cyclosporine solutions prepared in non-PVC containers other than glass. This study was conducted to provide data on the stability of I.V. cyclosporine in polypropylene-polyolefin containers. Also, since most hospital pharmacies prepare I.V. medications in a syringe format, the stability of I.V. cyclosporine in polypropylene syringes was tested.

Cyclosporine is a highly lipophilic (log P 2.92) and noncharged cyclic peptide of 11 amino acid residues {Wishart DS, 2008 #1197;Wishart DS, 2006 #1198}. Its solubility in water is as low as 0.04 mg/mL at 25 °C {Muller, 2006 #1179}. Furthermore, cyclosporine is a very stable molecule. Degradation of the cyclosporine molecule has been investigated under several stress conditions {Hassan MM, 1987 #1199;Bonifacio, 2009 #1011;Kumar, 2001 #1017}, and up to five known degradation products have been reported; however, significant degradation has been observed only under acidic conditions {Kumar, 2001 #1017}. Several high-performance liquid chromatography (HPLC) methods have also been studied to validate cyclosporine's stability {Bonifacio, 2009 #1011;Kumar, 2001 #1017;Jacobson, 1993 #1174;Ptachcinski, 1986 #1127;Shea, 1989 #1173}. All these methods are acetonitrile or tetrahydrofuran based. Due to cost and ecologic considerations, there is a need for the development of a methanol-based HPLC method.

The stability of I.V. cyclosporine in PVC containers has been previously studied {Ptachcinski, 1986 #1127;Shea, 1989 #1173}. A significant amount of drug can be lost by adsorption to PVC, with the extent of drug loss depending on the concentration, the vehicle, and the container. It was reported that cyclosporine 2 mg/mL in 5% dextrose injection remained stable for 24 hours in PVC bags, while the stability of cyclosporine in 0.9% sodium chloride injection decreased after only 6 hours {Ptachcinski, 1986 #1127;Shea, 1989 #1173} .

In previous studies, the physical stability of cyclosporine was evaluated by visual inspection {Jacobson, 1993 #1174}. Due to the micellar nature of the cyclosporine I.V. preparation, dynamic light scattering (DLS) is the method of choice for evaluating physical

changes; DLS can measure the nanometer-scale micelles and allow the monitoring of physical stability over time. Combined with visual observation, this method can be used to investigate the physical stability of cyclosporine injection and detect changes that would otherwise not be visible.

The objective of the study described here was to investigate the chemical and physical stability of cyclosporine in 5% dextrose injection and 0.9% sodium chloride injection stored in polypropylene syringes or polypropylene-polyolefin bags.

3.3 Methods

3.3.1 Sample preparation

Cyclosporine for injection (0.2 and 2.5 mg/mL) was aseptically prepared by diluting I.V. cyclosporine [a] with 0.9% sodium chloride [b] or 5% dextrose injection.[c] The diluted preparations (60 mL) were then transferred in 60-mL polypropylene syringes[d]. Additional diluted solutions (100 mL) were prepared in empty polypropylene-polyolefin containers[e]. All samples were prepared in triplicate. Containers were stored at 25 ± 2 °C and sampled (1.4 mL) at time zero and after 3, 7, and 14 days.

3.3.2 HPLC assay method

The optimized method to assay cyclosporine by HPLC was to use an ultrafast liquid chromatography system consisting of a vacuum degasser[f] , an autosampler[g] , a quaternary-flow solvent-delivery system [h], a C18 column[i] , a column oven[j] , a diode-array detector[k], and the LC Solution software (version 1.21, Shimadzu Corporation, Kyoto, Japan). The isocratic mobile phase consisted of a mixture of HPLC grade methanol and water (86:14, by volume) at a flow rate of 1 mL/min. The column was heated to 50 °C. Injection volumes of 20 and 2 µL were used for 0.2- and 2.5-mg/mL samples, respectively. The run time of 20 min is set up for analysis. Quantitation was performed by integration of the area under the curve at a detection wavelength of 203 nm at a retention time of about 8.9 minutes.

Each analysis was confirmed by a second injection, with an acceptance specification of < 1% of difference reported as the average of both analyses. Peak purity was also

confirmed by calculating the similarity index between 190 and 250 nm, with an acceptance specification of > 99.99%.

Seven calibration standard solutions were prepared by dissolving cyclosporine powder[l] with HPLC-grade methanol to concentrations of 0.05–0.40 mg/mL. The standards were assayed by HPLC with injection volumes of 20 µL, and a seven-point calibration curve was obtained by linear regression (r^2 = 0.9999). Another calibration curve was similarly prepared using seven solutions of concentrations of 0.5–4.0 mg/mL and an injection volume of 2 µL (r^2 =0.9999). The calibration standards were prepared using pure methanol rather than a mixture of water, alcohol, and polyoxylated castor oil; this facilitated the complete dissolution of cyclosporine and also prevented any degradation during the preparation of the calibration samples.

The precision of the method was assessed by evaluating the interday and intraday relative standard deviations (RSDs). The intraday and interday RSD values were 0.32% (n = 5) and 0.33% (n = 6), respectively, for cyclosporine solutions of 0.247 mg/mL in methanol. The interday RSDs for samples of 0.2 mg/mL in 5% dextrose injection and 0.9% sodium chloride injection were 0.12% (n = 5) and 0.36% (n = 5), respectively (injection volumes of 20 µL); the values were 0.57% (n = 5) and 0.12% (n = 5) for 2.5 mg/ in 5% dextrose and 0.9% sodium chloride (injection volumes of 2 µL), respectively. For interday RSD evaluations, the samples were injected in duplicate on five consecutive days (i.e., n = 5 denotes five times the average of two injections).

The method was demonstrated to be stability-indicating by stress degradation of cyclosporine. Based on previously reported stress degradation results,7 acidic degradation was conducted to generate the relevant cyclosporine degradation products. Stress degradation testing was conducted on I.V. cyclosporine to rule out any peak overlap between the cyclosporine and the excipients, their degradation products, or the degradation products of cyclosporine. The test sample was prepared by adding I.V. cyclosporine solution (0.1 mL) to hydrochloric acid (5 mL, 1 N) which was then heated at 80 °C for four hours, cooled at room temperature, neutralized with sodium hydroxide (5 mL, 1 N), diluted with methanol to about 0.25 mg/mL, and filtered (0.2 mm); the sample was then assayed by

HPLC. The resulting chromatogram was compared with the chromatograms obtained from a solution of pure cyclosporine A (0.25 mg/mL in methanol) and reconstituted I.V. solutions of cyclosporine (0.20 mg/mL in 0.9% sodium chloride and 5% dextrose injection). No peak overlap was observed with the main cyclosporine peak (Figure 3.1).

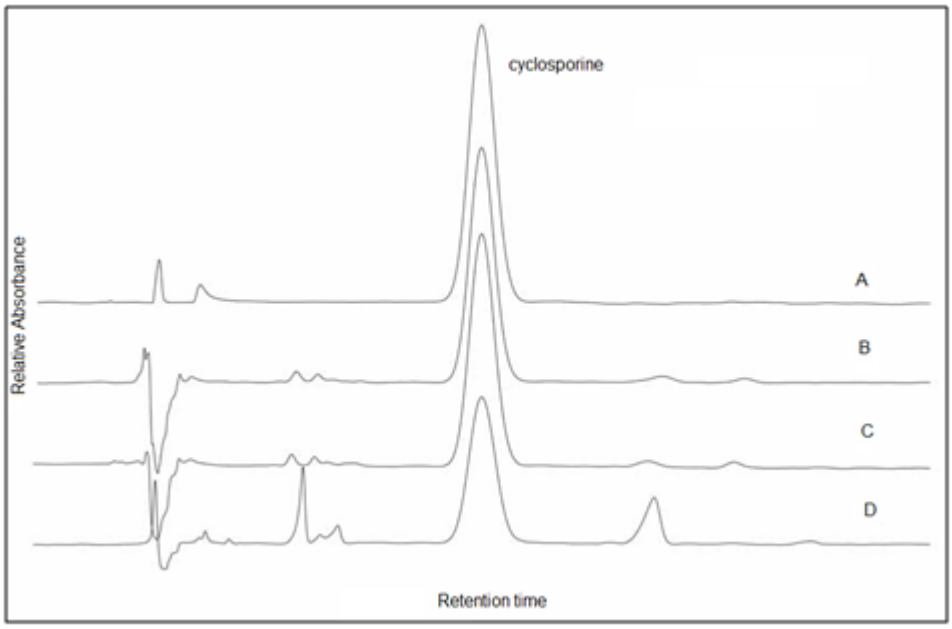

Figure 3.1 Representative chromatograms of (A) pure cyclosporine solution in methanol (0.25 mg/mL), (B) I.V. cyclosporine reconstituted in 0.9% sodium chloride (0.20 mg/mL), (C) I.V. cyclosporine reconstituted in 5% dextrose injection, and (D) I.V. cyclosporine solution submitted to forced degradation in acidic conditions (0.25 mg/mL).

3.3.3 DLS testing

The particle size of test solutions was measured with a DLS system^{m} , The refractive index and viscosity of water were used in the calculation of the particle size (1.330 and 0.8872 mPa·s). The reference sample was polystyrene (refractive index, 1.590). The measurements were performed in disposable cuvettes with a sample volume of 1.4 mL at 25 °C. The attenuator was set to 9, the measurement position was set to 4.65 mm, and the measurement duration was 55 seconds. Due to the instrument's limit of detection, particle

sizing could only be performed on 2.5-mg/mL samples; the 0.2-mg/mL samples were too diluted to be evaluated using this instrument.

3.3.4 Sample analysis

Samples of 1.4 mL were aseptically withdrawn at time zero and after 3, 7, and 14 days. The samples were assayed immediately without filtration or dilution. Concentrated samples (2.5 mg/mL) were evaluated visually, by DLS, and by HPLC (injection volume, 2 mL). The diluted samples (0.2 mg/mL) were evaluated visually and by HPLC (injection volume, 20 mL). Visual inspections were performed with the unaided eye. The test solutions were visually examined in normal fluorescent light for color change and evidence of precipitation. The DLS measurements were performed in triplicate; the HPLC measurements were performed in duplicate, with acceptance specifications of <1% of the difference between both injections and calculated cyclosporine peak purity of >99.99% (based on the similarity index between 190 and 250 nm). The sterility of the cyclosporine solutions was not evaluated in this study.

3.3.5 Data analysis

The stability of cyclosporine was determined by evaluating the percentage of the initial concentration remaining at each time point. Stability was defined as a recovery of >90% of the initial concentration of cyclosporine, with a variation of <10% of the initial micelle average size (as indicated by DLS).

3.4 Results and discussion

As shown in Table 3.1, recovered cyclosporine stored in polypropylene-polyolefin bags was > 98% for all samples after 14 days at 25 °C (5% dextrose injection, 0.9% sodium chloride injection, 0.2 and 2.5 mg/mL). Furthermore, visual inspection did not find any evidence of physical change. The average particle size and the polydispersity index were unchanged during the study for the 2.5-mg/mL samples in both 0.9% sodium chloride and 5% dextrose injection (Table 3.2). The 0.2-mg/mL sample could not be evaluated because the concentration was below the limit of detection of the method; however, because a concentration of 2.5 mg/mL is considered a worst-case scenario in terms of physical

stability, no changes would be expected for the 0.2-mg/mL samples, as confirmed by visual inspection. Based on these results, no significant adsorption or chemical or physical change occurred when I.V. cyclosporine solution was stored in polypropylene–polyolefin containers for a period of 14 days at 25 °C.

Table 3.1 Stability of I.V. Cyclosporine in Polypropylene-Polyolefin Bags[a]

Norminal Cyclosporine Concentration and Diluent	Mean ± S.D Initial Cyclosporine Concentration (mg/mL)	Mean ± S.D Initial Concentration Remaining		
		Day 3	Day 7	Day 14
0.2 mg/mL 5% dextrose	0.207 ± 0.001	98.9 ± 0.6	99.1 ± 0.6	98.7 ± 0.8
0.2 mg/mL 0.9% sodium chloride	0.206 ± 0.001	99.7 ± 0.3	99.8 ± 0.2	99.7 ± 0.3
2.5 mg/mL 5% dextrose	2.49 ± 0.02	99.4 ± 0.7	99.0 ± 0.8	99.3 ± 0.7
2.5 mg/mL 0.9% sodium chloride	2.50 ± 0.01	99.2 ± 0.3	99.2 ± 0.4	99.4 ± 0.5

[a] Calclations are for triplicate samples, each sample was assayed in duplicate (n = 3×2)

Table 3.2 Micelle Size Distribution of I.V. Cyclosporine 2.5 mg/mL Stored in Polypropylene–Polyolefin Bags[a]

Diluent	Mean ± S.D. Average Particle Size (nm) [mean ± S.D. Polydispersity Index]			
	Initial	Day 3	Day 7	Day 14
5% dextrose	13.77 ± 0.28 [0.13 ± 0.05]	13.85 ± 0.12 [0.07 ± 0.04]	14.24 ± 0.26 [0.11 ± 0.06]	14.23 ± 0.23 [0.11 ± 0.07]
0.9% sodium chloride	12.90 ± 0.05 [0.06 ± 0.01]	12.92 ± 0.02 [0.03 ± 0.01]	13.19 ± 0.16 [0.07 ± 0.02]	13.07 ± 0.18 [0.07 ± 0.01]

[a] Calculations are triplicate samples; each sample was assayed in triplicate (n = 3×3)

Cyclosporine recovery of >95% for each sample stored in polypropylene syringes after seven days at 25 °C (5% dextrose injection, 0.9% sodium chloride injection, 0.2 and 2.5 mg/mL) was evaluated by HPLC. No physical change was detected by visual inspection. However, after only one day an impurity appeared on the HPLC chromatogram at a relative retention time of 1.45 (relative to the cyclosporine peak). After seven days, the polypropylene syringe stability study was terminated, as the impurity had increased significantly. This new impurity only appeared in the polypropylene syringe study and was never observed in the polypropylene–polyolefin container study.

Further investigations were performed to determine the origin of the impurity. A placebo solution corresponding to cyclosporine 2.5 mg/mL in 5% dextrose injection was prepared using polyoxyethylated castor oil, alcohol, and 5% dextrose injection, and the black rubber component of the syringe plunger was immerged in the solution. The same impurity was detected after one day of exposure at 25 °C (Figure 5.1 and 5.2). It was concluded that an ingredient used in the manufacture of the black rubber component of the syringe plunger could be leached by the ingredients of the I.V. cyclosporine preparation. Even though the presence of additives did not appear to have a detrimental influence on the chemical stability of cyclosporine, the leached additive could pose a toxicity hazard. Therefore, this kind of polypropylene syringes with rubber component cannot be recommended for the storage of I.V. cyclosporine solutions.

As polypropylene syringes are commonly used to transfer I.V. cyclosporine during the preparation of I.V. cyclosporine solutions, an additional test was performed to evaluate

whether any additive leached during short periods of contact with polypropylene. Solutions of undiluted I.V. cyclosporine (50 mg/mL) of 0.4 and 1 mL were stored for 10 minutes in 1- and 5-mL polypropylene syringes. Each sample was prepared in triplicate, diluted with 5% dextrose injection to a total volume of 20 mL, and assayed by HPLC (injection volume, 100 µL). The leaching additive could not be detected; therefore, polypropylene syringes appeared to be adequate for sampling and transferring I.V. cyclosporine solutions when the period of contact was less than 10 minutes.

3.5 Conclusion

Samples of I.V. cyclosporine solutions of 0.2 and 2.5 mg/mL diluted in 0.9% sodium chloride injection or 5% dextrose injection and stored at 25 °C in polypropylene–polyolefin bags were physically and chemically stable for at least 14 days. When stored in polypropylene syringes, the I.V. cyclosporine solutions were contaminated by an impurity within 1 day; however, the short-term (*i.e.,* ≤10 minutes) use of the syringes for the preparation and transfer of I.V. cyclosporine solution is considered safe.

3.6 Materials and Instruments

[a] Cyclosporine 50 mg/mL solution, Novartis, East Hanover, NJ, lot S0034 and lot S0040.

[b] Baxter Corp., Mississauga, Ontario, Canada, lot W9F22C2.

[c] Baxter Corp., Mississauga, Ontario, Canada, lot W9C26C3.

[d] Luer-Lok, Becton-Dickinson, Mississauga, Ontario, Canada.

[e] Aviva 250 mL capacity bags, Mississauga, Ontario, Canada.

[f] DGU-20, Shimadzu Corporation, Kyoto, Japan.

[g] SIL-20AC, Shimadzu Corporation.

[h] LC-20AD, Shimadzu Corporation.

[I] Zorbax, SB-C18, 4.6 × 250 mm, Agilent technologie, Mississauga, Ontario,Canada.

[j] CTO-20AC, Shimadzu Corporation.

[k] SPD-M20A, Shimadzu Corporation.

[l] Cyclosporine 98%, AK Scientific Inc., Union City, CA, lot H890.

[m] Zetasizer NS, Malvern Instruments Ltd.,Malvern, Worcestershire, U.K.

3.7 Acknowledgements

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Chapter four

4 Research paper: adsorption of intravenous cyclosporine injections on the surface of ethylene-vinyl acetate containers

Mengqing Li[1], Jean-Marc Forest[2], Christian Coursol[3], and Grégoire Leclair[1]

[1]Factulty of Pharmacy, Université de Montréal, Montreal, Quebec, Canada, H3C 3J7

[2]B.Pharm. M.Sc., is Pharmacist, Ste-Justine Hospital, Montreal,Quebec, Canada, H3T 1C5

[3]B.Pharm., M.Sc., is Pharmacist, Royal Victoria Hospital,

McGill University Health Center, Montreal, Quebec, Canada, H3A 1A1

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4.1 Abstract

The stability of cyclosporine solutions in 0.9% sodium chloride injection (NS) or 5% dextrose injection (D5W) stored in ethylene-vinyl acetate (EVA) containers was evaluated and the adsorption of cyclosporine to EVA surface was studied. The cyclosporine solutions (0.2 and 2.5 mg/mL) were aseptically prepared by injecting Sandimmune® IV in prefilled EVA containers and stored at 25°C. Chemical stability was measured using a stability-indicating high-performance liquid chromatography (HPLC) method. Physical stability was assessed by visual inspection and a dynamic light scattering (DLS) method. Based on the chemical and physical stability data, EVA containers can be used to store cyclosporine injections of 0.2 mg/mL in D5W and NS injections for 7 days. Concentrated 2.5 mg/mL cyclosporine solutions in D5W and NS injections can be stored in EVA bags at least 14 days. However, adsorption of cyclosporine on EVA containers was observed. The adsorption was studied on the test solutions which were first prepared in a glass beaker and then transferred into EVA containers. The adsorption kinetics was evaluated using three classic models (external diffusion model, Weber-Morris model, modified Weber-Morris

model) and two novel numerically resolved adsorption models based on Fick's first law. The results showed that the internal diffusion may be the rate-limiting step. The modified Weber-Morris model could successfully describe the adsorption process (0.2 mg/mL cyclosporine in NS and D5W) with good correlation coefficients (> 0.97) and low variation (predicted vs observed) of < 9%. However, the rapid adsorption events on surface sites were not taken into account. In this study, two novel diffusion models were established regarding rapid adsorption, contact surface area and volume of solution. The models were numerically resolved and very good fitting results were achieved with variation < 8%. The model based on internal diffusion could be applied to predict long-term drug stability with variation < 2%.

4.2 Introduction

Cyclosporine is a neutral and hydrophobic cyclic undecapeptide. It is a potent immunosuppressive agent used to reduce the risk of rejection following allogenic organ transplantations. Intravenous cyclosporine for injection contains cyclosporine (50 mg/mL), polyoxyethylated castor oil (650 mg/mL) and ethanol (qs). According to the product monograph, this latter preparation requires dilution to concentrations between 0.5 mg/mL to 2.5 mg/mL with 0.9% sodium chloride injection (NS) or 5% dextrose injection (D5W) prior to administration. Dilutions as low as 0.2 mg/mL are routinely used in pediatric hospitals. Diluted preparations should be slowly administered by intravenous infusion over 2 to 6 hours {Novartis, 2011 #380}.

Due to its physicochemical properties, cyclosporine is prone to adsorption on the surface of plastic containers or tubings, resulting in inadequate dosing {McLeod, 1992 #382}. Polyvinyl chloride (PVC) containers and tubings are not acceptable for intravenous cyclosporine as cyclosporine is significantly adsorbed on PVC surfaces. The extent of drug loss depends on concentration, diluent and container composition. For instance, cyclosporine adsorption on the surface of PVC containers is more important when it is diluted in NS and is less important when it is diluted in D5W {Shea, 1989 #388;Ptachcinski, 1986 #383}. In addition, the leaching of plasticizer di-2-ethyl hexyl phtalate (DEHP) from PVC containers to parenteral solutions could constitute a health hazard {Allwood, 1996

#7;FDA, 2011 #392}. The polyoxyethylated castor oil contained in intravenous cyclosporine injections can extract phthalates from PVC containers and may constitute another limitation for the use of PVC container with cyclosporine injections (Novartis 2011). Glass containers are not a convenient alternative. They are breakable, are not flexible like plastic containers and venting is required to avoid pressure change during filling or emptying. Thus, non-PVC plastic containers are the only practical remaining option for the preparation of intravenous cyclosporine. Very little information is available regarding the stability of cyclosporine in such containers. Our group previously reported the stability and compatibility of intravenous cyclosporine injections in polypropylene-polyolefin containers (PP-PO, Aviva®) and PP syringes (Luer-Lok®) {Li, 2011 #394}. Cyclosporine injections (0.2 mg/mL and 2.5 mg/mL) in D5W and NS were physically and chemically stable for a period of at least 14 days in PP-PO containers. However, storage in PP syringes with rubber plunger could not be recommended. Some additives were found to leach from the rubber within 24 h because of the presence of polyoxyethylated castor oil.

The absence of plasticizers in ethylene-vinyl acetate (EVA) containers is beneficial for the storage of parenteral preparations {Driscoll, 2006 #173;Trissel, 2003 #219}. Even if EVA containers are readily available, only limited stability and compatibility data are currently reported for these containers. One of these studies reports a significant insulin loss during infusion because of drug adsorption {Doglietto, 1989 #395} {Doglietto, 1989 #395}. Insulin and cyclosporine present some structural similarities as the former is a small peptide and the latter is a small cyclic peptide. Therefore, adsorption of cyclosporine to EVA surfaces is a concern and its significance will depend on cyclosporine properties, EVA properties, diluent, contact surface area, concentration and storage interval.

The objective of this study is to investigate the compatibility of cyclosporine injections in D5W and NS with EVA containers.

4.3 Methods

4.3.1 Real conditions stability study

The samples used for the real conditions stability study were aseptically prepared at Ste-Justine Pediatric Hospital (Montreal, Canada) using their standard procedures. The cyclosporine test solutions (0.2 and 2.5 mg/mL) in 100 mL D5W[a] and NS[b] were prepared in triplicate by injecting Sandimmune® IV[c] in prefilled EVA containers[d]. The containers were then transferred to Université de Montréal to be stored at 25°C (23-27°C) in an incubator. Aliquots (1 mL) were aseptically withdrawn 12 h after the preparation (initial time point) and after 3, 7 and 14 days. These samples were evaluated by HPLC, DLS and visual inspection.

4.3.2 Adsorption study

Cyclosporine test solutions (0.2 and 2.5 mg/mL, 350 mL) were prepared in glass beakers by diluting Sandimmune® IV[c] with D5W[a] and NS[b]. Each preparation was then transferred in 3 empty EVA containers[d] using a 50 mL-glass syringe (100 mL per container). The containers were stored at 25°C (23-27°C) in an incubator. Aliquots (1 mL) were aseptically withdrawn from the beaker prior to the transfer in the EVA container and also after 5 min, 4 h, 8 h, 12 h, 24 h and 72 h following the transfer. These samples were evaluated by HPLC and visual inspection.

4.3.3 HPLC assay

Content assay was performed using a stability-indicating HPLC method which was previously reported by our group (Li, Forest et al. 2011). Triplicated injection volumes of 20 µL and 2 µL were utilized for cyclosporine solution samples of 0.2 and 2.5 mg/mL, respectively.

4.3.4 Dynamic Light Scattering

Due to the surfactant nature of polyoxyethylated castor oil, the cyclosporine solutions are micellar. The micelle size distribution of test solutions was measured in water by dynamic light scattering (Zetasizer NS, Malvern Instruments Ltd.). Triplicated

measurements were performed in low-volume disposable cuvettes (0.5 mL) at 25°C. An attenuator value of 8 or 9, a measurement position of 4.65 mm and duration of 60 s were set for each measurement. Due to the sensitivity of the instrument, particle sizing could only be performed on 2.5 mg/mL samples. The samples of 0.2 mg/mL were too diluted to be evaluated by this instrument.

4.3.5 Data analysis of HPLC

The stability of cyclosporine was determined by evaluating the percentage of the initial concentration remaining at each time point. Stability was defined as a recovery of > 90% of the initial concentration of cyclosporine, with a variation of <10% of the initial micelle average size (as indicated by DLS).

4.3.6 Visual inspection

Visual inspections were performed with the unaided eye to check if color change and precipitation occurred.

4.3.7 Data analysis of DLS

Stability was defined as the absence of any visual evidences of instability, a recovery of more than 90% of the initial concentration measured by HPLC and a variation of less than 10% in the initial size measured by DLS. Furthermore, DLS polydispersity index larger than 0.5 was considered unacceptably large {Patravale, 2004 #1161}. Averaged results are reported with their standard deviation (mean ± sd). HPLC and DLS measurements were repeated 3 times for each analyzed sample. Statistical significance of differences was evaluated using the Student's T-test and a $p < 0.05$ was considered significant.

For the adsorption study, the adsorbed mass of cyclosporine (m_i) on EVA containers was calculated for each time interval (i) using the measured concentration (c_i), the residual volume after sampling (v_i), the measured initial concentration (c_0), the initial volume (v_0=100 mL) and the sampled volume (v_s=1 mL). Based on the principle of mass conservation (Equation 1), the sum of the adsorbed mass (m_i), the mass in solution within the container ($c_i \times v_i$) and the cumulative mass in the sampled solutions (*summation term*)

will always remain equal to the initial mass ($c_0 \times v_0$). As illustrated by Equations 2 and 3, the adsorbed mass (m_i) can easily be calculated, and normalized (q_i) per unit of container surface area (s=410 cm^2).

(1) $$c_0 \times v_0 = m_i + c_i \times v_i + \sum_{j=1}^{i} c_j \times v_s$$

(2) $$m_i = c_0 \times v_0 - c_i \times v_i - \sum_{j=1}^{i} c_j \times v_s$$

(3) $$q_i = m_i / s$$

4.4 Results and discussion

4.4.1 Real conditions stability study

Usually, the injection preparations should be monitored in the range of 90-110% of intended concentrations. As reported in Table 4.1, for the 2.5 mg/mL solutions, the concentrations at the initial time point were 2.47 mg/mL and 2.45 mg/mL in D5W and NS. These concentrations remained stable for at least 2 weeks. Furthermore, the micelle size distribution was not affected for the duration of the study (Table 4.2). No visual evidences of instability were observed at any time point. Therefore, cyclosporine injection was stable at least 2 weeks when diluted in D5W or NS and stored at 25°C in EVA containers.

The 0.2 mg/mL solutions demonstrated evidences of incompatibility (Table 4.1). Initial concentrations were unusually low due to very small amount of sampling. Nevertheless, the concentrations fell in the acceptable range of 90-110% of the targeted concentration (0.2 mg/mL) : 0.185 mg/mL in D5W and 0.186 mg/mL in NS, respectively.The concentrations decreased with time and fell below 90% of initial after 2 weeks. Even if concentration decreased more than 10%, no new impurities were detected by HPLC, suggesting adsorption as the root cause of this incompatibility. No visual evidence of degradation was observed at any time point. Based on these results, EVA containers could be considered acceptable to store 0.2 mg/mL cyclosporine injections in NS and D5W for a period of 1 week at 25°C to maintain a concentration of cyclosporine above 90% of the initial concentration. However, clinically significant adsorption of cyclosporine with the EVA container was observed at this concentration after 2 weeks. As this

phenomenon was not observed with polypropylene-polyolefin (PP-PO) containers (Li, Forest et al. 2011), these latter containers are highly preferable to store cyclosporine injections.

Cyclosporine adsorption on EVA containers was slightly reduced in NS compared to D5W. Interestingly, this result differed from previously reported adsorption results with PVC containers, in which the adsorption of cyclosporine in NS solutions was more significant than in D5W solutions (Ptachcinski, Logue et al. 1986; Shea, Ptachcinski et al. 1989). It can be assumed that the adsorption mechanism is closely related to vehicles. D5W and NS differ in ionic strength. It has been reported that a greater ionic strength in solution may decrease adsorption when electrostatic interactions are involved between the solute and the polymer {Palmgren, 2006 #481}. Considering the chemical structure of EVA and cyclosporine, the intermolecular interaction maybe predominated by electrostatic dipole-dipole attraction or hydrogen interaction, which arise from polar groups such as -C=O in EVA and -COOH, $-NH_2$ in cyclosporine. The competing ions in NS may reduce cyclosporine adsorption on EVA.

4.4.2 Adsorption study

The stability study was repeated under slightly different conditions to model the kinetics of adsorption for the 0.2 mg/mL solutions and to confirm the absence of clinically relevant adsorption for the 2.5 mg/mL solutions. This adsorption study was therefore designed to better control the variables of the experiments. The solutions were first prepared in a glass beaker and then each solution was transferred to 3 EVA containers. This allowed the accurate preparation of a larger volume of solution and also allowed determination of a real initial concentration prior to any adsorption event.

Results of the adsorption study are listed in Table 4.3. Cyclosporine solutions in D5W and NS decreased to 98.6% and 99.4%, respectively after only 5 min of contact time with EVA container. This suggested rapid adsorption occurred during the very first minutes of contact. This difference was statistically significant in both cases. After 3 days of storage, similar cyclosporine recovery was observed for both the real conditions study and the adsorption study (Tables 4.1 and 4.3).

Again, no significant adsorption was observed for the 2.5 mg/mL solutions after 3 days, confirming the results of the real conditions stability study. Assuming the adsorption saturated after 2 weeks, the saturated adsorption in 0.2 mg/mL D5W injection is estimated as 2.27 mg in 100 mL EVA bag. Generally, the adsorption amount should increase with the equilibrium concentration, thus, the saturated adsorption in 2.5 mg/mL should be much higher than 2.27 mg. Based on it, the concentration should be less than 99% of initial for 2.5 mg/mL injections. Such difference should be captured by HPLC. However, no significant decrease in concentration was observed in this study, indicating that negligible amount of adsorption occurred in high concentration of injections. It has been reported that surfactant polysorbate 20 is effective to reduce protein and peptide adsorption to glass and polypropylene surface because of greater surfactant-surface interactions than drug-surface interactions {Duncan, 1995 #404}. Similarly, the greater concentration of polyoxyethylated castor oil in the 2.5 mg/mL injections compared to 0.2 mg/mL injectins, maybe associated with a reduced adsorption of cyclosporine on EVA surfaces.

As pictured in Figure 4.1, the adsorption process involves three steps resulting in the actual adsorption or uptake: (1) diffusion from the bulk to the surface of the interfacial liquid film (bulk diffusion); (2) diffusion through the interfacial liquid film (external diffusion); and (3) diffusion within the polymeric membrane (internal diffusion). This model of adsorption is an adaptation for polymeric membranes of the intraparticle diffusion model {Igwe, 2007 #405}. The intraparticle diffusion model was developed to describe the adsorption of small molecules to inorganic or resin beads {Wu, 2009 #439;Plazinski, 2009 #440}. The rate of adsorption is controlled by its slowest step. For physical adsorption, adsorption is very rapid and the rate is controlled by one of the diffusion steps {Alzaydien, 2009 #469;Sekine, 2002 #420}. Considering timescale, viscosity and Brownian motion, diffusion in the bulk did not limit the rate of adsorption for the current study. Therefore, the rate of adsorption was controlled by external or internal diffusion.

The adsorption kinetics of cyclosporine 0.2 mg/mL in D5W and NS in EVA containers was evaluated using three classical adsorption models and two numerically resolved adsorption models based on Fick's first law. The five models are summarized in

Table 4.4 along with calculated parameters. The goodness of the fit for each model was evaluated using the coefficient of variation of the root mean squared errors (CVRMSE).

4.4.2.1 External diffusion model

When external diffusion controls the rate of adsorption, the adsorbed amount is equal to the transfer of solute across the liquid film according to Fick's first law. The rate of external diffusion is illustrated by Equation 4 where d_e ($cm^2 \cdot h^{-1}$) is the diffusion coefficient in the interfacial liquid film, l_e (cm) is the thickness of the interfacial liquid film, v (cm^3) is the volume of solution, s (cm^2) is the surface of the polymeric membrane and t (h) is the time. c_s and c ($mg \cdot cm^{-3}$) correspond to the concentration at the surface of the membrane and in the bulk of the solution respectively, both vary with time. An external diffusion parameter (k_e, h^{-1}) is defined from d_e, l_e and v to simplify the problem. Assuming very fast uptake, c_s can be assumed to be zero and Equation 4 can be integrated as Equation 5.

(4) $$-\frac{\partial c}{\partial t} = \frac{d_e\, s\, (c - c_s)}{v\, l_e} = k_e\, c, \text{ for } k_e = \frac{d_e\, s}{v\, l_e} \text{ and } c_s = 0$$

(5) $$-ln\, \frac{c}{c_0} = k_e\, t$$

The validity of this model can be verified by linear regression of a plot of $-ln\ (c/c_0)$ as a function of t. The slope of this regression corresponds to the external diffusion constant k_e. This calculation was performed and resulted in an R^2 of 0.3448 for the 0.2 mg/mL solution in D5W and an R^2 of 0.5473 for the 0.2 mg/mL solution in NS. These poor correlation coefficients as well as the poor CVRMSE (Table 4.4) suggested external diffusion was not the rate limiting step.

4.4.2.2 Weber-Morris model for internal diffusion

The Weber-Morris model is a mathematical approximation describing adsorption when internal diffusion is the rate-limiting step. In this model, the amount of adsorption is proportional to $t^{1/2}$ {Ozer, 2006 #472;Qiu, 2009 #484}. The Weber-Morris model is illustrated by Equation 6 where q ($mg \cdot cm^{-2}$) is the amount of adsorption at time t (h) and k_i ($mg \cdot cm^{-2} \cdot h^{-0.5}$) is the internal diffusion constant.

(6) $$q = k_i t^{1/2}$$

When the plot of the amount of adsorption (q) versus the square root of time (t) is linear and passes through the origin, internal diffusion is the only rate-limiting step. The linear fitting results are shown in Table 4.4. R^2 is 0.9669 and 0.9012 for 0.2 mg/mL cyclosporine in NS and in D5W. However, this was not the case for cyclosporine 0.2 mg/mL in EVA containers as significant adsorption was rapidly observed resulting in CVRMSE > 10% (Table 4. 4).

4.4.2.3 Modified Weber-Morris model

Ozer *et al.* (Ozer, Akkaya et al. 2006) investigated the biosorption of dyes on green algae and also found the linear regression did not pass through the origin. They proposed the modified Equation 7. This equation was more accurate when adsorption rate was influenced both by internal and external diffusion processes. The parameter m ($mg \cdot cm^{-2}$) was introduced by Igwe (Igwe and Abia 2007) and reflects the rapid contribution of external diffusion, however, the parameter m is not clearly defined in terms of adsorption mechanism. Rapidly occurring events could include readily accessible adsorption sites at the surface of the polymeric membrane. Larger values of m are representative of significant occurrence of adsorption at the surface sites. (Wu, Tseng et al. 2009).

(7) $$q = k_i t^{1/2} + m$$

With regards to the current study, a linear regression was performed on the plots of the adsorbed amount of cyclosporine for the 0.2 mg/mL solution in D5W and the 0.2 mg/mL solution in NS against the square root of time (Figure 4.2). Good correlation coefficients and CVRMSE (Table 4.4) were achieved in both cases showing internal diffusion was the rate limiting step with occurrence of adsorption on readily available sites.

4.4.2.4 Numerical resolution of the combined external and internal diffusion model

We propose a comprehensive model of adsorption taking into account a rapid contribution of surface sites (q_0), an external diffusion constant (k_e) as well as an internal diffusion constant (k_i). This model was constructed from the differential equations describing the external and the internal diffusion as derived from Fick's first law of diffusion. The rate of external diffusion is illustrated by Equation 8 where d_e ($cm^2 \cdot h^{-1}$) is

related to the diffusion coefficient in the interfacial liquid film and l_e (cm) is the thickness of the interfacial liquid film c_s and c (mg·cm^{-3}) correspond to the concentration at the surface of the membrane and in the bulk of the solution respectively, both vary with time. The external diffusion parameter (k_e, cm·h^{-1}) is defined from d_e and l_e to simplify the problem.

(8) $$\frac{\partial q}{\partial t} = \frac{-d_e\,(c_s - c)}{l_e} = -k_e(c_s - c), \text{ for } k_e = \frac{d_e}{l_e}$$

The rate of internal diffusion was similarly defined (Equation 9). For internal diffusion, a front of diffusion was assumed to progress within the polymeric membrane. The length of this front of diffusion (l_i, cm) varied with time. Concentrations where assumed equal to zero at position l_i and equal to c_s at the surface of the polymeric membrane. The adsorptive capacity (ai, mg·cm^{-3}) of the polymeric membrane was defined from the quantity of drug it can adsorb (q, mg·cm^{-2}) per unit of length (l_i, cm), and is related to the chemistry and structure of the polymeric membrane. d_i (cm^2·h^{-1}) is the diffusion coefficient within the containers. Thus, k_i (mg·cm·h^{-1}) was defined from ai and d_i and was assumed constant during the adsorption process.

(9) $$\frac{\partial q}{\partial t} = \frac{-d_i\,(0 - c_s)}{l_i} = \frac{k_i\,c_s}{q}, \text{ for } ai = \frac{q}{l_i} \text{ and } k_i = ai\,d_i$$

Both differential equations (Equations 8 and 9) can be combined to obtain Equation 10.

(10) $$\frac{\partial q}{\partial t} = k_e\left(c - \frac{\frac{\partial q}{\partial t}q}{k_i}\right) = k_e\left(c_0 - \frac{q\,s}{v} - \frac{\frac{\partial q}{\partial t}q}{k_i}\right), \text{ for } c = c_0 - \frac{q\,s}{v}$$

In order to take into account the adsorption sites which are readily available at the surface of the polymeric membrane, q_0 (mg·cm^{-2}) was not assumed null. Therefore c_0 (mg·cm^{-3}) was defined from the concentration of the preparation (c_{prep}) and q_0 as illustrated by Equation 11.

(11) $$c_0 = c_{prep} - \frac{q_0\,s}{v}$$

Equations 10 and 11 can be combined as Equation 12. This equation was numerically resolved as an attempt to evaluate the contribution of each mechanism to the total adsorption observed for cyclosporine in EVA containers.[e]

(12) $$\frac{\partial q}{\partial t} = k_e \left(c_{prep} - \frac{q_0\, s}{v} - \frac{q\, s}{v} - \frac{\frac{\partial q}{\partial t} q}{k_i} \right)$$

As reported in Table 4.4, very good CVRMSEs were obtained using this mathematical model. CVRMSE was not affected by k_e as long as extremely large values were used suggesting again that external diffusion was not a limiting factor of adsorption rate.

4.4.2.5 Numerical resolution of the internal diffusion model

Considering the negligible contribution of external diffusion, an internal diffusion-only model was defined from Equation 9 resulting in Equation 13. Equation 13 was numerically resolved to determine the parameters k_i and q_0 for this model of diffusion.[e]

(13) $$\frac{\partial q}{\partial t} = \frac{k_i\, c_s}{q} = \frac{k_i}{q}\left(c_{prep} - \frac{q_0\, s}{v} - \frac{q\, s}{v} \right) \text{ for } c_s = c = c_0 - \frac{q\, s}{v} \text{ and } c_0 = c_{prep} - \frac{q_0\, s}{v}$$

This model of diffusion very accurately described the adsorption of cyclosporine on the surface of EVA containers (Table 4.4). Like the modified Weber-Morris model, the internal diffusion model uses only two parameters (k_i and q_0). This latter model is usually applied to the adsorption of solute from a liquid to solid particles and was not reported yet for the adsorption of pharmaceutical compounds to membranes. The proposed internal diffusion model also takes into account the rapid adsorption events resulting from readily accessible adsorption sites. Also, the proposed model takes into account the volume of solution, the surface of the polymeric membrane and the initial concentration of the preparation to evaluate the stability of the drug. These three important variables are overlooked by the modified Weber-Morris model. Furthermore the proposed internal diffusion model was numerically resolved and not only an approximation of Fick's first law of diffusion.

4.4.2.6 Validation of the adsorption model

Equation 13 and its previously optimized parameters were utilized to build a predictive model of adsorption of cyclosporine in NS and D5W. Results from the real conditions stability study were compared to data generated using this model. As shown in Table 4.5, differences between the predicted and the observed data were very small. Demonstration of the accuracy of this model was performed by calculating the coefficient of variance of the root of mean squared error.

4.5 Conclusion

Based on chemical and physical stability data, EVA containers can be used to store cyclosporine injections of 0.2 mg/mL in D5W and NS injections for 7 days. Concentrated 2.5 mg/mL cyclosporine solutions in D5W and NS injections can be stored in EVA bags at least 14 days. However, adsorption of cyclosporine on EVA containers was observed. The study of the adsorption kinetics showed that the internal diffusion may be the rate-limiting step.

The modified Weber-Morris model could successfully describe the adsorption process. In this study, a novel internal diffusion model was established regarding rapid adsorption, contact surface area and volume of solution. This model was numerically resolved and very good fitting results were achieved.

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4.7 Footnotes

[a]Sandimmune IV, 50 mg/mL, Novartis Canada, lot S0040, exp MAR 2013.

[b]Dextrose 5% injection, Baxter Canada, Lot W1C09B0, EXP JUN 2012.

[c]Normal saline injection, Baxter Canada, Lot W1C25A0, EXP SEP 2012.

[d]EVA containers, 250 mL, Baxa, Lot 757280, EXP DEC 2012.

[e]Equation simplification, numerical resolution and parameter optimization using Mathematica 8.0 (Wolfram Research, Inc.) are illustrated in the supplementary materials.

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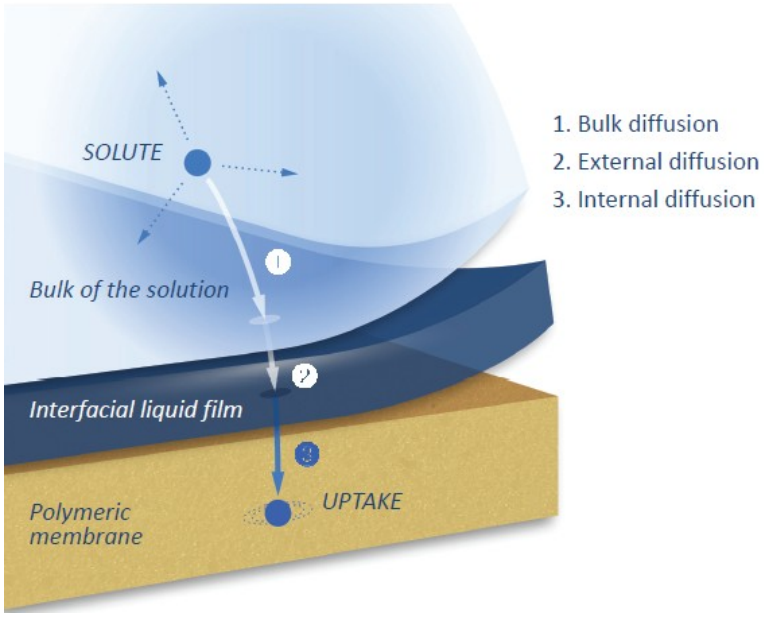


Figure 4.1 Adsorption process

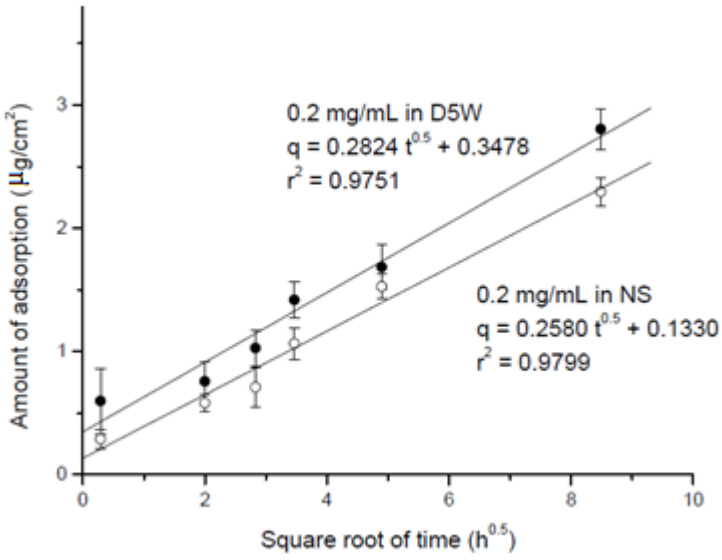


Figure 4.2 Modified Weber-Morris adsorption model

Table 4.1 Real conditions stability study[a]

Solution	Initial concentration[b] (mg/mL)	Fraction of initial concentration recovered		
		3 days (25°C)	7 days (25°C)	14 days (25°C)
0.2 mg/mL in D5W	0.1854 ± 0.0060	95.9% ± 0.2%	92.0% ± 0.4%	87.5% ± 0.2%
0.2 mg/mL in NS	0.1860 ± 0.0101	95.8% ± 0.4%	92.6% ± 0.7%	89.1% ± 0.8%
2.5 mg/mL in D5W	2.472 ± 0.047	100.1% ± 0.5%	100.6% ± 0.6%	100.3% ± 0.3%
2.5 mg/mL in NS	2.451 ± 0.046	100.1% ± 0.3%	100.6% ± 0.3%	101.4% ± 0.4%

[a] Each value is the average result of three containers.

[b]Initial sampling was performed after 12h.

Table 4.2 Micelles size distribution by dynamic light scattering

Solution	Initial[a]	Micelle size distribution		
		3 days (25°C)	7 days (25°C)	14 days (25°C)
2.5 mg/mL in D5W[b]				
Z-Avg (nm)	13.71 ± 0.09	14.24 ± 0.26	14.06 ± 0.07	14.09 ± 0.09
PDI	0.042 ± 0.010	0.056 ± 0.027	0.035 ± 0.005	0.032 ± 0.008
2.5 mg/mL in NS[b]				
Z-Avg (nm)	12.86 ± 0.05	12.83 ± 0.05	12.71 ± 0.09	12.71 ± 0.03
PDI	0.022 ± 0.006	0.029 ± 0.005	0.026 ± 0.005	0.017 ± 0.001

[a]Initial sampling was performed after 12h.

[b]Average of triplicated preparations and triplicated analyses (n=3x3)

Table 4.3 Adsorption study

Solution	Initial concentration[a] (mg/mL)	Fraction of initial concentration recovered					
		5 min (25°C)	4 h (25°C)	8 h (25°C)	12 h (25°C)	1 day (25°C)	3 days (25°C)
0.2 mg/mL in D5W	0.1992 ± 0.0004	98.8% ± 0.5%	98.4% ± 0.3%	97.9% ± 0.1%	97.0% ± 0.1%	96.5% ± 0.2%	94.0% ± 0.1%
0.2 mg/mL in NS	0.2056 ± 0.0003	99.4% ± 0.1%	98.8% ± 0.0%	98.6% ± 0.3%	97.8% ± 0.2%	96.9% ± 0.1%	95.3% ± 0.1%
2.5 mg/mL in D5W	2.5128 ± 0.0076	100.1% ± 0.4%	100.0% ± 0.2%	100.0% ± 0.1%	100.4% ± 0.1%	100.4% ± 0.1%	100.5% ± 0.1%
2.5 mg/mL in NS	2.4994 ± 0.0054	100.3% ± 0.1%	100.0% ± 0.2%	100.2% ± 0.2%	100.2% ± 0.1%	100.3% ± 0.2%	100.4% ± 0.3%

Table 4.4 Adsorption models

	Equation	Cyclosporine 0.2 mg/mL in NS	Cyclosporine 0.2 mg/mL in D5W
External diffusion model	$-\frac{\partial c}{\partial t} = k_e c$	k_e = 7.81e-4 h^{-1} R^2 =0.5473 CVRMSE = 43.7%	k_e = 9.82e-4 h^{-1} R^2 =0.3448 CVRMSE = 45.6%
Weber-Morris model	$q = k_i t^{1/2}$	k_i =2.82e-4 mg cm^{-2} $h^{-0.5}$ R^2 =0.9669 CVRMSE = 11.3%	k_i =3.46e-4 mg cm^{-2} $h^{-0.5}$ R^2 =0.9012 CVRMSE = 16.7%
Modified Weber-Morris model	$q = k_i t^{1/2} + m$	k_i = 2.58e-4 mg cm^{-2} $h^{-0.5}$ R^2 =0.9799 m = 1.33e-4 mg cm^{-2}	k_i = 2.82e-4 mg cm^{-2} $h^{-0.5}$ R^2 =0.9751 m = 3.48e-4 mg cm^{-2}

		CVRMSE = 8.82%	CVRMSE = 8.40%
External and internal diffusion model	$\frac{\partial q}{\partial t} = k_e\left(c_{prep} - \frac{q_0\, s}{v} - \frac{q\, s}{v} - \frac{\frac{\partial q}{\partial t}\, q}{k_i}\right)$	k_e > 1.93e-2 cm h^{-1} k_i =1.93e-7 mg $cm^{-1}h^{-1}$ q_0 = 2.59e-4 mg cm^{-2} CVRMSE = 7.99%	k_e > 2.80e-2 cm h^{-1} k_i =2.80e-7 mg cm^{-1} h^{-1} q_0 = 5.53e-4 mg cm^{-2} CVRMSE = 5.81%
Internal diffusion model	$\frac{\partial q}{\partial t} = \frac{k_i}{q}\left(c_{prep} - \frac{q_0\, s}{v} - \frac{q\, s}{v}\right)$	k_i = 1.93e-7 mg cm^{-1} h^{-1} q_0 = 2.59e-4 mg cm^{-2} CVRMSE = 7.99%	k_i = 2.80e-7 mg cm^{-1} h^{-1} q_0 = 5.53e-4 mg cm^{-2} CVRMSE = 5.81%

CVRMSE: Coefficient of variation of the root of mean squared error (predicted q vs. observed q)

Table 4.5 Validation of the adsorption model

Solution		Concentration (mg/mL)				
		Initial	12 h	3 days	7 days	14 days
0.2 mg/mL in D5W						
Measured		N/A	0.185 ± 0.006	0.178 ± 0.005	0.170 ± 0.005	0.162 ± 0.005
Predicted		0.188	0.183	0.177	0.171	0.165
Error			0.002	0.001	-0.001	-0.003
CVRMSE	1.11%					
0.2 mg/mL in D5W						
Measured		N/A	0.186 ± 0.010	0.178 ± 0.010	0.172 ± 0.011	0.166 ± 0.010
Predicted		0.187	0.183	0.178	0.173	0.16
Error			0.003	0.000	-0.001	-0.002

CVRMSE	1.09%

CVRMSE: Coefficient of variation of the root of mean squared error (predicted c vs. observed c)

Chapter Five

5 Discussion

The intravenous cyclosporine Sandimmune® I.V. (Novartis) contains cyclosporine (50 mg/mL), polyoxyethylated castor oil (Cremophor EL) 650 mg/mL and ethanol 32.9% (by volume) {Novartis, 2011b #485}. Due to its hydrophobic nature, the adsorption of cyclosporine on plastic containers or administration sets is a potential issue {McLeod, 1992 #382}. Additionally, some additives in the container or sets may be leached due to higher concentration of Cremophor EL in the formulaion {Ptachcinski, 1986 #488}. Currently, non-PVC containers are widely used for the preparation of intravenous cyclosporine. It is imperative to evaluate their compatibility with cyclosporine injections.

In the present project, a new stability-indicating HPLC method was developed and validated for the current stability study. There is numerous non-PVC containers used for preparation of intravenous cyclosporine administration. PP-PO bags, EVA bags and PP syringes are plasticizer- free and widely used clinically. This study has assessed the compatibility of these containers with intravenous cyclosporine injections, and attempted to provide adequate information required for clinical practice.

5.1 Development of an HPLC method

Several cyclosporine analytical methods have been reported. In brief, these methods include reverse phase HPLC (RP- HPLC) methods employing acetronitrile- or tetrahydrofuran-based mobile phases {Kumar, 2001 #1017}. In this study, a methanol-based HPLC method was developed. Compared to the current USP method, this method used a mixture of methanol and water as mobile phase, which is more cost-effective and environmental favourable {Ankur M. Desai , 2011 #1195;Ankur M. Desai , 2011 #1195}.

The main challenge of RP-HPLC method for cyclosporine analysis is the broadening peak due to inter-conversion of multiple conformations of cyclosporine, which sometimes

impacts separation efficiency {Nishikawa T., 1994 #486}. The USP HPLC method recommends theoretical plates > 700 and a tailing factor < 1.5. In our study, the methanol-based method was optimized by changing the ratio of methanol/water, and column temperature. The appropriate conditions were determined as: methanol/water was 86:14; column temperature was 50 ^{o}C, in which the theoretical plate number and tailing factor are 1309, 1.052, respectively, indicating that a good separation could be achieved. The precision and accuracy assessments verified that the method had good repeatability and reproducibility. The intraday and interday RSD of repeated injections were 0.32% and 0.33%, meeting the criterion < 1% regulated in USP and ICH guidelines. Accuracy was validated by standard additions. In the USP and ICH regulations, the acceptable criterion of recovered standard for final drug product is 98-102%. In this method, the recovered standard cyclosporine was between 99.80% and 100.1% (Velagaleti 2007).

In the method development, the stress degradation tests on both cyclosporine and intravenous cyclosporine were performed under acid condition. As shown in Figure 2.1, no interference occurred with cyclosporine after acid degradation. In the chromatogram of degraded intravenous cyclosporine, some new peaks (at 5.3 min and 12.7 min) appeared due to the degradation of Cremophor EL in the formulation. However, these peaks did not interfere with the cyclosporine peak. Peak purity was checked by PDA detector. The purity index of all cyclosporine peaks (190 nm to 600 nm) in the current study was higher than 0.999, confirming no significant impurity coeluted with cyclosporine.

According to USP and ICH guidelines, the method was validated to demonstrate stability-indicating, including suitability test, validation of specificity, linearity and range, detection limit, precision, accuracy and robustness. Based on these results, this method is suitable for stability tests of intravenous cyclosporine or routine assays. This method has been successfully used to identify impurity leached from syringe rubber, and studied cyclosporine stability in PP-PO bags and the adsorption in EVA containers.

5.2 Stability of intravenous cyclosporine stored in polypropylene syringes with rubber plunger

In our preliminary stability tests, intravenous cyclosporine preparations of 0.2 and 2.5 mg/mL in D5W and NS injections were prepared and transferred to polypropylene syringes (60 mL, Luer-Lok). By HPLC assays, the recovered cyclosporine in each sample was > 95% after 1 week stored at 25 °C, no significant physical changes were observed by visual inspection and particle size analysis. However, as shown in Figure 5.1 and Figure 5.2, a new impurity peak appeared at relative retention time of 1.45 (relative to the cyclosporine peak) after 24 h (performed on Waters HPLC system consisting of LDC Analytical ConstaMetric 3000 pump, Waters 717 plus autosampler, Waters 486 UV detector).

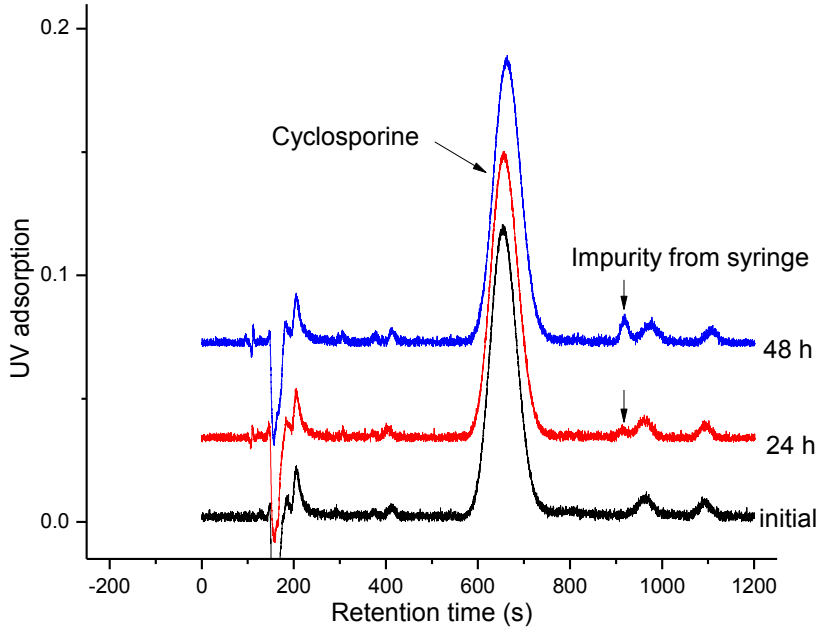


Figure 5.1 HPLC chromatograms of intravenous cyclosporine 0.2 mg/mL in D5W stored in syringe at time zero, 24 h and 48 h.

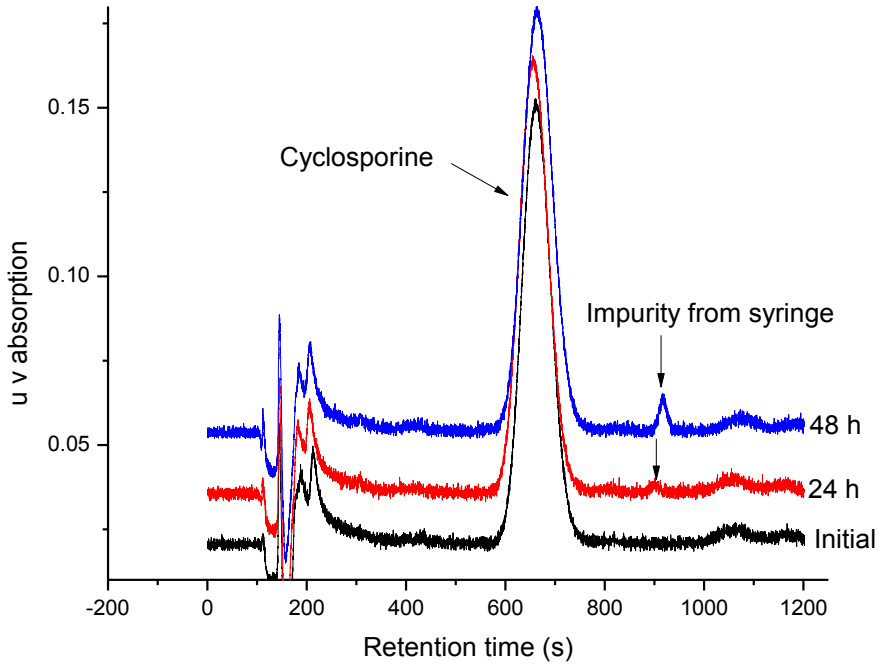


Figure 5.2 HPLC chromatograms of intravenous cyclosporine 0.2 mg/mL in NS stored in syringe at time zero, 24 h and 48 h.

As the impurity significantly increased with time, the polypropylene syringe stability study was terminated after 7 days. In the preliminary stability study on polypropylene polyolefin plastic containers, the new impurity peak was never observed. The impurity may not originate from plastic materials. The syringes used in this study are composed of plastic part-polypropylene and black part-latex-free rubber, and have a silicone lubricant coating in the inner surface. If the impurity is from silicone oil, the dissolution of oil would be fast and the impurity concentration would not increase with contact time. It has been reported that surfactant- polysorbate 80 could leach additives from rubber stoppers in glass vials {Yu, 2010 #1144}. Therefore, plunger black tips may be the potential impurity origin. In order to confirm this hypothesis, a placebo solution corresponding to cyclosporine 2.5mg/mL in 5% dextrose injection was prepared using polyoxyethylated castor oil

(Cremophor EL), alcohol, and 5% dextrose injection, and the rubber plunger tip was removed from syringe and immerged in the solution.

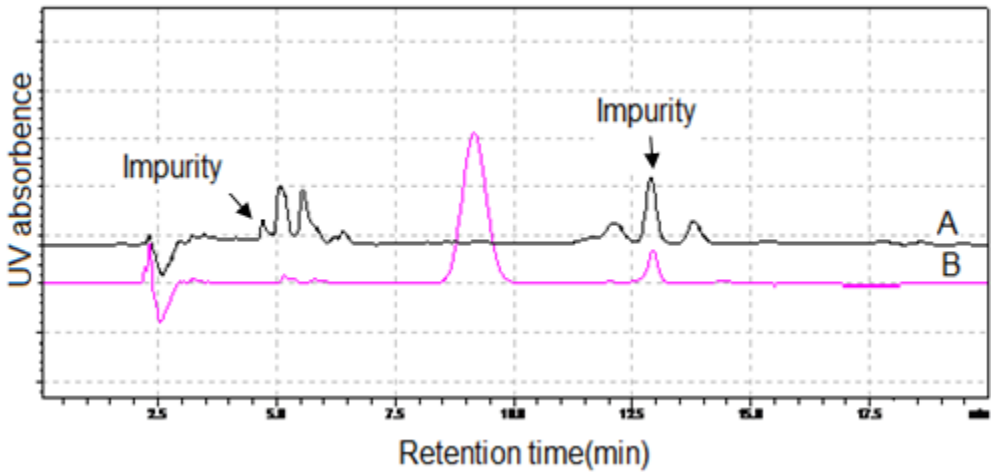


Figure 5.3 HPLC chromatograms of intravenous cyclosporine (2.5 mg/mL in D5W) stored in syringe after 1 week (B) and placebo solution (Cremophor EL, ethanol, D5W) after exposition to syringe plunger tip for 24h (A).

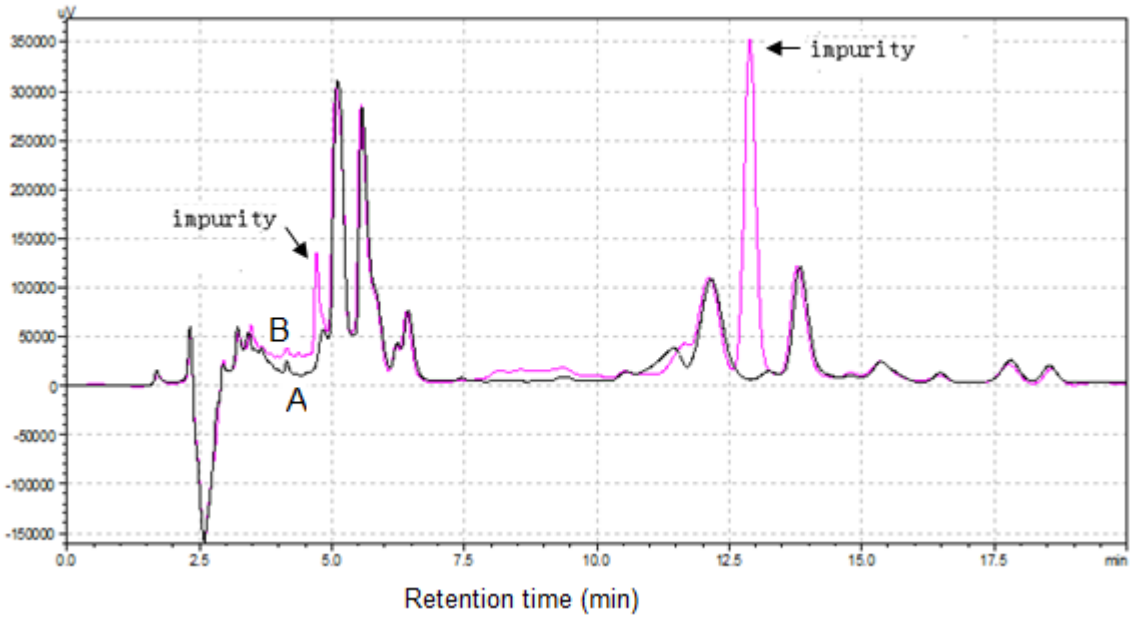


Figure 5.4 HPLC chromatograms of initial placebo solution (A) and after exposition to syringe plunger tip for 24 h (B)

As illustrated in Figure 5.3 and Figure 5.4, the same impurity was detected only after one day of exposure, indicating the impurity was leached from the rubber tip. Moreover, another impurity peak was observed at retention time 4.5 min, which was not detected in intravenous cyclosporine preparations due to low sensitivity. It was concluded that some ingredients used in the manufacture of the black rubber of the syringe plunger could be leached by Cremophor EL. Even though the presence of impurities had no detrimental influence on the chemical stability of cyclosporine, the impurities constitute a potential toxicity hazard. The potential leachables maybe oligomers, antioxidants, and curing agents {Yu, 2010 #1144}. Considering the complexity of rubber composition, it is necessary to investigate the leachables when surfactant consisting formulations are contacting with rubber materials.

As plastic syringes are commonly used to transfer intravenous drug in preparation of intravenous solutions, their compatibility with drug products could pose a health issue, especially if the formulation comprises surfactants. Thus, an additional test was performed to verify whether any additive leached from syringes during short periods of contact with intravenous cyclosporine. Undiluted intravenous cyclosporine (50 mg/mL) of 0.4 and 1 mL were stored in 1- and 5-mL polypropylene syringes for 10 minutes (each sample was prepared in triplicate). The test samples were diluted with 5% dextrose injection to the concentration about 1 mg/mL of cyclosporine, and then assayed by HPLC with large volume of injection (100 μL, 25 times of amount of cyclosporine injected in general assay). As shown in Figure 5.5, the leaching impurities could not be detected. Thus, these syringes can be considered to be safe for sampling and transferring intravenous cyclosporine when the contact time is less than 10 minutes.

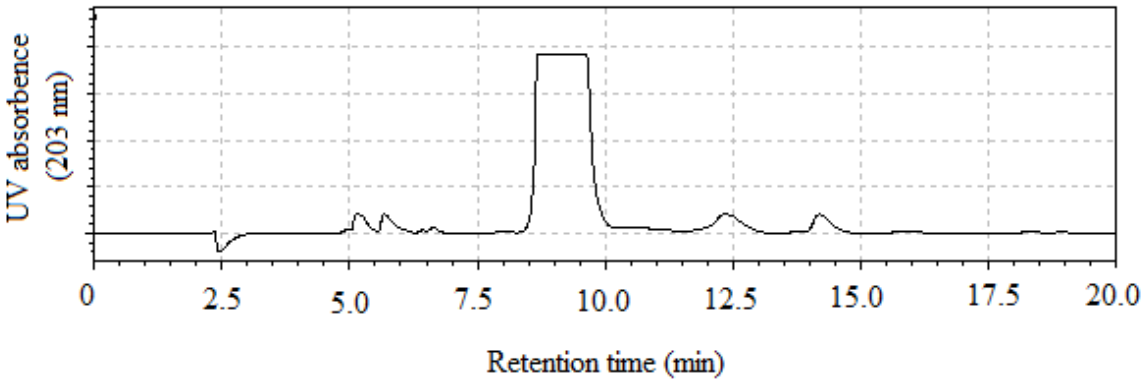
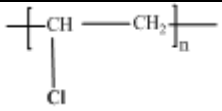
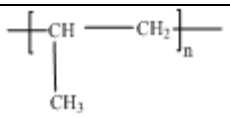
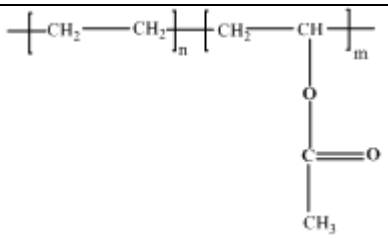


Figure 5.5 HPLC chromatogram of intravenous cyclosporine stored in syringe after 10 min diluted with D5W injection to 1 mg/mL, injection volume 100 µL)

5.3 Stability of intravenous cyclosporine stored in polypropylene-polyolefin (PP-PO) and Ethylene-vinyl acetate (EVA) bags

As discussed in Chapter 3, for all samples (0.2 mg/mL, 2.5 mg/mL in D5W and NS), the remaining cyclosporine was > 98% of initial after stored in PP-PO bags for 14 days. No changes were detected in the chromatograms after 14 days, illustrating intravenous cyclosporine was chemically stable during the storage at least 14 days. When stored in EVA bags for 14 days, the recovered cyclosporine was < 90% for diluted samples (0.2 mg/mL in D5W and NS) due to drug adsorption. Interestingly, no significant change in concentration was observed for the 2.5 mg/mL solutions. Surfactants are effective to reduce protein and peptide adsorption to PP surfaces (Duncan, Lee et al. 1995). In the 2.5 mg/mL solutions, the concentration of polyoxyethylated castor oil is 32.5 mg/mL, greater concentration of polyoxyethylated castor oil may reduce the adsorption of cyclosporine.

Table 5.1 Polymer structure of containers

Material	Structure	Cyclosporine adsorption
PVC		Significant
PP		No
EVA		Significant

The adsorption of hydrophobic drug to plastic is mainly driven by hydrophobic interactions; poorly soluble drugs are prone to partition on hydrophobic surface rather than in aqueous solution. Electrostatic interactions and hydrogen bonding also play a role in drug adsorption {Kambia, 2005 #1193;Palmgrén, 2006 #6}. Assuming the hydrophobic interactions play the major role, the adsorption of cyclosporine would occur on PP-PO surface, which is more hydrophobic than EVA surface. While in this study, significant adsorption was observed on EVA containers other than on PP-PO containers, implying that the adsorption is presumably dominated by electrostatic interactions caused by dipole-dipole interaction or hydrogen-bonding. Even though cyclosporine is a highly hydrophobic

peptide, polar groups such as carbonyls and amides allow strong interaction with container surface.

As shown in Table 5.1, EVA is a copolymer of ethylene and vinyl acetate. Ester groups in vinyl acetate units have potential to interact with cyclosporine by hydrogen bond or dipole-dipole attraction; while for PP-PO multilayer containers, PP polymer presents on the contact surface as out layer, similar interactions did not occur due to lack of polar groups. The adsorption of intravenous cyclosporine on PVC has been reported {Ptachcinski, 1986 #488}, the cyclosporine (2 mg/mL in NS) loss was > 8% after stored for only 24 hours. Beside, significant loss of cyclosporine injections in D5W and NS occurred when administrated through PVC sets. The presence of polar group-chloride may induce strong interactions with cyclosporine by dipole interactions.

Drug adsorption depends on the vehicle and composition. Surfactants can be preferably adsorbed to polymer surface and inhibit drug adsorption. Ion strength and pH values also affect adsorption profiles {Palmgrén, 2006 #6}. In this study, it was found that cyclosporine adsorption in D5W is higher than in NS. On the contrary, more cyclosporine was adsorbed on PVC containers in NS than in D5W vehicle {Ptachcinski, 1986 #488}, indicating the adsorption mechanisms on these two solvents are different.

5.4 Reference

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Palmgrén, J. J., J. Mönkkönen, et al. (2006). "Drug adsorption to plastic containers and retention of drugs in cultured cells under in vitro conditions." European Journal of Pharmaceutics and Biopharmaceutics **64**(3): 369-378.

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Yu, X., D. DeCou, et al. (2010). "A Study of Leachables for Biopharmaceutical Formulations Stored in Rubber-Stoppered Glass Vials." Biopharm International **23**(4): 26-+.

6. Conclusion

In this work, a methanol-based HPLC method has been developed and validated according to the guidance regulated in FDA, USP and ICH. The method was cost-effective and environmental favourable compared to the current acetonitrile-based USP method. This method was demonstrated to be stability-indicating and successfully applied in study of intravenous cyclosporine stability, examining leachables from syringe rubber.

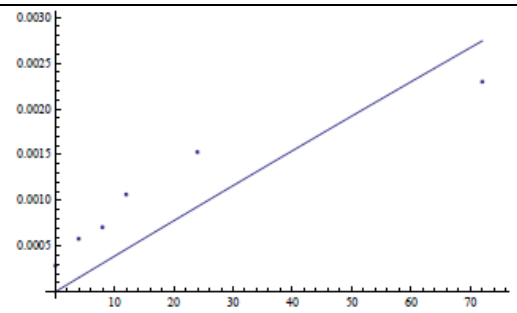
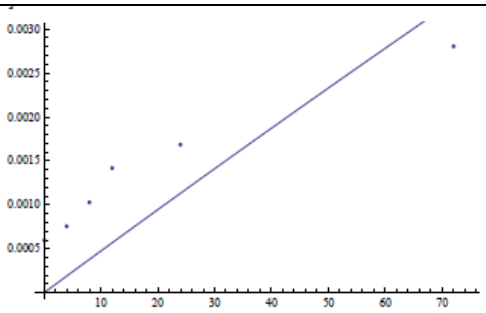
The compatibility of intravenous cyclosporine (0.2 mg/mL; 2 mg/mL in D5W or NS injections) with three types of non-PVC containers (PP-PO bags, PP syringes, EVA bags) has been investigated. Polypropylene–polyolefin (PP-PO) bags are the best choice. All preparations are stable for at least 14 days at 25 °C. As stored in polypropylene syringes with black rubber, some impurities were leached from rubber plunger tip. Therefore, polypropylene syringes with rubber parts could not be recommended. However, these syringes were safe for preparation and transfer of intravenous cyclosporine solution if the contact time is < 10 minutes. Based on these results, it is proposed that the stability of surfactant- involved formulations should be highlighted when contacting with rubber packaging materials.

EVA containers can be used to store cyclosporine of 0.2 mg/mL in D5W and NS injections less than 7 days and preparations of 2.5 mg/mL for 14 days. However, for diluted cyclosporine injections (0.2 mg/mL), significant adsorption on EVA bags was observed. The adsorption study showed that the internal diffusion maybe the rate-limiting step. The modified Weber-Morris model could successfully describe the adsorption process. In this study, a novel diffusion model based on internal diffusion was proposed regarding rapid adsorption, contact surface area and volume of solution. The model was numerically resolved and successfully used to predict long-term stability with variation < 2% (the remaining concentration of cyclosporine measured vs the value predicted based on the model).

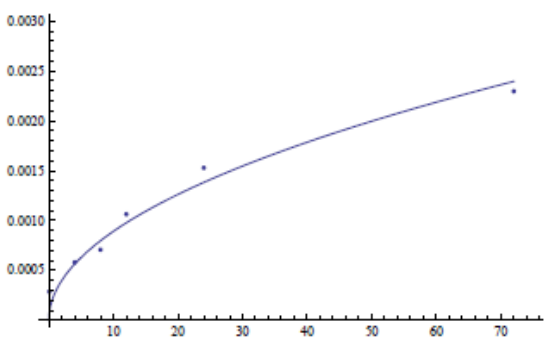
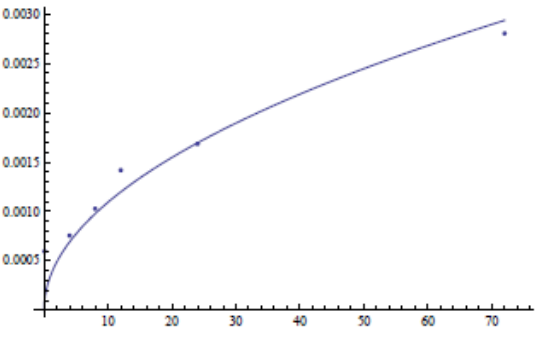
Appendix
Supporting information of Chapter 4

Fitting results of various models

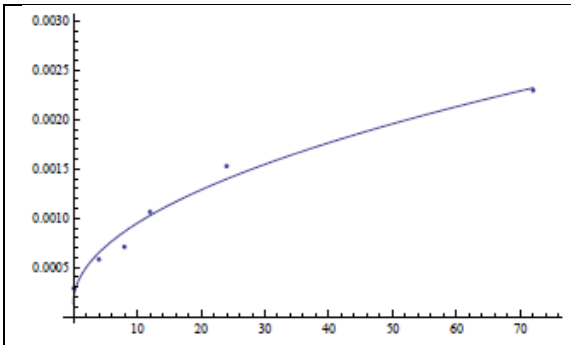
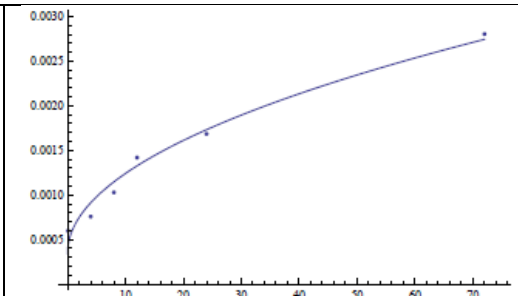
1. External diffusion model

	
Adsorption amount vs contact time (cyclosporine injections in NS)	**Adsorption amount vs contact time (cyclosporine injections in D5W)**

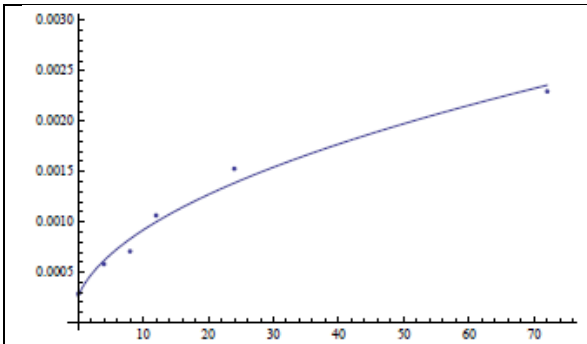
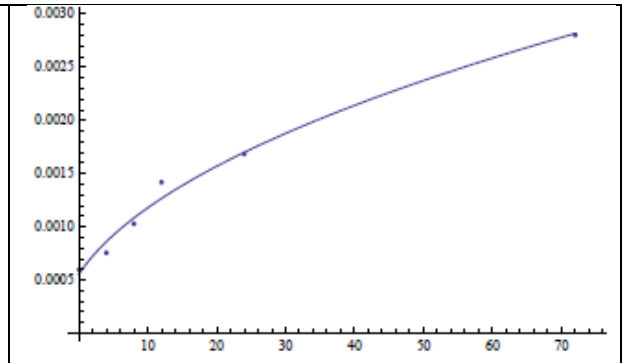
2. Weber-Morris model

	
Adsorption amount vs contact time (cyclosporine injections in NS)	**Adsorption amount vs contact time (cyclosporine injections in D5W)**

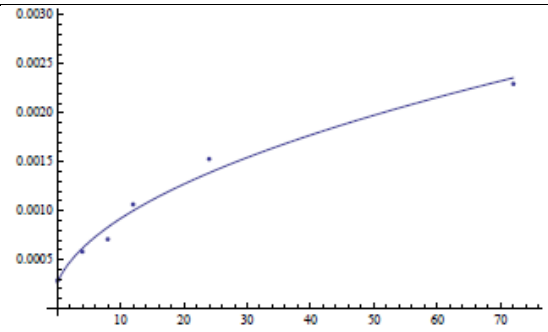
3. Modified Weber-Morris model

	
Adsorption amount vs contact time (cyclosporine injections in NS)	**Adsorption amount vs contact time (cyclosporine injections in D5W)**

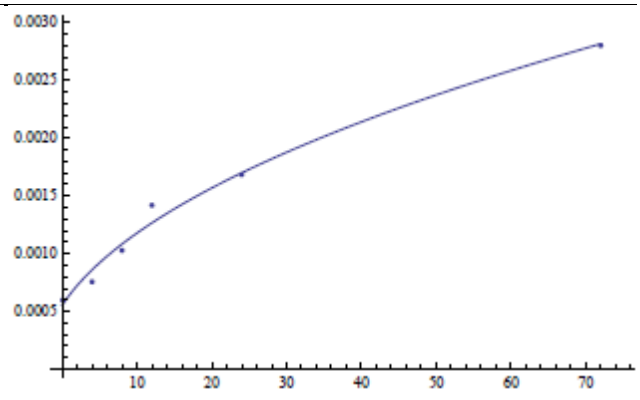
4. Numerical Resolution of External+Internal Diffusion Model

	
Adsorption amount vs contact time (cyclosporine injections in NS)	**Adsorption amount vs contact time (cyclosporine injections in D5W)**

5. Numerical Resolution of Internal Diffusion Model



Adsorption amount vs contact time (cyclosporine injections in NS)



Adsorption amount vs contact time (cyclosporine injections in D5W)