Preformulation and Formulation of Steroids and Assessment of an Electronically Modulated *Intravaginal* Device for Induced Calving or Oestrous Synchronization of Cattle

A thesis submitted in fulfilment
of the requirements for the degree of
Doctor of Philosophy in Biotechnology
At the Department of Engineering

by

Ali Abdi Ismail

The University of Waikato
Te Whare Wānanga o Waikato

July 2006

Abstract

Drug delivery technology is currently advancing faster than at any time in biotechnology history. The challenge of drug delivery is to achieve a controlled release of therapeutic agents over an extended period. Controlled release potentially offers significant advantages over conventional dosage forms, by eliminating both under- and overdosing while maintaining a desired range of drug concentrations. An existing drug regimen for induced calving produces a declining blood profile that does not mimic the naturally occurring, gradually increasing, cortisol blood level around parturition effectively, causing animal health issues. Likewise, while the existing progesterone controlled release systems for oestrous control successfully synchronise oestrus, it is however, associated with reduced fertility and as such other drugs have to be administered to improve the fertility during oestrus. Therefore, there is a need for a drug delivery system that is capable of delivering multiple drugs at various times and patterns. This research aimed to investigate, characterize, identify steroids with high absorption rates through vaginal mucosa and evaluate the potential of an electronic drug delivery system for the delivery of steroids for either the control of the bovine oestrous cycle or induced calving.

In order to identify steroids with high absorption rates across the vaginal mucosa, an *in vitro* permeation method was developed to screen selected steroids for their ability to permeate artificial and biological membranes. The steroids were pre-formulated to enhance their solubility and permeation through these membranes. Analytical UV and HPLC assays to characterise the pure and formulated steroidal compounds were also developed and validated. An assessment of an *intravaginal* Electronically Modulated Intravaginal Device (EMID) for the control of the bovine oestrous cycle or induced calving was carried out. Five different release assessment methods were investigated and critically evaluated in order to identify the most appropriate release assessment method for the EMID. These were: 1) the Drug Dissolution Test, 2) a weight loss method, 3) the dispensed weight method, 4) the determination of piston travel distance method, and 5) the rod expulsion from the EMID method. The methods investigated were critically evaluated in

terms of ease of use and automation, reproducibility and cost/time savings. Optimisation of various components and construction materials of the EMID were also investigated. Animal trials were carried out using the original EMID (manufactured from polypropylene polymer) and modified inserts (manufactured from high density polyethylene polymer) to determine their retention rate in the animals. Accelerated stability testing of progesterone in suspension, oestradiol-17β tablets, cloprostenol as a powder blend and the driving mechanism of the EMID were examined.

The flux of the steroids was evaluated through poly-ε-caprolactone and excised cow mucosa membranes using side-by-side permeation cells.

Results indicated that progesterone followed by dexamethasone acetate and dexamethasone valerate showed higher permeability values through vaginal mucosa compared to dexamethasone or its other analogues. The weight loss method of the EMID proved to be an easy and appropriate method to measure the release rate from the EMID. A high density polyethylene polymer was identified as the most ideal body material for the insert compared to polypropylene body. Also double O-ring silicone, Elastollan WYO 1388-5 and solid silicone pistons were found to be amongst the best pistons tested and all performed well compared to other piston materials. There was a low retention rate with either the original EMID or the modified inserts. Further modification of retention wings of the EMID did not improve the retention rate, but a good blood profile response was obtained from cows treated with the complete EMID containing formulated progesterone. The formulations and driving mechanism were found to be stable under the tested conditions.

Therefore, the EMID has potential for commercial application of induced calving or oestrous control *per vaginum* administration, reliant on improvement of its retention mechanism.

Acknowledgements

I would like to thank the Foundation for Science, Research and Technology who provided me with a Technology Industry Fellowship (TIF) without which I could not have pursued this degree. Thanks to the New Zealand Vice-Chancellors' Committee for awarding me the funds to attend and present a research paper at the Annual Meeting of Controlled Release Society in 2004. Special thanks to Dr Bob Welch for initiating this research and providing valuable instructions, theory and thoughts on induced calving. Thanks to Dr Craig Bunt for providing instruction on the theory and practice of electronically modulated *intravaginal* drug delivery. Many thanks to both the Materials and Process Engineering Department of the University of Waikato and InterAg who made this project possible and also for their financial support to attend Australian Pharmaceutical Science Association and Formulation and Delivery of Bioactive Conferences. Thanks also to InterAg for providing the vast majority of materials, resources, chemicals, equipment, laboratories and electronic drug delivery inserts used in this project. I am also grateful to Dexcel who facilitated the animal experiments. Thanks to all of the members of the Ruakura and the University of Waikato Animal Ethics Committees who reviewed and approved animal trial experiments. Also thanks to those international scientists who proof read various chapters and provided valuable comments on my thesis - Dr Keith Ellis (InterAg), Dr Jim Peterson (Agresearch, Ruakura Research Centre) and Dr Rod Walker (Rhodes University, South Africa). Special thanks go to my Industry Chief Supervisor, Dr Michael Rathbone, for his invaluable mentorship and seemingly limitless input of his time and expertise and to my University Chief Supervisor, Dr Kim Pickering for keeping a watchful eye on my progress.

Finally, thanks to all my family members, especially my wife Maryan Muse for her encouragement and unconditional moral support.

Publications arising from this thesis

1. A.A. Ismail, M.J. Rathbone, C.R. Ogle, C.R. Bunt, K.L. Pickering & C.J. Fee. Poly-ε-caprolactone as model membrane in side-by-side cells by Proceeding of the Annual Conference of the Australian Pharmaceutical Science Association, 3-5 December 2003, Bondi, NSW, Australia. pp.108.

2. A.A. Ismail, M. Rathbone, C. Ogle, C. Bunt, K. Pickering & C. Fee. Comparison of Several Approaches to *in Vitro* Drug release Determination for an Electronic Drug Delivery System. Proceeding of the 6th Annual Conference of the Formulation and Delivery of Bioactive, 12th & 13th February 2004, Otago University, Dunedin, New Zealand.

3. A. Ismail, M. Rathbone, C. Ogle, C. Bunt, K. Pickering & C. Fee. Characterisation of an Electronic Drug Delivery Insert. Proceeding of the 31st Annual Conference of the Controlled Release Society, 12-16 June 2004, Honolulu, Hawaii, USA. Abstract/Poster #182.

4. A. A. Ismail, M. Rathbone, C. Ogle, C. Bunt, K. Pickering & C. Fee. Development and Evaluation of Several Release Assessment Methods for an Electronically Modulated Drug Delivery System. Proceeding Joint SCENZ/FEANZ/SMNZI Conference, July 2004: "Engineering Revolution". Waikato University, Hamilton, New Zealand. pp.155-157.

Table of contents

List of tables

List of figures

Preformulation and Formulation of Steroids and Assessment of an Electronically Modulated *Intravaginal* Device for Induced Calving or Oestrous Synchronization of Cattle

A thesis submitted in fulfilment
of the requirements for the degree of
Doctor of Philosophy in Biotechnology
At the Department of Engineering

by

Ali Abdi Ismail

The University of Waikato
Te Whare Wānanga o Waikato

July 2006

Abstract

Drug delivery technology is currently advancing faster than at any time in biotechnology history. The challenge of drug delivery is to achieve a controlled release of therapeutic agents over an extended period. Controlled release potentially offers significant advantages over conventional dosage forms, by eliminating both under- and overdosing while maintaining a desired range of drug concentrations. An existing drug regimen for induced calving produces a declining blood profile that does not mimic the naturally occurring, gradually increasing, cortisol blood level around parturition effectively, causing animal health issues. Likewise, while the existing progesterone controlled release systems for oestrous control successfully synchronise oestrus, it is however, associated with reduced fertility and as such other drugs have to be administered to improve the fertility during oestrus. Therefore, there is a need for a drug delivery system that is capable of delivering multiple drugs at various times and patterns. This research aimed to investigate, characterize, identify steroids with high absorption rates through vaginal mucosa and evaluate the potential of an electronic drug delivery system for the delivery of steroids for either the control of the bovine oestrous cycle or induced calving.

In order to identify steroids with high absorption rates across the vaginal mucosa, an *in vitro* permeation method was developed to screen selected steroids for their ability to permeate artificial and biological membranes. The steroids were pre-formulated to enhance their solubility and permeation through these membranes. Analytical UV and HPLC assays to characterise the pure and formulated steroidal compounds were also developed and validated. An assessment of an *intravaginal* Electronically Modulated Intravaginal Device (EMID) for the control of the bovine oestrous cycle or induced calving was carried out. Five different release assessment methods were investigated and critically evaluated in order to identify the most appropriate release assessment method for the EMID. These were: 1) the Drug Dissolution Test, 2) a weight loss method, 3) the dispensed weight method, 4) the determination of piston travel distance method, and 5) the rod expulsion from the EMID method. The methods investigated were critically evaluated in

terms of ease of use and automation, reproducibility and cost/time savings. Optimisation of various components and construction materials of the EMID were also investigated. Animal trials were carried out using the original EMID (manufactured from polypropylene polymer) and modified inserts (manufactured from high density polyethylene polymer) to determine their retention rate in the animals. Accelerated stability testing of progesterone in suspension, oestradiol-17β tablets, cloprostenol as a powder blend and the driving mechanism of the EMID were examined.

The flux of the steroids was evaluated through poly-ε-caprolactone and excised cow mucosa membranes using side-by-side permeation cells.

Results indicated that progesterone followed by dexamethasone acetate and dexamethasone valerate showed higher permeability values through vaginal mucosa compared to dexamethasone or its other analogues. The weight loss method of the EMID proved to be an easy and appropriate method to measure the release rate from the EMID. A high density polyethylene polymer was identified as the most ideal body material for the insert compared to polypropylene body. Also double O-ring silicone, Elastollan WYO 1388-5 and solid silicone pistons were found to be amongst the best pistons tested and all performed well compared to other piston materials. There was a low retention rate with either the original EMID or the modified inserts. Further modification of retention wings of the EMID did not improve the retention rate, but a good blood profile response was obtained from cows treated with the complete EMID containing formulated progesterone. The formulations and driving mechanism were found to be stable under the tested conditions.

Therefore, the EMID has potential for commercial application of induced calving or oestrous control *per vaginum* administration, reliant on improvement of its retention mechanism.

Acknowledgements

I would like to thank the Foundation for Science, Research and Technology who provided me with a Technology Industry Fellowship (TIF) without which I could not have pursued this degree. Thanks to the New Zealand Vice-Chancellors' Committee for awarding me the funds to attend and present a research paper at the Annual Meeting of Controlled Release Society in 2004. Special thanks to Dr Bob Welch for initiating this research and providing valuable instructions, theory and thoughts on induced calving. Thanks to Dr Craig Bunt for providing instruction on the theory and practice of electronically modulated *intravaginal* drug delivery. Many thanks to both the Materials and Process Engineering Department of the University of Waikato and InterAg who made this project possible and also for their financial support to attend Australian Pharmaceutical Science Association and Formulation and Delivery of Bioactive Conferences. Thanks also to InterAg for providing the vast majority of materials, resources, chemicals, equipment, laboratories and electronic drug delivery inserts used in this project. I am also grateful to Dexcel who facilitated the animal experiments. Thanks to all of the members of the Ruakura and the University of Waikato Animal Ethics Committees who reviewed and approved animal trial experiments. Also thanks to those international scientists who proof read various chapters and provided valuable comments on my thesis - Dr Keith Ellis (InterAg), Dr Jim Peterson (Agresearch, Ruakura Research Centre) and Dr Rod Walker (Rhodes University, South Africa). Special thanks go to my Industry Chief Supervisor, Dr Michael Rathbone, for his invaluable mentorship and seemingly limitless input of his time and expertise and to my University Chief Supervisor, Dr Kim Pickering for keeping a watchful eye on my progress.

Finally, thanks to all my family members, especially my wife Maryan Muse for her encouragement and unconditional moral support.

Publications arising from this thesis

1. A.A. Ismail, M.J. Rathbone, C.R. Ogle, C.R. Bunt, K.L. Pickering & C.J. Fee. Poly-ε-caprolactone as model membrane in side-by-side cells by Proceeding of the Annual Conference of the Australian Pharmaceutical Science Association, 3-5 December 2003, Bondi, NSW, Australia. pp.108.

2. A.A. Ismail, M. Rathbone, C. Ogle, C. Bunt, K. Pickering & C. Fee. Comparison of Several Approaches to *in Vitro* Drug release Determination for an Electronic Drug Delivery System. Proceeding of the 6th Annual Conference of the Formulation and Delivery of Bioactive, 12th & 13th February 2004, Otago University, Dunedin, New Zealand.

3. A. Ismail, M. Rathbone, C. Ogle, C. Bunt, K. Pickering & C. Fee. Characterisation of an Electronic Drug Delivery Insert. Proceeding of the 31st Annual Conference of the Controlled Release Society, 12-16 June 2004, Honolulu, Hawaii, USA. Abstract/Poster #182.

4. A. A. Ismail, M. Rathbone, C. Ogle, C. Bunt, K. Pickering & C. Fee. Development and Evaluation of Several Release Assessment Methods for an Electronically Modulated Drug Delivery System. Proceeding Joint SCENZ/FEANZ/SMNZI Conference, July 2004: "Engineering Revolution". Waikato University, Hamilton, New Zealand. pp.155-157.

Table of contents

List of tables

List of figures

1 Introduction to induced calving and oestrous control

Induced calving and oestrous control are farm management tools used to achieve the following: 1) to make cows calve prematurely, 2) to bring a large number of animals into oestrus at a predetermined time, 3) to reduce the calving period of the herd to the shortest period possible, and 4) to achieve high conception rates at the end of the mating period (Nation, 1997; Holmes & Garcia, 1999; Compton, 2005a). In New Zealand, seasonal calving is a prevailing zootechnical strategy used to synchronise the time of calving with the increase in pasture growth and the decrease in feed demand at the drying-off season with the reduction in pasture growth. Oestrous synchronisation is used to induce oestrus in a large proportion of a herd within a short period in order to eliminate the need of the oestrus detection, a process that is normally difficult and time consuming (Jemmeson, 2000). Both induced calving and oestrous synchronisation involve administering hormones to control either the pregnancy or reproductive cycle of cattle. In the case of induced calving, hormones are administered using intramuscular injections whereas for oestrus synchronisation, hormones can be administered either by intramuscular injections or through the use of intravaginal devices or subcutaneous implants. The existing drug delivery systems used for induced calving lead to health issues for the animals such as high mortality rates of the newborn calf and the maternal cow endometrial disease due to retained foetal membranes (Stevens *et al*., 2000). Drug delivery systems for oestrous synchronisation are designed to deliver a single drug at one time, however, for oestrous synchronisation to be effective, several drugs must be administered to improve fertility. A review of the New Zealand dairy farming industry with respect to current induced calving and oestrous synchronisation protocols are elaborated herein.

1.1 The New Zealand dairy farming industry

The majority of New Zealand dairy cows calve once a year, in a concentrated pattern in late winter-early spring (Nation, 1997; Holmes & Garcia, 1999). This seasonal calving allows the increase in the herd's feeding requirements after

calving to coincide with the increase in pasture growth during springtime. At this time, the cows need extra energy to cope with the commencement of lactation and the resumption of the reproductive cycle. The lactation period usually lasts about 300 days in a year to allow for a dry period of at least 60 days before next calving (Holmes & Garcia, 1999). However, there are a significant number of cows that calve later than the desired calving time, and conceive late in the breeding season, thereby making it difficult to bring these cows forward to the optimal calving season (Welch, 1973; Welch *et al*., 1979; Compton, 2005b). Late calving cows are more likely to be dried off while they are still milking heavily and, therefore, would have a shorter lactation period and result in a poorer return for the farmer. In order to control the uneven calving time among the cows in the same herd and to make the lactation period coincide with the availability of pasture, production induced calving was introduced into New Zealand in 1973.

1.2 Induced calving

The normal gestation period of dairy cattle ranges between 278 and 290 days (Allen *et al*., 1976). However, this period may be shortened by terminating the pregnancy through drug intervention. Therefore, the incidence of late calving cows can be reduced by prematurely induced calving (Welch *et al*., 1979). These cows can then be brought back in synchronisation with the rest of herd to the normal conception date of the following season. This practice enables seasonal calving patterns to be maintained among dairy herds and gives the late calving cows the opportunity to become pregnant in time to calve naturally at an optimal time. Other possible reasons for induced calving include: 1) grouping calving at a precise time to ensure adequate care and shelter, 2) extending the mating season, 3) reducing calf size and incidence of dystocia (difficult labour) and 4) terminating undesired pregnancy for medical reasons (Allen *at al*., 1976; Gordon, 1996).

1.2.1 Practical aspects of induced calving

Parturition has been induced using exogenous pharmacological agents while mimicking the hormonal changes during the last few days of cow pregnancy (Adams, 1969). Administration of synthetic corticosteroids to the pregnant cow

results in expulsion of the foetus from as early as two months before the end of the pregnancy. A similar situation to natural parturition can be seen if dexamethasone is injected into pregnant cows to induce parturition (Kindahl *et al*., 2004). Corticosteroids alone or in combination with prostaglandins are also used for the induction calving (Refsdal, 2000). For instance, parturition can be induced reliably in a cow by administration of a single glucocorticoid (e.g., dexamethasone) treatment of the animals after about day 255 and, less reliably, as early as day 235 of gestation (Gordon, 1996). Historically, corticosteroids have been used systematically in over 80% of New Zealand dairy herds to control the birth, to induce premature parturition during the third trimester of pregnancy, and to condense calving patterns into periods of 6-12 weeks (Macmillan, 2002). Corticosteroid preparations used to induce premature calving are divided into two categories depending on the time they take to induce calving, *i.e*., short-acting and long-acting (McGowan *et al*., 1975). The short-acting drugs (i.e., dexamethasone and flumethasone) induce parturition within two to three days after administration, but have two major disadvantages: firstly the number of animals which respond to the treatment varies markedly from herd to herd, secondly the animals that do respond have a high incidence of retained placenta. The longer acting drugs (e.g., dexamethasone trimethylacetate) are more widely used and are usually insoluble forms of the short acting drugs (McGowan *et al*., 1975). They induce calving approximately 15 days after injection. Some reports have suggested that the problem of undesired side effects such as retained placenta are worse with short-acting formulations (Peters & Poole, 1992). When it is desirable that calving should be induced in cows within the last two or three weeks of gestation, a single injection of a number of short-acting preparations (corticosteroids administered as a free alcohol or soluble ester) of dexamethasone and flumethasone have shown to produce reliable and predictable results (Gordon, 1996).

Calving induction and the use of corticosteroids (e.g., long- and short-acting corticosteroids) or/and cloprostenol (prostaglandin analogue) to induce parturition is well documented in the literature (Adams, 1969; Agrawal *et al*., 1993; Allen & Herring, 1976; Barth *et al*., 1978; Beardsley *et al*., 1976; Davis *et al*., 1979; Evans & Wagner, 1976; Fairclough *et al*., 1975; Fairclough *et al*., 1981b; Fulkerson &

McDowell, 1975; Garverick *et al*., 1974; Hansen & Christiansen, 1971; Jackson, 1979; MacDiarmid, 1979; McFeely & Ganjam, 1976; Morton & Butler, 1995; Murray *et al*., 1981; Murray *et al*., 1982; Murray *et al*., 1984; Nasser *et al*., 1994; O'Farrell & Crowley, 1974; Kaker *et al*., 1984; Peters & Poole, 1992; Schmitt *et al*., 1975; Terblanche *et al*., 1976; Terblanche & Labuschagne, 1980; Wagner *et al*., 1974; Welch, 1973; Welch *et al*., 1973; Welch *et al*., 1977; Welch *et al*., 1979).

Induction of parturition by injections of prostaglandin $F_{2\alpha}$ (PGF) alone or its analogue has been suggested to be non-physiological as the foetal cortisol levels obtained from the induced calving cows were not ideal to mimic the blood profiles obtained from the naturally calving cows (McGowan *et al*., 1975; Königsson, 2001).

1.2.2 Effect of induced calving on milk production

Induced calving can have both direct and indirect effects on subsequent milk production. The direct effect can be seen by comparing the difference in subsequent milk production between induced and non-induced cows that calve at the same time. The indirect effect is the difference in subsequent milk production due to altered calving dates (Morton & Butler, 1995a). While studies found induced calving increased milk production by increasing the number of days in milk, the induced cows produced 9% less than cows that calved naturally on the same day (Morton & Butler, 1995a).

1.2.3 Effect of induced calving on reproductive performance

One of the biggest challenges that New Zealand dairy farmers face is to improve the reproductive performance of their herds (Compton, 2005a). Induced calving minimises the adverse effects of declining reproductive performance in dairy herds on the sustainability of seasonally concentrated calving (Macmillan, 2002). Results of induced calving on cows reproductive performance found no effects of induced calving on 3-week submission rates (Morton, 2000). In contrast, results from Morton & Butler, (1995b) and McDougall, (2001) indicate an 8.3%

reduction in the induced cows' 28-day pregnancy rate. The induced late calving cows however, got back into calf much sooner after calving than their earlier calving herd mates (Stevens *et al*., 2000). This means that the difference in calving dates between induced and late calving cows is shorter in the subsequent season.

1.2.4 Animal welfare issues arising from induced calving

Induced calving has now become an animal welfare issue due to high calf mortality rates, the stress it places on the cow and associated animal health problems (Stevens *et al*., 2000). For instance, the problem of retained foetal membranes (RFM) is associated with induced calving. Table 1.1 presents the effects of calving induction on RFM, stage of pregnancy and the formulation used.

The incidence of clinical disease and mortality of induced cows were high relative to their non-induced herd mates (Morton & Butler, 1995c). The corticosteroids used for induction, suppress the immune system of the cow and may reduce its ability to combat disease. Additionally, corticosteroids may cause high mortality rates in induced calves compared with naturally born calves (75% versus 7%, respectively) (Morton & Butler, 1995d). Calf mortality rates vary considerably depending on the timing of induction and the drug regimen used.

Table 1.1. Rate of RFM incidences extracted from the literature due to the induced calving.

Formulation	Phase of gestation	RFM rate	Reference
Dexamethasone or flumethasone	Last 1-2 weeks	54%	(Wagner *et al*., 1974)
Opticortenol (dexamethasone trimethylacetate) with or without prednisolone	Last 1-6 weeks	8.5%	(O'Farrell *et al*., 1974)
PGF analogue (Cloprostenol)	Last 5 weeks	5.7-28.2%	(Day, 1977)
Opticortenol and bethamethasone in different combinations	2 weeks	16%	(Welch *et al*., 1977)
Dexamethasone	Last week	52%	(Chew *et al*., 1978)
Opticortenol/dexamethasone isonicotinate	Close to calving	25%	(Welch *et al*., 1979)
A mixture of 20 mg dexamethasone phenylpropionate and 10 mg dexamethasone phosphates 1st shot and ± 0.5 mg of cloprostenol (after 10-11 days) as 2nd shot	Last 2 weeks	53%	(Kaker *et al*., 1984)
Opticortenol (1st shot) and cloprostenol (2nd shot)	Last 5-12 weeks	15%	(Morton & Butler, 1995d)
Dexamethasone isonicotinate and dexamethasone sodium phosphate	Close to calving	8.6%	(Verkerk *et al*., 1997)

1.2.4.1 Other dexamethasone applications and its misuses in livestock animals

Although dexamethasone is frequently used for the induction of parturition, it is also used in cattle either alone or in combination with antibiotics for the treatment of inflammatory diseases (Toutain *et al*., 1982; Calvarese *et al*., 1994). In addition, dexamethasone is the most abused corticosteroid (De Wash *et al*., 1998). Dexamethasone is used illegally as a growth promoter and has been administered to cattle through livestock food or by injection, in order to achieve mass gains.

Similarly, corticosteroids such as betamethasone or its esters have been detected in injection sites. This is unfortunate because the consumption of meat contaminated with injection sites can be a considerable threat to human health. Public opinion rejects the use of growth promoter substances such as corticosteroids in animal fattening (Antignac *et al*., 2001). Consequently, analytical techniques have been developed, in order to control their illegal use as growth promoters in meat producing animals and to insure food safety. The use of growth promoters has never been allowed in Europe and maximal residue levels have only been established for dexamethasone, which are 0.75 ng/mL in liver, 0.5 ng/mL in muscle and 0.3 ng/mL in milk.

1.2.4.2 Dexamethasone residue in milk and plasma

The levels of dexamethasone in the milk of lactating cows shows the same profiles as the levels in plasma, but the concentration of dexamethasone in milk decreases more rapidly compared to the plasma concentrations because of the shorter half-life of dexamethasone in milk compared to the plasma (Dilova *et al*., 1999). Dilova *et al*. (1999) showed that the clearance of dexamethasone from the milk was 5 days. In another study, the concentration of dexamethasone in both milk and plasma of cows treated with 25 mg dexamethasone trimethyl acetate containing radiolabelled molecules showed similar profiles (Fairclough *et al*., 1981a). The concentration of dexamethasone in both milk and plasma were still detectable at around 0.05 ng/mL, 30 days post injection.

Another study showed that dexamethasone traces were still detectable from plasma and affected cortisol levels of the treated cows up to 52 days post injection (Toutain *et al*., 1982). Even though the drug concentrations were below the maximal residue levels permitted, there was still some concern relating to the residue risk of some long acting corticosteroids. Consequently, the New Zealand Food Safety Authority (NZFSA) defined meat and milk-withholding periods, 35 and 12 days, respectively, where there has been the use of long acting corticosteroids (NZFSA, 2006). Finally, social or marketing pressures have limited the use of calving induction, which is now discouraged on animal welfare grounds and has possible consequences for the marketing of New Zealand dairy

products (Macmillan, 2002). This reflects that the modern induced calving does not control the drug residue of long-acting corticosteroids after calving, and consequently, there are animal health concerns due to the undesired side effects arising from the treatment. Corticosteroid release needs to be controlled during and after treatment in order to maintain the desired drug blood responses to mimic naturally occurring profiles around parturition. Safer induced calving in which the prevailing undesired side effects are reduced to minimum is yet to be developed. As discussed earlier, the induced calving involves termination of pregnancy and, therefore, a brief discussion of recent advances in the physiology and endocrinology of bovine pregnancy and parturition is further discussed in the following sections.

1.2.5 Introduction to endocrinology of cattle reproduction

After reaching the age of puberty, cows are either cycling, pregnant or recovering from pregnancy to recycle again. Several hormones produced by the hypothalamic-pituitary-ovarian axis and the uterus, control cattle reproduction (Gordon, 1996). The hypothalamus, pituitary gland and ovary coordinate the reproductive cycle by secreting hormones directly into the blood. The hypothalamus secretes Gonadotrophic Releasing Hormone (GnRH), which in turn stimulates the anterior pituitary to secrete two gonadotrophic hormones (Follicle Stimulating Hormone, FSH and Luiteinizing Hormone, LH). LH and FSH control ovarian functions. FSH initiates maturation of ovarian follicles while LH induces ovulation and luteinization of corpus luteum (CL) cells known as granulosa and thecal cells. At the ovaries, the follicles produce oestrogens (primarily oestradiol-17β) and CL produces progesterone. Both oestradiol-17β and progesterone regulate the reproductive cycle through a series of feed back mechanisms acting on the hypothalamus and pituitary gland. The uterus produces PGF which causes luteolysis at the end of the oestrous cycle or pregnancy (Kindahl *et al*., 2004; Goff, 2004). The luteolysis process can be prevented by production of antiluteolytic signals, such as interferon factors produced in the presence of the embryo (Mann *et al*., 1998; Ivell *et al*., 2000). The interferon factors prevent PGF release from the endometrial epithelial cells, therefore CL function is maintained and progesterone is released during the pregnancy.

1.2.5.1 Physiology and endocrinology of bovine pregnancy and parturition

Steroidal hormones associated with cow pregnancy are mainly progesterone and oestrogens (Kindahl *et al*., 2002). The progesterone level, which is used to indicate the stage of a cow's reproductive cycle, is near zero during oestrus. When a cow conceives, the progesterone levels rise and remain high for the rest of the gestation period preventing further reproductive cycles occurring during pregnancy (Kindahl *et al*., 2002; Peters & Lamming, 1983). If conception does not happen, the progesterone level increase, reaches a peak level, and then declines to near zero as the cow returns to oestrus once again. Progesterone plays the dominant role in the maintenance of pregnancy. Progesterone originates from the CL and, later on in pregnancy, is supplemented by further progesterone from the placenta and maternal adrenal glands (Kindahl *et al*., 2004). It is a known fact that the bovine adrenal glands contribute to progesterone production and are capable of maintaining pregnancy following an ovariectomy at 215 days of gestation (Wendorf *et al*., 1983). The concentration of oestrogens in bovine blood plasma remain low during the early period of pregnancy and increase gradually throughout mid- to late gestation (Hirako *et al*., 2003). Oestrogens, which are mainly produced from the placenta and ovaries in the maternal cow during gestation, start to increase between days 70 and 265 of pregnancy (Kindahl *et al*., 2004). Oestrogens play an essential role in the maintenance of pregnancy and the initiation of parturition. Oestrogens have several derivatives and the major biologically active compounds are oestradiol-17β, oestrone and oestriol. Oestrone sulphate, oestrone in its conjugated form, is the major oestrogen that occurs in the pregnant cow. Oestrone sulphate blood level begins to increase around day 50 of pregnancy and gradually increases until day 80 (Hirako *et al*., 2003). However, oestradiol-17β and oestrone blood levels remain low until day 80. Oestrone sulphate levels are monitored during pregnancy and foetal wellbeing (Kindahl *et al*., 2004).

1.2.5.1.1 Hormonal blood profiles of the foetus and maternal cow at late pregnancy and around parturition

Maintenance of pregnancy in the cow is dependent upon the balance of several hormones maintained by interactions between the cow, the placenta and the foetus (Kindahl *et al*., 2002). At late pregnancy and around parturition, the decreased levels of progesterone prior to the time of parturition and the increased levels of oestradiol-17β and PGF on the day of parturition appear to be crucial (Kornmatitsuk *et al*., 2002). A gradual reduction of progesterone concentration is seen in the last 2-3 days prior to parturition followed by an even more rapid decline in progesterone until delivery, a process known as pre-partial luteolysis (Fairclough *et al.,* 1981b). PGF plasma levels increase in late pregnancy and the highest levels are reached during parturition (Königsson, 2001). Hypothetical profiles of the foetal and maternal hormonal changes around calving period of cattle are shown in Figure 1.1. Initially, the foetal cortisol levels start to rise significantly at less than four days before parturition (Fairclough *et al.,* 1981b). The cortisol rise continues in the next two days and then reaches a maximum level the day before parturition. PGF levels on the other hand start to rise only two days before parturition, and the progesterone level starts to drop dramatically just several hours before parturition and the oestrogen levels continue to rise until on the day of parturition (Figure 1.1).

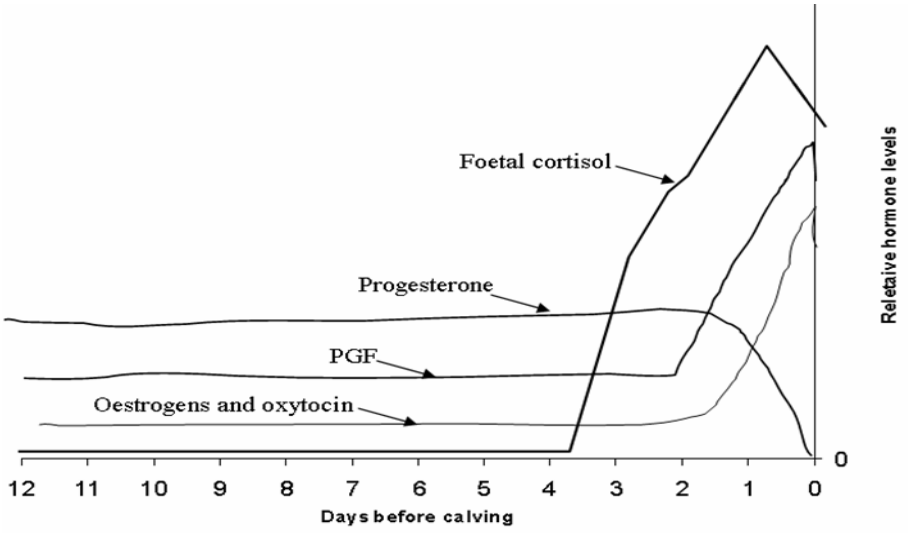


Figure 1.1. Hypothetical foetal cortisol and maternal hormone changes around calving

1.2.5.1.2 Mechanism of naturally occurring parturition

Parturition is a natural event that involves stress and pain for the cow (Hydbring *et al*., 1999). Cortisol is a naturally occurring stress hormone that is produced by the adrenal gland and released during stress and pain (Kindahl *et al*., 2004). It is generally accepted that the signal for the initiation of parturition originates in the foetus and not the maternal cow (Taverne *et al*., 2002). A functionally intact foetal hypothalamus-pituitary-adrenal axis (HPA) dictates the initiation of spontaneous parturition (Young *et al.,* 1996). The mechanism of this trigger is not well understood, but it might be due to stress caused by the foetus reaching its critical size and the lack of space in the uterus. This stress exerts considerable pressure on the foetal hypothalamus to release corticotrophin releasing hormone (CRH). CRH and adrenocorticotrophic hormone (ACTH) together with cortisol are involved in the initiation of parturition in the foetus (Kindahl *et al*., 2004). ACTH is secreted from the anterior pituitary in response to CRH from the hypothalamus. ACTH stimulates secretion of cortisol from the adrenal cortex. A typical diagram representing a hypothesis for the mechanism of initiation of parturition in the cow is shown in Figure 1.2. The release of cortisol induces the 17α-hydroxylase enzyme in the placenta to start the conversion of progesterone to oestrogen (Kindahl *et al*., 2004). Therefore, the level of progesterone falls and oestrogen increases in the blood. Cortisol also regulates PGF synthesis (Kindahl *et al*., 2004) such that cortisol induces enzymes, which promote PGF synthesis (Goff, 2004).

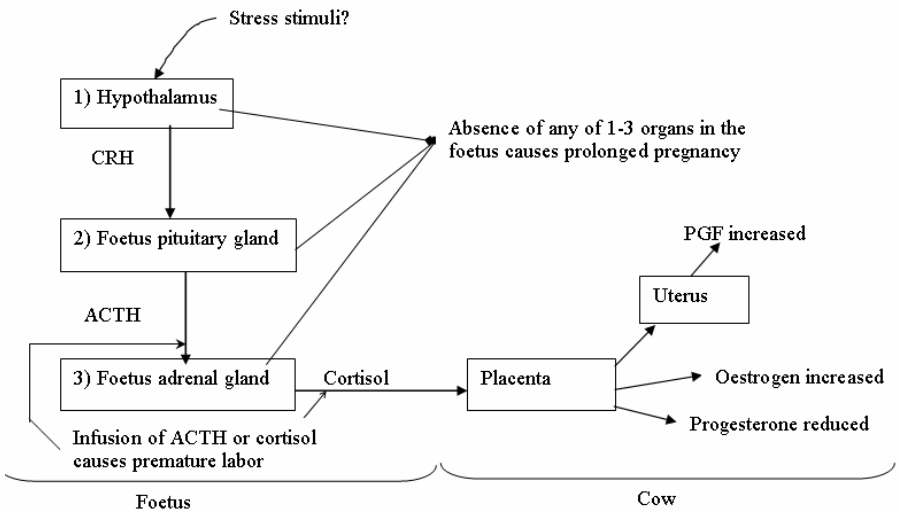


Figure 1.2. Representation of the mechanism of initiation of parturition in the cow.

In the meantime, the reproductive organs of a pregnant cow undergo discrete changes during gestation. The vulva enlarges, becoming noticeable around the seventh month of gestation. The cervix remains tightly closed and is sealed by a mucus plug. Relaxation of the pelvic ligaments occurs gradually during gestation and becomes more noticeable with approaching parturition (Gordon, 1996).

Relaxin, together with oestrogen compounds, soften the cervix, opens the birth canal, and stimulates the release of PGF from the endometrium (Kindahl *et al*., 2004). Softening of the cervix is due to changes in collagen fibres. These changes lead to loss of the mucus plug followed by dilation and opening of the cervix as well as the stretching of the vagina, which in turn causes neural impulses to the brain through ascending pathways and stimulates the secretion of oxytocin from the pituitary gland, causing the uterine smooth muscles to contract. This makes the uterus become more excitable and starts contractions to propel the foetus into the vaginal canal (Kindahl *et al*., 2004). Therefore, sequential events, some of which are cervical ripening and dilatation, activation of uterine musculature, attainment of the appropriate posture by the foetus, expulsion of the foetus and detachment and expulsion of the placenta, make up the process of natural parturition (Gordon, 1996). The placental detachment process initiates several weeks before parturition and reaches completion prior to the day of parturition (Königsson, 2001). The final detachment occurs along the interface borders between the foetal and the maternal parts of the placenta.

1.2.5.2 Parturition failures

Complex foetal endocrine dysfunction can lead to growth retardation of the foetus (Hafner *et al.,* 1991). For instance, hypoplasia of the anterior pituitary gland and adrenal cortex dysfunction suggest that a failure in the initiation of parturition results from a foetal deficiency of ACTH and cortisol hormone (Figure 1.2). The lack of foetal brain development can cause prolonged pregnancy since the hypothalamus does not release CRH which triggers the release of ACTH from pituitary (Wintour *et al*., 1996). In addition, surgical disconnection of the pituitary from the hypothalamus (hypophysectomy) prevents parturition (Poore *at al.,* 1999). Furthermore, some studies have found that an ingestion of certain species

of teratogenic plants (i.e., Lupinus formosus and Lupinus arbustus) during specific gestational periods harm the unborn calf, which leads to birth defects and the need for cows induction (Panter *et al.,* 1998; Keeler *et al.,* 1976).

1.2.5.3 Drug intervention to prolong or to shorten pregnancy

Pregnancy can be prolonged or shortened through drug intervention. For example, when pregnant cows are given norgestomet, a progesterone analogues, ear implants, the pregnancy was prolonged and within 36-47 hours after the removal of the implants, the expulsive stage of calving will start (Janszen *et al.,* 1990). This is due to progesterone withdrawal, which is a prerequisite for the normal parturition process. On the opposite end of the spectrum, an intrafoetal infusion of dexamethasone can shorten pregnancy and induce premature parturition in cattle at six weeks before full term (Fairclough *et al.,* 1981b). A study showed that dexamethasone infusions are more effective when given to the foetus rather than the maternal cow. Intrafoetal infusion of prostaglandin can also induce premature parturition in sheep and does so by activating the foetus HPA axis (Young *et al.,* 1996). An infusion of ACTH to hypophysectomised foetuses can also induce labour (Figure 1.2). Similarly, an infusion of cortisol to foetus can lead to premature parturition (Ingram *et al*., 1999). Feeding pine needles to pregnant cows in late gestation causes incidences of premature parturition due to a change of cortisol, progesterone and oestrogens metabolism (Short *et al.,* 1993). In fact, the feeding of pine needles shortened pregnancy by about two weeks and induced a premature normal rise in cortisol and estradiol-17β associated with parturition (Short *et al.,* 1989). Foetal cortisol triggers the parturition process and causes dramatic changes of oestradiol-17β, PGF and progesterone levels in the maternal cow. The levels of cortisol and other hormones in both foetus and the maternal cow close to parturition will be elaborated in the following section.

1.2.5.4 Foetal cortisol, oestrone and oestradiol-17β levels during normal calving

Fairclough and others (1975) investigated plasma cortisol concentrations in the foetus and the maternal cow near full term. Foetal cortisol levels increased

progressively from around 10 ng/mL, 9 days before term, to a maximum 60-100 ng/mL at parturition. This six to eight fold increase in foetal cortisol concentrations over the last 10 days of pregnancy was also reported in other studies. For instance, McGowan *et al*. (1975) found that the foetal cortisol levels were less than 10 ng/mL three weeks before normal parturition, then rose to a peak of up to 80 ng/mL on the day of parturition. In another study, it was found that foetal cortisol rose slowly from 5.0 ± 0.7 ng/mL at 20 days to 9.3 ± 3.0 ng/mL at 10 days before term and then progressively increased to a mean of 74 ng/mL (Hunter e*t al*., 1977), whereas, the foetal oestrone and oestradiol-17β concentrations showed little or no change toward term and were both less than 50 pg/mL.

1.2.5.5 Maternal cortisol, oestrone, oestradiol-17β and PGF levels of normal calving

The maternal cortisol levels remained below 20 ng/mL up to the day of parturition (Fairclough *et al*., 1975; McGowan *et al*., 1975). The maternal utero-ovarian oestrogen levels rose slowly from 20 to 10 days pre-partum and then increased more rapidly reaching peak levels of 2.9 ± 0.6 ng/mL for oestrone and 1.4 ± 0.3 ng/mL for oestradiol-17β, 1 to 4 days before delivery (Hunter e*t al*., 1977). The maternal progesterone concentrations declined towards term and showed a rapid decrease over the last 36-48 hours before calving. This was concomitant with a gradual rise of PGF until the last 24 hours where there was a dramatic increase, reaching peak levels (5.7 ± 0.6 ng/mL) during labour. During normal parturition, cortisol concentrations increase and continue to rise during foetal expulsion, peak until levels are altered when the calf is born. Levels return to preparturition values one day after calving (Hydbring *et al*., 1999).

1.2.6 Hormonal blood levels associated with induced calving

Kaker *et al*. (1984) investigated hormonal profile changes between induced and spontaneous calving cows during and after the induction of parturition using dexamethasone with or without the aid of PGF and found: 1) there were no major differences in progesterone, oestradiol, and prostaglandin concentration between

the induced and non-induced cows before or after the treatment, 2) treated cows, which retained foetal membranes, had lower progesterone values one to two days before calving compared to their non-induced counterparts, and 3) PGF concentrations steadily increased in the treated cows in contrast to the dramatic increase that occurred in non-induced cows in the last two days of pregnancy. The study concluded that the pre- and post-partum hormonal profiles and fertility were similar in induced and non-induced cows. Perhaps this similarity between the two groups was because the cows were almost close to their normal calving time and, therefore, the induction programme would have no major impact on them. In both spontaneous and induced calving, maternal plasma progesterone levels fall before oestrogen levels rise suggesting that the induced treatment might mimic the physiological mechanisms by which the foetus induces parturition in cattle. However, cortisol, the trigger hormone or the dexamethasone levels, were not monitored in this study. In another study, the effect of the infusion of dexamethasone into foetal calves, which induced premature parturition in six cows (at day 240 of gestation) on the maternal and foetal hormonal changes were investigated (Fairclough *et al*., 1981b). Three infusion rates of 0.1, 1.0 or 10 mg/day, which induced calving in 12, 9 and 3 days, respectively, were tested. The maternal hormonal changes observed for all cows were similar. Progesterone levels declined and both oestrogen and PGF concentrations increased before parturition. The foetal plasma cortisol levels were reduced by the higher infusion rates (1.0 or 10 mg/day) during the entire infusion. At the lower infusion rate (0.1 mg/day), there was a rise in foetal cortisol concentration from 5 to 60 ng/mL during the last 2-3 days before parturition. This level of cortisol is similar to that found at naturally occurring delivery as previously discussed. Dexamethasone had no effect on the foetal oestrogen levels at the lowest infusion rate, but at the rate of 1.0 mg/day or greater there was a marked rise in the foetal plasma levels of oestrogen. The study also showed that the effect of dexamethasone infusion on hormonal changes was dose dependent (Fairclough *et al*., 1981b).

1.2.6.1 The pharmacokinetics of dexamethasone in cows

The pharmacokinetics of dexamethasone were studied in four non-lactating dairy cows all of which received dexamethasone and dexamethasone isonicotinate via

the intravenous or the intramuscular route (0.1 mg/kg) (Toutain *et al*., 1982). Following intravenous administration, the half-lives were similar (5.6 and 4.9 hours, respectively) for dexamethasone and dexamethasone isonicotinate. The peak plasma concentrations reached maximum values within one-minute post injection and bioavailability was approximately 75%-80%. The drug was largely distributed in the tissues and only a fraction of the intravenous dose reached the systemic circulation as intact drug (Toutain *et al*., 1982). The plasma concentrations of both formulations showed a very rapid and high blood response (very sharp initial concentrations can be toxic to the animal subjects) with ranges from 500 to 600 ng/mL, followed by a three phase declining profile. The first and second phases were rapid (0-16 and 16-60 minutes, respectively) whereas the final phase was prolonged and showed the elimination of the drug up to 24 hours later. This study found that the two formulations produced similar results, 1) showing dexamethasone isonicotinate releases dexamethasone as the active compound, and 2) the ester bond hydrolysis was very rapid and complete since the dexamethasone concentration in plasma reached the highest level within one minute. Like wise, following intramuscular administration of dexamethasone or dexamethasone isonicotinate (0.1 mg/kg) in the same number of cows, the half-lives showed no significant difference between the two formulations (6.6 and 6.1 hours). For both formulations the plasma concentrations initially increased, the peak plasma concentrations (ranges between 42 and 44ng/mL) were reached at 3 to 4 hours post injection and bioavailability was approximately 70%. The plasma concentrations reached a plateau level and subsequently the concentrations decreased as observed at the post intravenous injection. Both the intravenous and intramuscular dexamethasone injections showed different plasma profiles. The dexamethasone was available in the blood circulation immediately after the intravenous administration, whereas with the intramuscular administered dexamethasone, absorption took place before the drug was available in the blood circulation. The half-lives of dexamethasone absorption range between 1.5 and 2.0 hours for the two formulations, respectively. The plasma concentration of dexamethasone in cows following the administration of dexamethasone esters showed that plasma dexamethasone concentrations were elevated for 3-4 and 7-8 days following intramuscular injection of soluble short-acting and insoluble long-

acting dexamethasone esters, respectively (Fairclough *et al*., 1981a). For instance, the plasma concentration of dexamethasone in cows injected intramuscularly with either 20 mg of dexamethasone trimethyl acetate or tributyl derivative reached peak levels of 0.6-1.1 ng/mL of dexamethasone in 2-6 days then declined to undetectable levels (<0.15 ng/mL) 14 days after the injection, whereas the equal dose of dexamethasone sodium phosphate resulted in the plasma concentrations of dexamethasone which increased sharply to maximum levels of 24-70 ng/mL within 2-20 minutes and fell to undetectable levels (<0.15 ng/mL) after 3 days. In the case of dexamethasone tributyl acetate the plasma concentration between the treated cows showed a similar pattern with maximum levels of 0.7-1.1 ng/mL occurring 1-7 days following the injection. In the case of dexamethasone sodium phosphate treatment the plasma concentration between the treated cows showed a considerable variation with a peak value ranging from 22-70 ng/mL. This study demonstrated that the insoluble ester formulations of dexamethasone could lead to a sustained release of dexamethasone into systemic circulation from intramuscular injections.

Dexamethasone pharmacokinetics in sheep using two injectable dexamethasone formulations-water suspension of dexamethasone associated with albumin microspheres (AM) and water-alcohol solution of dexamethasone were studied (Dilova *et al*., 1999). Following intramuscular injection of a water suspension containing dexamethasone-AM (at 1 mg active substances/kg of animal body weight), a plateau of plasma concentration of 23-25 ng/mL was observed within the animal blood levels between 72-120 hours post injection, and then subsequently the concentration decreased. Dexamethasone blood levels showed a gradual decrease until six days post injection. In contrast, the blood plasma concentration of dexamethasone obtained after injection of 1 mg dose/kg of animal body weight containing water or alcohol solution, which reached a short time peak of 55-59 ng/mL at about 3 hours post injection and then concentration decreased drastically and returned to the baseline concentration within several hours. This study demonstrated that a sustained release of dexamethasone for a period of a week could be achieved when dexamethasone is associated with MA.

1.2.6.2 Current dexamethasone delivery systems for inducing calving and its shortfalls

To imitate the natural slow rise of cortisol during parturition, a two-injection schedule has been developed for current induction protocols (Verkerk *et al*., 1997). The current treatment is based on injection of late pregnant cows with insoluble esters of dexamethasone from which the drug is released over two weeks after the injection and followed by a further boost injection of soluble dexamethasone. The initial priming dose of a long- acting steroid, followed some days later by injection of a short- acting formulation, was developed to produce a reliable and predictable response. Between the two injections the treated cows may or may not respond to the first long acting steroid. The first injection, an intramuscular injection of a long acting depot corticosteroid such as of 21 mg dexamethasone isonicotinate (Voren AP, Boeringer-Ingelheim (NZ) Ltd; I1) initiates the induction process. Cows that have adequate udder development and vulva enlargement after 10-15 days receive a further intramuscular injection, of 20 mg dexamethasone sodium phosphate (Dexadresin V, Pharmaco (NZ) Ltd; I2), which results in parturition typically within 3-4 days. If udder development is not adequate, an additional I1 is administered. During the treatments, all relevant information is recorded, and all cows receiving I2 are examined at 3-5 days post-partum to determine the presence of retained foetal membranes (Verkerk *et al*., 1997).

Presently, limited data exists on dexamethasone pharmacokinetics in plasma due to current calving practices. However, studies demonstrated dexamethasone blood levels followed by the intramuscular injection of insoluble (long-acting) or soluble (short-acting) dexamethasone esters differed from cortisol levels found during natural delivery. For instance, the long-acting preparations produced increase of plasma dexamethasone concentrations in the treated cows within a week following the intramuscular injection then declined to undetectable levels within two weeks after the injection (Fairclough *et al*., 1981a). The short acting preparations resulted in the plasma concentrations of dexamethasone, which increased sharply to reach maximum levels within a short time and fell to undetectable levels after 3 days. An ideal dexamethasone preparation for induced

calving should produce dexamethasone plasma levels that slowly increase, continue to rise around parturition and peak when the calf is born. Finally, the concentration of dexamethasone in plasma should return to preparturition levels after calving.

Based on the foregoing studies, dexamethasone formulations need to be frequently administered by injection or infusion in order to maintain the desired hormonal blood levels until calving occurs. In addition, dexamethasone activity depends on the route of administration i.e., intramuscular versus intravenous injection (Toutain *et al*., 1982) and the type of dexamethasone esters - dexamethasone-21 trimethyl acetate versus dexamethasone-21 sodium phosphate used (Fairclough *et al*., 1981a). Suffices to say that both short and long-acting dexamethasone preparations showed declining profiles and dexamethasone levels were not maintained within a desired range during the treatment. This is the short fall of the current treatment and hence, finding a controlled drug release system to achieve a sustained dexamethasone release during the treatment would be advantageous.

1.2.6.3 Some conceptually developed dexamethasone delivery systems

An injectable biodegradable sustained release formulation was developed when drug compounds were entrapped in cross-linked bovine serum albumin microbeads (Lee *et al*., 1981). The bovine serum albumin (BSA) is used to bind many drugs. This binding is reversible and retards drug release from an injection site until the albumin is degraded by enzyme proteolysis.

Dilova *et al.* (1999) has developed a preparation of BSA microspheres associated with dexamethasone. BSA microspheres containing dexamethasone were manufactured by means of heat denaturation. About 0.1 g of dexamethasone was suspended in 10 mL of a sodium phosphate buffer and 1 g of BSA was dissolved in the suspension. 100 mL of sunflower oil was then added to the suspension and the mixture was emulsified at 1000 rpm. The resulting emulsion was added to 400 mL of preheated sunflower oil. Heating and stirring were maintained for 60 minutes at 100°C. The suspension was then allowed to cool to 25°C. After

filtration and washing with ether, the microspheres were stored at 4°C until used. An evaluation of the potential use of albumin microspheres as a drug delivery system to provide sustained release of dexamethasone *in vivo* has been investigated (Dilova *et al*., 1999). *In vivo* dexamethasone release from albumin microspheres was achieved for a period of a week, but non-linear release over time and declining profiles were inevitable. Therefore, another route of dexamethasone delivery is essential as intramuscular and intravenous routes failed to produce a linear drug release over time.

1.2.6.4 The vagina as a route for dexamethasone delivery

The vagina as a route of drug delivery has been known since ancient times (Hussain & Ahsan, 2005). The vaginal route offers numerous advantages as a site for drug delivery, such as convenient access, prolonged retention of formulations, a great permeation area, high vascularization, relatively low enzymatic activity, and the avoidance of hepatic first-pass metabolism. The level of interest in both local and systemic vaginal drug delivery systems has increased considerably within recent years (Bernkop-Schnürch & Hornof, 2003). In addition, various auxiliary agents some of which are permeation enhancers, such as bile salts or solubility-enhancing agents including cyclodextrins, have been developed for vaginal use to improve drug solubility and bioavailability at the site of delivery.

1.2.6.4.1 Intravaginal controlled drug delivery

The vagina has been recognized as a potential route for progesterone administration since the mid-Seventies when it was used to control oestrous cycles in cattle (Rathbone & Macmillan, 2004). Progesterone was homogenously incorporated into a silicone based type-matrix to control drug release at a predefined and reproducible rate for prolonged periods. Drug release from the polymer matrix followed a diffusion controlled process based upon Fick's Law (Rathbone *et al*., 2001). Initially the diffusion process starts with the elution of the solid drug near the surface of the polymer matrix. This leads to the formation of a drug depletion zone within the matrix over time. The depletion zone becomes larger and larger and the drug requires more time to diffuse to the interface

between the delivery system and the site of delivery. This increased diffusion path causes a non-linear dependence of drug release with extended time. In addition, dexamethasone compounds are ionisable unlike progesterone (a nonionisable compound), and might exhibit low solubility in a silicone matrix and a better solubility in a hydrophilic poly-ε-caprolactone polymer (Section 2.4.1.7.3).

However, the passive diffusion controlled mechanism of polymeric matrix delivery systems may be replaced by active control, such as a battery driven pump as part of an intravaginal device. More recently, IBD (Intelligent Breeding Device) and EMID (Electronically Modulated Intravaginal Device) for delivering multiple drugs simultaneously have been conceptually developed (Rathbone & Macmillan, 2004). Both the IBD and EMID use a gas controlled delivery system, which is capable of delivering multiple drugs at various times and in various patterns. A detailed comparison of the similarities and differences of the two delivery systems are elaborated on in Chapter Four (Sections 4.1.1 and 4.1.2). Most recently, drug delivery scientists have also developed an electronically controlled drug delivery system that is designed for use in the vagina of dairy cows (Cross *et al*., 2004). Unlike IBD and EMID, this device has electronic components which are built from off-the-shelf parts based on a 16-bit microcontroller and a 433.92 MHz radio transceiver placed inside a syringe. The microcontroller reads and logs sensor data and controls drug release, which is accomplished using electrolytic gas production from a gas cell the current of which is controlled by the microcontroller. Similarly, to the IBD and EMID devices, the produced gas pressure propels the syringe piston and releases the formulation. Delivery from the device could be remotely controlled from the animal or overridden via the wireless link to deliver an arbitrary and complex variable-rate profile of a drug. However, as the time the present research was carried out only IBD and EMID were available.

1.2.6.5 Hypothesis for possible solutions for induced calving

The electronically controlled drug delivery technologies could be programmed to deliver an increasing amount of drug over time, thus producing a slowly rising blood profile that would mimic closely that seen naturally. The new drug delivery system, which could deliver dexamethasone intravaginally to its desired site of

action in a controlled way to maintain effective levels for a desired period, would be an advantage. The technology may provide prolonged delivery of the drug while maintaining its blood concentration within therapeutic limits, thereby achieving a desired release profile of dexamethasone until parturition. The device could be loaded with formulated long- acting dexamethasone suspension and the rate of drug release that could be adjusted by controlling the rate of gas production by the battery driven pump.

1.2.6.5.1 Sub-hypothesis for drug delivery

As demonstrated, different dexamethasone formulations produced varied blood profiles using the same route (Toutain *et al*., 1982). The best dexamethasone candidate for induced calving must be identified and the goal is to identify a dexamethasone analogue with high absorption rates across the vaginal mucosa. This may be achieved by:

- developing an *in vitro* permeation method to screen dexamethasone analogues to monitor their ability to permeate non-biological and biological membranes.
- formulating dexamethasone analogues to enhance their aqueous solubility and permeation through these membranes.
- developing UV and HPLC assays to characterise the formulated dexamethasone compounds.
- assessing the intravaginal drug delivery system for suitability to deliver dexamethasone formulation and also to develop an appropriate release assessment method for the device.
- conducting some preliminary experiments of *in vivo* animal work.
- assessing the outcomes and recommend future work.

1.3 Oestrous synchronisation in cattle

Oestrous synchronisation is the manipulation of the reproductive cycle such that all treated cows come into oestrus at a predefined period with normal fertility (Kesler *et al*., 1995). Oestrous synchronisation involves administration of hormones, which are delivered in a synchronous fashion and that are released in a similar manner to by the endogenous endocrine system thereby simulating the blood level changes of these hormones in a normal oestrous cycle in cows with the aim of controlling the oestrous cycle (De Rensis & Peters, 1999; Rathbone *et al*., 2001). More specifically, oestrous synchronisation involves the manipulation of ovarian activity so that the time of ovulation can be predicted without the need for oestrous detection. The blood level concentration changes of progesterone and oestradiol during oestrous cycle regulates reproduction and oestrus behaviour of cows.

Reliable oestrus detection in cows requires several periods of intensive daily observation and is, therefore, time consuming and expensive for dairy farmers (De Rensis & Peters, 1999). The average rate of oestrus detection is about 50% with wide variation amongst dairy farmers. This may lead to a large proportion of animals being artificially inseminated while they are not in oestrus. In addition, different farmers have different objectives in what they wish to achieve among their dairy cows and some may want to have as many cows as possible becoming pregnant by artificial insemination (AI) within a particular time span or to have calving occur a short period of time. Some other reasons why farmers would consider controlling the oestrous cycle in their dairy cattle (Rathbone *et al*., 2000) include: 1) reducing the time and labour costs in detecting a reliable oestrous time, 2) allowing for more cost effective implementation of timed AI programmes, 3) increasing the use of AI with fresh, transported or frozen semen in order to use desirable genes, 4) allowing seasonal breeding at optimum time in the season and 5) maximising reproductive efficiency with ovulation control programmes to provide producers with substantial opportunities to reduce production cost and enhance profitability.

Ideally, farmers want to achieve a predetermined time for effective mating with normal fertility without having to carry out oestrous detection of their herds. In addition, for cows to become pregnant and to avoid any failures of insemination, oestrus must be accurately identified (Gordon, 1996). The identification of a precise method of inducing and synchronising oestrus and ovulation in cows would be an important advance in cattle management so that cattle may be artificially inseminated at predetermined times (De Rensis & Peters, 1999). The methods of controlling the oestrous cycle, recent advances of the physiology and endocrinology of bovine oestrous cycle are discussed in the following sections.

1.3.1 Physiology and endocrinology of bovine post-partum

Cows are normally pregnant for 285 out of 365 days of a year, leaving only 80 out of 365 days to conceive following calving to maintain an optimal calving pattern (Nation, 1997). In addition, cows require several weeks to recover from calving and begin a new cycle. During this period, cows undergo a change in physiological state from pregnancy to the initiation of lactation and resumption of a fertile oestrous cycle. The period after calving is known as post-partum, which is defined as the interval from parturition to complete uterine involution (Lewis, 1997). During this period, the uterus shrinks to resume its pre-pregnancy size and ovaries regain their cyclical activity (Kindahl *et al.,* 2004; Nation, 1997). Uterine involution may require 30 days post-partum followed by a return of the oestrous cycle (Nation, 1997). The post-partum period is divided into three sub-periods (Lewis, 1997):

1. Puerperal, the interval from parturition until the pituitary gland becomes responsive to GnRH which lasts approximately 7 to 14 day,
2. Intermediate, the interval from the time that the pituitary gland becomes responsive to GnRH to the first post-partum ovulation,
3. Postovulatory, the interval from the first ovulation to complete uterine involution.

The post-partum anoestrus duration or the interval of sexual inactivity between the parturition and the following oestrous period varies considerably among cows with many contributing factors (i.e., parity, breed, health, nutrition level, body

condition, etc.) and has been reviewed by Nation (1997). Furthermore, the first ovulation is often not accompanied by oestrus, in which case, the first expression of oestrus accompanies the second ovulation, post-partum. In addition, as Nation (1997) indicated, some cows may not show any oestrous cycle for at least 60 days because of the prevalence of post-partum anoestrus. This leaves only a small chance for cows to become pregnant again, during which time they must show oestrus so they can be efficiently mated.

1.3.2 Endocrinology of the bovine oestrous cycle

The oestrous cycle is controlled by hypothalamic-pituitary-ovarian axis, where the ovarian oestradiol acts on receptor cells in the hypothalamus to secrete GnRH, which in turn, causes the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. LH and FSH promote steroid synthesis, ovarian follicular growth and ovulation. GnRH plays a crucial role in regulating ovarian activity during the normal oestrous cycle of a cow, as well as in initiating ovarian activity prior to the onset of puberty and after periods of anoestrus. The bovine oestrous cycle is generally divided into four subsequent phases and are referred to as proestrus, oestrus, metoestrus, and dioestrus, on the basis of the hormonal changes that occur (Hafs *et al*., 1976). The oestrous cycle is also divided into two major phases generally known as the follicular and luteal phases, on the basis of the different structures present in the ovary (Evans, 2003; Rathbone *et al*., 2001). The follicular phase is comprised of proestrus and oestrus and lasts from 3 to 7 days. The proestrous and oestrous periods are primarily controlled by oestrogen levels and are associated with growth of the follicle and, therefore, are called the follicular phase of the cycle. The metoestrous and dioestrous periods are associated with growth of the corpus luteum (CL), are primarily under the influence of progesterone and called the luteal phase of the cycle.

1.3.2.1 Proestrus

Proestrus is the period of the menstrual cycle that begins from the time of regression of the CL to the onset of the new oestrous cycle. The proestrus period

lasts 2-6 days during which time a dominant ovulatory follicle matures. During this maturation period, the dominant follicle produces increasing amounts of -17β oestradiol. When the -17β oestradiol levels peak in the presence of undetectable levels of progesterone, the proestrous period ends and oestrus commences (Rathbone *et al*., 1998).

1.3.2.2 Oestrus

Oestrus is the period of sexual receptivity in the cow and is characterised by the animal being willing to be mounted by other herd mates, both male and female (Gordon, 1996). Other behavioural signs of oestrus include vulvar swelling, restlessness, clear vaginal mucus discharge, and bellowing. Oestrus is the period of receptivity of a cow to a male. The oestrous period lasts for 8-30 hrs (Rathbone *et al*., 1998) or 12-24 hrs (Downey, 1980). The high circulation concentrations of -17β oestradiol that coincides with a low concentration of progesterone and initiates a surge in LH concentrations that usually exceeds 30 ng/mL (Rathbone *et al*., 1998), which, in turn, ruptures the follicle destined for ovulation.

1.3.2.3 Metoestrus

Metoestrus is the period that begins from the cessation of oestrus to the early development of the CL. The metoestrous period ranges between 0-5 days starting from day 0, on which ovulation takes place and is characterised by the formation of the CL (Figure 1.3). Cells of the granulosa and theca internal layers of the ruptured ovarian follicle form the CL (Gordon, 1996). These granulosa and theca internal layers start to transform and differentiate by forming large and small luteal cells, respectively. The former cells secrete progesterone and oxytocin and are responsive to PGF whereas the latter cells secrete progesterone and are responsive to LH. During the metoestrous period progesterone levels start to increase beginning on day 3 or 4 from around 1 ng/mL to 4 ng/mL (Rathbone *et al*., 1998).

Figure 1.3 presents idealised changes in blood progesterone, oestradiol and LH during the bovine oestrous cycle (Hafs *et al*., 1976).

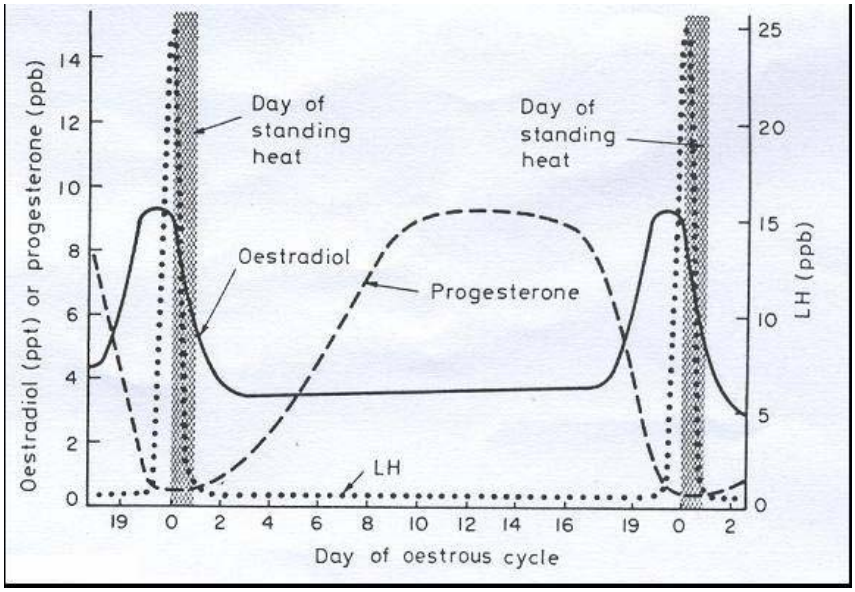


Figure 1.3. Changes in hormone concentrations during the cow's inter-oestrous cycles (Hafs et al., 1976).

1.3.2.4 Dioestrus

Dioestrus is the period of the oestrous cycle when the CL is fully functioning and lasts for 6-18 days. During this period, the progesterone concentration produced mainly by the large luteal cells, rapidly increases from 6 ng/mL and reaches 10 ng/mL by days 8 or 9 and remain elevated for the duration of the CL life span (Gordon, 1996; Rathbone *et al*., 1998; Rathbone *et al*., 2001; Mann *et al.,* 1998; Downey, 1980; Hafs *et al*., 1976) before starting to decline on day 18 (Figure 1.3).

1.3.2.5 Follicular development during the oestrous cycle

The selection of a single dominant follicle, which attains the ability for final maturation and ovulation during the oestrous cycle, culminates in ovarian follicle growth in the cattle (Mihm *et al*., 2002). Ovarian follicular development occurs in a wave-like pattern (Evans, 2003). Two or three waves of follicular development occur during the oestrous cycle (Soboleva *et al*., 2000). Each wave consists of a group of growing follicles, classified into larger dominant and smaller follicles. Over several days, one follicle grows larger than the others and is selected to

become the dominant follicle while the smaller follicles regress (Fortune, 1993). The first follicular wave emerges on day 1 of the cycle and the selection of the dominant follicle takes between days 2 and 3 of the cycle and the follicle becomes dominant between days 4 and 5 (De Rensis & Peters, 1999). The dominant follicle reaches its maximum size between days 6 and 7 of the cycle. This is followed by a period of relative stability between days 6 and 10 and then it decreases in size and is no longer identifiable by day 15. This loss of the dominant follicle coincides with emergence of a new follicle wave, preceded by a transient increase in FSH. The second dominant follicle emerges on day 10 of the cycle and will ovulate 11 days later.

Animals with three follicular waves, the first, second and third follicular waves emerge on days 1, 9 and 16, respectively. The third dominant follicle ovulates 7 days later (De Rensis & Peters, 1999). Therefore, oestrous cycles with cows with two follicular waves are 2 days shorter than the three wave cycles (21 versus 23 days). However, it was found that cattle populations differ in the relative proportion of cows exhibiting two wave versus three wave cycles. Some publications have reported that 80% of cows have three wave cycles whereas others have also found that about 80% of cows have two wave cycles. It is clear that the first dominant follicle does not ovulate during the normal bovine oestrous cycle. Yet the dominant follicle that develops in the presence of functioning CL will undergo atresia due to the absence of a frequent LH pulse pattern inhibited because of high progesterone concentrations. Therefore, only the dominant nonatretic follicle that is present when the CL regresses has the opportunity to ovulate.

1.3.2.6 Luteolysis

The three hormones responsible for the initiation of luteolysis are progesterone, oestradiol and oxytocin (Goff, 2004). Together they regulate the uterine secretion of PGF that causes regression of the CL. Progesterone can regulate PGF secretion such that prolonged exposure of the endometrium to progesterone promotes the endometrial accumulation of arachidonic acid, a precursor of PGF and the cyclooxyginase enzyme which are necessary for the synthesis of PGF. On the

other hand, progesterone also exerts a suppressive effect on PGF secretion by blocking oxytocin receptor gene expression during the early and mid-luteal phases of the oestrous cycle (Ivell *et al*., 2000). The progesterone block to oxytocin receptors is lost after 12 days of continuous exposure, possibly due to the loss of progesterone receptors (Goff, 2004). At the end of the luteal phase, oxytocin triggers a positive feed back loop on the endometrial oxytocin receptor mediating the release of PGF, which feeds back to the CL to cause the secretion of more oxytocin and to induce luteolysis. During the luteal phase, there is an increase of oestradiol and oestradiol receptors, whereby the activated oestradiol receptors stimulates the up regulation of oxytocin receptors. The oestradiol effect is dependent on progesterone exposure to the endometrium because the effect is only observed after the endometrium has been primed by progesterone for a certain period of time. At the end of the luteal phase, the endometrium becomes responsive to oxytocin to initiate the excitability and the contraction of the myometrium. Therefore, progesterone controls the timing of luteolysis by mediation through the control of endometrial oxytocin receptors.

1.3.3 Introduction to control of the oestrous cycle

The main methods of controlling the oestrous cycle are through the manipulation of the life span of the CL by either prolonging the luteal phase or by use of progestagens or by inducing premature luteolysis by prostaglandins. Manipulation of the follicular phase by lengthening/shortening the luteal phase or inducing premature luteolysis using pharmacological hormone treatments can control the oestrous cycle (De Rensis & Peters, 1999). GnRH or human chorionic gonadotrophin (hCC) has recently been used for the manipulation of follicle development to achieve precise synchrony of ovulation. Oestrus has been synchronised in cattle with progestorone-oestradiol or progesterone-PGF combinations (Odde, 1990).

1.3.3.1 Oestrous control with PGF

Traditionally, single drug therapy for oestrous control was established once PGF was identified as the natural luteolytic hormone in cattle (Lauderdale, 1975). The

luteal phase can be terminated or shortened prematurely by intramuscular or vulvar subcutaneous injection of PGF. This compound causes regression of the CL and a decrease in circulating progesterone, thereby allowing the resumption of gonadotrophic stimulation to release GnRH which, in turn causes a surge of LH and FSH to initiate follicular development and maturation so that responding cows should show oestrous signs 2-4 days after treatment (Rathbone *et al*., 2001). The limitation of this method is that only 50-70% of the treated animals responded to a single administration as PGF injections can only be effective during the luteal phase of the cycle. Fertility of oestrus after PGF treatment did not differ from that of untreated animals (Odde, 1990). Assuming cows have a 21-day oestrous cycle there might be two populations of cows, one which would be without CL while the other group might have a fully functioning CL (i.e., from day 5 onwards of the oestrous cycle) at the time of the treatment. All cows that have CL, are potentially responsive to PGF injection, which will cause regression of CL, reduction of blood levels of progesterone, and return to oestrus (Rensis and Peters, 1999). However, PGF treatment is ineffective during the follicular phase of the first 5 days of the oestrous cycle (Lucy, 2001). Moreover, cows injected between days 7 and 8 or 15 and 16 of the cycle showed oestrus earlier than those injected between days 12 and 14. It was suggested that the considerable variation in the interval from PGF treatment to oestrus and ovulation could be attributed to the status of the follicular wave at the time of treatment. Finally, oestrus is not synchronised precisely using PGF treatment since animals were detected in oestrus over periods of 5 days (De Rensis and Peters, 1999). Therefore, two PGF injections 11 days apart are necessary to improve synchrony of oestrus. Double PGF injections 11 days apart applied to heifers induced a high oestrous response (80-90% responding between 2-4 days), but it was less precise in the lactating cows (Gordon, 1996).

1.3.3.2 Oestrous synchronisation with progesterone

Progesterone or its analogues have been used in oestrous synchronisation programmes for cattle for many years (Whisnant & Burns, 2002). These include 1) feeding in meal, 2) injection in oil or other media, 3) subcutaneous implants, 4) by way of intravaginal devices such as PRID (Progesterone Releasing Intravaginal

Device, which consist of a stainless steel coil and looped nylon structure covered by a layer of progesterone impregnated silastic), CIDR™-B (Controlled Internal Drug Release Device, which is made of a T-shaped nylon structure covered by a layer of progesterone impregnated silastic) or sponges (polyurethane sponges containing progesterone) (Gordon, 1996).

The main biological functions of progesterone include oestrous suppression and inhibition of a pre-ovulatory LH surge during the insertion of the progesterone releasing device in the animal (Odde, 1990). The oestrous cycle resumes for a cycling cow upon the removal of a device (Kesler & Favero, 1995). Rathbone *et al*. (2000) have reviewed several progesterone delivery methods and their administration routes (i.e., intramuscular injection, oral administration through the diet, ear implants, and intravaginal administration) for oestrous control of domesticated livestock.

1.3.3.3 Commercial progesterone releasing intravaginal inserts for the oestrous control

Commercially available progesterone releasing intravaginal drug delivery systems include PRID, CIDR™-B, Cuemate™, INVAS (Intravaginal Application System comprising a polypropylene spine, which is covered by progesterone matrix), Rajamehendran rubber tubing, containing progesterone and oestradiol-17β and sponges. These devices are inserted intravaginally and are usually left in place for between 7 and 12 days. Progesterone is released slowly from the device and absorbed into the system via the vaginal mucosa, the luteal phase condition will be maintained entirely by progesterone absorbed from the inserts. This will prevent oestrus and ovulation for the treatment period and all treated cows should commence a follicular phase immediately after removal of the device (Rathbone *et al*., 2001).

It is essential that an intravaginal device is retained in an animal during the treatment. Retention rates of the PRID and CIDR™-B are reported to be 95 and 99.2%, respectively (Rathbone *et al*., 2000). The CIDR™-B is further elaborated because of the particular interest of this thesis. The CIDR™-B has been

commercially available since 1987 (Rathbone *et al*., 2002). A product containing 1.92 g of progesterone (the same drug load of PRID) was initially formulated to deliver progesterone for a 12-day period based on the knowledge that progesterone provides potent suppression of oestrus and ovulation following insertion. The removal of a device causes a rapid drop in progesterone concentration in the blood circulation and promotes oestrous synchronisation within a herd and allows for mass artificial insemination to take place. The amount of progesterone released during a 15-day treatment period was highly repeatable and is dependent on the initial progesterone content (Macmillan& Peterson, 1993). In this study, however, individual treated animals showed wide variations in plasma progesterone concentrations. The progesterone blood levels achieved using commercially approved progesterone releasing products, CIDR™-B as an example, provided blood progesterone levels less than those observed (4-5 ng/mL from Rathbone *et al*., 2002) during the natural mid-luteal phase (9-10 ng/mL see Figure 1.3) (Rathbone *et al*., 2001). To date oestrous synchronisation programmes have been shortened to 7-8 days. In this period, a sustained progesterone delivery that provides a minimum plasma progesterone concentration of 2 ng/mL over the last four days of treatment is required (Rathbone *et al*., 1998). Since oestrous synchronisation programmes are shortened, a high residual drug load in the CIDR™-B after removal is inevitable. Therefore, reduction of the initial progesterone load in the CIDR™-B or reusing the CIDR™-B more than once without compromising the effective plasma profile to induce oestrous synchrony may be an advantage.

1.3.3.3.1 Re-engineering or reuse of CIDR™-B

The residual amount of progesterone remaining in a used CIDR™-B after removal was investigated (Ogle, 1999). Ogle (1999) found that the residual amount of progesterone was dependent upon the length of insertion and the initial progesterone load in the CIDR™-B. Reducing the initial progesterone load would lead to a reduction in the cost of raw material (progesterone) and less environmental concerns for the disposing of waste into the environment.

The re-engineering of commercially available CIDR™-B inserts while reducing the drug load was investigated using a shorter treatment duration of 7 days (Rathbone *et al*., 2002). Both the initial drug load (from 1.92 to 1.34 g) and the residual drug remaining in the product after removal (from 1.31 to 0.72g) were reduced. In addition, the lower drug load CIDR™-B showed the same progesterone plasma levels as the commercially available CIDR™-B.

The blood profiles of the two CIDR™-B products fluctuated between 3 and 3.5 ng/mL for the last six days of treatment (Rathbone *et al*., 2002). However, there was a clear difference in the plasma progesterone profiles between the commercial and re-engineered (approx 5-6 ng/mL versus 4-4.5 ng/mL, respectively) CIDR™-B products prior to day 2. This can be explained by the higher drug load product having a higher drug concentration gradient between the surface of the CIDR™-B and the surrounding vaginal lumen fluids of the cow at the time of insertion compared to the lower drug load product. In addition, a comparative study of pregnancy rates following fixed time AI in beef cattle was investigated (Colazo *et al*., 2004) using CIDR™-B inserts that have been used more than once. In this study heifers were given a new or once used CIDR™-B (CIDRs were sterilised by autoclaving before reuse). Overall pregnancy rate was not affected by either new or once-used CIDRs in heifers treated with a new or once used CIDR™-B (48.3% of 145/300 heifers versus 46.2% of 146/316 heifers). In the same study, cows were given once used and twice used CIDRs. Overall pregnancy rate of cows treated with once used CIDR™-B showed a higher pregnancy rate than those treated with a twice-used CIDR™-B (62.4% of 113/181 versus 48.4% of 88/182 cows, respectively). Furthermore, cows treated with twice used CIDR's in the same cow did not significantly improve the pregnancy rates compared to a single twice used CIDR™-B.

1.3.3.3.2 Cuemate intravaginal inserts for the oestrous control

Cuemate™ is a drug-releasing insert administered intravaginally to cattle. It contains 1.56 g of progesterone used for oestrous control purposes. It consists of a semi-rigid wishbone onto which are threaded two progesterone containing silastic

cylindrical pods which have an enclosed cap end that locks the pods in place on the wishbone (McDougall *et al*., 2004). An injection moulding process is used to manufacture the progesterone impregnated fluted pods of the Cuemate™. The wishbone shaped spine function facilitates the administration, retention and removal of the insert (Rathbone & Macmillan, 2004). There appears to be more than one type of commercially available Cuemate™ and two types of Cuemate™ with a drug load difference (1.56 g versus 1.4 g of progesterone) have been reported (McDougall *et al*., 2004; Hanlon *et al*., 2002, respectively). Cuemate™ is reported to offer excellent animal comfort, efficacy and the ability to re-use the carrier "wishbone" thereby reducing costs to consumers (Hanlon *et al*., 2002). The Cuemate™ consists of two main components, the carrier body and two treatment pods. The carrier body called a "wishbone" is used to hold the pods inside the vagina and is re-usable, being made from a plastic that can be sterilised between treatments. The two "treatment pods" are attached to the arms of the wishbone. The pods contain progesterone (1.56 g or 1.4 g, 10 % w/w) that is formulated into a silicone matrix.

In a study of the comparison of the efficacy of Cuemate™ and CIDR™-B a large scale clinical trial (in 423 cows) for the treatment of anovolatory anoestrus was conducted (Hanlon *et al*., 2002). In this study, 51% and 49% cows were treated with Cuemate™ and CIDR-B™, respectively. All inserts were removed within six days of insertion, and 24 hours later, each cow was given an intramuscular injection of 1 mg oestradiol benzoate. This trial resulted in no significant difference between the 3-day submission rate for cows treated with a Cuemate™ or CIDR-B™ (92 % versus 94 %) or the conception rate to the induced oestrus (41 % versus 43 %). This result clearly demonstrated that Cuemate™ was as good as CIDR-B™ in this particular study.

1.3.3.4 Conceptual drug releasing intravaginal inserts for oestrous control

There are some developed intravaginal drug delivery technologies for progesterone and oestradiol such as IBD, PCL-CIDR (progesterone releasing

intravaginal device, which consists of progesterone homogenously dispersed in a poly-ε-caprolactone matrix) and EMID (Rathbone *et al*., 2000). These drug delivery systems have been viewed by Rathbone *et al*. (2000). The EMID is critically evaluated in this research (Chapter Four).

1.3.3.5 Subcutaneous drug delivery systems for oestrous control

Ear implants of progestagen are available on the market that can efficiently release norgestomet (17α-acetoxy-11β-methyl-19-norpreg-4-ene-20, dione), which is a modified 19-norprogesterone (Kesler & Favero, 1995). Norgestomet implantation in cows for oestrous synchronisation have been reported (Kesler *et al*., 1995; Kesler & Favero, 1995; Kesler & Favero 1996; Kesler *et al*., 1997). Lower doses of the active pharmaceutical ingredient are required because norgestomet is more potent than progesterone (Kesler *et al*., 1995). Examples of these implants are CRESTAR® (silicon implant) and SYNCRO-MATE-B® (hydron implant). Hydron implants are manufactured with norgestomet homogeneously distributed through the hydron, whereas silicon implants contain dispersed crystalline norgestomet in a micro reservoir of aqueous PEG 400 within a matrix of polymerised silicone (Rathbone *et al*., 2000). The norgestomet content of both the hydron and silicon implants is 6 mg (Kesler *et al*., 1995). Both the hydron and silicon implants are cylinders of approximately 2.6 mm in diameter and 2.0 cm in length. These implants are inserted under the loose skin at the base of the ear using a special implantation device. Hydron implants and silicon implants have shown different *in vitro* drug release profiles. Silicon implants release norgestomet in a more homogenous and linear manner, while hydron implants release high quantities in the first two days and the quantity released decreases during the following days (Kesler *et al*., 1995). The implant is removed after 9 days by making a small incision and forcing it out. An *in vivo* study comparing the efficacy of hydron and silicon implants demonstrated that synchronised pregnancy rates were greater for hydron than silicon implants (53% versus 44% respectively) (Kesler *et al*., 1995). However, the requirement of aseptic technique during administration and the need for minor surgery to remove the implant to produce a precipitous decline in progesterone levels have resulted in this product having limited practical application (Rathbone *et al*., 2000).

Compared with the single hormone treatment for oestrous control, the progesterone treatments achieved excellent precision with the onset of the oestrus but with lowered fertility rates, whereas PGF treatments achieved less precision in onset of oestrus as well as variation in the fertility of individual (Rathbone *et al*., 1998). Other hormones such as oestradiol and GnRH have also been used alone to alter the length of the oestrous cycle, but none of these has yet been successfully adapted into single hormone oestrous control programmes like those involving progesterone or PGF, due to their variable effects.

However, single hormone treatment using progesterone has only been used to a limited extent due to route specificity and poor fertility rates. Progesterone cannot prevent the development of persistent dominant follicles during treatment, but can prevent only oestrus and ovulation. Synchrony of oestrus following the use of progesterone alone was not sufficient for success with a fixed time of insemination, and therefore, other drugs are necessary to co-administer with progesterone (Whisnant *et al*., 1999). At the end of the treatment, any persistent dominant follicle must be induced to ovulate, otherwise, this would lead to poor fertility (Rathbone *et al*., 2002). Advances in endocrinology knowledge have demonstrated that the poor fertility associated with the commercial products could be overcome using shorter treatment durations with the addition of co-administered multiple hormones. For instance, >90% versus 85% of cattle can be induced to enter oestrus between 36-60 hours versus 24 hours of the end of treatment when using intravaginal inserts or subcutaneous ear implant progesterone releasing systems, with or without administering oestradiol benzoate, at the end of the treatment period (Cavalieri *et al*., 2004).

Therefore, to achieve adequate oestrous synchronisation without compromising fertility a combination of multiple drugs administered at different rates and times throughout the treatment period is needed. For instance, additional compounds are administered at insertion or removal of a progesterone releasing device. It is well established that inclusion of oestradiol with progesterone appears to intensify the negative feedback on LH secretion compared to progesterone administered alone (Kesler & Favero, 1995). Other reasons for combined treatment are that the CL does not lyse in response to PGF until the fifth day following oestrus onwards and

oestradiol therapy increases the sensitivity of the CL to luteolytic PGF. Delivering more than a single active in combined synchronised luteolysis and ovarian follicle wave patterns could be achieved (Macmillan & Peterson, 1993).

1.3.3.6 Combination of progesterone and oestradiol for the oestrous control

A slow release formulation that involves a single intramuscular injection (i.m.) of biodegradable progesterone microspheres (progesterone + oestradiol benzoate) for controlling bovine oestrus and inducing puberty in beef heifers has been studied (Whisnant & Burns, 2002). These microspheres contained 625 mg of progesterone and 50 mg of oestradiol benzoate in poly-(DL-Lactide-co-glycolide 75:25) microspheres prepared using an emulsion based process (Rathbone *et al*., 2000). Progesterone plasma concentration patterns showed a rapid increase and sharp decline that may allow for oestrous synchrony. This treatment produced progesterone plasma concentrations (4-5 ng/mL) similar to that in the naturally occurring luteal phase. The follicular development and turnover were not determined in this study. About 75% of the treated cows were observed in oestrus over a two-day period. In addition, some heifers treated with a microsphere formulation containing oestradiol benzoate alone were observed in oestrus on day 1 and 2. Also, an induced earlier onset of luteal activity in perpubertal beef heifers with a consistent pattern of progesterone plasma concentrations was observed (Whisnant & Burns, 2002). Similarly, as reviewed by Rathbone *et al*., 2000, a biodegradable sucrose acetate isobutyrate delivery system (SABER Mate B (Bovine)) to control the release of deslorelin acetate was developed for the use of oestrous control.

Combined therapies allowed treatment durations of 10 and 12 days using the subcutaneous and intra-vaginal routes, respectively (Odde, 1990; Gordon, 1996). Progesterone administered for 14 to 20 days was effective in synchronising oestrus, but this was associated with lower fertility rates (Odde, 1990). Duration of the treatment was reduced and oestrous synchronisation improved by combining progesterone with an oestradiol. SYNCHRO-MATE®-B is a typical example of progesterone-oestradiol combination. A combination of progesterone

with either oestradiol benzoate or potent progestogens with oestradiol valerate has been used for oestrous control (Rathbone *et al*., 1998). Synchronising the stage of the follicle wave using a combination of either oestradiol or GnRH at the start of treatment with progesterone (PRID or CIDR™-B for 10-12 days) or the subcutaneous norgestomet (ear implants for 9-10 days) is ideal. Attaching a small gelatin capsule containing oestradiol benzoate into the inner surface of the PRID or CIDR™-B has also been developed as a combination drug therapy for oestrous control. Cows show oestrus within a few days of removal of the implant or withdrawal of the devices. Effective treatments suppress GnRH secretion from the hypothalamus so long as progesterone administration is maintained, but the release of GnRH resumes immediately following removal of the implant or withdrawal of PRID/CIDR™-B because of the rapid decline of progesterone concentration. Therefore, all the treated animals begin a follicular phase at the same time.

More recently, McDougall *et al.*(2004) studied the effect of Cuemate™ progesterone dose titration and oestradiol benzoate on plasma progesterone concentration and follicle dynamics in anovulatory anoestrus post-partum pasture-fed dairy cattle. Cuemate™ containing a normal dose (1.56 g) of progesterone or a modified Cuemate™ containing double (3.12 g) or triple (4.68 g) the normal progesterone dose with (2 mg) or without oestradiol benzoate for 8 days was used. McDougall *et al.*(2004) found that increasing the progesterone dose was associated with an increase in plasma progesterone concentration during the time of device insertion. However, plasma progesterone concentration for each of the three doses showed similar shaped profiles with increasing levels from the lowest to the highest dose. The average values of plasma progesterone of from day 1 up to day 8 were 1.2 ± 0.2, 2.0 ± 0.2 and 2.9 ± 0.2 ng/mL for normal, double and triple Cuemate™ progesterone doses, respectively. McDougall *et al.* (2004) also determined that there was no effect of oestradiol benzoate at the time of Cuemate™ insertion on plasma concentration and concluded that a delay in the subsequent follicle wave and more rapid follicle growth were associated with the increasing progesterone dose and oestradiol benzoate treatment.

Combinations of progesterone releasing products and PGF improved synchronisation rates in cows to 84% as compared to 57% for PGF alone (Lucy, 2001). Other researchers found similar results after cows were treated with progesterone for 7 days prior to PGF administration (Macmillan & Peterson, 1993). This occurs as all cows have a CL that has developed for at least 7 days as progesterone treatment suppresses any naturally occurring luteolysis, but does not affect any follicular development. PGF treatment ensures regression of any CL and therefore a new cycle begins. Other attempts have been made using injections of PGF or oestradiol benzoate or both in conjunction with a progesterone delivery device, PRID (Whisnant *et al*., 1999). It was found that the combination of progesterone, oestradiol benzoate and cloprostenol treatments resulted in significantly higher synchrony of ovulation and pregnancy rates compared with all other treatments. Oestrus synchrony of inclusion of 1 mg of oestradiol benzoate or 5 mg of -17β oestradiol with progesterone and cloprostenol was equally effective, and there were no differences between heifers and cows.

Furthermore, a combination system of PGF and/or GnRH (Rathbone *et al*., 2001) has also been developed. The administration of GnRH induces ovulation and/or luteinization of the dominant follicle. Given that the time for a follicle to become dominant is around 8 days and dominance normally lasts about 4 days, injection of PGF 6-7 days after GnRH induces luteolysis allowing a new follicle to ovulate. A comparative study of oestrous synchronisation rates using double PGF injections, GnRH + PGF and human chorionic gonadotropin (hCG) +PGF was investigated (Coyan *et al*., 2003). Application of a GnRH or hCG prior to a PGF injection provided better oestrous synchronisation rates than two injections of PGF. This was explained by hCG (it exerts a primarily LH effect with very little of the FSH effect) and GnRH which cause ovulation or initiation of a new follicular wave. It is possible that a fully functioning CL, which is responsive to PGF treatment, is formed after 7 days of hCG and GnRH treatment. This type of treatment was effective because it controls both the follicular and luteal phases of the oestrous cycle.

1.3.3.7 Oestrous synchronisation with PGF and GnRH

As effective oestrous synchronisation of the oestrous cycle requires the control of both luteal and follicular functions, a combination of three injections of GnRH, PGF and GnRH (on days 0, 7 and 9, respectively) was developed to synchronise the follicular wave and timing of ovulation followed by a fixed time for AI. Using this method known as Ovsynch, ovulation occurs between 24 to 32 hours after the second GnRH injection. The rate of oestrous synchronisation was between 87 to 100% of lactating dairy cows. Two modifications of the Ovsynch protocol, which are referred to as Co-Synch and Select Synch have also used extensively in postpartum beef cows (Patterson *et al.*, 2003). Co-Synch is similar to Ovsynch, and the sole difference is that cows are inseminated when the second GnRH injection is administered. Select Synch differs in that cows do not receive a second GnRH injection as in the Ovsynch protocol and they are inseminated 12 hours after oestrus is detected. However, these treatments are very expensive because of the increased number of times the animal is handled and the associated veterinarian cost needed for herd administration.

1.3.3.8 Current oestrous control programme and its deficiency

A combination of multiple drugs has become necessary to achieve affective oestrous control. The current practice of oestrous control treatment is based on the control of both luteal and follicular functions and protocols as found in the recent literature are as follows (Patterson *et al*., 2003; Rathbone *et al*., 2001; Cross *et al*.,2004): On day 0 CIDR™-B is inserted to release progesterone to inhibit ovulation and oestradiol benzoate is injected intramuscularly to induce follicular atrasia. The device is removed at the end of the treatment, followed by an intramuscular injection of PGF to regress any CL. Oestradiol benzoate is injected intramuscularly on day 9 to initiate a new oestrous cycle.

Therefore, there is a need for a drug delivery system that enables the delivery of multiple drugs at appropriate times. The delivery should perform a sustained release of progesterone for 7 or 8 days, as well as providing an immediate release dose of oestradiol followed by a delayed release dose of a PGF one day before removal of the device. The current drug delivery system for oestrous control is

based on a single drug delivery programme, and therefore, it cannot control the luteal and follicular functions using the same delivery system. Further research is needed to improve current oestrous control programmes.

It is the aim of this research to assess an electronic drug delivery system for the delivery of progesterone, oestradiol and PGF, for the control of the bovine oestrous cycle.

2 Preformulation properties of selected steroids useful for administration via the intravaginal route

2.1 Introduction

Preformulation is the physicochemical characterisation of the solid and solution properties of compounds (Steele Gerry, 2004). Preformulation studies performed on a drug compound in order to produce information to enable the formulation of a stable and pharmaceutically sound drug dosage form. The physicochemical properties of a variety of steroidal compounds for the purpose of determining their suitability for incorporation into a novel intravaginal drug delivery system were determined. Although information could be found in the literature on the preformulation properties of numerous steroids, much information was lacking for the specific steroids investigated in this study. Therefore, an investigation into the physicochemical characteristics of the steroids investigated in this research was conducted.

Preformulation typically involves establishing qualitative and quantitative techniques to determine the physicochemical properties of the steroids investigated in this research (Table 2.1). Some key physicochemical properties which are integral and need to be determined during drug preformulation stages are solubility, lipophilicity, ionisation constant (pKa) and permeability (Avdeef, 2001; Kerns & Di, 2004). High solubility and lipophilicity are both crucial for drug permeability across biological membranes. A low solubility is detrimental to absorption after drug administration and can cause false low estimates of compound activity in bioassay (Kerns & Di, 2004). A high hydrophilic drug can have poor passive transcellular permeability. In addition, increasing lipophilicity of a drug reduces aqueous solubility and therefore the lipophilicity and solubility may tend to cancel one another in terms of increased permeability (Schoenwald & Ward, 1978).

Table 2.1 summarises the physicochemical properties that must be elucidated and why tests are performed.

Table 2.1. Physicochemical tests carried out during preformulation studies.

Test/activity	Reason for test/activity	Reference	How determined
HPLC analysis	For quantitative analysis	(Steele Gerry, 2004)	Experimental determination
UV spectroscopy	For quantitative analysis		Experimental determination
Infra Red (IR) spectroscopy	For identification purposes	(Steele Gerry, 2004) (Cohen, 1973)	Experimental determination/ Literature
Elemental analysis	To define empirical formula	(Steele Gerry, 2004)	Literature
pKa	Extent of ionisation is important for solubility, stability and drug absorption	(Steele Gerry, 2004)	Literature
Log_{10} of partition coefficient (Log P)	Degree of lipophilicity is important for drug absorption	(Ritschel, 1988; Steele Gerry, 2004)	Literature
Apparent partition coefficient	Apparent partition coefficient is important for drug absorption		Experimental determination
Solubility	Solubility is important for drug absorption	(Ritschel, 1988; Steele Gerry, 2004)	Experimental determination
Molecular weight	MW is important for drug transport through membranes	(Ritschel, 1988; Steele Gerry, 2004)	Literature
Retention times on HPLC (C_{18})	Ranking the drugs in order of their lipophilicity	(Veith *et al*., 1979; Kerns & Di, 2004)	Experimental determination
Differential Scanning Calorimetry	To determine melting point of pure drugs or complexes	(Clas *et al*., 1999)	Experimental determination

The common steroid molecule consists of a four-fused ring structure, three with six carbons and one with five carbons in the ring (Figure 2.1). All steroids are derivatives of this common structure (Dawson *et al*., 1986). The four ring structures are conventionally lettered as indicated in Figure 2.1 (A, B, C & D). The carbon position of a steroid compound is conventionally numbered according to the system listed for cholestane (Figure 2.1). There are usually methyl groups on carbon positions 10 and 13, and often an alkyl group at position 17.

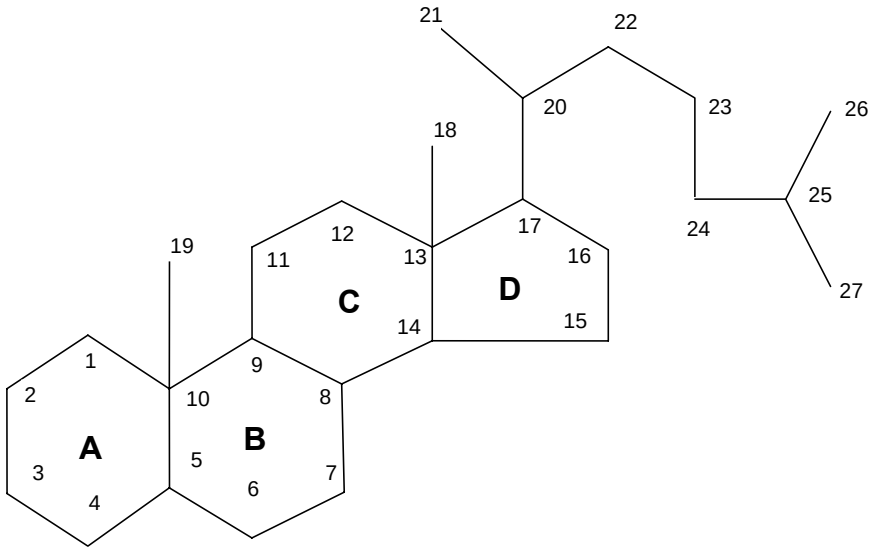


Figure 2.1. Numbering of steroid carbon atoms of cholestane molecule.

Different steroids contain different functional groups attached to different positions on the molecule. For instance, some steroids studied in this research have ester groups at different carbon positions of the steroid molecule. Dexamethasone acetate and dexamethasone isonicotinate have acetate and isonicotinate groups on carbon position 21, respectively. Likewise, dexamethasone valerate has a valerate group on carbon, position 17 and dexamethasone dipropionate has propionate group on both carbon positions 17 and 21. Oestradiol benzoate has a phenolic group on carbon position 3.

Steroids occur naturally in humans and animals (Osamu Nozaki, 2001) and analogues can be produced synthetically. As previously discussed in Chapter One,

steroids are extensively used throughout the veterinary pharmaceutical industry in farm animals for the control of reproduction (e.g., progesterone and oestradiol), parturition (e.g., dexamethasone) and growth promotion (e.g., oestradiol). Testosterone is used in dogs for the purpose of chemical castration. However, the use of steroids in farm animals will be the focus of this research.

Steroids are relatively insoluble in water. To increase steroid solubility and bioavailability of steroids, cyclodextrin molecules can be used (Loftsson & Brewster, 1996). Cyclodextrins are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose containing a relatively hydrophobic central cavity and hydrophilic outer surface. The most common pharmaceutical application of cyclodextrins is to enhance solubility in aqueous solution (Loftsson & Brewster, 1996). The most common cyclodextrins are α-cylodextrin, β-cyclodextrin and γ-cylodextrin, which consist of six, seven, and eight glucopyronose units, respectively.

The physicochemical properties of the most commonly used cyclodextrins and some of their derivatives are summarised in Table 2.2.

The size of the cyclodextrin cavity is crucial to increase steroid solubility and bioavailability (Loftsson, 1995). For example, α-cylodextrin has the smallest cavity which can only be used for small molecules, whereas β-cyclodextrin is the most useful for most drugs of average size range and γ-cylodextrin can be used for large molecules. However, a low aqueous solubility of β-cyclodextrin limits its use (Loftsson, 1995).

The molecular structure of the parent cyclodextrins (α-cylodextrin, β-cyclodextrin and γ-cylodextrin) has been modified to form cyclodextrins derivatives which are more soluble than their parent cyclodextrins.

This has involved alkylation (methyl and ethyl derivatives) or hydroxylation (hydroxypropyl and hydroxyethyl derivatives) of the parent cyclodextrin compounds listed inTable 2.2.

Table 2.2. Natural cyclodextrins and some of their derivatives that are currently used in pharmaceutical products (Loftsson & Brewster 1996; Loftsson & Stefansson 2002).

Cyclodextrin type	Substitution[1]	MW[2]	Solubility in water (mg/mL)[3]	Central cavity diameter (Å)[4]
α-Cyclodextrin	-	972145	-	4.7 - 5.3
β-Cyclodextrin	-	1135	18.5	6.0 - 6.5
2-Hydroxypropyl-β-cyclodextrin	0.65	>1400	600	
Randomly methylated β-cyclodextrin	1.8	>1312	500	
β-Cyclodextrin sulfobutyl ether sodium salt	0.9	>2163	500	
γ-Cyclodextrin	-	1297	232	7.5 - 8.3
2-Hydroxypropyl-γ-cyclodextrin	0.6	>1576	500	

[1]Average number of substituents per glucopyranose repeat unit. [2]MW given by the supplier, or the calculated value based on the average degree of substitution, for the water-free substance. [3]Solubility in pure water at 25°C. [4]Loftsson (1995).

2-Hydroxypropyl-β-cyclodextrin (HPβCD) was selected for use due to its high solubility (Table 2.2) in aqueous solution and its potential use to improve the bioavailability of various steroids (Usayapant & Iyer, 1999; Loftsson *et al.,* 2003; Loftsson *et al.,* 1994), thereby favourably altering permeation through biological and non-biological membranes.

2.2 Steroid permeation through non-biological and biological membranes using side-by-side cells

Side-by-side cells are used to screen compounds for their ability to permeate membranes. It is well documented that both non-biological and biological membranes have been used in side-by-side cells (Friend, 1992; Haigh & Smith, 1994; Zhang & Robinson, 1996). Membranes can also be classified according to their characteristics and barrier function resulting in four main groups (Loftsson *et al*., 2003). These four different types of membranes are: 1) lipophilic membrane barriers to drug delivery from aqueous vehicles through the membrane, 2) porous membranes that are permeable to free drug but impermeable to drug/cyclodextrin complexes, 3) porous membranes that are permeable to both free drug and

drug/cyclodextrin complexes and 4) membranes which possess both aqueous diffusion barrier and lipophilic membrane barriers towards drug permeation.

Both resistance of the lipophilic membrane and that of the adjacent aqueous boundary layer have controlled passive drug penetration across biological and non-biological membranes (Loftsson *et al*., 2003). Cyclodextrins can enhance drug delivery through artificial and biological membranes (Loftsson *et al*., 2003). Composition of a drug formulation, and the physicochemical and physiological composition of a membrane, will determine what type of enhanced effect is obtained. For instance, cyclodextrins will enhance drug delivery through diffusion control or aqueous diffusion and lipophilic controlled barriers (e.g., fish skin or hairless mouse skin membranes). The effect of cyclodextrin on *in vitro* drug permeation through biological membranes has been investigated (Lopez *et al*., 2000). It was found that cyclodextrin increased the amount of dexamethasone acetate transferred across hairless mouse skin with respect to free dexamethasone acetate in water alone.

However, biological membranes suffer from a disadvantage in that they require external preparation prior to use and the viability decreases over time. Biological membranes also require sensitive analytical techniques such as HPLC to differentiate the drug from extraneous material that may leach out of the membranes. In contrast, artificial membranes offer the advantage of needing little or no preparation, are easier to use, do not require complex analytical techniques and do not deteriorate during use. However, to be of any value, they should be usable as an alternative to biological membrane and capable of predicting the permeability of compounds *in vivo*. Poly-ε-caprolactone (PCL) is a polymer that is readily available, easily fabricated into thin flat sheets and low in cost (Pitt, 1990). Silicone polymer is also known to be useful for drug-loaded films that can be applied to the skin, implanted subcutaneously or inserted into several of body cavities (Juliano, 1980). In this research, permeation rates and permeability coefficients of the steroids with PCL, silicone, synthetic skin membrane and excised mucosa were investigated. An artificial model membrane was selected from the non-biological membranes for screening the steroids.

2.3 Methods

2.3.1 Steroids

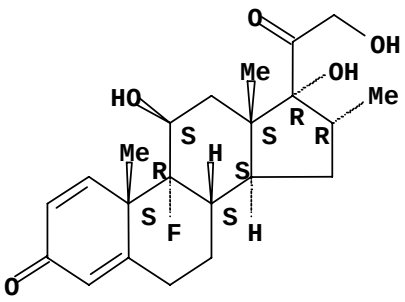
The steroids investigated in this research are listed in Table 2.3.

Table 2.3. Drugs, grade, suppliers, molecular formula and molecular weight of compounds used in the study.

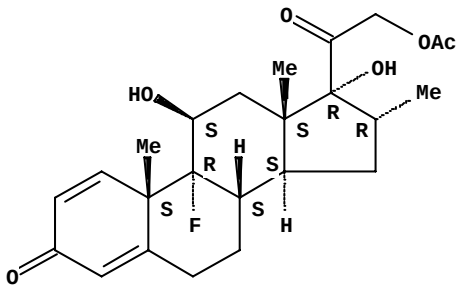
Drug	Grade	Supplier	Company & Country	Molecular formula	Molecular weight	CAS
Dexamethasone	USP micronized	Australian generic Pty ltd	Crystal Pharma, Spain	$C_{22}H_{29}FO_5$	392.47	50-02-2
Dexamethasone acetate				$C_{24}H_{31}FO_6$	434.5	1177-87-3
Dexamethasone isonicotinate				$C_{28}H_{32}FNO_6$	497.6	2265-64-7
Dexamethasone dipropionate				$C_{28}H_{37}FO_7$	504.6	55541-30-5
Dexamethasone valerate				$C_{27}H_{37}FO_6$	476.577	33755-46-3
Progesterone	USP Grade	Pharmacia	USA	$C_{21}H_{30}O_2$	314.47	57-83-0
Oestradiol-17β	BP 2002 Ph.Eur.4	Gedeon Richter Ltd	Budapest Hungary	$C_{18}H_{24}O_2$	272.4	50-28-2
Oestradiol-benzoate				$C_{25}H_{18}O_3$	376.5	50-50-0

These compounds were selected based on what is currently used in the veterinary field and upon their availability from suppliers.

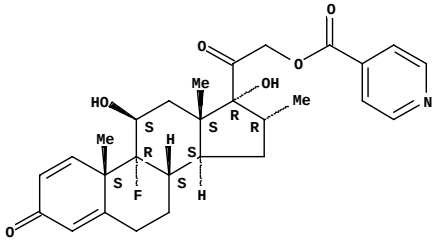
The chemical structure of the steroids used in this study are shown in Figure 2.2.



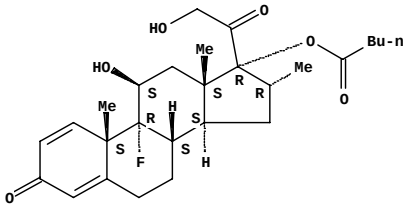
a) Dexamethasone, CA Index Name: Pregna-1,4-diene-3,20-dione, 9-fluoro-11,17,21-trihydroxy-16-methyl-, (11β,16α)-(9CI).



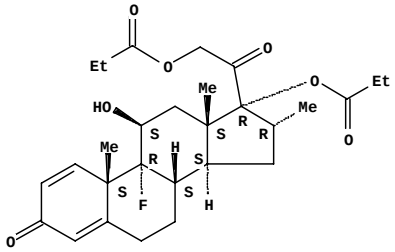
b) Dexamethasone acetate, CA Index Name: Pregna-1,4-diene-3,20-dione, 21-(acetyloxy)-9-fluoro-11,17-dihydroxy-16-methyl-, (11β,16α)-(9CI).



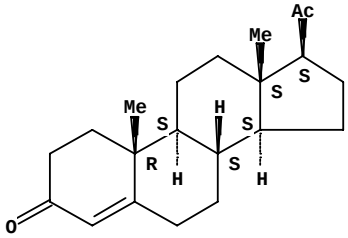
c) Dexamethasone isonicotinate, CA Index Name: Pregna-1,4-diene-3,20-dione, 9-fluoro-11,17-dihydroxy-16-methyl-21-[(4-pyridinylcarbonyl)oxy]-, (11β,16α)- (9CI)



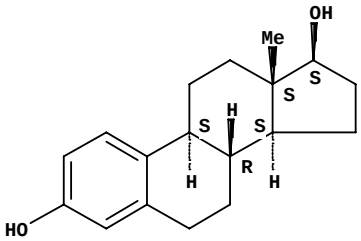
d) Dexamethasone valerate, CA Index Name: Pregna-1,4-diene-3,20-dione, 9-fluoro-11,21-dihydroxy-16-methyl-17-[(1-oxopentyl)oxy]-, (11β,16α)- (9CI)



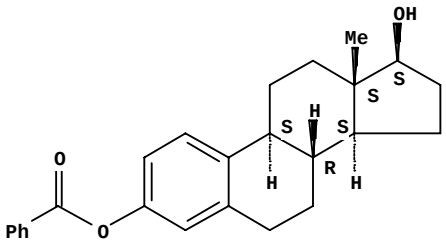
e) Dexamethasone dipropionate, CA Index Name: Pregna-1,4-diene-3,20-dione, 9-fluoro-11-hydroxy-16-methyl-17,21-bis(1-oxopropoxy)-, (11β,16α)- (9CI)



f) Progesterone, CA Index Name: Pregn-4-ene-3,20-dione (9CI)



g) Oestradiol 17-β, CA Index Name: Estra-1,3,5(10)-triene-3,17-diol (17β)-(9CI).



h) Oestradiol benzoate, CA Index Name: Estra-1,3,5(10)-triene-3,17-diol (17β)-, 3-benzoate (9CI).

Figure 2.2. Chemical structures of (a) Dexamethasone (b) Dexamethasone acetate (c) Dexamethasone isonicotinate (d) Dexamethasone valerate (e) Dexamethasone dipropionate (f) Progesterone (g) Oestradiol-17β and (h) Oestradiol benzoate. These figures were obtained via www.scifinder.com.

2.3.2 Analytical methods

2.3.2.1 Infra Red (IR) spectrometry

IR spectroscopy was performed to confirm the identity of all steroids used in this research. An infrared spectrometer (Bomen, MB104, A.I. Scientific, New Zealand) with an Attenuated Total Reflectance attachment and Flat Plate Crystal was used. Three scans were performed on each sample with the instrument resolution set at 4 cm^{-1} and spectral range set at 3600-6000 cm^{-1}. For each run, sufficient sample was used to completely cover the ATR crystal.

2.3.2.2 Melting point determination by Differential Scanning Calorimetry (DSC)

DSC analysis was carried out according to the method described by Vianna *et al*. (1998). DSC measurements were conducted using a differential thermal analysis instrument (TA Instruments, DSC 2920, USA) linked to a data analysis station (Thermal Solution 1.2J, TA Instrument). DSC thermal analysis was performed in order to define the melting point of pure and formulated steroidal compounds used in this research.

All the DSC traces for the melting point of each sample were analysed by measuring heat flow differences between the sample and a reference. Samples of materials of weight 4-7 mg were accurately weighed in hermetic sealed aluminium pans and scanned over a temperature range of 25-270 °C at a heating rate of 10°C/minute and with an air flow rate of 50 mL/minute. An empty sealed hermetic aluminium pan was chosen as the reference sample. A graph of heat flow (mW) versus temperature (ºC) was plotted from the generated data. The intersection of the extrapolated baseline prior to the transition with the extrapolated leading edge of the transition of the heat flow versus temperature graph was taken as an extrapolated onset melting temperature (Clas *et al*., 1999). The peak temperature (T_m) was also obtained from the graphs for all the steroids.

2.3.2.2.1 Oestradiol-cyclodextrin complexation

Complexes were prepared for DSC from freeze-dried drug/cyclodextrin mixtures (HPβCD or γCD [(Wacker-Chemie GMBH, Germany]), which were obtained from saturated drug solutions of steroids in 5% w/v cyclodextrin buffer (pH 5.0) at 39ºC. The mixtures were allowed to equilibrate in a water bath (Grant OLS200 Shaking Water Bath, Grant Instruments Ltd, UK) at 39ºC at 50 strokes per minute for a week. The mixtures were centrifuged (Beckman, Avanti™ 30 Centrifuge, Beckman Instruments, Inc., USA) at 5700 rpm for 15 minutes. The supernatants were removed from the aqueous drug/cyclodextrin sediments and freeze-dried.

Solid complexes of oestradiol benzoate or oestradiol-17β with γCD or HPβCD were obtained from saturated solutions of oestradiol benzoate or oestradiol-17β in the presence of the appropriate cyclodextrin (5% w/v cyclodextrin in PBS at pH 5.0). The supernatant was removed and freeze-dried to obtain a dry powder for DSC analysis. Physical mixtures were obtained by direct mixing of either the oestradiol benzoate or oestradiol-17β and cyclodextrin and mixing at a ratio of 1:4 drug to cyclodextrin. Approximately 0.200 and 0.210 grams of oestradiol-17β were weighed in separate glass bottles and mixed with 0.800 grams of γCD or 0.840 grams of HPβCD, respectively. Similarly, 0.269 and 0.290 grams of oestradiol benzoate and 1.076 grams of γCD or 1.160 grams of HPβCD, were prepared in the same manner.

2.3.2.3 Solubility of the steroids

Solubility was determined at 39ºC by adding excess drug to the aqueous phase (5% w/v HPβCD in PBS, pH 5.0) and equilibrated in a water bath at 50 strokes per minute for 7 days. At periodic times samples were taken from the solution to provide evidence that equilibration had occurred. All samples were extracted from the supernatant using a syringe and passed through a 0.22 μm Millipore filter (preheated to 39ºC in an oven) with the first 2 mL being discarded. The sample was then appropriately diluted and analysed by UV spectroscopy at the peak of maximum absorbance (λ_{max}) of the steroids. The blank was 5% w/v HPβCD in pH 5.0 PBS. Determination of solubility values was performed in duplicate samples on three different days for each steroid.

2.3.2.3.1 Apparent association/dissociation constant (K_c) of drug-HPβCD complex

In an aqueous cyclodextrin solution, when a drug forms a complex with cyclodextrin, the drug molecule, in part or in whole, is taken up into the somewhat lipophilic cylodextrin cavity (Loftsson *et al.,* 2003). Then, the free drug molecule goes into equilibrium with the drug-cylodextrin complex. The equilibrium constant (association/dissociation constant) is K_c. The solubility of steroids was determined in various concentrations of HPβCD (0, 2.5, 5.0, 7.5, 10.0 and 12.5% w/v) in PBS (pH 5.0) at 39ºC as described in Section 2.3.2.3. All samples were extracted from the supernatant using a syringe and passed through a 0.22 μm Millipore filter (preheated to 39ºC in an oven) with the first 2 mL being discarded. The sample was then appropriately diluted and analysed by UV at the λ_{max} of the relevant steroid. A blank was used that contained the same concentration of HPβCD in pH 5.0 PBS as the solution studied. The solubility of each drug versus the HPβCD concentration was plotted to determine the slope. A plot of drug solubility versus cyclodextrin is called a phase solubility diagram (Loftsson *et al*., 2002; Loftsson *et al*., 2003). The phase solubility diagrams are used to calculate, the apparent association/dissociation constant (K_c). The linear phase solubility diagrams are thought to indicate that the drug-cylodextrin complexes are first order with respect to cyclodextrin and first or a higher order with respect to the drug (Loftsson *et al*., 2002). If a water-insoluble drug molecule forms a complex with one cyclodextrin molecule, K_c (1:1), then K_c of the complex can be determined by the phase solubility method. When the aqueous solubility of the drug shows a linear increase when plotted as a function of the cyclodextrin concentration, K_c can be determined from the initial linear slope and the solubility of the drug in water (C_o), according to Equation 2.1 (Babu & Pandit 2004).

$$K_c = \frac{Slope}{C_o(1 - Slope)}$$ **Equation 2.1.**

2.3.2.4 Partition coefficients

A partition coefficient is a measure of the relative hydrophobicity of a compound (Kerns & Di, 2004). Generally, the partition coefficient is calculated from the relative distribution of a compound between a water immiscible organic solvent (e.g., octanol) and an aqueous buffer at varying pH. For instance, the octanol-water partition coefficient of a compound is the ratio of the concentration of the compound in octanol and in aqueous buffer at equilibrium and at a specified temperature and pH (Kerns, 2001).

2.3.2.4.1 Octanol: 5% w/v HPβCD buffer (pH 5.0)

Partition coefficients were determined using the shake-flask method (Pharmaceutical Society of Great Britain Department of Pharmaceutical Sciences, 1979; Kerns, 2001). Partition coefficients were determined between octanol and 5% w/v HPβCD buffer (pH 5.0) at 39ºC in triplicate on two different days for each of the steroids investigated in the study. The absorbance of each steroid was measured using UV spectrophotometer.

Pre-saturation of the two phases was carried out prior to the experiment. The two phases (*n*-octanol and the aqueous HPβCD-containing buffer) were pre-saturated in a separating funnel at 39ºC in an oven for six days. Pre-saturated aqueous phase was used to make a known concentration of drug solution and 25 mL of this was added to a 100 mL bottle. The appropriate volume of organic phase was then carefully added on top of the aqueous phase. The bottle contents were then gently shaken to equilibration in a water bath maintained at 39ºC for 24 hours ($t = \infty$). At equilibration, absorbance values (Abs^{∞}) were determined. Apparent partition coefficients were calculated according to Equation 2.2.

$$Pc = \left(\frac{Abs^{0} - Abs^{\infty}}{Abs^{\infty}}\right)r \qquad \textbf{Equation 2.2.}$$

Where P_c = partition coefficient, r = phase volume ratio (aqueous /organic phase).

2.3.2.4.2 PCL: 5% w/v HPβCD in PBS buffer (pH 5.0) partition coefficients

Partition coefficients of the steroids were also determined by the shake-flask method between the PCL membrane and 5% w/v HPβCD in PBS buffer (pH 5.0) at 39ºC in triplicate on two different days for each of the compounds investigated in the study. The absorbance of the drug analogue was measured using a UV spectrophotometer. Very thin PCL sheets were prepared by placing PCL beads between two flat metal plates which were compressed between the jaws of a bench vice. The PCL sheets obtained were then cut into small pieces and used in the study.

Partition coefficients were determined as described using Equation 2.2 except that r = phase weight (aqueous phase weight/PCL sheets weight) in this case.

2.3.2.5 Ionisation constant and partition coefficient (octanol/water) values

The charge state that a molecule exhibits at a particular pH is characterised by the ionization constant (pKa) of that molecule (Avdeef, 2001). Unless otherwise stated the pKa and partition coefficient in octanol/water values of the steroids studied in the thesis were found via www.scifinder.com. According to this web address, the pKa values were calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67 (© 1994-2003 ACD).

2.3.2.6 Side-by-side cell permeation

2.3.2.6.1 Apparatus description

The side-by-side diffusion cells used in this study were obtained from Crown Glass Co. Inc. (Somerville, New Jersey, USA). They are comprised of two half cells (donor and receptor) that were assembled in a purpose built rack which had a threaded screw on one side and when tightened, allowed a pressure seal to be made against the membrane and the half-cells. Each half-cell had a volume of 3.4 mL and provided a surface area for permeation of 0.785 cm^2. The donor cell was

loaded with excess steroid in 5% w/v HP-β-cyclodextrin [(2-Hydroxypropyl-β-cyclodextrin (Wacker-Chemie GMBH, Germany)] in pH 5.0 phosphate buffer. The receptor cell was filled with 5% w/v HP-β-cyclodextrin in pH 5.0 phosphate buffer. The contents of each half-cell were stirred with small magnetic stirring bars (Spinbar, Stirring Bar, Teflon, Flea Micro, 7 x 2 mm., Pequannock, NJ, USA), which were rotated from the outside of the cell by a magnetic stirrer. Shallow round depressions in the interior of the cell held the stirrers close to but not touching, the membrane's surface. The permeation experiments were conducted at 39ºC using a thermal instrument (Thermomix 1419 Pump, KMS Ltd., NZ) jacketing of the cells to supply recirculating water to maintain temperature control. The thermal jacket extended all the way to the membrane surface. A diagram of the side-by-side apparatus used in the study is shown in Figure 2.3.

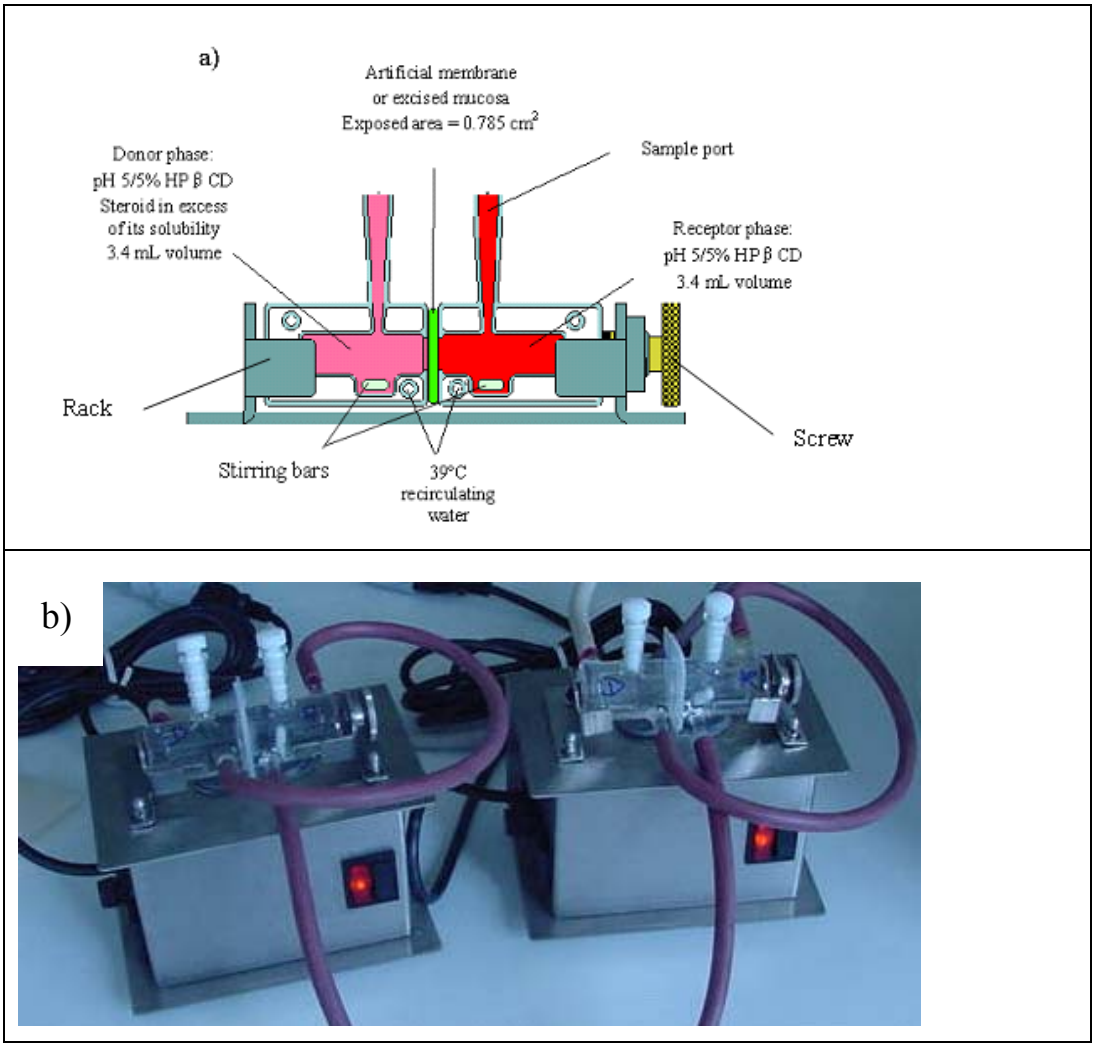


Figure 2.3. Side view schematic (a) and actual used in the study (b) of side by-side cell.

Samples of the receptor phase were taken at defined time intervals, with a disposable plastic pipette (Total Lab System Ltd, Auckland, NZ) and accurately weighed (Sartorius Analytical Balance Type BP110S, Biolab Scientific Ltd, NZ) and then, if required, diluted with an appropriate volume of buffer before analysis by UV spectrophotometry (Beckman DU® 640, Beckman, USA). For each sample, an equal volume of buffer was added to the receptor phase and correct selection of the aqueous phase composition ensured that sink conditions prevailed in the receptor phase throughout the duration of the experiment.

A single drug candidate and the selected model membrane were used to identify the factors that may affect drug permeation, such as, membrane thickness, temperature, pH and hydroxypropyl-β-cyclodextrin concentration in the donor phase.

2.3.2.6.2.1. Selection of non-biological membrane

Initial studies investigated several different types of membranes including silicone (150 μm thickness), synthetic skin (50 μm thickness) and PCL (140 μm thickness).

2.3.2.6.2.2. Synthetic skin

Synthetic skin membrane was obtained directly from the manufacturers (CoTran™ 9702 (2 mil, 9% vinyl acetate) 3M Pharmaceuticals, USA).

2.3.2.6.2.3. Silicone

Silicone membranes were obtained from DEC Manufacturing, (558 Te Rapa Road, Hamilton, NZ) and were manufactured using a purpose-built mould by injection moulding degassed 50:50 mixtures of Part A and Part B liquid silicone and curing at a temperature of 190ºC. Following manufacture, membranes were characterised by randomly sampling 10 membranes and their thickness determined using a micrometer screw gauge (Shardlow Micrometers Ltd, Sheffield, England).

2.3.2.6.2.4. Poly-ε-caprolactone

PCL membranes were manufactured in-house using two highly polished aluminium plates of dimensions (165 x 100 x 11.5 mm), which were separated using feeler gauges to give a membrane thickness of 140 μm (Figure 2.4). There were some occasions where different membrane thicknesses were manufactured by using feeler gauges of different thicknesses.

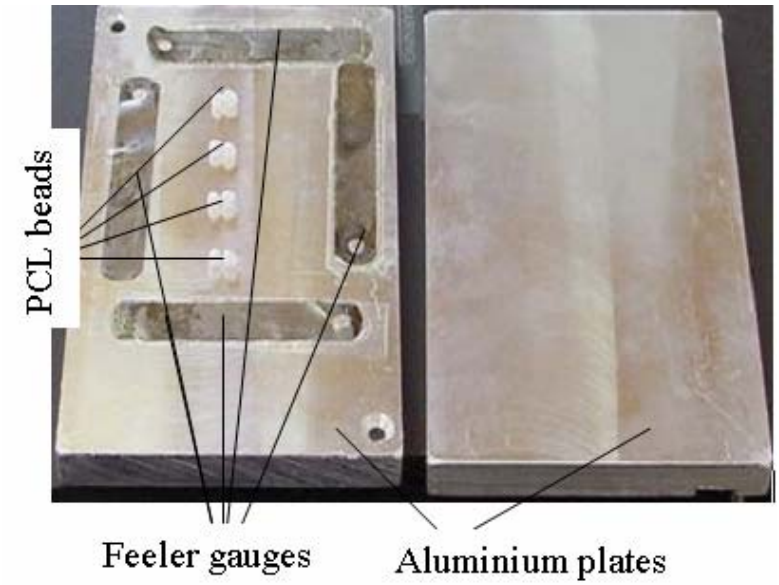


Figure 2.4. Photograph of plates, PCL bead and feeler gauges used to manufacture PCL membranes.

PCL beads were placed on to the surface of one of the plates (Figure 2.4) and heated to 120ºC in a bench top oven (Sunbeam Bake and Grill, BT020A model, Sunbeam, New Zealand) for 30 minutes to ensure they were molten. The plate was removed and the second plate (also preheated to 120ºC) was placed on top of the first. The two plates were then squeezed between the jaws of a vice and the plates compressed together. A screw knob on one side of the vice was tightened against the plates to ensure that uniform pressure is applied on the membrane in each time. They were left under pressure until the plates equilibrated to ambient temperature (approximately 60 minutes). At this point, the plates were separated and the membrane removed. The thickness of the membrane was determined using a micrometer screw gauge by measuring five different points on the membrane and averaging the five values (Figure 2.5).

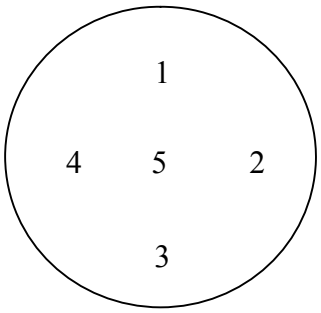


Figure 2.5. Diagram showing positions that thickness measurements were made on the PCL membrane.

The average thickness measured from over five points of the PCL membrane segments is shown in Appendix-A and a typical PCL manufactured membrane is shown by Figure 2.6.

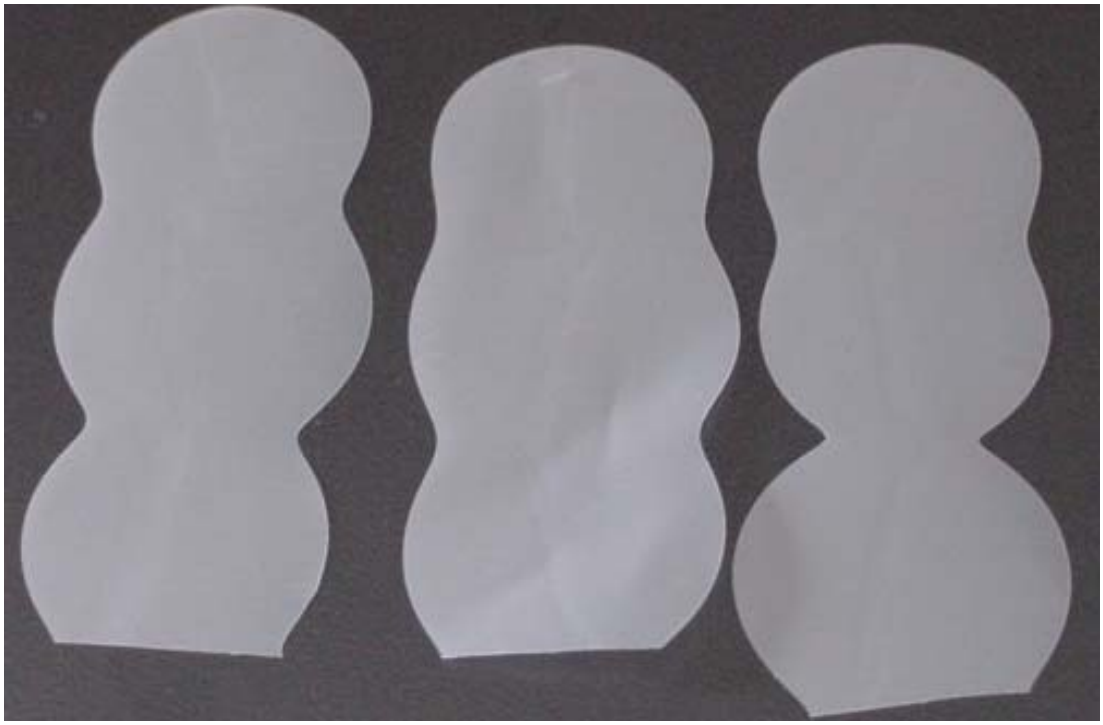


Figure 2.6. Photograph showing some PCL membranes .

2.3.2.6.2 Stability of membrane

PCL membranes were subjected to scanning electron micrography (SEM) to determine if changes in the surface properties of the membrane (as an indication of the stability of the membrane) during the permeation experiment occurred. The surface of the membrane was compared to the surface properties of a freshly prepared membrane.

2.3.2.6.3 Effect of changing conditions

The following conditions were changed to examine their effect upon dexamethasone permeation through PCL membranes of 140 μm thickness:

1. Membrane thickness: 70 to 200 μm thickness.
2. Temperature: 25, 30, 35 and 39ºC.
3. Cyclodextrin concentration: 1.25, 2.5, 5, 7.5% w/v in PBS/pH 5.0.
4. pH of the donor phase: 5, 7.4, 10 in 5% w/v in PBS.
5. Sensitivity: 140, 141, 145, 146, 148 μm thickness.

2.3.2.6.4 Screening of compounds through non-biological membranes

Unless otherwise stated, the permeation rates of all compounds studied in this research were determined using PCL membranes (~140 μm) and the chosen conditions (39ºC and 5% HPβCD in PBS at pH 5.0) in side-by-side cells. The aim was to rank the studied steroids in order of their permeation rates.

2.3.2.6.5 Excised cow vaginal mucosa (biological membranes)

The permeation rates of dexamethasone, dexamethasone acetate, dexamethasone valerate, dexamethasone isonicotinate, dexamethasone dipropionate and progesterone were also determined using the side-by-side cell apparatus and excised vaginal mucosa. Excised vaginal mucosa was used as the membrane separating the two half-cells. The mucosa was obtained in a fresh state from Ruakura Abattoir (Hamilton, NZ), put in a clean plastic bag, transferred to the laboratory on ice. The membrane was used fresh or kept in a freezer (-18°C) and thawed to room temperature prior to use. The mucosa was dissected from the majority of the connective tissue without damaging it by cutting along the outside length of the fresh vaginal sample with a scalpel until it reached the inner section. This opened up and exposed the vaginal mucosal surface. The sample was then averted to expose an excised mucosa with relatively low degree of folding. The vaginal mucosa was then laid uppermost. Excess mucus was removed. The edge of the mucosa was grasped with fine forceps, stretched and cut between the lamina propria and the mucosa by allowing the tissue to hung while cutting submucosa using surgical blades (Swann Morton Ltd, Sheffield England). The blade was held in parallel position to the inner surface (submucosal side of the

mucosa) to cut the mucosa, which then could be peeled away easily in sheet form. A 6.0 cm diameter piece of the mucosal sheet was then taken and the thickness of the dissected piece measured at five positions (see Figure 2.5) using a micrometer screw gauge to determine thickness. This was then cut into two equal parts and mounted onto the side-by-side cells with the surface of the vaginal mucosa facing towards the donor phase. The average thickness of the tightly stretched dissected mucosa was found to be between 1.0 and 1.2 mm.

The side-by-side cell study was performed under sink conditions over 10-12 hours. The donor cell was loaded with excess steroid in 5% w/v HP-β-cyclodextrin in pH 5.0 phosphate buffer. The receptor cell was filled with 5% w/v HP-β-cyclodextrin in pH 5.0 phosphate buffer. The temperature was maintained at 39ºC.

A 1.0 mL sample was taken at selected time intervals (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 hours) and analysed for drug concentration by a validated HPLC assay for each steroid such that changes in the drug concentration in the receptor phase and rate of transfer through mucosa could be monitored. An equal volume (1.0 mL) of 5 % w/v HP-β-cyclodextrin in pH 5.0 phosphate buffer was added to replace the volume that had been removed.

The peaks of the HPLC chromatogram generated from the analysed sample response were compared to those obtained the drug standards to see the effect of the mucosa on the drug. The concentration of a steroid transferred across the mucosal membrane at specific time in the receptor phase was calculated using the peak area and a standard calibration curve (peak area versus concentrations). The cumulative amount permeated drug appearing in the receptor phase (μg) versus time (hours) was plotted and analysed as described in Section 2.3.2.6.2 to determine the permeation rates of the studied drugs.

2.3.2.6.6 Analysis of data obtained using side-by-side cells

This study was carried out to investigate if there is any correlation between the physicochemical properties of the studied steroids (i.e., partition coefficient, solubility, HPLC retention time and/or molecular weight) and permeation

rates/permeability coefficients through the PCL model membrane and excised vaginal mucosa.

Permeation rates and permeability coefficients across the PCL membrane were calculated for each steroid and their values were plotted against their respective log ocatanol-buffer partition coefficients.

In addition, the permeation rate data generated using the selected model membrane will be evaluated to predict the permeation rate of selected steroids across excised vaginal mucosa in side-by-side cells.

The permeation rate was determined from the slope of the cumulative amount of steroid appearing in the receptor phase as a function of time (t) when steady-state transfer was occurring as follows. Note the membrane flux (which is the amount of steroid that can be passed through a square unit of area) can be calculated from the permeation rate divided by the surface area (0.785 cm^2). The slope is the cumulative amount of the tested steroid over time ($\Delta Q/\Delta t$). Therefore from knowledge of the slope, the initial solubility of the steroid in the donor phase, C_0, and the surface area available for transfer, A, the permeability coefficient, P can be calculated from (Artursson & Karlsson, 1991; Civiale *et al*., 2004) Equation 2.3.

$$\frac{\Delta Q}{\Delta t} = PAC_0 \qquad \textbf{Equation 2.3.}$$

Therefore, the permeability coefficient can be calculated by rearranging Equation 2.3 to give:

$$P = \frac{Slope}{AC_0} \qquad \textbf{Equation 2.4.}$$

However, for a water-insoluble steroid (when C_0 is practically impossible to experimentally determine) the permeability of some steroids across PCL membrane was alternatively determined using Equation 2.5 (Martin et al., 1983).

$$P = \frac{KD}{h}$$ **Equation 2.5.**

K is the partition coefficient of a drug from aqueous solution to PCL membrane, h is the thickness of the membrane (cm) and D is the drug diffusion coefficient (cm^2/sec) that was determined according to Equation 2.6.

$$D = \frac{h^2}{6t_{lag}}$$ **Equation 2.6.**

t_{lag} is the lag time, which is the point of intersection when the steady state portion of the line of the cumulative amount penetrated (mg) through the membrane versus time (hours) extrapolated to the time axis (x axis). The latter equation can only be used when *K*, *D* and t_{lag} are independently determined (Martin *et al*., 1983).

2.4 Results

2.4.1. Analytical methods

2.4.1.1 Infra Red

Table 2.4 shows the experimentally determined wave numbers for IR analysis of the steroids used in this study.

Table 2.4. Wave numbers for IR analysis of the steroids used in this study.

Drug	Wave number (cm^{-1})
Dexamethasone	620; 705; 852; 893; 913; 953; 981; 1033; 1054; 1092; 1135; 1243; 1270; 1299; 1395; 1437; 1451; 1621; 1664; 1706
Dexamethasone acetate	608; 698; 739; 871; 968; 1022; 1055; 1079; 1118; 1186; 1255; 1300; 1364; 1413; 1435; 1450; 1608; 1673; 1731; 2947
Dexamethasone valerate	626; 698; 813; 831; 892; 938; 980; 1031; 1066; 1256; 1306; 1394; 1453; 1609; 1617; 1662; 1715; 1728
Dexamethasone isonicotinate	626; 705; 829; 848; 902; 983; 1044; 1063; 1117; 1288; 1411; 1604; 1622; 1663; 1723; 1743
Dexamethasone dipropionate	686; 806; 881; 895; 926; 1040; 1062; 1082; 1114; 1242; 1257; 1308; 1393; 1414; 1607; 1620; 1662; 171; 1750; 3301
Progesterone	638; 687; 778; 871; 893; 948; 1162; 1204; 1227; 1278; 1328; 1356; 1385; 1614; 1661; 1698
Oestradiol-17β	619; 784; 818; 872; 916; 928; 1011; 1054; 1230; 1249; 1281; 1355; 1497; 1585; 1608; 2862; 2934; 2959
Oestradiol benzoate	605; 620; 685; 704; 786; 815; 894; 1023; 1063; 1129; 1174; 1213; 1262; 1378; 1450; 1495; 1723; 3552

2.4.1.2 Melting point by DSC

The extrapolated onset melting temperature was obtained by extrapolating the base line and the leading edge of the transition peak and finding their intersection. The resultant peak temperature (T_m) and literature values for the steroids used in the study are shown in Table 2.5.

Table 2.5. Extrapolated onset melting temperature, melting peaks and literature values (ºC).

Drug	Experimentally determined extrapolated onset melting temperature (ºC)	Experimentally determined melting peak temperature (ºC)	Literature melting point (^{0}C)
Dexamethasone	261.32	268.52	262-264[1]
Dexamethasone acetate	207.71	210.18	215 – 221[1]
Dexamethasone isonicotinate	267.28	269.54	250 – 252[1]
Dexamethasone dipropionate	205.22	206.99	Not found
Dexamethasone valerate	180.50	184.11	180[2]
Progesterone	130.53	130.70	126-131[3]
Oestradiol-17β	174.07	176.61	173-179[3]
Oestradiol benzoate	198.08	201.04	190-198[4]

The Merck Index, 2001; [2]Vianna *et al*., 1998); [2]Product specification data sheet and [3](United States Pharmacopeial Convention.Committee of Revision., 1994). [4](Clarke *et al*., 1986).

Table 2.5 demonstrates that the literature and experimentally determined DSC values for steroids tested are very similar.

2.4.1.3 Oestradiol-cyclodextrin complexation

DSC thermograms of oestradiol-17β and oestradiol benzoate complexes and physical mixtures with HPβCD or γCD are shown in the following thermograms together with comparison of the thermograms of the pure compounds.

Disappearance of the original endothermic peaks of oestradiol benzoate and oestradiol-17β is clearly seen in the complex thermograms (Figure 2.7). This might be due to possible complex formations between oestradiol benzoate or oestradiol-17β and HPβCD or γCD. In addition, the shape change of the original endothermic peak of γCD is obvious in the oestradiol benzoate or oestradiol-17β-γCD complex samples.

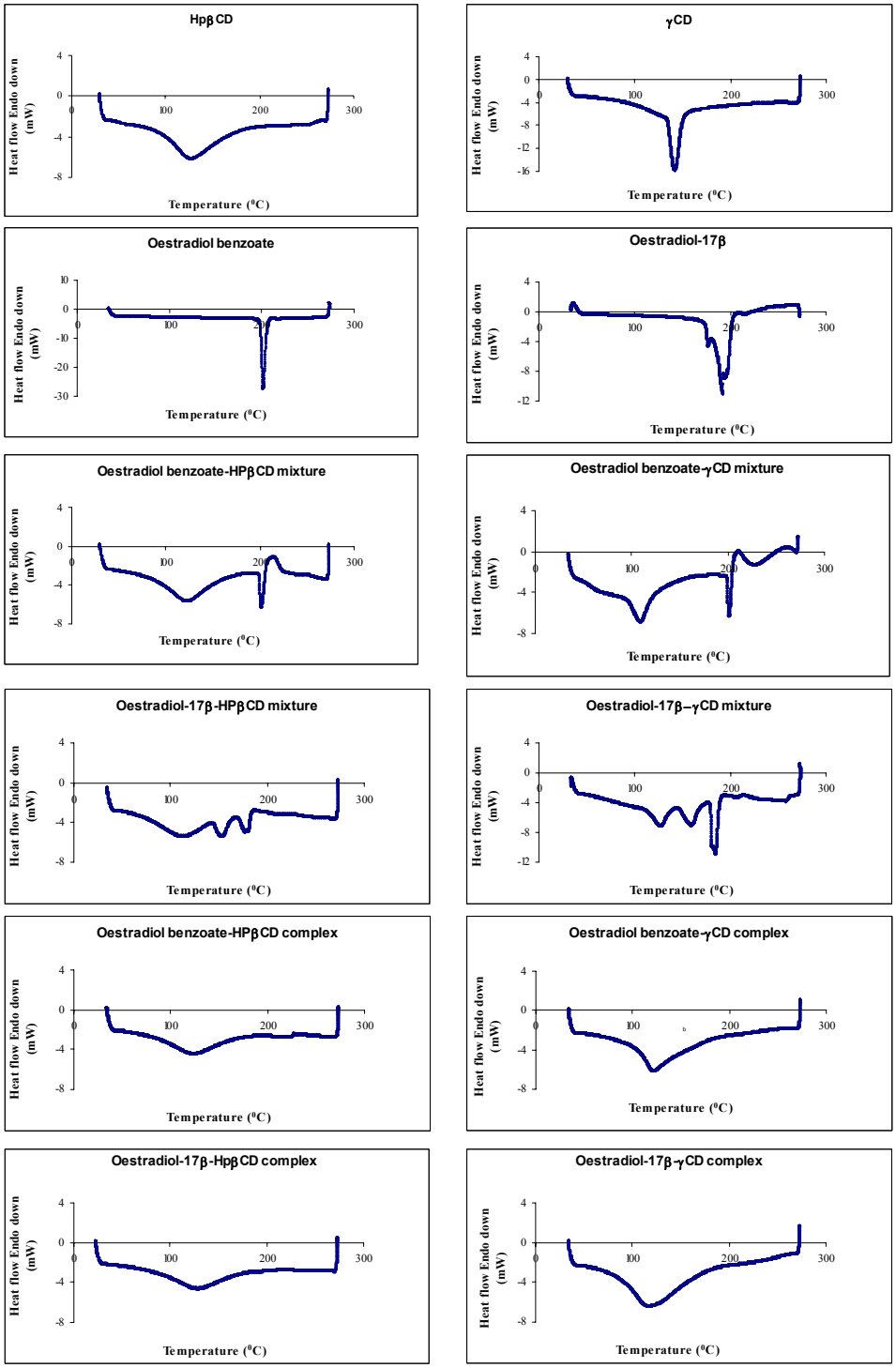


Figure 2.7. Thermograms of pure HPβCD, γCD, oestradiol-17β, oestradiol benzoate, their physical mixtures and complexes.

The extrapolated onset melting peak temperatures (T_m) for the oestradiol/cyclodextrin complexes investigated in the study are shown in Table 2.6. Literature values of the melting peak temperatures of γCD and HPβCD were obtained as 151.0 and 105.1, respectively (Vianna *et al.*, 1998).

Table 2.6. Experimentally determined melting peaks for the steroid-cyclodextrin complex or mixture.

Sample name	Experimentally determined melting peak values (˚C)
γCD	141.34
HPβCD	127.03
Oestradiol benzoate γCD mixture	109.18 & 200.97
Oestradiol benzoate HPβCD mixture	122.60 & 201.25
Oestradiol 17β γCD mixture	127.19 & 159.14
Oestradiol 17β HPβCD mixture	117.26 & 176.90
Oestradiol benzoate γCD complex	122.02
Oestradiol benzoate HPβCD complex	124.27
Oestradiol 17β γCD complex	117.35
Oestradiol 17β HPβCD complex	128.81
Oestradiol 17β	176.61
Oestradiol benzoate	201.04

There are shifts of the original endothermic peaks of oestradiol benzoate and oestradiol-17β in the case of oestradiol benzoate or oestradiol-17β and HPβCD or γCD complexes. This might be due to potential complex formation of oestradiol benzoate or oestradiol-17β and HPβCD or γCD.

2.4.1.4 Solubility of the steroids

The solubility of the steroids investigated in the study in PBS buffer (pH 5.0) and with 5% HPβCD in PBS buffer are shown in Table 2.7.

Table 2.7. The solubility of various steroids in PBS (pH 5.0) and 5% HPβCD/PBS buffer (pH 5.0). (Mean ± SD; N=6).

Steroid	PBS (pH 5.0) Solubility (µg/mL)	Solubility (µg/mL) 5% HPβCD/PBS
Dexamethasone	108.1 ± 4.8	4232 ± 44
Dexamethasone acetate	23.9 ± 1.1	1372 ± 33
Dexamethasone isonicotinate	-	366 ± 2.8
Dexamethasone valerate	12.4 ± 1.2	282 ± 11.1
Dexamethasone dipropionate	-	138 ± 2.2
Progesterone	10.7 ± 1.0	3711 ± 63.2
Oestradiol benzoate	-	149.9 ± 3.8
Oestradiol-17β	-	2483 ± 27

-Cannot be determined.

It was impossible to determine the solubility values of dexamethasone isonicotinate, dexamethasone dipropionate, oestradiol benzoate and oestradiol-17β in PBS (pH 5.0) alone due to insufficient solubility in the buffer and low UV sensitivity.

2.4.1.4.1 Apparent association/dissociation constant (K_c) values of steroid-HPβCD complexes

The solubility phase diagram of the steroids in PBS buffer (pH 5.0) with 0-12.5% HPβCD at 39ºC are shown in Figure 2.8. The solubility was expressed in moles to allow a comparison with literature values.

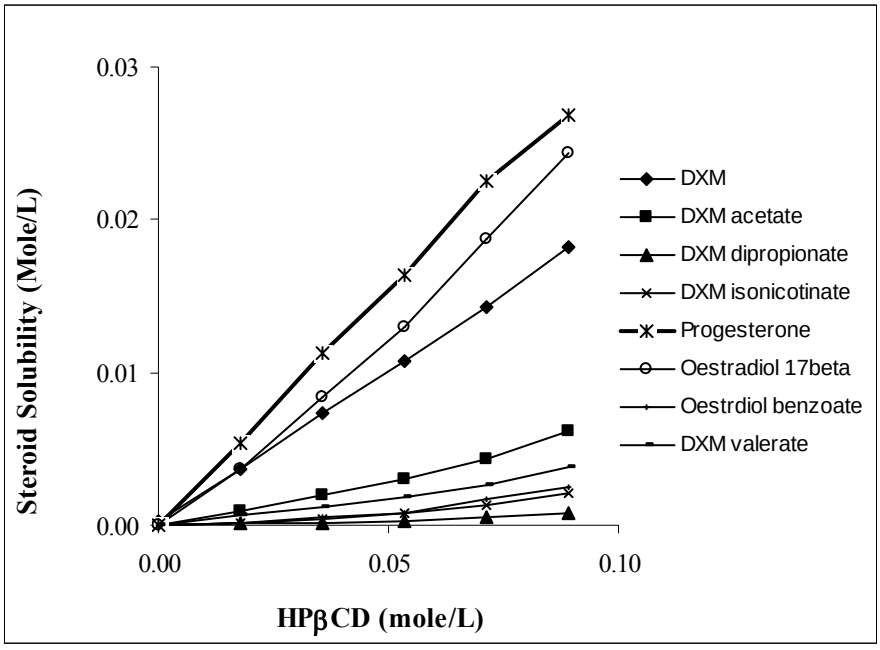


Figure 2.8. Solubility phase diagrams in 0 - 12.5% w/w HPβCD in PBS (pH 5.0) of steroids used in this study (DXM = dexamethasone).

The apparent K_c values of the steroid-HPβCD complex calculated from the initial linear portion of the slope of the phase solubility diagram and the initial solubility in the buffer (PBS/pH 5.0) using Equation 2.1, are shown in Table 2.8. The *Kc* values of dexamethasone isonicotinate, dexamethasone dipropionate, oestradiol-17β and oestradiol benzoate could not be calculated because the initial solubility (C_0) value of these steroids could not be determined in the buffer alone.

Table 2.8. Apparent K_c (association/dissociation constant) values and slopes of solubility diagrams of the drug-HPβCD complexes of steroids used in this study (Mean + SD; N=6).

Steroid	Slope (M/M)	K_c (M^{-1})
Dexamethasone	0.200 ± 0.005	892 ± 26
Dexamethasone acetate	0.059 ± 0.001	1121 ± 101
Dexamethasone valerate	0.037 ± 0.001	1480 ± 148
Progesterone	0.330 ± 0.019	16470 ± 1408

The apparent K_c values increase from dexamethasone to progesterone (Table 2.8). The lipophilicity of these compounds increases with the same manner of the apparent K_c values (Table 2.10).

2.4.1.4.2 Apparent partition coefficients (octanol: 5%HPβCD/PBS pH5.0)

The apparent partition coefficients of the steroids investigated in the study are shown in Table 2.9.

Table 2.9. Apparent partition coefficient (P_{app}) of steroids used in the study.

Steroid	Day 1 Mean ± SD (N=3)	Day 2 Mean ± SD (N=3)	Mean (N=6)	SD	Log P_{app}
Dexamethasone	6.78 ± 0.31	7.3 ± 0.59	7.04	0.26	0.85
Dexamethasone acetate	43.12 ± 0.36	40.97 ± 0.47	42.05	1.07	1.62
Dexamethasone isonicotinate	115.5 ± 3.6	105.5 ± 13.7	110.50	5.00	2.04
Dexamethasone valerate	206.88 ± 26.58	226.38 ± 28.27	216.63	9.75	2.34
Dexamethasone dipropionate	363.37 ± 22.10	389.7 ± 34.19	376.535	13.17	2.58
Progesterone	77.74 ± 3.36	80.22 ± 4.71	79.11	4.29	1.90
Oestradiol benzoate	624.23 ± 10.49	599.73 ± 34.94	612.94	28.20	2.79
Oestradiol-17β	41.88 ± 0.36	39.68 ± 2.13	40.80	1.86	1.61

The retention times or lipophilicity ranking order using reversed phase HPLC for the steroids investigated are shown in Table 2.10.

Table 2.10. Retention time/lipophilicity order of the steroids investigated in the study.

Drug name	Retention time (minutes) on HPLC (C_{18})
Dexamethasone	6.01
Dexamethasone acetate	8.13
Oestradiol-17β	8.72
Dexamethasone isonicotinate	9.94
Dexamethasone valerate	14.50
Dexamethasone dipropionate	14.70
Progesterone	17.50
Oestradiol benzoate	24.30

2.4.1.5 Apparent partition coefficients (PCL: 5% HPβCD/PBS pH 5.0)

The apparent partition coefficient values (Mean ± SD, N=6) of steroids between PCL and pH 5.0 PBS buffer used in the study are shown in Table 2.11.

Table 2.11. Apparent partition coefficient values of steroids between PCL and 5%HPβCD/PBS at pH 5.0.

Steroid	Day 1 Mean ± SD (N=3)	Day 2 Mean ± SD (N=3)	P_{app}(Steroid/PCL)	Log P_{app}
Dexamethasone isonicotinate	9.27 ± 0.509	8.28 ± 0.509	8.78 ± 0.587	0.94 ± 0.03
Dexamethasone valerate	23.24 ± 0.02	23.20± 0.40	23.29 ± 1.68	1.37 ± 0.03
Dexamethasone dipropionate	67.17 ± 0.421	70.52 ± 0.421	68.86 ± 2.44	1.84 ± 0.02
Progesterone	1.46 ± 0.26	1.89 ± 0.09	1.67 ± 0.289	0.22 ± 0.08
Oestradiol benzoate	31.97 ± 0.33	23.75 ± 0.11	27.87 ± 4.14	1.44 ± 0.07

The apparent partition coefficient values of dexamethasone, dexamethasone acetate and oestradiol-17β could not be determined because these steroids were insufficiently soluble in to the PCL membrane.

2.4.1.6 Ionisation constant (pKa)

The pKa and partition coefficient (octanol/water) values of the steroids investigated in the study are shown in Table 2.12.

Table 2.12. pKa and partition coefficient values of the steroids investigated in the study.

Steroid	pKa[1]	Partition coefficient (log P)
Dexamethasone	12.14 ± 0.70	2.1± 0.58
Dexamethasone acetate	12.05 ± 0.20	2.6 ± 0.58
Dexamethasone isonicotinate	11.93 ± 0.70; 2.85 ± 0.10	3.3 ± 0.59
Dexamethasone valerate	12.67 ± 0.20	4.0 ± 0.58
Dexamethasone dipropionate	12.93 ± 0.20	4.2 ± 0.59
Progesterone	N/A	4.0
Oestradiol-17β	10.37 ± 0.20	4.1 ± 0.25
Oestradiol benzoate	18.0[2]	6.2 ± 0.36

[1]Obtained using Advanced Chemistry Development (ACD) Software Solaris V4.67 (© 1994-2003 ACD). [2]Spidey, Alchemist-Anabolic Mod (2003).

Table 2.12 shows that progesterone is non-ionisable compound, but oestradiol and dexamethsone compounds are ionisable. Additionally, dexamethasone analogues have similar pKa values between 11.9 and 12.9.

2.4.1.7 Permeation studies

2.4.1.7.1 Side-by-side cells (non biological membranes)

2.4.1.7.2 Manufacture of membrane

The silicone membranes manufactured by DEC Manufacturing are shown in Figure 2.9.

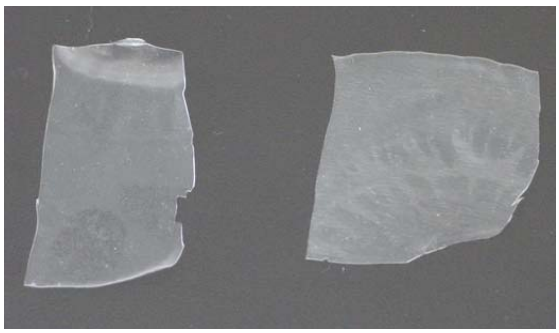


Figure 2.9. Silicone membranes manufactured by DEC Manufacturing.

The average thickness measured from over nine points of the silicone membrane segments is shown in Appendix-B.

2.4.1.7.3 Effect of membrane type

Individual values for dexamethasone permeation through PCL (~140 μm), silicone (~150 μm) and synthetic skin (50 μm) membranes are shown in Appendix-C1. Also individual values and rates of permeation for dexamethasone acetate through PCL (~140 μm), silicone (~150 μm) and synthetic skin (50 μm) membranes are shown in Appendix-C2.

The summarised results for the initial studies assessing permeation of dexamethasone and dexamethasone acetate across several different types of membranes including silicone (150 μm thickness), synthetic skin membrane (50 μm thickness) and PCL (140 μm) are shown in Table 2.13.

Table 2.13. Effect of membrane composition upon the rate of permeation of dexamethasone and dexamethasone acetate.

Drug candidate	Membrane type	Membrane thickness (μm)	Permeation rate (μg/h)	Average permeation rate (μg/h)
Dexamethasone base	Silicone	150	0.089; 0.103; 0.094; 0.082	0.092 ± 0.008
	Synthetic skin	50	0.057; 0.069; 0.058; 0.044	0.057 ± 0.009
	PCL	140	2.00; 2.35; 1.90; 2.42	2.17 ± 0.222
Dexamethasone acetate	Silicone	150	0.348; 0.374; 0.372	0.365 ± 0.012
	Synthetic skin	50	0.064; 0.083 0.069	0.072 ± 0.008
	PCL	140	2.78; 3.09 2.61	2.83 ± 0.199

The permeation rates of dexamethasone and dexamethasone acetate were higher in PCL compare to silicone and synthetic skin membranes (Table 2.13).

2.4.1.7.4 Stability of membrane

Figure 2.10 shows the SEM photographs of the surface properties of the PCL membrane at the end of a 7-day run compared to the surface of a freshly manufactured membrane. There is no any obvious surface property difference between the used and the fresh PCL membrane.

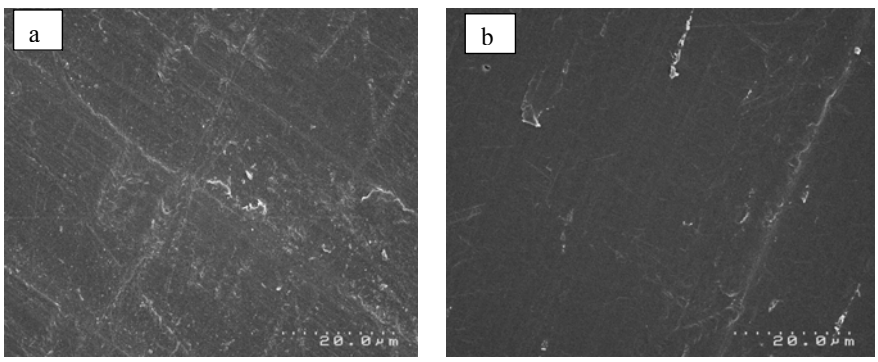


Figure 2.10. SEM of the surface of the membrane at the end of the long run (a) compared to the surface properties of a freshly prepared membrane (b).

2.4.1.7.5 Effect of changing conditions

The effect of changing conditions on dexamethasone permeation through PCL membranes of 140 μm thickness is shown in Table 2.14.

Table 2.14. Effect of the PCL membrane thickness with single or duplicate runs on the cumulative amount permeated per unit area and permeation rates of dexamethasone.

Parameter	Variable	*Flux (μg/h/cm^2)	Permeation rate (μg/h)	Average permeation rate (μg/h)+SD
Membrane thickness(μm)	70	5.29; 4.23	4.15; 3.32	3.74 ± 0.42
	83	3.92; 3.46	3.08; 2.72	2.90 ± 0.18
	86	3.69	2.90	2.90
	98	4.28	3.36	3.36
	102	3.44	2.70	2.70
	104	3.90	3.06	3.06
	114	3.44	2.70	2.70
	121	2.98	2.34	2.34
	130	3.03	2.38	2.38
	138	2.87;3.24	2.25; 2.54	2.40 ± 0.15
	139	3.24, 2.87	2.54; 2.25	2.40 ± 0.15
	140	2.39; 3.08 2.41; 2.73	1.88; 2.42; 1.89; 2.14	2.08 ± 0.22
	141	2.55; 2.99	2.00; 2.35	2.18 ± 0.18
	145	2.42; 3.08	1.90; 2.42	2.16 ± 0.26
	148	2.39	1.88	1.88
	170	1.76	1.38	1.39
	180	1.40	1.10	1.10
Temperature	25°C	0.64; 0.66	0.50; 0.52	0.51 ± 0.01
	30°C	0.99; 1.11	0.78; 0.87	0.82 ± 0.05
	35°C	1.58; 1.34	1.24; 1.05	1.15 ± 0.10
	39°C	2.96; 2.36	2.32; 1.86	2.09 ± 0.23
Cyclodextrin concentration	1.25 w/v	2.04; 1.84	1.60; 1.44	1.52 ± 0.08
	2.5 w/v	2.33; 2.37	1.83; 1.86	1.85 ± 0.02
	5 w/v	2.97; 2.47	2.33; 1.94	2.14 ± 0.20
	7.5% w/v	3.04; 3.16	2.39; 2.48	2.44 ± 0.05
pH of the donor phase	5.0	3.08; 2.42	2.42; 1.90	2.16 ± 0.26
	7.4	2.36; 2.11	1.85; 1.66	1.76 ± 0.10
	10.0	3.15; 2.29	2.47; 1.80	2.14 ± 0.34
pH of the receptor phase	10	3.11; 2.66	2.44; 2.09	2.27 ± 0.18

*NB: the flux was calculated using the permeation rate value divided by area of 0.785 cm^2.

From Table 2.14 it can be seen that the much thicker PCL membranes (≥150 μm) showed lower permeation rates, and therefore diffusion of dexamethasone across the thicker membrane would take longer. The thinner membranes (≤120 μm) showed higher permeation rates with some considerable variation. Improving permeation rates with less variation and need of reasonable time of diffusion across the membrane, 140 μm thick PCL was considered for further study. The

permeation rate showed dependence on temperature and cyclodextrin concentration but there was little or no effect due to variation in pH.

2.4.1.7.6 Screening of steroids across PCL membrane

The flux and permeation rate of various dexamethasone analogues through 140 μm thick PCL membranes is shown in Table 2.15.

Table 2.15. Flux and permeation rates of the different steroids across PCL membrane.

Drug	Flux (μg/h/cm^2)	Permeation rate (μg/h)	Average permeation rate (μg/h) ±SD	Permeation rate ratio relative to dexamethasone
Dexamethasone	2.55; 2.99 2.42; 3.08	2.00; 2.35; 1.90; 2.42	2.17 ± 0.22	1
Dexamethasone isonicotinate	0.71; 0.66	0.56; 0.52	0.543 ± 0.02	0.3
Dexamethasone isonicotinate (pH 7.4)	0.80; 0.87 0.83; 0.82	0.63; 0.68; 0.65; 0.64	0.649 ± 0.02	
Dexamethasone valerate	2.01; 2.46	1.58; 1.93	1.76 ± 0.18	0.8
Dexamethasone dipropionate	2.79; 2.70	2.19; 2.12	2.16 ± 0.04	1.0
Dexamethasone acetate	3.55; 3.94 3.32	2.79; 3.09; 2.61	2.83 ± 0.20	1.3
Oestradiol benzoate	5.20; 5.52; 4.55; 5.04	4.08; 4.33; 3.57; 3.96	3.99 ± 0.27	1.8
Oestradiol-17β	7.12; 6.20 7.66; 7.46	5.59; 4.87; 6.01; 5.86	5.58 ± 0.44	2.6
Progesterone	34.87; 34.32; 26.42; 30.28	27.37; 26.94; 20.74; 23.77	24.71 ± 2.68	11.4

The permeation rate ratio relative to dexamethasone of the tested steroids across the PCL membranes is shown in Table 2.15. Progesterone and dexamethasone isonicotinate showed the highest and the lowest values across the PCL membrane, respectively.

The diffusion coefficient and permeability coefficient values using Equation 2.4, Equation 2.5 and Equation 2.6 of various steroids through 140 μm thick PCL membrane are shown in Table 2.16.

Table 2.16. Diffusion coefficient and permeability coefficient values of various steroids through 140 mm thick PCL membrane (Mean ± SD).

Drug name	$D^{\dagger}$ (cm^2/sec) X 10^{-7}	Permeability coefficient (P_{app})‡ X 10^{-4} cm/sec	Permeability coefficient (P_{aap})‡‡x 10^{-4} (cm/sec)
Dexamethasone	4.8 ± 1.1	0.1 ± 0.01	N/A
Dexamethasone acetate	2.6 ± 0.3	0.42 ± 0.04	
Dexamethasone valerate	1.2 ± 0.2	0.51 ± 0.1	1.7 ± 0.2
Progesterone	6.5 ± 1.0	8.2 ± 1.0	0.55 ± 0.08
Dexamethasone dipropionate	2.4 ± 0.1	N/A	11.2 ± 2.8
Dexamethasone isonicotinate	3.3 ± 1.4		1.5 ± 0.5
Oestradiol benzoate	8.2 ± 0.1		19.3 ± 7.5

†Equation 2.6; ‡Equation 2.4; ‡‡Equation 2.5.

Permeability coefficients of the steroids studied across PCL membrane varied among different steroid analogues. There is a tendency of the permeability coefficient to increase with higher lipophilicity compounds such as progesterone, dexamethasone dipropionate and oestradiol benzoate (Table 2.16).

2.4.2 Side-by-side cells (biological membrane)

2.4.2.1 Screening of steroids across excised mucosa

Table 2.17 shows the flux and permeation rates of the different steroids across excised vaginal mucosa. It was found that some steroids, which have an ester group on carbon position 21 such as dexamethasone acetate, dexamethasone isonicotinate and dexamethasone dipropionat, were found to hydrolyse. Dexamethasone was identified as a secondary compound of ester hydrolysis product of dexamethasone acetate and dexamethasone isonicotinate.

Table 2.17. Flux and permeation rates of the different steroids across excised vaginal mucosa.

Drug name	Flux [ester] (μg/h/cm^2)	Permeation rate [ester] (μg/h)	Average permeation rate [ester] (μg/h) ±SD
Dexamethasone*	16.92; 15.40 21.26; 15.34	13.28; 12.09; 16.69; 12.04	13.53 ± 1.89
Dexamethasone acetate	14.31; 16.42 8.39; 18.01	11.23; 12.89; 6.59; 14.14	11.21 ± 2.86
Dexamethasone isonicotinate	0.84; 0.68 0.54; 0.41	0.66; 0.53; 0.42; 0.32	0.48 ± 0.12
Dexamethasone valerate*	7.12; 6.28 8.37; 7.99	5.59; 4.93 6.57; 6.27	5.84 ± 0.63
Dexamethasone dipropionate	0.60; 0.82 0.43; 0.55	0.47; 0.64 0.34; 0.43	0.47 ± 0.11
Progesterone*	14.27; 11.11 21.03; 11.55	11.20; 8.72 16.51; 9.07;	11.38 ± 3.11

*Compounds not hydrolysing.

Table 2.18 shows the permeation rates of the ester hydrolysis product of dexamethasone acetate and dexamethasone isonicotinate across excised vaginal mucosa. The ester hydrolysis of dexamethasone dipropionate were observed on the chromatogram but not quantified due to not having a reference compound (e.g., dexamethasone-17-propionate). The peak due to the ester hydrolysis product was well separated from the principal peak due to dexamethasone dipropionate.

Table 2.18. Permeation rates of the ester hydrolysis product (dexamethasone) obtained by dexamethasone acetate and dexamethasone isonicotinate across excised vaginal mucosa.

Drug name	Permeation rate [dexamethasone] (μg/h)	Average permeation rate [dexamethasone] (μg/h) ±SD
Dexamethasone acetate	1.91; 1.36; 1.85; 1.33	1.61 ± 0.27
Dexamethasone isonicotinate	0.66; 0.53; 0.64; 0.62	0.61 ± 0.05

Table 2.19 shows permeability coefficient values of the different steroids across excised vaginal mucosa. These values were calculated using Equation 2.4.

Table 2.19. Permeability coefficients of the studied steroids across excised mucosa.

Drug name	Permeability coefficient (P_{app})[1] (cm/sec x 10^{-5})	Average permeability coefficient [ester] (cm/sec x 10^{-5}) ±SD
Dexamethasone	4.3; 4.0; 5.5; 3.9	4.4 ± 0.72
Dexamethasone acetate	16.6; 19.1; 9.8; 20.9	16.6 ± 4.9
Dexamethasone valerate	16.0; 14.1; 18.7; 18.0	16.7 ± 2.1
Progesterone	37.0; 28.8; 54.6; 30.0	37.6 ± 12.0

Progesterone and dexamethasone showed the highest and the lowest permeability values across the excised mucosa, respectively. This is possibly due to the relative molecular size and degree of lipophilicity of progesterone compared to dexamethasone, dexamethasone acetate and dexamethasone valerate which may all contribute to the ranks of permeability values.

2.5 Discussion

2.5.1 Confirmation of identity of the active ingredients

In order to confirm the identity of the steroids used in the study, experiments were conducted using IR and DSC analysis. The IR spectrum was analysed and the principle peaks were compared to those found in the literature for the same compounds or their chemical structures. The comparison is shown in Table 2.20.

Table 2.20. Literature values and experimentally determine IR values for the steroids used in this study.

Drug name	Experimentally observed principle Wave numbers (cm^{-1})	Literature wave numbers (cm^{-1})
Dexamethasone	852; 893; 1033; 1054; 1135; 1621; 1664; 1706	852; 893; 1040; 1150; 1620 1670; 1660; 1700 [1,2]
Dexamethasone acetate*	871; 968; 1055; 1608; 1673; 1731; 2947	852; 893; 1040; 1610; 1670; 1700; 2960-2850
Dexamethasone valerate*	831; 892; 1031; 1394; 1609; 1617; 1662; 1715	852; 893; 1040; 1395; 1610; 1660; 1670; 1700
Dexamethasone isonicotinate*	848; 983; 1044; 1604; 1622; 1663; 1723;	852; 893; 1040; 1610; 1660; 1670; 1700
Dexamethasone dipropionate*	881; 895; 926; 1040; 1257; 1662; 1701; 1750; 3301	852; 893; 1040; 1260; 1610; 1660; 1670; 1700; 1755-1725 3200-3600
Progesterone	871; 1204; 1227; 1278; 1614; 1661; 1698	872; 1209; 1232; 1614; 1662; 1700 [2,3]
Oestradiol-17β	818; 872; 1011; 1054; 1355; 1497; 1585; 1608; 2862	818; 872; 1011; 1054; 1355; 1497; 1585; 1608; 2862[4]
Oestradiol benzoate	704; 1063; 1129; 1262; 1723	704; 1075; 1220; 1250; 1724 [2,3]

[1](Cohen, 1973) and [2](Pharmaceutical Society of Great Britain.Department of Pharmaceutical Sciences, 1979); [3](Clarke *et al*., 1986) and [4](Salole, 1986). *Dexamethasone ester chains wave numbers from literature were obtained using infrared correlation charts by Weast (1988).

The close agreement between the literature values and the experimentally determined IR values seen in Table 2.20 indicates that each of the steroids used in the study were as described by the supplier. The DSC traces were analysed and

endothermic melting peaks shown by the thermographs were compared to the reported melting point peak values for the steroids used in the study. Literatures values and experimentally determined DSC values for the steroids used in the study are shown in Table 2.5.

Melting is an endothermic process in which the sample takes in a net quantity of heat (Clas *et al*., 1999). A 100% pure crystalline compound shows a sharp melting endothermic change. The presence of impurities or crystalline defects lead to a broadening of the melting endotherm and to a lowering of the melting temperature. According to the literature, the onset temperature where the transition temperature starts to deviate from the baseline can describe a melting endotherm. The extrapolated onset melting temperature is reported as the temperature at the intersection of the extrapolated baseline prior to the transition with the extrapolated leading edge of the transition. The endothermic melting peak is defined as the peak temperature (T_m). Extrapolated onset temperature and literature values shown in Table 2.5. The close agreement between the literature values and the experimentally DSC values seen in Table 2.5 again indicates that each of the steroids used in the study were pure as described by the supplier.

2.5.2 Oestradiol-cyclodextrin Complexation

Complexation leads to changes in the physicochemical properties of the steroids used in this study. The extrapolated onset melting peak temperatures for the oestradiol/cyclodextrin complexes investigated in the study are shown in Table 2.6.

DSC thermograms showed endothermic peaks corresponding to the melting points of oestradiol benzoate, oestradiol-17β, γCD and HPβCD in physically mixed samples (Table 2.6), as shown in Figure 2.7. This indicates that there was no potential interaction between γCD or HPβCD and the oestradiols.

The endothermic peaks for oestradiol benzoate or oestradiol-17β were absent in the thermograms from samples prepared from saturated solution. However, γCD and HPβCD melting peaks were always present with slight shifts. Broadening and

lowering of the melting temperatures seen for oestradiol and cyclodextrin are due to complex formation. The broadening is much more pronounced in the case of γCD than HPβCD. Presumably this is due to a different central cavity diameter (7.5-8.3 for γCD, 6.0-6.5 Å for HPβCD) (Loftsson *et al*., 1996; Loftsson *et al*., 2003). It is possible that the drug has more central cavity space to interact with when γCD is used compare to HPβCD.

The disappearance of the oestradiol benzoate or oestradiol-17β melting peaks in the complex samples showed that there was a possible interaction between the drug and cylodextrin. This might be due to γCD or HPβCD oestradiol complexes being in an amorphous state. A similar explanation was given (Babu & Pandit, 2004) when inclusion of complexes of bupranolol with cyclodextrin were characterised by DSC and the melting point of bupranolol was found to completely disappear.

However, further analysis of the pure samples and their complexes using X-ray diffractometry might explain the presence of the amorphous state further.

2.5.3 Solubility and association/dissociation constants (K_c)

The use of the drug carrier cyclodextrin is to improve the solubility and bioavailability of the steroids across artificial or/and biological membranes (Lopez *et al*., 2000). The phase-solubility diagram of dexamethasone in aqueous HPβCD solution was investigated (Loftsson *et al*., 1994). The solubility increased linearly as a function of HPβCD concentration with a slope of less than unity and, thus, formation of a 1:1 complex could be assumed. The K_c value of the dexamethasone-HPβCD inclusion complex was determined to be 1230 M^{-1}.

The phase-solubility diagram in aqueous HPβCD (0-12.5%w/w in PBS/pH 5.0) solution of steroids used in this study has been p shown in Figure 2.8 (see Section 2.4.1.4.1). The solubility of all studied steroids was found to increase with an increase in HPβCD concentration. This is in agreement with data reported in the literature (Usayapant & Iyer 1999). The increase in aqueous solubility of the steroids is evidence of formation of a water-soluble drug HPβCD (1:1) inclusion

complex (Loftsson *et al*., 1994). In addition, a slope of less than unity (K_c) suggests the formation of a drug-cyclodextrin (1:1) complex. Moreover, the disappearance of the oestradiol benzoate or oestradiol-17β melting peaks in DSC scans of HPβCD complexes samples showed that there was interaction between the steroids and cyclodextrin (see Section 2.5.2).

The ranking order of solubility profile dependency on HPβCD content increase was progesterone, oestradiol-17β, dexamethasone, dexamethasone acetate, dexamethasone valerate, oestradiol benzoate, and dexamethasone isonicotinate and dexamethasone dipropionate (Figure 2.8). It seems that structural complexity (perhaps presence of attached groups on steroid compound such as ester chains), size and the initial solubility of these steroids all contribute to give different solubility diagram profiles.

It was found that the total amount of drug in solution is a function of both the intrinsic solubility of the drug in the absence of cyclodextrins and of the complexation constant, K_c, and sometimes the two effects can cancel out (Stella *et al*., 1999). In addition, the solubility of most drugs increases with increasing temperature. West (2003) reported that the body temperature of a cow might vary between 38.9 and 39.4ºC. In addition, any product resulting from this research would be intravaginally administered into cows. It was therefore decided to conduct the preformulation experiments at a temperature of 39ºC. According to the literature, the formation rate constant of the drug/cyclodextrin complex was found to be close to that of a diffusion controlled process and weakly dependent on structure of the drug and K_c, while the dissociation rate constant was very structure sensitive and inversely proportional to K_c (Stella *et a*l., 1999).

The linear phase solubility diagrams of dexamethasone and dexamethasone acetate K_c values (Table 2.8) are in agreement with data reported in the literature. In this study, the K_c values for complex formation were 892 and 1121 M^{-1} versus literature values of 1230 (Loftsson *et al*., 1994) and 2240 M^{-1} (Usayapant *et al*., 1991) for dexamethasone and dexamethasone acetate, respectively.

In principle, drug/cyclodextrin complexes may dissociate due to a decrease in K_c as the complex is exposed to a pH, which changes the charge status of either the drug or the cyclodextrin (Stella *et al*., 1999). Additionally, binding of drugs to cyclodextrins has been shown to be an exothermic process (ΔH is negative). Hence, any increase in temperature results in a weakening of the complex, and thus an increase in the free fraction of drug. This can explain lower values of K_c of the studied steroids compared with values of 39°C versus 23-25°C, respectively.

2.5.4 Side-by-side-cells and using non-biological membranes

Various non-biological membranes were investigated in side-by-side cell experiments. Synthetic skin of known thickness (50 μm) was used as received. However, the membranes that were manufactured by DEC Manufacturing or in-house were characterised in terms of their thickness and uniformity of thickness. The silicone membranes manufactured by DEC Manufacturing (Figure 2.9) were shown to exhibit a uniform thickness with an average thickness of 150 ± 8 μm ranging from 140.0 to 166 μm. PCL membranes manufactured in-house for use in the study (Figure 2.6) were also shown to exhibit a uniform thickness with an average of 143.1 ± 5.0 μm ranging from 138.0 to 148 μm.

Initially, the effect of membrane composition on the permeation of two compounds (dexamethasone and dexamethasone acetate) was studied in order to identify a membrane for use in future experiments. Three membranes were investigated: silicone, synthetic skin and PCL. Several criteria were identified that the membrane should meet for use in these studies. These included: (i) ready availability or ease of manufacture; (ii) sufficiently cheap to purchase or manufacture; (iii) exhibition of a reproducible thickness; (iv) stability for the duration of the side-by-side cell studies (up to 7 days); (v) permeability to the steroids used in the study. Each of the membranes investigated fulfilled criteria (i) to (iv), however, the membranes were observed to vary in their permeability toward steroids. Permeation rates for dexamethasone and dexamethasone acetate across the membranes were ordered from the slowest to the fastest as follows synthetic skin followed by silicone followed by PCL (Figure 2.11).

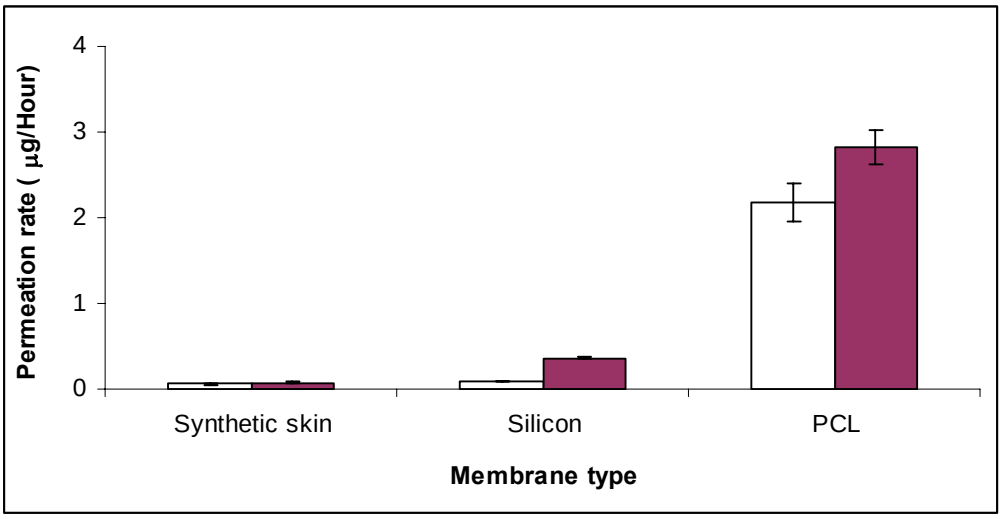


Figure 2.11. Rate of permeation (Mean ±SD, N = 4) for dexamethasone (□) and dexamethasone acetate (▪) through silicone, synthetic skin and PCL membranes.

The high permeability of PCL led to it ultimately being chosen as a membrane for use in this reseasrch, since it resulted in high steroid concentrations appearing in the receptor phase thus allowing for easy and accurate detection of the steroids by a simple UV analytical method. The high permeability of PCL also meant that the duration of an experiment could be reduced (compared to the other membrane types) and yet still provides an accurate estimate of the permeation rate.

However, in contrast to silicone membrane or synthetic skin, the PCL membrane is a degradable polymer. Therefore, it was important to show that the membrane was not degrading over the time course of an experiment. An experiment was initiated using dexamethasone and dexamethasone acetate that studied the effect of 7 days exposure of the membrane to the aqueous phase used in the side-by-side cell studies on the stability of the PCL membrane. SEM photomicrographs revealed that freshly manufactured and exposed membranes had the same surface characteristics suggesting that no degradation had taken place.

In addition, the linearity ($R^2 \geq 0.9996$) of the appearance of the steroids in the receptor phase suggests that no degradation of the membrane is occurring during the course of the experiment (Figure 2.12).

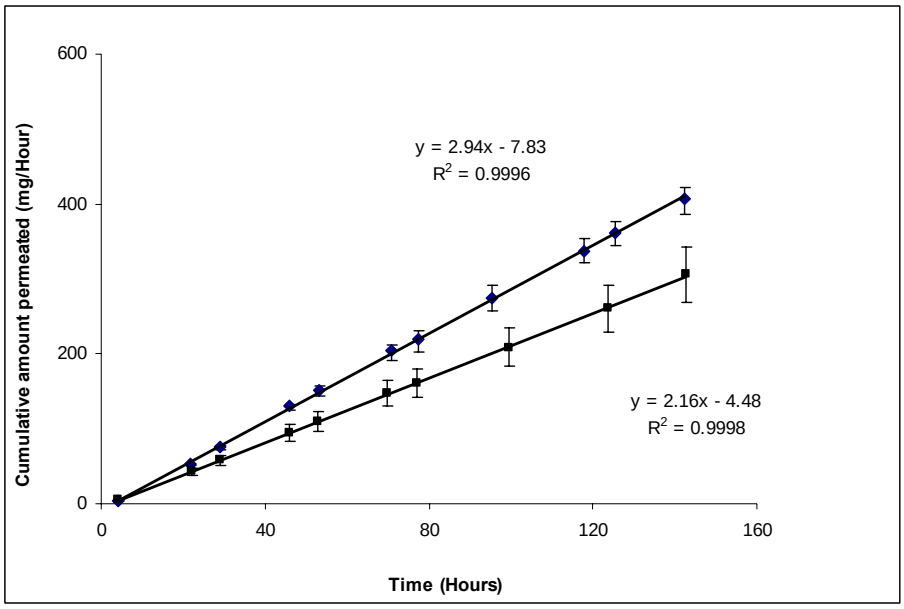


Figure 2.12. Rate of permeation (Mean ±SD, N = 4) for dexamethasone acetate (◆) and dexamethasone (■) through PCL membranes.

The effect of changing conditions in the donor and receptor phases upon dexamethasone permeation through PCL membranes of 140 μm thickness was studied. As the thickness of the membrane increased, the rate of permeation decreased (Figure 2.13).

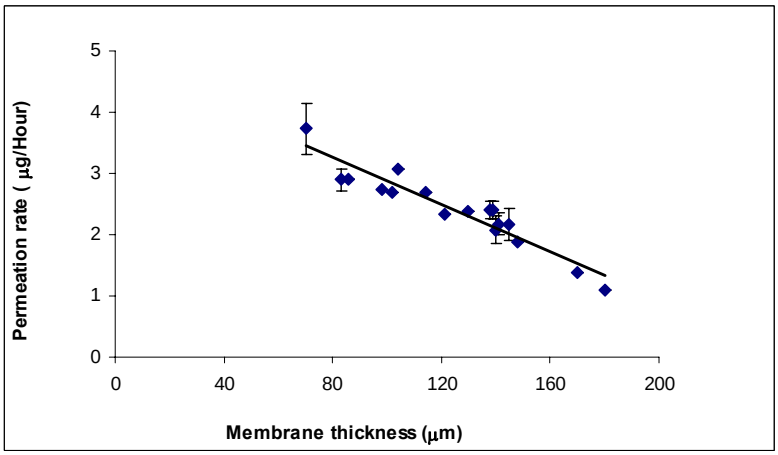


Figure 2.13. Effect of PCL thickness on dexamethasone permeation rate through PCL membranes.

The temperature of the donor and receptor phase also affected the rate of permeation with increasing temperature increasing permeation rate (Figure 2.14).

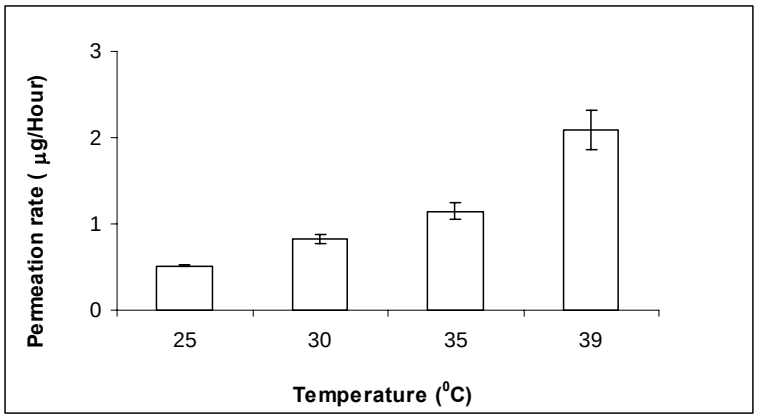


Figure 2.14. Effect of temperature on dexamethasone permeation rates.

Cyclodextrin concentration was also seen to affect the rate of permeation of dexamethasone. As the concentration of cyclodextrin was increased in the donor phase, the rate of permeation increased (Figure 2.15). Since passive diffusion is driven by high drug concentration in the aqueous vehicle, the increase of dexamethasone in solution can explain the increase of dexamethasone permeation through the PCL membrane. This was in agreement with what is reported in the literature (Loftsson *et al*., 1994).

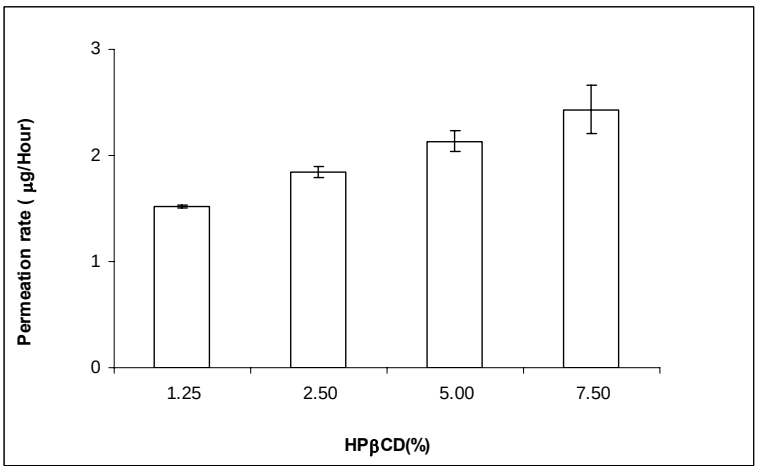


Figure 2.15. Effect of cyclodextrins (HPβCD) concentration on dexamethasone permeation rates.

Changing the pH of the donor phase (while keeping the receptor phase pH at 5) had no effect on the rate of permeation of dexamethasone (Figure 2.16).

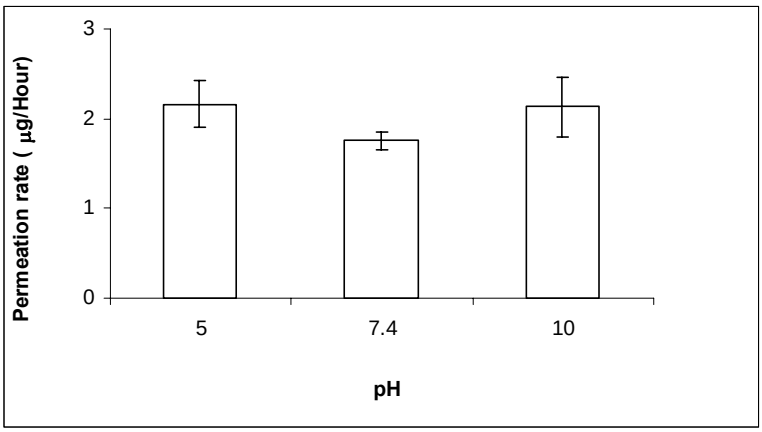


Figure 2.16. Effect of donor and receptor phases' pH (5 vs. 5; 7.4 vs. 5; and 10 vs. 5 for the donor and receptor phases respectively) on dexamethasone permeation rates.

In addition, changing the pH of the receptor phase to pH 10 (from pH 5) had no effect upon permeation rate. It is expected that ionised species penetrate poorly through the membranes compared with non-ionised species (Mashru *et al.*, 2005). The effect of pH on the rate of permeation would be expected to be negligible due to the fact that dexamethasone has a pKa value greater than 12.0 which is at least two or more pH units away from the range of the tested pHs.

Figure 2.17 shows the rate of permeation of different steroids through PCL membranes.

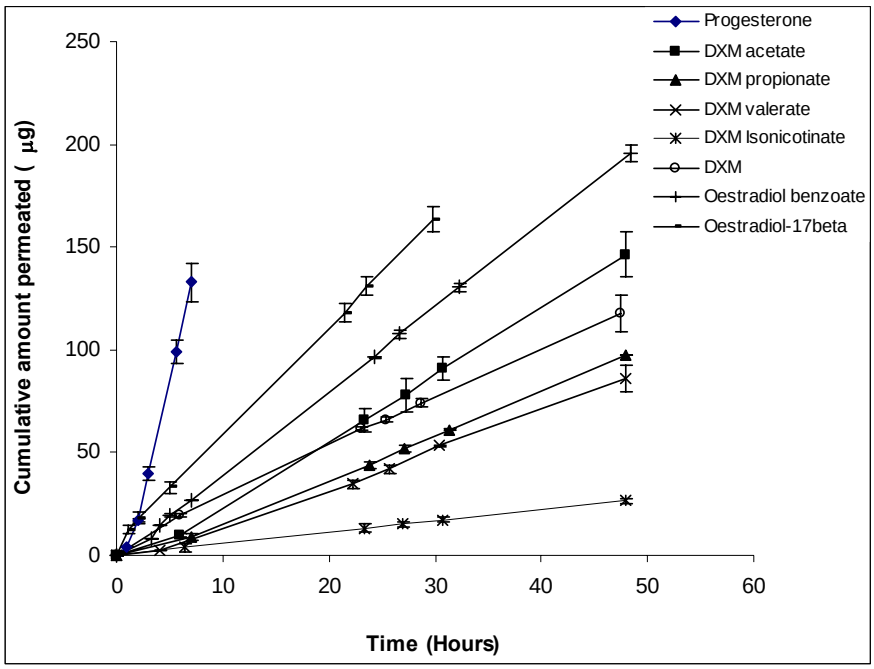


Figure 2.17. Effect of steroids on rate of permeation profiles through PCL membranes (Mean ± SD, N=2).

The permeation rate profiles of the various steroids significantly varied. Progesterone showed the highest permeation rate of all tested steroids. There is a trend of decreasing permeation rate from oestradiol-17β, oestradiol benzoate to dexamethasone analogues (Figure 2.17). Haigh & Smith (1994) have reported that the steroid partition coefficient between donor or receptor phase and the membrane is the most important variable that can affect the permeation process. The different permeation rate profiles could be explained by different solubility, lipophilicity, relative molecular size and molecular shape among the steroids. The permeation rate is function of the steroid concentration in the donor phase as well as the partition coefficient (Schoenwald & Ward, 1978) between the steroid and the membrane.

Figure 2.18 shows the relationship between permeation rates versus partition coefficient expressed in LogP at octanol: pH 5/5%HPβCD, retention time on

HPLC (C_{18}), solubility at pH 5/5%HPβCD and partition coefficient at pH 5/5%HPβCD/PCL membrane of some steroids. It can be seen from Figure 2.18 that the permeation rates of these compounds did not show any obvious trend with their physicochemical properties.

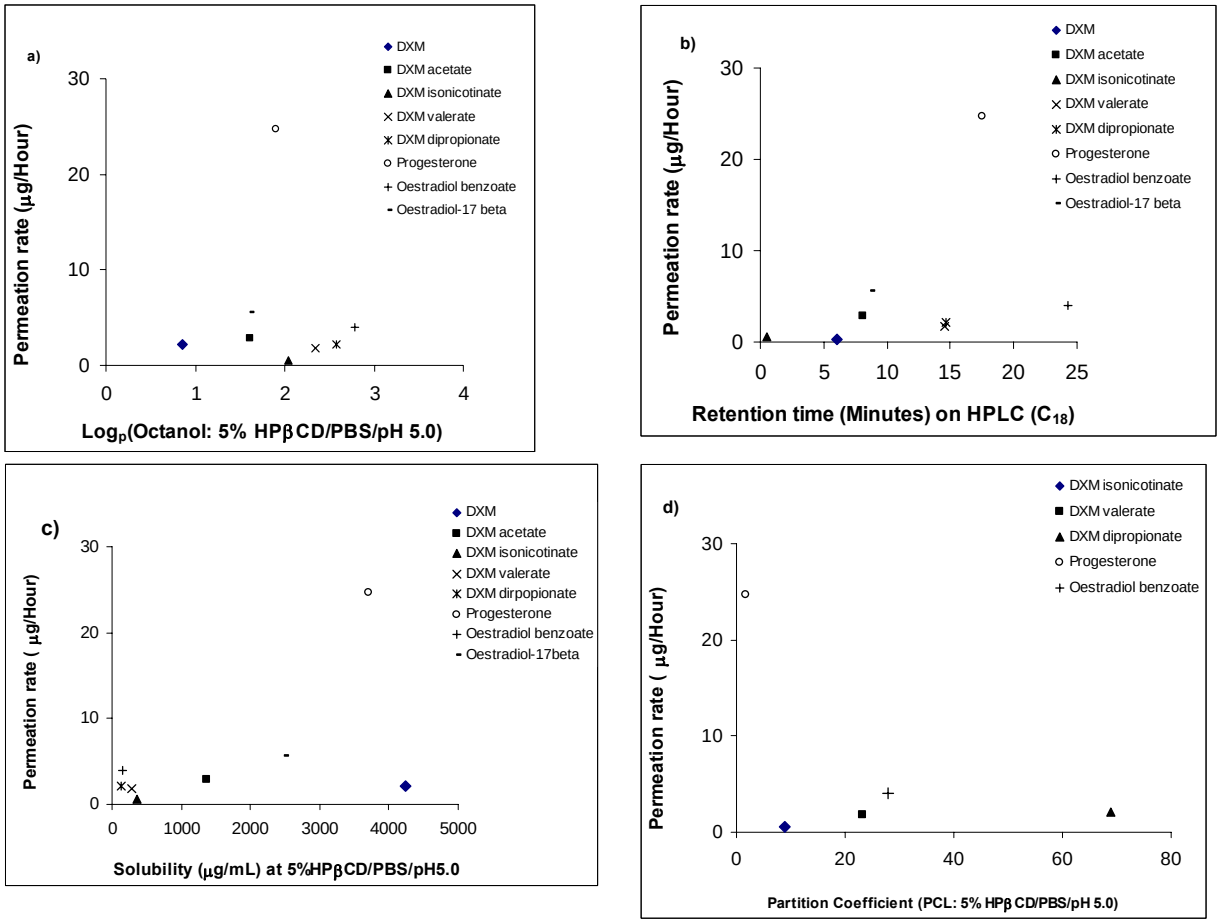


Figure 2.18. Permeation rates through PCL membranes versus partition coefficient (a), retention time on HPLC (C18) (b), solubility (c), and partition coefficient (pH 5/5%HPbCD)/PCL membrane (d) of some steroids.

Figure 2.19 shows the relationship between permeability coefficients versus partition coefficient (LogP of octanol: pH 5/5%HPβCD), retention time on HPLC (C_{18}), solubility at (pH 5/5%HPβCD) and partition coefficient (pH 5/5%HPβCD)/PCL membrane of some steroids.

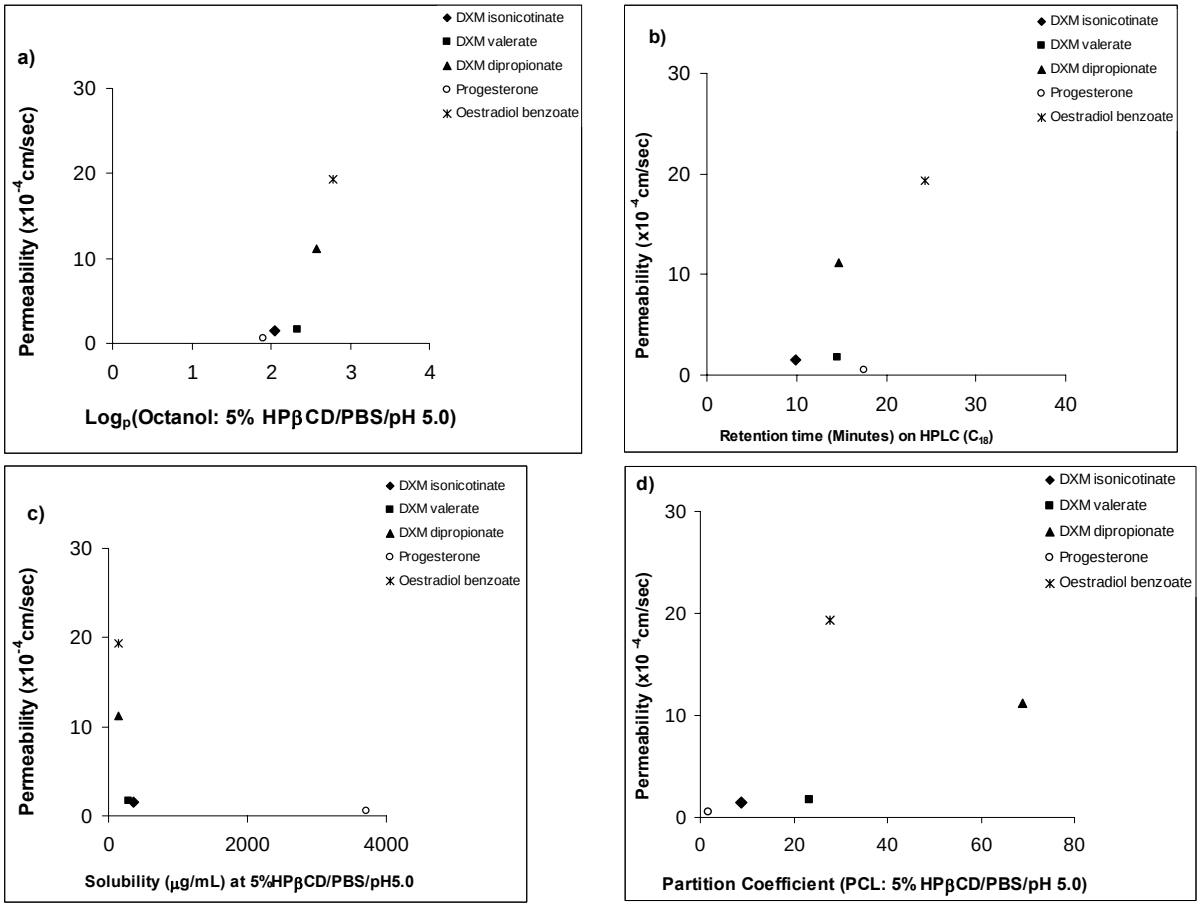


Figure 2.19. Effect of partition coefficient (LogP)(pH 5/5%HPbCD) (a), retention time on HPLC (C18) (b) solubility at (pH 5/5%HPbCD) (d), and partition coefficient (pH 5/5%HPbCD)/PCL (d) upon the permeation rates through PCL of some steroids.

It can be seen from Figure 2.19(a-b) that as the LogP and retention time of the compound increase, so does its permeability coefficient. However, other physicochemical properties did not show any obvious correlation with the permeability coefficients of the steroids. Both the partition coefficient and retention time indicate the lipophilicity of the steroids. Since the partition coefficients and retention time showed a correlation with the permeation rates, the PCL appears to be non-porous and lipophilic membrane. The PCL membrane appears to offer some rate-limiting step to steroids permeation. This type of barrier function is inherited to the membrane for which only dissolved free drug molecules will have to partition from the aqueous donor to the membrane (Loftsson *et al*., 2003). Therefore, the lipophilicity between the membrane and drug reflects the ability of the drugs to leave the donor phase and partition into the membrane (Figure 2.19a). The use of PCL as a model membrane in side-by-side

cell experiments results in the advantage that compounds rapidly permeate through it, resulting in the need for less sensitive analytical techniques to be developed and potentially only short experimental times can be used to generate sufficient data to provide a reliable estimate of the permeation rate. In addition, the PCL membrane can be used for showing a sufficient discriminatory power between steroidal dosage forms.

2.5.5 Side-by-side-cells using excised mucosal membranes

All of the steroids studied permeate across excised mucosal membranes with varying permeation rates. A typical curve of the cumulative amount of steroid permeated through excised mucosa over time (i.e., dexamethasone valerate) is shown in Figure 2.20a. Some other examples are shown in Appendix-D. A linear ($R^2 \geq 0.9986$) relationship was obtained when the total amount of each steroid analogue in the receptor phase was plotted against time. Therefore, the dexamethasone analogues and progesterone permeation through excised mucosa transport can be described by zero order kinetics. Figure 2.20a exhibits a lag time (the time before establishing steady state concentration across membrane) which was not used in the calculation of the linear portion of the curve to determine the slope of the line. An example of a typical curve that is used for permeation rate calculations of the steroids studies is shown in Figure 2.20b.

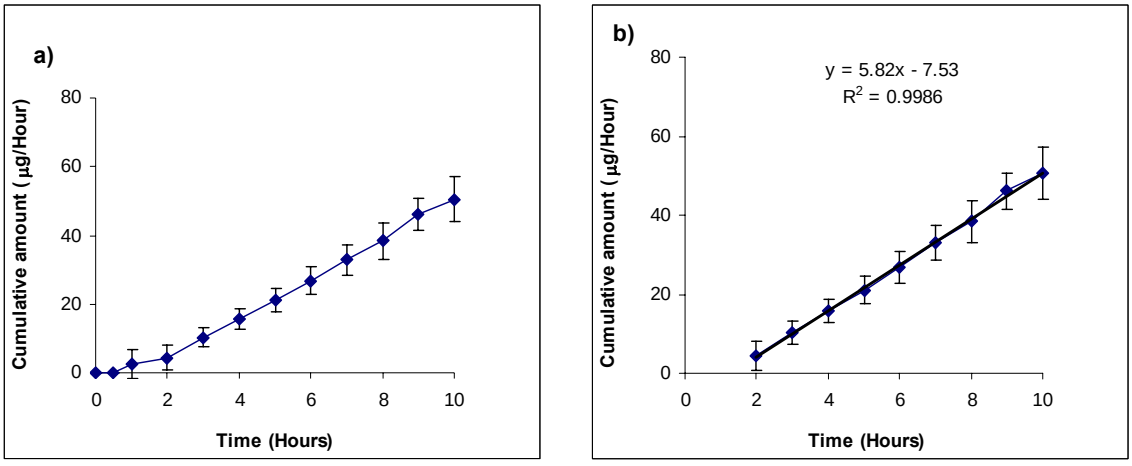


Figure 2.20. A typical cumulative amount of dexamethasone valerate across excised mucosa membrane (Mean ±SD, N=4) including (a) and excluding the lag phase (b).

Some steroids showed higher permeation rates than others, as previously shown in Table 2.17. This could be a possible combination of the steroid lipophilicity and the concentration gradient between the two sides (donor and receptor phase) of the membrane. Dexamethasone acetate, dexamethasone isonicotinate and dexamethasone dipropionate showed ester bond hydrolysis when the excised mucosal membrane was used. In addition, no secondary product was found when dexamethasone acetate and non-biological membranes (i.e. silicone or PCL membrane) were used in side-by-side cells. Therefore, it is possible that some esterase enzymes are present in the vaginal mucosa membrane which are specific enough to cleave the ester bond at the carbon 21 position of dexamethasone acetate, dexamethasone isonicotinate and dexamethasone dipropionate. This possibility is supported by other researchers who found that dexamethasone ester hydrolysis was occurring in biological tissues due to esterase enzymes (Lopez *et al*.,2000 & Civiale *et al*., 2004). In addition, other researchers found that dexamethasone was released from dexamethasone isonicotinate or dexamethasone sodium phosphate due to ester hydrolysis and was available in the systemic circulation immediately following intravenous or intramuscular administration (Toutain *et al*., 1982; Welch *et al*., 1973; Fairclough *et al*., 1981a).

Interestingly, the dexamethasone ester on the carbon 17 position (i.e. dexamethasone valerate) did not show any secondary metabolic products. This suggests the presence (in the excised mucosa) of a stereo-specific enzyme, which only cleaves at a specific ester bond. From the chemical structure of dexamethasone esters on the carbon 21 position, which is more exposed to enzymatic hydrolysis reactions, seems to be more susceptible to the hydrolysis unlike position 17 esters, which is perhaps due to possible steric hindrance and stereospecifity of the enzymes causing the hydrolysis. Further evidence is that one secondary product was found from dexamethasone dipropionate, which has two ester bonds on carbon position, 17 and 21. The carbon position 21 ester bond was likely to be cleaved perhaps due to its lack of steric hindrance and thereby susceptibility to the hydrolysis as shown by the ester hydrolysis on carbon position 21 of dexamethasone acetate or dexamethasone isonicotinate.

Figure 2.21 shows the correlation of permeation rates between PCL and excised mucosa. It can be seen from Figure 2.21 the permeation rate values using PCL cannot predict the rate of permeation through excised mucosa for the same steroid, and therefore, the PCL was not an ideal model membrane to choose for predicting the rate of permeation through excised mucosa.

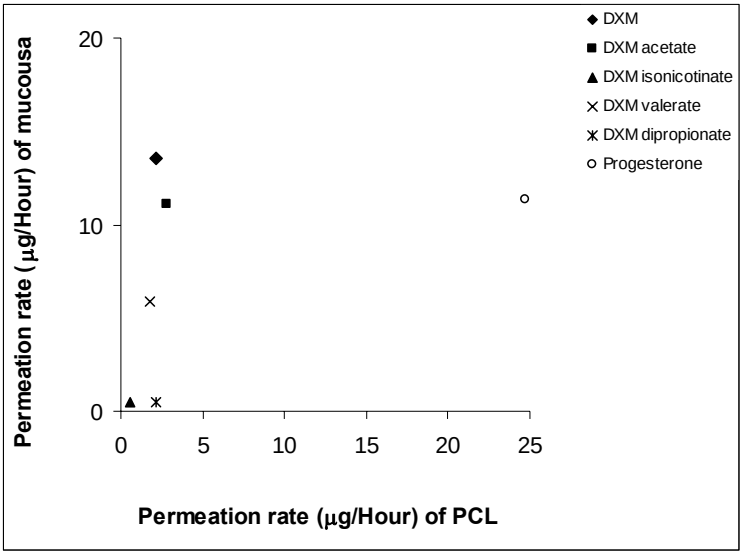


Figure 2.21. Correlation of permeation rates between PCL and excised mucosa.

Figure 2.22 shows the relationship between permeation rates and log solubility in a pH 5/5% HPβCD solution through the excised mucosa of some steroids. It can be seen that as the solubility of the compound increases so does its permeation rate. The steroid compound can be sheltered inside the HPβCD cavity, become more soluble and creating a large concentration gradient between the donor and receptor phases and thereby improving the rate of permeation.

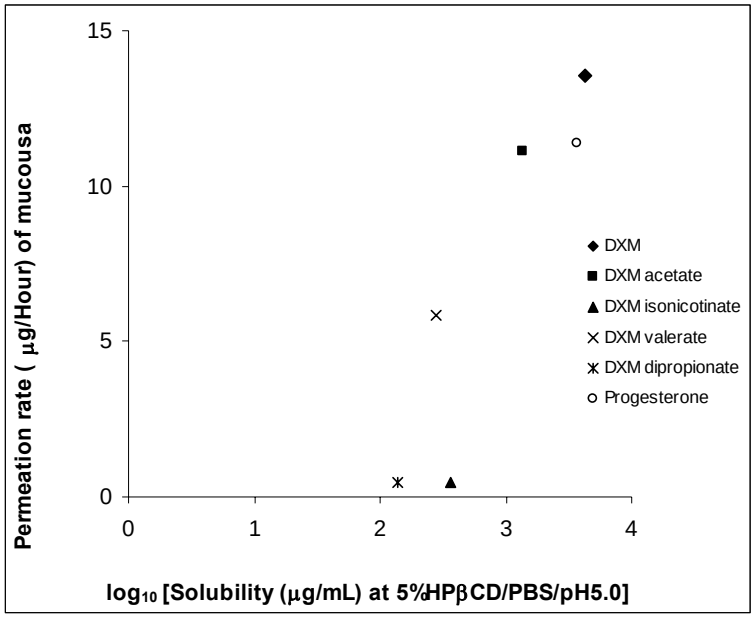


Figure 2.22. Effect of solubility upon the permeation rate of some steroids.

Table 2.21 presents the permeability coefficients and their permeability relative to that for dexamethasone using Equation 2.4.

Due to the poor solubility of dexamethasone isonicotinate and dipropionate in the PBS buffer, the permeability coefficient values were impossible to determine using Equation 2.4.

Table 2.21. Permeability coefficients and their ranking order of some steroids through excised mucosa.

Drug name	Average permeability coefficient [ester] (cm/sec x 10^{-5}) ±SD	Permeability coefficient ratio relative to dexamethasone
Dexamethasone	4.4 ± 0.72	1
Dexamethasone acetate	16.6 ± 4.9	4
Dexamethasone valerate	16.7 ± 2.1	4
Progesterone	37.6 ± 12.0	9
Dexamethasone isonicotinate Dexamethasone dipropionate	Impossible to be determined	

The permeability coefficients of the tested steroids through excised mucosa are ranked from the highest to the lowest as follows: progesterone followed by dexamethasone valerate/dexamethasone acetate followed by dexamethasone as

shown in Table 2.21. The rank order of the permeability coefficients seems to follow increasing order of lipophilicity.

Figure 2.23 shows the effect of molecular weight on the permeation and permeability rates through the excised mucosa of some steroids. A trend shows the permeation and permeability values decreased with increasing of the steroid molecular weight. For instance, the steroids with lower molecular weights, such as progesterone and dexamethasone (DXM), showed high permeation rates compare to those with higher molecular weights such as DXM dipropionate and DXM isonicotinate (Figure 2.23a). This finding was in agreement with reported information that demonstrated the transport of a drug molecule through living membranes is a size dependent (Ritschel, 1988).

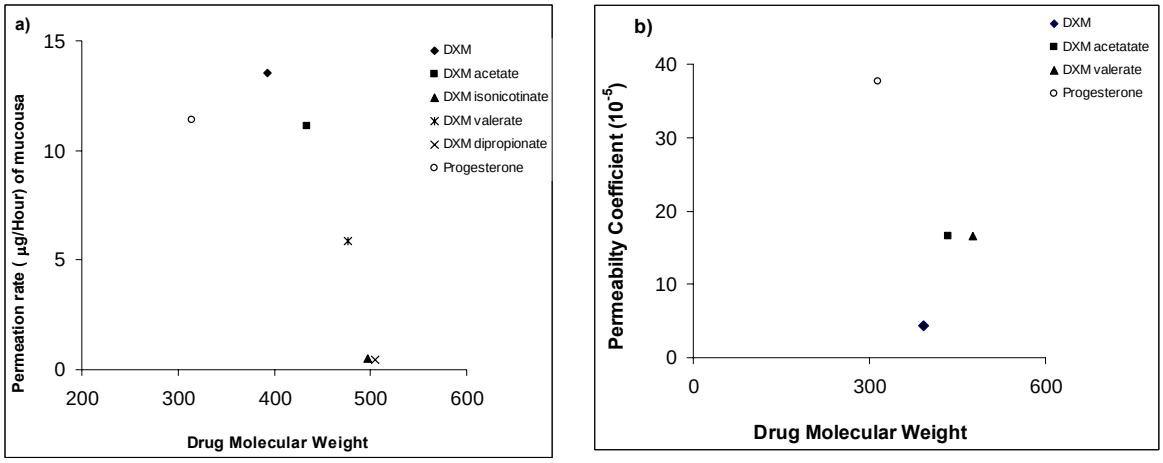


Figure 2.23. Effect of molecular weights on permeation (a) and permeability values (b).

A close agreement of the progesterone permeability coefficient through excised vaginal mucosa obtained in this study and the literature value (37.6 versus 69 x 10^{-5} cm/sec, respectively) was obtained (Chien, 1992). Similarly, dexamethasone and dexamethasone acetate permeability coefficient values obtained in this study showed the same order of magnitude with the literature values across a Caco-2 cells monolayers (4.4 versus 1.3 x 10^{-5} cm/sec for dexamethasone) (Artursson & Karlsson, 1991) or/and bovine conjunctival epithelial cells (4.4, 16.6 versus 0.1, 1.8 x 10^{-5} cm/sec for dexamethasone and dexamethasone acetate, respectively) (Civiale *et al*., 2004).

Ester bond hydrolysis of dexamethasone acetate, dexamethasone isonicotinate and dexamethasone dipropionate has been observed. Dexamethasone valerate was not hydrolysed and showed a relatively good permeability coefficient through vaginal mucosa. Dexamethasone acetate existed in two forms, which are dexamethasone and dexamethasone acetate. The latter dexamethasone ester permeability was good as per dexamethasone valerate. However, both the two forms of dexamethasone acetate are active drugs.

2.6 Conclusion

The IR spectrum and DSC results were found to be in close agreement with those found in the literature for the same compounds. The solubility phase diagrams for the different steroids showed different profiles due to their different physicochemical properties.

An *in-vitro* steroid permeation technique was developed and validated using side-by-side cells with non-biological membranes.

A simple method using tools made in-house was developed to manufacture PCL membranes with defined thicknesses. The PCL membrane used for the extended study appeared very similar under the SEM to a similar unused membrane, indicating that it had retained its integrity. The permeation rate remained constant over the 7 days for which permeation was monitored, supporting visual findings of membrane integrity. The effect of temperature on the permeation rate showed a positive relationship between increased temperature and the permeation rate. Changing the pH resulted in no significant change to the permeation rate, whereas increasing cyclodextrin concentration resulted in a small increase in the rate of dexamethasone permeation. Solubility and apparent partition coefficient values of the dexamethasone analogues, progesterone, and oestradiols (in 0-12.5%HPβCD at 39°C inPBS/pH5.0) were determined and found to be comparable to the available literature values.

Dexamethasone analogues and progesterone could be easily permeated through PCL and excised vaginal mucosal membranes. In addition, oestradiol-17β and

oestradiol benzoate could be also easily absorbed and permeated through PCL. Reproducible and linear permeation rates of the steroid studied were obtained from side-by side cell experiments using the PCL and excised mucosa membranes.

The potential advantage of PCL as a model membrane in side-by-side cell experiments arises from the rapidity with which compounds permeate through it, resulting in the need for only short experimental times to generate sufficient data to provide a reliable estimate of the permeation rate. It is also cheap to purchase and easily fabricated in the laboratory into thin flat sheets. However, experiments conducted using dexamethasone and its analogues, oestradiol-17β, oestradiol benzoate, and progesterone, suggest that PCL is not a useful model membrane since it is a poor predictor of permeation rate across (excised) vaginal mucosa.

The excised mucosa was permeable to the tested steroids with some differences in degree of permeation. Bothe molecular weight and solubility of the steroid affected the rate of permeation. The rate of permeation increased with increasing the solubility of the steroid. Conversely, both rate of permeation and permeability values decreased with increasing the molecular weight of the steroid.

The ester group on the carbon 21 position for dexamethasone acetate, dexamethasone isonicotinate and dexamethasone dipropionate were hydrolysed. The ester group of dexamethasone valerate did not show any hydrolysis. Dexamethasone acetate and dexamethasone valerate showed higher permeability values across vaginal mucosa compare to other tested dexamethasone analogues and therefore, they could be used for induced calving *pervaginum* administration.

3 Analytical methods

3.1 Introduction

Initially an analytical UV spectrophotometric (Spectrophotometer DU® 640, Beckman, USA) method was developed and validated for all steroids studied in this research. This analytical method was used for *in vitro* permeation experiments to measure steroid permeation across non-biological (140 μm thick poly-ε-caprolactone, 150 μm thick silicone and 50 μm thick synthetic skin) membranes as described in Chapter Two (Section 2.3.2.6). The use of the UV method was considered because it is fast, easy and inexpensive compare to HPLC.

The same analytical UV method was used for the determination of dexamethasone concentration from the EMID (electronically modulated intravaginal device) using an *in vitro* Drug Dissolution Method as described in Chapter Four (Section 4.2.6.2) .

However, it was found that using biological tissues such as excised vaginal mucosa requires sensitive analytical techniques such as HPLC to differentiate the analyte from extraneous material that may come off the membrane. Therefore, the development and validation of an HPLC method was essential.

In addition, HPLC assays for progesterone, oestradiol-17β and cloprostenol stability were developed. All dosage forms of these three compounds were evaluated by assessing their chemical and physical stability and the results of this stability study are presented in Chapter Five (Section 5.3.5).

3.2 Materials and methods

3.2.1 List of reagents

The following reagents which were used: Sodium dihydrogen orthophosphate 1-hydrate, di-Sodium hydrogen orthophosphate anhydrous, ammonium acetate, HCl (36%) and NaOH (BDH laboratory supplies Poole, BH15 1TD, England),

hydrogen peroxide (H_2O_2 100 volume 27%, Andrew Industrial Ltd, New Zealand), 2-Hydroxy-propyl-β-cyclodextrin, HPβCD, (Wacker-Chemie GMBH, Germany), Hydroxy-propylmethylcellulose (Shin-Estsu Chemical Co., Ltd, Japan), HPLC grade methanol (Scharlau, Chemie, S.A), alcohol [specially denatured alcohol, SDA] Mobil, NZ) and deionised water. The following steroids were used: dexamethasone, dexamethasone acetate, dexamethasone isonicotinate, dexamethasone dipropionate, dexamethasone valerate, progesterone, oestradiol-17β and oestradiol benzoate (all of which were described in Chapter Two) and cloprostenol sodium (Everlight Chemical Industrial Corporation, Taiwan). The reagents used were analytical grades.

3.2.2 List of equipment

Ultrasonic bath (Soniclean Pty. Ltd, Australia), UV (Spectrophotometer DU® 640, Beckman, USA), four-place analytical balance (Sartorius Analytical Balance Type BP110S, Biolab Scientific Ltd, NZ), HPLC system comprised of a Waters® 1525 Binary HPLC Pump, Waters® 2996 Photodiode Array Detector, Waters® 717 Plus Autosampler fitted with a Column heater (Waters, Milford, MA, USA). Empower® software (Waters®, Milford, MA, USA), 0.20 µm membrane filter (Minisart®-plus, Sartorius Corporation, Germany). Hanson Dissolution Testing Apparatus (Hanson SRII8 Dissolution Test Station equipped with water bath, dissoette and autosampling apparatus, Hanson Instruments, USA).

3.2.3 Analytical methods

3.2.3.1 UV spectroscopy

3.2.3.1.1 Preparation of 5% HPβCD in PBS (pH 5.0)

Phosphate buffer of pH 5.0 was prepared in deionised water following the procedure given by Lentner (984) from a combination of sodium dihydrogen orthophosphate 1-hydrate (66 mM $NH_2PO_4 \cdot H_2O$, 9.2 g/L) and di-Sodium hydrogen orthophosphate anhydrous (66 mM N_2HPO_4, 9.5 g/L) 99:1 buffer ratio.

Following manufacture of the buffer, 5% w/v (50 g/L) HPβCD was added and dissolved using ultrasound for 30 minutes.

3.2.4 Determination of the wavelength of maximum absorbance (λ_{max})

The λ_{max} of the steroids were determined by scanning (UV Spectrophotometer) a 20 μg/mL solution of each steroid in 5% HPβCD in PBS (pH 5.0) or deionised water/ethanol over the wavelength range 200–400 nm. From this scan, the peak of maximum absorbance was identified.

In addition, the effect of buffer composition on the λ_{max} for dexamethasone was also determined using various buffers to investigate the ruggedness of the UV method. The buffers with different pH were prepared using the procedure given by Lentner (984). These buffers were phosphate buffer (pH 5.0), phosphate buffer (pH 7.4), 1-5% HPβCD in PBS (pH 5.0), 5% HPβCD in PBS (pH 7.4), 10% ethanol in PBS (pH 5.0), 25% ethanol in PBS (pH 5.4), 50% ethanol in PBS (pH 5.8), 10% ethanol/0.1% gel (HPMC) in PBS (pH 5.0). Another reason why this experiment was carried out was to investigate the effect of the different buffers (in the release media) on the delivery rate of the EMID (Section 4.2.6.2) using the *in vitro* drug dissolution method.

3.2.5 Preparation of calibration standards

Calibration standards of the steroids were prepared from a stock solution that was prepared by accurately weighing 0.0500 g of the specific steroid in a 50 mL volumetric flask using a four decimal place analytical balance and filling up to the mark with alcohol. The mixture was subjected to ultrasound for 20 minutes until all drugs were completely dissolved.

Standards were prepared by serial dilution of the stock solutions with buffer (5% HPβCD PBS at pH 5.0) to make working standards between 0 and 50 μg/mL. Working standards differed between different steroid analogues due to their different solubilities.

3.2.6 Validation of UV spectrophotometer assay

The UV assay was validated according to linearity and range, accuracy, precision and limits of quantitation using methods described by the United States Pharmacopoeia (USP, 1994).

3.2.6.1 Linearity and range

Calibrations curves of absorbance values verus concentrations were produced for each of the steroids investigated. This process was repeated to assess the accuracy and precision of the linearity and range. Linear regression of the slopes of the calibration curves was compared to determine the linearity of the working standards for each steroid. The linearity and the ranges of all steroid analogues were also determined in conjunction with accuracy and precision. A relative standard deviation (%RSD) from the true values was calculated for both lower and upper range concentrations. In order to have a suitable level of precision and accuracy, the percentage RSD for lower and upper range concentrations were chosen to be ≤10 and 5%, respectively. The results are reported in Table 3.6.

3.2.6.2 Accuracy

For each steroid, validation standards with three different concentrations (i.e., 4.0, 30.0 and 40.0 μg/mL) were prepared and analysed in triplicate on three different days and their concentrations were calculated from the linear regression line of a standard calibration curve made on the same day. The deviation of the calculated results from their true concentrations was determined to assess the accuracy of each method. These results are reported in Table 3.6.

3.2.6.3 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (USP, 1994). Precision may be considered at three levels: repeatability (intra-assay precision), intermediate precision (within different days) and reproducibility (between laboratories). Executing repeat absorbance readings of a single standard

solution and determining the relative standard deviation of the results about the mean value assessed repeatability. Intermediate precision of all steroid analogues were determined in conjunction with the accuracy, linearity and ruggedness experiments from relative standard deviations data of intra-day and inter-day reproducibility of calibration standards for these parameters. These results are reported in Table 3.6.

3.2.6.4 Limit of quantitation

To determine the limit of quantification of the UV assay 1, 2 and 3 μg/mL solutions of dexamethasone, its analogues and progesterone were accurately prepared in duplicate and their absorbance values determined against the blank (5% HPβCD PBS at pH 5.0). The concentrations of the standards were calculated against the linear regression line of a standard calibration curve made on the same day. This procedure was performed in duplicate on three different days. In order to have a suitable level of precision and accuracy, the %bias from true values for lowest standard concentration was set to be ≤15% for quantitation limit of the tested steroids. These results are presented in tables of Appendix-F.

3.3 HPLC

The HPLC assay was validated by assessment of its linearity and range, accuracy, precision, limit of quantitation and limit of detection by the methods described by Ogle (Ogle, 1999).

3.3.1 HPLC System

Chromatography was performed on an HPLC system comprised as listed in Section 3.2.2. All data were assessed using Empower® software. Before any analyses were carried out, the column was equilibrated with the mobile phase until a constant temperature of 30ºC was reached. A column oven was used to control the temperature. The HPLC running conditions are as shown in Table 3.1.

Table 3.1. HPLC running conditions.

Parameter	Value
Blank volume	20 μL
Sample injection volume	20 μL
Temperature	30ºC
Mobile phase (methanol and deionised water)	70:30
Detection wavelength was monitored at	242 nm dexamethasone, its analogues and progesterone
Column type and Guard column	Phenomenex® (Phenomenex (NZ) Ltd)
Column description	Luna 5 μm C_{18}
Column dimensions	250 x 4.6 mm
Guard column description	C_{18} (ODS, Octadecyl), SecurityGuard™
Guard column dimensions	4.0 x 3.0 mm
Flow rate	1 mL/min
Pressure	2200-2500 p.s.i
Analytical run time	10-30 minutes (varied with the drug analogues)

3.3.2 Preparation of standard solutions for the HPLC standard curve

Initially a stock solution of a specific steroid was prepared by accurately weighing 0.0100 g of crystalline drug analogue using a four decimal place analytical balance. To the drug, 100 mL of alcohol was carefully added and made up to 100 mL volume. The mixture was subjected to ultrasound until the crystals had completely dissolved. The concentration of the resultant standard solution was 100 μg/mL. Working standards of between 0.1 and 5.0 μg/mL were then prepared from the stock solution by diluting with alcohol using precision pipettes. The working standard solutions were filtered using a 0.20 μm membrane filter prior to HPLC analysis.

3.3.3 Calibration curves

For each steroid, the peak area of the chromatograms versus the concentrations of the standards was plotted to obtain the calibration curves. Equations of the computed regression lines and correlation coefficients were also calculated. Once

standard curves with very good linearity and reproducibility (inter-day and intra-day reproducibility) were obtained, they were used to calculate any unknown sample concentrations. A fresh standard calibration curve was made on the same day as the unknown samples were analysed.

3.3.4 HPLC validation

3.3.4.1 Linearity and range

The linearity of dexamethasone and progesterone responses were determined by analysing two sets of calibration standards (0.0, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0 μg/mL) prepared and analysed on two different days and performing linear regression on the results. Analysis of standards at 0.2, 2.0 and 4.0 μg/mL and comparison of their concentrations against the standard curve determined the range of workability for the method for these steroids. The linearity of dexamethasone acetate, dexamethasone valerate, dexamethasone dipropionate and dexamethasone isonicotinate was determined by analysing three sets of calibration standards (0.0, 0.4, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μg/mL) and performing linear regression on the results. Analysis of standards at 0.2, 2.0 and 5.0 μg/mL and comparison of their concentrations against the standard curve determined the range of workability for the method for these steroids.

3.3.4.2 Accuracy

Accuracy was determined by analysing standards at 0.2, 2.0 and 4.0 μg/mL for dexamethasone and progesterone and at 0.4, 2.0 and 5.0 μg/mL for the other steroids. Accuracy was established by calculating the concentrations against the standard calibration curve made on the same day and assessing the %bias of the calculated results from their true concentrations.

3.3.4.3 Precision

Precision was established by comparing the experimentally determined concentrations of replicate 0.2, 2.0 and 4.0 μg/mL dexamethasone and progesterone standards against the standard curve and the relative standard

deviations of the results about their means were determined. For the remaining steroids, standards of 0.4, 2.0 and 5.0 μg/mL were used.

3.3.4.4 Limit of quantitation

The limit of quantitation was established by determining the background response of the method from multiple blank determinations, followed by analysis of standards near the detection limit. The limit of quantitation was determined using 0.1 and 0.2 μg/mL for dexamethasone and progesterone and 0.1, 0.2, 0.4 μg/mL for the remainder of the steroidal compounds.

3.4 HPLC stability indicating assays for progesterone, oestradiol-17β and cloprostenol

3.4.1 HPLC stability assay parameters

The progesterone HPLC stability assay was provided by DEC Manufacturing, along with recommendations for its verification (Personal communication). The chromatographic conditions for the HPLC stability assay are listed in Table 3.2.

Table 3.2. Chromatographic conditions for the analysis of progesterone

Parameter	Value
Blank volume	10 μL
Sample injection volume	10 μL
Column temperature	30ºC
Mobile phase (methanol: deionised water	60:40
Detection wavelength	254 nm
Column type	Waters SymmetryShield™ RP_{18} (Symmetry® Column)
Guard column	Waters SymmetryShield™ RP_{18} (Syntery™ Guard)
Column dimensions	250 x 4.6 mm, 5 μm
Guard column dimensions	20 x 3.9 mm, 5 μm
Flow rate	1.2 mL/min
Pressure	2200–4000 p.s.i.
Analytical run time	70 minutes

The same HPLC system and method as described in Table 3.2 for the analysis of progesterone was used for the analysis of oestradiol-17β with the exception that

the detection wavelength was 280 nm. In addition, the same HPLC method for progesterone described in Table 3.2 was used for cloprostenol with the following changes. The flow rate was 1.0 mL/minute. The detection wavelength was monitored at 280 nm. The mobile phase remained as 60% methanol, 40% aqueous but the aqueous phase comprised 15 mM ammonium acetate in deionised water. In addition, the run time was shortened to 10 minutes.

3.4.1.1 Verification of progesterone assay

Verification of the progesterone HPLC stability indicating assay was achieved by assessment of linearity, accuracy and precision. Data were generated from working standard solutions, prepared as described in 3.4.1.1.1., on three different days.

Criteria for acceptance of the method revalidation are shown in Table 3.3.

Table 3.3. Criteria to be met for acceptance of method verification

Parameter	Specification
Linearity	$R^2 \geq 0.999$
Range	Low standard %bias <20% and High standard %bias <2%
Intercept	-80000 >intercept <80000
Intercept	<2% of the target peak area
Accuracy	%bias <2%
Precision	Injection repeatability <1.5%RSD

3.4.1.1.1 Preparation of progesterone working standards for calibration curves

The working standards were manufactured by accurately weighing 0.1700, 0.1380, 0.1100, 0.0500 and 0.0030 g of progesterone crystalline powder. Each sample was transferred into a volumetric flask using a glass funnel. The sample was dissolved in an aliquot of alcohol with the aid of ultrasonification for 30 minutes. The solution was made up to 200 mL volume with ethanol. The resultant concentrations were 850, 690, 550, 250 and 15 µg/mL, respectively. The working standards were filtered using a 0.20 µm membrane filter and an approx 0.40 mL

of the filtrate was collected and transferred into 1.0 mL polyethylene snap cap vials (Waters, MA, USA) prior to analysis by HPLC. The corner of each vial cap was pierced with a small needle (Monoject®, polypropylene hub hypodermic needles, Fabrique Par, Sherwood medical, USA) prior to loading into the autosampler carousel, to prevent injection sample pressure causing irreproducible results. Calibration standards were analysed in triplicate, from the lowest to the highest concentration for all analytical runs.

3.4.1.2 Verification procedure

Mean peak areas of the analysed standards were plotted against concentration. The linearity, intercept and correlation coefficient values were determined by linear regression analysis performed on each set of calibration standards on each separate day. The accuracy of each calibration standard was determined by interpolation of the mean peak area (N=3) from the calibration curve generated for that day to determine the concentration of that standard. The percent bias was then determined using Equation 3.1.

The %RSD of peak areas of triplicate injections was used to determine injection repeatability precision. A portion of the 690 µg/mL standard prepared in Section 3.4.1.1.1 was stored separately labelled as a QC standard. This solution was filtered independently and injected in triplicate to provide a further estimate of the accuracy and precision of the assay. Percent bias and injection repeatability were calculated for the QC standard following interpolation of the data from the calibration curve generated on the same day.

Inter-day precision was determined by calculating the %RSD of three independent sets of working standards analysed on different days.

3.4.2 Development and validation for oestradiol-17β

This method was intended to be applied to the assay of 2.5 mg oestradiol-17β tablets. The proposed sample preparation for the assay involved crushing 10 tablets and then dissolving the resulting powder in 100 mL alcohol. This would

provide a target oestradiol-17β concentration of 250 μg/mL. Experiments were conducted to define linearity, range and limit of quantitation based around the selected target concentration. In order to develop a stability indicating assay for oestradiol-17β, specificity was considered essential in order to confirm the suitability of the chromatographic conditions in the presence of degradation products and/ or other interferences.

3.4.2.1 Forced degradation stress reactions in acidic, basic conditions and H_2O_2

Acidic, basic and peroxide alcoholic solutions were prepared. To make 2 M HCl solution, 11.25 mL of the concentrated HCL solution (Section 3.2.1) was transferred in a 1000.0 mL flask and the volume was made with alcohol. Similarly, 2% v/v H_2O_2 in alcohol was prepared in the same manner using 74 mL of the concentrated H_2O_2 in alcohol. To make 2 M alcoholic NaOH, 80 grams of NaOH granules were dissolved in 200 mL of water. Then, 20.0 mL of this solution was transferred in a 100.0 mL flask and the volume was made with alcohol. In addition, dilute stress solutions were made using their respective 2 M HCl, 2 M NaOH and 2% v/v H_2O_2 solutions.

Forced degradation studies were performed on oestradiol-17β in the presence of acid, base and oxidative conditions. 0.5 g of oestradiol-17β crystalline powder was dissolved in either 100 mL of 0.1 or 2 M alcoholic HCl, 100 mL of 0.1 or 2 M alcoholic NaOH, or in 100 mL of 1% or 2% v/v H_2O_2 in alcohol in 200 mL Schott bottles at 40ºC for two hours. Samples were withdrawn at the following predetermined time points: 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes. Prior to HPLC analysis, each sample was diluted with alcohol (1:9 dilution) and passed through a 0.2 μm nylon filter.

3.4.2.2 Linearity, range, limit of quantitation and limit of detection

Working standard solutions were prepared as described in Section 3.4.2.2.1 and injected in duplicate from lowest to highest concentration. Mean peak areas were plotted against concentration.

Linearity, intercept and correlation coefficient values were determined from the linear regression analysis performed on the calibration standards. Regression analysis was performed leaving out successive low level and high standards to determine an acceptable linear range.

The accuracy of each calibration standard was then determined by taking the mean peak area of the duplicate injections and applying the calibration curve equation generated with the chosen linear range standards to give a theoretical concentration of the standard. Percent bias was determined by Equation 3.1.

$$\%\text{bias} = \frac{C_t - C_a}{C_a} \times 100 \qquad \textbf{Equation 3.1.}$$

where C_t and C_a are the theoretical and actual concentrations, respectively.

The percentage assay recovery of analysed samples was calculated using Equation 3.2 as follows.

$$\%\ \text{calculated} = \frac{C_a}{C_t} \times 100 \qquad \textbf{Equation 3.2.}$$

The %RSD of peak areas of duplicate injections was used to determine injection repeatability precision. The quantitation limit was determined by the lowest concentration of oestradiol-17β that showed a quantifiable peak response on the chromatogram within ≤20% bias. The detection limit was determined by the lowest concentration of oestradiol-17β, which produced a peak response on the chromatogram but was impossible to quantify with an acceptable precision and accuracy.

3.4.2.2.1 Preparation of oestradiol-17β working standards for calibration curves

The working standards were manufactured by accurately weighing 0.0100, 0.0200, 0.0250, 0.0300, 0.0400, 0.0500 and 0.0600g of oestradiol-17β crystalline powder using a four decimal place analytical balance. Each weighed sample was transferred carefully through a glass funnel into a 100 mL volumetric flask and dissolved in alcohol with the aid of ultrasound for 30 minutes. The solution was then made up to 100 mL with ethanol. The resultant standard concentrations were 100, 200, 250, 300, 400, 500 and 600 µg/mL, respectively. Low level standards at 1, 2, 5 and 10 µg/mL were prepared by appropriate dilution from the 500 µg/mL standard. Prior to injection onto the HPLC column, the working standards were filtered through a 0.20 µm membrane filter and approximately 0.40 mL of the filtered sample was poured into 1.0 mL vials.

Before each vial was loaded into the carousel from the lowest to the highest concentration standards, each corner of the snap cap was pierced with a small needle as previously described in Section 3.4.1.1.1, to prevent injection sampler pressure causing irreproducibility.

3.4.2.3 Method validation

The specifications determined for method validation are defined in Table 3.4.

Table 3.4. Method parameters and specifications to be met for method validation.

Parameter	Specification
Linearity	$R^2 \geq 0.999$
Range	Low standard %bias <20% and high standard %bias <2%
Intercept	<2% of the target peak area
Accuracy	%bias <2%
Precision	Injection repeatability <1.5%RSD
Limit of quantitation	%bias <20% (5 ppm)
Limit of detection	Concentration where peak observed but %bias >20%

3.4.2.3.1 Validation of linear range of assay

Oestradiol-17β working standards of 5, 10, 100, 200, 300, 400 and 500 µg/mL and a separate 250 µg/mL QC standard were prepared as described in Section 3.4.2.2.1 on each of three separate occasions. Standards were injected in duplicate. Mean peak area was plotted against concentration and linear regression was performed to determine slope, intercept and correlation coefficient of the calibration curves. The accuracy of each calibration standard on each day was then determined by taking the mean peak area of the duplicate injections and applying the calibration curve equation generated on that day to give a theoretical concentration of the standard. Bias and injection repeatability were calculated as described in Section 3.4.2.2. Bias and injection repeatability were also calculated for a QC standard according to the calibration curve generated on each day.

3.4.3 Development and validation for cloprostenol

This method was intended to assay 100 mg amounts of a 0.75% w/w cloprostenol sodium formulation dissolved in 10 mL deionised water. From this, a target concentration of 75 µg/mL was established. Experiments were conducted to define linearity, range, limit of quantitation and limit of detection based around the selected target concentration. In order to develop a stability indicating method for cloprostenol sodium, specificity was considered in order to confirm suitability of the chromatographic conditions in the presence of degradation products.

3.4.3.1 Forced degradation stress reactions in acidic, basic conditions and H_2O_2

Cloprostenol powder of 0.01 g was weighed and dissolved in equal volume of 100 mL of 1M HCL, 1M NaOH or 0.1%, 1% and 10% v/v H_2O_2 in deionised water in separate small Schott bottles. The samples were put in a dry oven at 40ºC for two hours. From each treatment, 9 samples were taken at specified time intervals for sample analysis (i.e., 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes) to qualitatively investigate drug degradation. These samples were passed through a 0.20 µm filter prior to analysis by HPLC.

3.4.3.2 Linearity, range, limit of quantitation and limit of detection

Working standard solutions were prepared as described in Section 3.4.3.2.1 and injected in duplicate from lowest to highest concentration. Mean peak areas were plotted against concentration. Linearity, intercept and correlation coefficient values were determined from the linear regression performed on the calibration standards. Regression was performed leaving out successive low and high level standards to determine an acceptable linear range. The accuracy of each calibration standard was then determined by taking the mean peak area of the duplicate injections and applying the calibration curve equation generated with the chosen linear range standards to give a theoretical concentration of the standard. The %RSD of peak areas of duplicate injections were used to determine injection repeatability precision as described in Section 3.3.4.3. Percent bias and calculated rates were then determined by the Equation 3.1 and Equation 3.2. The lowest concentration of cloprostenol produced a peak response on the chromatogram, but was impossible to quantify with acceptable precision and accuracy determined the detection limit. The quantitation limit was determined by the lowest concentration of cloprostenol sodium that showed a quantifiable peak response on the chromatogram within ≤20% bias.

3.4.3.2.1 Preparation of cloprostenol standard solutions for the HPLC standard curve

Stock solutions of cloprostenol were prepared by accurately weighing 0.0100, 0.0150, 0.0250, 0.0300, 0.0350 and 0.0450 g of crystalline powder in separate small weighing boats using a four decimal place analytical balance. The weighed drug was transferred through a funnel into 50 mL volumetric flasks while rinsing with deionised water carefully and made up to 50 mL. The mixture was subjected to ultrasound until the crystalline powder was completely dissolved. The theoretical concentrations of resultant standards solution were 200, 300, 500, 600, 750 and 900 μg/mL, respectively. Also 5, 10 and 100 μg/mL standards were manufactured from the 500 μg/mL standard solution with appropriate dilutions.

3.5 Results

3.5.1 UV spectrophotometric profile

The experimentally determined λ_{max} in 5% HPβCD in PBS/pH 5.0 of the compounds used in the study are shown in Table 3.5.

Table 3.5. λ_{max} of the steroids used in the study of the UV assay.

Drug	**λ_{max}**
Dexamethasone	242
Dexamethasone acetate	
Dexamethasone isonicotinate	
Dexamethasone dipropionate	
Dexamethasone valerate	
Progesterone	246
Oestradiol benzoate	233
Oestradiol-17β	217

In addition, the different buffer compositions or pH had no effect on λ_{max} of dexamethasone. Regardless of any of the buffer systems used, the experimentally determined λ_{max} of dexamethasone was remaining unchanged (242 nm). Therefore, this indicated the robustness of the wavelength used.

3.5.1.1 Linearity and range

Concentration versus absorbance values were plotted from data for the two sets of calibration standards to make two standard curves for all steroids studied (Figure 3.1). This data for the calibration working standards was generated to enable determination of slope, linearity and range of the UV method.

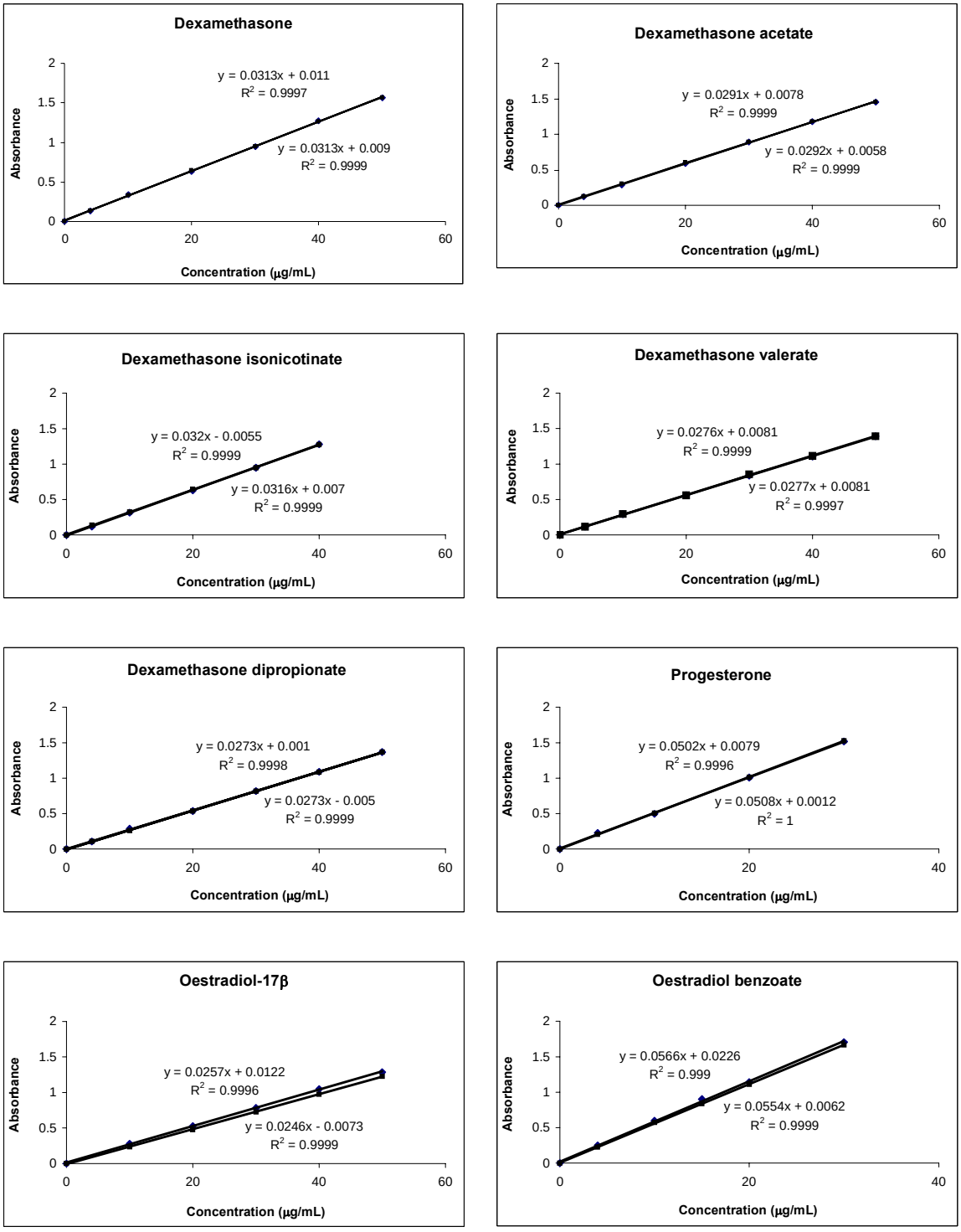


Figure 3.1. Standard calibration curves for the steroid studied to enable determination of inter-day reproducibility of slope, linearity and range of the UV assay.

All data fitted very well to the linear regression lines (R^2 ≥0.999) with reproducible slope values for each steroid studied (Figure 3.1).

3.5.1.2 Accuracy and precision

The assessment of accuracy of the UV method for all steroids studied was conducted in conjunction with assessment of the precision of the method. The inter-day reproducibility of accuracy (% bias) and precision (%RSD) of the lower (4 μg/mL), middle (30 μg/mL) and high (40 μg/mL) range is shown in Table 3.6. The accuracy (%bias) and precision (%RSD) values presented in here were calculated from an average of two replicates of the standard with the lowest accuracy and precision of the sated range.

Table 3.6. The inter-day reproducibility of accuracy (% bias) and precision (%RSD) of the lower (4 μg/mL), middle (30 μg/mL) and high (40 μg/mL) range standards.

Drug	Precision(%RSD)	Accuracy(%bias)
Dexamethasone	≤2.4	≤1.3
Dexamethasone acetate	≤0.7	≤3.7
Dexamethasone isonicotinate	≤2.4	≤3.1
Dexamethasone valerate	≤1.4	≤10.0
Dexamethasone dipropionate	≤2.9	≤3.0
Progesterone	≤6.2	≤5.0
Oestradiol benzoate	≤2.6	≤6.0
Oestradiol-17β*	≤3.2	≤1.0

* The lower standard was10 μg/mL.

In addition, the inter-day reproducibility of accuracy (≤3%bias) and precision (≤2.1%) of the middle and high standards were found to be very accurate and more precise than the lower range standard for the steroids studied (Appendix-E). This is because the lower standard is closer to quantitation limit than the middle and high standards. The raw data of inter-day reproducibility of accuracy and precision of the lower, middle and high ranges for each steroid is shown in tables of Appendix-E.

3.5.1.3 Quantitation limit of the UV assay

The data for quantitation limit of each steroid was determined to define the lowest concentration of the drug with acceptable accuracy and precision for the true value against the standard calibration curves. The quantitation limit of the UV assay of the steroids studied was 1, 1, 2, 2, 2 and 3μg/mL for dexamethasone, dexamethasone acetate, dexamethasone isonicotinate, dexamethasone valerate,

dexamethasone dipropionate and progesterone, respectively. The inter-day reproducibility for lowest standard concentration proved to have the %bias of ≤15% from true values for quantitation limit of the tested steroids. The raw data of quantitation limit of the UV assay is shown in tables of Appendix-F.

3.5.2 HPLC assay

A typical chromatogram for the various steroids investigated in the thesis is shown in Figure 3.2. Some peaks reflecting the solvent front (mobile phase or junk materials) were eluted out of the column before 4 minutes of HPLC analytical run time. The retention time of dexamethasone peak appeared at 6.0 minutes. All other steroids were eluting out of the column between 8 and 24 minutes due to their different lipophilicity. The steroids were analysed on the HPLC system one at time. All steroids studied showed very good peak shape and a stable baseline as shown below.

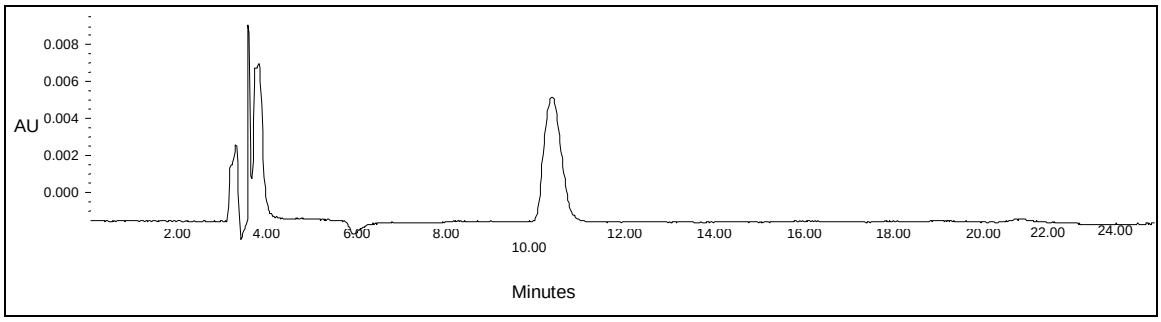


Figure 3.2. A typical chromatogram for the steroids used in the study.

The retention times of the steroids used in this study applied on the HPLC running conditions was previously presented in Chapter Two.

3.5.2.1 Linearity and range

Two calibration curves obtained on two different days of the validation procedure for steroids studied are presented in Figure 3.3. These were used to enable determination of slope, linearity and range of the HPLC method.

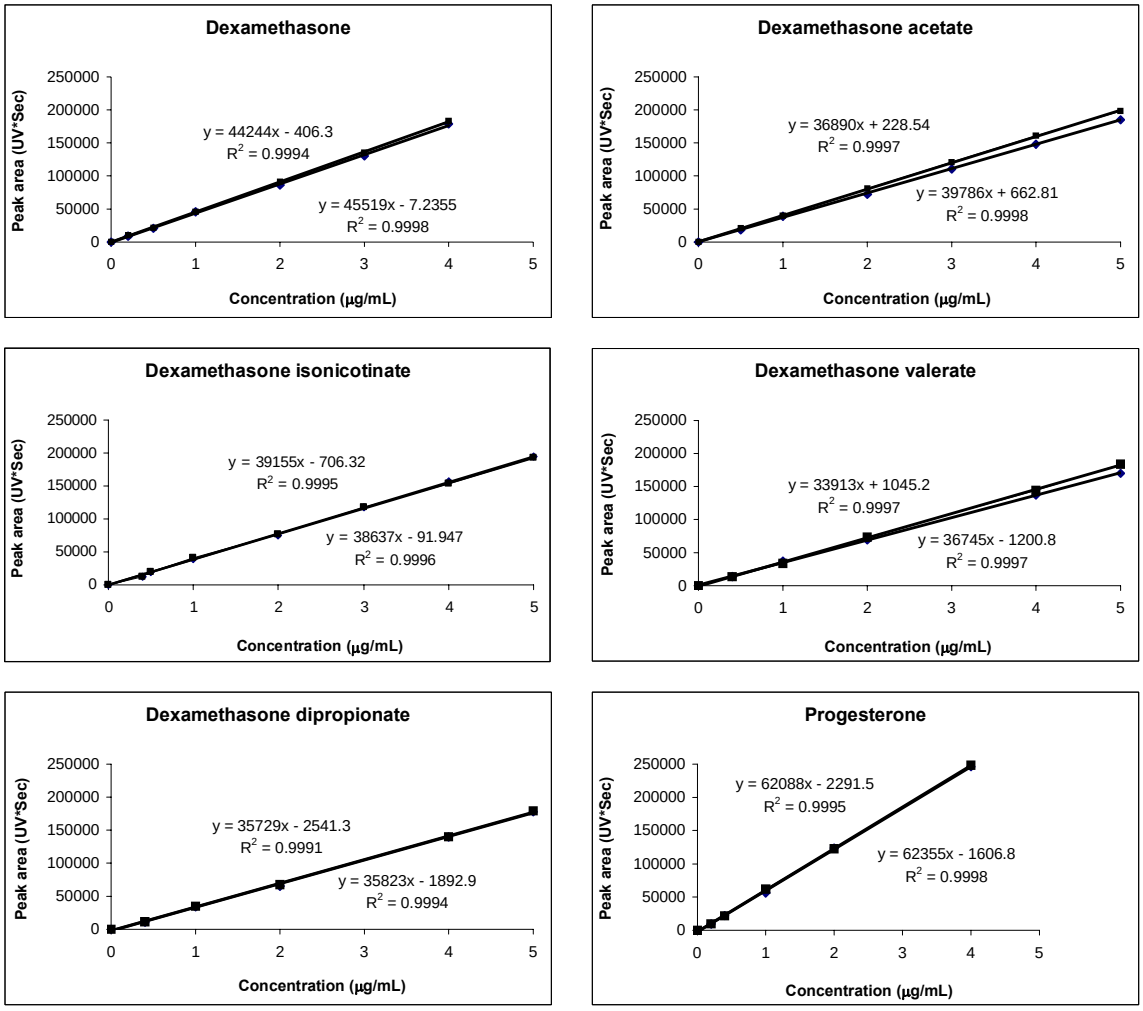


Figure 3.3. Standard calibration curves for the steroids studied to enable determination of inter-day reproducibility of slope, linearity and range of the HPLC assay.

All data fitted very well to the linear regression lines (R^2 ≥0.9991) with reproducible slope values for each steroid studied (Figure 3.3).

3.5.2.2 Accuracy and precision

The inter-day reproducibility of average three replicates used to determine the accuracy and precision of the lower, middle and high range (0.2 - 5 µg/mL) of the HPLC methods for the various steroids studied were ≥94.7% and ≤4.8%, respectively. The raw data is presented in tables of Appendix-G.

3.5.2.3 Limit of quantitation

The limit of quantitation standards for steroids studied were 0.2, 0.2, 0.4, 0.4, 0.4, and 0.2 μg/mL for dexamethasone, dexamethasone acetate, dexamethasone isonicotinate, dexamethasone valerate, dexamethasone dipropionate and progesterone, respectively. Data acquired for determination of limit of quantitation are presented in tables of Appendix-H.

3.5.3 HPLC stability indicating assays for progesterone, oestradiol-17β and cloprostenol

3.5.3.1 Progesterone HPLC stability indicating assay parameters

The method was successfully verified to meet the recommended validated parameters as shown in Table 3.3.

All progesterone working standards were carefully and accurately manufactured.

Figure 3.4 shows the inter-day reproducibility of the regression equation for progesterone standard curves for working standards of three different days. The regression equations showed very linear ($R^2 \geq 0.9998$) and reproducible slopes (21444-21682) in the three different days (Figure 3.4).

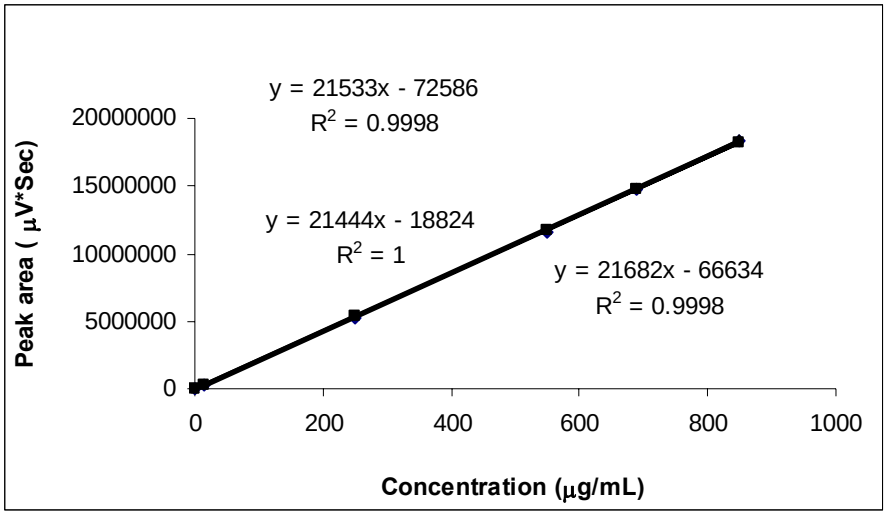


Figure 3.4. Standard calibration curves for progesterone to enable determination of inter-day reproducibility of intercept, linearity and range of the HPLC stability assay.

Table 3.7 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards of day 1. The results show the HPLC method was accurate and precise.

Table 3.7. Mean peak area, %RSD, actual concentration and %bias of calibration standards of day 1.

Standard concentration (µg/mL)	Mean peak area[1] (µV*Sec)	SD	%RSD	Actual Concentration[2] (µg/mL)	%Bias[3]
0	0	0	0	3.4	0
15	281553	2789	1.0	16	-9.6
250	5252212	13092	0.2	247	1.1
550	11600288	27446	0.2	542	1.4
690	14774707	26441	0.2	690	0.1
850	18365260	79436	0.4	856	-0.7
QC standard	15037038	27517	0.2	702	-1.7

[1]N=3. [2]Calculated from Y=21533x-72587. [3]Equation 3.1.

Table 3.8 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards of day 2.

Table 3.8. Mean peak area, %RSD, actual concentration and %bias of calibration standards of day 2.

Standard concentration (µg/mL)	Mean peak area[1] (µV*Sec)	SD	%RSD	Actual Concentration[2] (µg/mL)	%Bias[3]
0	0	0	0	0.09	0
15	291355	2821	1.0	14	3.6
250	5330818	9565	0.2	249	0.2
550	11777318	48665	0.4	550	0.0
690	14777453	31408	0.2	690	0.0
850	18211639	55668	0.3	850	0.0
QC standard	14787781	258376	1.7	690	-0.1

[1]N=3. [2]Calculated from Y= 21444-18824. [3]Equation 3.1.

The above results show the HPLC method was accurate and precise (Table 3.7 - Table 3.8).

Table 3.9 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards of day 3.

Table 3.9. Mean peak area, %RSD, actual concentration and %bias of calibration standards of day 3.

Standard concentration (µg/mL)	Mean peak area[1] (µV*Sec)	SD	%RSD	Actual Concentration[2] (µg/mL)	% Bias[3]
0	0	0	0	3.1	0
15	283814	1311	0.5	16	-7.8
250	5317585.3	7849	0.1	248	0.7
550	11690731	45941	0.4	542	1.4
690	14858758	45151	0.3	688	0.2
850	18510291	41375	0.2	857	-0.8
QC standard	15074696	72960	0.5	698	-1.2

[1]N=3. [2] Calculated from Y= 21682-66634. [3]Equation 3.1.

Table 3.10 shows the inter-day reproducibility of the mean peak areas of working standards from three independent calibration curves on three different days.

Table 3.10. Inter-day reproducibility of the mean peak areas of working standards from three independent calibration curves[1] on three different days.

Concentration (µg/mL)	Day 1	Day 2	Day 3	Mean of the three days	SD	%RSD
	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)		
0	0	0	0	0	0	0
15	281553	291355	283814	285574	4191	1.5
250	5252212	5330818	5317585	5300205	34364	0.6
550	11600288	11777318	11690731	11689446	72278	0.6
690	14774707	14777453	14858758	14803639	38991	0.3
850	18365260	18211639	18510291	18362397	121941	0.7

[1]Tables 3.7, 3.8 and 3.9. [2]N=3.

The inter-day reproducibility of progesterone working standards from the three independent calibration curves made on the three different days is very precise (RSD ≤1.5%) over the stated range of standards between 15 and 850 µg/mL.

3.5.3.2 Oestradiol-17β HPLC stability indicating assays

3.5.3.2.1 Forced degradation studies in acid, alkali and H_2O_2

Oestradiol-17β was stable at 40ºC for two hours in 0.1 M alcoholic HCl, 0.1 M alcoholic NaOH and 1% v/v H_2O_2. In addition, the compound was stable in 2M alcoholic NaOH and there was no evidence of any degradation of the product on the chromatograms. In contrast 2M alcoholic HCl and 2% v/v H_2O_2 induced degradation of the oestradiol-17β. Degradation products were observed on the chromatograms (Figure 3.5). However, none of these degradant peak products interfered with the peak of interest. Oestradiol-17β was found to be completely unstable in 2% v/v H_2O_2 and the peak representing oestradiol-17β completely disappeared from the chromatogram.

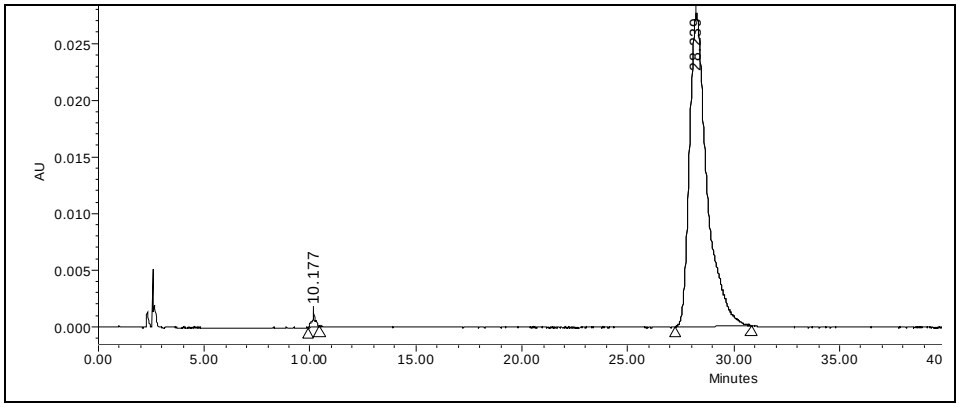


Figure 3.5. Typical chromatogram showing the degradation peak (10.177 minutes) arising after exposure of oestradiol-17β to 2M HCl.

3.5.3.2.2 Linearity, range, limit of quantitation and limit of detection

The data for linearity range, precision and accuracy of oestradiol-17β using working standards from 0 to 600 µg/mL is shown in Table 3.11. In addition, this table shows the quantitation and detection limit of the tested working standards for the method. The %RSD values of working standards between 10 and 600 µg/mL were ≤0.7% and 6.5% including 5 µg/mL (Table 3.11). The lower working standards of 1 and 2 µg/mL of oestradiol-17β have shown high %RSD (low precision) values of 55.8 and 15.8, respectively. In order to achieve the acceptable linear range of the assay the two lowest standards were left out.

Table 3.11. Linearity range, precision and accuracy of oestradiol-17β working standards from 0-600 µg/mL of working standards in conjunction with quantitation and detection limit of the tested standards for oestradiol-17β.

Theoretical concentration (µg/mL)	Mean peak area (µV*Sec)	SD	%RSD
0	0	0	0
1	4311	2406	55.8
2	7668	1211	15.8
5	17194	1112	6.5
10	36413	93	0.3
100	330660	602	0.2
200	674252	449	0.1
300	1003351	3330	0.3
400	1350768	7200	0.5
500	1639807	8303	0.5
600	1840727	12891	0.7

The results of regression analysis performed by leaving out successive low level and high standards determined the acceptable linear range is shown in Figure 3.6b.

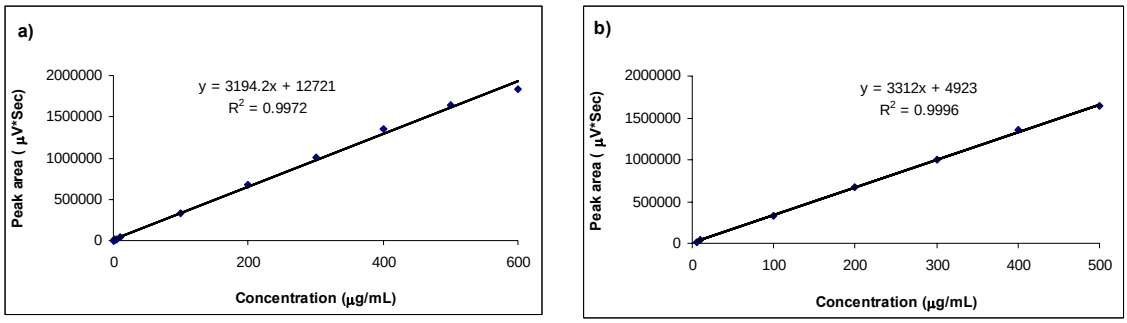


Figure 3.6. Standard calibration curve for oestradiol-17β 0 and 600 µg/mL(a) and 0 and 500 µg/mL (b) to enable determination the acceptable linear range of the HPLC stability assay.

Furthermore, the linearity (R^2) of regression analysis increased from 0.9972 to 0.9996 by leaving out the higher (600 µg/mL) standard as shown in determined the acceptable linear range of the assay (Figure 3.6b).

Table 3.12 shows the accuracy and precision of calibration standards concentrations calculated the regression line equation (Y = 3312x + 4923 and R^2 = 0.9996) for oestradiol-17β.

Table 3.12. Accuracy and precision of calibration standards concentrations calculated against their regression line equation (Y = 3312x + 4923 and R^2 = 0.9999) for oestradiol-17β.

Theoretical concentration (µg/mL)	Mean peak area (µV*Sec)	Actual concentration (µg/mL)	% Bias
0	0	-1	0
5	17194	4	-20
10	36413	10	-4.9
100	330660	98	-1.6
200	674252	202	1.0
300	1003351	301	0.5
400	1350768	406	1.6
500	1639807	494	-1.3

The %bias values of all working standards between 10 and 500 µg/mL were ≤4.9% and for the lowest standard (5g/mL) is not more than 20%. Therefore, the calibration standard concentrations were accurate and precise. All oestradiol-17β working standards were carefully and accurately manufactured.

3.5.3.2.3 Validation of linear range of assay

Figure 3.7 shows the inter-day reproducibility of the regression equation for oestradiol-17β standard curves for working standards of three different days. The regression equations showed very linear (R^2 ≥0.999) and reproducible slopes (3323-3386) for the three different days (Figure 3.7).

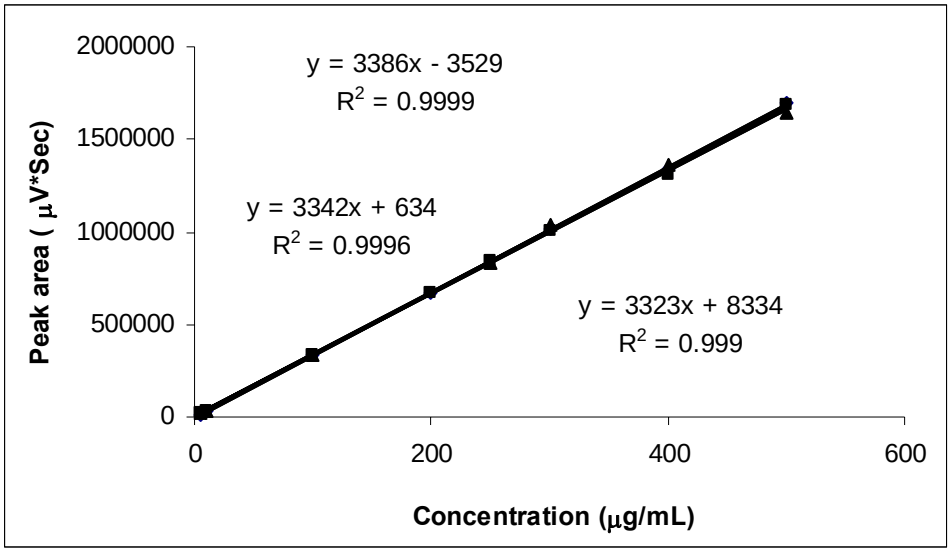


Figure 3.7. Standard calibration curves for oestradiol-17β to enable determination of intra-day reproducibility of slopes, linearity and range of the HPLC stability assay.

Table 3.13 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards of day 1. The %bias values of all working standards between 5 and 500 µg/mL were ≤5% except in one occasion.

Table 3.13. Mean peak area, %RSD, actual concentration and %bias of calibration standards of day 1.

Theoretical concentration (µg/mL)	Mean peak area[1] (µV*Sec)	SD	% RSD	Actual Concentration[2] (µg/mL)	% Bias[3]
0	0	0	0	1	0
5	14242	1863	13.1	5	5.0
10	34360	544	1.6	11	11.9
100	330728	600	0.2	99	-1.3
200	674709	339	0.1	200	0.2
300	1012315	4211	0.4	300	0.0
400	1340423	3582	0.3	397	-0.8
500	1697791	1721	0.1	502	0.5
QC standard	804870	1794	0.2	239	-0.05

[1]N=2. [2]Calculated from Y = 3386x - 3529 and R^2= 0.9999. [3]Equation 3.1.

Table 3.14 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards of day 2. The %bias values of all working standards between 5 and 500 µg/mL were ≤0.01%.

Table 3.14. Mean peak area, %RSD, actual concentration and %bias of calibration standards of day 2.

Theoretical concentration (µg/mL)	Mean peak area[1] (µV*Sec)	SD	% RSD	Actual Concentration[2] (µg/mL)	% Bias[3]
0	0	0	0	0.2	0
5	17595	214	1.2	5	0.01
10	34443	465	1.3	10	0.01
100	330738	754	0.2	99	-0.01
200	674429	331	0.0	202	0.01
250	839851	239	-0.0	251	0.00
300	1009691	2039	0.2	302	0.01
400	1312730	4649	0.4	393	-0.02
500	1684520	1075	0.1	504	0.01
QC standard	839851	3293	0.4	251	0.4

[1]N=2. [2]Calculated from Y= 3342x+634 and R^2 = 0.9996. [3]Equation 3.1.

Table 3.15 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards of day 3. The %bias values of all working standards between 5 and 500 µg/mL were ≤0.3%. The above results show the HPLC method was accurate and precise.

Table 3.15. Mean peak area, %RSD, actual concentration and %bias of calibration standards of day 3.

Theoretical concentration (µg/mL)	Mean peak area[1] (µV*Sec)	SD	% RSD	Actual Concentration[2] (µg/mL)	% Bias[3]
0	0	0	0	-2.5	0
5	19449	779	4.0	3	-0.33
10	35054	1346	3.8	8	-0.20
100	338506	7715	2.3	99	-0.01
250	831552	3204	0.4	248	-0.01
300	1038400	16611	1.6	310	0.03
400	1357373	1097	0.1	406	0.01
500	1638280	3366	0.2	491	-0.02
QC standard	831551	11956	1.4	248	-0.9

[1]N=2. [2]Calculated from Y = 3323x+8334 and R^2 = 0.999. [3]Equation 3.1.

Table 3.16 shows the inter-day reproducibility of the mean peak areas of working standards from three independent calibration curves on three different days. The %RSD values of all working standards between 10 and 500 µg/mL were ≤1.1%. The lowest standard of 5µg/mL showed 15% of RSD value which is still within the acceptable range as previously described in Table 3.4 .

Table 3.16. Inter-day reproducibility of the mean peak areas of working standards from three independent calibration curves[1] on three different days.

Theoretical concentration (µg/mL)	Day1	Day2	Day3	Mean of the three days	SD	%RSD
	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)		
0	0	0	0	0	0	0
5	14242	17595	19449	17095	2639	15.4
10	34360	34443	35054	34619	379	1.1
100	330728	330738	338506	333324	4488	1.3
200	674709	674429	-[3]	674569	198	0.0
250	-[3]	839851	831552	835701	5868	0.7
300	1012315	1009691	1038400	1020135	15872	1.6
400	1340423	1312730	1357373	1336842	22536	1.7
500	1697791	1684520	1638280	1673530	31241	1.9

[1]Tables 3.13, 3.14 and 3.15. [2]N=3. [3]Standards not prepared on that day.

The inter-day reproducibility of oestradiol-17β working standards from the three independent calibration curves made on the three different days is very precise (RSD ≤1.7% except 5 µg/mL quantitation limit standard) over the stated range of standards between 5 and 500 µg/mL (Table 3.16).

3.5.3.3 Cloprostenol HPLC stability indicating assay

3.5.3.3.1 Forced degradation stress reactions in acidic, basic conditions and H_2O_2

Neither the 1 M aqueous HCl nor the 1 M aqueous NaOH induced any degradation to occur in the cloprostenol when subjected to 40ºC for two hours. Very rapid cloprostenol degradation was observed in the case of exposure of cloprostenol to 10% v/v H_2O_2. No degradation of cloprostenol was observed when cloprostenol was exposed to 0.1 % v/v H_2O_2. When 1% v/v H_2O_2 was used the peak of interest was observed to get smaller, however, no degradation peaks were observed and no interference of the peak representing cloprostenol was detected.

3.5.3.3.2 Linearity, range, limit of quantitation and limit of detection

The data for linearity range, precision and accuracy of cloprostenol using working standards from 0 to 900 µg/mL is shown in (Table 3.17). In addition this table shows the quantitation and detection limit of the tested working standards for the

method. The precision of injection reproducibility is ≤3.4% of RSD between 5 and 900 µg/mL standards.

Table 3.17. Linearity range, precision and accuracy of cloprostenol working standards from 0-900 µg/mL of working standards in conjunction with quantitation and detection limit of the tested standards for cloprostenol.

Concentration (µg/mL)	Mean peak area (µV*Sec)	SD	%RSD
0	0	0	0
5	7656	261	3.4
10	17037	401	2.4
100	162634	2685	1.7
200	331061	2234	0.7
300	516057	2595	0.5
500	856564	2008	0.2
600	1040790	6101	0.6
750	1231565	9265	0.8
900	1442398	859	0.1

The results of regression analysis performed by leaving out successive low level as well as high standards shown in Figure 3.8b determined the acceptable linear range in Table 3.18.

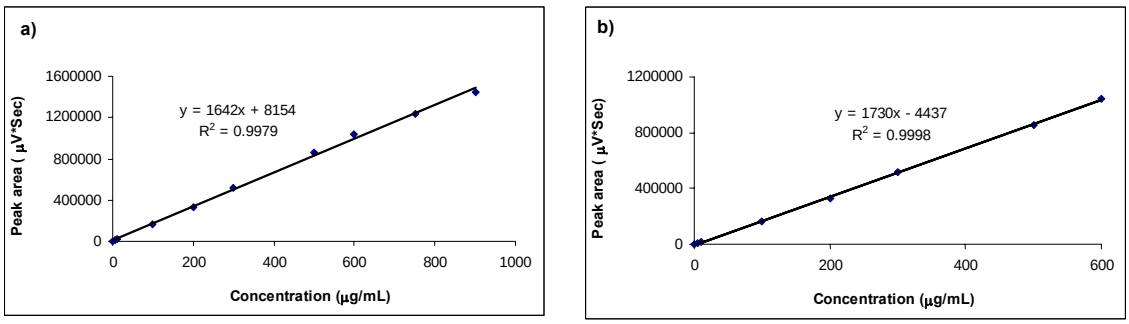


Figure 3.8. Standard calibration curves for cloprostenol 0-900 µg/mL (a) and 10-900 µg/mL (b) to enable determination linearity and range of the HPLC stability assay.

The results of regression analysis performed by leaving out successive low and high level standards to determined the acceptable linear (R^2 ≥0.9998) range for cloprostenol was between 10 and 600 µg/mL standards Figure 3.8b. All cloprostenol working standards were carefully and accurately manufactured.

3.5.3.3.3 Validation of linear range of assay

Table 3.18 shows the accuracy and precision of calibration standards concentrations calculated against their regression line equation (Y= 1730-4437 and R^2 = 0.9998) for cloprostenol. Table 3.18 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards of day 1.

Table 3.18. Accuracy and precision of calibration standards concentrations calculated against their regression line equation (Y= 1730-4437 and R^2 = 0.9998) for cloprostenol.

Theoretical concentration (µg/mL)	Mean peak area[1] (µV*Sec)	SD	% RSD	Actual Concentration (µg/mL)	% Bias[2]
10	17037	401	2.35	12	20.0
100	162634	2685	1.65	97	-3.4
200	331061	2234	0.67	194	-3.0
300	516057	2595	0.50	301	0.3
500	856564	2008	0.23	498	-0.5
600	1040790	6101	0.59	604	0.7

[1]N=2. [2]Equation 3.1.

The %bias values of all cloprostenol working standards between 10 and 600 µg/mL were ≤20% and therefore the calibration standard concentrations were accurate and precise (Table 3.18). Figure 3.9 shows the inter-day reproducibility of the regression equation for cloprostenol standard curves for working standards of three different days. The regression equations showed very linear (R^2 ≥0.9998) and reproducible slopes (1713 - 1733) in the three different days (Figure 3.9).

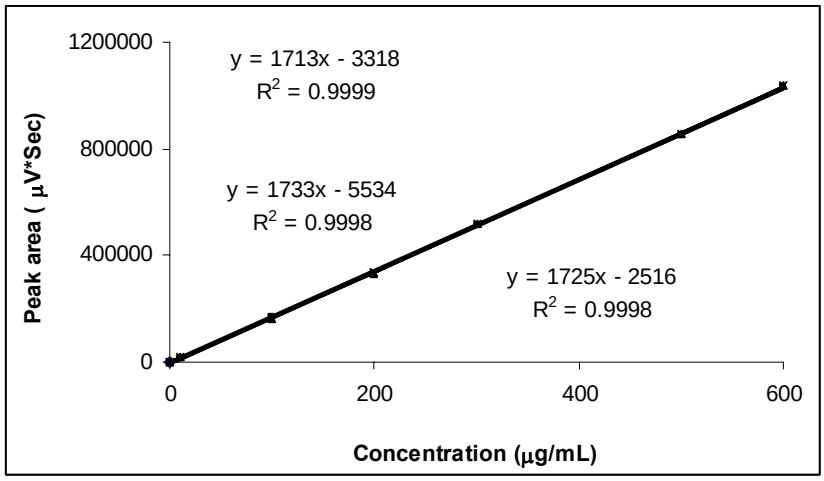


Figure 3.9. Standard calibration curves for cloprostenol to enable determination of inter-day reproducibility of intercept, linearity and range of the HPLC stability assay on three different days.

Table 3.19 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards for cloprostenol on day 2.

Table 3.19. Mean peak area, %RSD, actual concentration and %bias of calibration standards for cloprostenol on day 2.

Theoretical concentration (μg/mL)	Mean peak area[1] (μV*Sec)	SD	% RSD	Actual concentration[2] (μg/mL)	% Bias[3]
10	16003	718	3.3	11	10.0
100	166135	843	0.5	95	4.7
200	333349	1237	0.4	194	3.0
300	511702	1620	0.3	299	0.3
500	849790	0	0.0	499	0.3
600	1028925	3120	0.3	604	-0.7
QC standard	601159	9544	1.6	352	0.03

[1]N=2. [2]Calculated from Y= 1713x-3318 and R^2 = 0.9999. [3]Equation 3.1.

Table 3.20 shows the mean peak area, %RSD, actual concentration and %bias of calibration standards for cloprostenol on day 3.

Table 3.20. Mean peak area, %RSD, actual concentration and %bias of calibration standards for cloprostenol on day 3.

Theoretical concentration (μg/mL)	Mean peak area[1] (μV*Sec)	SD	% RSD	Actual concentration[2] (μg/mL)	% Bias[3]
10	20201	700	3.4	14	13.2
100	162772	962	0.6	96	-4.2
200	336830	799	0.2	197	-1.6
300	518832	3289	0.6	302	0.7
500	857401	431	0.1	499	-0.3
600	1036228	1051	0.1	602	0.4
QC standard	588340	6990	1.2	343	2.7

[1]N=2. [2]Calculated from Y= 1725-2516 and R^2 = 0.9998. [3]Equation 3.1.

Table 3.21 shows the inter-day reproducibility of the mean peak areas of working standards from three independent calibration curves on three different days for cloprostenol.

Table 3.21. Inter-day reproducibility of the mean peak areas of working standards from three independent calibration curves[1] on three different days for cloprostenol.

Theoretical concentration (µg/mL)	Day 1	Day 2	Day 3	Mean of the three days	SD	%RSD
	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)	Mean peak area[2] (µV*Sec)		
0	0	0	0	0	0	0.0
10	16003	17037	20201	17747	2187	12.3
100	166135	162634	162772	163847	1983	1.2
200	333349	331061	336830	333746.67	2905	0.9
300	511702	516057	518832	515530.33	3594	0.7
500	849790	856564	857400.5	854584.83	4173	0.5
600	1028925	1040790	1036228	1035314.3	5985	0.6

[1]Tables 3.18, 3.19 and 3.20. [2]N=3.

The inter-day reproducibility of cloprostenol working standards from three independent calibration curves on three different days is very precise (RSD ≤1.2% except 10 µg/mL standard which is 12.3% for which was selected to be a quantitation limit standard) over the stated range of standards between 10 and 600 µg/mL (Table 3.21).

3.6 Discussion

3.6.1 Developed and validated analytical methods

The UV assays and their parameters developed in this thesis are compiled in Table 3.22.

Table 3.22. Combined parameters developed and validated for the steroids studied in this thesis for the UV assay.

Drug	λ_{max} [1]	Slope (≥)	linearity (R^2 ≥)	Range (μg/mL)		Quantification limit (μg/mL)
				Lower limit	Upper limit	
Dexamethasone	242	0.031	0.9997	4	50	1
Dexamethasone acetate		0.029	0.9999	4	50	1
Dexamethasone isonicotinate		0.032	0.9999	4	40	2
Dexamethasone valerate		0.028	0.9997	4	50	2
Dexamethasone dipropionate		0.027	0.9998	4	50	2
Progesterone	246	0.050	0.9996	4	30	3
Oestradiol benzoate	233	0.055	0.999	4	30	Not determined
Oestradiol-17β	217	0.025	0.9996	10	50	

[1] 5% HPβCD in pH 5.0/PBS.

All UV assays were shown to be linear within the stated range, precise and accurate. In addition, experiments performed using dexamethasone suggested that these assays are robust with respect to buffer composition as shown in section 3.4.1.

In addition, the inter-day reproducibility of accuracy and precision of the lower (4 μg/mL), middle (30 μg/mL) and high (40 μg/mL) range were also accurate and precise for the stated range of each steroid as previously explained in section 3.4.1.2.

Though the UV method is not specific, it is fast, easy and inexpensive compare to the HPLC method.

The HPLC assays and their parameters developed in this thesis are compiled in Table 3.23.

Table 3.23. Combined parameters developed and validated for the steroids studied in this thesis for the HPLC assay.

Drug	Peak time (minutes)	Slope (≥)	linearity (R^2 ≥)	Range (μg/mL)		Detection limit (μg/mL)	Quantification limit (μg/mL)
				Lower limit	Upper limit		
Dexamethasone	6.01	44244	0.9994	0.2	4	0.1	0.2
Dexamethasone acetate	8.13	36890	0.9997	0.5	5	0.1	0.2
Dexamethasone isonicotinate	9.94	38637	0.9995	0.4	5	0.2	0.4
Dexamethasone valerate	14.50	33913	0.9997	0.4	5	0.2	0.4
Dexamethasone dipropionate	14.70	35729	0.9991	0.4	5	0.2	0.4
Progesterone	17.50	62088	0.9995	0.2	4	0.1	0.2

All HPLC assays were shown to be linear within the stated range, precise and accurate (Table 3.23). The HPLC method is specific and selective. However, the HPLC is time consuming (longer analytical run time), complex (programme set-ups) and expensive (cost of consumables) compare to UV method.

3.6.2 HPLC stability indicating assays for progesterone, oestradiol-17β and cloprostenol

Verification of the existing stability indicating HPLC assay for progesterone supplied by DEC Manufacturing QC Laboratory was successfully achieved (Table 3.24). All specification limits were met and therefore the method is revalidated. The generation of the three independent standard curves plotted using the data

from Figure 3.4, respectively, provided data that met the predefined verification specifications.

Progesterone retention time was the same between laboratories at approximately 27 minutes and the method linearity, slopes, intercepts, accuracy and precision of the 15 to 850 µg/mL range were very reproducible between the DEC and InterAg laboratories. The inter-day reproducibility data of the verified method (Table 3.10) and the method precision of target concentration were excellent (≤1.5 %RSD, for an average of 0.2, 1.7 and 0.50%, RSD = 0.8%).

Table 3.24. Method parameters, specifications and experimentally determined values for progesterone method verification.

Parameter	Specification limits	Experimentally determined values in this research
Linearity[1]	R^2 >0.999	R^2 ≥0.9998
Range[2]	Low range %bias <20% High range %bias <2%	Low = 15 µg/mL (%bias = 9.6, 3.6 and 7.8%) High = 850 µg/mL (%bias = 0.74, 0.0 and 0.8)
Intercept[1]	Minimum:-80000, Maximum:80000	-72587, –18824 and -66634
Accuracy[2]	%bias <2%	1.7, 0.1 and 1.2 %bias at QC standard
Precision[2]	Injection repeatability <1.5%RSD	0.2, 1.7 and 0.50 %RSD at QC standard

[1]Figure 3.4. [2]Tables 3.7, 3.8 and 3.9.

Development and validation of a stability indicating assay for oestradiol-17β was successfully achieved by adopting a slight modification to the method supplied by DEC QC Laboratory for progesterone (change of analytical wavelength). This is because oestradiol-17β absorbs at different wavelength from progesterone. A similar wavelength was used by previous researchers using HPLC stability assay methods for either oestradiol-17β or one of its analogues. Kotiyan & Vavia (2000) and Segall et al. (1999) used the analytical wavelength of 280 and 286 nm for oestradiol-17β and oestradiol valerate, respectively.

The peak representing oestradiol-17β appeared at 28 minutes and was shown through forced stability studies to remain independent of the degradation peak. The additional peak appeared on the chromatogram of oestradiol-17β exposed to 2M HCl due to formation of degradation product as shown in Figure 3.5. This was explained by the fact that oestradiol-17β molecule is prone to dehydration in acidic conditions. For instance, in 1M HCl at 70ºC for 2 hours caused oestradiol-17β degradation as it was investigated by Kotiyan & Vavia (2000). This occurs due to loss of a molecule of water on 17-β-hydroxyl group of oestradiol-17β to form degradation product. In the same study, oestradiol-17β was stable in basic conditions. This also is in accord with absence of any additional peak indicating decomposition of oestradiol-17β samples which were exposed in basic conditions in our study. The generation of three independent standard curves of Figure 3.7, respectively provided data that validated the method shown in Table 3.25. The specification limits were met and therefore this validates the method.

Table 3.25. Validated parameters for oestradiol-17β HPLC stability indicating assay.

Parameter	**Specification limits**	**Experimentally determined values in this research**
Linearity[1]	$R^2 \geq 0.999$	$R^2 \geq 0.999$
Range[2]	Low range %bias <20% High range %bias <2%	Low range = 5 µg/mL (%bias = 5.0, 0.01 and -0.33%) High range = 500 µg/mL (%bias = 0.5, 0.01 and -0.02 %)
Intercept[1]	Minimum:-80000 Maximum: 80000	-3530, 634 and 8334
Accuracy[2]	%bias <2%	-0.05, 0.4 and -0.09% (%bias at QC standard)
Precision[2]	Injection repeatability <1.5%RSD	0.2, 0.4 and 1.4 % (%RSD at QC standard)

[1]Figure 3.7, [2]Tables 3.13, 3.14 and 3.15.

The method linearity, slopes, intercepts, accuracy and precision of the 5 to 500 µg/mL range was very reproducible. The inter-day reproducibility data of the verified method (Table 3.16) and the method accuracy and precision of target concentration were excellent (≤1.4 %RSD) and (≤0.4%bias) as seen in Table 3.25.

Development and validation of a stability indicating assay for cloprostenol was successfully achieved by adopting several modifications to the method supplied by DEC QC Laboratory for progesterone (change of analytical wavelength, flow rate and pH of mobile phase). Cloprostenol has different physicochemical properties from progesterone or oestradiol-17β and therefore different chromatographic conditions were required.

The peak representing cloprostenol appeared at 6 minutes and was shown through forced stability studies to remain independent of degradation peaks. No additional peak, indicating degradation, appeared on the chromatograms. The generation of three independent standard curves (Figure 3.9, respectively) provided data that validated the method shown in Table 3.26. The specification limits were met and therefore this shows that the method is validated.

Table 3.26. Validated parameters for cloprostenol HPLC stability indicating assay.

Parameter	**Specification limits**	**Experimentally determined values in this research**
Linearity[1]	$R^2 \geq 0.999$	$R^2 \geq 0.9998$
Range[2]	Low range %bias <20% High range %bias <2%	Low range of 10 µg/mL (%bias = 20, 10 and 13%) High range of 600 µg/mL (%bias = 0.7, - 0.7 and 0.4%)
Intercept[1]	Minmum: -80000 Maximumn:80000	-4437, -3318 and -2516
Accuracy[2]	%bias <3%	(%bias = 0.03 and 2.7%)
Precision[2]	Injection repeatability <1.5%RSD	1.6 and 1.2 %RSD at target concentration

[1] Figure 3.8b and Figure 3.9, [2] Tables 3.18, 3.19 and 3.20.

The method linearity, slopes, intercepts, accuracy and precision of the 10 to 600 µg/mL range was very reproducible. The inter-day reproducibility data of the validated method (Table 3.21) and the method precision of target concentration were excellent (≤1.5%RSD).

3.7 Conclusions

UV and HPLC analytical methods were developed and validated for *in vitro* permeation and Drug Dissolution Testing (UV only) experiments on the steroids studied in this research. In both methods, assays were shown to be linear within the stated range, precise and accurate.

In addition, the HPLC stability assays for progesterone were revalidated and for oestradiol-17β and cloprostenol were developed and validated. All the HPLC stability assays were also shown to be linear within the stated range, precise and accurate. In addition, there were no additional peaks, which were not originally apparent, indicating no degradation interfering in the stability assays of the tested drugs.

4 Assessment of an electronically controlled drug delivery technology for veterinary applications

4.1 Introduction

A veterinary controlled drug delivery technology provides for dosing animals with a drug or drugs at predefined and reproducible rates, often for prolonged periods of time. These systems can provide significant benefits compared to single injection dosage forms including (Rathbone *et al*., 2000):

- the ability to tailor or to integrate the administration of health and production oriented pharmaceuticals around the constraints of farm management systems,
- a reduction of stress to the animal by decreasing the number of times the animal is handled,
- financial benefits to the animal's owner resulting from reduced veterinarian cost, optimisation of employees time by reducing the time needed for herding administration,
- improved therapeutic outcomes,
- the maintenance of drug levels within a desired range,
- increased patient compliance,
- increased cost benefits to the end user.

A common means of controlling the delivery of a drug is through the use of a polymer. The drug is incorporated into either a natural or synthetic polymer matrix and is slowly released at a rate that is dependant on the physicochemical properties of both the drug and the polymer. These systems offer several advantages:

- they are cheap to manufacture,
- a variety of polymers are available and the release profile can therefore be tailored to meet a particular need.

However, they also have certain disadvantages, which include:

- once manufactured the release rate cannot usually be adjusted,
- once manufactured it is difficult to modify the duration of release,
- the technology is usually route specific and cannot be transferred,
- each technology is often developed for a specific active drug, and the polymeric system technology is often not applicable to other drugs,
- the release profile from simple polymeric systems declines with time, resulting in non-linear release of drug over time and declining blood profiles.

Amongst the ways scientists have attempted to overcome these problems is the development of systems that use electronic devices to control the rate and duration of drug delivery. In such cases, drug delivery control resides in the electronic circuitry, and is not dependant upon the properties of the drug. Such systems offer versatility in:

- adjustable release rates,
- adjustable durations of release,
- the capability of being designed for several different routes,
- applicability to a wide range of drugs,
- sustaining high blood profiles as a result of uniform and linear release of drug over time.

Examples of electronically controlled devices used for farm animals include the SMARTT1® Intelligent Breeding Device (IBD) and the Electronically Modulated Intravaginal Device (EMID). Most of this thesis is concerned with the EMID. A description of SMARTT1® is provided for comparative purposes.

4.1.1 The SMARTT1® IBD

The SMARTT1® IBD is shown in Figure 4.1.

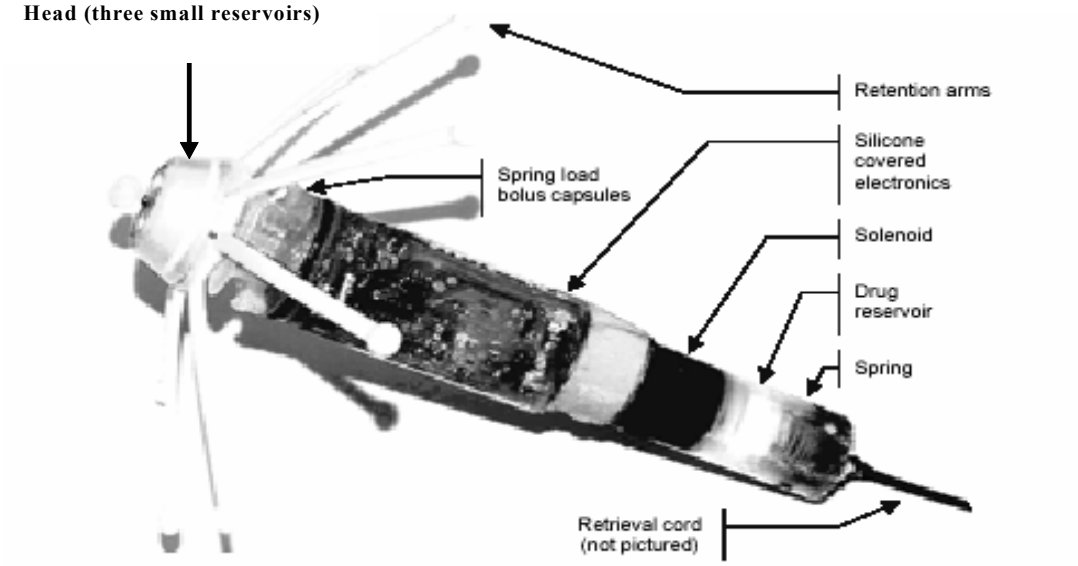


Figure 4.1.SMARTT1® Intelligent Breeding Device (Cross, 2002).

The IBD was developed in New Zealand for the purpose of controlling the oestrous cycle in cattle (Rathbone *et al.*, 1998; Cross, 2002). This device comprises three main sections, an outer plastic sheath is designed to protect the inner compartment, which comprises an electronic chip and board, four drug reservoirs, a retention mechanism and a tail (Figure 4.1). The retention mechanism has an umbrella configuration shape and is located at the head of the device.

The main drug reservoir is located at the bottom of the device, and contains 5 mL of a progesterone formulation. This reservoir opens directly to a small orifice at the top of the device (Cross, 2002) via a small bore stainless steel tube that opens to the exterior at the flat face. The other three reservoirs, each of which has a maximum capacity of 0.45 mL, are located near the head of the device and contain either oestradiol or prostaglandin. Tight fitting santoprene rubber seals are used to prevent drug solutions from leaking from their reservoirs during storage

and while in the animal. There is a movable piston and spring between the main rubber seal and the bottom of the device.

The remainder of the device comprises a circuit board and batteries. The circuit board contains a controlling chip, a power-on indication LED and a quartz timer (Rathbone *et al.*, 1998; Cross, 2002). Drug release from the reservoirs is controlled electronically according to a pre-programmed electronic chip. The small orifice of the inner compartment reservoir is opened and closed by a switch mechanism that is operated by a solenoid, and drug can only leave the reservoir when the switch is in the open position. The batteries and an electronic timing system of the device control a solenoid that operates a valve, which in turn, controls the drug release slowly and continuously over a 10 day period under the influence of the spring pressurized piston.

Unlike other conventional drug delivery systems that are currently on the market such as the PRID and CIDR™-B, the IDB technology offers the advantage of incorporating multiple drugs required for oestrous control of cattle within a single device.

However, because the batteries and electronics take up so much room and there is limited room available for the main reservoir, there are restrictions on the capacity of the drug reservoirs of the device. Also the nature of the spring loaded controlled orifice release mechanism is such that any small blockage could adversely affect dosage after that point and could undermine practical application of the IDB technology (Cross, 2002). Finally, the IBD has a limited volume capacity and a very complex design. Unlike the IBD technology, the EMID offers the advantage of having a large volume reservoir and a less complex design.

4.1.2 EMID description

4.1.2.1 Development prior to this study

The EMID is shown in Figure 4.2. The EMID is assembled from injection moulded plastic components.

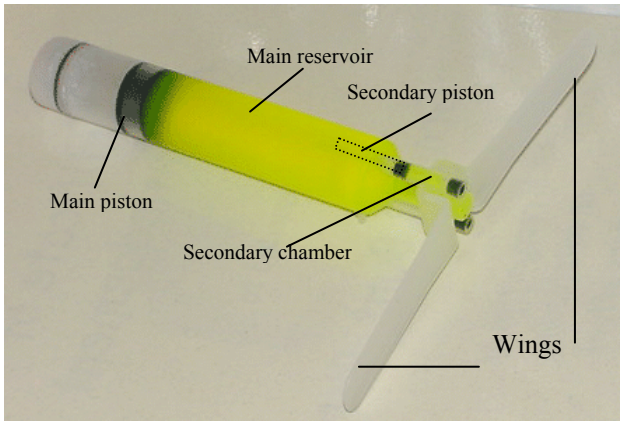


Figure 4.2. EMID for the delivery of progesterone, oestradiol benzoate and prostaglandin.

It is noteworthy that the final EMID arrangement has tinfoil adhered onto the outside of the body (not shown). The main reservoir of the EMID inner compartment has a 30 mL capacity and an outlet area of 0.22 cm^2 whereas the secondary chambers have a total volume of 100 mL each and an individual outlet area of 1.1 cm^2. Tight fitting santoprene rubber bungs seal the small chambers at the top and bottom (Figure 4.2).

The progesterone formulation is dispensed from the device by a gas-producing battery located in the back of the EMID. A resistor in contact with the battery controls gas release and as the gas is produced, it accumulates and creates a pressure on the piston. The piston then moves forward and dispenses known volumes of the formulation from the main reservoir of the EMID. Part way through the process, pistons in the secondary chambers are activated by the primary piston and cause the secondary chambers to open, thereby releasing oestradiol benzoate and/ or prostaglandin.

Similar to the IBD technology, the EMID offers the advantage of incorporating the multiple drugs that are required for oestrous control of cattle within a single device.

However, in contrast to the IBD the main reservoir has a large volume enabling more scope for formulation strategy. In addition, the large outlet orifices associated with the large reservoir and secondary chambers are less susceptible to blockage as compared to the IDB technology.

The EMID appears to offer the advantages that are required for the complex and demanding requirements of delivering the drugs associated with either oestrous control or induced calving. Therefore, the objective of this chapter was to critically evaluate the EMID for its application for either oestrous control or for the induction of calving.

4.1.2.2 Evaluations made during this study programme

Early in the *in vivo* animal work, it became apparent that there were a number of parameters of the EMID which did not maintain consistency. In particular, it appeared that many of the physical components of the EMID were prone to act differently or even to fail when placed in the vagina compared to simple *in vitro* air or aqueous environments. The piston was sticking in the barrel and the tinfoil was separating from the plastic resulting in a non-linear release profile. In addition, the EMID was not being retained for the full duration of the treatment and caused a high incident of vaginal damage among treated animals. These observed and unexpected failures of the technology led to the need to reinvestigate independently and in multi-factorial combinations, each component of the device (e.g. piston material, body composition, gas-cell function, and retention wings), and the properties of the payload which was to be released. Also development of an *in vitro* release test to correlate EMID release rates from *in vitro* with *in vivo* rates was investigated.

4.2 Description of the assembled EMID and its components

A list and general description of the main components of the EMID are shown in Figure 4.3.

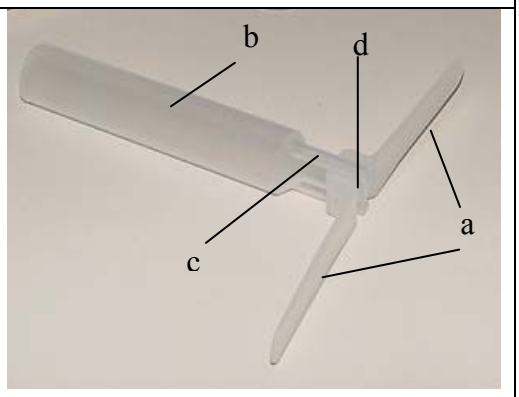
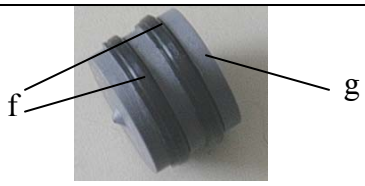
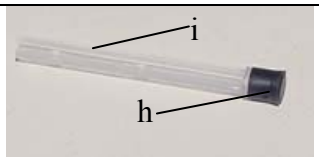
Component material	Description	Photograph
Main body (typically made from polypropylene (PP), high density polyethylene (HDPE) or like materials)	Retention wings (a) Body and main reservoir (b) Secondary reservoirs (c) Outlet orifice (d)	
Primary piston comprising o rings (made from a variety of silicone or other plastic materials) and carrier (polyvinyl chloride (PVC)	The silicone piston comprising silicone O-rings (f) positioned over the carrier (g)	
Secondary piston carrier (made from PP or HDPE) and piston (santoprene)	Secondary piston (h) is located over the piston carrier (i)	
Plug (made from PP or HDPE) and O-ring (silicone).	Comprises an O-ring seal over a plastic plug	
Cap (made from PP or HDPE)	Plastic end cap that locates over the plug	
Gas cell unit	Battery (gas cell) and the resistor (the wire)	

Figure 4.3. EMID components.

The rate of release from the EMID is controlled by the rate of gas production, which was proportional to, and controlled by, the applied current (Bunt *et al*., 1998). The resistor is placed in series with the battery (Varta 4690 Simatec, Switzerland), thereby influencing the current (Equation 4.1.). Gas produced from the reaction $6H_2O \rightarrow 2H_{2(g)} + O_{2\,(g)} + 4H^+ + 4OH^-$ is a function of the amount of current and time as follows:

$$\text{Gas produced (moles)} = \text{current (A)} \times \text{time (s)} \times \frac{1\,\text{Farad}}{96500} \times \frac{\text{mol product}}{\text{mol electrons}} \qquad \textbf{Equation 4.1.}$$

All EMID components were manufactured and supplied by a technical injection moulding company (Millennium Plastics, Te Rapa, Hamilton).

4.2.1 Assembly of the EMID

All EMIDs were easy to assemble (Section 4.2.1.1), although care had to be taken to follow the defined procedure. In addition, friction welding required the need to remove a very fine film of silicone lubricant that was deposited from the piston as it was pushed up to the top of the EMID to ensure a successful welding process. This was easily removed by use of a tissue and 10% toluene in ethanol. Toluene dissolves and removes the silicone lubricant.

4.2.1.1 Assembly of the EMID

To assemble the EMID the following steps were followed.

1. A thin layer of silicone lubricant (Dow Corning 360 medical fluid, 350 CST Dow Corning Corporation, Midland, USA) was applied onto the surface of each of the silicone o-rings that make up the primary piston.
2. The lubricated primary piston was inserted into the body by pushing it through the open back of the EMID and positioned at the head of the device.
3. The plug was friction welded onto the back of the body.
4. Fix the O-ring onto the plug and apply a surface coating of lubricant.

5. Where appropriate apply tinfoil (Sellotape, New Zealand) around the outside of the body. The tinfoil is used to reduce gas diffusion through the polymer.
6. Fill the EMID with the formulation.
7. Seal the two small chambers with the secondary pistons.
8. Attach the resistor to the gas cell (Varta 4690 Simatec, Switzerland) and load into the back of the EMID.
9. Push the cap onto the plug and fix it in place by screwing it into position.

The final assembled EMID is shown in Figure 4.4.

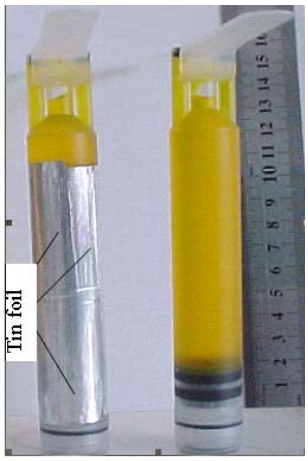


Figure 4.4. Assembled EMID with or without tinfoil.

4.2.1.2 Measurement of the EMID function

For most biological applications, a test procedure is required, which monitors the release of the particular bioactive material from the device. This issue is addressed near the end of this chapter (Section 4.4), but for evaluation of the EMID, a simple mechanical means of monitoring the device performance involved the measurement of either the piston movement relative to a fixed point over time, or, the measurement of weight loss over time.

"Due to the greatly differing types of technology involved, the methodology and results of each part are presented as sub-sections in this chapter."

4.2.2 *In vitro* characterisation the component parts of the EMID

4.2.2.1 Determination of the effect of piston material on the *in vitro* release rate

4.2.2.1.1 Methods

The effect of piston material type and piston design on the in *vitro* release rate was determined using the weight loss method. Polypropylene EMIDs covered in tinfoil containing 2% hydroxypropyl methylcellulose (HPMC) gel (Grade 60SH-10000 USP [Shin_Etsu Chemical Co., Ltd, Chiyoda-ku, Japan]) were fitted with a resistor of 750 Ohms. Experiments were conducted at 40ºC/75%RH and the release rate was monitored for 12 days.

The different piston designs are shown in Figure 4.5. Piston designs were classified into three major types which were: type A (original type, double O-ring piston), type B (solid silicone) and type C (various pistons over a polypropylene carrier).

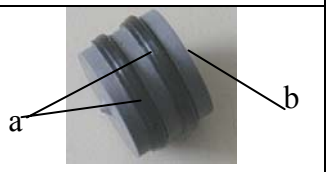
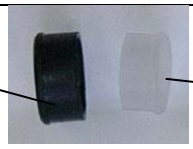
Description	Piston design	Photograph
A) Original design (Double silicone O-ring type)	Silicone O rings (a) positioned over the carrier (b)	
B) Solid type	Solid piston	
C) Piston over polypropylene carrier	Piston (various materials) (c) polypropylene carrier to provide support (d)	

Figure 4.5. Different piston designs investigated in this study.

The different piston materials used in this study are shown in Table 4.1.

Table 4.1. Different piston materials used in the study.

Piston type	Piston material (Trade name)	Material composition (polymer)
A) Original type	Double O-rings silicone piston	Silicone (Q74840 Dow coning)
B) Solid type	Solid silicone	Silicone
C) Piston over a polypropylene carrier	Santoprene (8211-35) + 10% polypropylene	Santoprene (90% and 10%polypropylene)
	Santoprene (201-73)	Santoprene (100%)
	Santoprene (8211-75) + 1% lube	Santoprene 99%+1%lubricant
	Santoprene (8211-87A)	Santoprene (100%)
	Santoprene (8211-87A) + 10% polypropylene	Santoprene (90% and 10% polypropylene)
	Reflex 123E	Polyurethane
	Polypropylene	Polypropylene (100%)
	Thermoflex 65A 1-800	Polyester
	Desmopan 192	Polyester urethane
	Hytrel 4069	Polyether ester
	Elastollan WYO 1388-5	Polyurethane
	Silicone	Silicone

Each combination of design and/or material was tested in triplicate. All of the raw weight loss data is presented in tables of Appendix-I.

4.2.2.1.2 Results and discussion

The effect of piston material on *in vitro* release rate for various materials is shown graphically in Figure 4.6 to Figure 4.7.

Figure 4.6 show the cumulative weight loss versus time of double silicone O-ring and solid silicone pistons.

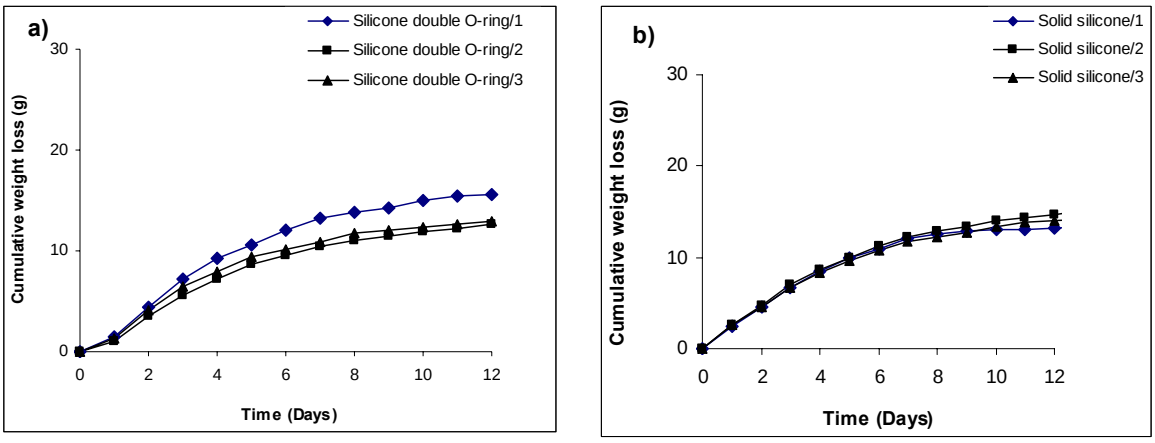
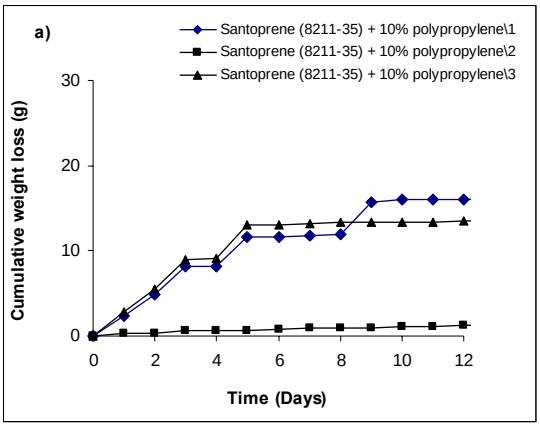


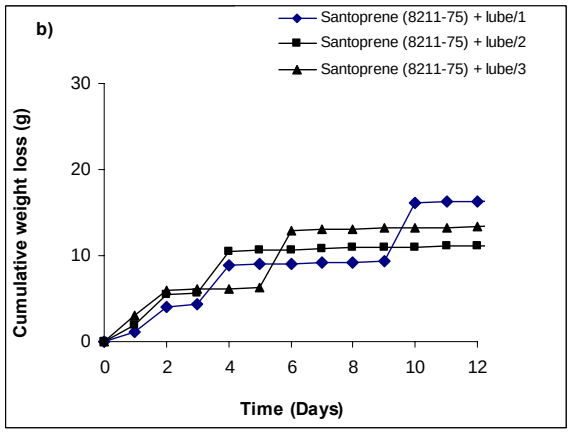
Figure 4.6. Cumulative weight loss over time for: a) double silicone O-ring pistons b) solid silicone pistons.

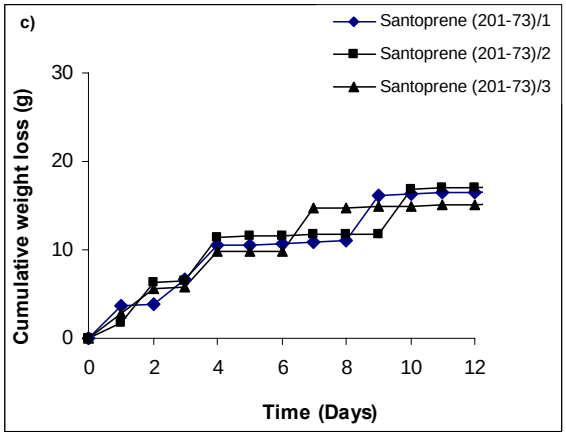
Both double silicone O-ring piston (Figure 4.6a) and solid silicone (Figure 4.6b) piston materials showed a typical and anticipated release profile. In both cases, all devices performed well despite a small release variation among the devices in the case of double O-ring silicone piston. Small gas bubbles were observed in the formulation in the case of the solid silicone pistons. These gas bubbles were attributed to the presence of a possible small leakage of gas between the barrel and the piston. However, these gas bubbles were negligible, as they were not growing much in size to cause any spontaneous discharge of the formulation.

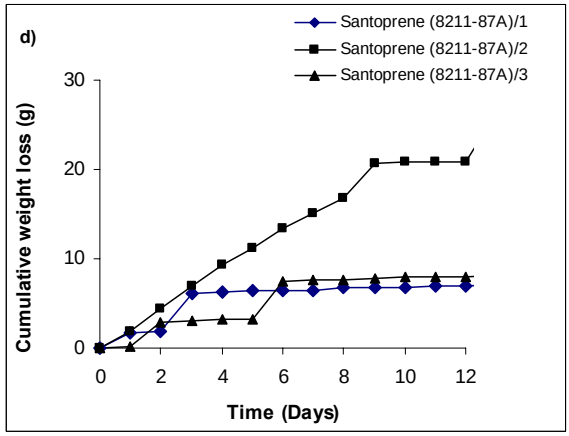
Neither of the two pistons delivered the entire payload contained in the EMIDs possibly due to friction caused by the tight fit between the piston and the barrel.

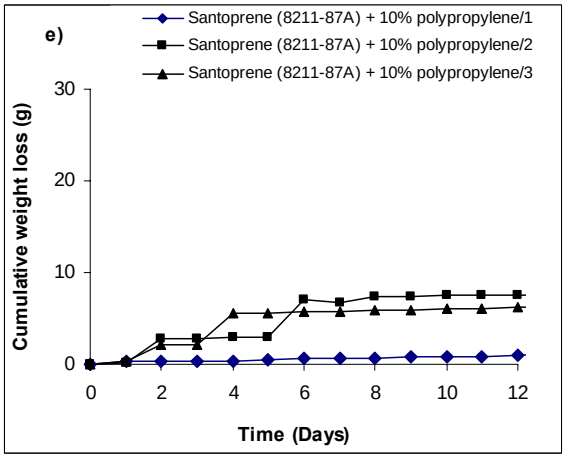
Figure 4.7 shows the cumulative weight loss over time of various pistons over a polypropylene carrier. These pistons were made of santoprene (a-e) and polypropylene (f) materials. The material compositions of the pistons from Figure 4.7g-l (Silicone, Reflex 123E/Elastollan WYO 1388-5, Thermoflex (65A 1-800)/Hytrel 4069 and Desmopan 192) were silicone, polyurethane, polyester, polyester urethane, respectively.

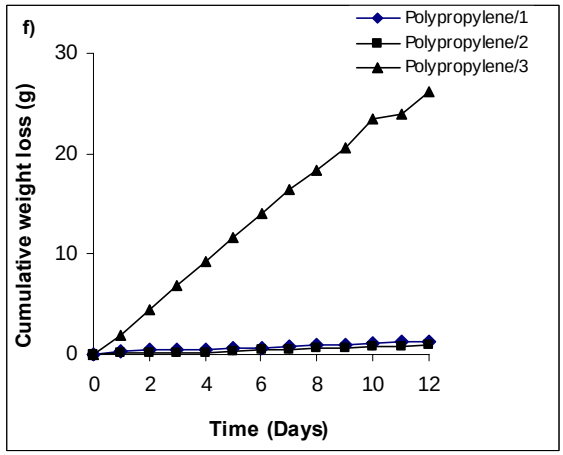

a)
Santoprene (8211-35) + 10% polypropylene\1
Santoprene (8211-35) + 10% polypropylene\2
Santoprene (8211-35) + 10% polypropylene\3
Cumulative weight loss (g)
Time (Days)

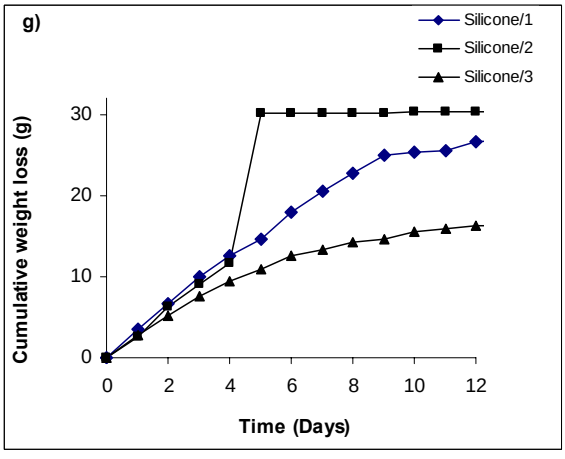

b)
Santoprene (8211-75) + lube/1
Santoprene (8211-75) + lube/2
Santoprene (8211-75) + lube/3
Cumulative weight loss (g)
Time (Days)

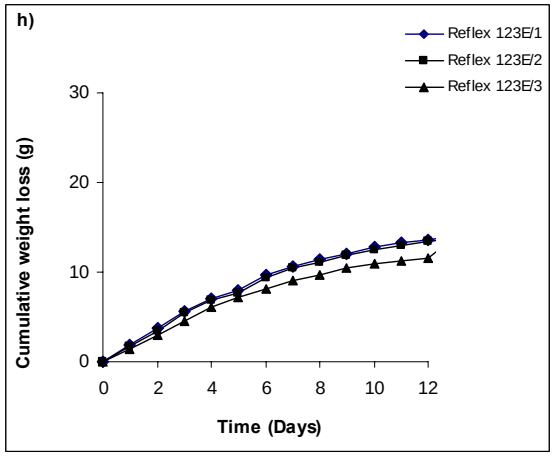

c)
Santoprene (201-73)/1
Santoprene (201-73)/2
Santoprene (201-73)/3
Cumulative weight loss (g)
Time (Days)


d)
Santoprene (8211-87A)/1
Santoprene (8211-87A)/2
Santoprene (8211-87A)/3
Cumulative weight loss (g)
Time (Days)


e)
Santoprene (8211-87A) + 10% polypropylene/1
Santoprene (8211-87A) + 10% polypropylene/2
Santoprene (8211-87A) + 10% polypropylene/3
Cumulative weight loss (g)
Time (Days)


f)
Polypropylene/1
Polypropylene/2
Polypropylene/3
Cumulative weight loss (g)
Time (Days)


g)
Silicone/1
Silicone/2
Silicone/3
Cumulative weight loss (g)
Time (Days)


h)
Reflex 123E/1
Reflex 123E/2
Reflex 123E/3
Cumulative weight loss (g)
Time (Days)

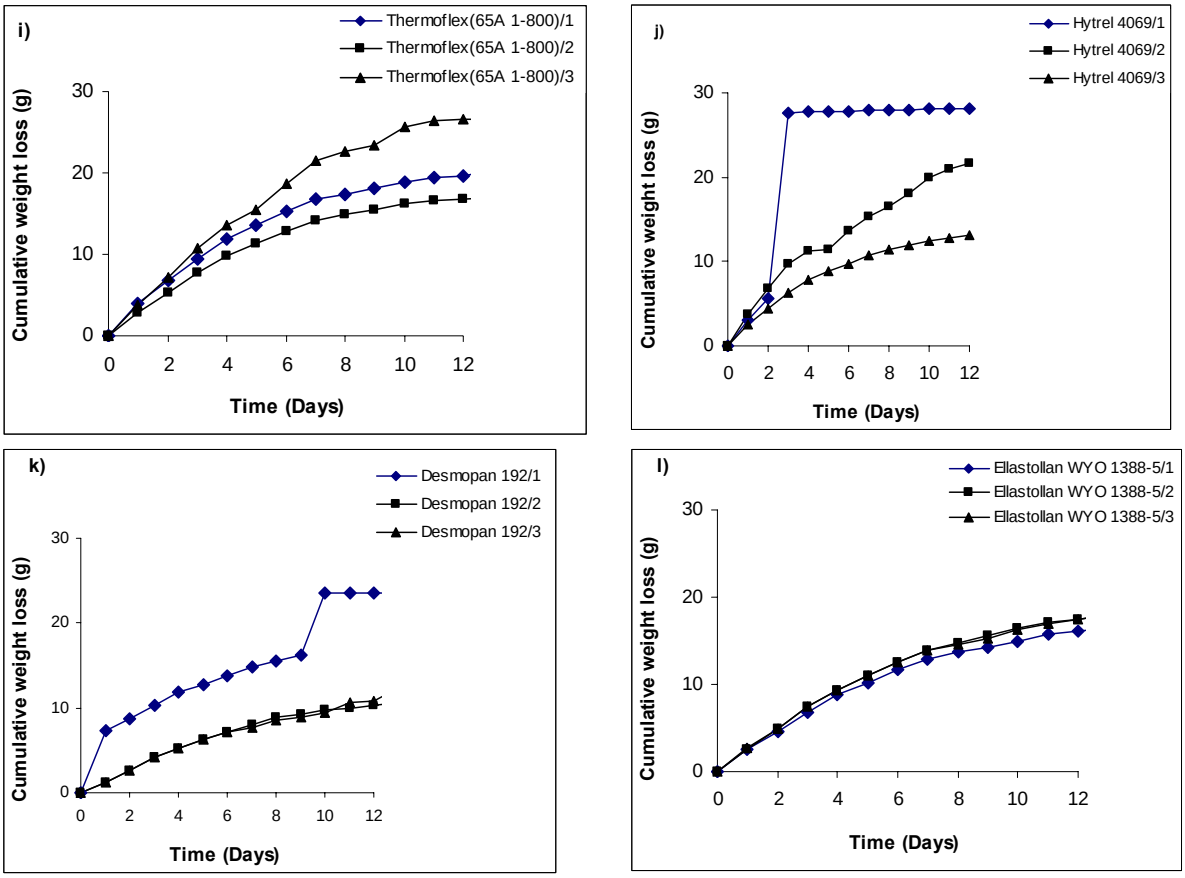


Figure 4.7. Cumulative weight loss over time for pistons over a polypropylene carrier containing a) santoprene (8211-35) + 10% polypropylene; b) santoprene (8211-75) + lube; c) santoprene (201-73); d) santoprene (8211-87A); e) santoprene (8211-87A) + 10% polypropylene; f) polypropylene pistons; g) silicone; h) reflex 123E; i) thermoflex (65A 1-800); j) hytrel 4069; k) desmopan 192 and l) elastollan WYO 1388-5 piston.

All the santoprene piston materials (Figure 4.7a-e) showed slip stick phenomenon with some degree of variation among them. Slip stick behaviour produced non-zero order of release profile due to the inconsistency of piston motion within the barrel, which in turn causes inconsistency of delivery rate of the payload over time.

For one device of samples represented in Figure 4.7a did not move at all due to piston sticking in the barrel (device # 2). This was checked by ensuring the pressure build up in the back chamber and tested at end of the experiment.

From Figure 4.7b and c it can be seen that all devices functioned despite showing signs of slip stick and some release variation among them. For one device of samples represented in Figure 4.7d did not move (device # 2) despite showing weight loss (apparently due to improper sealing between the piston and the barrel, because gas was seen to move past the piston and cause spontaneous discharge of the formulation).

From Figure 4.7e it can be seen that one out of the three pistons (device # 1) failed to move at all due to the piston becoming stuck. In addition, an inadequate seal between the piston and the barrel created gas leakage into the formulation chamber for one of the two working devices but this did not affect the weight loss profile.

None of the three polypropylene pistons moved (Figure 4.7f), despite one of them showing a linear weight loss versus time. This piston (device # 3) showed gas leakage between the piston and the body, which caused spontaneous formulation discharge.

Polypropylene carrier pistons showed the highest friction of all (due to the fact that none of the three pistons moved at all), followed by santoprene (8211-87A) + 10% polypropylene pistons. On the other hand, it can be seen that all the pistons in Figure 4.7g-l did not exhibit any slip-stick characteristics, possibly due to their less coefficient friction compared to the pistons made of either santoprene or polypropylene materials.

One out of three devices performed well (device # 3) in Figure 4.7g for the silicone rubber with piston carrier. The other two had improper sealing between the piston and the barrel. Gas leakage from the back chamber went through the formulation to cause spontaneous discharging. One device became completely exhausted by day 5 (device # 2). Formulation also leaked into the back chamber near the gas cell.

In Figure 4.7h all three devices for Reflex 123E piston material showed the desired profile and performed well. From Figure 4.7i it can be seen that for

Thermoflex (65A 1-800) one out of three (device # 3) had gas leakage due to improper sealing between the piston and the barrel. This caused a slight increase of discharge of the formulation. The improper sealing was due to the piston and its carrier breaking into parts. However, two of the three pistons performed despite some release variation between them.

From Figure 4.7j it was observed that for Hytrel 4069 piston two out of the three devices (devices 1 and 2) had gas leakage into the formulation due to improper sealing between the piston and the barrel. One device became completely exhausted by day 3 (device # 1) due to gas leakage from the back chamber that went through the formulation causing a dramatic spontaneous discharge. In addition, one (device # 2) out of two failed devices caused formulation leakage into the back chamber. One out of the three pistons performed well.

From Figure 4.7k it was observed that for Desmopan 192 piston one (black diamond) out of three had gas leakage between the piston and the barrel due to improper sealing between them. Two out of the three pistons performed well giving very similar release profiles.

From Figure 4.7l it can be seen that for Elastollan WYO 1388-5 piston all three devices performed well giving very similar release profiles compare to silicone pistons (Figure 4.6). It appears to be a very good material for this purpose.

However, none of the pistons (Figure 4.7) delivered the entire payload contained in the EMIDs perhaps due gas diffusion through the body wall, resulting in progressively lower internal pressure acting on the piston (see Section 4.2.6). The total amount of the payload delivered varied among the piston materials (i.e., Thermoflex (65A 1-800) versus santoprene (8211-87A) + 10% polypropylene for 18g versus 7g) perhaps due to the coefficient frictional difference of their materials. Therefore, pistons made of santoprene or polypropylene materials were not recommended for further study due to the slip stick behaviour or high friction inheritance. Table 4.2 summarises piston performance including the presence/absence of slip-stick behaviour, function and release rate of the initial linear portion of the release profile of the tested pistons.

Table 4.2. Summarised overall effect of piston material, slip-stick behaviour, function and release rate of initial linear portion of the slope of the tested pistons.

Piston type	Piston Material (Trade name)	Graph displays slip-stick behaviour Yes or No	Function	Slope (g/Hour, Mean ± SD)
Original type	Double O ring silicone piston	No	****	2.0 ± 0.2
Piston over polypropylene carrier	Santoprene (8211-35) + 10% polypropylene	Yes	*	2.4 ± 0.2
	Santoprene (201-73)	Yes	*	2.2± 0.2
	Santoprene (8211-75) + lube	Yes	*	1.8 ± 0.6
	Santoprene (8211-87A)	Yes	*	1.9 ± 0.6
	Santoprene (8211-87A) + 10% polypropylene	Yes	*	1.0 ± 0.6
	Reflex 123E	No	***	1.6 ± 0.1
	Polypropylene	Possibly	*	-2.4
	Thermoflex 65A 1-800	No	***	2.7 ± 0.4
	Desmopan 192	No	**	1.6 ± 0.6
	Hytrel 4069	No	**	2.1 ± 0.4
	Elastollan WYO 1388-5	No	****	2.2 ± 0.1
	Silicone	No	**	2.6 ± 0.5
Other types	Solid silicone	No	****	2.0 ± 0.0

****Excellent; ***good; **poor; *very poor. –none of the pistons moved despite weight release.

Double O-ring silicone, Elastollan WYO 1388-5 and solid silicone pistons were deemed to be amongst the best pistons tested and all performed well with a smooth release profile (no slip-stick behaviour), and allowed for a consistent rate of release (2.0 ± 0.2, 2.2 ± 0.1 and 2.0 ± 0.0 g/day, respectively) for the initial trial period.

Consideration of the above results leads to the conclusion that double O-ring silicone pistons may be the preferred material and design for optimum device performance. This type had demonstrated smooth motion (no slip-stick characteristics), producing good rate of release. In addition, the double O-ring silicone pistons were in abundance compared to other materials. Therefore, further study was focused on this piston. However, it is recognised that not all possible materials and designs were tested in this study, and that there may indeed be even more beneficial combinations that could be used in future work and/or any commercial development of this product.

After piston material selection (double O-ring silicone piston), the effect of resistors on the release of the EMID was investigated.

4.2.3 Determination of the effect of electrical resistance on *in vitro* release rate

4.2.3.1 Methods

The effect of electrical resistance on *in vitro* release rate was determined using the weight loss method. Polypropylene EMIDs covered in tinfoil containing 2% HPMC gel, were fitted with resistors of various resistances (4.7, 270, 560, 750, 1000 and 1300 Ohms). The use of the selected resistors can make possible to monitor the rate of release of the payload from the EMID between hours and weeks (Table 4.3). Experiments were conducted at 40ºC/75%RH and release rate was monitored for 25 days (4.7 Ohms for hours). The pistons used in the study were the silicone double O-ring type. Each combination of design and/or material was tested in triplicate.

4.2.3.2 Results and discussion

Figure 4.8 shows the effect of resistor values between 4.7 and 1300 Ohms on the delivery rate profile of EMIDs covered with tinfoil. Each of the curves in Figure 4.8 has a similar (hyperbolic) shape except in the case of the 4.7 Ω resistor (the entire payload had been exhausted within 4-5 hours). Table 4.3 shows the initial slope of the delivery rate for each resistor. The main differences among the resistors are a difference in the initial slope (Table 4.3 and Figure 4.8).

Table 4.3. Effect of resistor values on the initial slope of delivery rate of the EMID (Mean±SD, n=3).

Resistor value	**4.7 Ω**	**270 Ω**	**560 Ω**	**750 Ω**	**1000 Ω**	**1300 Ω**
Delivery rate (g/day)	166.2 ± 6.1	6.3 ± 0.2	3.0 ± 0.4	2.2 ± 0.3	1.6 ± 0.1	1.2 ± 0.1

The final asymptote for each of the curve also varies with difference resistance values (Figure 4.8). Lower resistor lead to a cumulative rate loss profile, which tended towards linearity until a plateau is reached when the entire payload had been exhausted and the piston, has reached the end of its travel. On the other hand, a high resistance (which is expected to provide a lower rate of gas production) clearly show a much slower initial rate and more obvious curvature. What is more,

the final asymptote corresponds to a much lower accumulated weight loss, with the piston not moving the whole length of the barrel.

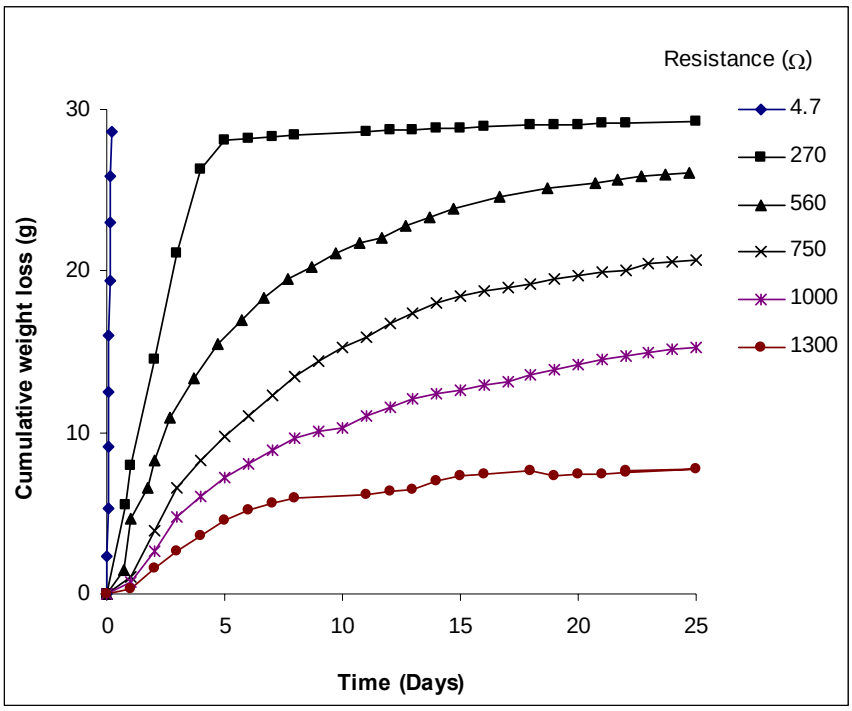


Figure 4.8. Effect of resistance on the release profile of EMIDs covered with tinfoil.

The different release rates of the formulation delivery are explained by the different resistors that control the gas production rate according to Equation 4.2 (Ohm's Law).

$$I = \frac{V}{R}$$ **Equation 4.2.**

Where the current (*I)* is directly proportional to the voltage (*V*) and inversely proportional to the resistance (*R*).

Given that, the total gas production from a battery should be independent of the resistance one might expect that the only difference will be in the rate of weight loss, not the absolute total loss (Cross, 2002). The clear discrepancy here suggests that in these systems, all of the gas is not always converted to payload expulsion. This in turn implies some loss of gas, either by leakage or by some other

mechanism. Great care was taken to ensure there were no gas leaks from any source, and the most obvious explanation for gas loss is that the hydrogen being generating in the gas chamber can in fact escape via diffusing through the walls of the barrel. This phenomenon is described in more detail in section 4.2.6, but suffices to say, the rate of gas diffusion, or rate of loss of gas through the barrel, would be proportional to the exposed surface area behind the piston, and this means that the diffusion effect will become steadily more important as the size of the gas chamber increases.

The only reliable way to compare the effect of resistance is to consider only the initial slopes of each of the lines in Figure 4.8 as in the early time the surface area of barrel available for diffusion is very small, and one can reasonably expect that all of the generated gas will be reflected in piston travel. Applying Equation 4.1, the reciprocal of the initial rate versus resistance was plotted to investigate if there is any correlation between the rate and the resistance. From Figure 4.9 it was found a linear relationship between the reciprocal initial linear portion of the release rate and the resistor values ($R^2 \geq 0.998$).

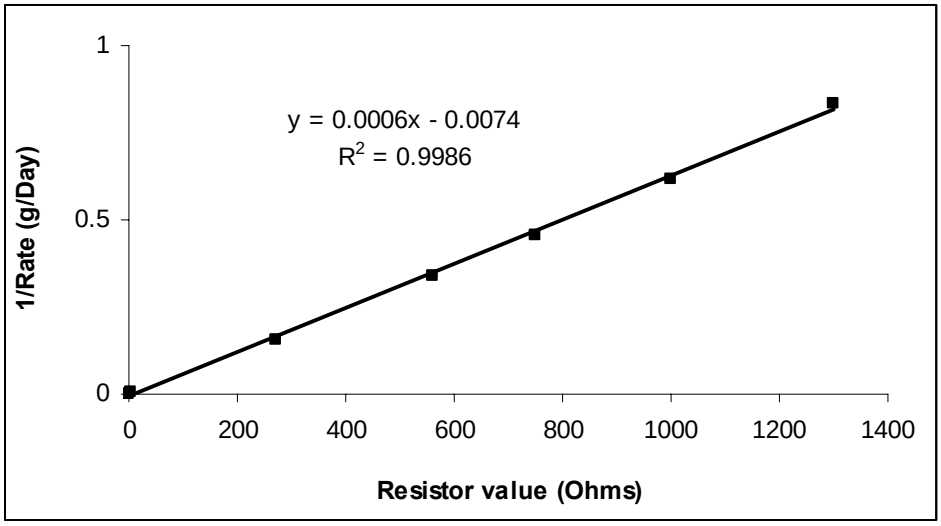


Figure 4.9. Effect of resistors on the reciprocal of the initial rate of the EMID.

The rate of release of the payload from the EMID can be predicted (between 4.7 and 1300 Ohms range) by interpolating the regression line equation for a known resistor (Figure 4.9).

The effect of body composition on the delivery of the EMID was also investigated.

4.2.4 Determination of the effect of body material on *in vitro* release rate

4.2.4.1 Methods

The effect of body material on *in vitro* release rate was determined using the weight loss method. Polypropylene EMIDs with and without tinfoil covering and high density polyethylene containing 2% HPMC gel were fitted with a 750 Ohms resistor. Experiments were conducted at 40ºC/75%RH and release rate was monitored for 25 days. The piston material used in the study was the silicone double O-ring type. Each combination of design and/or material was tested in triplicate.

4.2.4.2 Results and discussion

The effect of polymer type on the formulation release is shown in Figure 4.10.

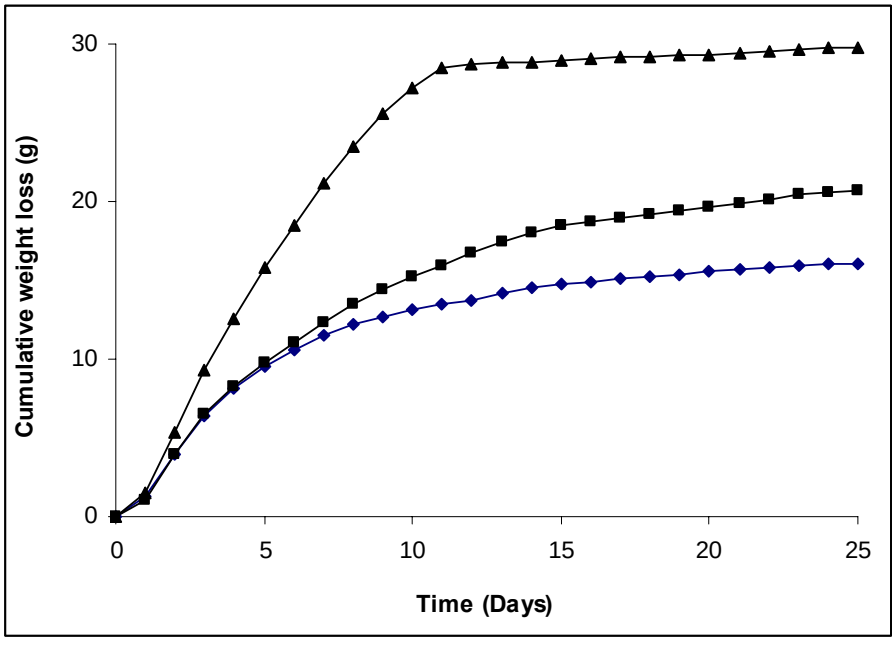


Figure 4.10. Effect of body composition of HDPE (▲) PP with tinfoil (■) and PP (♦) on EMID delivery rate profile.

Body material was found to influence delivery rate profile from the EMID. A near linear release rate was shown by HDPE until complete exhaustion coincident with the piston reaching the end of the barrel. The small weight change observed over the final 10 days was thought to be due to water evaporation from the residual payload remaining in the orifice housing.

In contrast, both PP barrels gave non-linear and non-exhaustive release profiles, although, the one with the tinfoil coating was similar to the HDPE device than the uncoated one. It is believed (see section 4.2.6) that this phenomenon is another demonstration of gas diffusion through the body wall, resulting in progressively lower internal pressure acting on the piston and therefore a reduced delivery rate. Covering the PP body of the insert with tinfoil appears to have inhibited gas diffusion to some extent, but did not completely prevent it.

Further supporting evidence of gas diffusion of leading to the having different profile release rates found from EMID fabricated from HDPE and PP polymers is shown in Table 4.4. The gas permeability was lower for HDPE than for PP polymer.

Table 4.4. Gas permeability differences between PP and HDPE plastics.

Polymer	**Gas permeability[1]([mm cm^3/cm^2(cm Hg) x 10^{10}]**		
	N_2	CO_2	O_2
PP	4.4	92	28
HDPE	3	45	10

[1](http://www.dynalabcorp.com/files/Use%20and%20Care%20of%20Plastics.pdf).

This table indicates that gas permeability values for N_2, CO_2 and O_2 are always higher in the case of PP material compare to HDPE polymer.

Based on these findings, HDPE polymer was selected as the best available body material for the EMID.

4.2.5 Gas diffusion

It was apparent that the EMID manufactured from PP material have non-linear and non-exhaustive release profiles possibly due to gas diffusion through the polymer. Therefore this experiment was carried out to evaluate possible of any gas diffusion through the PP EMID.

4.2.5.1 Method

In order to investigate gas diffusion through the PP EMID, experiments were conducted using two devices (active and control PP EMIDs) which were fully immersed in water. Another active device manufactured from HDPE was also made for comparison. Each active EMID (PP or HDPE) was loaded with a gas cell and a resistor (270 Ω). The EMIDs were inserted into three separate bottles. The three bottles were subjected to ultrasound to remove any dissolved gas in the water for 10 minutes. The bottles were placed on the bench (with lids on) and observed regularly to see any gas bubbles appearing on the surface of the EMIDs. Images were taken at appropriate times (e.g., when piston reached at the head of the EMID within seven days) to show the gas diffusion of PP EMID (Figure 4.11).

4.2.5.2 Results and discussion

There were no gas bubbles seen with either the control or the active HDPE EMID. On the other hand, the PP active EMID became covered in gas bubbles, the number of which increased over time. These gas bubbles are clearly seen in Figure 4.11.

The gas bubbles observed from this experiment, together with the previous release rate profile shown by PP EMID covered with tinfoil from the previous experiment (Figure 4.10) (steeper linear release profile compare with PP no tinfoil on) provide supporting evidence of the concept of gas diffusion. Putting tinfoil on the PP EMID reduced the gas diffusion problem, in a manner not dissimilar to using other plastic materials (e.g., HDPE) which are known to be less prone to gas diffusion.

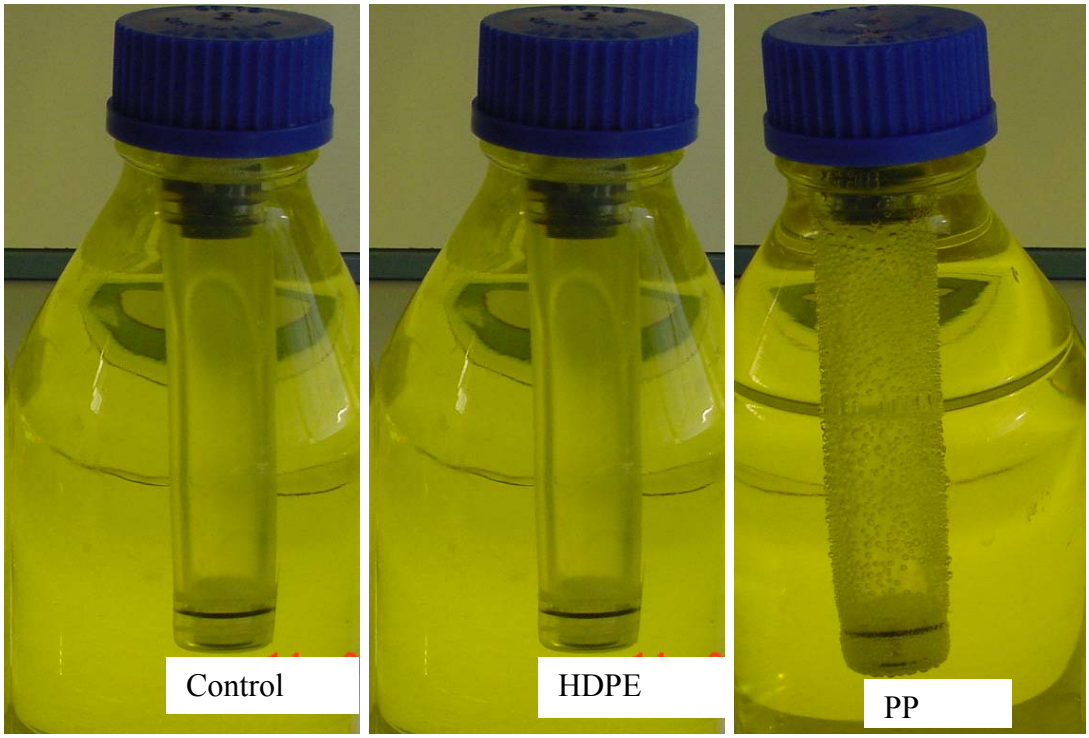


Figure 4.11. Control, HDPE and PP showing gas bubbles on EMID manufactured from PP material.

4.2.6 Analytical method to measure drug release from EMID

During technology development studies described in the previous sections, weight loss or piston travel measurements were the simplest and most appropriate ways to measure the drug release rate from EMID. However, any product that might result from this research will need to demonstrate compliance with a variety of pharmaceutically accepted procedures.

An establishment of an *in vitro* drug release method is a key parameter that is used to assess in both the formulation development and quality control of a final product. The USP Apparatus 1 (basket method) and 2 (paddle method) are the most widely used by which the pharmaceutical industry monitors drug release from controlled release products. Up to this date, there has been no critical assessment of any methods to determine the release of formulation from the EMID. Therefore, a pharmaceutical acceptable test procedure using the USP paddle method was evaluated in comparison with the simple physical measure used above.

4.2.6.1 Preparation of formulations used *in vitro* drug release method

The drug/gel formulations used in the drug dissolution *in vitro* release method comprised a gel comprising dexamethasone (0.5 mg/mL) dissolved in 2% HPMC in a 10% v/v of ethanol/water continuous phase.

The gel was prepared by dissolving 0.15 g dexamethasone in 30 mL of ethanol (SDA, Mobil, NZ). Next, 270 mL of water was added while stirring vigorously using a blade mixer. Then 6.0 g of HPMC was added slowly to the mixture and stirred for 7 to 8 hours. This formulation was used as the payload for series of EMIDs in the dissolution method.

4.2.6.2 Establishment of *in vitro* drug release method

The *in vitro* release from an electronic insert was conducted using a Hanson Dissolution Testing Apparatus (Section 3.2.2). The paddle method with some modifications was used (Figure 4.12 and Figure 4.13). The method involved a 1 litre nominal capacity flask that contained the receptor phase (500 mL of degassed 10% alcohol/pH 5.0 phosphate buffer). The 10% of the alcohol in the release media composition was to achieve sink conditions for the drug solubility. Experiments were conducted at 39ºC with the receptor phase stirred at 100 rpm using a paddle stirrer positioned 25 mm from the bottom of the flask. A specially designed lid was manufactured to hold the EMID in the same position on each run (Figure 4.12).

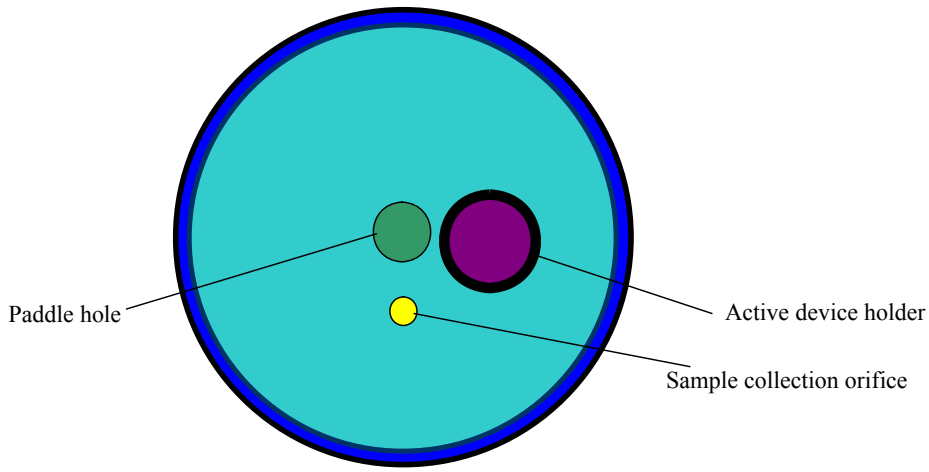


Figure 4.12. Lid designed to hold the EMID in the same position on each run.

For this study and for comparison with other measurements, all EMIDs were made with PP barrels coated with tinfoil. Prior to mounting the insert into the holder of the lid, the wings of the EMID were cut off. The outlet of the insert was initially positioned 60 mm from the bottom of the flask (fully immersed in the release medium).

The final physical set-up is shown in Figure 4.13.

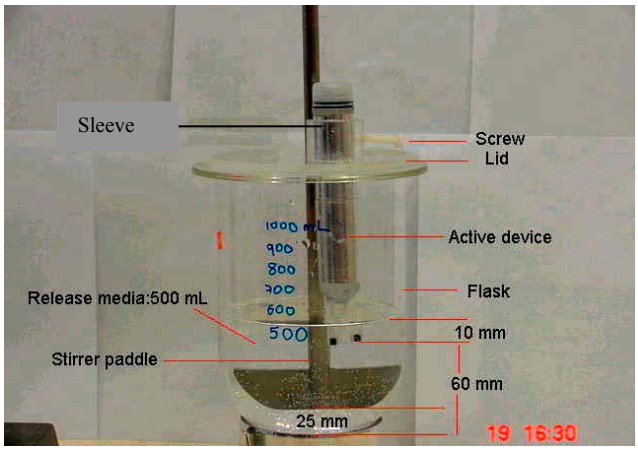


Figure 4.13. The final experimental set-up for the *in vitro drug* release test.

To perform a run 30 g of the dexamethasone formulation was loaded into each of six replicate PP EMIDs covered in tinfoil. A resistor (4.7 Ω) was used to control gas production in each. This resistor value was chosen since it resulted in full expulsion of the EMID contents within 4-5 hours, thereby ensuring the test could be completed in a working day. Initially the samples were taken over 5 hours, but then routinely taken at 0, 0.5, 1.0, 1.5, 2.0, and 3.0 hours. This represents over 70% of the drug release from the EMID and yet avoids any complication which might result from accumulation of drug around the outlet orifice. A 1.0 mL sample was taken at each time point and analysed for drug concentration using a UV spectrophotometer monitored at 242 nm. The analytical UV method is previously described in Chapter Three (Section 3.2.3.1).

For the initial 5 hour tests, the cumulative amount of drug dispensed from the EMIDs versus time was plotted between 0 and 5 hours, to examine the rate of

release profile (slope). This showed a curved end of the release profile due to the piston reaching the end of the EMID and causing a full exhaustion of the material contained in EMID Figure 4.14.

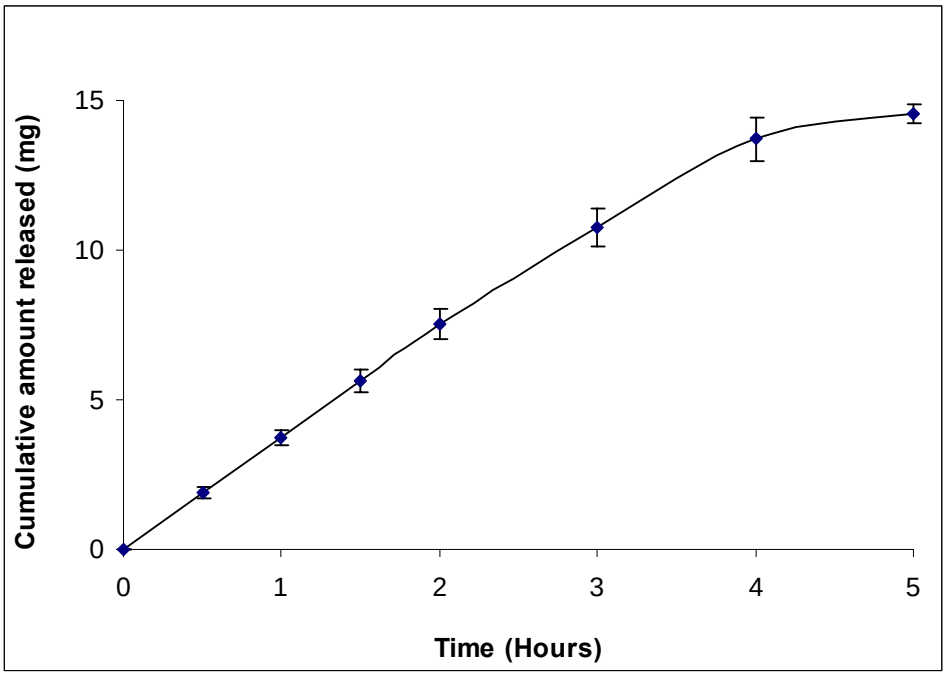


Figure 4.14. Extended run up to EMID exhaustion using the *in vitro* drug release method (Mean ± SD, n=6).

As is clearly seen in Figure 4.14, the amount of the drug dispensed at 4 and 5 hours was very similar (13.7 ± 0.7 mg and 14.5 ± 0.3 mg) and the rate of release profile reached plateau values. This was lowering the slope values (tables of Appendix-J) and therefore, the last two sampling points (4 and 5 hours) were omitted from further work.

The cumulative amount of drug dispensed from the EMIDs versus time was plotted between 0 and 3.0 hours, to determine the slope and linearity of release (R^2). The mean slope of six replicates and coefficient correlation were 3.6 mg/Hour and 0.998, respectively.

The rate of release of drug from the EMID can be calculated from this data, knowing the known concentration of drug in the payload. For instance, 3.6 mg of dexamethasone was dispensed in an hour from the payload of 0.5mg/mL drug

concentration. This indicates 7.2 mL/hour for the release rate from the EMID (e.g., the slope value divided by the drug concentration in the payload).

4.2.6.3 Effect of external and internal parameters on release rate of the EMID

The effect of stirring speed, paddle position, temperature, pH of the release media, alcohol strength and volume of the release media on drug release from the PP EMID covered in tinfoil was investigated. The vertical position of the EMID could be varied and in most cases the outlet orifice was well immersed in the release medium (Figure 4.13) but in other cases was just touching or staying outside in the release medium. In addition, the effect of the EMID outlet orifice position in the release medium was also investigated. Table 4.5 shows the variables and the values used in the study.

Table 4.5. The parameters and their ranges used *in vitro* drug release method.

Parameter	Tested value
Paddle distance from the bottom of the flask (mm)	10
	25
Position of EMID relative to the release media (mm)	Immersed
	Touching
	Outside
Temperature (ºC)	35
	39
Degassing the dissolution medium	Not degassed
	Degassed
pH of the release media	1.60
	5.0
	7.4
Composition of the release media (%alcohol)	25
	50
Stirring speed (rpm)	50
	150
Volume (mL)	500
	750
	1000

4.2.6.3.1 Data analysis

All data were analysed as described above by using linear regression and conversion from cumulative amount to an absolute release rate. The release rate results obtained from all parameters were statistically compared using a single ANOVA variance method.

4.2.6.4 Results and discussion

4.2.6.4.1 Effect of environmental parameters on release rate

Zero-order release rates from the EMID determined under various external and internal parameters in the *in vitro* drug release method are shown in Table 4.6. In this study there were no EMID failures.

Table 4.6. Results for the different variables investigated *in vitro* drug release method (Mean±SD, N=6).

Parameter	Value	Average rate of release (mL/hour ± SD
Paddle distance from the bottom of the flask (mm)	10	7.3 ± 0.3
	25	7.2± 0.3
Position of EMID relative to the release media (mm)	Immersed	7.3 ± 0.3
	Touching	7.3 ±0.3
	Outside	6.9 ± 0.4
Temperature (ºC)	35	7.2 ± 0.5
	39	7.4 ± 0.5
Degassing	Degassed	6.9 ± 0.4
	Not degassed	7.6 ± 0.7
pH of the release media	1.60	7.8 ± 0.7
	5.0	7.3 ± 0.3
	7.4	7.2 ± 0.2
Composition of the release media (%alcohol)	25% SDA	7.7 ± 0.5
	50% SDA	7.5 ± 0.4
Stirring speed (rpm)	50	7.2 ± 0.3
	150	7.2 ± 0.5
Volume (mL)	500	7.0 ± 0.3
	750	7.3 ± 0.4
	1000	7.3 ± 0.2

There are no statistically significant differences among any of the tested parameters. This indicates that the rate limiting step factor in the EMID was the

rate of gas production (i.e., driving mechanism) which is in turn controlled by the rate of gas (oxygen and/or hydrogen) produced (see Figure 4.8) during electrolysis which is proportional to the applied current (Bunt *et al*., 1998).

Since gas production is independent of external factors, the release rate from the insert also remains independent of external factors.

The *in vitro* drug release method was complex and involved analytical equipment. Previously simpler methods (weight loss and piston travel) were used to measure the release rate of EMIDs. It is important to demonstrate that the simpler methods gave results consistent with the drug release method. Therefore, in addition to the *in vitro* release method, four other methods were investigated.

4.3 Other *in vitro* release tests for EMID

The four alternative methods included assessing weight loss, weight dispensed, piston and rod travel.

4.3.1 Preparation of payload used

The formulation was the same as for the *in vitro* drug release method but with the exclusion of drug and alcohol content. The payload was a 2% w/v HPMC gel in water (Section 4.2.6.1).

4.3.2 Establishment of the other methods

The release rate from PP EMID's covered in tinfoil containing 4.7 Ω resistors was monitored with each of the above methods. In each case, the EMID was loaded with 30 g of 2% HPMC gel and experiments were conducted at 40ºC/75%RH in an oven (Contherm Cooled Incubators, Contherm Scientific Ltd, New Zealand). The release of gel monitored using the different methods at selected time points (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 hours) by measuring the cumulative weight loss (g), amount released (g), piston movement (cm) or rod travel distance (cm). The amount released or distance moved versus time (hours) was plotted and converted

to release rate using appropriate parameters such as the payload density and physical dimensions of the EMID to determine the rate of release. For instance, to allow comparison between these four methods and *in vitro* drug release method, all release rates were expressed in the same units (slopes were expressed in mL/Hour). Therefore, the release rates for weight loss and weight dispensed were converted from g/hour to mL/hour by multiplying the density of the 2% HPMC gel (1.02 g/mL) whereas, for the piston travel and rod travel methods, the rates were converted from cm/hour to mL/hour by multiplying by the cross sectional area of the EMID (3.14 cm^2).

4.3.2.1 Methods

4.3.2.1.1 Weight loss

Weight loss involves the measurement of the weight loss of the EMID over time.

4.3.2.1.2 Piston travel

This involved the measurement of the piston position relative to a fixed point within the EMID over time.

In order to evaluate piston position along the barrel a very small window was cut along the length of the EMID in the tinfoil to allow the piston to be seen without significantly compromising the gas diffusion and its travel distance measured (Figure 4.15).

Figure 4.15. PP EMID covered in tinfoil showing a window along the length of the EMID in the tinfoil to allow the piston to be seen.

4.3.2.1.3 Weight dispensed

This involves regular collection and weighing of gel dispensed from the EMIDs. EMIDs were arranged to lie horizontally over the edge of collection bottles in which the dispensed gel was collected and weighed.

4.3.2.1.4 Rod travel

This involved determination of the distance of travel of a rod that had been inserted into the barrel through the release orifice and which was touching the piston. The rod was protruding through the release orifice while maintaining contact with the piston. The protruded length of the rod was measured relative to the orifice of the EMID over time.

4.3.2.2 Results and discussion

Each method gave linear (≥0.998) dependence of release versus time (Figure 4.16-Figure 4.17) and provided similar release rate values (Table 4.7).

An example of typical weight loss and weight dispensed release profile is shown in Figure 4.16. The mean slope for six replicates for each case was 6.7 and 6.5 g/hour for weight loss and weight dispensed, respectively.

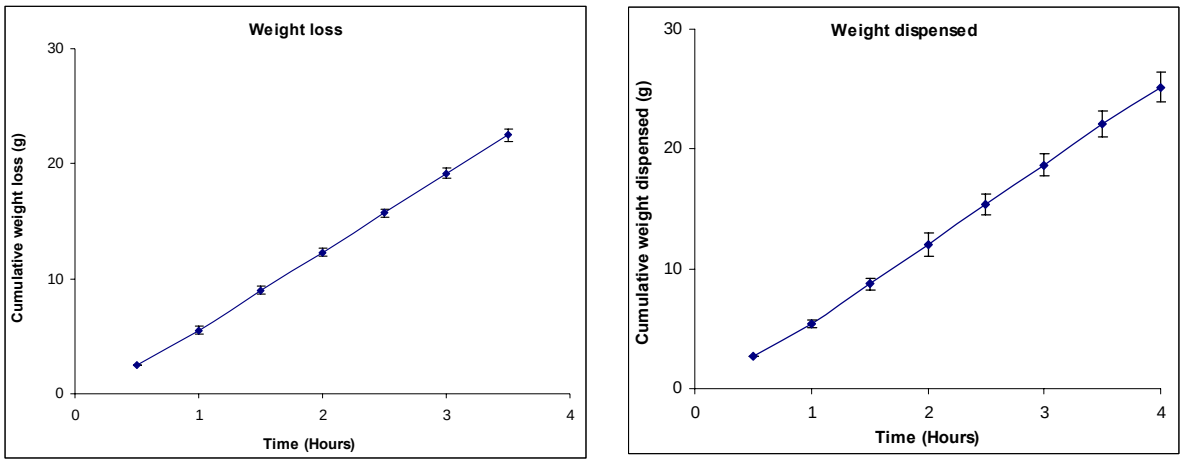


Figure 4.16. Typical cumulative weight loss and weight dispensed *in vitro* release test of EMID (Mean ± SD, n=6).

An example of typical piston and rod travel release profile is shown in Figure 4.17. Mean slopes for six replicates were 2.20 and 2.05 cm/hour for piston and rod travel, respectively.

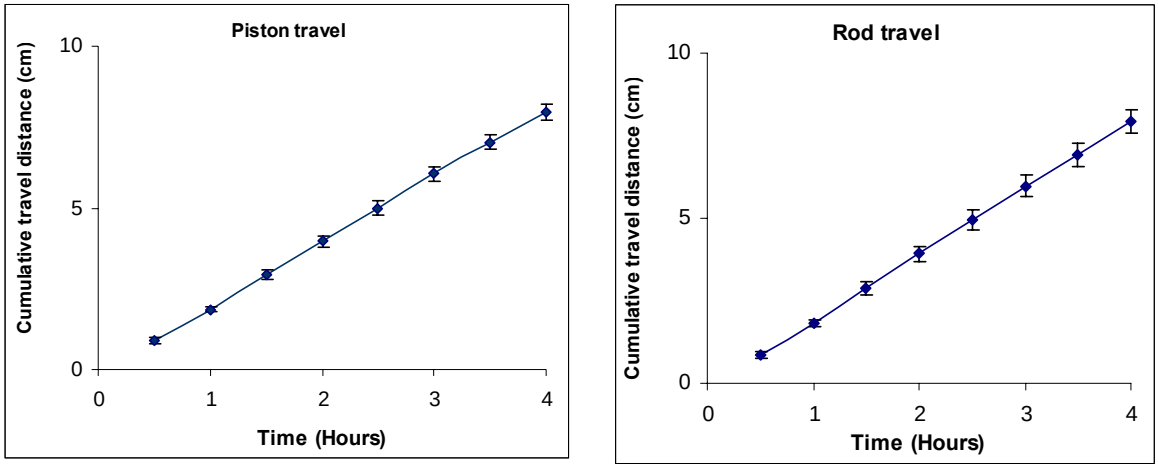


Figure 4.17. Typical cumulative piston and rod travel *in vitro* release test of EMID (Mean ±SD, n=6).

These alternative release assessment methods provided similar estimates to the *in vitro* drug dissolution for the release rate of the formulation from the EMID (each method was shown to produce results that correlated well with the *in vitro* dissolution method) (Table 4.7).

Table 4.7. Summarised rate of release (mL/Hour) of EMID by different methods.

Replicate #	***In vitro* drug dissolution***	**Weight loss**	**Weight dispensed**	**Piston travel**	**Rod travel**
1	6.3	6.1	6.3	6.8	6.0
2	7.0	6.7	7.0	6.7	6.7
3	7.2	7.2	6.6	6.2	5.9
4	7.0	7.0	5.9	6.3	6.3
5	7.1	6.6	7.0	6.2	6.7
6	6.5	6.8	6.1	6.7	6.8
Release rate (mL/Hour, Mean ±SD)	6.9 ± 0.4	6.7 ± 0.4	6.5 ± 0.5	6.5 ± 0.3	6.4 ± 0.4
Statistical analysis	**P = 0.23**				

*EMID was positioned outside of the release media.

The statistical comparisons of the release rate of HPMC gel from the EMID demonstrated that there was no significant difference among the different methods used (P = 0.23) (Table 4.7). Each method provides a reliable estimate of the release rate of the EMID. The way in which the EMID works means that there is always an "initiation" period, during which the battery function is changing from gas (oxygen) consumption to gas (hydrogen) generation and also it takes a while for the gas pressure to build up to move the piston. During this time, graphs of release rate over time will exhibit some form of a lag phase (initiation phase). In order to reduce the demand on the *in vitro* drug dissolution apparatus, and to reduce as far as possible the numbers of samples need for further analysis, all EMIDs tested in this way were subjected to a short period of pre-observation, before being inserted in to the bath. In this case, once it was established that material was being extruded through the orifice, the excess material was wiped from the surface and the EMID place into the bath. It was at this point in time that a zero reference point was established. With all the alternative methods, zero time was taken as the point at which the gas chamber was sealed. The end result is that the *in vitro* drug dissolution data points clearly progress from the origin, whereas graphical data for the other methods all demonstrate a lag phase, which was ignored in the calculations. Typical example of curve showing a lag phase for the other methods is shown in Appendix-K. All five methods developed were compared in terms of ease of use, cost, time, automation capability, need for analytical equipment, reproducibility, limitations and need for consumables. The results and comparison are shown in Table 4.8.

Table 4.8. Comparison between different *in vitro* release methods for the EMID.

Criteria	**Method**				
	***In vitro* drug dissolution**	**Weight loss**	**Weight dispensed**	**Piston travel**	**Rod travel**
Ease of use	Many set up steps	Easy			
Need for analytical equipment	UV spec	Balance		Calliper	
Cost	Expensive	Low			
Time	Extra time for sample analysis	Real time			
Automation capability	Yes	Possibly	Yes	Possible	
Reproducibility	Very good			Good	
Limitation	None			EMID must be clear	None
Consumables	drug and release media	N/A			

All methods provided a similar estimate for the rate of formulation release. However, in terms of operation and set-up, the *in vitro* drug dissolution method was relatively complex and involved expensive analytical equipment. The remaining four methods were much simpler to perform and did not involve complicated set-ups or expensive equipment. The conclusion that can be drawn from this study was that release rate determinations for the EMID did not need to be performed using the complex and expensive *in vitro* drug dissolution equipment. Reliable estimates of the release rate could be determined by much simpler and less expensive methods. The simplest, cheapest and easiest method proved to be weight loss (Table 4.8).

4.4 Physical characterisation of the EMID manufactured from HDPE

Although the early work of this thesis was based on the EMID manufactured from PP, observation of the PP polymer highlighted the need to replace this with an alternative plastic material. HDPE material was chosen for further study because of its lower gas diffusion properties and near linear release rate of the formulation over time (Section 4.2.5). The complete description of the EMID (e.g., retention wings and drive mechanism) manufactured from PP EMID (original insert) was previously described in Section 4.2. As well as changing the plastic, the opportunity was taken to modify the wing design shape to rounded wing tips (Figure 4.18) because the EMID manufactured from PP caused severe damage on vaginal mucosa membranes. This was attributed to abrasions caused by not having rounded end wing tips for the PP EMID.

The EMID insert manufactured from HDPE was manufactured using rounded wing tips (Figure 4.18).

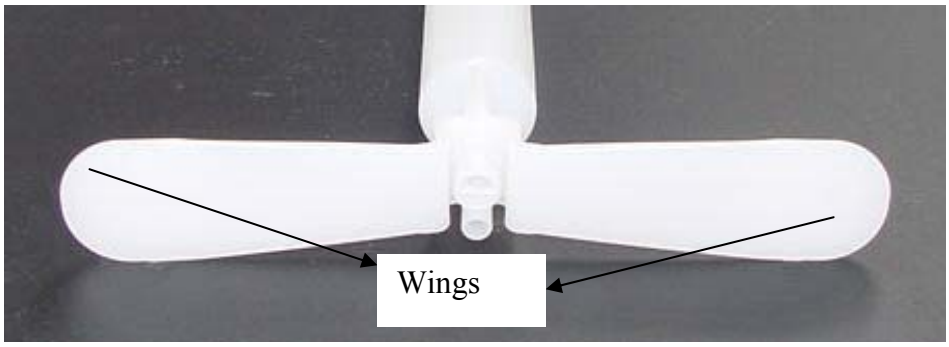


Figure 4.18. Close up of wing shape of EMID manufactured from HDPE.

To compare PP and HDPE EMIDs, the original 2% HPMC formulation was used as a payload (Section 4.3.1). Additionally, in the HDPE studies, the opportunity was taken to explore the effect of varying the HPMC concentration in the payload.

4.4.1 Methods

4.4.1.1 Retention mechanism and site safety determination

Since a different plastic (HDPE) from PP was used to make this EMID, *in vivo* characterisation was carried out to investigate the retention mechanism and safety at the site of administration. This involved monitoring whether the EMID remains in the animal during the length of treatment without damaging the vaginal mucosa membranes.

The EMIDs were placed into the vagina of 12 cattle and monitored for 10 days. The retention rate and site safety of the EMIDs were monitored during the treatment. Cows underwent a visual inspection of the vagina on days 5 after removal of the EMIDs. If there was no damage on the mucosa, they were reinserted. On day 10, all animals underwent another visual vaginal inspection for any mucosal damage.

The EMID manufactured from HDPE was retained reasonably well (at the half way of the length of the treatment) but caused a high incidence of vaginal damage (Section 4.5.2.1.1). This led to a modification of the EMID retention mechanism, which involved cutting the wings off, and attaching the EMID to a commercially

available CIDR™-B as shown in Figure 4.19. These resultant devices were observed in 30 cattle over 16 days.

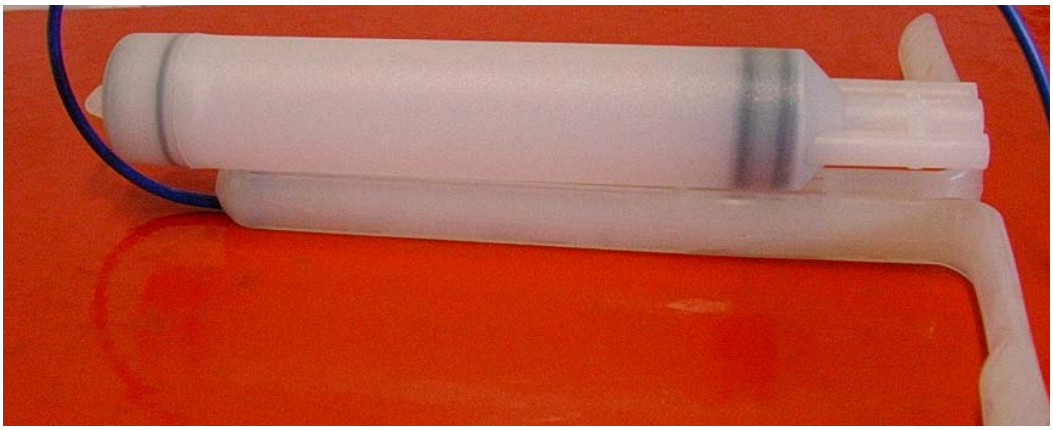


Figure 4.19. Picture of the EMID manufactured from HDPE attached to a commercially available CIDR™-B.

Due to a poor retention rate (Section 5.4.2.1.2) of the modified HDPE EMID strapped onto a loaded CIDR™-B, a further modification of the EMID retention mechanism was made. This involved attaching blank PCL wings (Supplied by InterAg) to the EMID after removal of the original wings (Figure 4.20). The wings were manually attached to the EMID using epoxy resin. The retention rate and site safety of this design were tested in 12 cows for 10 days.

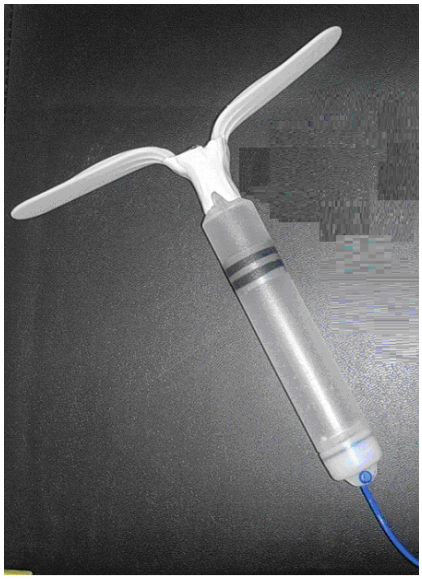


Figure 4.20. HDPE EMID with attached PCL wings.

EMIDs containing 2% HPMC gel were fitted with a 750 Ω resistor (this resistor was expected to provide a complete release of the payload material over 10 days). The piston material used in the study was the silicone double O-ring type. Cows underwent a visual inspection of the vagina after 5 days involving removal of the EMIDs. EMIDs were reinserted into the cows after examination.

4.4.1.2 Preparation of payload formulations

Formulations of 1, 2 and 3% w/v HPMC were prepared by weighing appropriate amounts of HPMC (9, 18 and 27 g), adding slowly to 900 mL water in a beaker and leaving to be stirred for 7 to 8 hours at room temperature using a star blade mixer (Heidolph, Nicholas Watson Victor Ltd, Germany).

In addition, three 4% w/v HPMC gel formulations containing 5 mg/mL, 15 mg/mL and 25 mg/mL progesterone were used in a drug release evaluation study. Each of these formulations was manufactured by first mixing a known amount of progesterone and water in a beaker using the star blade mixer and the slow addition of the HPMC until a uniform suspension product was achieved.

All formulations were left to stand at room temperature for two days prior to loading EMIDs to ensure complete removal of any gas bubbles from the payload. The gas bubbles were formed during the manufacturing of the formulation.

4.4.1.3 Effect of different viscosity and different orifice size on release profiles of the EMID

It is apparent that cows move during their grazing time. They acquire different postures such as standing, running, sitting, jumping, mounting, etc. All these activities create a movement, which may disturb and shake the EMID payload and thus disturb the release profile. In addition, it is expected that the viscosity of the payload and the size of the outlet orifice of the EMID may affect the release profile. For instance, a large orifice EMID containing water (as a payload) is more likely to lose the payload compared to a high viscosity payload and small orifice EMID, when both the EMIDs are introduced by similar movements. The modified

EMID manufactured from HDPE attached to a commercially available CIDR™-B as shown in Figure 4.19, was used.

The aim was to investigate the effect of orifice diameter and gel viscosity (%HPMC) upon *in vivo* release rate of the payload.

Using the modified insert, *in vivo* release rate was determined by administering EMID's into the vaginas of cows and removing them at specified (daily) time points. Upon removal, the EMIDs were washed, dried and then weighed. In addition, the piston position was also measured. The EMID's were then re-placed into the vagina of the same cow where they remained until due for removal.

In practice, the recovery times were staggered to accommodate animal welfare considerations. On removal, the EMIDs were cleaned and inspected. The inspection process included weighing and noting piston position prior to re-insertion. The EMID's were removed for analysis then re-inserted 5 times over 16 days, with all EMID's being removed on day 16.

The interaction between viscosity and orifice surface area on *in vivo* release rate was determined. HDPE EMIDs contained either water or HPMC gel at 1, 2 or 3% and were fitted with a 1000 Ω resistor (this resistor is expected to provide a complete release of the payload material over 15 days). Release rate was monitored for 16 days. The piston material used in this study was the silicone double O-ring type. Orifice surface areas were altered by changing the diameter of the outlet orifice of the large reservoir of the EMID (Figure 4.21). This was achieved by either removing the central cross-likes structure or inserting a sleeve into the orifice. The resultant orifice surface areas were then 0.354 (large) or 0.194 (small) cm^2 compared to the original 0.22 cm^2 (medium). Each of the four formulations (water, 1, 2 and 3%HPMC) were tested using these orifice sizes. All combinations were tested in duplicate for water and 3%HPMC whereas triplicate measurements were used for 1 and 2%HPMC. Any technology failures and the causes were carefully observed and recorded.

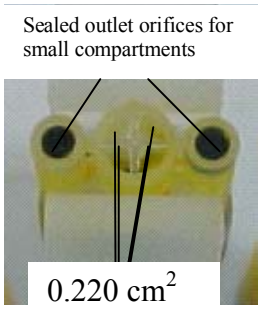


Figure 4.21. Outlet orifice area of a normal EMID.

4.4.1.4 Drug release evaluation (progesterone dose titration study)

Ovariectomised (ovex), non lactating cows aged between 3 to 4 years were enrolled from a herd of 14 animals that were located at Dexcel, Hamilton, NZ. The cows weighed between 500 and 700 kg.

The three formulations containing 5 mg/mL, 15 mg/mL and 25 mg/mL progesterone were used in the study. Each formulation was tested in quadruplicate EMIDs manufactured from HDPE which had attached to blank PCLwings (Figure 4.20) and containing 30 g of the formulation. This resulted in three treatments each with a total expected progesterone load of 150, 450 and 750 mg per EMID. All EMIDs were fitted with a 750 Ω resistor. These were administered into the vagina of 12 cattle for 8 days. The progesterone plasma concentration of the treated ovex cows was monitored over time.

Plasma samples were taken at 1, 2, 3, 4, 5, 6, 7 and 8 days and 24 hours post removal. The blood samples were collected in evacuated tubes containing heparin. Plasma was separated by centrifugation (1500 x g for 15 min) and stored at or below -12^{o}C for subsequent progesterone analysis. Concentrations of progesterone in plasma were determined by direct radioimmunoassay using a commercial solid phase I^{125} label (Coat-a-Count, DPC, Los Angeles, California, USA).

4.4.2 Results and discussion

4.4.2.1 Retention rate and safety determination of the EMID

4.4.2.1.1 HDPE EMID

The EMID manufactured from HDPE was retained reasonably (80%) well but caused a considerable of vaginal damage. This resulted in a decision to terminate the study on day 5.

4.4.2.1.2 Modified insert strapped with CIDR™-B

The animal experiment retention rate of the EMID was poor throughout the study period. Only 13 out of 30 EMIDs were retained throughout the study period. The poor retention was due to a number of factors, some of which might be due to mechanical complication such as increased size and weight (i.e., too bulky) of the EMID strapped with CIDR™-B. Therefore, the second round of this study was not conducted.

4.4.2.1.3 Second modification of EMID with attached PCL wings

Retention at day 5 was 100% and all vaginal inspections found no observations to report. By day 10, four of the EMIDs had lost their tails. The EMIDs that did not lose their tails showed little reaction by day 10, of the EMIDs that had lost their tail there were minor irritations observed.

Subsequently, during “drug evaluation trial”, poor retention was again observed using the HDPE EMIDs attached to PCL wings. In this case, it was recognised that the wings were in a “Y” shape, not the “T” shape used previously. Only six cows retained the EMIDs during the treatment of progesterone dose titration.

The retention rate data for each EMID over an eight day insertion period is shown in Table 4.9.

Table 4.9. The retention rate for each EMID dose in ovex cows.

EMID insert dose (mg/mL)	Number inserted	Number retained	Retention rate
5	4	1	25
15	4	2	50
25	4	3	75

The lower retention rate of this trial compared to the previous experiment indicates attaching the PCL wings to the EMID was not reproducible between the sequencial trials. Some explanation of this could be some possibility due to small changes in wing orientation (changes from the original T-shape to a Y shape), leading to the EMID's beocoming dislodged from the animal. It appears that the angle of the wings to the body must be maintained close to 90º, rather than expanding to 120° as was seen with a number of the EMID's in the dose titration work.

4.4.2.2 *In vivo* release rates using different viscosities and orifice sizes

Only 16 EMIDs were retained in the cows for a sufficient period to collect EMID weight and piston position data for further analysis. A plot of the EMID's cumulative mass against time (Figure 4.22) shows that EMIDs followed the same trend and achieved a similar mass loss. However, the low viscosity payload EMIDs with a large or medium orifice resulted in loss of the payload material (Figure 4.22a). This loss occurred within the first 4 days of the insertion period.

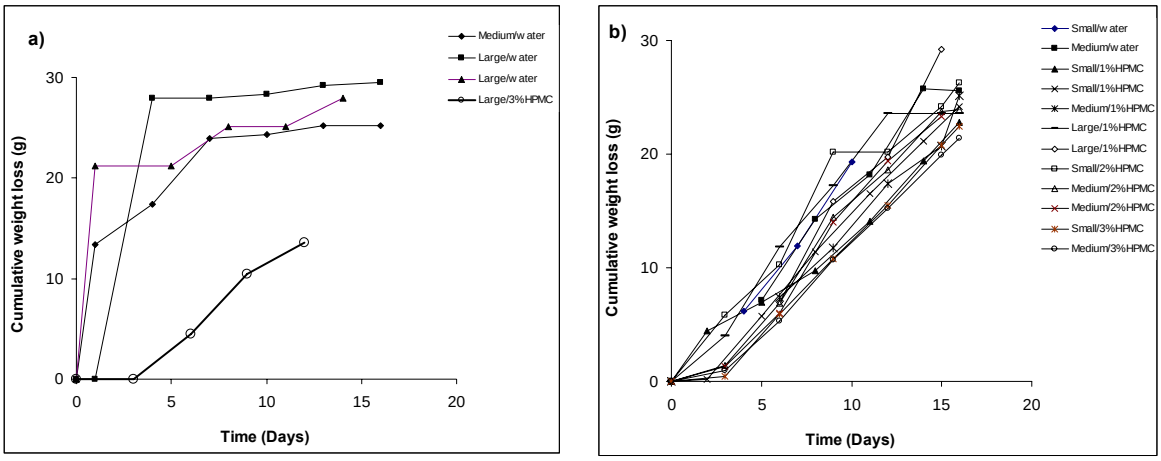


Figure 4.22. Cumulative weight loss of the EMIDs during the insertion period of different viscosities and orifice sizes.

Plots of cumulative piston travel distance versus time show different sensitivities of the measuring systems compared to the weight loss method. Figure 4.23 shows most of the EMIDs followed similar trends and achieved a similar piston travel distance. The daily piston travel rate (or weight loss) was clearly not linear for the whole insertion period. The piston travel data suggests a fast initial rate, followed by a slower and possibly steady state rate. On the other hand, the weight loss data may indicate an initial lag followed by a more steady rate.

The piston travel versus time profile demonstrates an independence of the outlet orifice and the viscosity of the payload on the release rate. The piston travel represents more the driving mechanism of the EMID but it cannot reflect whether the EMID lost its payload. The weight loss method may be used reliably to measure the performance of the EMIDs when low viscosity and large orifice size EMIDs are used, since, this method directly measures what contains the EMID at specified time point.

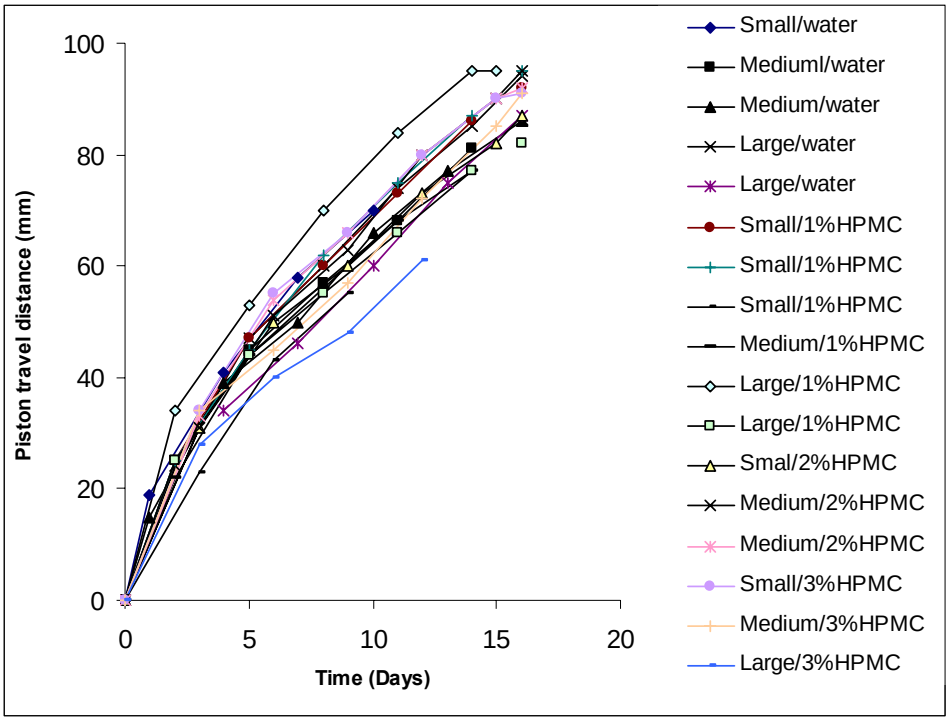


Figure 4.23. Individual piston position within the EMIDs during the insertion period of different viscosities and orifice sizes.

Because of the design limitation (relating to animal ethics issue) together with the non-linearity exhibited by both the weight loss and piston travel methods, there are insufficient data for reasonable analysis.

4.4.2.3 Progesterone dose titration study

Table 4.10 shows the results of the progesterone concentration in plasma at steady state for the treated cows. The progesterone concentration over time shows that each of the three doses produced different responses in the plasma profiles from the ovex cows. It was expected that higher doses would produce higher plasma progesterone responses (Table 4.10). The individual cow progesterone plasma profiles over time are presented in figures of Appendix-L.

Table 4.10. Concentration of progesterone in plasma at steady state for the treated cows for each of the 3 doses used (Mean ± SD).

Progesterone plasma concentration (ng/mL)	**5mg/mL**	**15 mg/mL**	**25 mg/mL**
	Cow 1) 0.38 ± 0.20, Cow 2) 1.42 ± 0.10	Cow 1) 2.03 ± 0.71	Cow 1) 3.7* ± 0.81 Cow 2) 3.30 ± 0.98 Cow 3) 2.3 ± 0.84

*on day 6 outlier point was excluded.

None of the doses showed what is known as a burst release (a characteristic of initial progesterone CIDR™-B release profile) phenomenon.

It is expected that progesterone suspension is dispensed from the EMID outlet orifice into the vaginal lumen of the cow. Then, progesterone partitions from the dispensed material into the vaginal fluids and from there into the mucosa, then the submucosa to the blood stream. The larger the area exposed to the progesterone absorption, the larger the amount of progesterone absorbed and permeated through the membrane would be. Therefore, it seems that the limiting factor of plasma progesterone levels is distribution of the suspension in the vaginal lumen solubility of progesterone. With this EMID, unlike a standard CIDR™-B, the drug is released only at one end. Therefore, only a small area of mucosa may have been exposed to an unevenly distributed progesterone suspension.

In addition, the dose with the higher concentration (25 mg/mL) would have a higher concentration gradient of progesterone between the dispensed material and the mucosa membrane in the vaginal lumen. This higher concentration would favour a high permeation of progesterone compared to lower dose progesterone (5 or 15 mg/mL). Each of the three doses showed sharp decline of progesterone plasma levels after 24 hours of EMID removal. This is explained by the short biological half-life of progesterone (Rathbone & Macmillan, 2004).

25 mg/mL doses led to plasma progesterone fluctuating between 2-5 ng/mL during the treatment except for one occasion. Any progesterone blood levels greater than 2 ng/mL can induce oestrous synchronisation among the dairy cows after the removal of the device (Rathbone *et al*., 1998). The EMID demonstrated a sustained release of progesterone up to 8 days. However, the retention mechanism of the EMID needs to be improved.

5 Stability testing

5.1 Introduction

Stability testing of all pharmaceutical products is crucial in determining their shelf lives. An active pharmaceutical ingredient must remain stable during handling, formulation, storage and administration of the drug product. The purpose of stability testing, as described by the International Conference on Harmonisation (ICH, 2003), is to produce evidence on how the quality of a drug product changes with time under the influence of a variety of environmental parameters such as temperature, humidity and light, and enables recommended storage conditions, retest periods and shelf lives to be established.

Various formulations used in the EMID, namely the progesterone suspension, oestradiol-17β tablets and a blended cloprostenol powder which have been previously developed (Bunt *et al.,* 2001; Bunt *et al.,* 1999; Burke *et al*., 1999, respectively) were manufactured and exposed to elevated temperature and humidity conditions (40°C/75%RH) for 4 weeks, during which time, the chemical and physical stability of each of the various formulations were determined. Stability-indicating assays were developed for each compound incorporated in the EMID (Section 3.4.). All dosage forms were evaluated by assessing their chemical and physical stability and the results are reported in this chapter. The analytical method should simultaneously identify and quantify the drug of interest as well as any degraded products. The appearance of any new peaks present on HPLC chromatograms, which were not originally apparent in freshly made samples were to be sought as these would reflect the presence of degradation products from the stored progesterone suspension, oestradiol-17β tablets and cloprostenol powder analysed by HPLC stability indicating assays.

In addition, the effect of 40°C/75%RH on battery functionality in the EMID was assessed.

5.2 Methods

5.2.1 Manufacture and assessment of progesterone suspension

5.2.1.1 Manufacture of progesterone suspension

The formulation composition of the suspension investigated in this stability trial is listed in Table 5.1.

Table 5.1. Composition of progesterone suspension.

Ingredients	Content % (w/w)
Progesterone USP	2.5
Hydroxypropyl methyl cellulose (HPMC) NF (60 SH 4000) Metolose	4.0
Distilled water	93.5
Total	100.0

The formulation was manufactured by first mixing the progesterone and distilled water in a beaker using a star blade mixer (Heidolph, Nicholas Watson Victor Ltd, Germany) at 1000 rpm speed followed by the slow addition of HPMC until a uniform (lacking of any clear lumps in the suspension) product was achieved. The resultant formulation was left to stand at room temperature for two days to ensure complete removal of any gas bubbles from the suspension. A thin foam layer is formed at the top of the suspension due to gas bubbles moving to the top of the suspension. This layer was removed using a spatula, prior to assessment of the suspension.

5.2.1.2 Assessment of progesterone suspension

A pharmaceutical suspension is a coarse dispersion in which insoluble particles are dispersed in an aqueous medium (Attwood, 2002). A measurement of the rate of sedimentation of a suspension, the final volume or height of the sediment and ease of its redispersion are used to assess the physical stability of a suspension (Billany, 2002).

A stable suspension is one in which the final volume or height of the sediment remains constant and the dispersed drugs retain their initial form and remain uniformly distributed for at least the period between shaking the container and removing a required amount considering that progesterone suspension is a finely distributed and dispersed as insoluble powder progesterone solid particles in 4% w/w HPMC gel in distilled water. For the chemical stability study, the drug was extracted from the matrix and analysed using the validated HPLC indicating assay.

5.2.1.2.1 Drug content uniformity

5.2.1.2.1.1. Selection of extraction solvent

Drug content was quantified by HPLC after extraction of the progesterone from the suspension formulation. Three extraction solvents were investigated for their ability to extract progesterone from the suspension formulation: alcohol, methanol/water (6:4) solution and 5% HPβCD in distilled water.

In order to determine the ability of the different solvents to extract progesterone from the formulation, a known quantity of progesterone was added to the formulation vehicle. Spiked progesterone samples were prepared by incorporating a known amount of progesterone (0.1380 g) into 5.3820 g of formulation base.

The total amount of formulation was quantitatively transferred into a 200 mL volumetric flask. The mixture was stirred using a magnetic stirrer flea (50 x 8 mm Magnetic Teflon Stirring Bar, Biolab, New Zealand) for 30 minutes. The mixture was subsequently subjected to ultrasound (Soniclean Pty. Ltd, Australia) for a further 30 minutes. The stirrer was removed and the solution was made up to 200 mL with the extraction solvent. Finally, the solution was filtered through a 0.20 µm nylon filter and the filtrate injected onto the HPLC column for analysis.

The efficiency of the extraction process was determined by calculating the percent bias and the percentage recovery using Equation 5.1 andEquation 5.2, respectively (where C_t and C_a are theoretical and actual concentrations, respectively) where the theoretical concentration of this extract was estimated as 690 µg/mL.

$$\% \text{ Bias} = \frac{C_t - C_a}{C_a} \times 100$$ **Equation 5.1.**

$$\% \text{ Recovery} = \frac{C_a}{C_t} \times 100$$ **Equation 5.2.**

The extraction efficiency was determined by analysis of spiked samples in triplicate for each extraction solvent.

5.2.1.2.2 Appearance

The appearance of the suspension was assessed visually by noting any changes in colour and the presence of any sedimented particles.

5.2.1.2.3 Suspension physical stability

The physical stability of the progesterone suspension was assessed by pouring 50 mL of progesterone suspension into a 50 mL graduated measuring cylinder. The suspension was vigorously mixed prior to pouring. In order to prevent evaporation of the vehicle, the measuring cylinders were covered with parafilm.

The suspensions were observed for evidence of sedimentation on standing for 4 weeks at (40°C/75%RH). Sedimentation was assumed to have occurred, when after visual examination, sedimented particles were observed at the bottom of the cylinder with the subsequent appearance of clear a supernatant.

5.2.1.2.4 Manufacturing assessment

The reproducibility of the manufacturing process was assessed by examining two separate batches of product produced on the same day. In addition, the consistency of the manufactured product was also evaluated by taking samples of known weight (5.5200 g) from bulk batches of the formulation from the top, middle and bottom of that batch. These samples were analysed by HPLC as

previously described in Chapter Three. The extraction efficiency of extracted samples was calculated using Equations 5.1 and 5.2.

5.2.2 Manufacture and assessment of oestradiol-17β tablets

5.2.2.1 Manufacture of oestradiol-17β tablets

Table 5.2 documents the oestradiol-17β tablet formulation that was subjected to stability testing.

Table 5.2. Composition of oestradiol-17β tablet.

Ingredients	Content % (w/w)
Oestradiol-17β	2.5
γ-cyclodextrin	3.75
Lactose	8.75
Microcrystalline cellulose (MCC) PH 101	9.75
Magnesium stearate	0.25
Total weight (mg)	25

The oestradiol-17β and excipients were accurately weighed using a four decimal place balance (Sartorius Analytical Balance, Type BP110S, Biolab Scientific Ltd, NZ). The drug and excipients (with the exception of magnesium stearate) were added to a plastic bag and blended by shaking for 10 minutes. Magnesium stearate was added and the blend mixed for a further 3 minutes, following which, tablets were manufactured using a TDP Single Punch Tablet Press (STH®, Shanghai Tianhe Pharmaceutical Manufacturing Factory, China). A sample of the blend was retained for assessment of blend uniformity.

A single batch of tablets (2400 tablets) was manufactured. The tablets were separated and collected in lots of one hundred and allocated a sequential number (1 to 24). The humidity and temperature of the room environment during the manufacturing process were (62.3-65.0 %RH) and (16.5-16.8ºC) respectively. The tablets were stored in plastic bags, in a cupboard at room temperature until they were assessed.

5.2.2.2 Assessment of oestradiol-17β tablets

Like any other dosage form, tablets should meet a number of specifications regarding their chemical, physical and biological properties. Details of tests and specifications for these properties can be found in official monographs in the United States Pharmacopoeias (USP, 1994) and some of the qualities that a tablet should have include:

- the correct dose as labelled,
- a consistent elegant appearance, weight and size,
- release of drug content in a controlled and reproducible way,
- be free from any contaminants and micro-organisms that could cause any harm to the user/patient,
- sufficient mechanical strength to withstand fracture and erosion during handling,
- be chemically, physically and microbiologically stable during its lifetime.

The physical and chemical parameters that should be examined during characterisation testing of a normal tablet include friability, weight, thickness, hardness, disintegration, assay, drug content uniformity and tablet dissolution.

5.2.2.2.1 Drug content uniformity

A known amount (0.4500 g) of a placebo blend was spiked with a known amount of oestradiol-17β (0.0500 g) in a 10 mL test tube to produce a powder of equivalent weight to 20 tablets. This powder was then mixed for 10 minutes using a spatula. The powder blend was transferred into a 100 mL volumetric flask by rinsing the test tube with the extraction solvent (alcohol). The solution was subjected to ultrasound (Soniclean Pty. Ltd, Australia) for 40 minutes. After sonification, the solvent was made up to 100 mL with alcohol. The resultant solution was then passed through a 0.20 μm filter and analysed using the HPLC method. The theoretical concentration of the oestradiol-17β in the resultant solution was 500 μg/mL. Three replicate spiked samples were analysed on three different days.

The same experiment was repeated using 0.2250 g of blend placebo spiked with 0.0250 g of oestradiol-17β to produce a blend of the equivalent weight of 10 tablets. The blend was subjected to the same extraction procedure as described for the 20 tablet blend. The theoretical concentration of the oestradiol-17β in the resultant solution was 250 µg/mL and once again, three replicate spiked samples were analysed on three different days.

In addition, the extraction method was applied to the tablets manufactured as previously described to assess if the compaction process had any affect upon extraction efficiency. Twenty tablets of oestradiol-17β were weighed, completely crushed and subjected to the same extraction procedure described above. The theoretical concentration of the oestradiol-17β in the resultant solution was 500 µg/mL.

The extraction efficiency for each experiment was calculated using Equations 5.1 and 5.2.

5.2.2.2.2 Appearance

The appearance of the tablets was assessed visually by noting any changes in the colour of the tablet and the surface morphology (e.g., smoothness, pitting).

Tablets from a randomly chosen bag were evaluated to define the standard physical properties of the tablets.

5.2.2.2.3 Friability testing

Friability was determined using a Model FT440C Friability Tester with a digital counter (Key International, Inc., NJ, USA). Twenty tablets were accurately weighed and rotated for 100 drop cycles after which they were gently brushed to remove any dust from their surface and reweighed. The weight difference at the beginning and after testing was used to determine the friability of the tablets.

5.2.2.2.4 Tablet weight, thickness, diameter and hardness testing

The weight of each tablet was measured using a four decimal place analytical balance. The thickness of each tablet was measured using digital callipers (Mitutoyo, CD-6″ CS, Mitutoyo Corp, Japan). The diameter and hardness of each tablet were measured simultaneously using the Erweka tablet hardness tester (Erweka GmbH, TBH 210, Total Lab System Ltd, Germany).

5.2.2.2.5 Tablet disintegration

Tablet disintegration was determined using a QC-21 disintegration test station (Model 1620 USP disintegration baskets 65-620-001, 1000 mL glass beaker 91-030-036, acrylic water bath 64-700-131, Hanson Research Corporation, CA, USA). The disintegration station was operated at a temperature of 37ºC using 800 mL of water in the glass beaker. The time taken for each lot of tablets (N=6) to disintegrate was recorded. The time of which the last tablet disintegrates and falls through the mesh was taken as the end point of the disintegration test.

5.2.2.2.6 Dissolution Testing

Dissolution testing of oestradiol-17β tablets was conducted in a sealed 200 mL glass bottle to prevent evaporation. The dissolution method was developed by taking tablets from the randomly chosen bag and assessing the effect of sampling time, receptor phase composition, shaking rate and volume of the dissolution media on the percent of drug released from a tablet. The final test parameters were selected based on the results of these experiments. Because the need of smaller volumes of the release media to dissolve 2.5 mg of the active, the widely used of compendial apparatus was not considered to conduct the dissolution testing for oestradiol-17β tablets.

The final test conditions selected are listed in Table 5.3. The method involved 50 mL of 10% HPβCD in pH 7.4 PBS being added to the 200 mL glass bottle and equilibrated to 39ºC. The tablet was dropped into the bottom of the bottle at time zero and dissolution was conducted in a shaking water bath (Grant Shaking Water Bath, Grant OLS 200, Grant Instruments England) at 50 strokes/minute. A sample

of the dissolution media was taken after 30 minutes of shaking. The sample was filtered using a 0.20 μm nylon filter before HPLC analysis.

Table 5.3.Dissolution conditions selected for oestradiol-17β tablets.

Parameter	value
Volume (mL)	50
Release media composition	HPβCD: PBS at pH5.0(1:9)
Temperature (ºC)	39
Speed (spm)	50
Dissolution runt time (minutes)	30

5.2.2.2.7 Manufacturing assessment

The consistency of manufacturing was assessed by examining samples from each of the 24 bags of product that were separated out during manufacture. The tests performed were appearance, friability, weight, thickness, diameter, hardness, disintegration, dissolution and drug content of the oestradiol-17β tablets. The number of samples for each bag was 20 tablets for friability, weight, thickness, diameter and hardness tests. For the disintegration test there were 6 sample tablets per a bag. Every second bag had 6 tablets and every third bag had 5 tablets for the dissolution and the drug content tests, respectively. The results were used to calculate the mean value and standard deviation where appropriate.

5.2.3 Manufacture and assessment of cloprostenol powder

Thus far, a physicochemical description of cloprostenol has not previously covered in this thesis. Therefore, the following is a brief description and application of cloprostenol according to British Pharmacopoeia (1993). Cloprostenol sodium is a synthetic analogue of dinoprost (prostaglandin $F_{2\alpha}$) and it is used as a luteolytic agent in veterinary medicine. Cloprostenol is an almost white amorphous hygroscopic (absorbs moisture from atmosphere) powder. Cloprostenol is freely soluble in water, ethanol (96%), and methanol; it is practically insoluble in acetone. Cloprostenol should be protected from light and moisture as described in BP (Veterinary) 1993.

5.2.3.1 Manufacture of cloprostenol powder

Table 5.4 documents the cloprostenol powder formulation that was investigated in this study.

Table 5.4. Composition of cloprostenol powder.

Ingredients	Content % (w/w)
Cloprostenol Sodium salt	0.75
Microcrystalline cellulose (MCC) PH 102	10.25
Lactose	88
Magnesium stearate	1.0
Total weight (mg)	100

The components of the powder formulation were accurately weighed using a four decimal place balance. The blend was prepared in a plastic bag using the following procedure. Half of the microcrystalline cellulose was blended by shaking with the active ingredient for five minutes; the balance of microcrystalline cellulose was then added to the blended mixture and was blended for a further five minutes; half of the lactose was added to the mixture and blended for a further five minutes; the balance of the lactose was then added and blended for a further five minutes; magnesium stearate was then added to the mixture and blended for three minutes.

5.2.3.2 Assessment of cloprostenol powder

5.2.3.2.1 Drug content uniformity

Drug content was quantified by HPLC assay after extraction of the cloprostenol from the powder blend. Cloprostenol was extracted by dissolving a known amount (0.1 g) in 9.9 g of deionised water and subjecting the mixture to ultrasound for 40 minutes. These samples were then passed through a 0.20 μm nylon filter before analysis using a validated HPLC method.

Validation of the extraction method was achieved by analysis of samples derived from a spiked blend that was accurately mixed using a mortar and pestle coupled

with a geometric dilution of the powders. This was repeated on six separate occasions with random samples being taken from the same spiked blend. Three different spiked blends were evaluated. The theoretical concentration of the cloprostenol in the resultant solution was 75 µg/mL.

In each case, the extraction efficiency for each experiment was calculated using Equations 5.1 and 5.2.

5.2.3.2.2 Angle of repose

For a powder dosage form, a measurement of powder cohesion, angle of repose is used to determine flow properties of the powder (Staniforth, 2002). The angle of repose is formed by the powder poured from a container on to a horizontal surface, which makes a conical heap. This is because the particles of the powder stack until the approach angle for subsequent particles joining the stack is large enough to overcome friction and they slip and roll over other until the gravitational forces balances the interparticle forces. The sides of the conical heap formed in this way make the angle of repose with the horizontal surface. The angle of repose is a characteristic of the internal friction/cohesion of the particles. Angles of repose greater than 50º have unsatisfactory flow properties, whereas minimum angles close to 25º correspond to having very good flow properties (Staniforth, 2002).

The angle of repose of the blend was determined by inverting and lifting vertically a 10 mL volumetric cylinder, which was previously filled with 10g of the powder blend, onto a petridish. This allows the powder to flow freely onto a petridish. The diameter and the height of the resultant conical heap of powder were measured to facilitate the calculation of the angle of repose (ɵ) using Equation 5.3.

$$\tan\theta = \frac{height(cm)}{radius(cm)}$$ **Equation 5.3.**

5.2.3.2.3 Appearance

The appearance of the powder was assessed visually by noting any changes in colour and any aggregation.

5.2.3.2.4 Manufacturing assessment

Manufacturing assessment was achieved by taking random samples of known weight (0.1000 g) from various regions of the bulk mixture and extracting the cloprostenol by the method described in Section 5.2.3.2.1. The extracts were then analysed by HPLC and the consistency of drug content over the batch was determined. This was performed using five replicates on three different days.

5.2.4 Manufacture of intravaginal product

EMID's (36 devices) were fabricated from HDPE material as described in Section 4.2.1 for this thesis. Progesterone suspension formulation was added to the large chamber, two oestradiol-17β tablets were placed in one of the small compartments at the head of the insert and 100 mg of cloprostenol powder was added to the other small compartment located at the head of the insert. A gas cell (Varta 4690 Simatec, Switzerland) was incorporated into the insert, but no resistor was added to activate the battery.

5.2.5 Stability assessment

A stability trial was conducted under accelerated conditions at 40ºC/75%RH using a temperature and humidity controlled oven (Contherm Digital Series, Cooled Incubator, Contherm Scientific Ltd, New Zealand). Progesterone suspension, oestradiol-17β tablets and cloprostenol powder and complete EMIDs, which loaded with associated formulations as described in (Section 5.2.4.1.), were all subjected to the stability trial conditions. All samples were stored for 4 weeks and samples were taken for analysis at time 0, 1, 2, 3 and 4 weeks.

5.2.5.1 Progesterone suspension, oestradiol-17β tablets and cloprostenol powder

Progesterone suspension was stored in 50 mL graduated measuring cylinders, the oestradiol-17β tablets were stored in open top plastic pottles and the cloprostenol powder was stored in 10 mL graduated measuring cylinders. Table 5.5 depicts the tests that were performed at each time point on each dosage form.

Table 5.5. Stability tests conducted on progesterone suspension, oestradiol-17β tablets and cloprostenol powder formulations investigated in this study.

Formulation	Test	Method reference	Samples at each time point
Progesterone suspension	Appearance	5.2.1.2.2.	2
	Suspension physical stability	5.2.1.2.3.	2
Oestradiol-17β tablets in open pottles (Type 1 and Type 2 Fast and slow disintegration*)	Appearance	5.2.2.2.2.	10
	Friability	5.2.2.2.3.	20
	Weight	5.2.2.2.4.	10
	Hardness		10
	Thickness		10
	Diameter		10
	Dissolution	5.2.2.2.2.	6
	Drug content	5.2.2.2.1.	5
Cloprostenol Powder	Appearance	5.2.3.2.3.	1
	Angle of repose	5.2.3.2.2.	1

*Two groups of the oestradiol-17β tablets were selected fast (Type 1) and slow (Type 2) disintegration on the basis of their disintegration time.

5.2.5.2 EMID product and associated formulations

Complete EMID's were stored horizontally on the metal trays of an oven. Table 5.6 depicts the tests that were performed at each time point on EMID.

Table 5.6. Stability tests conducted on formulations and battery functions of the EMID investigated in this study.

Product	Test	Method reference	Samples at each time point
EMID associated formulations	Progesterone drug content and degradation products	5.2.1.2.1.	5
	Oestradiol-17β drug content and degradation products	5.2.2.2.1.	5
	Cloprostenol drug content and degradation products	5.2.3.2.1.	5
	Battery function	5.2.5.2.1.	4

5.2.5.2.1 Determination of the effect of storage on the battery

The effect of storage (accelerated conditions at 40ºC/75%RH) on the battery shelf life was tested. At end of the treatment period, a 750 Ω resistor was placed in series with the battery, thereby influencing the current and starting the gas production as previously described in Chapter Four (Section 4.2.2.1). The rate of release of the formulation was determined by the weight loss method as previously described in Chapter Four Section 4.2.4.1. A comparison of the rates of release of EMIDs using the batteries subjected to the storage was made.

5.2.5.3 Criteria of stability testing for a veterinary product

Acceptance criteria of the mean recovery of the active drug in a veterinary product should be within the following ranges as shown in Table 5.7 (Australian Pesticides & Veterinary Medicines Authority, 2004). The sample matrix, the sample processing procedure and the analyte concentration affect the expected mean recovery.

Table 5.7. Acceptable mean recovery of active constituent of a veterinary product.

% Active/impurity content	Acceptable mean recovery (%)
≥10	98-102
≥1	90-120
0.1-1	80-120
<0.1	75-125

In this study, a deviation of greater than or equal to 10% from the labelled claim of oestradiol-17β (10%) tablets is regarded unacceptable. Progesterone (2.5%) and cloprostenol (0.75%) mean recovery should be within the ranges of 90-120% and 80-120%, respectively (Table 5.7).

Stability of progesterone greater than two years old under the accelerated conditions at 40°C/75%RH was verified in previous studies on the CIDR™-B product (Ogle, 1999). In the CIDR™-B, progesterone is homogenously dispersed in a silicone matrix by injection moulding, at a high temperature (190ºC). Despite the high temperature, progesterone compound was very stable according to a personal communication from DEC International CIDR™-B experts. Likewise, oestradiol-17β tablets prepared from extruded solid dispersion process in 60ºC has also been found to be a stable product (Hulsmann *et al.,* 2000).

Previously, progesterone in aqueous suspension, oestradiol-17β in a tablet formulation and cloprostenol powder blend in EMID for veterinary application have never been investigated for stability. Therefore, a validated stability-indicating assay was used for each compound (oestradiol-17β, cloprostenol and progesterone) incorporated in the EMID to asses the presence of additional peaks appearing on the HPLC chromatograms which would indicate decomposition (Section 3.4). The dosage forms were regarded as chemically stable products if the tested products retained their chemical and labelled claim as well as their original physical properties such as appearance, uniformity, suspendibility and dissolution after they were exposed to the stability trial.

5.3 Results and discussion

5.3.1 Manufacture and assessment of progesterone suspension

5.3.1.1 Manufacture of progesterone suspension

All progesterone suspensions used in this study were successfully manufactured using the method described 5.1.2.1.1. The process was successful in terms of the criteria used for the assessment.

5.3.1.1.1 Drug content

5.3.1.2.1.1. Selection of extraction solvents

The results of the effect of extraction solvent type on progesterone extraction efficiency from spiked samples in 4% HPMC containing 690 µg/mL of progesterone is shown in Figure 5.1 for alcohol, mobile phase (60:40 of methanol: water) and 5%HPβCD (Mean±SD).

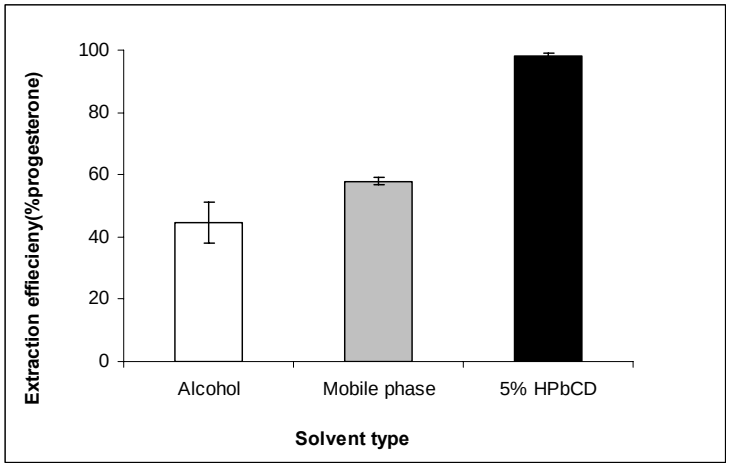


Figure 5.1. Effect of solvent type on progesterone extraction recovery from progesterone suspension.

Extraction of progesterone from the suspension was shown to be dependant on the extraction solvent used (Figure 5.1). Alcohol and the mobile phase were not as efficient at extracting progesterone (45 ± 7% and 58 ± 1%, respectively) from the

suspension as 5% HPβCD in deionised water. The latter showed an extraction efficiency of 98 ± 1%. Therefore, the 5% HPβCD extraction solvent was used for further study.

5.3.1.1.2 Appearance

All suspensions appeared homogenous and white in appearance after manufacture and stored at room temperature for two days.

5.3.1.1.3 Physical stability

The defined manufacturing process resulted in an elegant white pharmaceutical suspension that appeared uniform.

5.3.1.1.4 Manufacturing assessment

Figure 5.2 shows the results of manufacturing reproducibility between two batches of progesterone 2.5 % w/w in 4% HPMC and the consistency of the final product of these batches. In both batches the average recovery percent of progesterone remained the same (100 ± 1) regardless of the sample position within a batch or the particular batch.

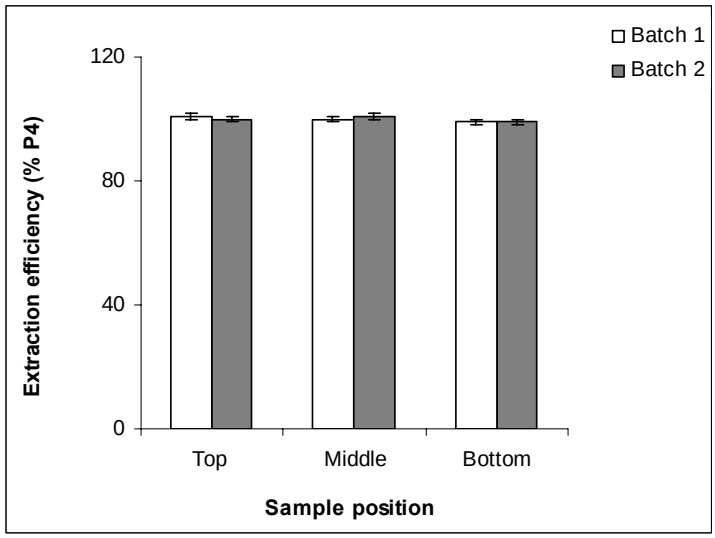


Figure 5.2. Extraction of progesterone from the suspension formulation used in this study from different sample positions of 2 different batches using 5% HPβCD as an extraction solvent.

The batch-to-batch reproducibility of the manufacturing process for the progesterone suspension was shown to be consistent.

5.3.2 Manufacture and assessment of oestradiol-17β tablets

5.3.2.1 Manufacture of oestradiol-17β tablets

All the tablets used in the study were successfully manufactured using the method described in 5.2.2.1.

5.3.2.2 Assessment of oestradiol-17β tablets

5.3.2.3 Drug content

Figure 5.3 shows the results of extraction efficiency of oestradiol-17β spiked into a placebo blend at two different concentrations (500 and 250 μg/mL) on three different days. In all three occasions, the extraction recovery was ≥ 97%, which shows that almost all drug content in the spiked samples at the two different concentrations was extracted efficiently. Therefore, the extraction efficiency of oestradiol-17β was very precise and reproducible.

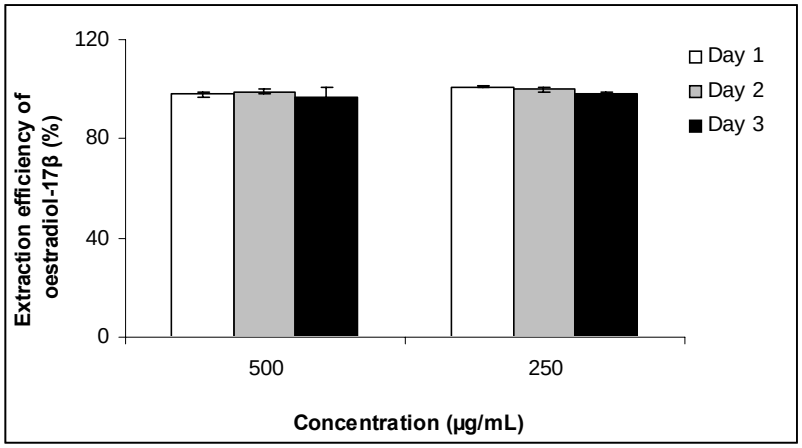


Figure 5.3. Extraction efficiency (%) of oestradiol-17β spiked into a placebo blend at two different concentrations on three different days.

In addition, Table 5.8 shows the extraction efficiency on two occasions of oestradiol-17β from 11 tablets each containing a theoretical amount of 2.5 mg of oestradiol-17β. On both occasions, the extraction recovery and bias were 103% and 3%, respectively, which also indicates that almost all drug content in the eleven tablets was extracted efficiently and so confirming that a reproducible and relatively precise extraction method for oestradiol-17β tablets had been developed.

Table 5.8. The extraction efficiency on two occasions of oestradiol-17β from 11 tablets each containing a theoretical amount of 2.5 mg of oestradiol-17β.

Replicate #	Theoretical concentration (μg/mL)	Actual Concentration (μg/mL)	% Recovery[1]	% Bias[2]
1	259	267	103	3
2	259	267	103	3
		Mean % Recovery	**103**	
		SD	**0**	

[1]Equation 5.2. [2]Equation 5.1.

5.3.2.3.1 Appearance

Tablets from the randomly selected bag were white in colour and had a smooth surface.

5.3.2.3.2 Friability, weight, thickness, diameter and hardness test

Tablets from the randomly selected bag provided an average friability value of 0.5%. The tablets had an average tablet weight, thickness, diameter and hardness values of 26 mg, 2.4 mm, 3.1 mm and 56.8 N, respectively (Table 5.9).

Table 5.9. Average weight, thickness, diameter and hardness values of oestradiol-17β tablets (N=20).

Parameter	Mean ± SD	% RSD	Range
Weight (mg)	26.0 ± 0.2	0.8	25.7 - 26.3
Thickness (mm)	2.4 ± 0.0	1.0	2.3 - 2.4
Diameter (mm)	3.1 ± 0.0	0.3	3.1-3.1
Hardness (N)	56.8 ± 4.6	8.1	50 - 65

The oestradiol-17β tablets from the randomly selected bag showed very precise (%RSD ≤0.8%) diameter and weight values (Table 5.9).

5.3.2.3.3 Tablet disintegration

Tablets from the randomly selected bag showed that the disintegration time of the oestradiol-17β was 20.6 minutes.

5.3.2.3.4 Drug dissolution

Table 5.10 shows the effect of dissolution time on percentage drug release from an oestradiol-17β tablet estimated on three different occasions. The sampling points were arbitrarily selected for assessment of dissolution from the randomly selected bag. The aim was to define the time at which at least 70% of the drug content of oestradiol-17β tablet was released under the operated conditions. In order to validate the dissolution method, the experiment was repeated on three different occasions under the same conditions. This validated method was then used to test the drug dissolution profile from the tablets that were subjected to accelerated testing.

Table 5.10. Effect of dissolution time on percentage of drug release from an oestradiol-17β tablet estimated on three different occasions. Receptor phase composition = 10% HPβCD in PBS at pH 7.4; Shaking rate = 50 strokes per minute (spm); Temperature = 39ºC.

Time (minutes)	Day			Average drug release (%)
	1	2	3	
0	0	0	0	0
15	18	19	16	18
30	33	32	34	33
60	34	42	42	39

The amount of drug released is affected by the time of exposure of the tablet to the release media (Table 5.10). There is a trend of increased percentage of drug release from an oestradiol-17β tablet over time.

Table 5.11 shows the effect of receptor phase composition on the percentage of drug released from an oestradiol-17β tablet after 30 minutes of shaking at 50 spm.

Table 5.11. Effect of receptor phase composition on percentage of drug release from an oestradiol-17β tablet after 30 minutes of shaking at 50 (spm); Temperature = 39ºC.

Receptor phase composition	% released
2.5% HPβCD in PBS at pH 7.4	23
10% HPβCD in PBS at pH 7.4	33
15% HPβCD in PBS at pH 7.4	27
10% SDA	5
25% SDA	5
50% SDA	21

The percentage of drug released from an oestradiol-17β tablet varied with different release media compositions. The 10% HPβCD in PBS at pH 7.4 release media gave the highest amount released (33%) compared to other release media used (Table 5.11). Table 5.12 shows the effect of stirring rate (50 and 100 spm) on percentage of drug release from an oestradiol-17β tablet.

Table 5.12. Effect of stirring rate on percentage of drug release from an oestradiol-17β tablet.

Stirring rate (spm)	% released
50	27
100	29

The stirring rate had little or no effect on the percentage of drug released from an oestradiol-17β tablet (Table 5.12).

Table 5.13 shows the effect of the receptor phase volume (50 and 100 mL) on the percentage of drug released from an oestradiol-17β tablet.

Table 5.13. Effect of receptor phase volume (50 and 100 mL) on percentage of drug release from an oestradiol-17β tablet.

Receptor phase volume (mL)	%released
50	27
100	23

The receptor phase volume affected the percentage of drug release from an oestradiol-17β tablet. Increasing of the receptor phase volume possibly leads to further dilution of the amount dissolved drugs. This would be likely to underestimation of the drug concentration (close to quantitation limit) in the receptor phase.

Tablets from the randomly selected bag provided an average (N=6) dissolution of 27 % drug released (Table 5.14).

Table 5.14. Percentage drug released of oestradiol-17β from tablets.

Bottle number	**% release**
1	20
2	22
3	29
4	36
5	27
6	27
Average	**27**
SD	**6**
% RSD	**21**

There is a relatively high variation of percentage release of oestradiol-17β among tablets obtained from the randomly selected bag (Table 5.14). This inconsistency of drug release profile of the tablets from the same bag could possibly be due to the lack of precision of compressive loading applied on the tablets during manufacturing.

5.3.3 Manufacturing assessment of oestradiol-17β tablets

5.3.3.1 Appearance

All of the 20 tablets tested in each bag (24 bags) were white in colour and had a smooth surface. The appearance of these tablets was tested against the appearance of oestradiol-17β tablets from the randomly selected bag.

5.3.3.2 Friability test

Weight loss in all bags before and after the friability test varied between 0 and 0.6% and did not exceed 0.6% in any bag. Therefore, the friability test of all bags did not differ from the friability (0.5%) of the randomly selected bag as previously described.

5.3.3.3 Tablet weight, disintegration and hardness testing

The average weight varied between 25 and 27 mg for all oestradiol-17β tablets from all bags except bags 2, 3 and 17 (Figure 5.4). Also, the oestradiol-17β tablets (N=20) from the same bag showed a small weight variation. The percentage RSD of the oestradiol-17β tablets from all bags was smaller or equal to 3.3% except bags 2 and 3.

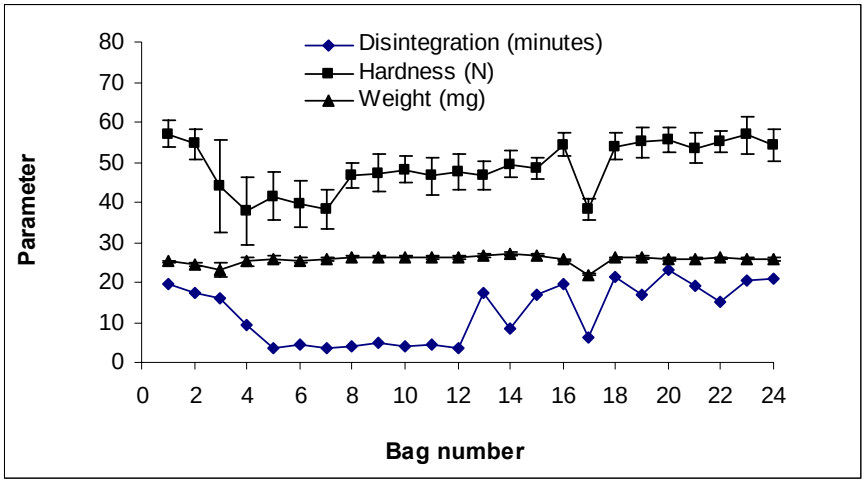


Figure 5.4. Oestradiol-17β tablet hardness, weight and disintegration values across all bag numbers (Mean±SD).

The average hardness for all of the tablets from the 24 different bags varied between 37.9 ± 8.4 and 57.1 ± 3.1 Newtons. Tablets within one bag also showed a variable range of hardness values. For instance, the tablets from bags 3 and 20 varied between 21 and 57 versus 52 and 59 Newtons, respectively. A pattern of hardness seems to have occurred during a manufacturing run such that after an initial drop, the hardness of the tablets appeared to gradually become harder with time (Bag 17 was regarded as an outlier).

Since the tablets were manufactured by manually turning the press of the machine, the dwell times of the punches in the die would be different resulting in different compression characteristics, and therefore this might have caused inconsistent compressibility of the tablets reflected from their hardness.

Figure 5.4 also shows the tablet disintegration results. The tablets from all bags showed variable disintegration times for the oestradiol-17β tablets from the 24 different bags (RSD of 57%). The disintegration time (minutes) of the oestradiol-17β tablets in the 24 different bags varied between 3.4 and 23 minutes. Tablet disintegration appeared to vary from bag to bag throughout the manufacturing process with little or no obvious trend. The pharmaceutical reasons for changes in tablet disintegration between tablets include poor blending and incomplete mixing of the disintegrants and changes in compaction pressure (Aulton, 2002). In this study, inconsistent compressibility loading during tablet manufacturing may affect disintegration time of the tablets.

The disintegration data resulted in the batch being divided into two types of tablets which are Type 1 (Bags 5-12) which were characterised by a fast disintegration with a disintegration time of less than 5 minutes and Type 2 (Bags 1-3, 13, 15, 16, 18-24) which were characterised by a slow disintegration with a disintegration time of greater than 15 minutes. A third type was also identified, which displayed an intermediate disintegration time between 5-15 minutes (Bags 4, 14 and 17) but these were not used for further study.

Figure 5.5 shows the average diameter and thickness of tablets for each bag. The diameter remained constant (3.0 mm) for all oestradiol-17β tablets. This was not a surprise as the inside diameter of the die remains the same during manufacturing, the tablet diameter is also expected to be unchanged. The average thickness varied between 2.3 and 2.5 mm for all oestradiol-17β tablets in all bags except bags 1, 2, 3 and 17. Also the oestradiol-17β tablets (N=20) from the same bag showed small thickness variation. The percentage RSD value for thickness was smaller or equal to 2.8% for the oestradiol-17β tablets from all bags. It is noteworthy that the thickness trend is very similar to that for the weight of the tablets. Possibly, some

changes of compaction pressure of the punches were applied during production of tablets from bags 1-3 and 17.

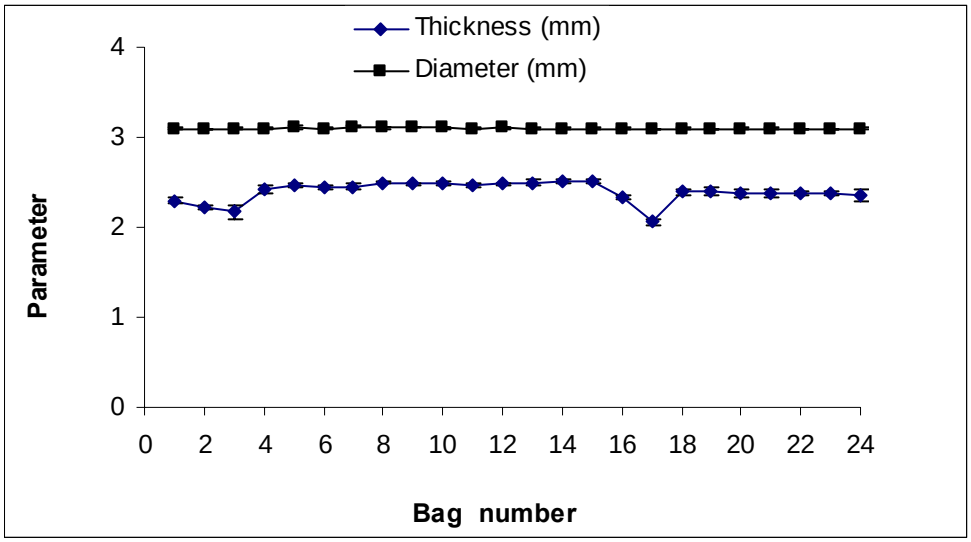


Figure 5.5. Oestradiol-17β tablet thickness and diameter values across all bag numbers (Mean±SD).

5.3.3.4 Drug dissolution

Table 5.15 shows the results of percentage dissolution release of oestradiol-17β tablets from 12 different bags (six tablets per bag). The tablets from 12 bags showed variable percentage dissolution release of the oestradiol-17β tablets (between 22±9 and 69±11 %) from the tested bags (RSD of 57%). The oestradiol-17β release of the tablets from all bags was greater or equal to 40% content except the tablets from bags of 21 and 23. Also, the oestradiol-17β tablets from within the same bag showed variable percentage dissolution release of the oestradiol-17β tablets (RSD of 7% and 43%). From Table 5.15 it can be seen that the fast disintegrating tablets from bags between 5 and 11 released at least 62% of their drug content. The slow disintegration tablets from bags between 1-3 and 11-23 released 45% or less of their drug content. The difference in percentage dissolution of oestradiol-17β tablets is probably due the different disintegration rates. For instance, the faster disintegration tablets are expected to release more drugs than the slow disintegration tablets under the same conditions. As previously explained in Section 5.3.3.3, the method of manufacture could also

affect the disintegration times and subsequently dissolution rates of the tablets. In addition, an inadequate mixing of the tablet blend could result in non-uniform distribution of the active and therefore this may result in variable dissolution rates.

Table 5.15. Percentage dissolution of oestradiol-17β tablets from 12 different bags.

Bag number	Replicate #						Average % release (Mean ±SD)	% RSD
	1	2	3	4	5	6		
1	31	48	38	52	35	66	45 ± 13	29
3	55	62	35	33	19	37	40 ± 15	38
5	75	79	-	61	53	76	69 ± 11	16
7	58	49	70	71	97	66	68 ± 16	24
9	64	63	65	54	66	61	62 ± 5	7
11	70	71	70	61	47	53	62 ± 10	16
13	40	47	49	71	53	32	49 ± 13	27
15	72	56	56	52	59	18	52 ± 18	35
17	38	65	47	46	46	57	50 ± 10	19
19	43	55	43	42	40	30	42 ± 8	20
21	19	23	27	8	20	36	22 ± 9	43
23	20	22	29	36	27	27	27 ± 6	21

It was concluded from Table 5.15 that the drug release was not constant across all the tested bags. This was attributed to inadequate mixing used to combine all the ingredients and the likely presence of segregation of the disintegrants.

5.3.3.5 Drug content

Figure 5.6 shows the results of percentage label claim of oestradiol-17β tablets from eight different bags (five tablets per bag). The tablets from the tested bags showed similar percentage of label claim between 93 and 106.4% of the oestradiol-17β tablets. Also, the tablets from the same bag showed an acceptable deviation (RSD ≤10%) from the label claim of the oestradiol-17β tablets. The overall percentage label claim of the oestradiol-17β tablets is 98 ± 4% for all tested bags, which is very promising. Since, the tablets were crushed and the active ingredient was completely dissolved in the receptor phase, their drug content is expected to be recovered.

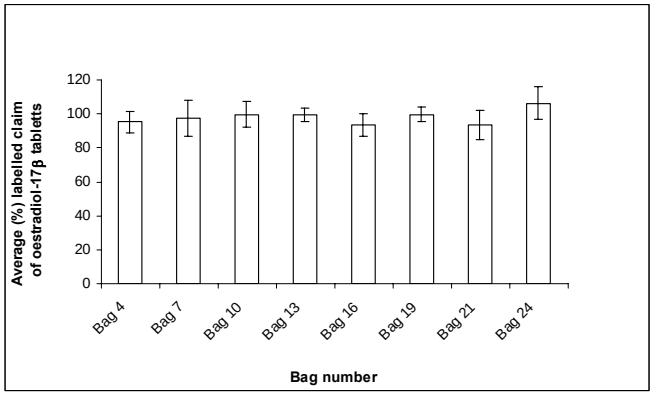


Figure 5.6. Oestradiol-17β content in tablets from a range of bags that cover the manufacturing run.

It was concluded from Figure 5.6 that the drug content was constant for the representative bags from the manufacturing run.

5.3.4 Manufacture and assessment of cloprostenol powder

5.3.4.1 Manufacture of cloprostenol powder

All cloprostenol powders used in the study appeared successfully manufactured using the method described 5.1.2.1.

5.3.4.2 Assessment of cloprostenol powder

5.3.4.2.1 Drug content of cloprostenol blend

Figure 5.7 shows the extraction efficiency of cloprostenol from a spiked blend manufactured using a mortar and pestle on three different days.

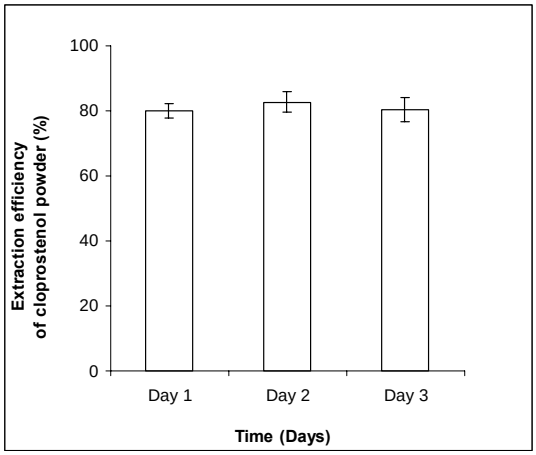


Figure 5.7. Effect of intra-day and inter-day extraction reproducibility on the extraction of cloprostenol from the powder blend.

Assessment of the manufactured blend revealed that it had uniform cloprostenol content (Figure 5.7). A reproducible extraction procedure for cloprostenol was developed and validated for which drug extraction efficiency was about 80% (80±2, 83±3 and 80±4%, for the three different days) of the expected theoretical value. This lower recovery of cloprostenol was attributed to an extraction problem.

5.3.4.2.2 Angle of repose

The angle of repose of the fresh blend was determined to be 31˚.

5.3.4.2.3 Appearance

The appearance of cloprostenol was white. No aggregation of the fresh blend was noted.

5.3.4.2.4 Manufacturing assessment

The cloprostenol content of the manufactured blend from five different regions of the mixture on three different days are shown in Figure 5.8. The cloprostenol powder manufactured using geometric mixing in a plastic bag resulted in inconsistency of the drug extraction efficiency (83±19, 90±16 and 84±15% for the three different days). The higher standard deviation shows inconsistent drug

content uniformity of the powder mixture. An inadequate mixing of the blend could result in non-uniform distribution of cloprostenol and therefore different samples of the blend might have different drug load and subsequently variable assay results. Proper blending and prevention of drug segregation are required for a successful manufacturing process (Wu *et al.,* 2000). It is a fact that formulation of low dose drugs can be very challenging and problems related to content uniformity may arise. This is likely due to the use of a plastic bag to mix the powders and lower drug dose (0.75% w/w). Variability of the drug content uniformity is due to inadequate distribution of low dosage drugs in powder (Greaves, Beasley, & Swarbrick, 1999). This inadequate homogeneity encountered with compositions containing lactose is due to the migration of the potent low dose drug that disrupts to achieve even distribution of the drug through the mixing. Shaking in a plastic bag was found to be an inadequate preparation method of cloprostenol powder.

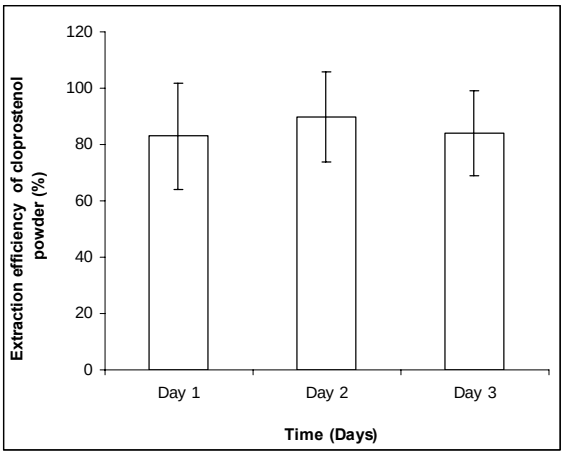


Figure 5.8. Effect of intra-day and inter-day extraction reproducibility on the extraction of cloprostenol from the powder blend.

5.3.5 Stability assessment of progesterone suspension, oestradiol-17β tablets and cloprostenol powder

5.3.5.1 Progesterone suspension

5.3.5.1.1. Appearance and progesterone suspension physical stability

The presence of any discoloration or sedimentation of the suspension was considered to indicate the physical stability of the product under operating conditions. All suspensions appeared white and did not discolour at any sample point (Table 5.5). The suspensions appeared homogeneous throughout the investigation. All suspensions remained physically stable. No sediment was observed and no supernatant occurred in any of the samples at any sample point. It was concluded that the progesterone suspension remained physically stable upon storage. This was due to the fact that the suspension contained a high concentration of suspending agent which produced a very viscous continuous phase preventing particle sedimentation.

5.3.5.2 Oestradiol-17β tablets

The previous tablet characterisation experiments elucidated that the tablet appearance, friability, weight, diameter and drug content were found to be relatively constant between different bags prior to the stability study. Disintegration time and percentage drug released varied among the bags. Two types of tables were selected, fast (Type 1) and slow disintegration (Type 2), on the basis of their disintegration time.

5.3.5.2.1 Appearance

All tablets appeared white and had a smooth surface at all sample points (Table 5.5). The appearance of the tablets did not change upon storage. There was no evidence of any surface changes implying that the elevated temperatures and humidity did not affect the visual appearance of any of the tablets.

5.3.5.2.2 Friability

Table 5.16 shows the friability of Type 1 and 2 tablets investigated. The tablet weight loss due to the friability test is insignificant. The friability value not greater than 1.0% is acceptable (USP, 1994). Therefore, the storage conditions do not appear to affect the susceptibility of the tablets to abrasion.

Table 5.16. Friability of Type 1and 2 tablets investigated in this stability study.

Friability	Sample time (weeks)				
	0	1	2	3	4
% weight loss(Bags of 5, 7 and 8)	0.2	1.0	0.7	0.2	0.6
% weight loss (Bags of 13, 15 and 19).	0.4	0.2	0.0	0.1	0.3

5.3.5.2.3 Weight

Tablet weight for both Type 1 and Type 2 oestradiol-17β tablets was observed to remain constant during storage (Table 5.17). Therefore, any potential absorption of water during storage was insignificant compared to the weight of the whole tablet. Table 5.17 shows the average weight (mg) and tablet weight precision (%RSD) of Type 1(fast) and Type 2 (slow) disintegrating tablets investigated in this stability study.

Table 5.17. Average weight (mg) and tablet weight precision (%RSD) of Type 1and Type 2 tablets.

Tablet types	Parameter	Sample time (weeks)				
Types	Weight (mg)	0	1	2	3	4
Type 1 (5, 7 and 8 bags)	Mean	26.0	26.3	26.3	26.1	26.0
	%RSD	2.8	1.0	2.0	2.0	2.0
Type 2 (13, 15 and 19 bags)	Mean	26.6	26.6	26.6	26.8	26.2
	%RSD	1.4	1.3	1.4	0.7	1.0

Both Type 1 and 2 tablets showed very close weight value. The average weight variation is similar for each type (RSD ≤2.8%).

5.3.5.2.4 Hardness

Table 5.18 shows the average hardness of Type 1 and Type 2 tablets.

Table 5.18. Average hardness of Type 1 and Type 2 tablets investigated in the stability study.

Tablet types	Parameter	Sample time (weeks)				
Types	Hardness (N)	0	1	2	3	4
Type1 (5, 7 and 8 bags)	Mean	42	41.1	42.0	37.9	37.3
	%RSD	9	11.8	11.2	9.7	12.5
Type 2 (13, 15 and 19 bags)	Mean	47	46.3	44.9	43.7	44.4
	%RSD	8	12.4	8.2	8.1	12.9

Tablet hardness for both Type 1 and Type 2 oestradiol-17β tablets was observed to decrease during storage. The percentage RSD fluctuates between 8 and 12% for the tested periods. Also hardness values of Type 1 tablets differed from Type 2. Both the Type 1 and 2 tablets showed some variation among them. However, overall this data suggested that there was no significant change in the tablet hardness on storage (Table 5.18).

5.3.5.2.5 Thickness Diameter

Average thickness and diameter of Type 1 and 2 tablets investigated in the stability study did not show any difference for the same type or between the two types. Tablet thickness for both Type 1 and Type 2 oestradiol-17β tablets was observed to remain constant during storage. This indicated that if any moisture penetrating the tablets when exposed to elevate humidity conditions, did not produce any swelling and did not significantly affect the thickness of the tablet.

5.3.5.2.6 Dissolution

The effect of storage on percentage release of oestradiol-17β tablets is shown in Table 5.19 for both types of tablets. The percentage drug released appear constant with time, and drug dissolution appears independent of storage. It can be seen from Table 5.19 that the general trend is for the Type 1 tablets to have a greater percentage drug release than the Type 2 tablets (≥69 versus ≤54%). A similar trend of percentage drug release was observed from the tablets prior to stability trial.

Table 5.19. Percentage released (%) of Type 1 and 2 tablets investigated in the stability study.

Tablet types	Parameter	Sample time (weeks)				
Types	Dissolution (%)	0	1	2	3	4
Type1 (5, 7 and 8 bags)	Mean	69	76	74	78	76
	%RSD	6	7	10	13	21
Type 2 (13, 15 and 19 bags)	Mean	51	48	40	54	45
	%RSD	15	55	18	27	21

The overall increase of drug release from the tablets subjected to the storage may have been due to an initial reaction to the humidity that remained constant after an initial exposure. Initial exposure may have impelled some absorption of water that resulted in a reaction with one of the components of the tablet causing them to bind more strongly with time, thereby affecting the dissolution of the tablets.

Table 5.20 shows the average drug content of Type 1 and 2 tablets. The average drug content varied between 91-99% and 94-104% for Type 1 and Type 2 tablets, respectively.

Table 5.20. Average drug content of Type 1 and Type 2 tablets investigated in the stability study.

Tablet types	Parameter	Sample time (weeks)				
Types	Drug content (%)	0	1	2	3	4
Type1 (5, 7 and 8 bags)	Mean	97	93	91	99	98
	%RSD	8	9	4	5	6
Type 2 (13, 15 and 19 bags)	Mean	100	102	95	104	94
	%RSD	4	8	4	7	5

The percentage RSD value is lower than 10% at any sample point for each type. The storage did not affect on the drug content the tablets contained in open pottles.

5.3.5.3 Cloprostenol powder

5.3.5.3.1 Appearance

No colour changes were observed in any of the samples at any sample point.

5.3.5.3.2 Angle of repose

The angle of repose of the cloprostenol samples was determined at all four sample points. It was observed that as the storage time increased, lumps appeared in the powder. For comparison purposes, the angle of repose of the samples in the presence of the lumps and after breaking the lumps was also determined.

The angle of repose at the four sampling times before and after breaking the lumps is presented in Table 5.21. The angle of repose of the clumped powder was greater than 25º at any sampling point. The declumped samples produced an acceptable angle of repose (approximately 25º).

Table 5.21. Effect of storage on the angle repose of cloprostenol powder before and after breaking the lumps.

Angle of repose	Week one	Week two	Week three	Week four
Clumped samples	34.1	29.8	45	31.2
Declumped samples	21.8	24.8	24.8	24.8
Difference	12.3	5	20.2	6.4

During storage the cloprostenol powder blend did not appear to change colour, but clumps of powder developed upon storage. The angle of repose was affected by the presence of clumps that formed on storage of the powder under accelerated conditions. The angle of repose was therefore determined both before and after destroying the clumps to make the powder blend homogenous in consistency. The angle of repose was variable and few conclusions can be drawn from this data. It is expected that the release of the drug from the powder and therefore from the delivery device would be affected by its ability to flow easily and freely.

Therefore, the ability of the drug to flow easily and freely would be expected to be affected by formation of the clumps.

5.3.6 Stability determinations of the whole EMID

EMIDS were successfully manufactured as previously described in Chapter Four. The complete EMID is assessed and the test procedures were described in Table 5.6 as previously described.

5.3.6.1 Progesterone drug content and degradation products in the EMID

The effect of storage conditions on the progesterone suspension contained inside the EMID and exposed to 40ºC/75%RH for four weeks is shown in Figure 5.9.

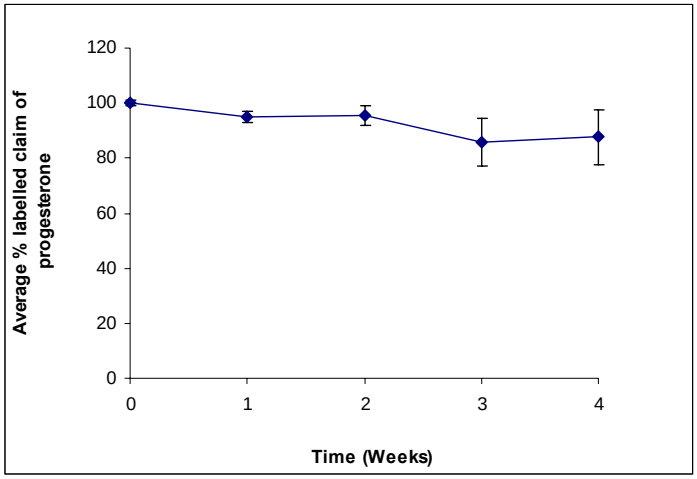


Figure 5.9. Effect of 4 weeks storage on progesterone content in the EMID.

The progesterone average percentage labelled claim recovery is within the acceptable range of 90-110% of the expected value at any time point of the 4 weeks study (Figure 5.9). There was an initial drop in progesterone content in the 3-week sample followed by a relatively stable period for the remaining week. There was no evidence of any degradation products and no extraneous peaks were seen on the chromatogrammes suggesting that no chemical degradation had occurred due to the storage conditions (Figure 5.10). Due to the large standard

deviation (±10) associated with the 3 and 4 week samples that this average value may not be at all significantly different to the samples of the remaining weeks.

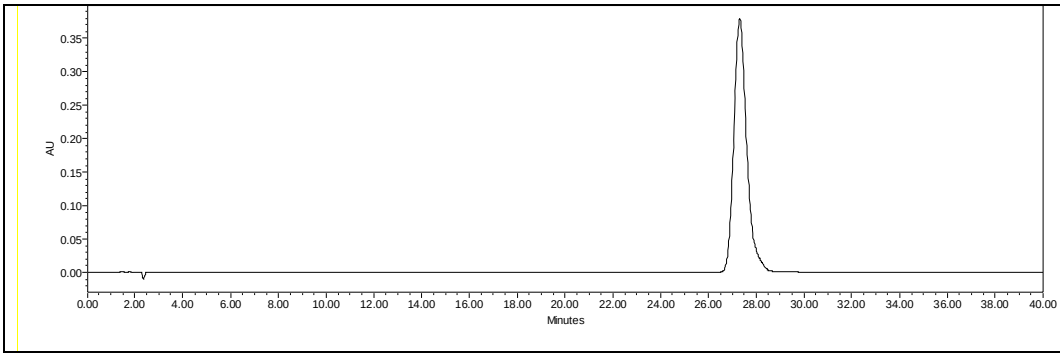


Figure 5.10. Effect of 4 weeks storage on progesterone peak chromatogram (i.e., week 4 sample).

The stability indicating assay showed no degradation products for stored progesterone samples. This is in agreement with literature which states that progesterone is very stable product (Ogle, 1999). The standard deviation of ±10 associated with samples of week 3 and 4 are possibly due to a change in the actual content of the suspension. It could be that progesterone starts to interact with polymer (HDPE) and the drug is partitioning into polymer chains or progesterone is interacting with the santoprene bung /piston material.

5.3.6.2 Oestradiol-17β tablets drug content and degradation products in the EMID

The amount recovered (%) compared to what is claimed on the label of the oestradiol-17β tablets contained within the EMID showed a slow decline upon storage (Figure 5.11). A trend shows that both Type 1 and Type 2 tablets followed the same profile of the drug content recovery in the samples of week 3 and 4. The Type 1 tablets showed a bit more pronounced decline than the Type 2 tablets with larger standard deviations. Within the EMID the tablet may absorb moisture and the drug that was in the solid state of the surface of the tablet would go into solution. The drug then may leak into the larger reservoir of the EMID from the solution or partition into the small seal rubber plastics and HDPE polymer. The

drug from the softer tablets is more likely to dissolve than the drug from the harder tablets. It was noted that some tablets (N=2) were found to be mushy or partially dissolved during recovery of the oestradiol-17β tablets contained in the EMIDs. This probably happened when a seal between the santoprene bung and the walls of the small compartments of the EMIDs was not attained and the moisture from the larger reservoir could leak into the small compartments. This would dissolve the tablets ingredients, which could leak into the larger reservoir of the device.

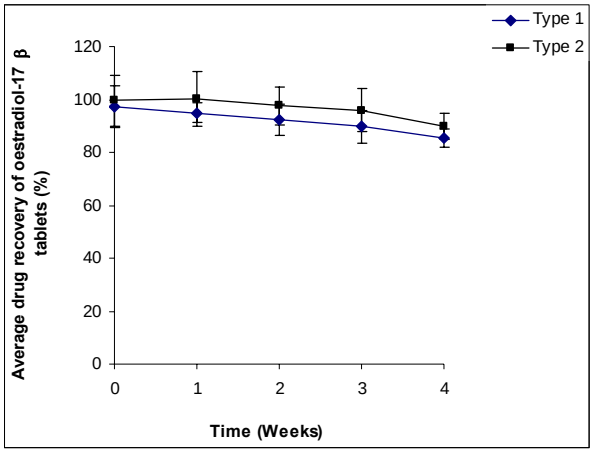


Figure 5.11. Effect of 4 weeks storage on the percentage of label claim for oestradiol-17β tablets contained inside an EMID.

The average drug recovery of oestradiol-17β from the tablets was not affected by storage with the mean recovery of oestradiol-17β remaining in the acceptable range of 90-110% of the expected value. Additionally, there was no evidence of any degradation products or any extraneous peaks seen on the chromatogrammes suggesting that no chemical degradation had occurred due to storage conditions (Figure 5.12). However, the assay value of the product proved that using oestradiol-17β tablets in EMID is feasible.

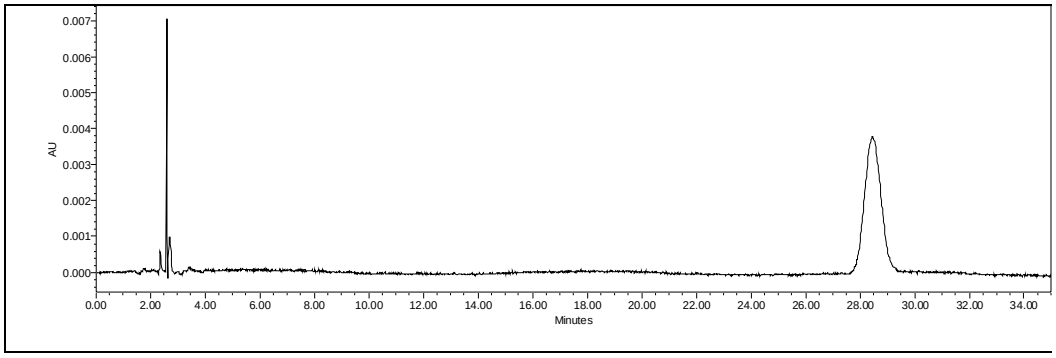


Figure 5.12. Effect of 4 weeks storage on oestradiol-17β peak chromatogram (i.e., week 4 sample).

This follows on from previous work on oestradiol-17β tablets manufactured from solid dispersion using melt extrusion at 60ºC, which were also found to be stable (Hulsmann *et al.,* 2000).

5.3.6.3 Cloprostenol drug content and degradation products in the EMID

The appearance of clumping is significant and indicates a possible adverse behaviour that could be detrimental to the EMID upon storage. It is noteworthy that recovered cloprostenol samples formed a rod shaped structure due to the shape of the small compartment of the EMID. Therefore, formation of cloprostenol powder clumps in the EMID would be expected. The mean recovery of cloprostenol remained in the acceptable range of 80-120% of the expected value (Figure 5.13).

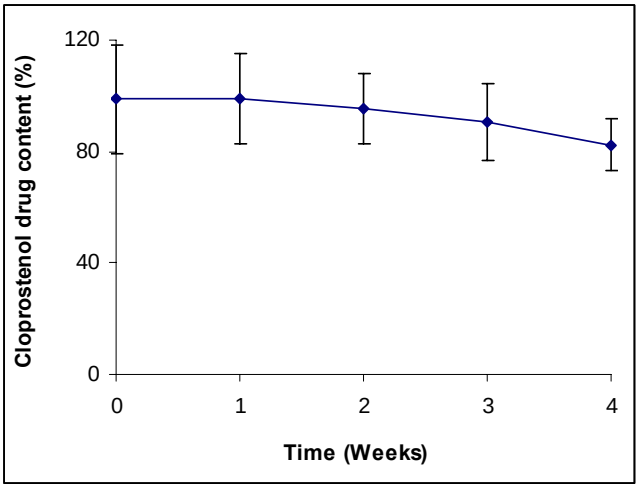


Figure 5.13. Effect of storage on the percentage of label claim for cloprostenol powder contained inside an EMID.

There was a slow decrease of percentage drug content observed for cloprostenol powder in the samples stored for 3 and 4 weeks. The decrease was however, much greater in week 4 compared to week 3. However, no extraneous peaks were seen on the chromatogram suggesting that no chemical degradation had occurred (Figure 5.14).

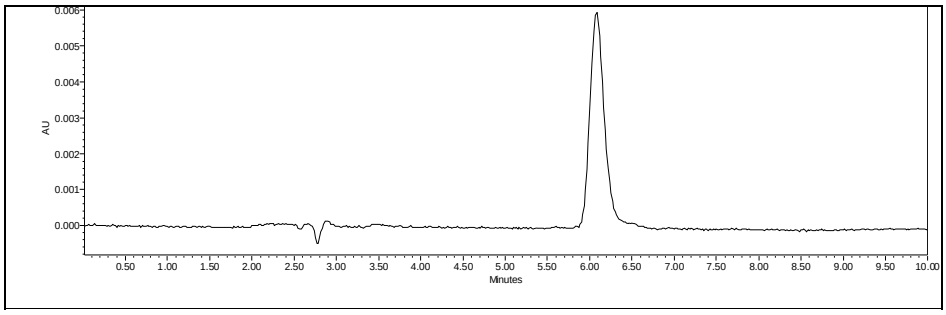


Figure 5.14. Effect of 4 weeks storage on cloprostenol peak chromatogram (i.e., week 4 sample).

Therefore, due to the large standard deviation associated with stored samples, the average value may not be at all significantly different to the 4 week samples. The larger standard deviation of cloprostenol was due to poor powder mixing and lower drug dosage. The average drug recovery of cloprostenol from the powder was not affected by the storage.

5.3.6.4 Effect of storage on battery functions in the EMID

All 16 gas cells worked well and showed that the gas cell was stable under the tested conditions (Table 5.22). The release rate of every individual gas cell remained the same (3 g/day) and exhibited a linear correlation of ≥0.996 when the cumulative weight loss was plotted against release time.

Table 5.22. Cumulative weight loss of progesterone suspension (g) versus release time (days) using batteries that had been stored for 4 weeks in an EMID at 40ºC/75%RH.

Day of release	Week one				Week two				Week three				Week four			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	1.8	1.5	1.6	2.4	1.8	1.7	1.5	1.8	1.8	1.9	2.2	1.7	1.5	1.6	1.8	1.8
2	5.5	5.1	5.2	5.2	5.6	5.5	5.2	5.5	5.6	5.5	6.5	5.4	5.4	5.4	5.6	5.3
3	9.1	8.2	8.1	8.7	8.9	8.9	8.4	8.8	8.8	8.8	10.3	8.9	8.9	8.9	8.9	8.8
4	12.3	11.2	11.2	12.1	12.1	12.2	11.3	12.1	12.1	11.8	13.8	12.2	12.3	12.2	12.1	12.3
5	15.8	14.2	14.2	15.6	15.3	15.8	14.4	15.3	15.2	15	17.2	15.4	15.5	15.4	15.2	15.1
6	18.5	16.8	16.8	18.6	17.9	18.5	17.1	18	17.8	17.6	20.3	18.1	18.5	18	18	17.9
7	21	19.1	19.1	21.5	20.5	21.3	19.6	20.6	20.2	20.1	23.1	20.7	21.3	20.6	20.5	20.4
Slope (g/day)	3.2	2.9	2.9	3.2	3.1	3.2	2.9	3.1	3.0	3.0	3.4	3.1	3.2	3.1	3.1	3.1
R^2	0.996	0.996	0.996	0.998	0.996	0.996	0.996	0.997	0.996	0.997	0.997	0.996	0.996	0.995	0.997	0.996

Table 5.22 shows the constant formulation release over time of all tested devices. This indicates that the battery is operating optimally and as good as fresh batteries. In this thesis (Section 4.2.4), the release rate of the fresh battery at 750 Ω was 3 g/day for HDPE EMID. The battery function was not affected by the storage.

5.4 Conclusion

Progesterone suspension, oestradiol-17β tablets and cloprostenol powder formulations subjected to accelerated testing outside of the EMID were found to be chemically stable. There was a tendency of a slow decrease in drug content of progesterone and oestradiol-17β contained in the EMID during the last two weeks of storage. However, this was not of concern as the overall percentage of the drug recovered was within acceptable range. Different compartments of the EMID should be properly sealed to prevent any cross contamination between different formulations. The Cloprostenol powder formed some clumps, which may affect the drug flow from the powder. The battery was found to be stable upon storage.

6 Conclusions and recommendations for future work

6.1 Conclusions

A simple method using tools made in-house was developed to manufacture PCL membranes with defined thicknesses. An *in-vitro* steroid permeation technique was developed and validated using side-by-side cells with PCL membrane. Dexamethasone analogues, oestradiol-17β and oestradiol benzoate and progesterone were found to be easily absorbed and permeated through PCL.

Excised mucosa was found to be permeable to the tested steroids with some differences in degree of permeation. Dexamethasone acetate, dexamethasone isonicotinate and dexamethasone dipropionate that their ester group attached on carbon position 21 were susceptible to ester hydrolysis. Dexamethasone valerate did not show any sign of hydrolysis. Progesterone followed by dexamethasone acetate and dexamethasone valerate showed higher permeability coefficients through vaginal mucosa compared to other dexamethasone analogues. Both the steroid molecular weight and solubility affected the rate of permeation. The permeation rate increased with increasing the solubility of a specific steroid. Conversely, the rate of the permeation and permeability coefficients decreased with increasing the molecular weight of the steroid.

The permeation rate data generated using the selected PCL model membrane could not predict the permeation rate of selected steroids across excised vaginal mucosa in side-by-side cells. However, the use of the PCL membrane might assist in allowing for discrimination among steroids dosage forms.

UV and HPLC analytical methods were developed and validated for characterising the tested steroids. In both methods, assays were shown to be linear within the stated range, precise and accurate. In addition, the HPLC stability indicating assays for progesterone, oestradiol-17β and cloprostenol were developed and validated. All the assays of HPLC stability assays were also shown to be linear within the stated range, precise and accurate. Progesterone suspension, oestradiol-17β tablets and cloprostenol powder formulations were successfully

manufactured and tested. Extraction solvents were developed for their ability to extract progesterone from the suspending agent, oestradiol-17β from the tablet matrix and cloprostenol from blended powder.

Weight loss was found to be the simplest, cheapest and easiest method to measure the release rate from the EMID. The body and piston materials were found to influence delivery rate profile from the EMID. A high density polyethylene polymer was identified as the most ideal body material for the insert. Likewise, double O-ring silicone, Elastollan WYO 1388-5 and solid silicone pistons were found to be amongst the best pistons tested, as they did not show any slip-stick behaviour and demonstrated a consistent rate of release for the initial trial period. The santoprene piston materials showed slip stick phenomenon with some degree of variation amongst them. Polypropylene carrier pistons showed the highest friction of all, followed by santoprene. Both santoprene and polypropylene materials showed a very poor performance.

In addition, the viscosity of the payload and orifice size was found to influence delivery rate profile from the EMID. The low viscosity payload with a large or medium orifice EMID resulted in loss of the payload material and thus disturbs the release profile.

The EMID can provide adjustable or versatile drug release profiles. The rate of release of the formulation from the EMID can be modified by controlling the rate of the gas production from the gas cell. Additionally, different drug concentrations in the formulation can produce different responses in the blood profiles. The EMID product including the battery and associated formulations was found to be stable upon storage.The EMID (manufactured from high density polyethylene polymer) produced a good response of blood profile from cows, which were treated with the complete EMID containing formulated progesterone, but it showed a low retention rate. Once the retention mechanism for the EMID is improved, the device would have potential for commercial application in induced calving and oestrous control of cattle via the *intravaginal* route.

6.2 Recommendations for future work

The EMID needs further improvement of its retention mechanism in order to stay in the animal. Not all possible materials and designs were tested in this research, and there may indeed be even more beneficial combinations of various components, construction materials and configurations of EMID that could be used in future work and/or any commercial development of this product. Further research is needed to find a lower gas permeability polymer than HDPE to get a more linear release profile for long periods of time, and to do further testing of Elastollan WYO 1388-5 and solid silicone pistons with different body materials. A low viscosity payload with a large or medium orifice should not be used for animal trials as this resulted in loss of the payload material. Different compartments of the EMID should be properly sealed to prevent any cross contamination between different formulations.

It is recommended that further animal trials are conducted using dexamethasone acetate formulation or dexamethasone valerate loaded into EMID for induced calving *per vaginum* administration. Also, it is recommended to screen flux of dexamethasone acetate or valerate (in suspension perhaps in 4% HPMC) through excised membrane using side-by-side cells. In addition, the same formulations could be administered into animals to produce drug blood responses and to investigate if there is an *in vitro* and *in vivo* correlation for the recommended steroids. This could lead a finding of an inexpensive *in vitro* system simulating and mimicking the barrier function of diffusion conditions in animals.

Further work for the EMID might be measuring pulsing precision of the secondary pistons to monitor the release of oestradiol-17β and cloprostenol together with progesterone for oestrous control to ensure that the EMID performs an immediate release of oestradiol, a sustained release of progesterone and a delayed release of a PGF at the appropriate times.

The EMID could also be explored for use as an intraruminal drug delivery system for livestock animals as the device would be expected to be retained in a closed volume.

References

Adams,W.M. (1969). The elective induction of labour and parturition in cattle. Journal of the American Veterinary Medical Association, **154** (3) 261-268.

Agarwal,S., Lakhchaura,B.D., Atheya,U.K., Dabas,Y.P., Sud,S.C. & Mishra,D.P. (1993). Levels of estrogen and progesterone in milk of animals induced into lactation by reproductive steroids and thyrotropic releasing hormone. Indian Journal of Experimental Biology, **31** (6) 554-556.

Alderborn,G. (2002). Tablets and compaction. In M. E. Aulton (Ed.), Pharmaceutics: The science of dosage form deign. (pp. 397-440). Leicester: Churchill livingstone.UK.

Allen,J.G. & Herring,J. (1976). The induction of parturition using dexamethasone. Australian Veterinary Journal, **52** (10) 442-445.

Antignac,Jean-Philippe., Le Bzec,Bruno., Monteau,Fabrice.,Poulain,Frédéric. & André Francois. (2001). Multi-residue extraction-purification procedure for corticosteroids in biological samples for efficient control of their misuse in livestock production. Journal of Chromatography B, **757**, 11-19.

Artursson,P. & Karlsson,J. (1991). Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochemical and Biophysical Research Communications, **175** (3) 880-885.

Attwood,D. (2002). Disperse systems. In M. E. Aulton (Ed.), Pharmaceutics: The science of dosage form design. (pp. 70-100). Leicester: Churchill Livingstone.UK.

Australian Pesticides & Veterinary Medicines Authority (2004). Guidelines for the validation of analytical methods for active constituent, agricultural and veterinary chemical products (pp, 1-20).

Avdeef,A. (2001). Physicochemical profiling (solubility, permeability and charge state). Current Topics in Medicinal Chemistry, **1** (4) 277-351.

Babu,R.J. & Pandit,J.K. (2004). Effect of cyclodextrins on the complexation and transdermal delivery of bupranolol through rat skin. International Journal of Pharmaceutics, 271, 155-165.

Bajema,D.H., Hoffman,M.P., Aitchison,T. E. & Ford S. P. (1994). Use of cow-side progesterone tests to improve reproductive performance of high-producing dairy cows. Theriogenology, **42** (5) 765-771.

Barth,A.D., Adams,W.M., Manns,J.C. & Rawlings,N.C. (1978). Induction of parturition in beef cattle using estrogens in conjunction with dexamethasone. Canadian Veterinary Journal, **19** (7) 175-180.

Beardsley,G.L., Muller,L.D., Garverick,H.A., Ludens,F.C. & Tucker,W.L. (1976). Initiation of parturition in dairy cows with dexamethasone. II. Response to

dexamethasone in combination with estradiol benzoate. Journal of Dairy Science, **59** (2) 241-247.

Billany,M. (2002). Suspensions and emulsions. In M. E. Aulton (Ed.), Pharmaceutics: The science of dosage form design. (pp. 334-359). Churchill Livingstone. Leicester:UK.

Bunt, C.R., Rathbone, M.J. & Burggraaf, S. (1999) "Vaginal active agent delivery procedures and formulations thereof". WO9965497.

Bunt, C.R., Rathbone, M.J., Ogle, C.R. & Morgan, S. (2001). "An electronically modulated bovine intravaginal device for the delivery of progesterone", Proceedings of the International Symposium on Controlled Release of Bioactive Materials, 28.

Burke,C.R., Bunt,C.R., Morgan,S.R., & Rathbone,M.J. (1999). Luteolytic efficacy of a prostaglandin-F2a analogue (cloprostenol) administered into the vagina of cattle Proceedings of the New Zealand Society of Endocrinology, Mount Manganui, New Zealand, p.9.

Calvarese,S., Rubini,P., Urbani,G., Ferri,N., Ramazza,V. & Zucchi,M. (1994). Experimental administration of 19-Nortestosterone and dexamethasone in cattle: Elimination of the two drugs in different biological matrices. Analyst, **119**, 2611-2615.

Cavalieri,J., Hepworth,G. & Macmillan,K.L. (2004). Ovarian follicular development in Holstein cows following synchronisation of oestrus with oestradiol benzoate and an intravaginal progesterone releasing insert for 5-9 days and duration of the oestrous cycle and concentrations of progesterone following ovulation. Animal Reproduction Science, **81** (3-4) 177-193.

Chew,B.P., Erb,R.E., Randel,R.E. & Rouquette,Jr.F.M. (1978). Effect of corticoid induced parturition on lactation and on prepartum profiles of serum progesterone and the estrogens among cows retaining and not retaining fetal membranes. Theriogenology, **10** (1) 13-25.

Chien,Y.W. (1992). Vaginal drug delivery and delivery systems. In Y. W. Chien (Ed.), Novel drug delivery systems. (pp. 529-584). Marcel Dekker, Inc., New York: .USA.

Civiale,C., Bucaria,F., Piazza,S., Peri,O., Miano,F. & Enea,V. (2004). Ocular permeability screening of dexamethasone esters through combined cellular and tissue systems. Journal of Ocular Pharmacology and Therapeutics, **20** (1) 75-84.

Clarke,E.G.C., Moffat,A.C. & Jackson,J.V. (1986). Clarke's Isolation and identification of drugs in pharmaceuticals, body fluids, and post-mortem material. (2nd edn.). Pharmaceutical Press. London. UK.

Clas,S.-D., Dalton,C.R. & Hancock,B.C. (1999). Differential scanning calorimetry: applications in drug development. Pharmaceutical Science & Technology Today, **2** (8) 311-320.

Cohen,E.M. (1973). Dexamethasone. In K. Florey & G. A. Brewer (Eds.). Analytical profiles of drug substances, **2** (163-197). Academic Press. New York. UK.

Colazo,M.G., Kastelic,J.P., Whittaker,P.R., Gavaga,Q.A., Wilde,R. & Mapletoft,R.J. (2004). Fertility in beef cattle given a new or previously used CIDR insert and estradiol, with or without progesterone. Animal Reproduction Science, **81** (1-2) 25-34.

Compton,C. (2005a). Inducing – sustainability of seasonal calving questioned. New Zealand Dairy Exporter, 36-36.

Compton,C. (2005b). Animal Health do the wheels fall off if you stop inducing cow? New Zealand Dairy Exporter, 57-58.

Çoyan,K., Ataman,M.B., Erdem,H., Kaya,A. & Kasikci,G. (2003). Synchronization of estrus in cows using double $PGF_{2\alpha}$, GnRH- $PGF_{2\alpha}$,and hCG- $PGF_{2\alpha}$ combination. Revue Med. Vet. **154**, 91-96.

Cross,P.S. (2002). External controlled and monitoring of intravaginal drug delivery in farm animals (MPhil Thesis). The University of Waikato.

Davis,D.L., Kesler,D.J., Jenkins,A.L., Garverick,H.A., Massey,J.W., Bierschwal,C.J. & Day,B.N. (1979). Induction of parturition in cattle with long and short acting corticoids and estradiol benzoate. Journal of Animal Science, **49** (2) 560-566.

Dawson,R.M.C., Elliott,D.C., Elliott,W.H. & Jones,K.M. (1986). Data for Biochemical Research. (3r^{d} edn.). Clarendon Press. Oxford .UK.

Day,A.M. (1977). Cloprostenol for termination of pregnancy in cattle. A) The induction of parturition. New Zealand Veterinary Journal, **25** (6) 136-139.

De Wash,Katie., De Brabander,Hubert., Courtheyn,Dirk. & Van Peteghem,Carlos. (1998). Detection of corticosteroids in injection sites and cocktails by MS^{n}. Analyst, **123**, 2415-2422.

Dilova,V., Konakchieva,R., Kanchev,L. & Dilov,P. (1999). Albumin microsphere as a drug delivery system for dexamethasone: pharmacokinetics in sheep, residue amount in cows and distribution in rats. Bollettino Chimico Farmceutico, **138** (9) 465-470.

Downey,B.R. (1980). Regulation of the estrous cycle in domestic animals- a review. Canadian Veterinary Journal, **21** (11) 301-306.

Evans,A. (2003).Characteristics of ovarian follicle development in domestic animals. Reproduction in Domestic Animals, **38** (4)240-246.

Evans,L.E. & Wagner,W.C. (1976). Bovine plasma oestrogens, progesterone and glucocorticoids during dexamethasone induced parturition. Acta Endocrinologica (Copenh), **81** (2) 385-397.

Fairclough,R.J., Hunter,J.T. & Welch,R.A. (1981a). Dexamethasone concentrations in plasma and milk of cows following the injection of long- and short-acting dexamethasone esters. Australian Journal of Biological Science, **34** 313-319.

Fairclough,R.J., Hunter,J.T., Peterson,A.J. & Welch,R.A. (1981b). Plasma concentrations of progesterone, oestrogens and prostaglandin F in maternal blood and corticosteroids and oestrogens in foetal blood of cows during dexamethasone-induced deliveries. Acta Endocrinologica (Copenh), **96** (3) 406-412.

Fairclough,R.J., Hunter,J.T., Welch,R.A. & Payne,E. (1975). Plasma corticosteroid concentration in the bovine foetus near term: short communications. Journal of Endocrinology, **65** (3) 139-140.

Fortune,J.E. (1993). Follicular dynamics during the bovine estrous cycle: A limiting factor in improvement of fertility. Animal Reproduction Science, **33** (1-4) 111-125.

Friend,D.R. (1992). In vitro skin permeation techniques. Journal of Controlled Release, **18**, 235-248.

Fulkerson,W.J. & McDowell,G.H. (1975). Artificial induction of lactation in cattle by use of dexamethasone trimethylacetate. Australian Journal of Biological Science, **28** (2) 183-187.

Garverick,H.A., Day,B.N., Mather,E.C., Gomez,L. & Thompson,G.B. (1974). Use of estrogen with dexamethasone for inducing parturition in beef cattle. Journal of Animal Science, **38** (3) 584-590.

Goff,A.K. (2004). Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle: minireview. Biology of Reproduction, **71**, 11-16.

Gordon,I.R. (1996). Controlled reproduction in cattle and buffaloes. CAB International. Wallingford.UK.

Hafner,A., Wanke,R., Schmidt,P., Hanichen,T. & Bise,K. (1991). Hypopituitarism as the possible cause of prolonged gestation in cattle. Tierarztl Prax, **19** (3) 258-262.

Hafs,H.D., Manns,J.G. & Lamming,G.E. (1976). Synchronisation of oestrus and ovulation in cattle. In H. Swan & W. H. Broster (Eds.), Principles of cattle production. (pp. 45-58). Butterworths. UK.

Haigh,J.M. & Smith,E.W. (1994).The selection and use of natural and synthetic membranes for in vitro diffusion experiments. European Journal of Pharmaceutical Sciences, **2** (5-6) 311-330.

Hanlon,D.W., Duirs,G.F., Beal,J.R. & Day,A.M. (2002). A new intra-vaginal progesterone-releasing device (Cue-Mate) for controlling oestrous cycle in cattle [Abstract]. Abstracts-XXII World Buiatrics Congress, 356-357. Germany.

Hansen,L.H. & Christiansen,I.J. (1971). Induction of parturition in cows with a corticosteroid (dexamethasone). Nordisk Veterinaer Medicin, **23** (3) 162-169.

Hirako,Makoto., Takahashi,Toru., Takahashi,Hitomi., Patel, Osman,V. & Domeki,Ikuo. (2003). Changes in plasma estrogen concentration during the first trimester of gestation in dairy cows: Comparison with the origin of embryo and fetal number. Japan Agricultural Research Quarterly, **37**, 195-200.

Holmes,C.W & Garcia,S.C. (1999). Effects of time of calving on the productivity of pasture-based dairy systems: a review. New Zealand Journal of Agriculture Research, **42**, 347-362.

Hulsmann, S., Backensfeld, T., Keitel, S. & Bodmeier, R. (2000). Melt extrusion--an alternative method for enhancing the dissolution rate of 17β-estradiol hemihydrate. European Journal of Pharmaceutics and Biopharmaceutics, **49** (237-242).

Hunter,J.T., Fairclough,R.J., Peterson,A.J. & Welch,R.A. (1977). Foetal and maternal hormonal changes preceding normal bovine parturition. Acta Endocrinologica (Copenh), **84** (3) 653-662.

Hussain,Alamdar & Ahsan,Fakhrul. (2005). The vagina as a route for systemic drug delivery. Journal of Controlled Release, **103**, 301-313.

Hydbring,E., Madej,A., MacDonald,E., Drugee-Boholm,G., Berglund,B. & Olsson,K. (1999). Hormonal changes during parturition in heifers and goats are related to the phases and severity of labour. Journal of Endocrinology. **160**, 75-85.

Ingram,J.N., Shaw,G. & Renfree,M.B. (1999). Cortisol in fetal fluids and the fetal adrenal at parturition in the Tammar Wallaby (*Macropus eugenii*). Biology of Reproduction, **60**, 651-655.

International Conference and Harmonization (2003). Guidance for industry Q1A(R2) stability testing of a new drug substances and products. U.S.Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, & Center for Biologics Evaluation and Research. Revision 2.(pp 1-22).

Ivell,R., Fushs,A-R., Bathgate,R., Tillmann,G. & Kimura,T. (2000). Regulation of the oxytocin receptor in bovine reproductive tissues and the role of steroids. Reproduction in Domestic Animals, **35** (3-4)134-141.

Jackson,P.G. (1979). Dystocia in heifers following induction of parturition using corticosteroids. Veterinary Records, **104** (4) 75.

Janszen, B.P., Bevers, M.M., Dieleman, S.J., van der Weyden, G.C. & Taverne, M.A. (1990). Synchronised calvings after withdrawal of norgestomet implants from cows treated near term with prostaglandin. Veterinary Records, **127** (16) 405-7.

Juliano,R.L. (1980). Drug delivery systems: characteristics and biomedical applications. Oxford University Press.UK.

Kaker,M.L., Murray,R.D. & Dobson,H. (1984). Plasma hormone changes in cows during induced or spontaneous calvings and the early post partum period. Veterinary Record, **115** (15) 378-382.

Keeler.R.F., Cronin,E.H. & Shupe,J.L. (1976). Lupin alkaloids from teratogenic and nonteratogenic lupins. IV. Concentration of total alkaloids, individual major alkaloids, and the teratogen anagyrine as a function of plant part and stage of growth and their relationship to crooked calf disease. Journal of Toxicology and Environmental Health, **1** (6) 899-908.

Kerns,E.H. & Di,L. (2004). Physicochemical profiling: overview of the screens. Drug Discovery Today: Technologies, **1** (4) 343-348.

Kerns,E.H. (2001). High throughput physicochemical profiling for drug discovery. Journal of Pharmaceutical Sciences, **90** (11) 1838-1858.

Kesler, D.J. & Favero, R.J. (1995). Estrus synchronization in beef females with norgestomet and estradiol valerate. Part 1: Mechanism of action. Agri-Practice, **16** (10) 6-11.

Kesler, D.J. & Favero, R.J.(1996). Estrus synchronization in beef females with norgestomet and estradiol valerate. Part 2: Factors limiting and enhancing efficacy. Agri-Practice, **17** (1) 12-17.

Kesler, D.J., Faulkner, D.B. & Nash, T.G. (1997). Norgestomet implantation in beef females. Large Animal Practice, **18** (3) 26-30.

Kesler,D.J.; Favero,R.J. & Troxel,T.R. (1995). A compararison of hydron and silicone implants in the bovine norgestomet and estradiol valerate estrus sunchronization procedure. Drug Development and Industrial Pharmacy, **21** (4) 475-485.

Kindahl,H., Kornmatitsuk,B. & Gustafsson,H (2004). The cow in endocrine focus before and after calving. Reproduction in Domestic Animals, **39**, 217-221.

Kindahl,H., Kornmatitsuk,B., Königsson,K. & Gustafsson,H (2002). Endocrine changes in late bovine pregnancy with special emphasis on fetal well-being. Domestic Animal Endocrinology, **23** (1-2) 321-328.

Königsson, K. (2001). Induced parturition and retained placenta in the cow: inhibition or prostaglandin $F_{2\alpha}$ synthesis and antibiotic therapy (PhD Thesis). Department of Obstetrics and Gynaecology. Swedish University of Agricultural Sciences. Uppsala. Sweden.

Kotiyan, P.N & Vavia, P.R (2000). Stability indicating HPTLC method for the estimation of oestradiol. Journal of Pharmaceutical and Biomedical Analysis, **22** (667-671).

Lauderdale,J.W.(1975).The use of prostaglandin in cattle. Annales de Biologie Animale Biochimie Biophysique **15**, 419.

Lee,T.K., Sokoloski,T.D. & Royer,G.P. (1981). Serum albumin beads: an injectable, biodegradable system for the sustained release of drugs. Science, **213** (4504) 233-235.

Lentner, C. (1984) In Geigy Scientific Tables, Vol 3, Physical Chemistry, pp.66.

Lewis,G.S. (1997). Symposium: Health problems of the postpartum cow. Journal of Diary Science, **80** (5) 984-994.

Loftsson,T. & Brewster,M.E. (1996). Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. Journal of Pharmaceutical Sciences, **85** (10) 1017-1025.

Loftsson,T.(1995). The effects of cyclodextrins on the chemical stability of drugs in aqueous solutions. Drug Stability, **1**, 22-33.

Loftsson,T., Fririksdóttir,H., Thórisdóttir,S. & Stefánsson,E. (1994). The effect of hydroxypropyl methylcellulose on the release of dexamethasone from aqueous 2-hydroxypropyl-cyclodextrin formulations. International Journal of Pharmaceutics, **104** (2)181-184.

Loftsson,T., Sigfússon,S.D., Sigurdsson,H.H. & Másson,M. (2003). The effects of cyclodextrins on topical delivery of hydrocortisone: the aqueous diffusion layer. Sciences Techniques et Pratiques Pharmaceutiques (S.T.P.Pharma Sciences), **13**(2) 125-131.

Lopez,R.F., Collett,J.H. & Bentley,M.V. (2000). Influence of cyclodextrin complexation on the in vitro permeation and skin metabolism of dexamethasone. International Journal of Pharmaceutics, **200** (1)127-132.

Lucy,M.C., Billings,H.J., Butler,W.R., Ehnis,L.R., Fields,M.J., Kesler,D.J., Kinder,J.E., Mattos,R.C., Short,R.E., Thatcher,W.W., Wettemann,R.P., Yelich,J.V. & Hafs,H.D. (2001). Efficacy of an intravaginal progesterone insert and an injection of $PGF_{2\alpha}$ for synchronizing estrus and shortening the interval to pregnancy in postpartum beef cows, peripubertal beef heifers, and dairy heifers. Journal of Animal Science, **79** 982-995.

MacDiarmid,S.C. (1979). Betamethasone alcohol suspensions for the induction of parturition in dairy cows: a comparison with dexamethasone trimethyl acetate. New Zealand Veterinary Journal, **27** (5) 86-89.

Macmillan,K.L. & Peterson,A.J. (1993). A new intravaginal progesterone releasing device for cattle (CIDR-B) for oestrous synchronisation, increasing pregnancy rates and the treatment of post-partum anoestrus. Animal Reproduction Science, **33** (1-4) 1-25.

Macmillan,K.L. (2002). Advances in bovine theriogenology in New Zealand. 1. Pregnancy, parturition and the postpartum period. New Zealand Veterinary Journal, **50** (3) 67-73.

Mann,G.E., Lamming,G.E. & Payne,J.H. (1998). Role of early luteal phase progesterone in control of the timing of the luteolytic signal in cows. Journal of Reproduction and Fertility, **113** (1) 47-51.

Martin,A.N., Swarbrick,J., Cammarata,A. (1983). Physical pharmacy: physical chemical principles in the pharmaceutical sciences (3rd Edn). (pp. 399-444). Lea & Febiger, Philadelphia, USA.

Mashru,R., Sutariya,V., Sankalia,M. & Sankalia,J. (2005). Transbuccal delivery of lamotrigine across porcine buccal mucosa: in vitro determination of routes of buccal transport. Journal of Pharmacy and Pharmaceutical Science,**8** (1)54-62.

McDougall,S. (2001). Effects of periparturient disease and conditions on the reproductive performance of New Zealand cows. New Zealand Veterinary Journal, **49**, 60-67.

McDougall,S., Compton,C.W.R. & Anniss,F.M. (2004). Effect of exogenous progesterone and oestradiol on plasma progesterone concentrations and follicle wave dynamics in anovulatory anoestrous post-partum dairy cattle. Animal Reproduction Science, **84** (3–4) 303-314.

McFeely,R.A. & Ganjam,V.K. (1976). Induction of parturition in farm animals. Annales de Recherches Veterinaires, **7** (2) 151-156.

McGowan,L.T., Welch,R.A. & Hunter,J.T. (1975). Studies on induced calving in cattle. Proceedings of the New Zealand Society of Animal Production, **35**, 92-96.

Mihm,M., Crowe,W., Knight,P. & Austin,E. (2002). Follicle wave growth in cattle. Reproduction in Domestic animals, **37** (4)191-200.

Morton,J.M. & Butler,K.L. (1995a). Reductions in milk production after induced parturition in dairy cows from commercial herds in southwestern Victoria. Australian Veterinary Journal, **72** (7) 241-245.

Morton,J.M. & Butler,K.L. (1995b). Direct effects of induced parturition on subsequent reproductive performance of dairy cows from commercial herds in southwestern Victoria. Australian Veterinary Journal, **72** (8) 293-295.

Morton,J.M. & Butler,K.L. (1995c). The effects of induced parturition on the incidence of clinical disease and mortality in dairy cows from commercial herds in south-western Victoria. Australian Veterinary Journal, **72** (1) 1-4.

Morton,J.M. & Butler,K.L. (1995d). The effects of induced parturition in dairy cows on the incidence of mortality in calves from commercial herds in southwestern Victoria. Australian Veterinary Journal, **72** (1) 5-7.

Morton,J.M. (2000). The InCalf Project- Some risk factors for reproductive performance in Australian dairy herds. Proceedings of the Australian and New Zealand Combined Dairy Veterinarians Conference, Massey University, Palmeston North, pp (43-61).

Murray,R.D., Nutter,W.T., Wilman,S. & Harker,D.B. (1984). Induction of parturition in cattle using dexamethasone and cloprostenol: economic performance and disease incidence after treatment. Veterinary Record, **115** (12) 296-300.

Murray,R.D., Nutter,W.T., Wilman,S.U. & Harker,D.B. (1982). Induction of parturition in a commercial dairy herd: clinical management and treatment. Veterinary Record, **111** (16) 363-365.

Murray,R.D., Smith,J.H. & Harker,D.B. (1981). Use of cloprostenol with dexamethasone in the termination of advanced pregnancy in heifers. Veterinary Record, **108** (17) 378-380.

Nasser,L.F., Bo,G.A., Barth,A.D. & Mapletoft,R.J. (1994). Induction of parturition in cattle: effect of triamcinolone pretreatment on the incidence of retained placenta. Canadian Veterinary Journal, **35** (8) 491-496.

Nation,D.P. (1997). Responses to the treatment of post-partum and pre-pubertal anoestrus in New Zealand dairy cattle (PhD Thesis). The University of Waikato. Hamilton, New Zealand.

New Zealand Food Safety Authority. (2006). Update-Review of long acting dexamethasone esters (http:/www.nzfsa.govt.nz/acvm/subject/vet/dex.htm, 14/1/2006).

Odde,K.G. (1990). A review of synchronization of estrus in postpartum cattle. Journal of Animal Science, **68**, 817-830.

O'Farrell,K.J. & Crowley,J.P. (1974). Some observations on the use of two corticosteroid preparations for the induction of premature calving. Veterinary Record, **94** (16) 364-366.

Ogle,C.R. (1999). Design, development and optimisation of veterinary intravaginal controlled release drug delivery systems (PhD Thesis). The University of Waikato. Hamilton, New Zealand.

Panter,K.E., Gardner,D.R. & Molyneux,RJ. (1998). Teratogenic and fetotoxic effects of two piperidine alkaloid-containing lupines (L. formosus and L. arbustus) in cows. Journal of Natural Toxins, **7** (2) 131-141.

Patterson,D.J., Kojima,F.N. & Smith,M.F. (2003). A review of methods to synchronize estrus in replacement beef heifers and postpartum cows. Journal of Animal Science, **81,** E166-E177.

Peters,A.R & Lamming,G.A. (1983). Hormone patterns and reproduction in Cattle. In Practice, **5,** 153-157.

Peters,A.R. & Poole,D.A. (1992). Induction of parturition in dairy cows with dexamethasone. Veterinary Record, **131**, 576-578.

Pharmaceutical Society of Great Britain Department of Pharmaceutical Sciences. (1979). The pharmaceutical codex :incorporating the 'British pharmaceutical codex'. (11th edn.). (pp. 646-648; 1008-1042) Pharmaceutical Press. London: UK.

Pitt,C.G. (1990). Poly-ε-caprolactone and its copolymers. In M. Chasin & R. Langer (Eds.), Biodegradable polymer as drug delivery systems. (pp. 71-120). Marcel Dekker, Inc., New York, USA.

Poore,K.R., Canny,B.J. & Young,I.R. (1999). Adrenal responsiveness and the timing of parturition in hypothalamo-pituitary disconnected ovine foetuses with and without constant adrenocorticotrophin infusion. Journal of Neuroendocrinology, **11**, 343-349.

Rathbone,M.J. & Macmillan,K.L. (2004). Application of controlled release science and technology: progesterone. Controlled Release Society Newsletter, **21**, 8-9.

Rathbone,M.J., Bunt,C.R., Ogle,C. R., Burggraaf, S, Macmillan,K.L., Burke, C.R. & Pickering,K.L. (2002). Reengineering of a commercially available bovine intravaginal insert (CIDR insert) containing progesterone. Journal of Controlled Release, **85** (1-3) 105-115.

Rathbone,M.J., Burns,P.J., Ogle,C.R., Burggraaf,S. & Bunt,C.R. (2000). Controlled release drug delivery systems for oestrus control of domesticated livestock. In M. J. Rathbone & R. Gurny (Eds.), Controlled release veterinary drug delivery: biological and pharmaceutical considerations. (pp. 201-228). Elsevier. New York. USA.

Rathbone,M.J., Kinder,J.E., Fike,K., Kojima,F., Clopton,D., Ogle,C.R. & Bunt,C.R. (2001). Recent advances in bovine reproductive endocrinology and physiology and their impact on drug delivery system design for the control of the estrous cycle in cattle. Advanced Drug Delivery Reviews, **50** (3) 277-320.

Rathbone,M.J., Macmillan,K.L., Inskeep,K., Burggraaf,S. & Bunt,C.R. (1998). Fertility regulation in cattle. Journal of Controlled Release, **54** (2) 117-148.

Refsdal,A.O. (2000). To treat or not to treat: a proper use of hormones and antibiotics. Animal Reproduction Science, **60-61**, 109-119.

Ritschel,W.A. (1988). Pharmacokinetic and biopharmaceutical aspects in drug delivery. In P. Tyle (Ed.), Drug delivery devices: fundamentals and applications. (pp. 30-79). Marcel Dekker, Inc., New York, USA.

Salole,E.G. (1986). Estradiol. Analytical Profiles of Drug Substances, **15**, 283-318.

Schmitt,D., Garverick,H.A., Mather,E.C., Sikes,J.D., Day,B.N. & Erb,R.E. (1975). Induction of parturition in dairy cattle with dexamethasone and estradiol benzoate. Journal of Animal Science, **40** (2) 261-268.

Segall, A., Hormaechea, F., Vitale, M., Perez, V. & Pizzorno, M.T (1999). Development and validation of a reversed-phase liquid chromatographic method for analysis of oestradiol valerate and medroxyprogesterone acetate in a tablet formulation. Journal of Pharmaceutical and Biomedical Analysis, **19** (803-808).

Shoenwald,R.D. & Ward,R.L. (1978). Relationship between steroid permeability across excised rabbit cornea and octanol-water partition coefficients. Journal of Pharmaceutical Sciences, **67** (6) 786-788.

Short, R.E., James, L.F., Staigmiller, R.B. & Panter KE. (1989). Pine needle abortion in cattle: associated changes in serum cortisol, estradiol and progesterone. Cornell Veterinary, **79** (1):53-60.

Short,R.E., Bellows,R.A., Staigmiller,R.B. & Ford,S.P. (1994). Pine needle abortion in cattle: effects of diet variables on consumption of pine needles and parturition response. Journal of Animal Science, **72**, 805-810.

Soboleva,T.K., Peterson,A.J., Pleasants,A.B., McNatty,K.P. & Rhodes,F.M. (2000). A model of follicular development and ovulation in sheep and cattle. Animal Reproduction Science, **58**, 45-57.

Staniforth,J. (2002). Powder flow. In M. E. Aulton (Ed.), Pharmaceutics: The science of dosage form deign. (pp. 197-211). Leicester: Churchill Livingstone. UK.

Steele Gerry. (2004). Preformulation prediction from small amounts of compound as an aid to candidate drug selection. In Gibson Mark (Ed.). Pharmaceutical preformulation and formulation: A practical guide from candidate drug selection to commercial dosage form. (pp. 21-95). Interpharm/CRC. London, UK.

Stella,V.J., Rao,V.M., Zannou,E.A., & Zia,V. (1999). Mechanisms of drug release from cyclodextrin complexes. Advanced Drug Delivery Reviews, **36** (1) 3-16.

Stevens Julian., Burton Lindsay. & Rendel John. (2000). Induced calving: small impact on profits, cause for concern among customers. Dairy Exporter, 52-55.

Taverne,M.A.M., Breeveld-Dwarkasing,V.N.A., van Dissel-Emiliani,F.M.F., Bevers,M.M., de Jong,R. & van der Weijden,G.C. (2002). Between prepartum luteolysis and onset of expulsion. Domestic Animal Endocrinology, **23** (1-2) 329-337.

Terblanche,H.M. & Labuschagne,J.M. (1980). Induced parturition in cattle. II. Plasma oestrogen and progesterone levels. Journal of the South African Veterinary Association, **51** (2) 101-103.

Terblanche,H.M., Kritzinger,L.J. & van Heerden,J.S. (1976). Induced parturition in cattle. 1. Clinical studies. Journal of the South African Veterinary Association, **57** (2) 113-115.

The Department of Health, Faculty of Public Health Medicine (1993). British Pharmacopoeia (Veterinary). pp.24. UK.

The Merck index (2001): An encyclopedia of chemicals, drugs and biologicals. (13th edn). Merck & Co., Inc. N.J. USA.

Toutain,P.L., Brandon,R.A., Alvinerie,M., Garcia-Villar,R. & Ruckebusch,Y. (1982). Dexamethasone in cattle: pharmacokinetics and action on the adrenal gland. Journal of Veterinary Pharmacology and Therapeutics, **5** (1) 33-43.

United States Pharmacopeial Convention.Committee of Revision. (1994). The United States Pharmacopeia; The National Formulary. (23rd revision edn.). Rockville, Md: United States Pharmacopeial Convention. USA.

Usayapant,A. & Iyer,B.R. (1999). The effect of 2-hydroxypropyl-beta-cyclodextrin on in vitro drug release of steroids from suppository bases. Drug Development and Industrial Pharmacy, **25** (3), 387-390.

Usayapant,A., Karara,A.H. & Narurkar,M.M. (1991). Effect of 2-hydroxypropyl-beta-cyclodextrin on the ocular absorption of dexamethasone and dexamethasone acetate. Pharmaceutical Research, **8** (12), 1495-1499.

Veith,G.D., Austin,N.M. & Morris,R.T. (1979). A rapid method for estimating log P for organic chemicals. Water Research, **13**, 43-47.

Verkerk,G.A., Goble,G., McDougall,S., Woods,B., Clark,B. & Macmillan,K.L. Induction of parturition in dairy cows. (1997). Anonymous. Hamilton, New Zealand: Dairy Research Corporation, Private Bag 3123, Hamilton.

Vianna,R.F.L., Bentley,M.V., Ribeiro,G., Carvalho,F.S., Neto,A.F., de Oliveira,D.C.R. & Collett,J.H. (1998). Formation of cyclodextrin inclusion complexes with corticosteroids: their characterization and stability. International Journal of Pharmaceutics, **167** (1-2) 205-213.

Wagner,W.C., Willham,R.L. & Evans,L.E. (1974). Controlled parturition in cattle. Journal of Animal Science, **38** (3) 485-489.

Weast, R. C. (1988). CRC handbook of chemistry and physics. In Robert C. Weast (Ed.). (Infrared correlation charts, F522-527). CRC Press,INC. USA.

Welch,R.A. (1973). Induction of parturition in cattle with corticosteroids. Journal of Reproduction and Fertility, **32** (2) 348

Welch,R.A., Crawford,J.E. & Duganzich,D.M. (1977). Induced parturition with corticosteroids: a comparison of four treatments. New Zealand Veterinary Journal, **25** (5) 111-114.

Welch,R.A., Day,A.M., Duganzich,D.M. & Featherstone,P. (1979). Induced calving: a comparison of treatment regimes. New Zealand Veterinary Journal, **27** (9) 190-194.

Welch,R.A., Newling,P. & Anderson,D. (1973). Induction of parturition in cattle with corticosteroids: an analysis of field trials. New Zealand Veterinary Journal, **21** (6) 103–108.

Wendorf, G.L., Lawyer, M.S. & First, N.L. (1983). Role of the adrenals in the maintenance of pregnancy in cows. Journal of Reproduction and Fertility, **68**, 281-287.

West,J.W.(2003). Effect of heat-stress on production in dairy cattle. Journal of Diary Science, **86** (6) 2131-2144.

Whisnant,C.S. & Burns,P.J. (2002). Evaluation of steroid microspheres for control of estrus in cows and induction of puberty in heifers. Theriogenology, **58** (6), 1229-1235.

Whisnant,C.S., Washburn,S.P. & Farin,P.W. (1999).Current concepts in synchronization of estrus and ovulation of dairy cows. Proceedings of the American Society of animal Science. Departments of Animal Science and Food Animal and Equine Medicine. North Caroline State University. (pp.1-8).

Wintour,E.M., Lewitt,M., McFarlane,A., Moritz,K., Potocnik,S., Rees,S. & Tangalakis,K. (1996). Experimental hydranencephaly in the ovine fetus. Acta Neuropathologica, **91** (5), 537–544.

Wu,Lei-Shu., Pang,Josephine., Chen,Jian-Ge., Hussain,M.A. (2000) Dry blending process scale-up for a very low dose drug candidate. American Association of Pharmaceutical Scientists PharmSciTech, **1** (3) Article 26.

Young,I.R., Deayton,J.M., Hollingworth,S.A. & Thorburn,G.D.(1996). Continuous intrafetal infusion of prostaglandin E_2 prematurely activates the hypothalamo-pituitary-adrenal axis and induces parturition in sheep. Endocrinology, **137** (6) 2424-2431.

Zhang,H., Robinson,J.R. (1996) In vitro methods for measuring permeability of the oral mucosa. In: Rathbone, M.J. (Ed.). Oral mucosal drug delivery. (pp. 85-100). Marcel Dekker, Inc. New York, USA.

Physical properties and chemical resistance of polymers (no date). Retrieved November 14, 2005 from http://www.dynalabcorp.com/files/Use%20and%20 Care%20of%20Plastics.pdf.

www.scifinder.com (accessed via the University Waikato Library Electronic Data base).

Spidey Alchemist-Anabolic Mod. (2003). Retrieved November 14, 2005, from http://www.superiormuscle.com/vbulletin/showthread.php?s=f3a6d6479671c44bc27e8f66422f1b93&threadid=22298.

http://www.dynalabcorp.com/files/Use%20and%20Care%20of%20Plastics.pdf

Appendix-A.The average thickness measured from over five points of the PCL membrane segments.

Table of thickness of the PCL membranes.

Membrane	Measurement position					Average (μm)	SD (μm)
	1	2	3	4	5		
1	150	150	140	150	150	148.0	4.0
2	140	140	140	140	140	140.0	0.0
3	150	150	140	145	145	146.0	3.7
4	140	140	140	145	140	141.0	2.0
5	145	145	145	145	145	145.0	0.0
6	150	150	140	140	145	145.0	4.5
7	140	140	135	142	142	139.8	2.6
8	140	140	138	140	140	139.6	0.8
9	138	140	140	140	140	139.6	0.8
10	140	140	138	140	140	139.6	0.8
11	140	140	140	140	140	140.0	0.0
12	135	142	140	140	142	139.8	2.6
13	140	140	145	135	140	140.0	3.2
14	138	140	145	135	140	139.6	3.3
15	142	142	145	140	145	142.8	1.9
16	145	135	135	142	140	139.4	3.9
17	155	142	130	145	145	143.4	8.0
18	150	135	135	140	140	140.0	5.5
19	140	140	140	140	140	140.0	0.0
20	135	142	140	140	142	139.8	2.6
21	145	142	140	145	142	142.8	1.9
22	145	135	135	142	140	139.4	3.9
23	150	140	135	140	140	141.0	4.9
24	140	145	145	145	140	143.0	2.4
25	148	150	140	142	145	145.0	3.7
26	145	140	140	150	142	143.4	3.8
27	150	135	130	148	148	142.2	8.1
28	140	140	135	135	140	138.0	2.4
29	150	135	130	148	140	140.6	7.6
30	142	150	145	140	140	143.4	3.8
31	145	145	140	140	145	143.0	2.4
32	140	135	140	140	140	139.0	2.0
33	140	140	140	140	140	140.0	0.0
34	140	140	140	140	140	140.0	0.0
35	150	150	140	145	150	147.0	4.0
36	142	135	140	148	140	141.0	4.2
37	145	140	130	135	140	138.0	5.1
38	135	135	140	140	145	139.0	3.7
37	149	152	149	140	150	148.0	4.1
38	144	145	145	155	146	147.0	4.0
39	150	150	142	140	150	146.4	4.5
40	150	142	150	150	152	148.8	3.5

41	150	140	140	150	150	146.0	4.9
42	145	145	152	150	148	148.0	2.8
43	150	155	140	140	150	147.0	6.0
44	152	142	138	150	148	146.0	5.2
45	145	150	140	135	142	142.4	5.0
46	135	148	150	140	145	143.6	5.5
47	135	138	148	150	142	142.6	5.7
48	150	140	140	148	145	144.6	4.1
49	145	138	140	150	145	143.6	4.2
50	140	150	150	145	150	147.0	4.0
51	145	145	150	150	148	147.6	2.2
52	140	142	150	150	148	146.0	4.2
53	148	142	140	150	145	145.0	3.7
54	142	152	152	145	150	148.2	4.0
55	140	145	139	132	140	139.2	4.2
56	150	150	145	150	150	149.0	2.0
57	145	142	135	130	140	138.4	5.3
58	130	145	142	135	145	139.4	6.0
59	140	140	142	150	142	142.8	3.7
60	135	150	145	135	145	142.0	6.0
61	140	140	148	150	145	144.6	4.1
62	140	140	148	150	140	143.6	4.5
63	145	150	148	145	150	147.6	2.2
64	145	140	148	150	145	145.6	3.4
65	140	150	150	145	148	146.6	3.8
66	135	130	140	148	140	138.6	6.0
67	145	148	140	138	145	143.2	3.7
68	150	140	140	150	145	145.0	4.5
69	145	150	140	145	150	146.0	3.7
70	142	138	145	150	140	143.0	4.2
71	145	152	150	145	150	148.4	2.9
72	140	135	140	150	140	141.0	4.9
73	145	150	140	140	145	144.0	3.7
74	148	140	140	150	145	144.6	4.1
Average (μm)	143.5	142.8	141.5	143.6	144.0	143.1	0.9
SD (μm)	5.1	5.4	5.2	5.4	3.8	5.0	0.6

Appendix-B. The average thickness measured from over nine points of the silicone membrane segments.

Table of Average thickness of the membrane segments of silicone membranes manufactured by DEC Manufacturing.

Sample	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9	No.10	Average	STD.DEV.
1	185	160	110	120	125	150	130	115	160	150	140.5	23.0
2	175	180	135	120	115	135	135	130	140	150	141.5	20.3
3	150	200	175	130	130	110	155	135	130	140	145.5	24.6
4	180	150	125	135	150	140	180	150	140	120	147.0	19.1
5	165	155	155	145	190	160	190	125	170	170	162.5	18.6
6	175	160	175	165	205	175	180	135	150	140	166.0	19.7
7	145	120	120	170	185	185	175	170	140	130	154.0	24.6
8	125	120	125	135	160	160	150	165	130	130	140.0	16.1
9	140	115	140	145	180	150	160	160	120	160	147.0	18.6
Average	160.0	151.1	140.0	140.6	160.0	151.7	161.7	142.8	142.2	143.3	**150.0**	**8.3**

Appendix-C1. Dexamethasone permeation throuugh PCL silicone (~150 μm), and synthetic skin (50 μm) membranes membrane

Table of Raw data for permeation of Dexamethasone through PCL membrane.

Run number	Time of sample points (hours)	Cumulative amount of dexamethasone permeated (μg)
1	4.25	5.96
	21.97	47.56
	28.89	64.50
	46.06	106.43
	52.89	122.94
	69.89	164.98
	76.89	180.13
	99.64	234.33
	123.64	291.85
	142.64	343.07
2	4.25	5.04
	21.97	38.77
	28.89	50.99
	46.06	82.85
	52.89	96.40
	69.89	129.67
	76.89	141.42
	99.64	183.32
	123.64	229.40
	142.64	269.40
3	4.00	6.57
	21.33	46.30
	45.33	105.06
	67.83	156.31
	71.13	160.90
	74.25	169.81
	92.00	215.73
4	4.00	4.76
	21.33	38.84
	45.33	83.81
	67.83	128.41
	71.13	135.22
	74.25	144.84
	92.00	183.26

Table of Raw data for permeation of Dexamethasone through silicone membrane.

Run number	Time of sample points (hours)	Cumulative amount of dexamethasone permeated (μg)
1	27.17	2.91
	48.03	4.72
	74.37	7.35
	95.70	9.10
	119.62	11.12
	142.37	13.19
2	27.17	3.53
	48.03	5.32
	74.37	8.18
	95.70	10.14
	119.62	12.48
	142.37	15.47
3	22.00	5.67
	46.00	7.13
	70.33	9.71

	94.08	13.44
	120.58	15.76
	167.66	18.49
4	22.00	4.59
	46.00	5.72
	70.33	8.31
	94.08	11.60
	120.58	12.88
	167.66	15.86

Table of Raw data for permeation of Dexamethasone through synthetic skin membrane.

Run number	Time of sample points (hours)	Cumulative amount of dexamethasone permeated (μg)
1	21.88	2.30
	46.63	4.10
	70.05	4.67
	94.80	6.25
	117.75	8.00
2	21.88	2.56
	46.63	4.91
	70.05	5.60
	94.80	6.68
	117.75	10.12
3	23.63	1.60
	47.72	3.39
	72.63	4.49
	96.00	5.51
	122.42	6.27
	144.42	7.08
4	23.63	1.71
	47.72	2.78
	72.63	4.92
	96.00	6.42
	122.42	7.00
	144.42	8.72

Appendix-C2. Dexamethasone acetate permeation throuugh PCL silicone (~150 μm), and synthetic skin (50 μm) membranes membrane

Table of Raw data for permeation of Dexamethasone acetate through PCL membrane.

Run number	Time of sample points (hours)	Cumulative amount of dexamethasone acetate permeated (μg)
1	4.25	4.42
	21.87	50.21
	29.20	72.74
	46.12	124.45
	53.17	144.12
	70.79	191.47
	77.54	202.14
	95.46	257.44
	117.88	321.29
	125.52	344.89
	142.27	386.16
2	4.25	4.54
	21.87	54.08

	29.20	77.61
	46.12	135.22
	53.17	158.70
	70.79	216.17
	77.54	236.38
	95.46	292.05
	117.88	353.85
	125.52	377.29
	142.27	425.99
3	5.50	6.80
	21.92	76.80
	28.59	90.53
	46.75	134.32
	69.42	193.38
	77.17	214.22
	94.59	260.01
	118.17	309.23

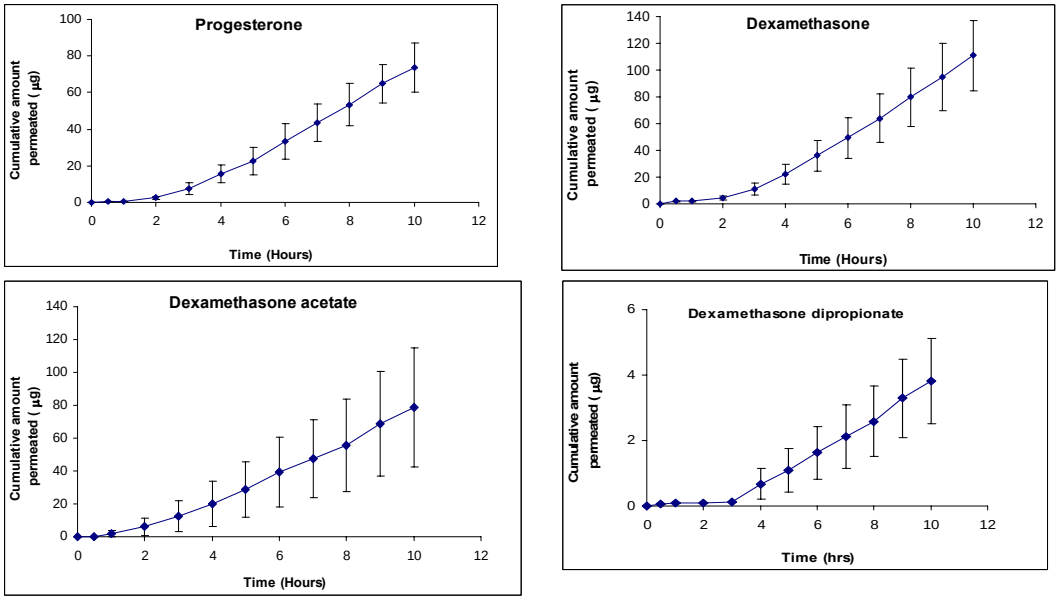
Table of raw data for permeation of Dexamethasone acetate through silicone membrane.

Run number	**Time of sample points (hours)**	**Cumulative amount of dexamethasone acetate permeated (µg)**
1	21.92	10.39
	49.17	20.33
	72.92	28.43
	95.42	35.82
	117.75	44.42
	141.25	51.81
2	21.92	10.15
	49.17	21.78
	72.92	29.66
	95.42	39.09
	117.75	46.50
	141.25	55.08
3	21.920	10.29
	46.750	17.16
	69.420	28.77
	94.590	37.22
	118.170	45.93
	142.670	54.11

Table of Raw data for permeation of Dexamethasone acetate through synthetic skin membrane.

Run number	Time of sample points (hours)	Cumulative amount of dexamethasone acetate permeated (µg)
1	24.00	1.79
	47.75	3.95
	71.92	6.12
	96.67	7.47
	121.25	10.10
	143.42	11.83
2	24.00	3.31
	47.75	5.44
	71.92	7.11
	96.67	8.29
	121.25	10.11
	143.42	11.83
3	19.88	2.92
	43.92	3.43
	70.63	4.77
	94.22	6.53
	119.80	8.65
	143.63	10.56

Appendix-D. The cumulative amount of the steroids permeated through excised mucosa over time



Figures of curves of the cumulative amount of progesterone, dexamethasone, dexamethasone acetate eter and dexamethasone dipropionate ester permeated through excised mucosa over time.

Appendix-E. Accuracy and precision of the UV assay

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for dexamethasone.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	3.93	3.96	3.90	3.93	0.024	0.62	-1.75
	2	3.99	4.16	3.75	3.97	0.17	4.24	-0.83
30 µg/mL	1	29.07	29.63	29.93	29.54	0.36	1.21	-1.52
	2	29.62	29.72	29.67	29.67	0.041	0.14	-1.10
40 µg/mL	1	39.47	38.99	38.78	39.08	0.29	0.74	-2.30
	2	40.53	39.40	39.85	39.93	0.46	1.16	-0.18

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for dexamethasone acetate.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	4.19	4.17	4.17	4.18	0.009	0.23	4.42
	2	4.06	4.14	4.17	4.12	0.046	1.13	3.08
30 µg/mL	1	29.62	29.99	29.82	29.81	0.151	0.51	-0.63
	2	29.45	30.02	29.85	29.77	0.239	0.80	-0.76
40 µg/mL	1	40.44	40.02	39.82	40.09	0.258	0.64	0.23
	2	40.18	39.92	39.85	39.98	0.142	0.36	-0.04

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for dexamethasone isonicotinate..

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	4.14	4.25	4.0	4.13	0.102	2.48	3.25
	2	4.05	4.25	4.06	4.12	0.092	2.23	3.00
30 µg/mL	1	29.7	29.89	30.02	29.87	0.131	0.440	-0.433
	2	29.75	30.2	30.02	29.99	0.185	0.617	-0.033
40 µg/mL	1	40.11	40.17	40.11	40.13	0.028	0.070	0.325
	2	40.17	40.04	39.89	40.03	0.114	0.286	0.083

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for dexamethasone valerate.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	4.48	4.50	4.43	4.47	0.03	0.76	11.73
	2	4.42	4.45	4.29	4.39	0.09	2.01	9.64
30 µg/mL	1	30.07	30.48	30.39	30.31	0.22	0.71	1.04
	2	29.93	30.37	30.48	30.26	0.29	0.95	0.87
40 µg/mL	1	40.09	39.04	39.71	39.61	0.53	1.34	0.98
	2	39.66	39.75	39.54	39.65	0.11	0.27	0.88

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for dexamethasone diproprionate.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	4.36	4.10	4.23	4.23	0.106	2.51	5.75
	2	3.99	3.92	4.23	4.05	0.133	3.28	1.17
30 µg/mL	1	29.71	30.07	30.49	30.09	0.319	1.06	0.30
	2	29.82	29.96	30.24	30.01	0.175	0.58	0.022
40 µg/mL	1	39.93	40.29	40.09	40.10	0.147	0.37	0.26
	2	39.96	39.74	40.02	39.91	0.120	0.30	-0.23

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for progesterone.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	3.80	4.35	4.31	4.15	0.250	6.03	3.83
	2	3.84	4.41	4.39	4.21	0.264	6.27	5.33
30 µg/mL	1	15.35	15.73	15.32	15.467	0.187	1.21	3.11
	2	15.17	15.69	15.24	15.367	0.230	1.50	2.44
40 µg/mL	1	20.09	20.99	20.14	20.41	0.4130	2.02	2.03
	2	20.16	21.08	20.08	20.44	0.4537	2.22	2.20

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for oestradiol benzoate.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	4.22	4.2	4.04	4.22	0.160	3.80	5.59
	2	4.16	4.22	4.18	4.24	0.060	1.35	5.97
30 µg/mL	1	14.5	15.31	15.16	14.99	0.350	2.35	-0.069
	2	14.57	15.22	15.28	15.02	0.320	2.15	0.149
40 µg/mL	1	20.49	20.25	20.01	20.25	0.200	0.97	1.25
	2	20.46	20.31	20.14	20.34	0.170	0.84	1.68

Table of Inter-day reproducibility of accuracy and precision of the lower, middle and high range for oestradiol-17β.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
4 µg/mL	1	10.02	10.67	9.86	10.18	0.350	3.44	1.83
	2	9.89	10.54	9.90	10.11	0.304	3.01	1.10
30 µg/mL	1	30.26	29.81	30.16	30.08	0.193	0.64	0.26
	2	30.40	29.65	30.10	30.05	0.308	1.03	0.17
40 µg/mL	1	39.99	39.27	40.00	39.75	0.342	0.86	-0.62
	2	40.06	39.17	39.93	39.72	0.393	0.99	-0.70

Appendix-F. The quantitation limit of the UV assay

Table of the UV quantitation limit for dexamethasone.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
1 µg/mL	1	1.14	1.02	1.05	1.07	0.07	6.10	7.02
	2	1.11	1.04	1.06	1.07	0.03	3.26	6.71
2 µg/mL	1	2.17	2.07	2.09	2.11	0.05	2.58	5.57
	2	2.17	2.08	2.09	2.11	0.05	2.29	5.57
3 µg/mL	1	3.23	3.11	3.11	3.15	0.07	2.13	5.06
	2	3.23	3.11	3.16	3.17	0.06	1.96	5.50

Table of the UV quantitation limit for dexamethasone acetate.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
1 µg/mL	1	1.09	1.06	1.12	1.09	0.03	2.88	8.88
	2	1.12	1.07	1.15	1.11	0.04	3.71	11.18
2 µg/mL	1	2.24	2.09	2.17	2.17	0.07	3.42	8.28
	2	2.18	2.12	2.14	2.15	0.04	1.64	7.29
3 µg/mL	1	3.18	3.07	3.17	3.14	0.06	2.01	4.71
	2	3.23	3.15	3.22	3.20	0.05	1.46	6.73

Table of the UV quantitation limit for dexamethasone isonicotinate.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
1 µg/mL	1	0.77	0.72	0.75	0.75	0.03	3.61	-25.19
	2	0.78	0.70	0.75	0.74	0.04	5.70	-25.82
2 µg/mL	1	1.84	1.74	1.79	1.79	0.05	2.83	-10.32
	2	1.80	1.72	1.81	1.78	0.05	2.87	-11.22
3 µg/mL	1	2.83	2.77	2.81	2.80	0.03	0.97	-6.56
	2	2.84	2.74	2.88	2.82	0.07	2.61	-6.07

Table of the UV quantitation limit for dexamethasone valerate.

Concentration (µg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
1 µg/mL	1	0.83	0.63	0.76	0.74	0.10	13.56	-25.67
	2	0.79	0.73	0.76	0.76	0.03	3.91	-24.16
2 µg/mL	1	1.83	1.72	1.78	1.78	0.06	3.16	-11.15
	2	1.83	1.77	1.83	1.81	0.03	1.84	-9.58
3 µg/mL	1	2.88	2.64	2.82	2.78	0.12	4.47	-7.36
	2	2.94	2.78	2.77	2.83	0.09	3.34	-5.61

Table of the UV quantitation limit for dexamethasone dipropionate.

Concentration (μg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
1 μg/mL	1	1.22	1.18	1.09	1.16	0.07	5.74	16.36
	2	1.25	1.20	1.15	1.20	0.05	4.13	19.66
2 μg/mL	1	2.20	2.15	2.15	2.17	0.03	1.42	8.30
	2	2.19	2.12	2.12	2.15	0.04	1.87	7.39
3 μg/mL	1	3.20	3.05	3.10	3.12	0.07	2.39	3.91
	2	3.18	3.23	3.06	3.16	0.09	2.86	5.29

Table of the UV quantitation limit for progesterone.

Concentration (μg/mL)	Replicate	Day 1	Day 2	Day 3	Mean	SD	% RSD	% Bias
1 μg/mL	1	0.64	0.66	0.60	0.63	0.03	4.91	-36.77
	2	0.64	0.64	0.60	0.62	0.02	3.76	-37.57
2 μg/mL	1	1.67	1.58	1.60	1.61	0.05	3.00	-19.28
	2	1.66	1.64	1.60	1.64	0.03	1.84	-18.22
3 μg/mL	1	2.62	2.56	2.56	2.58	0.03	1.33	-13.96
	2	2.62	2.59	2.60	2.60	0.02	0.62	-13.29

Appendix-G. Accuracy and precision of the HPLC assay

Table of the analysis of accuracy and precision for dexamethasone (N=3).

Sample concentration (μg/mL)	Actual concentration (μg/mL)	SD	% RSD	% Bias	% Recovery
0.2	0.190	0.000	0.000	5.00	95.00
2	2.123	0.00	0.222	-6.17	106.17
4	4.093	0.078	1.90	-2.33	102.33

Table of the Analysis of accuracy and precision validation standards for dexamethasone acetate HPLC assay validation (N=3).

Sample concentration (μg/mL)	Actual concentration (μg/mL)	SD	% RSD	% Bias	% Recovery
0.4	0.420	0.020	4.76	-5.00	105.0
2	2.05	0.005	0.244	-2.25	102.25
5	4.74	0.005	0.106	5.30	94.70

Table of the Analysis of accuracy and precision for dexamethasone valerate (N=3).

Sample concentration (μg/mL)	Actual concentration (μg/mL)	SD	% RSD	% Bias	% Recovery
0.4	0.400	0.020	5.0	0.0	100.00
2	2.02	0.070	3.47	-1.0	101.00
5	5.09	0.025	0.492	-1.7	101.70

Table of the Analysis of accuracy and precision for dexamethasone isonicotinate (N=3).

Sample concentration (μg/mL)	Actual concentration (μg/mL)	SD	% RSD	% Bias	% Recovery
0.4	0.380	0.007	1.94	0.503	94.97
2	1.95	0.008	0.388	1.31	97.38
5	4.74	0.001	0.014	6.51	94.79

Table of the Analysis of accuracy and precision for dexamethasone dipropionate (N=3).

Sample concentration (μg/mL)	Actual concentration (μg/mL)	SD	% RSD	% Bias	% Recovery
0.4	0.400	0.005	1.176	0.004	99.96
2	2.07	0.006	0.312	-1.774	103.55
5.0	5.19	0.016	0.315	-4.637	103.71

Table of the Analysis of accuracy and precision for progesterone (N=3).

Sample concentration (μg/mL)	Actual concentration (μg/mL)	SD	% RSD	% Bias	% Recovery
0.2	0.180		0.004	2.39	9.83
2	2.14		0.009	0.42	-6.99
4.	4.12		0.186	4.53	-2.92

Appendix-H. The limit of quantitation of the HPLC assay

Table of the Data for determination of the limit of quantitation for dexamethasone (N=3).

Sample concentration (µg/mL)	Calculated (µg/mL)	SD	% RSD	% Bias	% Recovery
0.0	0.0	0.0	0.0	00.0	0.0
0.1	0.0	0.0	0.0	100.0	0.0
0.2	2.12	0.00	0.22	-6.17	106.17

Table of the Data for determination of the limit of quantitation for dexamethasone acetate (N=3).

Sample concentration (µg/mL)	Calculated (µg/mL)	SD	% RSD	% Bias	% Recovery
0.0	0.0	0.0	0.0	00.0	0.0
0.1	0.0	0.0	0.0	100.0	0.0
0.2	0.19	0.11	11	5	95
0.4	0.40	0.027	2.7	0.0	100

Table of the Data for determination of the limit of quantitation for dexamethasone isonicotinate (N=3).

Sample concentration (µg/mL)	Calculated (µg/mL)	SD	% RSD	% Bias	% Recovery
0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	0.0	0.0	100.0	0.0
0.2	0.0	0.0	0.0	100.0	0.0
0.4	0.371	0.062	6.2	7.25	92.75

Table of the Data for determination of the limit of quantitation for dexamethasone valerate (N=3).

Sample concentration (µg/mL)	Calculated (µg/mL)	SD	% RSD	% Bias	% Recovery
0.0	0.0	0.0	0.0	00.0	0.0
0.1	0.0	0.0	0.0	100.0	0.0
0.2	0.0	0.0	0.0	100.0	0.0
0.4	0.41	0.02	2.1	2.5	97.5

Table of the Data for determination of the limit of quantitation for dexamethasone dipropionate (N=3).

Sample concentration (μg/mL)	Calculated (μg/mL)	SD	% RSD	% Bias	% Recovery
0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	0.0	0.0	100.0	0.0
0.2	0.0	0.0	0.0	100.0	0.0
0.4	0.37	0.2	2	7.5	92.5

Table of the Data for determination of the limit of quantitation for progesterone (N=3).

Sample concentration (μg/mL)	Calculated (μg/mL)	SD	% RSD	% Bias	% Recovery
0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	0.0	0.0	100.0	0.0
0.2	0.19	0.2	2	5	95

Appendix-I

Table of the Weight loss using Double silicone O-rings silicone piston

Time (Days)	Replicate		
	1	**2**	**3**
0	0	0	0
1	1.5	1	1.3
2	4.4	3.5	4.1
3	7.2	5.6	6.5
4	9.2	7.2	8
5	10.6	8.7	9.4
6	12	9.6	10.2
7	13.2	10.4	10.9
8	13.8	11	11.7
9	14.3	11.5	12.1
10	15	11.9	12.4
11	15.4	12.2	12.7
12	15.6	12.7	12.9
13	16.3	13.1	13.2

Table of the Weight loss using Santoprene (8211-35) + 10% polypropylene.

Time (Days)	Replicate		
	1	**2**	**3**
0	0	0	0
1	2.4	0.3	2.8
2	4.8	0.3	5.5
3	8.1	0.6	8.9
4	8.1	0.6	9.1
5	11.6	0.6	13
6	11.7	0.8	13.1
7	11.8	0.9	13.2
8	11.9	0.9	13.3
9	15.7	0.9	13.3
10	16	1.1	13.4
11	16	1.1	13.4
12	16	1.2	13.5
13	16.1	1.2	13.5

Table of the Weight loss using Santoprene (201-73).

Time (Days)	Replicate		
	1	**2**	**3**
0	0	0	0
1	3.7	1.7	2.8
2	3.8	6.28	5.7
3	6.7	6.5	5.8
4	10.5	11.4	9.8
5	10.6	11.5	9.9
6	10.7	11.6	9.9
7	10.9	11.7	14.8
8	11	11.8	14.8
9	16.2	11.8	14.9

10	16.3	16.9	15
11	16.5	17	15.1
12	16.5	17.1	15.1
13	16.6	17.2	15.2

Table of the Weight loss using Santoprene (8211-75) + lube.

	Replicate		
Time (Days)	**1**	**2**	**3**
0	0	0	0
1	1.2	2	3
2	4.1	5.5	5.9
3	4.3	5.6	6.1
4	8.9	10.5	6.2
5	9	10.6	6.3
6	9.1	10.7	12.9
7	9.2	10.8	13
8	9.2	10.9	13.1
9	9.3	10.9	13.2
10	16.2	11	13.2
11	16.3	11.1	13.3
12	16.3	11.1	13.4
13	16.4	11.2	13.5

Table of the Weight loss using Santoprene (8211-87A).

	Replicate		
Time (Days)	**1**	**2**	**3**
0	0	0	0
1	1.7	1.8	0.1
2	1.8	4.4	2.9
3	6.1	7	3.1
4	6.3	9.4	3.2
5	6.4	11.2	3.2
6	6.5	13.4	7.5
7	6.5	15.1	7.7
8	6.7	16.7	7.7
9	6.7	20.6	7.8
10	6.7	20.8	8
11	6.9	20.8	8
12	7	20.8	8
13	7.1	26.3	8.1

Table of the Weight loss using Santoprene (8211-87A) + 10% polypropylene.

	Replicate		
Time (Days)	**1**	**2**	**3**
0	0	0	0
1	0.3	0.2	0.3
2	0.3	2.8	2.1
3	0.4	2.8	2.1
4	0.4	2.9	5.5
5	0.5	3	5.6
6	0.6	7.1	5.7

7	0.7	6.8	5.8
8	0.7	7.3	5.9
9	0.8	7.4	5.9
10	0.9	7.5	6
11	0.9	7.5	6.1
12	1	7.6	6.2
13	1.1	7.6	6.3

Table of the Weight loss using Reflex 123E.

	Replicate		
Time (Days)	**1**	**2**	**3**
0	0	0	0
1	1.9	1.7	1.4
2	3.7	3.4	2.9
3	5.7	5.4	4.6
4	7.1	6.9	6.1
5	7.9	7.7	7.2
6	9.7	9.4	8.2
7	10.6	10.4	9
8	11.4	11.1	9.7
9	12.1	11.8	10.4
10	12.8	12.5	10.9
11	13.3	12.9	11.3
12	13.6	13.4	11.6
13	14	13.8	13.9

Table of the Weight loss using PP pistons.

	Replicate		
Time (Days)	1	2	3
0	0	0	0
1	0.3	0.1	1.9
2	0.4	0.1	4.5
3	0.4	0.2	6.9
4	0.5	0.2	9.3
5	0.6	0.3	11.6
6	0.7	0.4	14
7	0.8	0.5	16.5
8	0.9	0.6	18.4
9	0.9	0.6	20.6
10	1.1	0.8	23.4
11	1.2	0.8	23.9
12	1.2	0.9	26.1

Table of the Weight loss using Thermoflex(65A 1-800).

Time (Days)	Replicate		
	1	**2**	**3**
0	0	0	0
1	3.9	2.9	3.7
2	6.7	5.2	7.1
3	9.5	7.7	10.7
4	11.8	9.8	13.5
5	13.6	11.3	15.5
6	15.3	12.8	18.7
7	16.8	14.2	21.5
8	17.4	15	22.6
9	18.1	15.5	23.4
10	18.9	16.2	25.7
11	19.4	16.6	26.4
12	19.6	16.8	26.6
13	20.4	17	26.6

Table of the Weight loss using Desmopan 192.

Time (Days)	Replicate		
	1	**2**	**3**
0	0	0	0
1	7.3	1.3	1.2
2	8.8	2.7	2.6
3	10.3	4.1	4.1
4	11.8	5.3	5.3
5	12.8	6.3	6.2
6	13.8	7.1	7.1
7	14.9	8	7.7
8	15.6	8.9	8.6
9	16.2	9.3	8.9
10	23.5	9.7	9.4
11	23.5	10	10.7
12	23.6	10.3	10.9
13	23.6	10.7	12.3

Table of the Weight loss using Hytrel 4069.

Time (Days)	Replicate		
	1	**2**	**3**
0	0	0	0
1	3.1	3.8	2.5
2	5.6	6.9	4.4
3	27.6	9.8	6.3
4	27.7	11.2	7.9
5	27.8	11.4	8.9
6	27.8	13.7	9.8
7	27.9	15.4	10.7
8	27.9	16.5	11.4
9	28	18	11.9
10	28.1	20	12.5
11	28.1	20.9	12.8

12	28.1	21.6	13.1

Table of the Weight loss using Elastollan WYO 1388-5.

	Replicate		
Time (Days)	**1**	**2**	**3**
0	0	0	0
1	2.5	2.6	2.7
2	4.6	4.9	5
3	6.8	7.4	7.5
4	8.8	9.4	9.3
5	10.2	11.1	11
6	11.7	12.5	12.6
7	12.9	13.9	13.9
8	13.7	14.7	14.6
9	14.3	15.6	15.3
10	15	16.5	16.3
11	15.8	17.2	17
12	16.1	17.5	17.4
13	16.5	18	18

Table of the Weight loss using Silicone rubber piston on piston carriers.

	Replicate		
Time (Days)	**1**	**2**	**3**
0	0	0	0
1	3.5	2.6	2.7
2	6.7	6.2	5.1
3	10	9	7.6
4	12.6	11.6	9.4
5	14.7	30.1	11
6	17.9	30.2	12.5
7	20.6	30.2	13.4
8	22.8	30.2	14.2
9	24.9	30.2	14.7
10	25.4	30.3	15.6
11	25.6	30.3	15.9
12	26.7	30.3	16.2
13	26.8	30.3	16.3

Appendix-J

Effect of the range time of the release on the slope value of the *in vitro* drug release method.

	Time (Hours)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Mean	SD	
	0	0	0	0	0	0	0	0	0	
	0.5	1.95	2.2	1.95	1.85	1.9	1.6	1.9	0.2	
	1	3.75	4.2	3.65	3.75	3.45	3.6	3.7	0.3	
	1.5	5.5	6	5.8	5.9	5	5.5	5.6	0.4	
	2	7.35	7.95	7.6	7.8	6.6	8.0	7.5	0.5	
	3	10.5	11.45	11.2	10.65	9.65	11.0	10.7	0.6	
	4	13.65	14.4	14.45	13.75	12.45	13.6	13.7	0.7	
	5	14.2	14.95	14.9	14.35	14.55	14.3	14.5	0.3	

Convert using the slope vale (mg/Hour) divided by the drug concentration in the payload (0.5 mg/mL)

mg/Hour	Range	mg/Hour						Mean (mg/Hour)	SD	**mL/Hour**
Slope	0-5	3.0	3.1	3.2	3.0	2.9	3.0	3.0	0.1	**6.1**
Slope	0-4	3.4	3.6	3.6	3.5	3.1	3.5	3.4	0.2	**6.9**
Slope	0-3	3.5	3.8	3.7	3.6	3.2	3.8	3.6	0.2	**7.2**
Slope	0.5-5	2.9	3.0	3.1	2.9	2.9	2.9	2.9	0.1	**5.9**
Slope	0.5-4	3.3	3.5	3.6	3.4	3.0	3.5	3.4	0.2	**6.8**
Slope	0.5-3	3.4	3.7	3.7	3.6	3.1	3.8	3.6	0.3	**7.2**

Appendix-K. An example of the other *In vitro* release methods

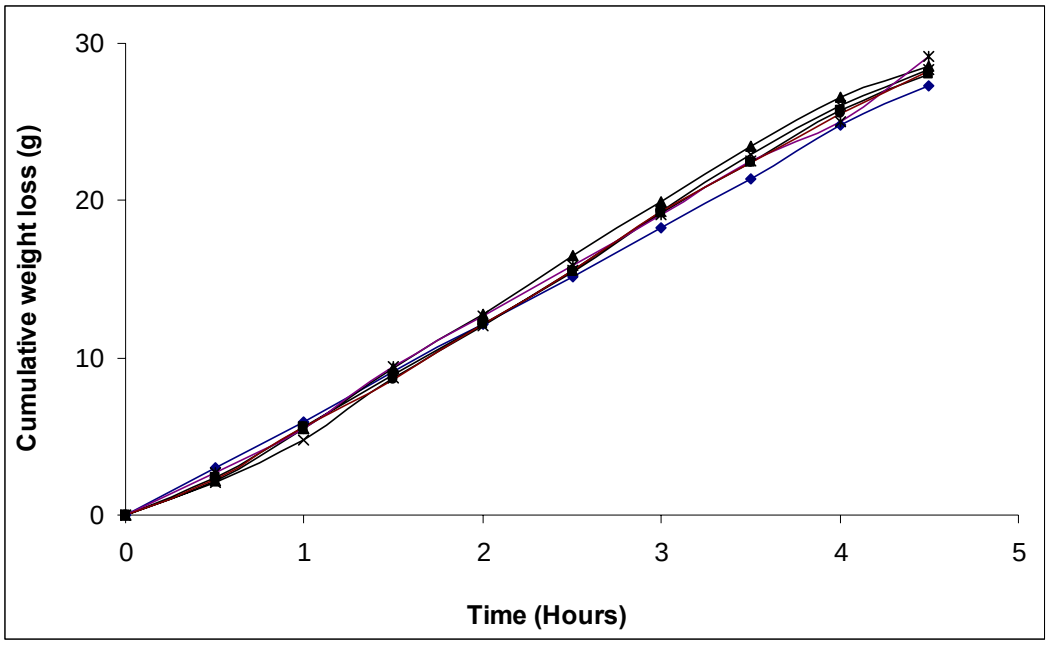
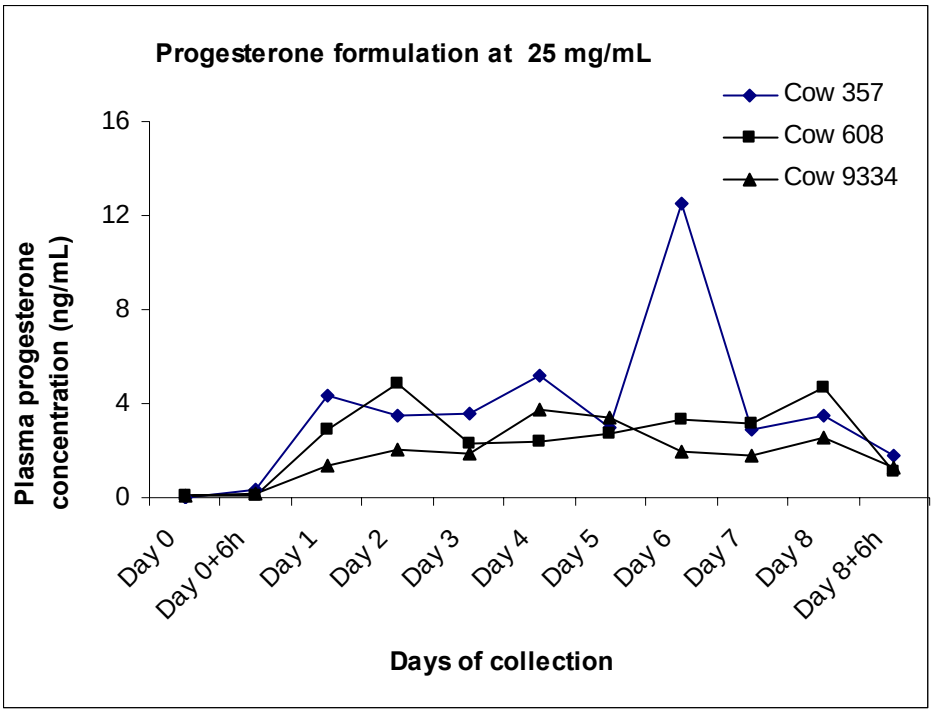
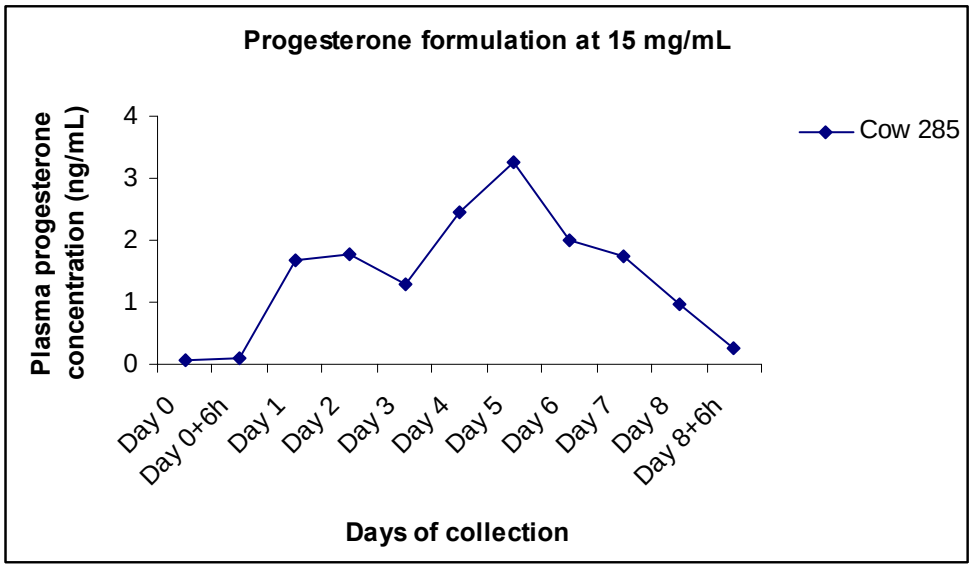
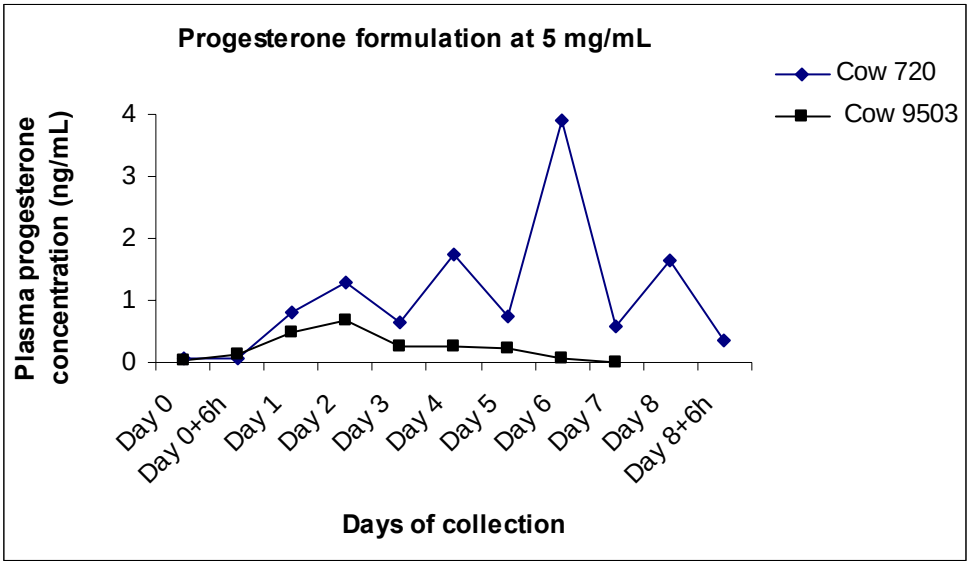


Figure of the typical example of curve showing a lag phase for the other methods.

Appendix-L. Individual cow progesterone plasma profile over time of the dose titration study.






Progesterone formulation at 5 mg/mL
Cow 720
Cow 9503
Plasma progesterone concentration (ng/mL)
4
3
2
1
0
Day 0
Day 0+6h
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 8+6h
Days of collection