In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pharmacology + Therapeutics

The University of British Columbia
Vancouver, Canada

Date August 31, 2001
Abstract

Genital herpes is a sexually transmitted disease affecting many women of childbearing age. Recurrences during childbirth can result in transmission to the neonate. The current strategy to prevent neonatal herpes has focused on the use of suppressive acyclovir therapy in late pregnancy. Issues pertaining to the safety, efficacy, and cost-effectiveness of this treatment were evaluated in our investigation. To examine the potential myelosuppressive effects of acyclovir use in late pregnancy, a clinical trial protocol was developed and implemented. A lack of enrolment in this placebo-controlled study led to inconclusive results. However, quantitative analysis of cord blood samples from the same patients revealed that although adequate blood levels are achieved with most patients at delivery, a longer duration of labor was correlated with lower levels. Inadequate levels of acyclovir could result in reactivation and viral shedding, thus endangering the neonate. Therefore, issues of noncompliance and vomiting during labor need to be addressed. For this investigation, a sensitive and rapid analytical assay with small volume requirements was developed and validated for the detection of acyclovir in plasma using capillary electrophoresis technology. With solid phase extraction and ultraviolet detection, the limit of quantification and the limit of detection was 20 ng/ml and 5.5 ng/ml, respectively. Suppressive acyclovir use in pregnancy was demonstrated to be cost-effective in a Canadian context, with most of the savings due to the avoidance of caesarean sections. This therapy was found to be especially cost effective in women with greater than 6 recurrences per year. Finally, the use of dendrimers as vaginal microbicides was proposed as an alternative strategy for the prevention of neonatal herpes. In vitro studies demonstrated that this class of compounds acts on both early and late stages of herpes simplex virus replication, making it a suitable candidate as an adjunct to existing oral antiviral therapy.
Table of Contents

Abstract .......................................................................................................................... ii

Table of Contents ........................................................................................................ iii

List of Tables ................................................................................................................ vii

List of Figures ................................................................................................................ ix

List of Abbreviations ...................................................................................................... x

Acknowledgments ......................................................................................................... xii

1. Background literature review .................................................................................... 1

1.1 Genital Herpes ........................................................................................................... 1

1.1.1 Epidemiology ......................................................................................................... 1

1.1.2 Clinical manifestations ......................................................................................... 2

1.1.3 Transmission .......................................................................................................... 3

1.1.4 Diagnosis .............................................................................................................. 4

1.1.5 Treatment ............................................................................................................. 6

1.1.6 Genital Herpes in Pregnancy .............................................................................. 7

1.1.7 Neonatal Herpes ................................................................................................ 9

1.1.8 Current Management Guidelines for Genital Herpes infections in Pregnancy ... 12

1.1.8.1 Antepartum maternal management ................................................................. 12

1.1.8.2 Caesarean Section .......................................................................................... 13

1.1.8.3 Postpartum neonatal management ................................................................. 14

1.2 Acyclovir .................................................................................................................. 15

1.2.1 Mechanism of Action .......................................................................................... 15

1.2.2 Efficacy and Safety of Acyclovir in the treatment of genital herpes in the non-

pregnant population .................................................................................................... 16

1.2.2.1 Episodic Therapy .......................................................................................... 16

1.2.2.2 Suppressive therapy ...................................................................................... 17

1.2.3 Acyclovir use in pregnancy ................................................................................ 18

1.2.3.1 Clinical Efficacy studies of suppressive acyclovir use in pregnancy ........... 20

1.3 Investigational Objectives ....................................................................................... 23

2. Adequacy of plasma acyclovir levels at delivery in a cohort of women on suppressive

acyclovir in pregnancy for the prevention of transmission to the neonate ................. 25

2.1 Introduction .............................................................................................................. 25

2.1.1 Neonatal hematological safety of acyclovir use ................................................. 25

2.1.1.1 Acyclovir use in neonates .............................................................................. 25

2.1.1.2 Fetal hematology .......................................................................................... 27

2.1.2 Pharmacokinetics of acyclovir in pregnancy ..................................................... 28
2.1.2.1 Placental kinetics ....................................................... 28
2.1.2.2 Fetal kinetics .......................................................... 30
2.1.2.3 Maternal kinetics ..................................................... 31
2.1.3 “University of Washington / University of British Columbia HSV & Pregnancy”
(UW/UBC) clinical trial ...................................................... 33
  2.1.3.1 Clinical trial protocol and objectives .............................. 33
  2.1.3.2 Neonatal haematological impact substudy .......................... 34
  2.1.3.3 Analytical assay pilot data ....................................... 35
2.2 Hypothesis ........................................................................ 36
2.3 Methods ........................................................................... 37
2.4 Results ................................................................................ 39
  2.4.1 Comparison of acyclovir vs. placebo in cord neutrophil count ................................. 39
  2.4.2 Acyclovir levels in cord and maternal blood .................................................. 39
  2.4.3 Correlation of cord venous acyclovir concentration with duration of labor ................. 40
2.5 Discussion ........................................................................... 46

3. Development and validation of a capillary electrophoresis assay for the quantification of
acyclovir levels in cord blood ...................................................... 50

  3.1 Introduction ......................................................................... 50
    3.1.1 Capillary electrophoresis .............................................. 50
    3.1.2 Analysis of acyclovir in plasma ...................................... 51
    3.1.3 Whole blood storage and preparation ............................... 52
  3.2 Hypothesis .......................................................................... 52
  3.3 Methods .............................................................................. 53
    3.3.1 Development of Assay Methodology ................................. 53
      3.3.1.1 Chemicals ............................................................. 53
      3.3.1.2 Preparation of plasma standards ................................. 53
      3.3.1.3 Solid phase extraction .............................................. 54
      3.3.1.4 Instrument and Separation Procedures ......................... 54
    3.3.2 Method Validation ......................................................... 55
    3.3.3 Method validation for acyclovir detection in blood samples ................................. 55
    3.3.4 Whole blood viability investigation .................................. 56
      3.3.4.1 Instrument and CE Procedure ..................................... 56
      3.3.4.2 Blood Sample Collection and Processing ....................... 56
      3.3.4.3 Plasma preparation .................................................. 57
      3.3.4.4 Recovery and Precision ............................................. 57
      3.3.4.5 Calibration Plot ...................................................... 57
  3.4 Results ................................................................................. 58
    3.4.1 Method development ..................................................... 58
      3.4.1.1 Sample preparation with SPE ....................................... 58
      3.4.1.2. HPCE Conditions ............................................... 58
      3.4.1.2.1 Separation .......................................................... 58
      3.4.1.2.2 Sample Stacking .................................................. 63
    3.4.2 Validation of assay ......................................................... 63
      3.4.2.1 Selectivity .............................................................. 63
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.2.2</td>
<td>Sensitivity</td>
<td>63</td>
</tr>
<tr>
<td>3.4.2.3</td>
<td>Recovery</td>
<td>64</td>
</tr>
<tr>
<td>3.4.2.7</td>
<td>Robustness</td>
<td>64</td>
</tr>
<tr>
<td>3.4.2.4</td>
<td>Linearity and Range</td>
<td>64</td>
</tr>
<tr>
<td>3.4.2.5</td>
<td>Accuracy</td>
<td>65</td>
</tr>
<tr>
<td>3.4.2.6</td>
<td>Reproducibility</td>
<td>65</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Validity of assay for qualitative detection of drug</td>
<td>71</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Whole blood storage investigation</td>
<td>73</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>76</td>
</tr>
<tr>
<td>4</td>
<td>Cost-effectiveness of suppressive acyclovir use in pregnancy</td>
<td>81</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>81</td>
</tr>
<tr>
<td>4.2</td>
<td>Hypothesis</td>
<td>83</td>
</tr>
<tr>
<td>4.3</td>
<td>Methodology</td>
<td>83</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Comparison of costs for caesarean section and vaginal delivery</td>
<td>83</td>
</tr>
<tr>
<td>4.3.1.1</td>
<td>BCCW data</td>
<td>83</td>
</tr>
<tr>
<td>4.3.1.2</td>
<td>OCCP data</td>
<td>84</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Costs pertaining to neonatal outcome</td>
<td>85</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Cost-effectiveness analysis of acyclovir prophylaxis in late pregnancy among women with recurrent genital herpes</td>
<td>86</td>
</tr>
<tr>
<td>4.4</td>
<td>Results</td>
<td>87</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Comparison of caesarean section and vaginal delivery costs</td>
<td>87</td>
</tr>
<tr>
<td>4.4.1.1</td>
<td>BCCW data</td>
<td>87</td>
</tr>
<tr>
<td>4.4.1.2</td>
<td>OCCP data</td>
<td>87</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Costs pertaining to neonatal outcome</td>
<td>90</td>
</tr>
<tr>
<td>4.4.3</td>
<td>Cost-effectiveness analysis of acyclovir prophylaxis in late pregnancy among women with recurrent genital herpes</td>
<td>90</td>
</tr>
<tr>
<td>4.5</td>
<td>Discussion</td>
<td>93</td>
</tr>
<tr>
<td>5</td>
<td>Vaginal microbicid.es as an alternative strategy for prevention of neonatal herpes – possible use of dendrimers</td>
<td>95</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>95</td>
</tr>
<tr>
<td>5.2</td>
<td>Hypothesis</td>
<td>98</td>
</tr>
<tr>
<td>5.3</td>
<td>Methods</td>
<td>99</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Compounds</td>
<td>99</td>
</tr>
<tr>
<td>5.3.2</td>
<td>Virus and cells</td>
<td>99</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Pre-treatment of cells with SPL-2999 prior to infection</td>
<td>99</td>
</tr>
<tr>
<td>5.3.4</td>
<td>Pre-mixing virus with SPL-2999</td>
<td>100</td>
</tr>
<tr>
<td>5.3.5</td>
<td>Addition of SPL-2999 at time of virus inoculation</td>
<td>100</td>
</tr>
<tr>
<td>5.3.6</td>
<td>Effect of SPL-2999 on infected cells</td>
<td>100</td>
</tr>
<tr>
<td>5.3.7</td>
<td>Effect of SPL-2999 on early step of virus replication</td>
<td>100</td>
</tr>
<tr>
<td>5.3.8</td>
<td>Data Analysis</td>
<td>101</td>
</tr>
<tr>
<td>5.4</td>
<td>Results</td>
<td>102</td>
</tr>
<tr>
<td>5.4.1</td>
<td>Antiviral effect of SPL-2999 determined by pretreatment of cells</td>
<td>102</td>
</tr>
</tbody>
</table>
List of Tables

Table 2.1. Baseline patient characteristics ................................................................. 42
Table 2.2. Comparison of mean neutrophil counts between the treatment groups .......... 42
Table 2.3. Plasma level of acyclovir in cord venous, cord arterial, and maternal blood samples ................................................................. 43
Table 3.1. Effect of pH of the phosphate wash buffer on SPE for the optimization of recovery of ACV from plasma ................................................................. 61
Table 3.2. Effect of increasing concentrations of NaCl on sample stacking in the quantification of acyclovir in plasma ................................................................. 67
Table 3.3. Accuracy of CE determination of acyclovir concentration in plasma .......... 69
Table 3.4. Intra-day and inter-day variability of the method for determining acyclovir concentrations ................................................................. 70
Table 3.5. Validation of CE determination of plasma levels of acyclovir (ACV) .......... 72
Table 3.6. Sensitivity, specificity, and predictive values for CE determination of plasma levels of acyclovir ACV) ................................................................. 72
Table 3.7. Effect of length of storage of whole blood at 4°C on determination of acyclovir concentration in plasma by capillary electrophoresis ................................................................. 74
Table 4.1. Average LDR and PP cost per delivery for the 1999/2000 fiscal year at BCCW .......... 88
Table 4.2. Average savings per vaginal delivery ................................................................. 88
Table 4.3. Average savings per vaginal delivery at OCCP hospitals ................................................................. 89
Table 4.4. Transmission rates, long-term prognosis odds, and costs of care pertaining to neonatal HSV transmission from mothers with a history of recurrent genital herpes .......... 91
Table 4.5. Cost savings per mother using acyclovir suppressive therapy ................................................................. 91
Table 4.6. Cost-effectiveness of suppressive acyclovir therapy in pregnant mothers with a history of recurrent genital herpes with <6 or ≥6 recurrences per year .......... 92
Table 5.1. Activity of SPL-2999 against HSV-1 in modified plaque reduction assays ................................................................. 103
Table 5.2. The antiviral effect of SPL-2999 on cells pre-treated with drug prior to addition of HSV-1 ................................................................. 104
Table 5.3. The antiviral effect of SPL-2999 on HSV-1 pre-mixed with drug prior to inoculation ................................................................. 105
Table 5.4. The antiviral effect of SPL-2999 on HSV-1 applied to cells at the same time. 107

Table 5.5. The antiviral effect of SPL-2999 on HSV-1 infected cells. 108

Table 5.6. The antiviral effect of SPL-2999 on HSV-1 determined by pre-incubating confluent Vero cells with various concentrations of drug for 1 h. 111
List of Figures

Figure 2.1. Plasma acyclovir concentration of each patient sample relative to the therapeutic range........................................................................................................... 44

Figure 2.2. Correlation of plasma levels of acyclovir in venous cord blood with duration of labor........................................................................................................... 45

Figure 2.3. Plasma acyclovir concentration vs. time after one 400 mg dose of acyclovir........ 49

Figure 3.1. Effect of pH of the phosphate wash buffer on SPE for the optimization of recovery of ACV from plasma.................................................................60

Figure 3.2. Effect of SDS on separation of acyclovir from other analytes in a plasma sample. 62

Figure 3.3. Effect of increasing concentrations of NaCl on sample stacking in the quantification of acyclovir in plasma.................................................................66

Figure 3.4. Demonstration of limit of quantification of acyclovir........................................68

Figure 3.5. Effect of length of storage of whole blood at 4°C on determination of acyclovir concentration in plasma by capillary electrophoresis...............................75

Figure 5.1. Molecular structure of the polylysine dendrimer SPL-2999 (formerly BRI-2999). 97

Figure 5.2. The antiviral effect of SPL-2999 on HSV-1 determined by pre-incubating confluent Vero cells with various concentrations of drug for 1 h.....................104

Figure 5.3. The antiviral effect of SPL-2999 on HSV-1 determined by pre-mixing virus with various concentrations of drug for 1 h..............................................105

Figure 5.4. The antiviral effect of SPL-2999 on HSV-1 determined by adding virus at the same time as drug..........................................................107

Figure 5.5. The antiviral effect of SPL-2999 on HSV-1 infected cells determined by adding drug 1 h after virus inoculation..........................................................108

Figure 5.6. The antiviral effect of SPL-2999 on HSV-1, for experiments where drug was applied at varying times.................................................................109

Figure 5.7. The antiviral effect of SPL-2999 on cells pretreated with drug, followed by HSV-1 incubation at 4°C.................................................................111
List of Abbreviations

ACOG – American College of Obstetricians and Gynaecologists
ACV – Acyclovir
ANC – Absolute Neutrophil Count
BCCW – British Columbia Children’s and Women’s Hospital
BID – Twice daily
CE – Capillary Electrophoresis
CMG – Case Mix Groups
CNS – Central Nervous System
DNA – Deoxyribonuclease Acid
HedU – 5-(2-hydroxyethyl)-2'-deoxyuridine
HIV – Human Immunodeficiency Virus
HPLC – High Performance Liquid Chromatography
HPβCD – Hydroxypropyl-β-cyclodextrin
HSV – Herpes Simplex Virus
LDR – Labor & Delivery Room
LOD – Limit of Detection
LOQ – Limit of Quantification
MS – Mass Spectrometry
NHANES – National Health and Nutrition Examination Surveys
OCCP – Ontario Case Costing Project
PCR – Polymerase Chain Reaction
PP – Post-Partum
QD – Once daily
RE – Relative Error
RIW – Resource Intensity Weighting
RSD – Relative Standard Deviation
SDS – Sodium Dodecyl Sulfate
SEM – Skin, Eyes, Mucous Membranes
SPE – Solid Phase Extraction
TCPC – Total Cost Per Case
TID – Three times daily
TK – Thymidine Kinase
USA – United States of America
UV – Ultraviolet
UW/UBC – University of Washington / University of British Columbia
Acknowledgments

I would like to thank my supervisor, Dr. Stephen Sacks for his time, energy, wit, and honesty in guiding me through this long but exciting journey. Dr. Deborah Money has provided great insight and has been a big help in providing the means through which I could establish a clinical investigation. Much thanks to Dr. David Godin for providing an honest evaluation of my thesis, especially for ensuring the integrity of my academic work. I would like to acknowledge the scientists at Viridae Clinical Sciences, especially Dr. Hung Vo and Dr. Yunhao Gong, who freely gave up their valuable time to mentor a basic science rookie. Special thanks to Paul Henning for his assistance with the acyclovir assay. The rest of the staff at Viridae Clinical Sciences were all a big help as well. I would also like to thank the study coordinators of the “HSV in pregnancy” study, Valencia Rempel, Bonnie Anderson, and Carole Rodger, for their help in carrying out the study protocol. Also, thanks to my fellow graduate students in the Department of Pharmacology & Therapeutics, who have provided much encouragement and camaraderie through the long rainy winters. I would like to acknowledge the financial support of the Science Council of British Columbia, which provided me with a G.R.E.A.T. Scholarship. Not to be forgotten is my church family, who has played a huge role in encouraging me to run a good race through these years. And finally, much love and admiration for my precious family who have cared for and nourished me physically, emotionally, spiritually, and mentally through the good times and the bad. I could not have completed this without any of you.
1. Background literature review

1.1 Genital Herpes

Herpes simplex virus (HSV) infections are among the most common infectious diseases in humans. Primary infection leads to a persistent state as latent infection in dorsal root ganglion, and reactivations result in symptomatic and/or asymptomatic recurrent infection. The two distinct serotypes of HSV are type 1 and type 2. Type 1 infection is associated with “above the belt” manifestations such as herpes labialis, adult herpes encephalitis, and herpes keratitis, while type 2 is more often associated with “below the belt” cases, such as herpes genitalis. Although HSV infection of either type is not usually life-threatening in immunocompetent individuals, perinatal transmission to the neonate has significant mortality and morbidity rates. Episodes, be they primary or recurrent in nature, of herpes simplex infections can be uncomfortable and disfiguring, and an unpleasant social stigma exists, especially for genital herpes. Mortality from HSV infection is primarily limited to cases of neonatal herpes, adult herpes encephalitis, and in the immunocompromised, where drug-resistance and severe mucocutaneous disease can lead to severe, life-threatening sequelae.

1.1.1 Epidemiology

Genital herpes is a sexually transmitted disease with persistent latent infection leading to recurrent genital lesions. It is one of the most common sexually transmitted diseases, and is

---

increasing in frequency to epidemic levels. Most cases (95%) are caused by herpes simplex virus type 2 (HSV-2) infection, although genital herpes due to infection of herpes simplex virus type 1 (HSV-1) is on the rise. According to the National Health and Nutrition Examination Surveys (NHANES) III, from 1988 to 1994, the seroprevalance of HSV-2 in persons 12 years or older in the USA was 21.9%, a 30% increase compared to data from NHANES II, which were collected from 1976 to 1980. In this survey, fewer than 10% of seropositive individuals reported a history of genital herpes infection. It is because of this high proportion of unrecognized cases, and the frequency of asymptomatic (absence of lesions) or subclinical (unrecognized lesions) shedding, that genital herpes continues to spread worldwide. Factors found to influence acquisition of HSV-2 include sex (women greater than men), race (blacks more than whites), marital status (divorced more than single or married), place of residence (city greater than suburb), and number of different sexual partners. The epidemiology of genital herpes infection specifically pertaining to reproductive age women will be discussed below.

1.1.2 Clinical manifestations

Disease manifestations of HSV are determined in large part by the site of virus inoculation. Reactivations occur via the sensory nerve pathways leading to the dorsal root ganglia affected by latent infection. Primary episode genital herpes presents with macules and papules on the

---

external genitalia and cervix, followed by vesicles, pustules, and ulcers. Lesions persist for about 3 weeks, and are associated with vulval pain, dysuria, vaginal discharge, and local lymphadenopathy. Furthermore, systemic symptoms such as myalgia and fever may be manifested. Primary infection is associated with larger quantities of virus replicating in the genital tract for a longer duration of time than with recurrent episodes.\textsuperscript{9,10} It is important to note that viral shedding from vulva and cervix can persist for some time even after healing of the primary lesion.\textsuperscript{11} Recurrent genital herpes is the second or subsequent episode of the clinical disease, and lesions may recur on the genitalia, perineum, or perianal areas, although approximately 10\% of patients will have manifestations of lesions on the buttocks or thighs. Recurrences are characterised by ulceration localised to a small area.

1.1.3 Transmission

Transmission of the herpes simplex virus requires physical contact of the virus with a susceptible site, such as mucosal membranes or abraded skin. While genital herpes transmission usually occurs through sexual contact, the virus does not need to come from a genital source. For example, the transmission of HSV-1 to the genital tract can be the result of oral sex, involving virus originating from an orolabial source.\textsuperscript{12} Likewise, transmission can occur to or from other

parts of the body, such as breasts or fingers. While autoinoculation is a possible mode of transmission, it is rare.\textsuperscript{13}

Virus can be shed at any time of the herpes infection, and may occur in the presence or absence of identifiable lesions or prodromal symptoms. First episodes of genital herpes have a longer duration of viral shedding than recurrent episodes. Shedding may also occur during the prodromal stage, characterised by symptoms of tingling, itching, burning, or pain. During asymptomatic periods, virus is shed on an average of 1\% of days and lasts 24 to 36 hours;\textsuperscript{14,15} therefore, it is possible that transmission can occur in the absence of symptoms.

1.1.4 Diagnosis

While both serology and culture techniques are available for the diagnosis of HSV infection, the lack of clinical expertise in recognising the symptoms of genital herpes makes this one of the most under- and mis- diagnosed sexually transmitted diseases. A genital herpes lesion may appear in varying shapes, sizes, and colours, and prodromal symptoms also vary in intensity and duration.

\textsuperscript{13} Nahmias AJ. Exogenous reinfection with herpes-simplex virus. NEJM 1971; 285:236.
While numerous serological assays are available for the detection of HSV antibodies, seropositivity that is not determined with type-specificity is often not useful clinically. In North America, the gold standard of serological assays is the Western blot, although the viral culture technique continues to be the most commonly used test for diagnosis. Using viral culture, virus specimen from active infections can be identified and differentiated in two or three days. Specimen from vesicular fluid of skin lesions can be inoculated onto a variety of tissue culture cell lines. However, since this method is dependent on successful recovery of virus from the infected site, there is a high possibility of false-negative results. Furthermore, antiviral treatment and late-stage lesion sampling are factors that decrease the chances of viral recovery. Viral DNA analysis using restriction enzyme techniques can be used for the fingerprinting of strains.\textsuperscript{16}

In recent years, polymerase chain reaction (PCR)\textsuperscript{17} for the enzymatic amplification of viral DNA has proved to be the most sensitive method to detect and differentiate HSV,\textsuperscript{18,19,20,21} especially for the detection of asymptomatic shedding. However, this technique has been under careful scrutiny as a reliable method of HSV diagnosis. Although the assay is much more sensitive than viral culture in detecting viral DNA,\textsuperscript{19} it is not known whether detection of viral DNA can be correlated with infectivity. Additionally, its limitations include increased cost and lack of

\textsuperscript{17} Hardy DA, Arvin AM, Yasukawa LL, et al. Use of polymerase chain reaction for successful identification of asymptomatic genital infection with herpes simplex virus in pregnant women at delivery. J Infect Dis 1990; 162:1031-5.
universal availability. Ironically, as time progresses, these assays may become less expensive and more accurate than culture and may replace it as the most commonly used method for diagnosis.

1.1.5 Treatment

The elimination of the herpes simplex virus from an infected host is not possible through any current forms of treatment. Even after several decades of research, no effective vaccines exist for this viral infection. Current treatment options for genital herpes are limited to the management of episodes and suppression of recurrences through oral antiviral agents. Intravenous formulations are only indicated in the immunocompromised, and effective topical agents are lacking.

Oral forms of the nucleoside analogues acyclovir, famciclovir, and valaciclovir currently dominate the antiviral market for the treatment of genital herpes. All three agents have been shown to effectively shorten the duration of both first and recurrent episodes. For patients with frequent recurrences, suppressive therapy with these agents can effectively and safely reduce the number of recurrences, although evidence for a reduction of transmission is lacking. Acyclovir is now off-patent and available through generic brands, resulting in a substantially lower cost than the other two competing agents. Acyclovir is more commonly prescribed than the other two combined. However, the dosing convenience of both famciclovir and valaciclovir over acyclovir may increase the popularity of these agents in the future.
1.1.6 Genital Herpes in Pregnancy

It is estimated that approximately 20% of reproductive age women are seropositive for HSV-2. In a recent survey of antenatal seroprevalence of HSV-2 in Canadian women, 17.3% of women aged 15 to 44 years were seropositive for HSV-2. Seroprevalence increases with age, from 7.1% for ages 15-19 years to 28.1% for ages 40-44 years. In the United States, a national survey conducted between 1988 and 1994 of over 13,000 persons 12 years of age or older revealed that approximately 25% of females are seropositive for HSV-2. This represents an increase from the 19% HSV-2 seroprevalence found in a similar survey conducted between 1976 and 1980. While such a large number are seropositive for HSV-2, only a small percentage of pregnant women have a history of genital herpes.

Not to be overlooked when considering genital herpes prevalence is the increasing number of women infected with the HSV-1 virus. A retrospective study of the prevalence of anogenital HSV isolates in the UK found that the ratio of HSV-1 and HSV-2 isolates in women ranges from 63-79% (compared to 16-39% for men) over a 13-year period. These results were supported by genital HSV-1 rates of 60%, 63%, 67.6%, and 53.8% in studies of women in both the UK and Japan. This surge in prevalence of genital HSV-1 infection can partly be explained by the increasing prevalence of unprotected oral sex. While genital HSV-1 infections are characterised

---

by lower rates of clinical recurrence, viral shedding, and transmission compared to those infected with HSV-2, \cite{28,29,30} the possibility of perinatal HSV-1 transmission to the neonate cannot be ignored. Regardless of the type of HSV involved, the above figures confirm that a high percentage of pregnant women are affected by genital herpes.

Primary HSV infection during pregnancy results in much higher rates of vertical transmission than recurrent HSV infection, and thus is an important factor to consider in the prevention of neonatal herpes. Acquisition of primary genital HSV-2 infection during pregnancy has been reported to be as low as 0.58% of uninfected women, \cite{31} although the 2.2%\cite{32} figure presented by another study is perhaps a better approximation. In that study, of women who acquired HSV during pregnancy, 30% acquired HSV in the first trimester, 30% in the second trimester, and 40% in the third trimester.\cite{32}

The recurrence rate for genital herpes is higher in pregnant women than in nonpregnant women, with the likelihood of recurrence increasing with gestational age.\cite{33} The prevalence of genital viral shedding at delivery ranges, depending on study methodology, between 0.01% and 0.6% of

\begin{thebibliography}{99}

\end{thebibliography}
all women, regardless of past history. Among untreated women with an episode of genital herpes at any time in pregnancy, 47% will have a recurrence within the last week of gestation, and 15% will have a lesion at delivery.\textsuperscript{41}

1.1.7 Neonatal Herpes

Neonatal herpes is one of the most serious consequences of HSV infection. The incidence of neonatal herpes is estimated to be approximately 7 per 100,000 live births, according to a critical review of the literature.\textsuperscript{42} The main cause of neonatal herpes infection is through contact with the herpes simplex virus at the time of delivery from the mother’s birth canal.\textsuperscript{43} Transmission occurs when the neonate comes into contact with infected secretions in the genital tract. Infection could theoretically also result from transplacental infection, and via ascending infection before labour or membrane rupture, although these cases are rare.\textsuperscript{44} A significant source of infection, in 5-10% of cases, is through an infected caregiver during the postpartum period, often transmitted from an

\begin{footnotesize}
\end{footnotesize}
orolabial infection. This risk can be eliminated through education of, and responsibility by, those who come into contact with neonates.

Due to the large numbers of pregnant women who are infected with HSV but have no prior history of genital herpes, there is a risk of asymptomatic shedding during labour. Identifying those at risk is difficult. Clinical tools, such as a detailed history of genital symptoms, may also not be helpful. In surveillance studies of neonatal HSV infections, only 22-27% of mothers of infected neonates had a history of genital HSV infection either before or during pregnancy.

Neonatal transmission occurs in 26-56% of infants delivered vaginally from mothers with first episode infections at term. The higher rate of vertical transmission from mothers with first episode infections relative to mothers with recurrent infections could be due to several factors, including longer duration (3 weeks) of virus shedding (compared to 2 to 5 days in recurrences), higher quantity of virus replicating in the genital tract, and lack of protective maternal antibody passage from mother to neonate. Transmission rates are substantially lower (~4%) for mothers with recurrent symptomatic episodes at delivery. The rate of a recurrent asymptomatic

---

transmission is even lower, at 0.04% of deliveries. In a recent survey, among the infants born to 9 women who acquired genital HSV infection shortly before labour, neonatal HSV infection occurred in four infants, one of whom died. Neonatal risk from mothers with established recurrent infection (positive HSV-2 Western blot in this study) is far lower. However, because recurrences account for the vast majority of active episodes, recurrent episodes are still responsible for a significant number of perinatal transmissions.

Neonatal HSV presentations are characterised as disseminated (45% of cases), central nervous system (CNS, 35%), or skin and mucous membrane disease (SEM, 20%). Mortality rates for treated patients with these presentations are 50%, 15%, and 0%, respectively. With CNS and disseminated disease, significant morbidity may result, with long-term neurological damage for up to half of the survivors. CNS disease involvement may present as seizures or cerebrospinal fluid pleocytosis, and the most common complications among survivors are mental retardation, chorioretinitis, seizures, and other CNS effects. The risk of severe complications increases with the length of delay in treatment, the degree of CNS involvement, and the number of recurrences the infant experiences in the first 6 months of life. SEM disease is characterized by classical vesicles, ulcerative skin lesions, conjunctivitis, or keratitis. Although it has the lowest morbidity rates of the three classifications of neonatal herpes, 14% of infants whose

---

initial HSV-2 infection appeared to be confined to SEM subsequently developed neurologic impairment,\(^5\) perhaps due to subsequent reactivation or dissemination to the CNS.\(^5\)

1.1.8 Current Management Guidelines for Genital Herpes infections in Pregnancy

1.1.8.1 Antepartum maternal management

While none of the available antiviral compounds indicated for genital herpes have received approval for use in pregnancy by the United States Federal Drug Administration or their manufacturers, acyclovir has been suggested by the American College of Obstetricians and Gynaecologists (ACOG) for both episodic and suppressive use in pregnancy.\(^5\) Oral acyclovir is recommended for the treatment of primary episodes of genital herpes in pregnancy to reduce the severity and duration of symptoms and viral shedding. Intravenous acyclovir is indicated in rare cases of disseminated HSV infection during pregnancy, due to the life-threatening nature (late- or un-treated mortality rate of 63\%)\(^5\) of this clinical manifestation.\(^5\) Episodic therapy with acyclovir for recurrences may also be considered. However, several studies have shown that the practice of obtaining weekly antepartum viral cultures from asymptomatic women with a history of genital herpes is not effective.\(^5\) In fact, this practice simply increased the number of caesarean deliveries without decreasing the incidence of neonatal herpes. Instead, current guidelines recommend the examination of the genital area upon presentation for delivery.

1.1.8.2 Caesarean Section

To reduce transmission rates to the neonate, the American College of Obstetricians and Gynaecologists has recommended that caesarean sections be performed on mothers presenting with genital herpes lesions at delivery, regardless of the length of time that the amniotic membranes may have been ruptured. However, this imposes risks to the mother and fails to properly address the differences in risk between first and recurrent episodes.

As a result of these efforts to prevent neonatal herpes, there is a high rate of caesarean section among women with recurrent genital herpes. In a study of births in Washington State, women entering pregnancy with a history of genital herpes or with their first symptomatic episode of HSV during pregnancy had a total caesarean rate of 62%, compared to 18% among women with no history of symptomatic genital herpes. Caesarean sections for suspected active recurrences are often unnecessary in mothers with established recurrent disease, given the low incidence of neonatal herpes infection in this population. In a decision-analysis, it was determined that the current policy of caesarean delivery for genital herpes lesions requires 1580 women with recurrent herpes to undergo caesarean sections to prevent one neonatal infection. Furthermore, this procedure does not guarantee the prevention of transmission, as 20-30% of cases of neonatal herpes simplex virus. Clin Infect Dis 1992; 15:1031-8.

---

60 Study protocol of the UBC/UW Project II “Acyclovir in pregnancy” study.
herpes occur in infants born by caesarean section. Evidently, in consideration of the morbidity and mortality associated with caesarean section compared to vaginal delivery, and in view of the facts presented above, alternatives to this approach to neonatal herpes prevention must be examined.

1.1.8.3 Postpartum neonatal management

Postpartum neonatal management plays an important role in the management of herpes in pregnancy. Regardless of the method of delivery, babies born to mothers who have had a recent or current episode of genital herpes should have HSV culture specimens collected from the conjunctiva, nasopharynx, and electrode surface sites. This, along with careful monitoring for skin or scalp rashes, unexplained illnesses, and seizures, and signs of sepsis, would facilitate early identification and management of neonatal infection. In cases of primary infection occurring at labour, intravenous acyclovir is probably warranted as a precautionary measure.
1.2 Acyclovir

1.2.1 Mechanism of Action

Acyclovir, an acyclic guanosine analogue, is a selective inhibitor of the replication of HSV types 1 and 2 and varicella-zoster virus. In virally infected cells, it is initially monophosphorylated by HSV-specific viral thymidine kinase (TK), then converted to its di- and tri-phosphate forms by cellular enzymes. The active form is acyclovir triphosphate, which lacks the 3'-hydroxyl group required to elongate the DNA chain. Acyclovir triphosphate competes with guanosine triphosphate as a substrate for viral DNA polymerase, thereby terminating viral DNA replication, and inactivating viral DNA polymerase. Since its discovery 20 years ago, acyclovir has become one of the most widely prescribed antiviral drugs in the world. It has proven to be effective and well tolerated for the treatment of genital herpes. As the first generation antiviral, it has had an excellent record of safety and high efficacy; however, poor oral absorption (bioavailability ~20%) and compliance issues (5 times daily regimen) have led to the development of newer agents.

---

1.2.2 Efficacy and Safety of Acyclovir in the treatment of genital herpes in the non-pregnant population

1.2.2.1 Episodic Therapy

Oral acyclovir 200 mg five times daily for 10 days (in USA, 5 days in Europe) effectively treats first episodes of genital herpes, reducing the duration of viral shedding, time to crusting, and time to healing. Acyclovir may also play a role in modifying the course of neurologic complications such as aseptic meningitis and urinary retention. Unfortunately, despite its efficacy, the requisite dosing frequency of oral acyclovir can be both inconvenient and embarrassing.

Oral acyclovir 200 mg five times daily for 5 days has been used in the episodic treatment of recurrent episodes of genital herpes. While the duration of viral shedding, time to crusting, and time to healing are reduced, studies show no improvements in the duration of symptoms nor the length of time to recurrence. In a large placebo-controlled randomized trial comparing acyclovir to valaciclovir, acyclovir was shown to reduce pain. Although oral acyclovir therapy provided a statistically significant benefit, the degree of clinical improvement perceived by the

---

patient is limited, and five times daily dosing is inconvenient. 1998 Genital Herpes Management Guidelines from the Centers for Disease Control and Prevention provides treatment options including acyclovir at the standard dose or 400 mg TID or 800 mg BID for 5 days. Neither dose has been properly studied in clinical trials.

1.2.2.2 Suppressive therapy

Acyclovir 400 mg BID is effective for continuous suppressive therapy for patients who have frequent recurrences. Daily administration reduces the frequency of recurrences by up to 80%, and 25-30% of patients had no further recurrences while taking acyclovir. Successful suppression can be maintained indefinitely with no evidence of substantial adverse effects. Reduction of the psychological morbidity of patients is an important benefit of suppressive therapy.

---

75 http://wonder.cdc.gov/wonder/prevguid/p0000480/entire.htm
Asymptomatic shedding of herpes simplex virus is thought to play a major role in transmission.\textsuperscript{12} Antivirals have been shown to significantly reduce asymptomatic and subclinical (includes shedding on days of prodrome) viral shedding when used as continuous suppressive therapy. In a crossover clinical trial of 34 women with a genital herpes history of less than 2 years, acyclovir 400 mg BID for 70 days was found to suppress subclinical shedding.\textsuperscript{85} Patients using acyclovir had significantly less (P<0.001) subclinical shedding, detected by viral isolation (0.3% of days), than patients in the placebo group (6.9% of days). This suppression of viral shedding is due to the inhibition of HSV replication in mucosal cells after reactivation of virus from its latent state in the dorsal root ganglion.

1.2.3 Acyclovir use in pregnancy

A pharmaco-epidemiologic study revealed that females of childbearing age accounted for more than half of the 2940 acyclovir users in a survey of the general medical practice of a large Health Maintenance Organization.\textsuperscript{86} Furthermore, the incidence of acyclovir use by females in the 15-44 age group (4.8 per 1000 person-years) was almost double that for males in the same age group (2.5 per 1000 person-years). This demonstrates both a higher need for genital herpes treatment in females and, more significantly, the popularity of acyclovir use among females of childbearing age. While the implications of this finding in terms of acyclovir use in pregnancy are not clear, perhaps the large numbers of women who use this drug outside of pregnancy would want to continue its use in pregnancy. Furthermore, the cessation of suppressive therapy and the lack of episodic therapy could result in further physical and emotional distress for the pregnant woman.

No teratogenicity has been observed in animal studies of acyclovir use in pregnancy when the drug was given at therapeutic levels. Pregnant rabbits and rats given subcutaneous injections of 15, 25, and 50 mg/kg/d of acyclovir did not exhibit embryotoxic or teratogenic effects. Even when doses of up to 450 mg/kg/d (30 times human therapeutic doses) were administered by gavage to mice, no adverse effects or toxicity appeared in reproduction or development over two generations. Glaxo Wellcome, the manufacturer of acyclovir, maintains an “Acyclovir in Pregnancy” registry, and there have been no reports of adverse effects due to its use in this setting. However, this registry only looks at birth defects due to acyclovir exposure, and does not record other possible side effects such as premature delivery, haematological changes, or other medical complications further into childhood.

As mentioned above, the American College of Obstetricians and Gynaecologists (ACOG) has recently released recommendations which included antiviral treatment for women with primary HSV during pregnancy, and consideration of antiviral therapy at or beyond 36 weeks of gestation in women at risk for recurrent HSV. While the ACOG mentions that studies have shown that acyclovir is safe to use during pregnancy without increasing the risk to the fetus, there is clearly a lack of randomized, placebo-controlled studies with appropriately powered sample sizes.

1.2.3.1 Clinical Efficacy studies of suppressive acyclovir use in pregnancy

Several groups have attempted controlled clinical studies to evaluate the safety and effectiveness of suppressive acyclovir therapy late in pregnancy. However, none of them had sample sizes adequate to allow definitive conclusions.

Stray-Pedersen was the first to report the results of a controlled clinical trial of acyclovir use in pregnancy. In a cohort of 92 women with a history of genital herpes before pregnancy, 46 were assigned to acyclovir 200 mg QID starting at one week before expected term, and 46 did not receive any treatment. Acyclovir was given for an average of 10 days, and no treated women had a symptomatic recurrence during the treatment period, nor was there viral shedding at delivery. On the other hand, 12 (26%) women in the control group had recurrences, and 9 (20%) of the 12 had caesarean section due to herpes, whereas none in the acyclovir group had a herpes-indicated caesarean. In both groups, 13% of subjects had a caesarean for obstetric reasons. None of the 92 infants acquired neonatal herpes, and no short-term side effects of acyclovir were observed.

In a subsequent report on the same cohort, 100 women with a history of genital herpes received acyclovir beginning 12 days before term, and 100 matched controls did not receive treatment. The control group had statistically significant higher rates of recurrences and caesarean sections (15%) than the control group (0%). Similar results were obtained in a report three years later, in which 150 patients administered acyclovir had significantly (P < 0.001) lower recurrence and

---

section rates (0%) than the matched untreated controls (14%). In both these studies, one patient in the untreated group had asymptomatic shedding at delivery. No infant in either group developed neonatal herpes.

Scott et al conducted a placebo-controlled, double-blinded study of 46 pregnant women with first episodes of genital herpes assigned to receive oral acyclovir 400mg or placebo TID beginning at 36 weeks until delivery. The acyclovir-treated group had a significantly reduced rate of clinical recurrence and caesarean section when compared with the placebo group. No woman treated with acyclovir had a caesarean delivery as a result of herpes, compared with 9 out of the 25 placebo recipients. No patient in either group experienced asymptomatic shedding at delivery, and none of the neonates acquired HSV infection. In a subsequent study conducted by Scott et al, 222 women with a history of genital herpes before pregnancy were randomized to receive acyclovir or placebo, and 92 in each group completed the study. 5 out of 92 acyclovir-treated women and 10 of 92 placebo-treated patients had a recurrence at delivery. In the intent-to-treat population, none of the 94 women treated with acyclovir experienced asymptomatic shedding at delivery, compared to three of 82 women on placebo who did.

In a two-centre randomized, placebo-controlled, study of 63 women with a history of genital herpes, subjects were given oral acyclovir 200 mg or placebo TID beginning at 36 weeks. Under different guidelines between the two centres, herpes-indicated caesarean sections were prevented.

---

performed in 25% of women in the placebo group compared with 13% of the acyclovir group. Furthermore, while 19% of the placebo-treated women had recurrences at delivery, only 6% of the acyclovir-treated group did.

A recent double-blind, randomized, placebo-controlled trial of oral acyclovir 400 mg TID beginning at 36 weeks revealed that acyclovir significantly reduced HSV lesions and viral shedding as detected by culture and PCR in late pregnancy. Only 5% of women dosed with acyclovir had a recurrent lesion at delivery, compared with 15% of women in the placebo group. Furthermore, women treated with acyclovir were less likely to have a positive PCR swab at delivery (4% compared to 25% for placebo). However, this study did not show statistical significance in the reduction of caesarean sections.

Unfortunately, no studies in late pregnancy suppression have been conclusive because of small sample size and/or failure to stratify by serologic status, asymptomatic shedding, or recurrence frequency rates. Intuitively, it would be expected that acyclovir suppression would reduce frequency rates of both symptomatic and asymptomatic recurrences. Furthermore, it is not known whether pharmacologic suppression would simply reduce symptomatic lesions to asymptomatic viral shedding. This could result in a potential problem whereby caesarean sections would be avoided at delivery not due to a reduction in transmission risk, but simply due to the absence of lesions. Nevertheless, more definitive details are needed.
Routine use of acyclovir in late pregnancy has become the standard of care in some communities. While it appears to be both desirable and cost-effective, this remains an unproven approach, with adverse effects unlikely, but potentially significant.

1.3 Investigational Objectives

Genital herpes is a disease approaching epidemic levels of infection worldwide, and affects women of childbearing age. Viral shedding at time of delivery can result in transmission of the virus to the neonate, causing potentially serious sequelae. Current strategies for the prevention of neonatal herpes recommend caesarean sections for mothers presenting with genital lesions at delivery. This strategy reduces, but does not eliminate, the incidence of neonatal herpes, and increases the potential for maternal morbidity and mortality. In the following chapters, we present investigations into therapeutic strategies for the prevention of neonatal herpes. Current investigations have focused on the suppressive use of acyclovir in late pregnancy to prevent recurrences at delivery. The objectives in this investigation are:

1) To determine cord and maternal plasma levels of acyclovir at delivery in a cohort of women treated with acyclovir in late pregnancy and to determine if adequate blood levels are achieved.

2) To develop and optimize a sensitive and rapid method for acyclovir detection in plasma using capillary electrophoresis.

3) To determine the cost-effectiveness of suppressive acyclovir use in late pregnancy in a Canadian context.
4) To examine the efficacy and mechanism of action of dendrimers as candidates for use alone, or in combination with acyclovir, as vaginal microbicides in the prevention of herpes simplex virus infection.
2. Adequacy of plasma acyclovir levels at delivery in a cohort of women on suppressive acyclovir in pregnancy for the prevention of transmission to the neonate

2.1 Introduction

2.1.1 Neonatal hematological safety of acyclovir use

2.1.1.1 Acyclovir use in neonates

Pharmacokinetic studies of acyclovir use in neonates have demonstrated the safety of acyclovir use,\textsuperscript{96,97} with no serious adverse effects even when accidentally overdosed.\textsuperscript{98} The drug appeared to be well tolerated by the neonates, although transient haematologic changes were noted during therapy in one of the aforementioned studies.\textsuperscript{97}

Several studies and case reports have also reported haematologic changes associated with acyclovir use. In a study of oral acyclovir suppressive therapy in 26 neonates,\textsuperscript{99} twelve (46\%) developed neutropenia (<1000 cells/mm\textsuperscript{3}). Ten of the patients had spontaneous recovery. Furthermore, in a study of acyclovir use in 88 neonates with HSV disease, 2 of 16 (13\%) patients on intermediate dose acyclovir (45 mg/kg/day) and 14 of 72 (19\%) patients on high dose acyclovir (60 mg/kg/day) had an absolute neutrophil count (ANC) of ≤1000 cells/mm\textsuperscript{3} during their 21-day therapy.\textsuperscript{100} In the first study, concurrent illness was documented in only 1 of the 12 patients, and additional medications that could account for the development of neutropenia were

\textsuperscript{98} McDonald LK, Corey L, Medelman PM, Opheim KE. Lack of toxicity in two cases of neonatal acyclovir overdose. Pediatr Infect Dis J 1989; 8:529-32.
not being administered, suggesting that acyclovir may have been the cause of the temporary myelosuppression. This myelosuppression could be attributed to an unknown effect of acyclovir on the bone marrow stem cells of the neonate.

There have also been other case reports of neutropenia associated with acyclovir administration in children and infants. In an infant with severe herpes simplex encephalitis, the discontinuation of acyclovir treatment led to the resolution of the neutropenia.\textsuperscript{101} However, the neutropenia returned when acyclovir therapy was reinstated. Similarly, in an 8-year-old child with neutropenia and thrombocytopenia, the cessation of chronic oral acyclovir treatment corresponded with the normalisation of his ANC and platelet count.\textsuperscript{102} In another case report, bone marrow was aspirated from a 7-year-old child treated with intravenous acyclovir who developed anaemia and mild leukopenia.\textsuperscript{103} The aspirate was found to contain decreased erythroblasts as well as increased promyelocytes and myelocytes, indicating the inability for progenitors to mature in the haematopoietic system.

One hypothesis to explain this phenomenon is that neonatal disseminated HSV infection has been associated with increasing renal and hepatic dysfunction.\textsuperscript{104} The myelosuppressive effects of acyclovir may be due to the neonate’s decreased ability to clear the drug, which is normally eliminated through renal and hepatic excretion. Outside of neonatal treatment, neutropenia has

\begin{flushleft}
\textsuperscript{100} Kimberlin DW, Jacobs RF, Powell DA, et al. The safety and efficacy of high-dose (HD) acyclovir (ACV) in neonatal herpes simplex virus (HSV) infections. Ped Res 1999; 45:165A. Abstract 963.
\end{flushleft}
been associated with acyclovir only in the presence of other myelosuppressive conditions such as in immunocompromised states\textsuperscript{71,105} or under myelotoxic drug therapy.

2.1.1.2 Fetal hematology

A study monitoring total and differential leukocyte counts in cord blood samples obtained by cordocentesis and elective caesarean section from normal fetuses of between 18 to 40 weeks' gestation revealed that the differential neutrophil count increased exponentially with gestation.\textsuperscript{165} From a mean value of 0.77 G/l at 31 weeks to 8.53 G/l at 40 weeks, the neutrophil count increases to become the most common leukocyte at term. This rise in neutrophils is paralleled by a reciprocal decrease in the erythroblast count. The changes in the number of neutrophils may reflect alterations in the pattern of differentiation of haematopoietic progenitors to meet changing fetal physiological priorities during gestation. Since the placenta acts as an effective barrier to most bacteria,\textsuperscript{106} the exponential rise in circulating neutrophils represents the acquisition of host defence mechanisms in preparation for extrauterine life. This reversal of erythroid and neutrophil production may reflect the change in the site of haematopoiesis, from the fetal liver to the fetal bone marrow, which occurs between the second and third trimesters.

Of importance to this discussion is the rise in neutrophil count in the last weeks of pregnancy, when the value of 2.60 G/l at 36 weeks rises to 8.53 G/l at 40 weeks. Clearly, the administration of potential myelosuppressive agents in the last weeks of pregnancy could have a significant impact on the neutrophil count in the fetus, and subsequently, in the term infant.

\textsuperscript{105} Bean B, Fletcher C. Neutropenia in immunocompromised patients receiving intravenous acyclovir. Program and abstracts of the 25\textsuperscript{th} interscience conference on antimicrobial agents and chemotherapy. American Society for Microbiology, 1985. 237.

\textsuperscript{106} Klein JO, Remington JS. Current concepts of infections of the fetus and newborn infant. In: Remington
Evidently, placental transfer of acyclovir during suppressive therapy could play a role in the suppression of myeloid proliferation in the fetus. This myelosuppression could lead to diminished neutrophil production, especially in the first three days post-partum (the neutrophil production cycle is between three to four days). The amount of safety data on neonates of mothers undergoing suppressive acyclovir therapy has been minimal. If approved for use in pregnancy for the suppression of genital herpes recurrences, acyclovir use could become widespread among the many pregnant individuals (approximately 20% of women of childbearing age are seropositive for HSV-2) who are affected by genital herpes. It is important to conduct a thorough evaluation of the neonatal safety of this drug.

2.1.2 Pharmacokinetics of acyclovir in pregnancy

2.1.2.1 Placental kinetics

Many drugs have been shown to cross the placenta and to appear in measurable concentrations in the fetal blood. Acyclovir is of relatively low molecular weight and is no exception. Although maternal blood does not mix freely with fetal blood, exchange occurs at capillaries of the placental villi by passive diffusion, through which the fetus also eliminates metabolic waste and drugs. The degree of protein binding of a drug is an important determinant of its movement across the placenta. During pregnancy, there is a gradual decrease in the concentrations of maternal albumin and an increase in the concentrations of fetal albumin. Drugs that bind poorly to plasma protein, such as acyclovir, reach higher concentrations in the fetus and in the amniotic fluid.

Several reports have demonstrated the transplacental passage of acyclovir. A report of acyclovir use in gestation revealed that in 18 cases, the acyclovir level in cord blood ranged from <0.5 to 1.23 μmol/L, while amniotic fluid samples contained <0.5 to 5.58 μmol/L. One case report of a pregnant woman treated for herpes encephalitis who underwent emergency caesarean section due to fetal distress showed that acyclovir was identified from urine samples taken from the infant, although neither the infant’s serum nor CSF samples contained detectable quantities of the drug. A second case report reported an acyclovir concentration of 2.95 μmol/L in cord blood obtained from a baby born to a woman with genital herpes, 13 hours after a maternal dose of 5 mg/kg.

The transport of acyclovir across the placenta has been investigated using normal term human placentas. At therapeutic concentrations, acyclovir was found to have an overall maternal-to-fetal compartment transfer rate of about 30% that of a freely diffusible marker. There was no placental metabolism, nor was there any apparent saturability of the transport system over a concentration range of 1 to 500 μg/ml. The fetal-to-maternal transfer had a rate that was slightly lower than the maternal-to-fetal rate, although the difference was not statistically significant. Since acyclovir uptake was inhibited by high concentrations of adenine and ganciclovir, but not by nucleosides, the authors concluded that the transport is by means of a carrier-dependent, nucleobase-type uptake of the drug. However, the existence of additional transport processes in contributing to the amount of drug in the fetus cannot be excluded.

In contrast to the 1.3 to 1 ratio of maternal to cord acyclovir plasma concentration reported in pharmacokinetic studies discussed below, there have been two case reports of newborn cord concentrations that exceeded maternal plasma concentrations. These case reports involve mothers who received intravenous acyclovir, and resulted in cord-maternal ratios of 1.25 and 1.45. Compounded by the phenomenon that acyclovir accumulates in breast milk at concentrations higher than those in the plasma, one cannot be certain whether there are other transport processes used by acyclovir.

2.1.2.2 Fetal kinetics

Placental metabolism, saturability of drug transport, lipid solubility, and degree of ionization all play large roles in determining the exposure of the fetus to drug. In a study of acyclovir pharmacokinetics in pregnancy, infants born to mothers who had received acyclovir during pregnancy did not accumulate acyclovir, and no acyclovir-related toxicity was reported. Due to ethical and logistical impracticalities, there have been no studies of acyclovir pharmacokinetics in the fetus. It is possible that approximations could be made with existing neonatal pharmacokinetic data.

A pharmacokinetic study of acyclovir use in critically ill neonates revealed that hepatic or renal dysfunction and prematurity are important determinants of acyclovir clearance. In healthy

neonates, clearance of acyclovir occurs at about one-third the rate of adults,\textsuperscript{117} although in general, neonatal pharmacokinetics is consistent with reports from adult studies.\textsuperscript{96} Infants with compromised renal function have a high risk for potential adverse effects due to drug accumulation and drug-related toxicities. Since premature infants have the same underdeveloped renal system as fetuses in the last 4 weeks of gestation, variable fetal clearance rates could be an issue in toxicity.

The metabolite of acyclovir, 9-carboxyhydroxymethyl guanine, which accounts for up to 14\% of a dose in persons with normal renal function, is more polar than acyclovir, and thus may not cross back to the mother through the placenta even when the concentration gradient favours such a transfer. This may result in metabolite accumulation in various fetal tissues. Whether or not this has any adverse effect is unknown. Acyclovir accumulation in the amniotic fluid, which at birth is reported to be three to six times the plasma concentration,\textsuperscript{110} may reflect the functionality of the fetal kidney to effectively excrete acyclovir.

2.1.2.3 Maternal kinetics

There have been three pharmacokinetic investigations of acyclovir use in human pregnancy to date. In 15 women given acyclovir 200mg or 400mg TID beginning at 38 weeks’ gestation until delivery, plasma levels and drug clearance were similar to those of control (nonpregnant) women.\textsuperscript{115} Acyclovir at both doses was well tolerated and did not cause adverse effects in the neonates. At steady state, fetal acyclovir plasma levels were shown to approximate the maternal plasma levels. This was later confirmed by a smaller study of five pregnant women administered

varying doses of acyclovir beginning at 37 weeks gestation.\textsuperscript{118} The maternal/fetal plasma concentration ratio was determined to be 1.3, suggesting little or no fetal accumulation of the drug. This concentration ratio is also consistent with passive diffusion. Acyclovir was found to be three to six times more concentrated in the amniotic fluid and placental tissue than in maternal and cord plasma, most likely due to excretion of acyclovir in the fetal urine. The investigators postulated that this accumulation in the amniotic fluid might be beneficial to the fetus, as it may act as a protective pharmacologic barrier to intrauterine transmission of virus.

In a more recent pharmacokinetic study, subjects received either oral acyclovir 400mg TID or its prodrug valaciclovir 500mg BID beginning at 36 weeks gestation.\textsuperscript{119} As with the first study, acyclovir was concentrated in the amniotic fluid, although there was no evidence of preferential fetal drug accumulation, as the maternal to cord vein plasma acyclovir ratio was 1.3 for subjects given acyclovir. Pharmacokinetic values and safety data obtained were similar to those reported previously. In fact, the increased blood volume of pregnant women, and other factors discussed below, surprisingly had little effect on acyclovir pharmacokinetics in any of the three clinical trials.

Maternal changes during pregnancy cause alterations in drug kinetics. A gradual increase in renal function translates to an increase in elimination rate for agents that are excreted by the kidney. Renal clearance accounts for approximately 75 to 80% of the total body clearance of acyclovir. Increases in blood volume (50%) and cardiac output (30%) lead to an increase in distribution

volume. Similarly, a decrease in serum albumin concentrations, leading to a decrease in the protein binding of drugs, also results in an increase of distribution volumes. However, acyclovir is one of the least protein-bound drugs (15%). The above-mentioned factors contribute to possible decreases in serum concentrations when compared to levels measured in nonpregnant women. The significance of these factors on the maternal kinetics of acyclovir is unknown. Nevertheless, the controlled environment of a pharmacokinetic study may not accurately depict real-life conditions during labor and delivery, where issues such as vomiting may present as significant barriers to optimal drug bioavailability.

2.1.3 “University of Washington / University of British Columbia HSV & Pregnancy” (UW/UBC) clinical trial

2.1.3.1 Clinical trial protocol and objectives

In 1995, with a grant from the United States National Institute of Health, the University of Washington began a study evaluating the effectiveness of acyclovir use in pregnancy for the prevention of viral shedding, symptomatic recurrences, and caesarean section. This was a randomized, double-blind, placebo-controlled trial of acyclovir 400 mg TID versus placebo beginning at 36 weeks gestation and continuing through labour until delivery. Healthy women with documented recurrent symptomatic genital HSV with no serious medical conditions, a normal 18-week ultrasound, and no known sensitivity to acyclovir, were recruited. The objectives of the study was to assess the effects of acyclovir on the rate of symptomatic genital HSV episodes and asymptomatic shedding among third trimester pregnant women, to attempt to decrease the caesarean section rate at term among women with recurrent genital herpes simples

---

virus infection in pregnancy by treatment with acyclovir from 36 gestation, to utilize PCR-based technology to determine if one can more precisely define women with sub-clinical shedding of HSV-2 at term, with and without acyclovir therapy, and to reduce the false-negative HSV culture rate. Since 1999, the University of British Columbia has collaborated with the University of Washington to have an additional satellite site of this clinical trial at the British Columbia Children’s and Women’s Health Centre. See Appendix I for the complete study protocol.

2.1.3.2 Neonatal haematological impact substudy

Using the cohort of women who are currently participating in the aforementioned UW/UBC clinical trial, a “neonatal haematological impact” substudy was originally developed and implemented to investigate the fetal myelosuppressive effects of suppressive acyclovir use in the third trimester of pregnancy. This substudy was initiated based on the hypothesis that the cord blood of neonates of mothers in the acyclovir treatment arm would have significantly fewer neutrophil counts when compared to the cord blood of neonates of mothers in the placebo arm. This hypothesis arose from clinical data from several clinical trials and case reports of neutropenia associated with acyclovir administration in children and infants. Because neutropenia due to diminished myeloid proliferative capacity is one of the factors that can contribute to a predisposition to bacterial infection in neonates, we felt that the consideration of this safety issue was important.

A protocol (Appendix II, and described in Methods section) was developed and implemented by the author with assistance from study coordinators Bonnie Anderson and Valencia Rempel. The

---

objectives of the investigation were to determine whether the venous cord neutrophil count is lower among neonates of mothers receiving acyclovir than those in the placebo group, and to determine whether a correlation exists between the venous cord to arterial cord acyclovir concentration ratio and the neutrophil count in neonates of the acyclovir group. Sample size calculations determined that 70 subjects were needed to achieve 0.05 significance at a power of 80%. Unfortunately, due to problems with subject recruitment, only four patients were enrolled in the first year of the study. Furthermore, logistic difficulties were encountered in the transport and delivery of cord blood to the hematology laboratory.

2.1.3.3 Analytical assay pilot data

In order to fulfill the analytical needs of the substudy, a capillary electrophoresis assay (Chapter 3) was developed and optimized to analyze the concentration of acyclovir in the cord blood. A pilot analysis of cord blood samples at delivery of a woman taking suppressive acyclovir in pregnancy revealed that the samples did not contain detectable amounts of acyclovir. The maternal to cord vein concentration ratio for acyclovir has been established to be 1.3 to 1. Therefore, since a detectable amount of acyclovir was not present in the cord blood, it is likely that amounts of acyclovir present in maternal blood may also be below the limit of detection, and thus below therapeutic levels. One possible explanation involves the compliance of the patient to the every-eight-hours dosing regimen on the day of delivery. At prescribed suppressive acyclovir regimens of 200mg five times a day, or even 400mg TID, doses may be missed during labor. The relatively short elimination half-life of acyclovir (2-3 hours), the possibility of lengthy labor times, and intrapartum pharmacokinetics may reduce drug levels at a time when the activity of the drug may be critical. Furthermore, factors such as vomiting and other gastrointestinal...
disruptions may decrease drug absorption. In the absence of efficacious drug levels, it is possible that reactivation of virus from latency can occur, resulting in viral shedding, thereby endangering the neonate.

Accordingly, we have decided to address this concern by looking at cord and maternal blood concentrations and their relationships to the duration of labor. Due to the lack of recruitment into the neonatal hematological investigation in reaching the required numbers for statistical significance, the inconsistency of substudy protocol adherence, and the new evidence suggesting the inadequacy of acyclovir plasma levels in cord blood, the hematological investigation presented here will examine both the neonatal hematological safety and the plasma levels of acyclovir as relating to the duration of labor.

2.2 Hypothesis

Infants of mothers treated with suppressive acyclovir in late pregnancy will have lower cord blood neutrophil levels than infants of mothers treated with placebo. Furthermore, in cases of noncompliance resulting from prolonged labor, mothers treated with acyclovir prophylaxis for herpes in pregnancy will have acyclovir levels in cord blood inadequate to prevent the acquisition of herpes in an exposed infant.

2.3 Methods

This was a double (investigator and patient)-blinded, placebo-controlled, and randomized study. Cord blood and, if possible, maternal blood samples of women who were participating in the "University of Washington / University of British Columbia HSV & Pregnancy" study were obtained. The samples were processed within three hours of delivery and haematological analyses of the samples were performed at the BC Women's Hospital. Determination of the acyclovir concentration was done at Viridae Clinical Sciences. The study was conducted between January of 2000 and June of 2001.

Subjects screened for the "University of Washington / University of British Columbia HSV & Pregnancy" study were asked for permission to use the umbilical cord blood from their placenta. Those women who chose to not participate in the randomized placebo-controlled study, but planned to take acyclovir as prescribed by their physician, were also asked to participate. The inclusion and exclusion criteria were the same as for the main study. Outcomes of interest included concentration of acyclovir in cord and maternal blood, and neutrophil count in cord blood. Baseline characteristics included maternal age, maternal ethnicity, gravidity, duration of labor, length of gestation, birthweight, and agpar score.

Cord blood samples were taken from the clamped umbilical cord immediately after delivery. One arterial and two venous samples were collected separately in EDTA-containing tubes. One venous sample was kept at room temperature for a maximum of three hours before delivery to the haematology lab at BCCW for hematological analysis. The other venous sample, along with the arterial sample, were stored at 4°C for a maximum of 72 hours, within which time this
sample was delivered to Viridae Clinical Sciences for determination of acyclovir concentration. The plasma component was separated by centrifugation at 1200rpm at 4°C, then stored at −70°C until electrophoresis processing. Please see Chapter 3 for the capillary electrophoresis assay methodology. A maternal blood sample was collected immediately after delivery and processed as described above for determination of acyclovir concentration.

Case report forms were used for recording all data collected in this study. The study coordinator completed case report forms by compiling information from the main study forms and faxed reports from the haematology and capillary electrophoresis laboratories. Due to the possible revelations from reports of acyclovir concentration in the case report forms, the investigators were kept blinded from the case report forms. The randomization code was broken to apportion patients into their respective treatment groups. The study protocol was approved by the Clinical Research Ethics Board, Office of Research Services and Administration, University of British Columbia, and the British Columbia’s Women’s Hospital and Health Centre Ethics Board. The full protocol and case report forms have been included as Appendix II.

Data analysis was performed using Microsoft Excel 2000 (Microsoft Corp., Seattle, WA, USA) Comparisons of baseline characteristics were performed by an unpaired 2-tailed t-test. Comparisons were made of the absolute neutrophil counts.
2.4 Results

A total of 14 subjects enrolled (Acyclovir arm, n = 10; Placebo arm, n = 4) and completed the neonatal hematology substudy. Data analysis was performed for baseline characteristics, plasma acyclovir concentration, and cord blood neutrophil count.

2.4.1 Comparison of acyclovir vs. placebo in cord neutrophil count

Except for the duration of labor, the two groups were well matched for baseline characteristics (Table 2.1). The acyclovir-treated group had a significantly (p = 0.013) longer mean duration of labor than the placebo-treated group. Due to the double-blind randomized nature of this study, this is an unexpected finding, and is unlikely to have been caused by acyclovir therapy.

Due to inconsistencies in adhering to study protocol, only 5 cord blood neutrophil counts were obtained in total (n = 3 for acyclovir group and n = 2 for placebo group). There was no statistically significant difference in neutrophil count between the two groups (Table 2.2). Surprisingly, the placebo group had a lower mean neutrophil count than the acyclovir group. However, these observations are difficult to interpret, given the small sample numbers.

2.4.2 Acyclovir levels in cord and maternal blood

A capillary electrophoresis assay was used to determine the concentration of acyclovir in cord and maternal blood samples (Table 2.3). The concentrations ranged between 36 and 1517 ng/ml (Figure 2.1). Only samples from 2 patients fell below the “therapeutic range” (see discussion section below). All others were within or above this range. One patient had an inexplicably high concentration of acyclovir, at 1517 ng/ml. The maximum plasma concentration for a 400 mg
dose of acyclovir is approximately 3 μM (~ 750 ng/ml; Figure 2.2). Assuming that this value is not due to analytical error, this suggests that the patient may have taken more doses than prescribed, or had a kinetics-related problem, such as with drug clearance.

Cord venous/arterial and maternal/cord venous ratios were determined for samples from the same subject (Table 2.3). Most venous/arterial ratios were close to 1.0 (range of 0.71 to 1.51), possibly due to error in sampling from the correct blood vessel within the umbilical cord. Due to the narrow diameter of the cord artery, it is possible to stick the needle through the arterial wall and accidentally sample the cord vein instead. The four maternal/venous cord ratios ranged between 0.72 and 5.88. However, since the time of maternal blood collection was not recorded, and due to the short half-life of acyclovir, even small delays between cord and maternal sampling could result in an inaccurate ratio.

2.4.3 Correlation of cord venous acyclovir concentration with duration of labor

For patients whose duration of labor was available, cord venous plasma concentrations, which are approximations of maternal plasma levels, were correlated with duration of labor (Figure 2.2). The Pearson’s correlation coefficient was determined to be -0.71, which demonstrates that there is a trend towards a linear correlation between duration of labor and cord venous level of acyclovir (p = 0.076 using a two-tailed t-test). This suggests that the duration of labor may have an effect on the acyclovir levels, due either to noncompliance with dosing regimen or malabsorption factors such as vomiting. It is also possible that acyclovir may have an effect on the duration of labor. However, there have been no reports in the literature on the correlation between acyclovir use and duration of labor, although none of the studies of acyclovir in
pregnancy have mentioned the duration of labor as a variable. Therefore, it is more likely that this correlation is due to noncompliance and malabsorption of drug.
Table 2.1. Baseline patient characteristics. Differences in characteristics were compared using an unpaired 2-tailed t-test.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Acyclovir (n=10)</th>
<th>Placebo (n=4)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years (range)</td>
<td>31.9 (19-39)</td>
<td>32.0 (27-37)</td>
<td>0.98</td>
</tr>
<tr>
<td>Race, % White</td>
<td>90</td>
<td>100</td>
<td>0.34</td>
</tr>
<tr>
<td>Mean education, years</td>
<td>15.3</td>
<td>15.3</td>
<td>0.97</td>
</tr>
<tr>
<td>Mean gravidity</td>
<td>1.9</td>
<td>1.5</td>
<td>0.44</td>
</tr>
<tr>
<td>Mean duration of labor, hours (range)</td>
<td>26.9&lt;sup&gt;a&lt;/sup&gt; (14-45)</td>
<td>13.8 (6-18)</td>
<td>0.013&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean infant birth weight, grams</td>
<td>3547</td>
<td>3629</td>
<td>0.76</td>
</tr>
<tr>
<td>Mean gestational age, weeks</td>
<td>39.9</td>
<td>39.3</td>
<td>0.53</td>
</tr>
<tr>
<td>Mean Agpar score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 1 min</td>
<td>7.2 (2-9)</td>
<td>8.3 (7-9)</td>
<td>0.25</td>
</tr>
<tr>
<td>At 5 min</td>
<td>9 (9)</td>
<td>9.5 (9-10)</td>
<td>0.18</td>
</tr>
</tbody>
</table>

<sup>a</sup>Duration of labor was obtained for only 8/10 subjects in the acyclovir treatment arm
<sup>b</sup>Duration of labor was the only significant difference between groups

Table 2.2. Comparison of mean neutrophil counts between the treatment groups. P-value was determined using an unpaired 2-tailed t-test.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Acyclovir (n=3)</th>
<th>Placebo (n=2)</th>
<th>p-value for comparison of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil count, G/l (mean)</td>
<td>12.31, 14.24, 10.36 (12.3)</td>
<td>9.60, 6.66 (8.1)</td>
<td>0.14</td>
</tr>
</tbody>
</table>
Table 2.3. Plasma level of acyclovir in cord venous, cord arterial, and maternal blood samples. Ratios of cord venous/arterial and maternal/venous cord levels.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[ACV] (ng/ml)</th>
<th>Cord Venous/Arterial</th>
<th>Maternal/Cord Venous</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 venous</td>
<td>242</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 arterial</td>
<td>245</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>2 venous</td>
<td>124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 arterial</td>
<td>131</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>3 venous</td>
<td>395</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 arterial</td>
<td>397</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>4 venous</td>
<td>177</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 arterial</td>
<td>175</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>5 venous</td>
<td>1517</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 venous</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 arterial</td>
<td>36</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>7 venous</td>
<td>295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 arterial</td>
<td>196</td>
<td>1.51</td>
<td></td>
</tr>
<tr>
<td>8 venous</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 arterial</td>
<td>59</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>9 venous</td>
<td>192</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 venous</td>
<td>111</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 maternal</td>
<td>80</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>11 venous</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 arterial</td>
<td>79</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>11 maternal</td>
<td>87</td>
<td>0.84</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1. Plasma acyclovir concentration of each patient sample relative to the therapeutic range.
Figure 2.2. Correlation of plasma levels of acyclovir in venous cord blood with duration of labor. Pearson correlation coefficient = -0.71 (p = 0.076).
2.5 Discussion

We have attained inconclusive results from the investigation of possible neonatal myelosuppressive effects of maternal suppressive acyclovir use. The sample number (5 in total between the 2 treatment groups) is too low even for statistical analysis, and falls far short of the sample size calculation of 70. Therefore, it is not possible at this time to make conclusions. In fact, the neonates of mothers receiving placebo appear to have a lower neutrophil count than the neonates of acyclovir-treated mothers. This is in opposition to the hypothesis that acyclovir can exhibit possible myelosuppressive effects on the fetus as manifested in lowered cord neutrophil levels.

The UW/UBC main study, which this substudy relies on for subjects, has experienced problems with subject recruitment, with an average of less than one enrolment a month. These recruitment difficulties partly arise from the patients’ unwillingness to risk being randomised into the placebo group when acyclovir is readily prescribed by doctors. Added to that is the commitment required for daily swabs and diary entry typical of many clinical trials in the genital herpes field. In fact, studies to date which have attempted to recruit adequate numbers of subjects to have enough power for clinical significance have experienced recruiting problems, and have fallen short of their recruitment goal. This includes studies both from Europe (only 63 out of a targeted 120 recruited over four years in the UK), and North America (only 222 out of a targeted 652 women over a period of 6 years).

In the literature, cord blood plasma levels of acyclovir serve as a good approximation of the maternal plasma levels, with a maternal to cord venous ratio of 1.3 to 1. However, results from our study do not match this. In our study, the maternal to cord venous ratios range from
0.72 – 5.88 to 1. As mentioned in the results section, this could be due to variability in the timing of maternal blood sampling. A linear regression analysis of the relationship between duration of labor and concentration of acyclovir in venous cord blood demonstrated that there is a possible negative correlation between these two variables (p = 0.076). However, due to the small sample size, this result is inconclusive.

Figure 2.3 is a graphical representation of the kinetics of a single dose of 400 mg acyclovir administered orally, containing an estimated “therapeutic range” encompassing 0.2 to 2.0 μM (or 50 to 500 ng/ml). (M. Reitano, personal communication) In our study, only 2 out of 10 patients receiving acyclovir had plasma levels below the “therapeutic range” (Fig. 2.1). This “therapeutic range” is an estimation based on the manufacturer’s preclinical data, an arbitrary number that has been utilized in the consideration of dosing in the clinical setting. This is far from a “magic number” above which recurrences will not occur. Rather, there is no clinical evidence that plasma levels above 0.2 μM (or 50 ng/ml) will guarantee against a clinical reactivation. According to Fig 2.3, with a dosing regimen of acyclovir 400 mg BID (every 12 h), the trough plasma ACV values do not fall below 0.5 μM (125 ng/ml). Nevertheless, clinical studies have shown that a regimen of acyclovir 400 mg BID, though somewhat effective, does not completely prevent recurrences.124

The subjects in this study are taking acyclovir 400 mg TID (every 8 h), which, according to a published pharmacokinetic study in pregnancy,115 should not fall below a steady-state trough value of 0.8 μM (200 ng/ml). If one applies 200 ng/ml as a cut-off for the minimum expected level of plasma acyclovir, then only 4 of 10 subjects achieve expected levels of acyclovir in their

---

plasma. Therefore, although only 2 patients had levels below 50 ng/ml, it is uncertain whether the levels of the other patients are adequate, and it is evident that optimal concentrations of acyclovir are not achieved in all patients.

These lower-than-expected plasma acyclovir values can be due to the duration and physical conditions of labor and delivery. Fig. 2.2 illustrates a possible negative correlation between duration of labor and plasma levels of acyclovir in cord blood. Non-compliance during a lengthy labor and delivery could be coupled with nausea, vomiting and other physiological disturbances that could decrease absorption of drug. If suppressive acyclovir use in pregnancy becomes the standard of care, the issue of compliance during labor could prove to be important. Outside the confines of a regulated pharmacokinetic study, levels of acyclovir at the time of delivery may be insufficient for the prevention of viral shedding. Further investigations need to be made into the clinical significance of the plasma acyclovir level at time of delivery and its effect on genital herpes disease manifestations.
Figure 2.3. Plasma acyclovir concentration vs. time after one 400 mg dose of acyclovir, plotted on a logarithmic scale. Shaded portion indicates estimated therapeutic range (0.2 – 2 μmol/L). (M. Reitano, personal communication)
3. Development and validation of a capillary electrophoresis assay for the quantification of acyclovir levels in cord blood.

3.1 Introduction

3.1.1 Capillary electrophoresis

Electrophoresis is a separation method based on the differential rate of migration of charged species in a buffer solution across which an electric field is applied. Electroosmotic flow dictates that cations migrate towards the negative electrode and anions migrate toward the positive electrode. Capillary electrophoresis (CE) involves the application of a high voltage through glass or silica capillaries. As with other analytical techniques, both fluorescence and absorbance is used in CE as detection methods.

The advent of capillary electrophoresis (CE) for the determination of drug levels in pharmacokinetics has provided investigators a viable alternative to high-performance liquid chromatography (HPLC). With its high separation efficiency and small sample volume requirements, CE is suitable for the monitoring of drug levels in plasma of children or in other situations in which there is a limited amount of sample available. Among other advantages CE has over HPLC are its high resolution, ease of operation, speed, and low consumable expense. While most pharmacokinetic investigations are still performed using HPLC, CE has gained popularity during recent years as a tool in the determination of drugs in biological fluids.\textsuperscript{125,126}

3.1.2 Analysis of acyclovir in plasma

Analysis of ACV in biological matrices has been made possible by a number of HPLC methods, with limits of detection ranging from 10 ng/ml to 60 ng/ml, employing different modes of detection such as fluorescence,\(^\text{127}\) direct ultraviolet (UV),\(^\text{128}\) and mass spectrometry (HPLC-MS).\(^\text{129}\) Sample preparation for these HPLC methods includes mainly a deproteinization step (using perchloric acid or organic solvents such as acetonitrile and methanol) and/or a solid phase extraction step (using a hydrophobic column), as well as other techniques such as ultrafiltration.\(^\text{130}\) There have been only two reported assays for acyclovir analysis in biological fluids using high performance capillary electrophoresis. These assays employed UV and amperometric modes of detection, achieving limits of detection in the low-\(\mu\)g/ml range.\(^\text{131,132}\)

A study comparing HPLC with CE for the determination of acyclovir in plasma and urine showed that the accuracy and reproducibility of both methods were similar.\(^\text{132}\) Pilot studies conducted at Viridae Clinical Sciences (Hung Vo, personal communication) have suggested that acyclovir determination using CE with UV detection is be capable of achieving a limit of quantification comparable to that of HPLC. Furthermore, the small sample volume requirements

---


of this assay make it an excellent tool for the analysis of acyclovir concentrations in cord blood, cerebrospinal fluid, and other pharmacokinetic samples from infants.

3.1.3 Whole blood storage and preparation

Clinical studies frequently require the collection and storage of blood samples for qualitative determination of the clinical compound of interest. The blood processing procedure is a major and essential task, consisting of centrifugation, aliquoting, and freezing of large numbers of blood samples. Whole blood is collected and stored in a variety of locations and schedules, including at remote sites and in the middle of the night. The investigation into plasma levels of acyclovir at delivery involves the determination of acyclovir in cord blood. Because the cord blood can only be collected immediately subsequent to the delivery, the times of collection are unpredictable and often inconvenient for immediate processing and analysis. Therefore, the determination of viable whole blood storage options is pertinent.

The following is a report of the development and validation of a capillary electrophoresis assay for the determination of acyclovir levels in plasma. The effect of length of storage of whole blood at 4°C on acyclovir determination is also evaluated.

3.2 Hypothesis

A novel plasma acyclovir assay that can be made available to clinicians for use in labour and delivery could be used to optimize antiviral prophylaxis strategies.
3.3 Methods

3.3.1 Development of Assay Methodology

The development and validation of the CE procedure for determination of acyclovir in cord blood was initiated and designed by the author in collaboration with staff at Viridae Clinical Sciences Inc. Here we include the methodology for the CE determination of acyclovir in plasma, as developed and optimized under the direction of Hung Vo, PhD, and with the assistance of Paul Henning at Viridae Clinical Sciences Inc.

3.3.1.1 Chemicals

Acyclovir and Borax were purchased from Sigma Chemical Co. (St. Louise, Mo, USA). The internal standard 5-(2-hydroxyethyl)-2'-deoxyuridine (HedU) was from Berry & Associates, Inc. (Dexter, MI, USA). Acetonitrile and methanol were HPLC grade from Fisher Scientific (New Jersey, NJ, USA). Hydroxypropyl-β-cyclodextrin (HPβCD) was obtained from Aldrich Chemical Co. (Milwaukee, MI, USA). The Oasis HLB solid phase extraction column and the vacuum manifold were from Waters (Milford, MA. USA). Uncoated capillaries were from Polymicro Technologies (Phoenix, AZ, USA). All other reagents were analytical grade. Water for the preparation of buffers and solutions was filtered and deionized using the ModuLab 2020 system from Continental Water System Co. (San Antonio, TX, USA). Pooled plasma was obtained from 20 healthy volunteers.

3.3.1.2 Preparation of plasma standards

Stock solutions of ACV (1.0 mg/ml) and HedU (0.5 mg/ml) were prepared and stored at −80°C. Serial dilutions of stock ACV and HedU were done in deionized water. Drug-free
plasma (600 μl), obtained by the pooling of plasma samples from over 20 volunteers, was spiked with 20 μl containing ACV (at final concentrations of 20, 100, 1000, 5000, and 10000 ng/ml) and HEdU (final concentration 1.0 μg/ml) to prepare the plasma standards.

3.3.1.3 Solid phase extraction

The Oasis solid phase extraction HLB column was conditioned with 2 ml of methanol followed with 2 ml of deionized water using a vacuum manifold. Pooled plasma sample (600 μl) was passed through the column under vacuum. The column was washed with 0.5 M phosphate buffer pH 7.4 containing 2 mM EDTA under high vacuum. Elution was achieved at flow rate of 0.2 ml/min using 200 μl of a solution containing 15% acetonitrile and 0.9% NaCl. The sample was stored at 4°C before analysis.

3.3.1.4 Instrument and Separation Procedures

Separations were performed on a Beckman MDQ automated capillary electrophoresis system (Beckman Instruments Inc., Missisauga, ON, Canada). Uncoated capillaries were used with internal diameters of 75 μm, external diameters of 360 mm and lengths of 60.2 cm (50 cm to detector). New capillaries were first rinsed with 1.0 M NaOH (10 minutes, 20 psi), followed by rinsing with the separation buffer (20 minutes, 20 psi). The new capillary was then left to equilibrate overnight in the separation buffer prior to operation. Each separation was preceded by a 1 min rinse with 1.0 M NaOH, a 1 min rinse with deionized water, followed by a 3 min rinse with the separation buffer. The sample was introduced using low-pressure (0.5 psi) injection for 100 seconds. All separations were carried out at 22°C using a voltage of 20 kV throughout the experiment. Detection of acyclovir was monitored at 254 nm using UV detection. Stock borate buffer (600 mM) was adjusted to pH 8.8 using HCl(aq) and was used
to dissolve SDS and HPβCD powders to make 90 mM borate pH 8.8 containing 175 mM SDS and 100 mM HPβCD with 0.2% NaCl, which is the separation buffer. The buffer was passed through a 0.22 μm filter (Sartorius AG, Gottingen, Germany) before analysis.

3.3.2 Method Validation

Method validation was performed by spiking 600 μl of plasma with 0, 20, 100, 1000, 5000, 10000 ng/ml of ACV and 1000 ng/ml of HedU. Validation samples were prepared with the SPE column and 50 μl was analyzed by CE as described in 3.3.1. Peak area was corrected by using the migration time of the analyte (area/migration time). Calibration was obtained by plotting the corrected peak-area ratios (ACV/HedU) versus ACV concentrations. Linear least-squares analysis was done using StatView (SAS, Cary, NC, USA) and the calibration plot was used to determine the concentrations of ACV in unknown samples. Intra-day accuracy, precision, recovery, linearity, and range were determined. The validation was repeated on different days to determine inter-day precision.

3.3.3 Method validation for acyclovir detection in blood samples

Plasma samples from the randomized placebo-controlled trial described in chapter 2 were obtained and the presence of acyclovir determined using CE by a treatment-blinded technician. The identity of the treatment was confirmed by the revelation of the randomization code, and comparisons between acyclovir detection and treatment identity were used to confirm the validity of the assay on cord blood samples. Specificity, sensitivity, and positive and negative predictive values were calculated based on comparing the identity of the treatment as determined by CE with the actual identity from the randomization code.
3.3.4 Whole blood viability investigation

3.3.4.1 Instrument and CE Procedure.

Separations were performed on an earlier generation CE procedure and instrument than the method described in section 3.3.1. A Beckman P/ACE 5500 automated capillary electrophoresis system (Beckman Instruments Inc., Missisauga, ON, Canada) was used for separation. Uncoated capillaries (Polymicro Technologies, Phoenix, AZ, USA) were used with internal diameters of 75 mm, external diameters of 360 mm and lengths of 57 cm (50 cm to detector). New capillaries were first rinsed with 1.0 M NaOH (5 minutes, 20 psi), followed by rinsing with the separation buffer BHE (20 minutes, psi). The new capillary was then left to equilibrate overnight in the separation buffer prior to operation. Each separation was preceded by a 1.5 minute rinse with 1.0 M NaOH, followed by a 3 minute rinse with the separation buffer BHE. The sample was introduced using low pressure (0.5 psi) injection for 99 seconds. All separations were carried out at 18°C and using a voltage of 30 kV throughout the experiment. Detection of acyclovir was monitored at 254 nm using a UV detector.

3.3.4.2 Blood Sample Collection and Processing

Blood samples (5.0 ml) were collected in polyethylene EDTA or heparinized tubes. Immediately after collection, the samples were spiked with 50 µl of an aqueous solution containing 100 µg/ml acyclovir. The tubes were stored at 4°C. Samples were centrifuged at times 0 (immediately), 8, 24 and 72 h after collection. Centrifugation was carried out at 1200 rpm at 4°C for 10 minutes. The plasma was separated and stored frozen at −20°C before CE analysis. Plasma was separated by centrifugation immediately after collection, and stored for 72 h before storage at −20°C.
3.3.4.3 Plasma preparation.

A 240 µl volume of the spiked plasma was mixed with 10 µl of an internal standard (guanosine at 100 µg/ml) before each analysis. Deproteinization of the sample was carried out by vortexing the plasma with 30 µl of 60% HPLC-grade perchloric acid. After 5 min at room temperature, the mixture was centrifuged at 13000 rpm for 5 min. To the supernatant was added slowly 40-45 µl of 6 N KOH until the solution pH is around 7-8 (testing done on pH paper). The solution was incubated at 4°C for 15 min, followed by centrifugation at 13000 rpm for 10 min, also at 4°C. The supernatant was filtered through a 0.22 µm membrane and stored at −20°C.

3.3.4.4 Recovery and Precision.

Analytical recovery of acyclovir was determined by comparing the peak heights of acyclovir in plasma with those obtained from direct injections of standards. Replicate samples were analyzed on separate days to determine the intra- and interassay precisions.

3.3.4.5 Calibration Plot.

Stock solution of acyclovir (0.5 mg/ml) was prepared by dissolving 25.0 mg ACV in deionized water in a 50 ml volumetric flask. The stock solution was stored at −20°C. Drug-free plasma was prepared as described above to make calibration solutions, which were prepared by serial dilutions of stock ACV in drug-free plasma over the concentration range of 50 to 50000 ng/ml. A calibration plot was obtained by plotting peak height of ACV to the concentration range of the analytes.
3.4. Results

3.4.1 Method development

This work was initiated and designed by the author under the supervision of Hung Vo, PhD, and developed and optimized by Paul Henning at Viridae Clinical Sciences Inc.

3.4.1.1 Sample preparation with SPE

Plasma samples were loaded directly onto a Waters Oasis HLB column that had been pre-conditioned with methanol and deionized water. Phosphate buffer (0.5 M) was used for washing the column instead of water to improve sample clean-up. A pH range of 7.4 to 9.5 was tested to optimize the recovery of ACV (Figure 3.1, Table 3.1). A buffered solution of 0.5 M phosphate at pH 7.4 yielded the best washing condition with the best recovery of the analyte. The elution buffer of 15% acetonitrile containing 0.9% NaCl was chosen because it provided the best ratio of analyte recovery to plasma interference while providing optimal conditions for sample stacking during HPCE separation (see below).

3.4.1.2. HPCE Conditions

3.4.1.2.1 Separation

Previously we have shown that the addition of HPβCD as a separation buffer additive can improve stacking of nucleosides to allow for greater sample concentration during electrophoresis (unpublished data). By including HPβCD (100 mM) in the separation buffer for analysis of ACV, excellent stacking was observed following a 100 s injection. While the inclusion of HPβCD in the buffer produced high theoretical plates, separation of ACV from
endogenous plasma interference was poor. By including SDS in the presence of HPβCD, ACV could be separated from plasma interference and high resolution could be achieved (Figure 3.2).
Figure 3.1. Effect of pH of the phosphate wash buffer on SPE for the optimization of recovery of ACV from plasma. The extraction column was washed with 0.5 M phosphate buffer of varying pH containing 2 mM EDTA under high vacuum. Separation was performed as described in methods section.
Table 3.1 Effect of pH of the phosphate wash buffer on SPE for the optimization of recovery of ACV from plasma.

<table>
<thead>
<tr>
<th>pH</th>
<th>$A_{254 \text{ nm}}$ (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>1.473</td>
</tr>
<tr>
<td>8.0</td>
<td>1.764</td>
</tr>
<tr>
<td>8.5</td>
<td>1.715</td>
</tr>
<tr>
<td>9.0</td>
<td>1.525</td>
</tr>
<tr>
<td>9.5</td>
<td>1.046</td>
</tr>
</tbody>
</table>
Figure 3.2. Effect of SDS on separation of acyclovir from other analytes in a plasma sample. Samples were separated using a 60 mM borate buffer pH 8.6 containing 100 mM HPβCD and a) 0 mM SDS, b) 125 mM SDS, and c) 150 mM SDS.
3.4.1.2.2 Sample Stacking

When using SDS in the presence of HPβCD, sample stacking was improved with the addition of NaCl and ACN in the sample. With 15% ACN in the sample, increasing concentrations of NaCl improved stacking up to an optimal concentration of 0.9% NaCl (Figure 3.3, Table 3.2).

3.4.2 Validation of assay

This work was initiated and designed by the author under the supervision of Hung Vo, PhD, and developed and optimized by Paul Henning at Viridae Clinical Sciences Inc.

3.4.2.1 Selectivity

The analytical peaks of ACV and HEdu were well resolved from one another and in plasma pooled from 20 people there were no interfering endogenous peaks (Figure 3.4). There was clear separation between ACV and the nearest plasma peak (resolution>2).

3.4.2.2 Sensitivity

The limit of quantitation (LOQ) was defined as the lowest drug concentration that can be determined with an absolute relative error (RE) and relative standard deviation (RSD) <20%. The lowest concentration that was tested that met this criterion was 20 ng/ml, with a RE of 15.6% and a RSD of 10.4% (n = 6). The limit of detection (LOD) was defined as the concentration giving rise to a signal that is three times the standard deviation of the blank, and was calculated to be 5.5 ng/ml.
3.4.2.3. Recovery

Recovery was determined by comparative analysis of extracted samples with standard samples prepared in the SPE elution buffer (15% ACN containing 0.9% NaCl) at concentrations of 20, 1000, and 10000 ng/ml \((n = 6)\). The average ACV recovery over the validation range was 102.9±14.2%.

3.4.2.7. Robustness

The assay was performed with separation buffer adjusted to pH 8.8±0.4 with no significant effect of the pH on the performance parameters.

3.4.2.4. Linearity and Range

Linearity was determined by plotting a standard curve from the ratio between the corrected ACV peak area to the corrected HEdU peak area \(versus\) the corresponding ACV concentration in plasma. Three different standard curves were obtained on three different days and all were found to be linear over the range of 20-10000 ng/ml by linear regression analysis \(\left( R^2 > 0.999 \right)\). Furthermore, the back-calculated mean values of the validation samples within this range were found to differ by less than 16% with the nominal values and the RSD of the validation samples were all less than 20%.
3.4.2.5. Accuracy

Accuracy of the assay was defined as the percentage of the systemic error, which was calculated as the agreement between the measured value of the validation samples and the true value as follows:

\[
\text{Accuracy} = \frac{\text{Measured Value} - \text{True Value}}{\text{True Value}} \times 100\%
\]

Accuracy values were determined over two days and were always within acceptable limits (<20%) at all validation concentrations (see Table 3.3).

3.4.2.6. Reproducibility

Reproducibility was tested on both the intra-day and inter-day reproducibility of the assay. Intra-day variability of the assay was determined by repeated analysis of the five validation samples on the same day (Table 3.4). Similarly, inter-day variability was determined by repeated analysis of the five validation samples on different days. The results summarized in Table 3.4 indicate that the assay was reproducible within the same day as well as between different days (RSD<20%).
Figure 3.3. Effect of increasing concentrations of NaCl on sample stacking in the quantification of acyclovir in plasma. Samples were separated using a 60mM borate buffer pH 8.6 containing HPβCD (100 mM), 150mM SDS, 15% ACN, and varying concentrations of NaCl.
Table 3.2. Effect of increasing concentrations of NaCl on sample stacking in the quantification of acyclovir in plasma.

<table>
<thead>
<tr>
<th>NaCl (%)</th>
<th>A254 nm (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>16.441</td>
</tr>
<tr>
<td>0.3%</td>
<td>15.551</td>
</tr>
<tr>
<td>0.6%</td>
<td>14.592</td>
</tr>
<tr>
<td>0.9%</td>
<td>18.424</td>
</tr>
<tr>
<td>1.2%</td>
<td>13.006</td>
</tr>
<tr>
<td>1.5%</td>
<td>12.335</td>
</tr>
</tbody>
</table>
Figure 3.4. Demonstration of limit of quantification of acyclovir. (A) ACV (20 ng/ml) and I.S. (1.0 μg/ml) were spiked in plasma and prepared and separated as indicated in the methodology section. (B) Separation of blank plasma.
Table 3.3 Accuracy of CE determination of acyclovir concentration in plasma, expressed as relative error (RE) in %. 600 μl of plasma was spiked with 1.0 μg/ml I.S. and 0, 20, 100, 1000, 5000, or 10000 ng/ml of ACV.

<table>
<thead>
<tr>
<th>Concentration added (ng ml⁻¹)</th>
<th>Concentration found (mean ± SD) (ng ml⁻¹)</th>
<th>Relative Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>23.1±2.4</td>
<td>15.6</td>
</tr>
<tr>
<td>100</td>
<td>107.3±11.6</td>
<td>7.3</td>
</tr>
<tr>
<td>1000</td>
<td>1089.3±28.4</td>
<td>8.9</td>
</tr>
<tr>
<td>5000</td>
<td>5176.8±114.1</td>
<td>3.5</td>
</tr>
<tr>
<td>10000</td>
<td>10160.6±352.5</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Table 3.4 Intra-day and inter-day variability of the method for determining acyclovir concentrations. 600 µl of plasma was spiked with 1.0 µg/ml I.S. and 0, 20, 100, 1000, 5000, or 10000 ng/ml of ACV on two separate days. RSD = Relative Standard Deviation.

<table>
<thead>
<tr>
<th>Concentration added (ng/ml)</th>
<th>Concentration found (mean±SD) (ng/ml)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Intra-day (n = 3)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>23.4±2.7</td>
<td>11.6</td>
</tr>
<tr>
<td>100</td>
<td>101.3±15.0</td>
<td>14.8</td>
</tr>
<tr>
<td>1000</td>
<td>1070.8±26.2</td>
<td>2.4</td>
</tr>
<tr>
<td>5000</td>
<td>5102.9±111.9</td>
<td>2.2</td>
</tr>
<tr>
<td>10000</td>
<td>10148.8±346.4</td>
<td>3.4</td>
</tr>
<tr>
<td><em>Inter-day (n = 3)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>23.1±2.4</td>
<td>10.4</td>
</tr>
<tr>
<td>100</td>
<td>107.3±11.6</td>
<td>10.8</td>
</tr>
<tr>
<td>1000</td>
<td>1089.3±28.4</td>
<td>2.6</td>
</tr>
<tr>
<td>5000</td>
<td>5176.8±114.1</td>
<td>2.2</td>
</tr>
<tr>
<td>10000</td>
<td>10160.6±352.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>
3.4.3 Validity of assay for qualitative detection of drug

To further validate the assay, 31 blood samples from a randomized placebo-controlled clinical trial were evaluated for presence of acyclovir (Table 3.5). Sensitivity, specificity, and predictive values were calculated for CE detection of acyclovir. CE results were compared against the "gold standard", which is the treatment identified by the breaking of the randomization code. CE was able to correctly predict the treatment arm for all 31 samples. Therefore, the sensitivity and specificity was determined to be 100%, and the positive and negative predictive values were also determined to be 100%.
Table 3.5. Validation of CE determination of plasma levels of acyclovir (ACV). Levels obtained by blinded CE investigator were matched against actual randomized treatment arm. BLD = Below limit of detection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Detection of ACV</th>
<th>Actual Treatment</th>
<th>Sample</th>
<th>Detection of ACV</th>
<th>Actual Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>6 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>1 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>6 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>12 venous</td>
<td>No</td>
<td>Placebo</td>
<td>7 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>12 arterial</td>
<td>No</td>
<td>Placebo</td>
<td>7 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>13 venous</td>
<td>No</td>
<td>Placebo</td>
<td>7 maternal</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>13 arterial</td>
<td>No</td>
<td>Placebo</td>
<td>15 venous</td>
<td>No</td>
<td>Placebo</td>
</tr>
<tr>
<td>2 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>15 arterial</td>
<td>No</td>
<td>Placebo</td>
</tr>
<tr>
<td>2 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>8 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>3 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>8 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>3 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>8 maternal</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>14 venous</td>
<td>No</td>
<td>Placebo</td>
<td>9 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>14 arterial</td>
<td>No</td>
<td>Placebo</td>
<td>10 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>4 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>10 maternal</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>4 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>11 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>5 venous</td>
<td>Yes</td>
<td>Acyclovir</td>
<td>11 arterial</td>
<td>Yes</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>11 maternal</td>
<td>Yes</td>
<td>Acyclovir</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6. Sensitivity, specificity, and predictive values for CE determination of plasma levels of acyclovir (ACV). Using data presented in table 3.5, calculated: Sensitivity = 100%, Specificity = 100%, Positive Predictive value = 100%, Negative Predictive value = 100%.

<table>
<thead>
<tr>
<th>Actual treatment as per randomization code</th>
<th>Acyclovir</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment as determined by CE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acyclovir</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Placebo</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>
3.4.4 Whole blood storage investigation

To determine the viability of short-term whole blood storage on analysis of acyclovir levels in plasma, whole blood was stored for various lengths of time before acyclovir spiking, separation of plasma, and determination of drug concentration. Results based on parallel experiments with samples from the same patient, are displayed in Table 3.7 and illustrated in Figure 3.5. P-values were determined using a 2-tailed unpaired T-test comparing the mean peak area of each storage time to the control mean (at 0 hours). Although differences in acyclovir concentration were present in all time points, only the 24 h storage sample produced statistically significant (p=0.034) differences in acyclovir concentration relative to the control sample, which was processed immediately after collection. These results suggest that whole blood storage for up to 72 hours after collection at 4°C will not significantly affect the concentration of acyclovir as determined by a capillary electrophoresis assay.
Table 3.7. Effect of length of storage of whole blood at 4°C on determination of acyclovir concentration in plasma by capillary electrophoresis. Whole blood was spiked with acyclovir after 0, 8, 24, and 72 h of storage and plasma concentrations determined by CE. The average adjusted acyclovir peak area was compared to the peak area at 0 h (control) using a 2-tailed unpaired t-test. 72* = spiking with acyclovir and separation of plasma performed at 0 h, then incubated at 4°C for 72 h before analysis. IS = Internal Standard, P/A = Peak Area, RSD = Relative standard deviation.

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>Sample</th>
<th>ACV P/A</th>
<th>Internal Standard</th>
<th>Adjusted Acyclovir Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P/A</td>
<td>Average P/A</td>
<td>Adjusted Ratio</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>29362</td>
<td>16473</td>
<td>0.876</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>27237</td>
<td>16196</td>
<td>0.861</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>28317</td>
<td>19599</td>
<td>1.042</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28063</td>
<td>17344</td>
<td>0.922</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>28921</td>
<td>20641</td>
<td>1.097</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>29155</td>
<td>19107</td>
<td>1.016</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>28227</td>
<td>18652</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>27190</td>
<td>20508</td>
<td>1.090</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>29026</td>
<td>20679</td>
<td>1.099</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30217</td>
<td>20538</td>
<td>1.092</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>27710</td>
<td>20146</td>
<td>1.071</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28461</td>
<td>21721</td>
<td>1.155</td>
</tr>
<tr>
<td>72</td>
<td>1</td>
<td>30298</td>
<td>16074</td>
<td>0.855</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>29886</td>
<td>15298</td>
<td>0.813</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>29560</td>
<td>18942</td>
<td>1.007</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>29609</td>
<td>16414</td>
<td>0.873</td>
</tr>
<tr>
<td>72*</td>
<td>1</td>
<td>29929</td>
<td>18672</td>
<td>0.933</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30022</td>
<td>18066</td>
<td>0.960</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30942</td>
<td>11398</td>
<td>0.606</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30677</td>
<td>13211</td>
<td>0.702</td>
</tr>
</tbody>
</table>
Figure 3.5 Effect of length of storage of whole blood at 4°C on determination of acyclovir concentration in plasma by capillary electrophoresis. Whole blood was spiked with acyclovir after 0, 8, 24, and 72 h of storage and plasma concentrations determined by CE. The average adjusted acyclovir peak area was compared to the peak area at 0 h (control) using a 2-tailed unpaired t-test. 72a = spiking with acyclovir and separation of plasma performed at 0 h, then incubated at 4°C for 72 h before analysis.
3.5 Discussion

A capillary electrophoresis assay for the determination of acyclovir concentration in cord plasma has been developed and validated. The limit of detection was determined to be 5.5 ng/ml, while the limit of quantification was determined to be 20 ng/ml. Several quantitative methods were used to confirm its validity, including the performance of validation tests on intra-day accuracy, inter-day accuracy, reproducibility, recovery, linearity, and range. The solid phase extraction procedure was optimized by using phosphate buffer at pH 7.4, because recovery was decreased at higher pH values and no significant improvement in plasma interference was observed with increasing pH. HPβCD was used in the separation buffer to increase sample stacking, thereby improving assay sensitivity. Furthermore, the addition of SDS to the separation buffer increased the resolution between acyclovir and interfering components of plasma.

For validation of use as a qualitative drug detection assay, the method was used to determine the identity of treatment of 31 plasma samples from a placebo-controlled clinical trial. While the specificity (ability to correctly identify persons without drug), sensitivity (ability to correctly identify persons taking drug), and predictive values were determined to be 100%, the low sample number may have influenced this statistic. Nevertheless, these results suggest that CE may be an useful assay for the identification of drug in plasma samples.

The storage of whole blood for up to 72 h was determined to not significantly affect the determination of acyclovir levels in plasma. The initial decrease in plasma acyclovir concentration between 0 and 24 h is probably due to uptake of the drug by red blood cells. The increase in concentration from levels obtained after storage for 24 h compared to levels obtained after storage for 72 h, on the other hand, is possibly due to lysis of these red blood cells during
storage, releasing the acyclovir into plasma, resulting in an increased level of drug in the plasma. Further experiments are needed to confirm red blood cell lysis during prolonged storage.

Therapeutic drug monitoring is the measuring of drug concentrations in biological samples commonly performed in a clinical setting. Commonly monitored agents are those in which plasma (or serum) drug concentrations outside a certain concentration range would result in either a greater risk of toxicity or reduced therapeutic efficacy. Acyclovir is an agent with a relatively large therapeutic index; however, when used as suppressive therapy during the late stages of pregnancy, its levels may fall below therapeutic levels during labor and delivery, as suggested in Chapter 2. Therefore, an assay for the optimization of dosing for this indication is needed. Effective, fast, and inexpensive drug monitoring tools may be needed for other therapies used in pregnancy.

The determination of drug concentration in biological matrices can be done using several methods including radioimmunoassay, fluorescence polarization immunoassay, enzyme-multiplied immunotechnique, and high-performance liquid chromatography (HPLC). Many of the more commonly monitored drugs can be measured by commercially available immunoassay kits, which are generally inexpensive and rapid. Therapeutic agents that do not have kits available, such as acyclovir, are commonly monitored using HPLC.

Capillary electrophoresis is a relatively new analytical tool that is proving to be increasingly useful in therapeutic drug monitoring. Separation of drugs can be achieved by taking advantage of differences in charge, hydrophobicity, size, and stereospecificity. Often, analytes can be separated with little or no preparation. These factors give CE several advantages over HPLC. Separation of drugs can be achieved by taking advantage of differences in stereospecificity.
Whereas an HPLC method would require specialized analytical columns, separation of enantiomers by CE can be achieved by adding compounds such as cyclodextrins to the separating buffer. As evidenced by this acyclovir assay, CE-based assays often have higher resolution and specificity as compared with HPLC.

Cost comparisons between CE and HPLC reveal that significantly less separating buffer is required in CE use, translating into decreases in consumable expense and waste disposal costs. Furthermore, the rapidity of the CE assay (5-20 minutes compared to 30-50 min for HPLC), and the low amount of sample preparation required also translates into lower technical labor costs. Although the cost of the analytical instrument is high, the cost of operation is much less than with HPLC.

A distinct advantage of CE over HPLC, as applicable to the investigation described in Chapter 2, is the small sample volume required for analysis. In this experiment, post-injection sample stacking was achieved through the addition of HPβCD. A typical injection volume for CE separations is in the low nanoliter (nl) range, whereas HPLC assays require volumes in the microliter (μl) range. Blood sampling volumes are limited in several clinical settings, including in the perinatal arena. Therapeutic drug monitoring in cord blood, neonatal capillary blood, or neonatal cerebrospinal fluid would require an assay that has very small sample volume requirements. For the monitoring of many drugs by HPLC, large amounts of samples – in the μl range – are needed. Capillary electrophoresis gives the potential to measure even smaller volumes, and the determination of concentrations in samples from neonatal airway surface fluid, vitreous humor, tears, and sweat may even be achieved.
Certainly, CE has its disadvantages as well. In general, HPLC has greater concentration sensitivity than CE, although with time, CE methods have been developed to overcome this disparity. For example, in our acyclovir assay, our limit of quantification of 20 ng/ml and limit of detection of 5.5 ng/ml is lower than that achieved by published HPLC methods. Our limits of quantification and detection are a vast improvement over published methods, which reported a limit of detection of 8.52 μg/ml. Additionally, concerns about the reproducibility of CE assays can be addressed by the use of an internal standard. In this experiment, HedU was employed as an internal standard. The intra-day and inter-day reproducibility studies (Tables 3.3 and 3.4) have revealed that with use of the internal standard, the relative standard deviation (RSD) can be reduced to under 20%.

Currently, the most exciting development in CE is as a potential bedside therapeutic drug-monitoring tool. Because CE separation is caused by electroosmotic flow, no mechanical pumping is required (as in HPLC). Add to this fact the small sample volumes required and the possibilities of direct sample injection, there is potential for use of portable small micro-chip based CE units to measure drug concentrations at the bedside. In this approach, on-chip sample injection and processing, followed by electrophoretic separation and analysis, would be all conducted in a time span of a few seconds. This would be a fast and convenient method of therapeutic drug monitoring, and is currently being studied using micromachining technology.

The development and validation of an effective analytical assay for the determination of acyclovir in plasma has been achieved using capillary electrophoresis. A limit of detection of 5.5 ng/ml and a limit of quantification of 20 ng/ml has been established. This is an improvement over existing HPLC methods of acyclovir quantification. The low volume sample requirement of CE makes this an ideal assay for use in perinatal investigations of pharmacokinetics or
therapeutic drug monitoring. In the study of suppressive acyclovir use in late pregnancy, this assay would be a useful tool for therapeutic drug monitoring and optimal dosing during labor and delivery, and could extend into drug monitoring in the neonate.
4. Cost-effectiveness of suppressive acyclovir use in pregnancy

4.1 Introduction

The usefulness of a pharmacological intervention is often bound by its cost-effectiveness. The current strategy for the prevention of transmission of herpes to the neonate involves the performance of caesarean sections on all women presenting with genital herpetic lesions at labour. It is estimated that the current strategy results in 1580 caesarean sections performed for every 1 case of neonatal herpes infection avoided, translating to a cost of $2.5 million per case of neonatal herpes prevented. At the same time, the increase in maternal morbidity due to the increase in caesarean sections results in an estimated 4 mothers dying for every 7 neonates saved from a herpes-related death. Suppression of recurrences of genital herpes with acyclovir in the weeks before delivery may be a more effective intervention.

There have been two cost-effectiveness analyses of acyclovir prophylaxis in late pregnancy to date, both done at USA institutions. Randolph et al used decision analysis and probabilities derived from the literature to estimate the clinical outcomes, and thus calculate the associated costs. They concluded that caesarean section for genital lesions at delivery would have a cost of more than $1.3 million per neonatal infection prevented and more than $3 million per neonatal death or disability prevented, whereas acyclovir prophylaxis for genital lesions at delivery followed by caesarean delivery would have a cost of over $493,000 per neonatal infection prevented and $1.1 million per neonatal death or disability prevented. However, it was also computed that with acyclovir prophylaxis during late pregnancy followed by caesarean delivery for genital lesions at delivery in women with recurrent genital herpes, 1818 women would be required to follow this strategy to prevent one neonatal infection.
Scott and Alexander\textsuperscript{134} used estimates of risk derived from literature reviews and prospective surveillance. They found that suppressive treatment with acyclovir in all patients with recurrent HSV could immediately save $186 per patient, with those who have frequent recurrences or first episodes benefiting even more ($391 and $455 per patient, respectively). However, acyclovir prophylaxis in patients who have a history of genital herpes with less than 6 recurrences a year would only save $14 per patient. It should be noted that these figures do not include indirect costs associated with caesarean deliveries. The studies cited are decision-analysis and risk-estimate-based and do not involve a randomized clinical trial.

These studies suggest that acyclovir prophylaxis of recurrences of genital herpes for the prevention of neonatal herpes appears to be more cost-effective than the current use of caesarean section. However, the above data are generated from costs in the USA health care system, and may not represent the situation in Canada. Although the increased morbidity, mortality, and psychological complications associated with caesarean sections cannot be calculated on a financial level, the cost data could serve as a useful tool in the consideration of the practicality of this strategy. Here, we evaluate the cost-effectiveness of acyclovir in a Canadian setting, using probability estimates from the literature and health care costs from sources within the Canadian health care system.

\textsuperscript{132} Randolph AG, Washington AE, Prober CG. Caesarean delivery for women presenting with genital herpes lesions. Efficacy, risks, and costs. JAMA 1993; 270:77-82.
4.2 Hypothesis

If clinical efficacy is similar to that of the non-pregnant individual, the use of acyclovir for the prevention of neonatal herpes in pregnancy would be cost-effective in the Canadian health care system.

4.3 Methodology

4.3.1 Comparison of costs for caesarean section and vaginal delivery

4.3.1.1 BCCW data

Hospital expenditures, delivery numbers, and caesarean section rates were obtained from British Columbia Children’s and Women’s Hospital (BCCW) finance department (for the 1999/2000 fiscal year. Hospital expenditures pertaining to cost differences affected by acyclovir therapy, such as labour and delivery room (LDR) and postpartum (PP), were obtained. The total expenditures by various LDR and PP departments (including Hospital Employees’ Union, British Columbia Nurses’ Union, the Healthy Beginnings Program, and Single Room Maternity Care) for the fiscal year 1999/2000 were summed and divided by the total number of deliveries to obtain the average LDR + PP cost per delivery. Resource intensity weightings (RIWs), calculated by dividing the number of weighted cases during the fiscal year by the number of cases, were used to determine the average LDR+PP costs per caesarean section and per vaginal delivery. The cost saving per caesarean section avoided was determined by subtracting the average cost of a vaginal delivery from the average cost of a caesarean section.

4.3.1.2 OCCP data

To determine the validity of the BCCW data on a national level, data from the Ontario Case Costing Project (OCCP)\textsuperscript{135} were used to calculate the cost difference between caesarean section and vaginal delivery. The OCCP was created by the Ontario Ministry of Health and the Ontario Hospital Association to develop information tools based on case cost data for hospital management and funding purposes. This project involves 32 Ontario hospitals that have implemented a standardized case costing methodology developed by the OCCP. Case mix groups (CMG) developed by the Canadian Institute for Health Information are used for classifying acute inpatient visits. Patients in a group have similar clinical characteristics and/or are expected to consume similar amounts of resources.

Data used in the calculations were obtained from a listing produced by the OCCP where the 1997 CMG was applied to 1995-96 typical cases. Total Costs Per Case (TCPCs) are used, which include both direct (e.g. nursing, laboratory, social work) and indirect (e.g. finance, housekeeping, food services, health records) costs. Costs include the expenses for salaries and benefits for all personnel, supplies and sundries, depreciation of equipment, and any other costs incurred by the unit in providing its services.

To compare the difference in cost between a caesarean section and a vaginal delivery, TCPCs for the two categories are weighted to account for the additional cost involved in complications. The TCPCs for neonatal hospital care of neonates >2500 g were used. The cost per caesarean section avoided was calculated by subtracting the cost per vaginal delivery from the cost per caesarean section.

\textsuperscript{135} http://www.occp.com
4.3.2 Costs pertaining to neonatal outcome

Costs associated with acute and long-term disability care due to neonatal herpes infection were calculated. Neonatal HSV infection transmission risks were obtained from a decision-analysis study by Randolph et al.^{133} Long-term prognosis odds for neonatal HSV infections were obtained from literature,^{51} acute hospital care costs were obtained from the OCCP,^{135} and life-time severe and moderate disability costs were obtained from a study of a population of children with cerebral palsy.^{136} Calculations of neonatal outcome cost per woman with recurrent genital herpes were divided into two groups, due to their difference in transmission rates – average costs per woman under suppressive acyclovir therapy in pregnancy, and average cost per woman receiving no treatment.

The cost per case for acute hospital care for infected neonates was multiplied by the rate of transmission from mothers with a history of recurrent genital herpes, giving an average acute care cost per mother with recurrent genital herpes. The cost per case for lifetime care was multiplied by the long-term prognosis odds of neonatal HSV infection and the transmission rates to give an average lifetime care cost per mother with recurrent genital herpes. Costs for severe disability and moderate disability were considered, whereas neonatal deaths and normal outcome were not, since these outcomes result in costs limited to hospital acute care. Acute and lifetime disability care costs were then summed for the two groups. The cost savings per mother using acyclovir were derived by subtracting the average cost per mother under acyclovir suppressive therapy from the average cost per mother not using acyclovir.


85
4.3.3 Cost-effectiveness analysis of acyclovir prophylaxis in late pregnancy among women with recurrent genital herpes

Women with a history of genital herpes can be divided into two groups: those who experience 6 or more recurrences per year and those who experience less than 6 recurrences per year. BCCW cost data computed in 4.3.1 were combined with treated and untreated transmission rates from a cost-effectiveness study published by Scott et al. The reduction in caesarean section rate due to acyclovir therapy was calculated by subtracting the recurrence rate at delivery of those on acyclovir therapy from the recurrence rates of those with no therapy. This reduction in rate was multiplied by cost savings per caesarean avoided, giving an average obstetric saving per mother on acyclovir therapy. The total savings per mother on acyclovir therapy was determined by adding the average obstetric savings per mother to the neonatal outcome cost calculated in 4.3.2, and subtracting from that figure the cost of acyclovir, which was taken to be $140, based on a cost of approximately $1.60 for a 400 mg tablet, taken three times daily for 4 weeks plus a $5 pharmacy fee.
4.4 Results

4.4.1 Comparison of caesarean section and vaginal delivery costs

4.4.1.1 BCCW data:
To determine the difference in cost between a caesarean section and a vaginal delivery at BCCW, the average hospital cost per delivery was calculated ($2427, Table 4.1). This cost was then multiplied by the RIW for the type of delivery to calculate the cost per case. Results demonstrate that the total hospital cost per caesarean delivery ($3606, Table 4.2) was $1600 higher per case than the total hospital costs per vaginal delivery ($2006, Table 4.2) at BCCW.

4.4.1.2 OCCP data:
To determine the difference in both indirect and direct costs between a caesarean section and a vaginal delivery at OCCP hospitals, average costs for different types of deliveries were weighted and added to average neonatal care costs. The total hospital cost per caesarean delivery ($3876, Table 3) was $1782 higher per case than the total hospital cost per vaginal delivery ($2094). The results reveal that the savings per caesarean avoided at BCCW ($1600) approximates the savings per caesarean avoided at OCCP hospitals ($1782).
Table 4.1. Average LDR and PP cost per delivery for the 1999/2000 fiscal year at BCCW, as calculated by adding total expenditures from all LDR and PP departments and dividing the sum by the total number of deliveries at BCCW.

<table>
<thead>
<tr>
<th>Department</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP - Hospital Employees' Union</td>
<td>$523000</td>
</tr>
<tr>
<td>PP - British Columbia Nurses’ Union</td>
<td>$5540000</td>
</tr>
<tr>
<td>Healthy Beginnings Program</td>
<td>$325000</td>
</tr>
<tr>
<td>LDR - Hospital Employees’ Union</td>
<td>$1567000</td>
</tr>
<tr>
<td>LDR - British Columbia Nurses’ Union</td>
<td>$7445000</td>
</tr>
<tr>
<td>Single Room Maternity Care</td>
<td>$1511000</td>
</tr>
<tr>
<td><strong>Total LDR+PP expenditures</strong></td>
<td><strong>$16911000</strong></td>
</tr>
</tbody>
</table>

| Total deliveries at BCCW                         | 6967     |
| Average LDR+PP cost / delivery                  | **$2427** |

Table 4.2. Average savings per vaginal delivery calculated from average cost per case multiplied by the RIW.

<table>
<thead>
<tr>
<th></th>
<th># cases</th>
<th>Weighted cases</th>
<th>RIW</th>
<th>Average cost per case</th>
<th>Weighted Cost per case (RIW * avg. cost)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All deliveries</td>
<td>6967</td>
<td>6211.68</td>
<td>0.892</td>
<td>$2427</td>
<td>$3606</td>
</tr>
<tr>
<td>Caesarean sections</td>
<td>1835</td>
<td>2430.487</td>
<td>1.32</td>
<td>$2427</td>
<td>$3606</td>
</tr>
<tr>
<td>Vaginal deliveries</td>
<td>5132</td>
<td>3781.193</td>
<td>0.737</td>
<td>$2427</td>
<td><strong>$2006</strong></td>
</tr>
</tbody>
</table>

Savings per caesarean section avoided = $1600
Table 4.3. Average savings per vaginal delivery at OCCP hospitals, derived from average delivery and neonatal care costs. OCCP 1995/96 typical cases and their Total Costs Per Case (TCPCs) were employed for calculations.

<table>
<thead>
<tr>
<th></th>
<th>TCPC</th>
<th># cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caesarean delivery</td>
<td>$2849</td>
<td>790</td>
</tr>
<tr>
<td>Repeat caesarean</td>
<td>$2351</td>
<td>532</td>
</tr>
<tr>
<td>Caesarean with complicating diagnosis</td>
<td>$3422</td>
<td>483</td>
</tr>
<tr>
<td>Repeat caesarean with complicating diagnosis</td>
<td>$2854</td>
<td>128</td>
</tr>
<tr>
<td><strong>Weighted average cost per caesarean delivery</strong></td>
<td><strong>$2855</strong></td>
<td><strong>1933</strong></td>
</tr>
<tr>
<td>Neonatal care (&gt;2500g) for caesarean-delivered newborn</td>
<td>$1021</td>
<td>1540</td>
</tr>
<tr>
<td><strong>Total hospital costs per caesarean delivery</strong></td>
<td><strong>$3876</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>TCPC</th>
<th># cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaginal delivery</td>
<td>$1566</td>
<td>8012</td>
</tr>
<tr>
<td>Vaginal after caesarean</td>
<td>$1654</td>
<td>346</td>
</tr>
<tr>
<td>Vaginal delivery with complicating diagnosis</td>
<td>$1757</td>
<td>2069</td>
</tr>
<tr>
<td><strong>Weighted average cost per Vaginal delivery</strong></td>
<td><strong>$1656</strong></td>
<td><strong>10115</strong></td>
</tr>
<tr>
<td>Neonatal care (&gt;2500g) for vaginal-delivered newborn</td>
<td>$438</td>
<td>8707</td>
</tr>
<tr>
<td><strong>Total hospital costs per Vaginal delivery</strong></td>
<td><strong>$2094</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Savings per caesarean section avoided = $1782**
4.4.2 Costs pertaining to neonatal outcome

To determine the cost-effectiveness of acyclovir treatment on neonatal outcome, transmission rates to neonates from mothers with a history of recurrent genital herpes were combined with acute and long-term disability costs per transmitted case (Table 4.4). The average neonatal outcome cost per mother with recurrent genital herpes on suppressive acyclovir treatment during pregnancy was $5.39, compared to $26.18 for a mother not on suppressive therapy. The largest financial burden from the risk of neonatal herpes for the average mother with recurrent genital herpes receiving no treatment was the lifetime care cost for severe disability ($20.03). The average financial savings from neonatal outcome per mother on suppressive acyclovir therapy was determined to be $20.79 (Table 4.5). Although the cost per neonatal infection outcome may be enormous, the low transmission rates from mothers with a history of recurrent genital herpes make this a small factor in the cost-effectiveness of acyclovir in pregnancy.

4.4.3 Cost-effectiveness analysis of acyclovir prophylaxis in late pregnancy among women with recurrent genital herpes

To determine the cost-effectiveness of suppressive acyclovir use in late pregnancy for women with genital herpes diagnosed prior to pregnancy and experiencing <6 recurrences per year, the sum of obstetrics and neonatal outcome cost savings was calculated, from which the cost of drug was deducted. Results demonstrate that acyclovir is cost-effective for women with genital herpes diagnosed prior to pregnancy and experiencing <6 recurrences per year at a savings of $40.79 per woman on acyclovir prophylaxis, and $312.79 for women experiencing ≥6 recurrences per year (Table 4.6).
Table 4.4. Transmission rates, long-term prognosis odds, and costs of care pertaining to neonatal HSV transmission from mothers with a history of recurrent genital herpes. Cost per mother is calculated as the product of the transmission rate and the cost per transmitted case.

<table>
<thead>
<tr>
<th>Transmission rates to neonate from mothers with a history of recurrent genital herpes</th>
<th>Costs per transmitted case</th>
<th>Average acute care cost per mother with recurrent genital herpes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ACV &amp; CS</td>
<td>0.00034</td>
<td>Acute hospital care for infected neonate</td>
</tr>
<tr>
<td>ACV therapy &amp; CS</td>
<td>0.00007</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neonatal HSV long-term prognosis odds</th>
<th>Costs per transmitted case</th>
<th>Average lifetime care cost per mother with recurrent genital herpes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonatal deaths</td>
<td>0.183</td>
<td>Limited to acute care costs</td>
</tr>
<tr>
<td>Severe disability</td>
<td>0.154</td>
<td>Lifetime care costs</td>
</tr>
<tr>
<td>Moderate disability</td>
<td>0.101</td>
<td>Lifetime care costs</td>
</tr>
<tr>
<td>Normal outcome</td>
<td>0.562</td>
<td>Limited to acute care costs</td>
</tr>
</tbody>
</table>

Table 4.5. Cost savings per mother using acyclovir suppressive therapy, as calculated by average cost of neonatal outcome per mother with recurrent genital herpes treated or untreated with acyclovir during pregnancy. Cost per mother is calculated by combining acute care and long-term disability costs (Table 4.4).

<table>
<thead>
<tr>
<th>Costs</th>
<th>Cost per mother with recurrent genital herpes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACV</td>
</tr>
<tr>
<td>Acute hospital care costs for infected neonate</td>
<td>$0.36</td>
</tr>
<tr>
<td>Life-time severe disability costs</td>
<td>$4.12</td>
</tr>
<tr>
<td>Life-time moderate disability costs</td>
<td>$0.90</td>
</tr>
<tr>
<td>Total care costs per neonatal herpes outcome</td>
<td>$5.39</td>
</tr>
</tbody>
</table>

Cost savings per mother using acyclovir = $20.79
Table 4.6. Cost-effectiveness of suppressive acyclovir therapy in pregnant mothers with a history of recurrent genital herpes with <6 or ≥6 recurrences per year, calculated as sum of obstetrics and neonatal outcome cost savings minus cost of acyclovir.

<table>
<thead>
<tr>
<th># of recurrences per year in mother</th>
<th>&lt;6</th>
<th>≥6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrence risks at time of delivery</td>
<td>No ACV</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Suppressive ACV therapy</td>
<td>0.05</td>
</tr>
<tr>
<td>Reduction in CS rate due to ACV therapy</td>
<td>0.10</td>
<td>0.27</td>
</tr>
<tr>
<td>Cost Savings per CS avoided</td>
<td>$1600</td>
<td></td>
</tr>
<tr>
<td>Average obstetric savings per mother on ACV therapy</td>
<td>$160</td>
<td>432</td>
</tr>
<tr>
<td>Neonatal outcome cost savings per mother on ACV therapy</td>
<td>$20.79</td>
<td></td>
</tr>
<tr>
<td>Cost of ACV</td>
<td>$140</td>
<td></td>
</tr>
<tr>
<td>Total savings per mother on ACV therapy</td>
<td>$40.79</td>
<td>$312.79</td>
</tr>
</tbody>
</table>
4.5 Discussion

Suppressive acyclovir therapy in pregnancy for the prevention of genital herpes recurrences and neonatal herpes infection is cost-effective in women with a history of genital herpes prior to pregnancy. The average cost saving for a woman with <6 recurrences per year is $40.79, and average cost saving for a woman with ≥6 recurrences per year is $312.79. The majority of cost savings is attributed to savings in perinatal costs due to the reduction in rate of caesarean sections. A minor cost saving is attributed to a reduction in rate of acquisition of neonatal herpes, leading to savings pertaining to both acute and lifetime costs of neonatal sequelae.

Differences in recurrence rates at delivery between acyclovir-treated and untreated individuals account for the disparity between the savings accorded by acyclovir prophylaxis in the two groups. The savings data from the two groups correspond with published data. For women with genital herpes diagnosed prior to pregnancy and experiencing <6 recurrences per year, the savings of $40.79 per treated patient is comparable to the $14.00 calculated in the Scott study, which used a similar methodology. The cost savings in the group of women with six or more recurrences per year, at $312.79 saved per treated patient, is also comparable to the $391.00 reported by Scott et al.

Surprisingly, using two different case costing systems in two different Canadian locations produced very similar results in both cost per caesarean section ($3876 in Ontario compared with $3606 for BCCW), costs per vaginal delivery ($2094 versus $2006), and savings per caesarean avoided ($1782 versus $1600). While these figures are dwarfed by the costs used for the published US studies, it should be noted that it is the differences in cost between the delivery procedures that matter, rather than the cost of the procedures themselves.
Calculations of costs of neonatal outcome reveal that due to the low risk of transmission from a mother with recurrent genital herpes, neonatal outcome costs per mother are relatively low compared with obstetrical costs associated with caesarean or vaginal delivery. However, if this cost-effectiveness analysis of acyclovir use included women presenting with primary HSV during pregnancy, neonatal outcome costs would have much higher impact, due to the much higher transmission rates among mothers with primary infection.

Evidently, acyclovir prophylaxis is cost-effective in all women with a history of genital herpes before pregnancy, although therapy in women with more frequent recurrences prior to pregnancy is more cost-effective. This analysis may actually underestimate the savings provided by suppressive acyclovir in pregnancy. Costs relating to long-term maternal morbidity due to caesarean section and future costs of repeat caesarean sections were not factored in. Furthermore, factors such as the psychological benefits of suppressive therapy and quality of life issues affected by a surgical delivery cannot be calculated in terms of cost. However, this analysis is limited by the use of recurrence risk and neonatal transmission estimates taken from literature. An analysis using transmission data from a randomized clinical trial would give a more accurate measure of the cost-effectiveness of acyclovir.
5. Vaginal microbicides as an alternative strategy for prevention of neonatal herpes – possible use of dendrimers

5.1 Introduction

The suppression of genital herpes recurrences is currently limited to the use of oral nucleoside analogue antivirals. Investigations into the prevention of neonatal herpes have been focused on the continuous use of these oral agents in the last weeks of pregnancy. Evidently, the inconvenience and costs of daily dosing in this time period is outweighed by the benefits afforded by antiviral treatment. However, a possibly preferable treatment for the prevention of neonatal herpes would be a drug that can be taken in a single intrapartum dose. This hypothetical treatment would need to be capable of preventing transmission of free virus to the neonate at the time of delivery.

Due to their mechanism of action, existing oral therapies fall short of this goal. In the case of acyclovir, therapeutic levels of the active triphosphate form need to be continuously present in the mucosal cells of the genital tract in order to prevent the replication of virus. Furthermore, since it acts on a late stage of the viral replication cycle (i.e., viral DNA transcription), the effectiveness of acyclovir is dependent upon its presence in a virally-infected cell. Reactivations from latently-infected cells are unpredictable. Accordingly, prevention of viral shedding from genital sites necessitates continuous dosing regimens, by definition. As observed in chapter 2, inadequate plasma drug levels can provide a window of opportunity for viral shedding. Evidently, there is a potential for more effective compounds to replace, or supplement, existing therapy in the prevention of perinatal transmission.
The transmission of sexually transmitted infections (STIs), including human immunodeficiency virus (HIV) infection, is a global public health concern. While condom use can reduce the propagation of these agents, there is a need for female-controlled methods of STI prevention. The development and use of microbicides, which could be used vaginally to prevent STIs, has been one proposed solution to this need. Such microbicides would act by disrupting or disabling organisms or block their entry into host cells by interfering with cell surface receptors, although disruption of normal vaginal flora would be suboptimal. Microbicidal activity in the physiological conditions of the female genital tract, ideally for several hours, is important for the prevention of sexual transmission of infectious agents.

Dendrimers (Figure 5.1) are a novel class of polyanionic macromolecules with antimicrobial activity. Individual dendrimers consist of a polyfunctional core layered with repeat units (eg., Polyamidoamine, polyamino acids, and polyethers), with separate functional groups capping the outer surface. These functional groups can be designed to interact with specific biological surfaces or receptors. Various dendrimers have demonstrated broad-spectrum antiviral activities against herpes simplex viruses (HSV), human immunodeficiency viruses (HIV), hepatitis B virus, respiratory viruses, and human papillomaviruses. This class of compounds is currently in development as potential vaginal microbicides. The properties of a vaginal microbicide – a topically applied agent capable of disabling virus or blocking virus entry into host cell, and free from local toxicity – would be a promising candidate for the prevention of intrapartum transmission of HSV. Furthermore, potential antibacterial and antifungal activity could also be an asset, since microbial infections of the genital tract increase the risk of HSV transmission.

Figure 5.1. Molecular structure of the polylysine dendrimer SPL-2999 (formerly BRI-2999). The molecule is synthesized by the addition of polylysine repeat units to a central benhydrylamine core group. The capping layer is naphthyl disodium disulfonate (n = 32).
SPL-2999 (formerly BRI-2999) is a dendrimer that has demonstrated in vitro efficacy against HSV (Starpharma Inc., personal communication). This compound is synthesized by adding polylysine repeat units to a central benzhydrylamine core group. The functional outer layer consists of naphthyl 3,6-disulfonic acid sodium salts. The mechanism of action of dendrimers has been postulated for HIV, involving inhibition of both early-stage (attachment and/or fusion) and late-stage (reverse transcriptase and integrase) replication. However, the mechanism of action against HSV has not been elucidated. Here, we present an in vitro evaluation of the efficacy and mechanism of action of SPL-2999 against HSV-1.

5.2 Hypothesis

Intrapartum vaginal installation of topical microbicides may represent an alternative pathway for neonatal herpes prevention allowing vaginal delivery. Furthermore, since acyclovir suppression provides incomplete protection from asymptomatic shedding, such an alternative may be useful as an adjunctive treatment to oral acyclovir.

---

139 Witvrouw M, Fikkert V, Pluymers W, et al. Polyanionic (i.e., polysulfonate) dendrimers can inhibit the replication of human immunodeficiency virus by interfering with both virus adsorption and later steps (reverse transcriptase/integrase) in the virus replicative cycle. Mol Pharmacol 2000; 58:1100-1108.
5.3 Methods

5.3.1 Compounds

Dendrimer SPL-2999 was synthesized by Starpharma Limited (Melbourne, Australia). The chemical formula of SPL-2999 is BHAlys15lys16(NHCSNHIaphth-3,6-diSO3Na)32 (BHA: benzhydrylamine) with molecular weight of 16,615 Da. The stock solution of SPL-2999 was prepared in minimum essential medium (MEM; GIBCO/BRL, Burlington, ON), supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

5.3.2 Virus and cells

HSV standard strains F (HSV-1) and G (HSV-2) (ATCC) were used in the experiments. Vero cells (African green monkey kidney cells, ATCC), a HSV susceptible cell line, were employed in the assays.

5.3.3 Pre-treatment of cells with SPL-2999 prior to infection

Confluent Vero cells in 6 well plates were incubated with 1.0 ml of various concentrations of SPL-2999 (final concentrations: 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 μg/ml) at 37°C for 1 h. 100 pfu virus was then added to the cells and the samples were incubated at 37°C for 1 h. After viral inoculum was removed, the cells were washed with PBS and overlaid with methylcellulose. The plates were incubated at 37°C for 2-3 days. When plaque size was adequate, cells were fixed with 1 ml formalin for 10 minutes and stained with 1 ml crystal violet for 10 minutes, and plaque count was performed.
5.3.4 Pre-mixing virus with SPL-2999

0.5 ml virus stock containing 100 pfu was mixed with 0.5 ml of various concentrations of SPL-2999. The mixtures were incubated at 37°C for 1 h, and used to inoculate confluent Vero cells in a 6-well plate. After incubating at 37°C for 1h, the viral inoculum was removed, the cells washed with PBS, and overlaid with methylcellulose. Plaque assay was performed as described above.

5.3.5 Addition of SPL-2999 at time of virus inoculation

0.5 ml virus stock containing 100 pfu was mixed with 0.5 ml of various concentrations of SPL-2999. The solution was immediately applied to confluent Vero cells. After incubating at 37°C for 1 h, the solution was removed, the cells washed with PBS, and overlaid with methylcellulose. Plaque assay was performed as described above.

5.3.6 Effect of SPL-2999 on infected cells

To test the efficacy of SPL-2999 on virally infected cells, confluent Vero cells in a 6-well plate were inoculated with 100 pfu virus per well and incubated at 37°C for 1 h. After viral inoculum was removed, the cells were washed with PBS and overlaid with methylcellulose containing various concentrations of SPL-2999. Plaque assay was performed as described above.

5.3.7 Effect of SPL-2999 on early step of virus replication

Confluent Vero cells were pre-cooled at 4°C for 1 h, then were pre-treated with various concentrations of SPL-2999 at 4°C for 1 h. 100 pfu virus was added to cells on ice and cells were incubated at 4°C for 2 h to allow virus to bind to the cell surface. Unbound virions were removed.
by washing with PBS x 3. The cells were then incubated at 37°C for 1 h to allow for virus uptake. 1 ml glycine solution (pH=2.2) was applied for 1 minute to the cells to strip off uninternalized virus from cell surface. The cells were then washed twice with PBS and once with MEM (containing no serum), and overlaid with methylcellulose for plaque assay.

5.3.8 Data Analysis

Experiments were performed in duplicate. Effects of compounds at varying concentrations were expressed as % of control ([mean plaque counts in drug treated wells]/[mean plaque counts in control wells]). The EC50 was calculated using linear regression analysis on Statview™ (SAS, Cary, NC).
5.4 Results

SPL-2999 demonstrated *in vitro* antiviral activity against HSV-1 (Strain F) in various modified plaque reduction assays (Table 5.1).

5.4.1 Antiviral effect of SPL-2999 determined by pretreatment of cells

To determine whether the antiviral effect of SPL-2999 involves interactions with the target cell, cells were pretreated with drug for 1 h, and washed with PBS before viral inoculation. This compound showed activity against the virus in this assay at an EC$_{50}$ of 1.0 μg/ml (Table 5.2, Figure 5.2). These results show that the activity of SPL-2999 involves interactions with the host cell, as opposed to agents that act solely by disabling the infectious agent before it reaches the cell. Antiviral activity was seen even though cells were washed with PBS after pretreatment with drug. This suggests that SPL-2999 may have inhibitory effects on the early stages of the viral replication cycle.

5.4.2 Pre-mixing virus with SPL-2999 prior to inoculation

To determine whether SPL-2999 has a detergent effect on the virus (i.e., disrupts or disables the virus prior to contact with the cell), the drug was incubated with the virus for 1 h. This virus/drug mixture was then applied to the target cells. An EC$_{50}$ of 0.9 μg/ml was achieved (Table 5.3, Figure 5.3), and is comparable with the EC$_{50}$ of the above experiment. This suggests that the detergent effect is not the primary mechanism of the antiviral activity of SPL-2999.
Table 5.1. Activity of SPL-2999 against HSV-1 in modified plaque reduction assays.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment of cells with SPL-2999 for 1 h prior to inoculation</td>
<td>1.0</td>
</tr>
<tr>
<td>Pre-mix virus with SPL-2999 for 1 h prior to inoculation</td>
<td>0.9</td>
</tr>
<tr>
<td>Addition of SPL-2999 at time of viral inoculation</td>
<td>1.8</td>
</tr>
<tr>
<td>Addition of SPL-2999 to virally infected cells 1 h after inoculation</td>
<td>18.5</td>
</tr>
<tr>
<td>Effect of SPL-2999 on early stages of viral replication</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Table 5.2. The antiviral effect of SPL-2999 on cells pre-treated with drug prior to addition of HSV-1, expressed as % of control.

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>0</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment of cells</td>
<td>100</td>
<td>93</td>
<td>27</td>
<td>69</td>
<td>67</td>
<td>54</td>
<td>16</td>
<td>11</td>
<td>9</td>
</tr>
</tbody>
</table>

Figure 5.2. The antiviral effect of SPL-2999 on HSV-1 determined by pre-incubating confluent Vero cells with various concentrations of drug for 1 h. After addition of 100 pfu virus for 1 h, the cells were overlaid with methylcellulose. The EC_{50} was determined to be 1.0 μg/ml.
Table 5.3. The antiviral effect of SPL-2999 on HSV-1 pre-mixed with drug prior to inoculation, expressed as % of control.

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>0</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment of cells</td>
<td>100</td>
<td>85</td>
<td>98</td>
<td>89</td>
<td>89</td>
<td>39</td>
<td>18</td>
<td>14</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 5.3. The antiviral effect of SPL-2999 on HSV-1 determined by pre-mixing virus with various concentrations of drug for 1 h. After addition of mixture for 1 h, the cells were overlaid with methylcellulose. The EC<sub>50</sub> was determined to be 0.9 μg/ml.
5.4.3 Addition of SPL-2999 at time of virus inoculation

To determine whether the antiviral effect of SPL-2999 is dependent upon the 1 h pretreatment with cells or pre-mixing with virus, a mixture of drug and virus was applied to target cells without time for incubation. Compared to the above-mentioned experiments, a two-fold reduction in potency (EC$_{50}$ = 1.8 µg/ml; Table 5.4, Figure 5.4) was observed. This suggests that the antiviral effect of SPL-2999 is not dependent on incubation time before contact with virus. This may also indicate that the compound is active either without internalization into cells or is internalized at an equal or faster rate than the virus.

5.4.4 Antiviral effect of SPL-2999 on HSV-1 infected cells

To determine the antiviral effect of SPL-2999 on infected cells, drug was applied to cells 1 h after inoculation. Compared to the antiviral effect on cells pretreated with drug, an approximately twenty-fold reduction in activity was (EC$_{50}$ = 18.5 µg/ml; Table 5.5, Figure 5.5). Figure 5.6 compares the potency of SPL-2999 when added 1 h before, at the same time of, and 1 h after virus inoculation. In comparing with the potency of SPL-2999 in experiments where drug was applied at or before time of inoculation, this suggests that this compound primarily acts on early stages of the virus replication cycle. However, since antiviral activity is still present, though reduced, in infected cells, this result does not rule out the possibility that SPL-2999 may also act on late stages of the virus replication cycle.
Table 5.4. The antiviral effect of SPL-2999 on HSV-1 applied to cells at the same time, expressed as % of control.

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment of cells</td>
<td>100</td>
<td>93</td>
<td>83</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 5.4. The antiviral effect of SPL-2999 on HSV-1 determined by adding virus at the same time as drug. The EC_{50} was determined to be 1.8 μg/ml.
Table 5.5. The antiviral effect of SPL-2999 on HSV-1 infected cells, expressed as % of control.

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>0</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment of cells</td>
<td>100</td>
<td>85</td>
<td>98</td>
<td>97</td>
<td>98</td>
<td>95</td>
<td>92</td>
<td>61</td>
<td>41</td>
</tr>
</tbody>
</table>

Figure 5.5. The antiviral effect of SPL-2999 on HSV-1 infected cells determined by adding drug 1 h after virus inoculation. The EC$_{50}$ was determined to be 18.5 μg/ml.
Figure 5.6. The antiviral effect of SPL-2999 on HSV-1, for experiments where drug was applied 1 h before inoculation with virus (P-1; $EC_{50} = 1.0 \mu g/ml$), drug was applied at the same time as viral inoculation (P0; $EC_{50} = 1.8 \mu g/ml$), and drug was applied 1 h after inoculation (P+1; $EC_{50} = 18.5 \mu g/ml$). Expressed as % control against varying concentrations (A) and log of varying concentrations (dose-response curve, B).
5.4.5 Effect of SPL-2999 on early steps of virus replication

To whether the antiviral activity of SPL-2999 involves the early steps of virus replication (i.e., virus entry into host cell), viral attachment was performed at 4°C. At this temperature, virions can only bind to cellular receptors, but cannot penetrate the plasma membrane. The temperature was raised to 37°C to allow for internalization of bound virions, and acidic glycine was used to strip off bound yet uninternalized virions. The EC\textsubscript{50} of SPL-2999 was determined to be 1.2 µg/ml (Table 5.6, Figure 5.7) under these conditions. Since the EC\textsubscript{50} in this experiment was comparable to above studies where virus internalization was not controlled, it is likely that SPL-2999 acts via the inhibition of a step involving the early steps of virus replication, possibly at the attachment, fusion, or adsorption stages.
Table 5.6. The antiviral effect of SPL-2999 on HSV-1 determined by pre-incubating confluent Vero cells with various concentrations of drug for 1 h, then performing viral inoculation at 4°C, expressed as % of control.

<table>
<thead>
<tr>
<th>Dose (µg/ml)</th>
<th>0</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment of cells</td>
<td>100</td>
<td>71</td>
<td>97</td>
<td>78</td>
<td>80</td>
<td>69</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 5.7. The antiviral effect of SPL-2999 on cells pretreated with drug, followed by HSV-1 incubation at 4°C. The EC₅₀ was determined to be 1.2 µg/ml.
5.5 Discussion

The dendrimer SPL-2999 demonstrated antiviral activity against HSV-1, at an EC\textsubscript{50} of approximately 1 µg/ml, determined by pretreatment of Vero cells by the compound prior to inoculation. A similar potency was achieved in an experiment where the compound was pre-mixed with virus prior to inoculation. In parallel experiments conducted by Gong et al\textsuperscript{140} examining the effect of SPL-2999 on HSV-2, an EC\textsubscript{50} of approximately 0.5 µg/ml was observed. In similar experiments published by Bourne et al\textsuperscript{141} the EC\textsubscript{50} of SPL-2999 (BRI-2999) in primary human foreskin fibroblast cells (HFF) was 1.9 and 2.4 µg/ml for HSV-1 and HSV-2, respectively. In this experiment, SPL-2999 was also observed to be effective on infected cells with an EC\textsubscript{50} of 18.5 µg/ml for HSV-1 and 6.3 µg/ml for HSV-2. The reduced potency of this compound on infected cells compared to pretreated cells suggests that SPL-2999 may act on an early stage of viral replication by blocking viral entry into cells.

To further investigate the mechanism of action of SPL-2999, an experiment studying the compound’s effects on the early stages of viral replication was performed, involving binding of virus to cells at 4°C, allowing internalization at 37°C, followed by the stripping of uninternalized virus in acidic conditions. The EC\textsubscript{50} (1.2 µg/ml) was observed to approximate that of the pretreatment experiment conducted without control of virus internalization. This is supportive evidence that SPL-2999 acts on an early stage of viral replication. This suspected mechanism of action is also supported by the experiments of Gong et al\textsuperscript{140} who used viral adsorption and penetration assays, combined with a time-of-addition experiment, to conclude that SPL-2999 blocks viral entry without binding to the cell surface.


Although the primary mechanism of action of SPL-2999 may be on the early stages of replication, its effectiveness on virally infected cells may suggest that a secondary mechanism of action may exist on later stages of replication. The CPE inhibition test conducted by Gong et al\textsuperscript{140} showed that 90-100\% of HSV-infected cells could be protected from virus CPE by the compound. Southern blot hybridization assays performed on HSV-2-infected cells treated with SPL-2999 revealed that 3 μg/ml of drug inhibits DNA synthesis in infected cells. Corresponding with our experiments, Bourne et al\textsuperscript{141} also observed both pre-infection and post-infection activity for this compound, suggesting a secondary mechanism of action further to the proposed early-stage inhibition. In light of the above data, SPL-2999 appears to also inhibit the late stages of HSV replication. However, the exact mechanism remains to be elucidated.

If applied in the vaginal tract in the intrapartum period, the toxicity of the compound, both to the mother and to the neonate, must be considered. In fact, local toxicity may lead to an increase in rate of disease transmission, due to the disruption of skin or mucosal barriers. In the above experiments with SPL-2999, the potencies of the pre-treatment and pre-mixing experiments were similar. This demonstrates that the antiviral activity of SPL-2999 is probably not due to a detergent-like action on the virus, as is the case for other microbicides in development. Therefore, the potential of high levels of cytotoxicity to epithelial cells, seen in detergent-like microbicides such as nonoxynol-9,\textsuperscript{142} should be minimal with SPL-2999. Cytotoxicity assays performed by Gong et al indicated that SPL-2999 has a high selectivity index.\textsuperscript{140} In neutral red dye uptake assays performed on Vero cells, the CC\textsubscript{50} (cytotoxic concentration producing 50\% of cell death) of SPL-2999 was found to be >1000 μg/ml. This is confirmed by the experiments of Bourne et al\textsuperscript{141} on HFF cells, where the CC\textsubscript{50} of SPL-2999 was demonstrated to be >100 μg/ml.

Bourne et al\textsuperscript{141} conducted an \textit{in vivo} evaluation of SPL-2999 in a murine model of genital herpes. This dendrimer reduced the incidence of disease compared to that in PBS-treated controls when applied intravaginally 20 s prior to virus challenge, and provided significant (p<0.0001) protection against infection. Additionally, significant protection remained when the time of application was extended to 30 min prior to virus challenge. This correlates with the \textit{in vitro} findings that the compound acts on an early stage of viral replication. There was no visible evidence of toxicity in mice after a single application of the compound. However, as opposed to \textit{in vitro} data suggesting efficacy against infected cells, SPL-2999 did not exhibit \textit{in vivo} activity when administered 30 minutes after viral challenge. Nevertheless, the prophylactic efficacy exhibited by SPL-2999 suggests that the compound is ideal for use as a topical microbicide.

Topical microbicides are compounds that are applied directly to the genital or rectal tract prior to intercourse to protect against the acquisition of sexually transmitted infections. Further to this usage, in the interest of this thesis, is the potential use in the prevention of intrapartum viral transmission. As HIV, HBV, and HSV are the most widely transmitted viral infections in the intrapartum setting, an agent protective against these infections would do more than simply reduce the incidence of neonatal disease. Such a compound would also reduce the rate of caesarean section by allowing for vaginal delivery by women affected by these diseases. Furthermore, its convenience as a topical application that only needs to be applied during labor and delivery makes it a suitable supplement to existing preventive agents indicated in HIV and HSV. Dendrimers such as SPL-2999 show much promise for use in this area. Its \textit{in vitro} effectiveness in preventing viral entry is coupled with its \textit{in vivo} ability to protect against viral challenge even for an extended period of time after application.
The current strategy for prevention of neonatal herpes focuses on preventing disease manifestations in the mother. Topical microbicides may prove to be an effective second line of protection against neonatal infection. Observations that viral shedding breakthroughs still occur at delivery in spite of suppressive therapy with acyclovir provide enough concern to give such an alternative strategy consideration. Undoubtedly, many questions remain. Issues such as neonatal and maternal toxicity, efficacy in the genital tract during prolonged labor, and stability amid changing physiologic conditions during labor will need to be addressed. Nevertheless, while much is still to be done in the preclinical development of dendrimers, the potential of this class of compounds for the prevention of neonatal herpes warrants further investigation.
6. Conclusions

Genital herpes prevalence has reached epidemic levels among women of childbearing age. Transmission to the neonate results in high rates of morbidity and mortality. Acyclovir is an effective agent for the pharmacologic suppression of genital herpes recurrences, but does not provide complete protection from transmission. We present investigations into issues of safety, efficacy, and cost-effectiveness of the use of acyclovir in late pregnancy to suppress recurrences at delivery for the prevention of transmission to the neonate.

Hematological safety concerns have prompted an investigation into possible myelosuppressive effects of acyclovir use in late pregnancy. Results from the investigation were inconclusive due to low sample size. The development and validation of an assay utilizing capillary electrophoresis for the determination of acyclovir concentration in plasma demonstrated that CE is a rapid and effective analytical tool achieving sensitivity lower than that for HPLC. Future uses in perinatal investigations are promising due to the low volumes required for CE assay.

Umbilical cord plasma levels collected for hematological testing were also analyzed for their plasma acyclovir levels. The concentrations of acyclovir in these plasma samples show large variability between subjects. For some, plasma levels are inadequate, possibly allowing a window of opportunity for the virus to replicate and shed, endangering the neonate. A negative correlation exists between the duration of labor and the level of acyclovir in the cord plasma. It is suspected that non-compliance during a lengthy labor and delivery could result in such low levels. Nausea and vomiting could also play important roles.

In nonpregnant adults, acyclovir is an effective agent for the pharmacologic suppression of genital herpes recurrences with an outstanding safety record. Although there has been a lack of
clinical trials with sufficient sample size, there have been no reports of adverse effects due to its use in pregnancy. Acyclovir is cost-effective in both USA and Canadian settings, especially in women with greater than 6 recurrences per year. Accordingly, this therapy is indicated for the prevention of caesarean sections. However, showing statistically significant benefit in the prevention of neonatal herpes may be too formidable a task.

There are certainly limitations to what acyclovir is able to do in the prevention of neonatal herpes. First of all, women with the highest risk of transmitting to the neonate are those who have their primary episode at delivery. Unless acyclovir is given to all women who are either seropositive for HSV or whose sexual partner is seropositive, therapeutic prophylaxis will not be possible for this cohort. Secondly, the transmission risk pertaining to those women with a history of genital herpes at pregnancy are low – 14% will have a lesion at delivery, and even without caesarean section, only 4% of those exposed infants will acquire HSV. Questions regarding the role of asymptomatic shedding suppression in pregnancy have not been answered in the context of pregnancy.

The use of dendrimers as microbicides in the prevention of sexually transmitted infections is promising. Its use as an alternative pathway for the prevention of neonatal herpes transmission has not been studied. Evidently, dendrimer SPL-2999 is effective against early and late stages of replication of HSV, and could prove to be a useful adjunct to standard oral antiviral therapy. Such a product, if proven effective in vivo, could aid in the fight against the intrapartum transmission of other viral infections as well.
The recent ACOG recommendation supporting the use of acyclovir in pregnancy will undoubtedly be accompanied by an increase in the number of pregnant women who will receive this therapy. Obviously, not every pregnancy will be as favorable to drug therapy as the ones selected to participate in a tightly controlled clinical trial. Indeed, the inexpensive cost of acyclovir and its world-wide popularity translates to potential use in millions of pregnancies. Caution must be employed until definitive safety data are obtained.
Bibliography


http://wonder.cdc.gov/wonder/prevguid/p0000480/entire.htm

http://www.occp.com


Study protocol of the UBC/UW Project II “Acyclovir in pregnancy” study.


Witvrouw M, Fikkert V, Pluymers W, et al. Polyanionic (i.e., polysulfonate) dendrimers can inhibit the replication of human immunodeficiency virus by interfering with both virus adsorption and later steps (reverse transcriptase/integrase) in the virus replicative cycle. Mol Pharmacol 2000; 58:1100-1108.


Appendix I

Randomized double-blinded trial of acyclovir vs. placebo for reduction in the rate of symptomatic recurrence of genital herpes infection and caesarean section among pregnant women (a.k.a. “UBC / UW Project II”)

Study Protocol

1. BACKGROUND

Herpes simplex virus (HSV) genital infection has been significantly rising in prevalence in the developed world (1). Neonatal HSV continues to be the most dire medical consequence of genital herpes (2). It occurs in approximately 1/3,000 to 1/7,000 live births as compared to 1/1,500 live births for Group B Streptococcal neonatal sepsis. In at least one study performed in the Seattle, WA area, the rate of neonatal HSV infection increased 10-fold from 1966 to 1982, from 2.4 to 28.2/100,000 live births. Similar findings are reported in Manitoba, Canada (3). In an effort to prevent neonatal HSV infection, there has been a remarkable increase in the rate of caesarean sections (CS) among women with recurrent genital herpes over the last two decades (4).

Among all pregnant women, 2.2% will have symptomatic recurrences of genital HSV infection during pregnancy(4). Among women with symptomatic genital herpes any time during pregnancy, 47% develop a symptomatic recurrent episode within one week prior to delivery, and 40% during labour who undergo CS. Overall, in a study of births in Washington state, 62% of women entering pregnancy with a history of genital HSV or with their first symptomatic episode of HSV during pregnancy had a total CS rate of 62% compared to 18% among women with no history of symptomatic genital HSV. The primary CS rate among women with symptomatic genital HSV was 59% compared to 10-20% among women with no history.

CS is associated with a 10-20 fold increased in morbidity and mortality for the mother compared to vaginal delivery (5). An alternative strategy directed at preventing recurrent genital HSV at term, acyclovir therapy, would reduce maternal morbidity significantly (6). Acyclovir pharmacokinetics among pregnant women are similar to those observed in other adult patients, and symptomatic recurrences were suppressed in 14 or 15 women enrolled in the study (7). Other studies have shown a 90% efficiency rate (8-10). Thus, if acyclovir were 90% or more effective in reducing recurrent genital HSV at term, the rate of symptomatic episodes at delivery could be reduced from 40% to approximately 4% with a proportional decrease in CS rate. However, this study has to be performed in a well-controlled fashion to evaluate the incidence not only of recurrent genital HSV and CS but also the incidence of symptomatic shedding among women on acyclovir compared to placebo.

This study has been underway at the University of Washington since 1995. As this study has progressed, there has been a tendency in many areas in North America to begin treatment of
women near term with anti-viral medication to suppress HSV outbreaks and decrease CS risks but this practice is not adequately evidence-based (11,12). We believe that the questions posed need to be answered in a clinical trial with sufficient power to determine the validity of this approach. The University of British Columbia is uniquely positioned to collaborate with the University of Washington, given the similarities of the population demographic and the large number of deliveries performed at Children’s & Women’s Health Centre of BC (C&W).

the safety of this study is supported by information from the Acyclovir in pregnancy registry that has not been able to demonstrate any specific toxicities to the fetus with the use of acyclovir in pregnancy (13).

2. OBJECTIVES

1. To assess the effects of acyclovir on the rate of symptomatic genital HSV episodes and asymptomatic shedding among third trimester pregnant women
2. To attempt to decrease the caesarean section rate at term among women with recurrent genital herpes simples virus infection in pregnancy by treatment with acyclovir from 36 gestation
3. To utilize PCR based technology to determine if one can more precisely define women with sub-clinical shedding of HSV-2 at term, with and without acyclovir therapy, and to reduce the false-negative HSV culture rate

3. DESIGN

This is a randomized, double-blind, placebo-controlled trial of acyclovir 400 mg q8h versus identical placebo beginning at 36 weeks gestation and continuing through labour until delivery.

Outcomes of interest include: the incidence of recurrent symptomatic HSV after study enrollment compared to the women’s history of recurrences during the pregnancy before enrollment; the percent of total cultures positive in each group; the comparison to PCR positive in each group; the number of symptomatic recurrences compared between the two groups; the number of positive cultures and symptomatic recurrences at delivery between the two groups; and the CS rate between the two groups. As well, infant outcomes will be compared including birthweight, gestational age at delivery, incidence of neonatal complications, neonatal laboratory abnormalities, and incidence of positive neonatal HSV cultures.

4. SUBJECTS

4.1 INCLUSION CRITERIA
- healthy pregnancy women at up to 36 weeks gestation with documented recurrent symptomatic genital HSV
- normal CBC, Creatinine
- no serious medical conditions
- normal 18-week ultrasound
- willingness to adhere to strict study protocol
- any condition that, in the opinion of the investigator, may influence the probability of needing CS

4.2 EXCLUSION CRITERIA

- known sensitivity to acyclovir
- serious medical conditions or abnormal ultrasound
- any condition that, in the opinion of the investigator, may influence the subjects ability to adhere to the study protocol

5. STATISTICS

5.1 SAMPLE SIZE CALCULATION

The sample sized required for 80% power in detecting the difference with a 2-tailed test at the 5% level are dependent on the CS rate. The calculations assume CS rates varying from 9-15%. At present the CS rate at C&W is approximately 20%. These calculations also assume a reduction in CS while on the drug of either 90% or 66%. Though two published pilot studies indicated a decrease in symptomatic recurrences of 90%, we feel this is overly optimistic and that with larger populations, a rate of reactivation with suppressive therapy comparable to that seen with non-pregnant women will become apparent.

5.2 SAMPLE SIZE CALCULATION FOR ACYCLOVIR SUPPRESSION

<table>
<thead>
<tr>
<th>OPTION</th>
<th>CS RATE</th>
<th>ASSUMED REDUCTION</th>
<th>TOTAL ENROLLMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15%</td>
<td>90%</td>
<td>158</td>
</tr>
<tr>
<td>B</td>
<td>11%</td>
<td>72%</td>
<td>376</td>
</tr>
<tr>
<td>C</td>
<td>9%</td>
<td>66%</td>
<td>568</td>
</tr>
</tbody>
</table>

As of June 12, 1997 there are 101 women enrolled at the University of Washington and the study remains blinded. There have been no adverse events. With combined annual delivery rates of the two sites of 8,330 and with 25-30% of the population HSV seropositive (of which 20% will report symptomatic disease), we should be able to enroll 130 - 150 women per year; we anticipate an enrollment of approximately 100-120 per year at C&W.

6. RECRUITMENT
6.1 POSTERS

Both posters will be distributed, together with a letter of invitation, throughout the Lower Mainland:
- OB care providers
- general practitioners
- prenatal care coordinators
- Public Health nurses
- Ultrasound labs
- STD clinics
- ambulatory clinics at C&W

6.2 INFORMATION NUMBER

“1-877-HERPES3 (1-877-437-7373)” (“herpes and pregnancy research info line”)

Women calling this line will receive a 2-minute outgoing message that will include the following information (see 1-877 line script):
- brief outline of purpose of the study
- inclusion criteria
- study coordinator contact information for scheduling (875-2345 *6390)
- referral information

7. ENROLLMENT

7.1 SCREENING

Women who call the contact number (875-2424 *6390) will either speak to the study coordinator directly or will leave a name and contact number for a return phone call. The Form A - Eligibility will be used to screen patients by phone.

Women with a primary outbreak during pregnancy will require an HSV antibody screen (this does not differentiate between HSV-1 and -2) and if possible an HSV culture (differentiates between HSV-1 and -2) to ensure eligibility. GPs will be contacted for copies of results for those who have had this done already. Those who have not been screened previously will have this done at a laboratory near them. Note that although Western Blot can identify HSV-1 or -2, it can take 6-8 weeks to perform (at Viridae), and will cost $25 at BCBio or Metro Labs for accessioning (not covered by Medical). Subjects’ OB providers will be contacted for ultrasound results when completed.

If time permits, schedule a screening visit to be conducted in the coordinator’s office. At this time the patient can have questions answered, meet Dr. Money, and get an idea of the time/effort commitment required for participation. The Enrollment Information Package can be given to eligible subjects at this visit, or posted if a screening visit is not possible.
Those who remain eligible and indicate consent to participate will be assigned a subject ID# and entered on the patient screening spreadsheet (excel/hsv.xls).

Subjects will be contacted at 34 weeks to schedule a 36 week visit; this date will be flagged on the patient screening spreadsheet. If subjects indicate they remain willing to participate, the Physician’s Notification Letter (doclttr) will be sent to the subject’s OB provider. Contact Bev Buchinski (2978, pgr 02405) 2 weeks prior to 36 weeks to arrange for a one-hour appointment in the contingency clinic. Clinics are open from 0900-1145, 1300-1700h. Women who make telephone contact at or close to 36 weeks may be booked in as there is usually space available for drop-ins each day.

Once the 36 week appointment has been scheduled, subjects will be instructed to come directly to BCW Admitting to get an addressograph (this will generate a chart at the Clinics) and then to the Clinics reception desk to report in.

Those who do not meet inclusion criteria but who may do so further in their pregnancy (i.e. those who have not had an outbreak in the past year, but may have one later in the pregnancy) should be advised to contact the coordinator if an outbreak occurs during their pregnancy, prior to 36 weeks gestation.

7.2 ENROLLMENT VISIT (36 WEEKS)

All subjects will be seen at C&W Diagnostics & Ambulatory Clinics for enrollment. Informed consent will be obtained at this time, and Dr. Money/Dr. Bebbington will be present for the initial examination.

Supplies needed:
- Informed Consent Form (2)
- lab supplies (bloodwork, cervicovulvar supplies, requisitions)
- take-home kit
- Subject Symptom Diary
- source document file

Obtain the following bloodwork: CBC (lavender top), chemistry, BUN, Creat (light red top), HSV serology (10ml red top Tube)

Complete Forms A-D. Demonstrate cervix and vulvar specimen technique and send enrollment specimens to lab / Viridae: bloodwork to Accessioning (2nd floor, BCCH) with C&W study requisition (C&W lab contact: Elvira Kozak 7521), and HSV serology and cervical specimens to Viridae via shuttle service. Call Viridae to ensure pickup at St. Paul’s Hospital and send specimens with ice pack (attn.: Paul Crosson 689-9404). Explain Patient Symptom Diary to subject. Identify BCBio lab closest to patient where she will be dropping of specimens.

Give patient prescription for the following: “one bottle study medication for UBC/UW Project II, patient number: 241 – (or something like that) “ and send them to outpatients’ pharmacy (BCCH) for pick-up.
After enrolling a subject, make telephone contact with the subject’s OB provider to determine if they are willing to participate in the trial. Those who are willing to participate will have an instruction package posted to them that includes the Physician Instructions (docinstr.doc) and extra supplies in the event a subject forgets to bring her supplies to a visit. Subjects will be required to deliver the specimens collected at her physician’s office to an appropriate drop-off lab. Subject appointments will be noted on the Subject Follow-up Table. If a physician is unable to perform the exam and obtain specimens, set up appointments for the subject to be seen at C&W Clinics once weekly for the duration of her pregnancy and enter these appointments on the subject screening spreadsheet.

7.3 STUDY REGISTRATION

Enrolled subjects will have registration paperwork kept in Admitting. Provide study notification stickers to be attached to the Prenatal 1 record as well as a purple study info sheet to be inserted into the prenatal paperwork. This is most easily done in person after the patient is seen for enrollment.

7.4 RANDOMIZATION & MEDICATION

Medication and placebo have been provided by the University of Washington herpes group (contact: Sheree Miller, PharmD).

Study drug and placebo will be dispensed through out-patients’ pharmacy. Patients will be given a study ID# sequentially starting at 241.

Reordering of medication will be arranged by Linda Morris (3263) at C&W Pharmacy.

Eligible, consenting subjects will be randomized by assignment of the study ID # (241-360). Randomization and blinding of the ACV and placebo will be performed by pharmacy. At the completion of the enrollment visit, subjects will be sent to the outpatient pharmacy to obtain their 5 weeks supply of study medication (*labeled “UBC/UW Project II”). Call pharmacy in advance to notify them of the subject’s arrival. The double-blind code will be kept by pharmacy, and a copy will be kept by the study coordinator in a sealed envelope identified by subject ID#.

8. WEEKLY FOLLOW-UP

8.1 TELEPHONE CONTACT

Subjects will be contacted by telephone weekly by the research nurse to confirm completion of the Subject Symptom Diary and medication accountability. If subjects are having trouble filling this form out, or if they have an outbreak, they are to be booked for a clinic visit at C&W, to be seen by the coordinator and Dr. Money (only for those with outbreaks).
8.2 WEEKLY VISITS

OB providers who will be performing weekly exams and specimen collection will be instructed to fax data collection forms (*Form I*) to the Study Coordinator (604-875-3212).

Subjects who will be seen at C&W for their weekly visits will be seen by the research coordinator in the Clinic (half hour visit). At this visit, a visual exam will be performed and specimens will be collected for HSV culture and PCR: a speculum exam will not be performed. The specimens will be transported to Viridae by the coordinator via shuttle service. Dr. Money will be paged (02426) to assess those who have clinical evidence of an outbreak. *Form I* will be completed at each weekly visit.

Subjects will be contacted by phone during the week prior to due date to be reminded to bring their *LDR Kit* when they present at C&W for delivery.

9. DELIVERY

9.1 ADMISSION

Subjects’ registration paperwork should have been “flagged” soon after enrollment. All patient registration papers are kept at Admitting. Assessment Room staff will contact the study coordinator when a subject is admitted: on weekdays, the coordinator can be paged (01691) or a message can be left at 6390; on nights and weekends, a message can be left at 6390.

Subjects presenting to C&W for delivery will bring their L&D Kit. Extras will be kept in the designated drawer in an exam room in Assessment for those who do not have them on admission; the drawer will be labelled “UBC/UW Project II.” Subjects will obtain a final C&S and PCR specimen in the assessment examining room. During weekdays, 0830-1630h, the study coordinator will be responsible for transporting of specimens to Viridae. On nights, specimens will be stored in the High Risk refrigerator for collection by porter and transporting by the coordinator the following morning. On weekends, specimens will be stored by C&W lab until Monday morning.

9.2 DELIVERY

Subjects will be given the usual pre-delivery care. Those with evidence or suspicion of genital HSV lesions will be offered cesarean section.

10. FOLLOW-UP

10.1 SUBJECT

*Forms F1 and F2* will be filled out by the coordinator. Subjects will be given the *Postpartum Information Form* and instructed on the signs and symptoms of neonatal HSV infection.
All subjects will be followed up by telephone by the study coordinator at postpartum week 1 and 2.

10.2 NEONATE

Forms G1 & G2 will be filled out by the coordinator. HSV cultures will be collected from the infants conjunctiva, nasopharynx, and electrode sites (opt) by the coordinator prior to discharge if the neonate is in-hospital during the week. For deliveries occurring Friday night - Saturday night, the cultures will be collected by attending. Any lesions or skin rashes will be documented.

Neonates of women who CULTURE POSITIVE AT DELIVERY will be seen at BCCH Infectious Disease Clinic by Dr. Simon Dobson for cultures, PCR, and physical examination.

REFERENCES

CONTACTS:

1. Kim Shearer (Jetty’s gone), Program Director, Low Risk Birthing (Delivery Suite): 2985
2. Clarice Perkins, Clinical Instructor, Assessment Rooms (2223 ... 6)
3. Bev Buchinski, Perinatal Clinical Educator, Outpatient Clinics (2978)
4. Pam O’Sullivan, Program Director, Diagnostics & Ambulatory Clinics (2382)
5. Theresa Ginn, Health Records (3202)
6. Diane Gulbranson, Admitting, Diagnostics & Ambulatory (2096)
7. Elvira Kozak, C&W lab (7521)
8. Paul Crosson, Viridae (lab supervisor) (689-9404)
9. Linda Morris, C&W pharmacy (3263)
10. Shih-chen Chen, PCR supplies, (206-667-6798)
11. Sylvia Berry, Project Coordinator, UW (206-543-9863)
12. Limei Fan (Budget coord) (206)667-6174 (4411) lfan@fhcrc.org
13. Anne Cent (lab super**) (206) 526-2088 (528-2793) acent@u.washington.edu
14. Stephen Sacks 689-9404 (5153) sacks@viridae.com
15. Rhoda Ashley lab head) (206) 526-2117 (2529) rashley@chmc.org
16. Anna Wald (MD, invest.) (206) 720-4340 (4371) annawald@u.washington.edu
17. David Koelle (“) (206) 667-6807 viralimm@u.washington.edu
18. Zane Brown (206) 543-3714 (3915) fx 616-9479 zbrown@u.washington.edu
19. Judy Zeh (data) (206) 720-4246 (4371) zeh@stat.washington.edu
20. Katie Link (data entry) (206) 720-4246 klink@u.washington.edu
21. Stacy Selke (data contact) (206) 720-4245 selke@u.washington.edu
22. Larry Corey (206) 667-6770 lcorey@u.washington.edu
23. Deborah Money 3075 (pgr. 02426) mccoll@interchange.ubc.ca
24. Jan Aura (RN) 543-5557 jaura@u.washington.edu
25. Sheree Miller (PharmD) (206) 598-6054 fax 598-4901 idssam@u.washington.edu
26. Meei-Li Huang, PhD (Mol. Dx Lab) 667-6803 fx 667-4411 meeili@u.washington.edu
APPENDIX II

Neonatal Haematological Impact of Acyclovir Use in Late Pregnancy

Money DM, Leung DT, Sacks SL, Bebbington MW
A double-blind, placebo-controlled, parallel group investigation of the haematological impact of third-trimester acyclovir suppressive therapy on the neonate.

Sponsor: National Institutes of Health (US)
University of Washington / University of British Columbia

Principal Investigator: Dr. Deborah M. Money
Division of Maternal-Fetal Medicine
Department of Obstetrics & Gynecology
British Columbia Women's Hospital

Co-Investigators: Daniel T. Leung
Dr. Stephen L. Sacks
Dr. Michael W. Bebbington

Study Coordinators: Valencia Rempel
Bonnie Anderson

Study site: British Columbia Women’s Hospital
4500 Oak St.
Vancouver, BC
Canada
V6H 1N1

Protocol Author: Daniel T. Leung
# TABLE OF CONTENTS

STUDY SYNOPSIS ............................................................................................................. 141

1. INTRODUCTION / RATIONALE ................................................................................. 142

2. OBJECTIVE .................................................................................................................. 143

3. STUDY PLAN ................................................................................................................. 144
   3.1 Study Design ............................................................................................................. 144
   3.2 Study Site and Duration .......................................................................................... 144

4. STUDY POPULATION .................................................................................................... 145
   4.1 Source and Number of Subjects .............................................................................. 145
   4.2 Inclusion/Exclusion Criteria .................................................................................... 145
   4.3 Baseline Characteristics ........................................................................................ 145

5. STUDY PROCEDURES AND ASSESSMENTS ................................................................. 37
   5.1 Screening ................................................................................................................ 146
   5.2 Collection of samples .............................................................................................. 146
   5.3 Haematological and Acyclovir Concentration Assessments .................................... 146

6. DATA MANAGEMENT .................................................................................................... 147
   6.1 Data Collection ........................................................................................................ 147

7. STATISTICAL CONSIDERATIONS AND ANALYSIS .................................................. 148
   7.1 Sample Size ............................................................................................................. 148
   7.2 Analysis .................................................................................................................. 148
   7.3 Breaking the Randomisation Code and Study Blinding ............................................. 148

8. REGULATORY REQUIREMENTS/LEGAL ISSUES ....................................................... 149
   8.1 Ethics Committee Approval ..................................................................................... 149
   8.2 Subject Informed Consent ....................................................................................... 149

9. PROTOCOL AMENDMENTS ......................................................................................... 150

10. References .................................................................................................................... 151
STUDY SYNOPSIS

A double-blind, placebo-controlled, parallel group investigation of the haematological impact of third-trimester acyclovir suppressive therapy on the neonate.

Objectives
To investigate the fetal myelosuppressive effects of suppressive acyclovir use in the third trimester of pregnancy.

Study Design
This is a double (investigator and patient)-blinded, placebo-controlled and randomized, parallel group study. Cord blood samples of women who are currently participating in the “University of Washington / University of British Columbia HSV & Pregnancy” study will be obtained. The samples will be processed by and haematological analysis of the samples will be performed at the BC Women’s Hospital. Determination of the acyclovir concentration will be done at Viridae Clinical Sciences.

Study site, timing, and investigator
The study will be conducted at the British Columbia Women’s Hospital between January and December of 2000. The principal investigator will be Dr. Deborah M. Money.

Subject numbers and analysis
Approximately 70 patients will initially be recruited into the study on an on-going basis. An interim analysis will be performed after data from samples of 15 patients of each treatment group is obtained. The mean neutrophil count of each treatment group will be compared, and the venous and arterial cord acyclovir concentration ratio will be correlated with the neutrophil count.
1. INTRODUCTION / RATIONALE

Bacterial infections are a major cause of morbidity and mortality in neonates, with an incidence of 1 to 10 per 1,000 live births and mortality rates ranging from 25% to 50%. Neutrophils are qualitatively and quantitatively the most important phagocytic cells defending the body against acute bacterial infection. They are formed in the bone marrow and use the blood stream as a means of transport. Progenitor cells are detected in bone marrow, blood, spleen, and cord blood. Neutropenia due to diminished myeloid proliferative capacity is one of the factors which contributes to a predisposition to bacterial infection in neonates.

Acyclovir is an acyclic guanine nucleoside analogue that is a selective inhibitor of the replication of HSV types 1 and 2 and varicella-zoster virus. Acyclovir triphosphate competes with guanosine triphosphate as a substrate for viral DNA polymerase, thereby terminating viral DNA replication. Although the safety of its suppressive therapy has been evaluated in pregnant individuals, the amount of safety data gathered on the neonates in the small studies published to date has been limited.

In a study of oral acyclovir suppressive therapy in 26 neonates, twelve (46%) developed neutropenia (<1000 cells/mm³). Ten of the patients had spontaneous recovery. Concurrent illness was documented in only 1 of the 12 patients, and additional medications that could account for the development of neutropenia were not being administered, suggesting that acyclovir may have been the cause of the temporary myelosuppression. Furthermore, in a study of acyclovir use in 88 neonates with HSV disease, 2 of 16 (13%) patients on intermediate dose acyclovir (45mg/kg/day) and 14 of 72 patients on high dose acyclovir (60mg/kg/day) had an absolute neutrophil count (ANC) of ≤1000 during their 21-day therapy. This myelosuppression could be attributed to an unknown effect of acyclovir on the bone marrow stem cells of the neonate. There have also been other case reports of neutropenia associated with acyclovir administration in children and infants.

Placental transfer of acyclovir during suppressive therapy could play a role in the suppression of myeloid proliferation in the fetus. This myelosuppression could lead to diminished neutrophil production, especially in the first three days post-partum (the neutrophil production cycle is between three to four days). The amount of safety data on neonates of mothers undergoing suppressive acyclovir therapy has been minimal. If approved for use in pregnancy for the suppression of genital herpes recurrences acyclovir use could become widespread among the many pregnant individuals (approximately 20% of women of child-bearing age are seropositive for HSV-2) who are affected by genital herpes. Evidently, it is important to conduct a thorough safety evaluation of the neonatal safety of this drug.

The neutrophil count from fetal cord blood from mothers undergoing suppressive acyclovir therapy will be compared with the neutrophil count from umbilical cord blood of mothers undergoing placebo. To determine the impact of possible fetal metabolism and clearance, the acyclovir concentrations of the umbilical venous and arterial cord blood will be analyzed using capillary zone electrophoresis, and correlations will be made with the haematological results. Extrapolations from these comparisons will be made to determine the neonatal myelosuppressive effects of acyclovir use in pregnancy.
2. OBJECTIVE

To investigate the fetal myelosuppressive effects of suppressive acyclovir use in the third trimester of pregnancy.

To determine whether the venous cord neutrophil count is lower among neonates of mothers under acyclovir therapy than those in the placebo group.

To determine whether a correlation exists between the venous cord to arterial cord acyclovir concentration ratio and the neutrophil count in neonates of the acyclovir group.
3. STUDY PLAN

3.1 Study Design

This is a double (investigator and patient)-blinded, placebo-controlled and randomized, parallel group study. Initially, approximately 70 cord blood samples of women who are currently participating in the “University of Washington / University of British Columbia HSV & Pregnancy” study will be obtained. The samples will be processed within three hours of delivery and haematological analysis of the samples will be performed at the BC Women's Hospital. Determination of the acyclovir concentration will be done at Viridae Clinical Sciences. On completion of 15 samples from each treatment group a formal interim analysis will be conducted. Outcome will be assessed based on a comparison of the mean neutrophil count of the two treatment groups, along with correlation of acyclovir uptake and neutrophil count in the acyclovir treatment group.

3.2 Study Site and Duration

The study will be conducted at the British Columbia Women's Hospital. The principal investigator will be Dr. Deborah Money, Division of Maternal Fetal Medicine, Department of Obstetrics & Gynecology. The study will be conducted between January and May of 2000.
4. STUDY POPULATION

4.1 Source and Number of Subjects

Subjects screened for the “University of Washington / University of British Columbia HSV & Pregnancy” study will be asked for permission to use the umbilical cord blood from their placenta.

4.2 Inclusion/Exclusion Criteria

The inclusion and exclusion criteria is the same as for the main study.

*Baseline Characteristics*
Baseline characteristics will include:
- Maternal age
- Maternal ethnicity
- Parity
- Maternal concomitant medications
- Duration of labor
- Length of gestation
- Maternal smoking status
- Complications during pregnancy
- Method of delivery
- Birthweight
- Neonatal sex
- Placental weight
- Cord blood pH
- Blood Pressure at delivery
- Agpar score
- Time of birth
5. STUDY PROCEDURES AND ASSESSMENTS

5.1 Screening

In the screening visit of the main study, the subject will be asked for informed consent to obtain blood samples from the umbilical cord after the delivery of the neonate. Those women not entering the placebo study, but planning to take acyclovir, will also be asked to participate.

5.2 Collection of samples

Cord blood samples will be taken from the clamped umbilical cord immediately after delivery. One arterial and two venous samples will be collected separately in EDTA-containing tubes. One venous sample will be kept at room temperature for a maximum of three hours before delivery to the haematology lab at BCCW for blood chemistry determination. The other venous sample, along with the arterial sample, will be stored at 4°C for a maximum of 72 hours, within which this sample will be delivered to Viridae Clinical Sciences for determination of acyclovir concentration.

Note: The cord arteries are very small in comparison to the vein, which can lead to difficulties in obtaining an arterial sample of adequate volume. Due to the narrow diameter of the artery, it is also possible to stick the needle right through the arterial wall and accidentally sample the vein.

5.3 Haematological and Acyclovir Concentration Assessments

One venous cord blood sample will be analysed by the haematology laboratory of BCCW to determine haematological variables. The other venous and arterial samples will be processed at Viridae Clinical Sciences, where the plasma component will be separated by centrifugation at 1200rpm at 4°C. The plasma sample will then be stored at −70°C until electrophoresis processing. Perchloric acid deproteinization will be used to precipitate proteins from the plasma, which will then be neutralized with potassium hydroxide, diluted using EDTA, filtered through 0.2μ centrifuge, then analysed for acyclovir content using capillary zone electrophoresis.
6. DATA MANAGEMENT

6.1 Data Collection

Case report forms will be used for recording all data collected in this study, together with screening and selection logs. The study co-ordinator will complete case report forms by compiling information from the main study forms and faxed reports from the haematology and capillary electrophoresis laboratories. Due to the possible revelations from reports of acyclovir concentration in the case report forms, the investigators will be kept blinded from the case report forms.
7. STATISTICAL CONSIDERATIONS AND ANALYSIS

7.1 Sample Size

Literature data shows that for normal fetuses of 40 weeks' gestation, the expected mean cord blood neutrophil count is approximately 8.5 G/liter. vii,viii In a recent clinical trial studying the pharmacokinetics of acyclovir in late pregnancy, ix the mean (n=3) cord blood neutrophil count for pregnancies under third trimester acyclovir suppression was 7.3 G/liter. x To detect statistically a treatment decrease of 1.2 G/liter (i.e. placebo = 8.5 vs acyclovir group = 7.3 G/liter) with a standard deviation of 2.0 and a power of 80% at the 0.05 significance level, requires 35 subjects per study group, for a total of 70 subjects.

7.2 Analysis

The neutrophil count from the two treatment groups, assuming a normally distributed population with possibly unequal variances and unequal sample sizes, will be analyzed using the two-sample t-test. The null hypothesis of a lower neutrophil mean from the acyclovir treatment group will be tested against a one-tailed alternative, with a type I error rate of 5%, and a type II error rate of 5%.

To determine whether acyclovir uptake/metabolism in the fetus correlates with neutrophil count, a linear regression analysis will be performed on the venous:arterial ratio of the acyclovir concentration in cord blood with the neutrophil count of that sample.

7.3 Breaking the Randomisation Code and Study Blinding

The randomisation will be decoded to apportion patients into their respective treatment groups in order to conduct the interim analysis, however, this will only be performed when all the key data for this analysis has been retrieved and audited. The investigators and patients will be blinded to the type of therapy received; however, the study co-ordinator, who will not have any interactions with the patients nor the investigators during the course of the trial, will not need to be blinded. As the investigator and patient are blinded, the technicians and study co-ordinator will need to keep the data hidden from the investigator until the breaking of the randomisation code. The actual study medication received by each group will not be unblinded until the study has been completed and all the data has been retrieved and audited and the database closed.
8. REGULATORY REQUIREMENTS/LEGAL ISSUES

8.1 Ethics Committee Approval

The final study protocol will be reviewed by the Clinical Research Ethics Board, Office of Research Services and Administration, University of British Columbia, and the British Columbia’s Women’s Hospital and Health Centre Ethics Board. Formal unconditional approval of the study must be obtained in writing before the study can commence. The approval must refer to the exact protocol title and number, and state the date of the review. The above ethics boards will be informed of, and may be required to approve, amendments to the protocol.

8.2 Subject Informed Consent

An addendum to the informed consent form of the main study will be used to obtain informed consent. It is the investigators’ responsibility to ensure that each subject gives informed consent before participating in the study. The Investigator will provide both a written and verbal explanation of the nature of the study, its purpose, procedures, duration and the potential benefits and risks of participation (addendum to main study consent form included in the Appendix). Subjects will be given the opportunity to ask questions and will be advised that they are free to refuse to take part in, or withdraw from, the study at any time. The medical care provided will not be affected by agreement or refusal to participate in the study. After this explanation, it will be necessary for the subject to voluntarily sign an Informed Consent Form which must be retained by the Investigator.
9. PROTOCOL AMENDMENTS

No modification to the study protocol will be allowed unless discussed in detail with the investigator and filed as an amendment to this protocol. Any modification to the protocol will be strictly adhered to and will apply to all subjects following approval by the ethics board.
10. References

* Personal communication with Deborah F Kimberlin of the University of Alabama.
Neonatal Haematological Impact of Acyclovir Use in Late Pregnancy

CASE REPORT FORMS

Principal Investigator:
Dr. Deborah M. Money

Co-Investigators:
Daniel T. Leung
Dr. Stephen L. Sacks
Dr. Michael W. Bebbington

Study Coordinators:
Valencia Rempel, Bonnie Anderson

Patient Study No. 152
Maternal Age at Delivery: [ ]

Maternal Race (✓ One):
- White [ ]
- Aboriginal [ ]
- Black [ ]
- Hispanic [ ]
- Oriental [ ]
- Mixed race [ ]
- East Indian [ ]
- Other [ ]

Education: [ ] years post-secondary

Annual household income:
- $≥50,000 [ ]
- $30,000-$50,000 [ ]
- $10,000-$30,000 [ ]
- $≤15,000 [ ]
- Prefer not to answer [ ]
- Unknown [ ]

# Persons in household depending on this income: [ ]

Smoking Status (✓ One):
- Non-smoker [ ]
- <5 Cigarettes / day [ ]
- >5 Cigarettes / day [ ]

Alcohol Use (✓ One):
- None [ ]
- ≤1 Drink / day [ ]
- >1 Drink / day [ ]

DATE: dd/mm/yy
Concurrent Medications (Including OTC medications):
Were any concurrent medications taken by the subject during the pregnancy? □ Yes □ No

If YES, record below:

<table>
<thead>
<tr>
<th>Drug (Trade name preferred)</th>
<th>Date started Or ✓ if started before trial</th>
<th>Date started Or ✓ if continued posttrial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR Pretrial</td>
<td>OR Posttrial</td>
</tr>
<tr>
<td></td>
<td>Dd/mm/yy</td>
<td>Dd/mm/yy</td>
</tr>
</tbody>
</table>

DATE: ...../...../.....

dd mm yy
Neonatal Haematology Substudy  

NEO-CRF3  

Pregnancy History  

**Gravidity** (including current pregnancy):  

**Parity** (excluding this pregnancy):  

**Live births:**  
- Term (≥38 weeks):  
- Preterm:  
- Neonatal Demise:  

**Pregnancies:**  
- Elective Abortion:  
- Spontaneous abortion:  
- Stillborn:  
- Ectopic pregnancies:  

**Weight:**  
- Pre-pregnant weight (lbs / kg):  
- Current weight (lbs / kg):  

DATE: ...../...../.....  
dd mm yy
Neonatal Haematology Substudy

Patient Study No. __________

NEO-CRF4

Labor and Delivery Information

Onset of labor:

DD / MM / YYYY HH:MM

Membranes ruptured:

DD / MM / YYYY HH:MM

Complete dilation:

DD / MM / YYYY HH:MM

Date and Time of Birth:

DD / MM / YYYY HH:MM

Fetal Monitor:  None □ External □ Scalp lead □ IUPC □

Membrane Rupture:  Spontaneous □ Artificial □ Unknown □

Amniotic fluid:  Clear □ Bloody □ Meconium □ Unknown □

Type of Labor:  Spontaneous □ Augmentation □ Induction □

No Labor □ Unknown □

# of Infants:  Singleton □ Multiple births □

Presentation:  Breech □ Vertex □ Other/twins □ Unknown □

DATE: ...../...../.....

dd mm yy
Complications during Pregnancy:

Complications during Labor:

Complications during Delivery:

Method of delivery (√ One): Spontaneous Birth ☐ Forceps ☐
Vacuum Extraction ☐ Caesarean Section ☐

Maternal Blood Pressure at Delivery (mmHg): Systolic: ☐
Diastolic: ☐

DATE: _____/_____/_____
   dd mm yy
Neonatal Sex (✓ One): Male □ Female □

Birthweight (g): □

Head circumference (cm): □

Length (cm): □

Gestational age (wks) (OB): □ Gestational age (wks) (PEDS): □

Size: AGA □ LGA □ SGA □ Unknown □

Agpar score: □ at 1 minute □ at 5 minutes

Neonatal Complications:

___________________________________________________________________________
___________________________________________________________________________
___________________________________________________________________________
___________________________________________________________________________

DATE: ...../....../.....
dd mm yy
CONFIDENTIAL!
Results must be hidden from Principal / Co-Investigators of the Main Study

Haematology Results:

<table>
<thead>
<tr>
<th>WBC</th>
<th>Platelets</th>
<th>Basophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>MPV</td>
<td>Metamyelocytes</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>Neutrophils</td>
<td>Myelocytes</td>
</tr>
<tr>
<td>MCV</td>
<td>Bands</td>
<td>Promyelocytes</td>
</tr>
<tr>
<td>MCH</td>
<td>Lymphocytes</td>
<td>Blasts</td>
</tr>
<tr>
<td>RDW-CV</td>
<td>Monocytes</td>
<td>Aty Lymphs</td>
</tr>
<tr>
<td>HgB</td>
<td>Eosinophils</td>
<td>NRBC</td>
</tr>
</tbody>
</table>

Comments:

Umbilical Arteriolar Acyclovir Concentration (µg/ml): __________

Umbilical Venous Acyclovir Concentration (µg/ml): __________

Date and Time of Plasma Separation: DD / MM / YYYY HH:MM

Cord Blood pH: __________

DATE: dd mm yy
Study Co-ordinator’s Statement

I certify that I have carefully examined and verified all entries in these Case Report Forms. All information entered onto these forms by myself and/or my associates are correct.

[Signature]

DATE: ....../....../......

dd mm yy