CANADIAN SOCIETY OF PHARMACOLOGY AND THERAPEUTICS

"CLINICALLY RELEVANT PHARMACOKINETIC CHANGES IN PREGNANCY"

MAY 27, 2011
MONTREAL, QUEBEC

SPONSORED BY:
PREGMEDIC

Pregmedic

PREGMEDIC, the Canadian Alliance for the Safe and Effective Use of Medications in Pregnancy and Breastfeeding, is a Canadian non-profit organization whose mission is to protect the health of pregnant women and their unborn babies by advocating for access to evidence-based information on the safe and effective use of medications during pregnancy and breastfeeding. Created in 2006 by representatives from health professions, academia, patients, regulatory and industry, Pregmedic aims at:

1. The creation of a Health Canada Expert Advisory Committee on Pregnancy and Breastfeeding;
2. The inclusion of women in clinical trials as well as pharmacokinetics and bioequivalence studies to be done in intended population;
3. A new labelling system to address drug use during pregnancy and breastfeeding;
4. The development of a national pregnancy exposure registry.

PREGMEDIC, l'Alliance canadienne pour une médication sécuritaire et efficace pendant la grossesse et l'allaitement, est un organisme canadien sans but lucratif qui a pour mission de protéger la santé de la femme et de son enfant à naître en militant pour l'accès à de l'information probante concernant l'utilisation de médicaments sécuritaires et efficaces pendant la grossesse et l'allaitement. Fondée en 2006 par des représentants de la communauté médicale, du milieu académique, de groupes de défense des patients, du secteur réglementaire et de l'industrie, Pregmedic vise :

1. La création par Santé Canada d'un comité consultatif expert en grossesse et allaitement;
2. L'inclusion des femmes dans les essais cliniques de même que la poursuite d'études de pharmacocinétique et de bioéquivalence dans la population visée;
3. Un nouveau système d'étiquetage des médicaments tenant compte de l'usage de ceux-ci durant la grossesse et l'allaitement;
4. Le développement d'un registre canadien sur les médicaments utilisés par les femmes enceintes.

For more information, please write to Pregmedic's Executive Director, at info@pregmedic.org
Pour plus d'informations, veuillez contacter la directrice exécutive de Pregmedic, à info@pregmedic.org

www.pregmedic.org

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SPEAKERS AND PRESENTATIONS

DAVID KNOPPERT, BScPhm, MScPhm, MSc(Clinical Epidemiology)

President, Pregmedic; Clinical Leader in Paediatric Pharmacy, London Health Sciences Centre, London ON; Assistant Professor, Departments of Paediatrics and Medicine, University of Western Ontario, London ON

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ANDRÉ B. LALONDE, MD, FRCPSC, FACS, FABOG, MSc (Health Policy, Planning and Financing)

Executive Vice-President, Society of Obstetricians and Gynaecologists of Canada, Ottawa ON; Clinical Professor of Obstetrics and Gynaecology, University of Ottawa, Ottawa ON, and McGill University, Montreal QC; Adjunct Professor, Health Administration, University of Ottawa, Ottawa ON

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IAIN J. MCGILVERAY, BSc (Pharm), PhD, FAAPS, FCSPS

Principal, McGilveray Pharmacon Inc, Ottawa ON; formerly Research Scientist, Bureau of Drug Research, and Chief, Biotherapeutics Division, Bureau of Biologics and Radiopharmaceuticals, Health Protection Branch, Health Canada, Ottawa ON

"BIOEQUIVALENCE STUDIES OF DRUGS PRESCRIBED MAINLY FOR WOMEN"
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GIDEON KOREN, MD, FRCPC, FACMT

Director, Motherisk Program, and staff pediatrician, Hospital for Sick Children, Toronto, Ontario; Richard Ivey Chair in Molecular Toxicology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario; professor of pediatrics, pharmacology, pharmacy and medicine, University of Toronto, Canada

"PHARMACOKINETICS IN PREGNANCY; CLINICAL SIGNIFICANCE"
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OFFIE P. SOLDIN, MSc (Pathology), PhD, MBA

Professor, Departments of: Oncology Division of Cancer Genetics and Epidemiology; Medicine, Division of Endocrinology and Metabolism; Obstetrics & Gynecology; Pharmacology and Physiology, Georgetown University Medical Center, Washington DC

"INNOVATIVE STUDIES IN WOMEN BY USE OF STABILIZED ISOTOPES IN PREGNANCY"
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JANINE R. HUTSON, BSc, MSc

Student in the MD/PhD Program and the Collaborative Program in Biomedical Toxicology, University of Toronto; Vanier Canada Graduate Scholar; Senior Editor, trainee section, Clinical and Investigative Medicine, official journal of the Canadian Society for Clinical Investigation; Co-Chief Editor of Pharmacology You See (textbook for pre-clerkship medical students being developed under the auspices of the University of Toronto Medical Society and the University of Cincinnati)

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SAFETY AND EFFICACY OF DRUGS IN PREGNANCY

David Knoppert

London Health Sciences Centre, London ON; Departments of Paediatrics and Medicine, University of Western Ontario, London ON

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ABSTRACT

Although most drugs are used to treat chronic or pregnancy-induced conditions during pregnancy and lactation, very few are studied in pregnant or breastfeeding women. The information we have on drugs taken during pregnancy and lactation is usually obtained after market approval through published case reports or case series and from pregnancy exposure or retrospective birth defect registries. Furthermore, generic drugs approved for use in this vulnerable population may be approved based on results from a male trial population. This disregards the changes that can occur during pregnancy which can affect the pharmacokinetics of drugs. In an effort to improve the information provided to prescribers, in 2008 the United States Food and Drug Administration proposed a change in product labelling where information from pregnancy exposure registries would be required. As of 2009, European Medicines Agency requires additional statements on use during pregnancy within drug labelling information. In Canada, it is anticipated that the efficacy and safety of drugs in pregnancy will be included under the Drug Safety and Effectiveness Network initiative, and that this will offer a unified approach for such assessments. Pregmedic, a non-profit organization for the advancement of safe and effective use of drugs in pregnancy, has presented a number of proposals and draft guidelines to Health Canada on the inclusion of pregnant women in pharmacokinetic studies and the establishment of registries for women who take drugs during pregnancy. Pregmedic advocates for ensuring that drugs indicated for women are studied in women.

Key Words: *Pregnancy, lactation, pharmacokinetics, registries, women, Pregmedic, advocacy*

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Introduction

Pregmedic is a non-profit organization for the advancement of safe use of drugs in pregnancy. The mission of Pregmedic is to advocate for the safe and effective use of medications in pregnancy and lactation. Our goals are to increase awareness of pregnancy issues at the government level through Health Canada; to require standard labelling of medicines for use in pregnancy and lactation; to provide practitioners and patients access to current and reliable information for decision-making during pregnancy and lactation; and to advocate for the development of patient registries or surveillance programs for medications used during pregnancy and breastfeeding.

Changes during Pregnancy

A number of physical changes occur during pregnancy, which in turn affect the pharmacokinetics of drugs.
These include:
- changes in total body weight and body fat;
- delayed gastric emptying and prolonged gastrointestinal transit time, which can affect the bioavailability of drugs;
- increased extracellular fluid and total body water, which can affect water-soluble drug kinetics, e.g., aminoglycosides;
- increased cardiac output as a result of increased stroke volume and maternal heart rate;

- increased blood flow to the organs, e.g., liver and kidney, thus drug clearance can be increased;
- decreased albumin concentration with decreased protein binding, affecting drugs that are highly protein bound; and
- altered hepatic enzyme activity, which can alter drug metabolism and interactions.

Odd Facts about Drugs in Pregnancy

Most drugs *are* used in pregnancy and lactation in order to treat chronic or pregnancy-induced conditions, such as high blood pressure, increased blood sugar, and infections; and most are used 'off label'. Very few drugs are studied for use during pregnancy or lactation, providing little guidance to physicians, pharmacists, and patients. Product monographs generally advise that drugs *should not* be used when women are pregnant and breastfeeding; as well, for reasons related to litigation, most pharmaceutical companies do not address the use of drugs during pregnancy. The information we do have is usually obtained after market approval through published case reports or case series and from pregnancy exposure or retrospective birth defect registries. Such reports are limited, representing only a fraction of the circumstances where drugs are used in pregnancy or lactation.

There is a significant difference in pharmacokinetics of drugs between men and women, and especially between men or non-pregnant women and women who are pregnant or lactating; yet bioequivalence studies include both men and women and report on the average findings from both genders. There is currently no requirement to disclose the exact population used in bioequivalence trials. Generic drugs approved for use in a vulnerable population, such as pregnant women, may be approved based on results from a male trial population. This disregards any of the changes that can occur during pregnancy (noted above), which can affect the pharmacokinetics of drugs.

The consequence of all these facts is that healthcare professionals are left with the burden of evaluating the risk or the benefit of using a medication during pregnancy or lactation.

Other Countries

What is happening in other countries? In the United States, the Food and Drug Administration (FDA) currently requires labelling according to preset categories for drug use in pregnancy (Table 1).[1] A few drugs are in categories A or B; some are clearly contraindicated during pregnancy; but most drugs are categorized under "C": Human data is lacking and animal studies have either not been conducted or have shown an adverse effect on the fetus.

TABLE 1 FDA Pregnancy Categories Summarized

Pregnancy Category	Description
A	Controlled studies in humans
B	Human data is reassuring (animal positive) or animal studies show no risk
C	**Human data is lacking - animal studies positive or not done**
D	Human data show risk; benefit may outweigh risk
X	Animal or human data positive

In 2008, an FDA proposal was made to amend the labelling regulations for drug use in pregnancy. Pregnancy information would move from the "Contraindications" section of the product monograph to the section "Use in Specific Populations".[2] Prescription drug labelling would then require information from pregnancy exposure registries, if applicable, a general statement about the background risk of fetal developmental abnormalities, clinical considerations, and a data component. Thus more information would be available to help the prescriber in decision-making.

On December 30, 2009, the FDA announced collaboration with researchers on the "Medication Exposure in Pregnancy Risk Evaluation Program", where data will be used from 11 U.S. health plan-affiliated research sites.[3]

In Europe, the European Medicines Agency (EMA) has a Guideline on the Exposure to Medicinal Products During Pregnancy: Need for Post-authorisation Data.[4] Furthermore, the Guideline on Risk Assessment of Medicinal Products on Human Reproduction and Lactation: From Data to Labelling,[5] which came into effect in January 2009, requires additional labelling information.

Examples of acceptable statements for use in the "Pregnancy" section of a product monograph, which provide more guidance for clinicians, are:

- Based on human experience (specify), Drug X is suspected to cause congenital malformation (specify) when administered during pregnancy.
- Drug X should not be used during pregnancy (specify trimester) unless the clinical condition of the woman requires treatment with Drug X.
- A moderate amount of data on pregnant women (between 300-1000 pregnancy outcome) indicate no malformative or feto/neonatal toxicity for Drug X.
- No effects during pregnancy are anticipated, since systemic exposure to Drug X is negligible.

Canada

In Canada, the Drug Safety and Effectiveness Network (DSEN) has been established as a program through the partnership of the Canadian

Institutes of Health Research (CIHR) and Health Canada. "New evidence generated via the DSEN will provide Health Canada with an important additional source of information for use in the ongoing assessments of drug products' safety risks relative to their therapeutic benefits. This evidence will also support decision-making on public reimbursement, and the safe and optimal prescribing and use of drugs within the Canadian health care system."[6] Certainly the efficacy and safety of drugs in pregnancy will be included under this initiative and will offer a unified approach across Canada for such assessments.

The priority actions undertaken by Pregmedic in Canada include the Draft Guideline for Inclusion of Pregnant Women in Pharmacokinetic Studies, which was presented to Health Canada in June 2009. Pregmedic also advocates for the adoption by Health Canada of the European Labelling Requirements for Pregnancy and Lactation; requests creation of registries for women who need to take drugs during pregnancy and for post-market surveillance studies; and advocates for ensuring that drugs indicated for women are studied in women.

So, although progress is being made in our country, there remains much work to be done, and action to be taken, in the field of drug safety in pregnancy.

Corresponding Author: dknopper@uwo.ca

REFERENCES

1. Adapted from: FDA Consumer magazine. Volume 35, Number 3, May-June 2001. http://www.perinatology.com/Archive/FDA%20CAT.htm (June 28, 2011).
2. U.S. Food and Drug Administration. News & Events. FDA News Release: FDA Proposes New Rule to Provide Updated Information on the Use of Prescription Drugs and Biological Products during Pregnancy and Breast-feeding. (May 28, 2008) http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2008/ucm116902.htm (July 15, 2011).
3. U.S. Food and Drug Administration. News & Events. FDA News Release: FDA, Health Organizations to Study Safety of Medications Taken During Pregnancy. New collaborative research program to study effects on mothers

and their babies. (December 30, 2009) http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm195934.htm (July 15, 2011).

4. European Medicines Agency. Guideline on the Exposure to Medicinal Products. www.ema.europa.eu/.../en_GB/document_library/Regulatory_and_procedural_guideline/2009/11/WC500011303.pdf (June 28, 2011).

5. European Medicines Agency. Guideline on Risk Assessment of Medicinal Products on Human Reproduction and Lactation: From Data to Labelling. 2008 July 24. http://www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500003307 (June 28, 2011).

6. Canadian Institutes of Health Research. About DSEN. In brief: The Drug Safety and Effectiveness Network (DSEN). Background. 2009 September 29. http://www.cihr-irsc.gc.ca/e/39389.html (June 28, 2011).

DRUGS INDICATED FOR USE DURING PREGNANCY

André B Lalonde

Society of Obstetricians and Gynaecologists of Canada, Ottawa ON; University of Ottawa, Ottawa ON, and McGill University, Montreal QC; Health Administration, University of Ottawa, Ottawa ON

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ABSTRACT

The Society of Obstetricians and Gynaecologists of Canada (SOGC) advocates that drugs used during pregnancy be tested exclusively in women. The SOGC holds the opinion that drugs to be used exclusively in men *or* in women should not be tested in a small number of men *and* women.

The SOGC, always cautious with the choice of pharmacological treatments recommended for use during pregnancy, welcomes the increased options resulting from the introduction of generic formulations of drugs shown to be bioequivalent to currently available brand name products. These formulations provide less expensive options to Canadian women in need of drug therapy. However, the Society does not believe that drugs should be substituted without the patient and the physician both agreeing to such a change. Generic substitutions of some products may mean a potentially clinically significant difference in drug dose, possibly resulting in a changed patient effect. Furthermore, substituting a product on the basis of price alone is not acceptable.

The SOGC, as an organization with the role of advising its members on clinical practice, calls on Health Canada to review its guideline on testing of drugs for vulnerable populations, especially pregnant women.

Key Words: *Bioequivalence, intrasubject variability, sex-related differences, pregnancy, Society of Obstetricians and Gynaecologists of Canada (SOGC)*

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Introduction

This presentation will introduce the discussions that have been underway at the Society of Obstetricians and Gynaecologists of Canada (SOGC) as regards the use of drugs in pregnancy. The Society's objectives on this topic include discussion of appropriate testing of drugs for use in men and women in Canada, and to advocate for testing of drugs used during pregnancy exclusively in women.

The SOGC is always cautious with the choice of pharmacological treatments recommended for use during pregnancy. The Society welcomes the increased choice of products resulting from the introduction of generic formulations of drugs that have been shown to be bioequivalent to currently available brand name products. These formulations provide less expensive options to Canadian women in need of drug therapy. According to Health Canada, 'bioequivalence' implies that the drug product has the same systemic exposure and effects, both therapeutic and adverse, as a reference product when administered to patients under conditions specified in the labelling. When testing bioequivalence, "An important objective in the selection of subjects is to reduce the intrasubject variability in pharmacokinetics that may be attributable to certain characteristics of the subject. Subjects should be assigned in such a way that the study design is balanced for any factors that are suspected to contribute to variability."[1]

Bioequivalence of Generic Drugs

Both Health Canada and the U.S. Food and Drug Administration (FDA) recognize that bioequivalence studies demonstrate significant variability between men and women. More than

30% of studies reviewed for bioequivalence would not have passed the required criteria for one sex when they did for the other, according to a review of trials by Chen et al.[2] Table 1 shows the intrasubject variability in men vs. women for 6 drugs, where not only are there differences within individuals' results, but the differences between the sexes range from about two- to six-fold.

TABLE 1 Intrasubject Variability in Men vs. Women in Bioequivalence Trials[a]

DRUG	MEN	WOMEN
Alprazolam	4.9%	29.4%
Cimetidine	15.8%	9.9%
Erythromycin	18.1%	25.7%
Naproxen (at low dose)	5.0%	9.1%
Nitroglycerine	21.3%	39.5%
NAPA (N-Acetylprocainamide) - Class III antiarrhythmic agent)	9.0%	4.4%

[a]Adapted from Chen M-L, et al. Clin Pharmacol Ther. 2000;68(5):510-521.[2]

In addition, the SOGC would like to advocate for testing of drugs within the appropriate test groups, so that drugs intended for use by women should only be tested in women. Presently in Canada, there is no regulation for preferring female over male subjects when studying drugs intended only for women. This could lead to serious errors when interpreting bioequivalence studies performed in men, or in combinations of male and female subjects. Hence, a generic formulation may be approved by Health Canada, based on an inadequate assumption that variability in the sexes is similar; and there is marked concern with the small patient numbers used for such testing. A recent SOGC survey showed that members believe that the introduction of a generic product has been tested in a few hundred to a thousand subjects. However, clinicians are very concerned when they learn that in some cases as few as 14 subjects are used to show bioequivalence. Furthermore, there is concern about the adequacy of results where the majority of subjects is male (e.g., of 20 subjects, only 4 are female), yet average results are used and then applied to the population as a whole. Also, although Health Canada requires that a generic formulation be bioequivalent to the innovator formula, where a drug is intended for treatment of pregnant women, the test population may include both men and women and the average results used. If a sex:formulation interaction occurs, the safety and efficacy of the generic formulation could not necessarily be considered equivalent to the reference formulation. These difficulties are even more evident for bioequivalence studies in pregnant women, which are almost non-existent.

There are currently no specific requirements for the approval of drugs intended for vulnerable populations, such as pregnant women. In addition, there are no requirements to disclose the exact population used for the determination of bioequivalence, which is a real concern for clinicians across Canada.

SOGC Opinions
The SOGC holds the opinion that drugs to be used exclusively in men *or* in women should not be tested in a small number of men *and* women. Drugs should be tested in an adequate number of men only or women only, depending on the demographic in which the drug is intended to be used. The SOGC also asks for higher subject numbers to be used in bioequivalence testing.

The SOGC does not believe that drugs should be substituted without the patient and the physician both agreeing to such a change. Generic substitutions of some products, e.g., very low dose

estrogen contraceptives, may mean a potentially clinically significant difference in drug dose, possibly resulting in a changed patient effect. Furthermore, substituting a product on the basis of price alone is not acceptable, especially for drugs that have been tested in very few subjects— sometimes as few as 15-20 patients and most often in both males and females.

CONCLUSIONS

The SOGC, as an organization with the role of advising its members on clinical practice, calls on Health Canada to review its guideline on testing of drugs for vulnerable populations, especially pregnant women, and would like to see a policy change established.

We recommend that testing standards be set at a minimum of 100 patients in order to detect intrasubject variability and to eliminate errors that can happen when transposing tests between males and females, especially for diseases or conditions that affect exclusively one sex or the other.

Call to Action

Healthcare professionals and women's groups are called to advocate that the Government of Canada establish an *ad hoc* committee to develop recommendations to change the way drugs intended for exclusive use in women, and particularly for use in pregnant women, are tested.

Corresponding Author: alalonde@sogc.com

REFERENCES
1. Health Canada. Drugs and Health Products. Guidance for Industry: Conduct and Analysis of Bioavailability and Bioequivalence Studies - Part A: Oral Dosage Formulations Used for Systemic Effects. (October 8, 1997) http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/prodpharma/bio-a-eng.pdf (July 15, 2011).
2. Chen M-L, Lee SC, Ng M-J, Schuirmann DJ, Lesko LJ, Williams RL. Pharmacokinetic analysis of bioequivalence trials: implication for sex-related issues in clinical pharmacology and biopharmaceutics. Clin Pharmacol Ther 2000;68(5):510-521.

BIOEQUIVALENCE STUDIES OF DRUGS PRESCRIBED MAINLY FOR WOMEN

Iain J McGilveray

McGilveray Pharmacon Inc, Ottawa ON; formerly Research Scientist, Bureau of Drug Research, Biotherapeutics Division, Bureau of Biologics and Radiopharmaceuticals, Health Protection Branch, Health Canada, Ottawa ON

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ABSTRACT

The basic components of pharmacokinetics are absorption, distribution, metabolism, and excretion. During pregnancy there may be changes in one or many of these components. Early drug studies did not include a representative proportion of women, however, researchers as well as regulators agree that studies on the sex differences in the disposition of drugs are important, but at what stage in the clinical trial process? Except for drugs used only in women, such as those for estrogen-dependent breast cancer, caution prevails and the differences are usually studied at phase 3. Studies in pregnant women are much rarer but some do get done, e.g., with antivirals and antimalarials, where the positive risk-benefit of these agents is the likelihood that fetal transfer of these drugs might help protect the fetus. Women are being included in pharmacokinetic studies for new drug applications in accordance with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), U.S. Food and Drug Administration (FDA), and Health Canada (HC) guidances. A new look at bioequivalence studies, to compare results in men and women, would help determine if interactions of formulation and gender are a problem.

Key Words: *Pharmacokinetics, bioavailability, bioequivalence, pregnancy, sex differences*

Introduction

The basic components of pharmacokinetics are described under the acronym ADME: absorption, distribution, metabolism, and excretion. When we speak of bioavailability, we speak about what happens from the point of drug administration to its absorption, and whether there are differences in absorption between products (comparative bioavailability = bioequivalence). In pharmacokinetics, when we consider plasma levels—and sometimes urine levels—we also look at all of the processes that affect these levels, such as metabolism and protein binding, which show changes in pregnancy, are to some extent different between the sexes, and are also different among various drugs. In determining bioavailability/bioequivalence we have to tease out absorption from the other physiological processes that are not product dependent.

Regulatory Guidelines

In 1993, the U.S. Food and Drug Administration (FDA) issued a guideline for the study and evaluation of gender differences in the clinical evaluation of drugs in order to involve more women in clinical trials;[1] before then, drugs were not well studied in women. Since 2004, the FDA has had a draft guideline in place: Pharmacokinetics in Pregnancy – Study Design, Data Analysis, and Impact on Dosing and Labeling.[2] In Canada, the Drugs Directorate issued a policy in September 1996 for the inclusion of women in clinical trials during drug development.[3]

A number of papers have reviewed participation of women in clinical trials and differences between the sexes as regards pharmacokinetics. Yang *et al*., in a study of the participation of women in clinical trials for new

drugs approved by the FDA between 2000 and 2002, reported "… overall participation by women and men was comparable, suggesting an improvement in including more women in clinical trials when compared with the previous FDA study evaluating women's participation from 1995 through 1999. As with the previous study, however, a significant underrepresentation of women in early phase trials and in certain areas, such as cardiovascular products, was observed and continues to be an issue of concern."[4] For new drugs, clinical pharmacology studies are reported according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Common Technical Documents (Section 2.7.2, with details in section 5).[5] Pharmacokinetics studies are often also reported under this category, with some analysis by gender; ICH Guideline E5 covers ethnic or special population data.

Reports on Pharmacokinetic Differences between the Sexes

In 2003, Schwartz published a paper on the influence of sex on pharmacokinetics.[6] He noted:

- Absorption was not significantly affected by sex, but that rates may be slightly slower in women.

- Bioavailability, for CYP3A substrates in particular, may be somewhat higher in women compared to men, resulting in greater exposure due to lower clearance.

- The role of sex on pharmacokinetics, when considered in conjunction with genetics, age, disease, and social habits is not yet known in the clinical setting and needs more study.

Beierle *et al.* reported that for the majority of investigated drugs in recent years, no, or only very minor, gender differences could be detected in pharmacokinetics or pharmacodynamics, and that their clinical significance seems very limited, i.e., seems rarely linked to treatment success or failure.[7] "Hence, it is undoubtedly necessary to include women in the clinical drug development process, but it seems questionable whether women of child-bearing capability should be exposed to potential risks in early phase I clinical trials."[7]

Soldin and colleagues, in their recent paper on sex differences in drug disposition, conclude: "Males and females may differ in specific drug

pharmacokinetics and pharmacodynamics. It is, therefore, essential to understand those sex differences in drug disposition and response, as they may affect drug safety and effectiveness."[8]

Researchers as well as regulators agree that studies on the sex differences in the disposition of drugs are important, but at what stage in the clinical trial process? Except for drugs used only in women, such as those for estrogen-dependent breast cancer, caution prevails and the differences are usually studied at phase 3. Studies in pregnant women are much rarer but some do get done, e.g., with antivirals and antimalarials, as the positive risk-benefit with these agents is the likelihood that fetal transfer of these drugs might help protect the fetus.

Alcohol Pharmacokinetics – An Example of Sex Differences in Drug Disposition

It has been known since antiquity that women are more susceptible than men to the effects of alcohol; and further, that fetal alcohol syndrome is a sad result of exposure. Some effects may be due to body mass, with higher blood levels more common in women. A small 1996 study found that "Dose-corrected values for AUC were on average 28% higher (p< 0.0001) in the women than in the men."[9] But the issue is more complex. One 2001 report noted "The gender difference in alcohol levels is due mainly to a smaller gastric metabolism in females (because of a significantly lesser activity of chi-ADH), rather than to differences in gastric emptying or in hepatic oxidation of ethanol."[10] Another review stated that "influences on alcohol elimination rate include gender, body composition and lean body mass, liver volume, food and food composition, ethnicity, and genetic polymorphisms in alcohol metabolizing enzymes."[11] More particularly, however, an "important determinant" was the allelic variants of the genes encoding the alcohol metabolizing enzymes, ADH and ALDH. Thus some women are less susceptible to the effects of alcohol, and even now we do not fully understand why.

Bioequivalence

Coming back to drug disposition, the main exposure metric of bioequivalence is absorption, which is affected by the formulation and in turn influences the plasma level and the area under the curve (AUC). However, it is metabolism that *primarily* influences the AUC. (See Table 1)

TABLE 1 Definitions

Bioavailability is a pharmacokinetic attribute. "It is defined as the rate and extent of absorption of a drug into the systemic circulation."[12] It is assessed by serial measurements of the drug in the systemic circulation, which provide a plasma concentration-time curve from which important pharmacokinetic parameters can be calculated, including the area-under-the-curve (AUC), the maximum observed concentration (C_{max}) and the time when C_{max} is reached (t_{max}).[12]

AUC provides an estimate of the amount of drug absorbed in the systemic circulation, while t_{max} reflects the rate of absorption. C_{max} is a more complex function, which, together with t_{max}, may reflect the rate of absorption."[12] AUC is a measure of total exposure; C_{max} is a measure of the rate of exposure.

Comparison of AUC values following oral vs. IV administration of the same active ingredient provides an estimate of the *absolute* bioavailability.[12]

Comparison of the test (T) and reference (R) product profiles of the drug provides an estimate of comparative bioavailability. T and R are said to be *bioequivalent* when the profiles are similar according to statistical assessment and by meeting stated standards.[12]

In Canada and the U.S., the general standard for AUC is that the 90% confidence interval (CI) of the geometric mean ratio (GMR) be within 80 and 125%. In the U.S., this is the same standard as for C_{max}. In Canada, the 90% CI of the GMR for C_{max} should be within 80 and 125%; however, for critical dose drugs (e.g., warfarin, phenytoin) the 90% CI of the GMR for AUC should be within 90 and 113% and the 90% CI for C_{max} should be within 80 and 125%. *[In July 2011, the FDA revisited bioequivalence of narrow therapeutic index (NTI) drugs and their advisory committee recommended tightening of the bioequivalence standards for these drugs.[13]]*

For oral drugs, the simplest absorption scenario is diffusion. This is dependent upon the environmental pH and the pKa of the molecule, but the process is typically much more complicated, often involving transporter-mediated absorption. Yet the main determinants of absorption remain the solubility of the drug released from the product and its pKa.

Bioequivalence implies that the drug product can be expected to have the same systemic effects (both therapeutic and adverse) as the reference product when administered to patients under the conditions specified on the label. For over 30 years, the premise was, and remains, that crossover studies on healthy volunteers can support this assumption. Health Canada states, "Drugs with uncomplicated characteristics can usually be tested in normal, healthy volunteers. The investigators should ensure that female volunteers are not pregnant or likely to become pregnant during the study."[12]

The FDA states, "We recommend that if the drug product is intended for use in both sexes, the sponsor attempt to include similar proportions of males and females in the study."[14] In addition, the FDA recommends having a representative sample, e.g., if the drug is to be used in the elderly, then a large proportion of the group should be elderly volunteers.

The numbers of test subjects are also important when testing for bioequivalence, taking into consideration the intrasubject coefficient of variation (CV). With highly variable drugs, the crossover study CV can be greater than 30%. Where the CV is under 15%, then 15 to 20 subjects may be a sufficient number for testing; where the CV is 30%, perhaps 80-100 subjects, all falling within the GMR range of 80 to 125%, would be needed to meet the standard. Figure 1 presents an example of failed bioequivalence tests for AUC. Despite the mean AUCs looking almost matched, the variability is high.

FIG. 1 Failed AUC Standard
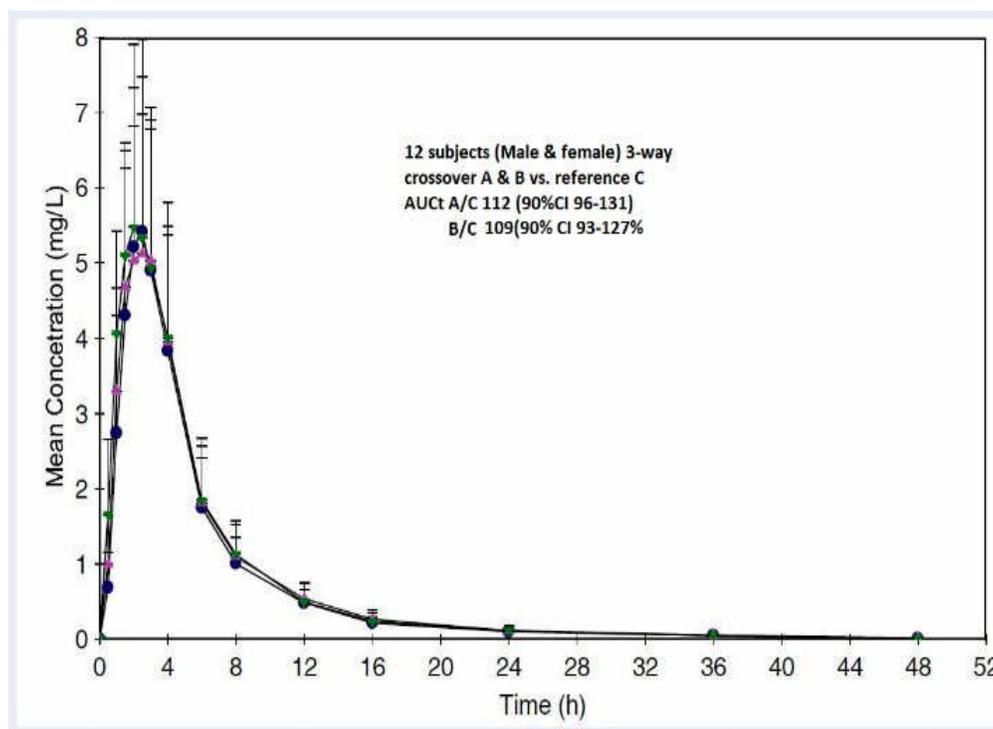
The crossover study reduces the variation (within-subject, rather than between-subject) compared to parallel studies in which each product is examined in different subjects (required for very long half-life drugs).

Another situation where there can be failed bioequivalence is in the case of formulation differences. For example, in the 1950s, it was found that the availability of poorly soluble griseofulvin was increased 50% by using a micronized formulation.

Women in Bioequivalence Studies

Chen *et al*., although noting that their "sample sizes for these studies were not chosen to examine the sex-related effects considered", reported that 26 bioequivalence studies performed between 1977 and 1995, with 20 or fewer subjects per study, found the AUC was higher 71% of the time and the C_{max} was higher more than 87% of the time in women.[15] Overall, female results were statistically higher for the reference product in 28% of the data sets. The frequency of statistically significant differences was lower when body weight was included in the statistical model, and

the authors noted that women tended to have higher variability. "The results of this study support recommendations of the 1993 FDA gender guideline that women not be excluded from bioequivalence studies."[15] Statistical examination of the data from the products tested for positive, body-weight corrected, sex-by-formulation interaction, showed higher C_{max} values in women for two transdermal nitroglycerin patches, where rate of exposure can be variable and patch size can have an effect, and for a formulation of erythromycin. These results are based on small samples against which to make recommendations. Ideally, the FDA would repeat such a review of bioequivalence studies to glean more information on gender differences.

Interestingly, the FDA has individual drug bioequivalence guidances, with the website (in May, 2011) listing 805 draft and 153 final guidances.[16] Most individual guidances recommend that subjects be "healthy males and non-pregnant females, general population", but the instructions for determining if a woman is pregnant (or lactating) are not standardized. Furthermore, about 15% of the guidances do not

mention pregnancy checks, including phenytoin - surprising, as it has been associated with birth defects. Breast cancer drugs, (e.g., anastrazole), vaginal preparations, oral contraceptives (e.g., norethindrone, etc.) and some hormones require women-only as subjects in their guidances. The exemestane guidance lists post-menopausal women as subjects. Guidances for drugs for prostate cancer and erectile dysfunction require study in men only. For progesterone, the guidance recommends healthy males and post-menopausal females are suggested (possibly due to endogenous interference in pre-menopausal women). The tamoxifen guidance recommends both men and women, as it is used in both sexes. In general the subject inclusion "recommendations" are reasonable. However, it would be useful to examine bioequivalence variations in men *vs.* women subjects, now that the FDA has more data.

Gender-Related Pharmacokinetics

Before the mid-1990s, between-gender pharmacokinetic differences were infrequently studied, largely due to the lack of regulatory requirements. Since then, more women have been included in clinical trials, as well as in the determination of pharmacokinetics of new drugs. Diclectin (doxylamine succinate 10 mg and pyridoxine hydrochloride 10 mg, delayed-release tablet) is one of those drugs.

A multiple dose pharmacokinetic study in 18 non-pregnant female subjects was sponsored by Duchesnay Inc.[17] An oral dose of 2 Diclectin tablets was given at 10 PM on Days 1 and 2, followed by multiple oral doses on Days 3 to 18,

according to the following schedule: 1 Diclectin tablet at 9 AM and 4 PM, and 2 tablets at 10 PM, under fasted conditions (at least 2 hours after eating). This is the maximum dose of 4 tablets daily as recommended in the product monograph.

This new study determined the pharmacokinetic parameters when Diclectin was administered to 18 healthy non-pregnant women in the recommended maximum dose regimen of doxylamine 40 mg/pyridoxine 40 mg per day, compared to a single 10/10 mg dose. Comparison of the first dose AUC with the final AUC, from time of dosing (0 h) to 24 hours post dose on day 18, provided an accumulation index (AI): $AI = AUC_{0-24, \text{day18}} \div AUC_{0-24, \text{day1}}$. The AI calculated from the study findings suggests an approximately 3-fold accumulation of doxylamine after multiple doses.

Pyridoxine is more difficult to research, due to its more complex metabolism. It is primarily metabolized in the liver, with the main active metabolite being pyridoxal 5'-phosphate (PLP). Other metabolites are pyridoxal (PYL), pyridoxamine (PYM), and pyridoxamine 5'-phosphate (PMP). The new data demonstrate that doxylamine and pyridoxine metabolites show clear dose accumulation after a total dose of 40 mg daily for 18 days. Some metabolites displayed 7-fold accumulation (see Table 2), along with increases in elimination half-life. The complex metabolism of pyridoxine, including reversible metabolism, presents difficulties in interpretation. The concern is the potential impact on the safety of patients, in view of anecdotal reports of patients taking off-label doses of Diclectin of up to 60 mg daily.

TABLE 2 Pyridoxine Kinetics[16]

Metabolite	$t_{1/2el(h)}$ Mean ± SD	AI AUC_{24} Day 18/Day 1
Pyridoxine (parent drug)	0.37 ± 0.16	1.59
PYL	2.14 ± 2.2	6.09
PLP	81.6 ± 42.1	3.98
PYM	3.1 ± 2.54	6.17
PMP	66.5 ± 51.3	6.67

For this presentation, there is not time to show other recent studies and to review doxylamine and pyridoxine bioequivalence information. However, women tended to be more variable (intrasubject CV%) than men and there appeared to be a gender difference in the effect of food; yet there were insufficient data to indicate a formulation interaction by gender. Nonetheless, the accumulation information from the first multiple dose study of this drug in women suggests such information is of concern, especially if higher doses are being used off-label.

CONCLUSIONS

Women are being included in pharmacokinetic studies for new drug applications in accordance with ICH, FDA, and HC guidances. Older drugs have been less studied and there are few studies available in pregnant women, other than for antivirals and antimalarials. A new look at bioequivalence studies, to compare results in men and women, would help determine if interactions between formulation and gender are a problem. It should be cautioned that body weight corrections do not remove all clearance differences.

Except for drugs used entirely in one gender, bioequivalence studies are supposed to include "representative numbers" of men and women. This may present a problem when bioequivalence studies are outsourced to offshore clinical research organizations, where cultural differences can affect gender participation in research studies.

We need to understand that questions remain about the effects of pregnancy, menarche, and menopause on pharmacokinetics, including bioavailability.

In the opinion of this speaker, bioequivalence is of less concern than are pharmacokinetics and the related drug effects. Furthermore, bioequivalence studies for drugs to be used exclusively in one gender are best studied in that gender only.

[It is interesting to note that the Health Canada Scientific Advisory Panel on Bioequivalence Requirements for Gender-Specific Drug Products (SAP-GSDP) noted in June 2011: "For the specific case of doxylamine succinate 10 mg and pyridoxine hydrochloride 10 mg, the panel recommended that the current practice of Health Canada to accept bioequivalence studies in only males, males and females or only females is acceptable."[18] As doxylamine succinate 10 mg with pyridoxine hydrochloride 10 mg is only indicated for prescribing to women, this recommendation is perhaps not in line with later remarks: "Panel members stated that cases certainly exist where bioequivalence studies do not require gender-specific samples, however, because of the nature of certain drugs; gender-specific samples are used (e.g., oral contraceptives). The members agreed that from a pragmatic standpoint, bioequivalence studies are occasionally done in gender-specific samples; the members acknowledged that Health Canada's current bioequivalence guidance already allows flexibility to accommodate these cases." [18] The final conclusion was: "while the panel does not view the issue as closed, presently there is no compelling scientific evidence to warrant gender-specific bioequivalence studies."[18]]

Corresponding Author: mcgilver@magma.ca

REFERENCES

1. U.S. Food and Drug Administration. Regulatory Information. Evaluation of Gender Differences in Clinical Investigations - Information Sheet: Guidance for Institutional Review Boards and Clinical Investigators. (Page last updated October 19, 2010) http://www.fda.gov/RegulatoryInformation/Guidances/ucm126552.htm (July 15, 2011).
2. U.S. Food and Drug Administration. Guidance for Industry. Pharmacokinetics in Pregnancy – Study Design, Data Analysis, and Impact on Dosing and Labeling. (October 2004) www.fda.gov/downloads/Drugs/.../Guidances/ucm072133.pdf (July 15, 2011).3.
3. Health Canada. Drugs and Health Products. Policy Issue from the Drugs Directorate: Inclusion of Women in Clinical Trials During Drug Development. (May 27, 1997) http://www.hc-sc.gc.ca/dhp-mps/prodpharma/applic-demande/pol/women_femmes_pol-eng.php (July 15, 2011).
4. Yang Y, Carlin AS, Faustino PJ, Motta MI, Hamad ML, He R, Watanuki Y, Pinnow EE, Khan MA. Participation of women in clinical trials for new drugs approved by the food and drug administration in 2000-2002. J Womens Health (Larchmt) 2009;18(3):303-10.
5. International Conference on Harmonisation of Technical Requirements for Registration of

Pharmaceuticals for Human Use (ICH). http://www.ich.org (July 15, 2011).

6. Schwartz JB. The influence of sex on pharmacokinetics. Clin Pharmacokinet 2003;42(2):107-21.

7. Beierle I, Meibohm B, Derendorf H. Gender differences in pharmacokinetics and pharmacodynamics. Int J Clin Pharmacol Ther 1999;37(11):529-47.

8. Soldin OP, Chung SH, Mattison DR. Sex differences in drug disposition. J Biomed Biotechnol. 2011;2011:187103. Epub 2011 Feb 23. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3051160/pdf/JBB2011-187103.pdf (July 16, 2011).

9. Ammon E, Schäfer C, Hofmann U, Klotz U. Disposition and first-pass metabolism of ethanol in humans: is it gastric or hepatic and does it depend on gender? Clin Pharmacol Ther 1996;59(5):503-13.

10. Baraona E, Abittan CS, Dohmen K, Moretti M, Pozzato G, Chayes ZW, Schaefer C, Lieber CS. Gender differences in pharmacokinetics of alcohol. Alcohol Clin Exp Res 2001;25(4):502-7.

11. Ramchandani VA, Bosron WF, Li TK. Research advances in ethanol metabolism. Pathol Biol (Paris) 2001;49(9):676-82.

12. Health Canada. Drugs and Health Products. Guidance for Industry: Conduct and Analysis of Bioavailability and Bioequivalence Studies - Part A: Oral Dosage Formulations Used for Systemic Effects. (Modified August 14, 2009) http://www.hc-sc.gc.ca/dhp-mps/prodpharma/applic-demande/guide-ld/bio/bio-a-eng.php (July 16, 2011).

13. Yu LX for the Advisory Committee for Pharmaceutical Science and Clinical Pharmacology. Approaches to demonstrate bioequivalence of narrow therapeutic index drugs. (July 26, 2011) http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/AdvisoryCommitteeforPharmaceuticalScienceandClinicalPharmacology/UCM266777.pdf (August 9, 2011).

14. U.S. Food and Drug Administration. Guidance for Industry, Bioavailability and Bioequivalence Studies for Orally Administered Drug Products - General Considerations. (March 2003, Revision 1) http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070124.pdf (August 10, 2011).

15. Chen ML, Lee SC, Ng MJ, Schuirmann DJ, Lesko LJ, Williams RL. Pharmacokinetic analysis of bioequivalence trials: implications for sex-related issues in clinical pharmacology and biopharmaceutics. Clin Pharmacol Ther 2000;68(5):510-521.

16. U.S. Food and Drug Administration. Drugs. Bioequivalence Recommendations for Specific Products. (Last Updated July 5, 2011) http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm075207.htm (July 16, 2011).

17. Duchesnay Inc. Data on file.

18. Health Canada Scientific Advisory Panel on Bioequivalence Requirements for Gender-Specific Drug Products (SAP-GSDP). Bioequivalence Requirements for Gender-Specific Drug Products (GSDP). (Record of Proceedings for meeting of June 2, 2011) http://www.hc-sc.gc.ca/dhp-mps/alt_formats/pdf/prodpharma/activit/sci-consult/gender-sexe/sapgsdp_gcsmss_m_bio-eng.pdf (August 9, 2011).

PHARMACOKINETICS IN PREGNANCY; CLINICAL SIGNIFICANCE

Gideon Koren

Motherisk Program, Hospital for Sick Children, Toronto, Ontario; Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario; University of Toronto, Canada

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ABSTRACT

In pharmacokinetics drug absorption, distribution, clearance, and bioequivalence are usually considered, but during pregnancy the most important variable is adherence or compliance. Pharmacokinetic changes during pregnancy that may lead to changes in maternal drug use are described through presentation of cases highlighting the relevance of these changes. Non-invasive methods of pharmacokinetic analysis, such as determining concentrations of drug in hair, are now being tested and used.

Pharmacokinetics are important, but one needs to consider the entire pregnant state and its circumstances when treating women. One treats people, not a "volume of distribution" or a drug level. Therapy should be individualized as much as possible, addressing kinetic changes in the context of dynamic alterations and the effects of underlying medical conditions. To ensure that women are not orphaned from advances in drug therapy, much more research is needed into the determinants of pharmacokinetic and pharmacodynamic changes in pregnancy.

Key Words: *Pharmacokinetics, pharmacodynamics, bioequivalence, pregnancy, hair analysis*

Introduction

The objectives of this presentation are to describe pharmacokinetic changes during pregnancy that may lead to changes in maternal drug use, through presentation of cases highlighting the relevance of these changes. In pharmacokinetics one usually considers drug absorption, distribution, clearance, and bioequivalence, but it is my view that during pregnancy the most important variable is adherence, or as it was called previously, compliance.

Adherence

Case Report - A 28-year-old woman with moderate-to-severe asthma was put on montelukast, discovering later that she was at 8 weeks of pregnancy. On learning of her pregnancy, she was herself fearful, and was also frightened by family members and women's magazines that noted drugs may be "bad for the baby". She therefore discontinued taking her medication at 16 weeks' gestation. At 19 weeks, she was brought to Emergency with a severe asthma attack and died 3 hours later from respiratory failure.

Ever since the thalidomide disaster, there have been high levels of sensitivity regarding teratogenic risks of drugs. In reality, very few drugs have been shown to cause malformations, many others are safe to take during pregnancy, while for quite a few we have insufficient information. Many women, and members of the public in general, believe that to avoid fetal malformations, women should take *nothing* during pregnancy. This leads women to not take medication as prescribed, even for life-threatening conditions. For example, at Motherisk we often counsel women suffering from depression who were told not to take their selective serotonin reuptake inhibitors (SSRIs) or serotonin-norepeinephrine reuptake inhibitors (SNRIs).or These women frequently stop their medication, despite being well managed, which often results in deeper depression, hospitalization, and suicide ideation and attempts. Such can be the cost of misperception.

As another example, Motherisk conducts many studies of poor adherence to drug therapy during pregnancy, using vitamins as the target treatment. Women who are keenly interested in participating are recruited and volunteer to participate in these pharmacokinetic/pharmacodynamic studies. Unfortunately, their adherence is quite variable: on average, they take only 53% of their study doses. Factors that can affect adherence during pregnancy can include whether a woman suffers from nausea and vomiting of pregnancy, i.e., not being able to keep down her medications, and medication tolerance: a smaller tablet size will be better tolerated, as will a low iron content in vitamins. Assessment of adherence is critical in evaluating poor response. The biggest variable in kinetics is whether the drug was taken in the first place.

With such poor levels of compliance, it is important for healthcare practitioners to investigate the reasons for them and to assist patients to take their medications. Counselling on the lack of teratogenic and other adverse fetal effects is crucial.

Absorption

These days, with delays in the timing of first pregnancies to a later age, there is a higher potential for mothers to have a chronic illness. The role of the underlying condition needs to be considered. For example, inflammatory bowel disease can be present in up to 5% of women during their productive years. Chronic bowel conditions can result in impaired absorption of nutrients, vitamin B_{12}, and potentially of medications.

In nausea and vomiting of pregnancy, there may be delayed absorption of drugs or incomplete dosing due to vomiting, so drugs may not be absorbed at all or absorbed only partially. Should another dose be given if vomiting occurs soon after the drug is taken? Should the dose be changed? Various disorders and conditions that affect drug absorption have important implications for drug dosing, kinetics, and efficacy.

Distribution

After absorption, a drug is distributed throughout the body into various compartments, depending on the nature of the molecule. A large increase in body weight can result in a relatively decreased dose per kilogram, and thus a decrease in steady state concentration.

Drug concentration is dependent on the dose per kg and on the clearance rate. In the third trimester there is a physiological decrease in serum albumin. Drugs which are highly albumin bound will then have decreased protein binding, be available to distribute further into other tissues/spaces, and thus have a larger volume of distribution; furthermore, more free drug will be available for elimination. Some may believe that an increased dose would then be required, given the larger volume of distribution, however, both the disease state and the pregnancy state need to be considered when adjusting drug dosing.

Elimination - Clearance Rate

Drug elimination in pregnancy has been an active area of study in the last two decades. It is acknowledged that there is increased activity of several CYP450 enzymes in late pregnancy (third trimester). This means that the elimination of drugs which are substrates to these enzymes will increase, and their steady state levels will therefore decrease. Below are some examples of the CYP450 isozymes and the drugs they affect.

CYP3A4 - is involved in the metabolism of protease inhibitors and midazolam. A woman receiving protease inhibitors before pregnancy for treatment of HIV/AIDS will require anywhere from 2 to 3 times the dose during pregnancy. Midazolam dosing may also need to be increased.

CYP2D6 - activity is increased, so that fluoxetine and other SSRIs/SNRIs will be more highly metabolized. However, dose changes may not be required for sertraline, which is an example of a drug that is metabolized to an active compound.

CYP2A6 - has nicotine as a substrate, and its metabolism is increased in late pregnancy. Smokers may find their habit or addiction worsened as their nicotine blood levels decrease. Women treated for smoking cessation with a nicotine patch may require higher doses in late pregnancy.

The activity of the cytochrome enzyme family is not generalizable in pregnancy, as some isozymes may have increased activity, while

others will have decreased activity. The production of active metabolites must also be kept in mind (e.g., sertraline, venlafaxine).

Examples of enzymes that have decreased activity in pregnancy are CYP1A2, involved in the metabolism of theophilline and caffeine, and CYP2C19, for which phenytoin is a substrate. These drugs will be eliminated more slowly during pregnancy, thus patients should be monitored for higher drug levels.

Besides these metabolic changes, for which the mechanisms remain unknown, there are changes in glomerular filtration rate (GFR) and hepatic blood flow. In late pregnancy, there is an increase of up to 50% in GFR. This results in increased clearance of renally eliminated drugs, such as lithium, digoxin, aminoglycosides. In addition, there is increased active renal tubular secretion. For example, p-glycoprotein, responsible for digoxin renal secretion and is the most studied renal transporter, is another clearance mechanism increased in late pregnancy.

There is also the situation where active metabolites are more highly renally eliminated, resulting in lowered drug effect. This is the case with morphine. It is metabolized to an active glucuronidated metabolite that is more highly cleared in late pregnancy, causing a lowered analgesic effect for the same drug dose.

Two groups of researchers recently published study results on oseltamivir kinetics in pregnancy.[1,2] Beigi and colleagues compared disposition of the drug in pregnant vs. non-pregnant women and found no changes in the area under the curve (AUC) of oseltamivir, but found a significant decrease in oseltamivir carboxylate, the active metabolite.[1] The active metabolite was cleared more quickly during pregnancy. Greer et al. compared three groups of pregnant women, 10 in each trimester.[2] They found no differences in AUC or in clearance of the active metabolite among the three subject groups and among the trimesters of pregnancy. Unfortunately, these results, although interesting, need further study, and the studies may require a power calculation to determine whether the number of subjects was sufficient to show significance.

Overall, the pregnant patient needs to be aware that in late pregnancy she could need higher doses of her medication(s). This may be counter-intuitive to her attempt to use fewer drugs or

lower doses. Where drug levels can be monitored, this should be done to determine appropriate dosing changes. For drugs which are not managed using drug levels, then clinical effects and adverse effects should be monitored and doses adjusted as needed—for example, in cases where symptoms are increased (drug effect is diminished), this may be due to increased drug clearance, whether that is due to pregnancy-related metabolic changes or to underlying genetic polymorphism. The minimum instructions to the patient would be to have her seek attention if she is experiencing toxicity or is having symptoms that are not being controlled.

Novel Methods to Study Metabolic Changes in Pregnancy

It is unrealistic to have a pregnant woman be admitted to hospital for the express purpose of monitoring her drug levels by our traditional method of taking repeated blood samples. Hair analysis, on the other hand, is non-invasive and can provide a history of drug and metabolite levels. Hair assay can provide the drug:metabolite ratio of specific drugs. Hair grows approximately 1 cm per month, and can therefore provide long-term information. This method of analysis has been the domain of forensic scientists, and we are learning from their research. Below are a couple of examples from our own studies.

Nicotine - We conducted a study to analyze the hair content of nicotine and of cotinine, its metabolite, in pregnant women who smoked throughout their pregnancy.[3] The hair was collected at the time of delivery and sectioned into segments representing the three trimesters. It was found that during pregnancy, nicotine had decreasing levels, whereas cotinine levels remained consistent: the cotinine to nicotine ratio being greater in late pregnancy. The lower levels of nicotine equated to a 50-75% increase in clearance. This may explain the failure of regular nicotine patch doses as an aid for smoking cessation in late pregnancy.

Venlafaxine - Venlafaxine has become a drug of choice for the treatment of depression in pregnancy. We found that in late pregnancy there was an increased production of the active metabolite.[4] Table 1 shows the results of a single patient, who took the same dose of drug

throughout her pregnancy and volunteered to have her blood levels drawn. This woman's blood levels in the third trimester fell to almost half of the first trimester levels, the trend for which was the same as for the AUC results. Given that she was compliant with taking her doses, this change could indicate either decreased absorption or increased clearance. The ratio of metabolite to parent drug was the reverse, with about double the blood level of metabolite to parent drug in the third trimester.

This subject's hair analysis results are shown in Table 2. From the 3rd month of pregnancy to the 9th month, there was a rise in metabolite:parent drug ratio, similar to what was found in the blood results. Hence, this method of analysis may replace the use of blood levels.

TABLE 1 Pharmacokinetics of Venlafaxine in Blood for A Sample Patient

Parameter	1st Trimester	3rd Trimester
C_{max} (ng/mL)	422	270
AUC (ng.h/mL)	3357	1965
Metabolite:Parent Drug Ratio	0.9	1.96

TABLE 2 Pharmacokinetics of Venlafaxine in Hair for A Sample Patient

Month of Pregnancy	Metabolite: Parent Drug Ratio
3	0.44
6	1.47
9	1.69
3 months post partum	0.74

Again, hair analysis is non-invasive. It can be used to determine population kinetics as well. Testing the hair of many women can serve to determine not only the change in drug concentrations over the term of pregnancy, but also the degree of change among different women. These changes could further be correlated to genotype.

Bioequivalence
Case Example - A company wishes to introduce a generic form of a drug for the treatment of pregnancy-induced biliary cholestasis. They compare their drug to the marketed compound by recruiting 20 men and studying bioequivalence. They claim that "although men may have different absorption or clearance - the comparison of 2 drugs in the same man is valid for women, because gender variability in bioequivalence is similar." But is the variability in both men and women truly the same?

Chen et al. showed that for many drugs gender variability may indeed be similar, but in others this may not be the case.[5] In their review of bioequivalence studies, 35% of drugs had differences in peak levels between men and women, and 13% showed differences in AUC. Overall, they reported that 28% of the data sets had a statistically significant difference between genders. It is clear that differences between men and women exist in the pharmacokinetics of some drugs. If variability may be different between women and men, then the results could be different depending on your volunteer group's ratio of males to females (e.g., 50:50 vs. 30:70). On top of this, we then need to consider that some drugs are indicated for use specifically in pregnancy…. Are the pharmacokinetic studies for these agents performed in pregnant women, in healthy non-pregnant female volunteers, in healthy male volunteers, or in a combination of male and female volunteers?

Pharmacodynamics

Assumptions for treatment, based on non-pregnant women, may not be valid. For example, during pregnancy we find:

- lower immunity in late pregnancy after a viral infection (e.g., varicella);
- lower protein binding;
- higher sensitivity to nausea and vomiting;
- more depression during the first trimester (due to morning sickness);[6]
- higher glucose levels due to corticosteroid hormones; and
- higher cardiac output, leading to more risk for heart failure in women with existing heart disease.

Furthermore, we need to be aware of the combination of pregnancy-related changes, metabolic changes *plus* genetic polymorphism, such as in the case of increased activity of CYP2D6 in late pregnancy, which will lead to different changes among ultrametabolizers, extensive metabolizers and slow metabolizers. There are also the interactions between a condition induced by pregnancy and an underlying disorder, as in the case of women with nausea and vomiting of pregnancy exacerbated by their underlying reflux disease.

CONCLUSION

We need to consider the entire person and their circumstances when we treat them. We treat people, not a volume of distribution or a drug level. Therapy should be individualized as much as possible, addressing kinetic changes in the context of dynamic alterations due to underlying medical conditions.

Corresponding Author: gkoren@sickkids.ca

REFERENCES

1. Beigi RH, Han K, Venkataramanan R, Hankins GD, Clark S, Hebert MF, et al. Pharmacokinetics of oseltamivir among pregnant and nonpregnant women. Am J Obstet Gynecol 2011;6(suppl):S84-8.
2. Greer LG, Leff RD, Laibl Rogers V, Robert SW, McCracken GH, Wendel GD Jr, et al. Pharmacokinetics of oseltamivir according to trimester of pregnancy. Am J Obstet Gynecol 2011;6(suppl):S89-93.
3. Klein J, Blanchette P, Koren G. Assessing nicotine metabolism in pregnancy--a novel approach using hair analysis. Forensic Sci Int 2004;145(2-3):191-4.
4. O'Brien L, Baumer C, Thieme D, Sachs H, Koren G. Changes in antidepressant metabolism in pregnancy evidenced by metabolic ratios in hair: a novel approach. Forensic Sci Int 2010;196(1-3):93-6.
5. Chen ML, Lee SC, Ng MJ, Schuirmann DJ, Lesko LJ, Williams RL. Pharmacokinetic analysis of bioequivalence trials: implications for sex-related issues in clinical pharmacology and biopharmaceutics. Clin Pharmacol Ther 2000;68(5):510-21.
6. Köken G, Yilmazer M, Cosar E, Sahin FK, Cevrioglu S, Gecici O. Nausea and vomiting in early pregnancy: relationship with anxiety and depression. J Psychosom Obstet Gynaecol 2008;29(2):91-5.

INNOVATIVE STUDIES IN WOMEN BY USE OF STABILIZED ISOTOPES IN PREGNANCY

Offie P Soldin

PregnaTox, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center; Departments of Oncology Division of Cancer Genetics and Epidemiology; Medicine, Division of Endocrinology and Metabolism; Obstetrics & Gynecology; Pharmacology and Physiology, Georgetown University Medical Center, Washington DC

ABSTRACT

Pharmacokinetic studies are conducted in order to determine drug absorption, distribution, metabolism and excretion. This knowledge serves to help determine the appropriate and timely use of medications and is also an important step in providing individualized therapeutics according to patient characteristics, such as disease state and genotypes of drug metabolizing enzymes. An innovative way of conducting pharmacokinetic research in pregnancy is presented, with the drug levothyroxine (LT4).

The stable, non-radioactive Carbon-13 isotope was used to prepare a derivative of LT4, which was then used to determine the pharmacokinetics of the drug in 9 pregnant women serving as their own controls. Of 9 study subjects, 6 returned to participate in the post partum (non-pregnant) portion of the study. The median time to peak blood level was determined to be at 8 hours post dose. The $AUC_{0-\infty}$ results were significantly higher during pregnancy than in the same woman approximately 6 months post partum. The increase in LT4 AUC during pregnancy could be attributed to a decrease in LT4 clearance. Additionally, a large variability in the pharmacokinetics of LT4 was found in pregnant women, and a relatively narrower range of variability in non-pregnant women. In order to solidify these findings, a larger group of patients is required. In addition, the mechanisms responsible for the gestational differences in pharmacokinetics need to be investigated.

Key Words: *Levothyroxine, stable isotope, pharmacokinetics, pregnancy*

Introduction

Pharmacokinetic studies are conducted in order to determine drug absorption, distribution, metabolism and excretion. This knowledge serves to help determine the appropriate and timely use of medications. This is also an important step in providing individualized therapeutics according to patient characteristics, such as disease state and genotypes of drug metabolizing enzymes. Presented here is an innovative way of conducting pharmacokinetic research in pregnancy, specifically with thyroxine.

Thyroxine (T4) is both a hormone and a therapeutic drug, synthetically produced as levothyroxine (LT4) for the treatment of hypothyroidism – underactivity of the thyroid gland. Thyroxine is essential for normal neural development of the fetus. Clinical evidence indicates that increased doses of T4 are often necessary in early pregnancy, yet it is unclear exactly when to start increasing the dose and how much to supplement. To study the drug's pharmacokinetics, conventional methods of analysis cannot differentiate between the administered drug and endogenous thyroxine. Furthermore, all pharmacokinetic studies are more complex and difficult to conduct during pregnancy, when the use of radiolabeled isotopes or ingesting high doses of the hormone are unethical.

Carbon-13 (^{13}C) is a stable isotope of carbon. It is non-radioactive (thus "stable") with a half-

life, if any, of greater than 0.5 billion years. It possesses no harmful or radiation-related effects. Tests have shown stable isotopes to be safe in newborn infants, and they are also safe in pregnant women. Stable isotopes have been used for over 30 years in studies with infants, children, and adults. The $^{13}C_6$-LT4 derivative of LT4 is highly stable and is not converted to the $^{12}C_6$-LT4 analog, i.e., the prescribed levothyroxine.

MATERIALS AND METHODS

The research protocol was approved by the Georgetown University institutional review board and written informed consents were obtained from all study participants. All women were recruited from Georgetown University Medical Center, where the studies were also conducted.

Hypothyroid women, rendered euthyroid by LT4 replacement, were recruited during pregnancy to participate in the study. Subjects were included in the study if they met the following criteria: women 18 years of age or older at time of consent, able to give written informed consent, euthyroid (LT4-treated hypothyroid) with no other serious illness, prescribed LT4 by their physician for therapeutic reasons during a pregnancy, and anticipating continuing LT4 medication post partum as prescribed by their physician. Subjects were excluded from the study for any of the following reasons: baseline hematocrit lower than 28.0%, TSH higher than 4.5 mIU/L, kidney dysfunction, or ingestion of any other drugs that affect the thyroidal axis, such as those that can alter TSH and thyroid hormone secretion, transport or metabolism.

Subjects were admitted into the General Clinical Research Center on the first day of each study period ("study day"). For each subject there were two study periods—one pre-delivery, then one within three to twelve months post partum, when maternal metabolism returns to normal. Only the LT4 dose on each study day was replaced by ^{13}C-LT4. The women remained on site for about 12 hours. On each of the following days of the pharmacokinetic study period (120 hours), the subjects continued taking the daily dose of their own LT4 (not the study $^{13}C_6$-LT4 preparation).

Participants were required to fast (other than water) for at least five hours prior to, and two hours following, the ingestion of the morning $^{13}C_6$-LT4 dose. They did not take any other medication within two hours of ingesting the study drug, to prevent any interference with the $^{13}C_6$-LT4 rate of absorption, and ate a simple breakfast based on eggs, toast, fruit, yogurt and tea, provided at the Research Center. The women were requested to discontinue taking any iron-containing multivitamins or pills at least one week before and throughout the study. All women were given their prescribed maintenance dose on the first day of the pharmacokinetic study. The subjects kept a record of the times and doses of their own LT4 taken daily during the study period.

The methods for the measurement of thyroid hormone and $^{13}C_6$-LT4 were developed in the Georgetown Bioanalytical Core Laboratory.[1-4] Blood samples were drawn before dose administration (0 h) and at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 48, 72, 96, and 120 h post dose. At most times, blood was drawn at the subject's home on Days 2, 3, 4, 5, 6, 7, and 14 by a study nurse.

The $^{13}C_6$-LT4 derivative was synthesized and purchased for this study from IsoSciences, LLC. 70 mcg and 100 mcg capsules of study material were prepared by a specialty compounding pharmacy, and the thyroxine content of the compounded capsules verified by HPLC analysis. The $^{13}C_6$-LT4 capsules were kept at controlled and monitored temperature and humidity until use. Quality control potency and stability testing were conducted biannually by Eagle Analytical Services, a specifically designated and independently licensed quality control laboratory. Any LT4 dose that was not a multiple of 70 or 100 mcg was supplemented by the Georgetown research pharmacists with a complementary dose of non-$^{13}C_6$-LT4 levothyroxine to total the subject's dose, while providing the highest possible portion of the dose as the stable isotope study material. For example, a woman prescribed a daily LT4 dose of 225 mcg received 210 mcg of $^{13}C_6$-LT4 (3 x 70 mcg) and an additional 12.5 mcg $^{12}C_6$-LT4 (half of a 25 mcg tablet), reflected in Table 1 as receiving 210 mcg for this study. Note that the results outlined in Table 2 reflect only the $^{13}C_6$-LT4 doses.

TABLE 1 $^{13}C_6$-LT4 Pharmacokinetic Profile for a Single Pregnant Subject

Sample #	Time (h)	$^{13}C_6$-LT4 (ng/mL)	Total T4 (mcg/dL)	Total T3 (ng/dL)
1	0.0	0.000	8.8	155
2	0.5	0.019	9.9	134
3	1.0	0.044	10.3	192
4	1.5	0.059	10.3	119
5	2.0	0.086	8.5	143
6	2.5	0.125	9.1	119
7	3.0	0.141	9.9	178
8	4.0	0.208	10.4	124
9	6.0	0.296	9.7	135
10	8.0	0.461	8.7	136
11	10	0.374	9.3	120
12	12	0.322	8.8	136
13	24	0.196	9.9	133
14	48	0.090	8.9	125
15	72	0.046	8.9	127
16	96	0.029	8.8	113

Serum concentrations of $^{13}C_6$-LT4 and T4 were measured using a validated, sensitive, and specific isotope dilution tandem mass spectrometry.[4] Pharmacokinetic assessments were conducted by standard two-stage approach, using non-compartmental techniques in WinNonLin (Version 5.1.1, Pharsight Corporation, Mountain View, CA). Calculated pharmacokinetic variables included peak concentration (C_{max}), time to peak concentration (T_{max}), $AUC_{0-\infty}$, clearance rate (defined as the ratio of dose administered and AUC), the apparent plasma terminal rate constant (λ), and the half-life of the terminal disposition phase ($t_{1/2}$) estimated by $\ln(2)/\lambda$. A combination of linear trapezoidal method during the ascending phase and log linear method during the descending phase was used for estimating AUC. Levothyroxine pharmacokinetic parameters in pregnant vs. non-pregnant women were compared using Wilcoxon signed-rank test using

significance level of $\alpha = 0.05$. The challenges were (1) to use a traceable form of LT4, and (2) the measurement of very low concentrations of T4, since this study design followed only a single dose of administered ^{13}C-LT4.

Pharmacokinetic Studies of Levothyroxine

The 9 women, whose data is reported here, were recruited during pregnancy. Their ages were 31 to 42 years, and all were euthyroid following LT4 replacement. The women were in various trimesters and were Caucasian (n=7), with Asian (n=1) and African American (n=1), (Table 1). To date we have recruited 16 subjects and are continuing the study. Target therapeutic TSH levels are maintained at around 1 mIU/L, and are trimester-specific during pregnancy. Of the 9 study subjects, 6 returned to participate in the post partum portion of the study approximately 3 to 12 months following delivery.

RESULTS

We observed a peak $^{13}C_6$-LT4 concentration (T_{max}) approximately 8 hours following $^{13}C_6$-LT4 dosing, much later than the previously reported 3 hours for LT4 T_{max}. Table 1 shows the blood level profile for one pregnant subject (data shown to Day 4), with peak $^{13}C_6$-LT4 level achieved at 10 hours.

Median LT4 pharmacokinetic parameters for our study subjects are summarized in Table 2 and again show that the median time to peak blood level at 8 hours. The maximum concentration is similar between the pregnant and non-pregnant groups, but the AUC and clearance rates are significantly different. The percentage change in $^{13}C_6$-LT4 AUC_{0-72} was compared between pregnant and non-pregnant women. It was found that the AUC was almost 50% higher in the women when they were at the pregnant state, compared to these same women postpartum.

The relationship between gestation week and the ratio of AUC in the pregnant vs. non-pregnant state is shown in Table 3, where an increase in the AUC with progression of pregnancy is demonstrated.

TABLE 2 $^{13}C_6$-LT4 Pharmacokinetic Measures Following Oral Administration

		T_{max} (h)	C_{max} (ng/mL)	AUC_{0-72} (ng h/mL)	Clearance (L/h)
Pregnant (n=9)	median	8.0	0.9	14.8	4.5
Non-pregnant (n=6)	median	8.0	0.7	10.5	7.0
AUC = area under the curve					

TABLE 3 $AUC_{13C-LT4}$ Relative to Week of Gestation

Week of Gestation	$AUC_{13C-LT4}$ pregnant/non-pregnant
13	1.03
16	1.11
22	1.33
25	1.11
30	2.50
36	4.15

DISCUSSION

We successfully conducted pharmacokinetic studies of a single dose of stable isotope-labelled LT4 ($^{13}C_6$-LT4), at patient-specific doses, in non-pregnant and pregnant women serving as their own controls.

The $AUC_{0-\infty}$ results were significantly higher during pregnancy than in the same woman approximately 6 months post partum. The increase in LT4 AUC during pregnancy could be attributed to a decrease in LT4 clearance.

It should be noted that LT4 is not metabolized by the cytochrome family of enzymes, where the activity of certain isozymes can change over the course of a pregnancy. LT4 is metabolized by deiodinases, the activity of which varies in each tissue. These preliminary results suggest that LT4 pharmacokinetics change significantly with gestational age. Additionally, there is a large variability in the pharmacokinetics of LT4 in pregnant women, and a relatively narrower range of variability in non-pregnant women.

Future Research

In order to solidify these findings, a larger group of patients is required. We are continuing to recruit patients, and are currently at n = 16. The mechanisms responsible for the gestational differences in pharmacokinetics need to be investigated. Whether these differences should necessitate dosing schedule changes during pregnancy should also be investigated further.

Acknowledgements

Conflict of Interest

The author declares no conflict of interest. Dr. Offie Soldin reports having served as a consultant for Abbott Laboratories.

Corresponding Author: os35@georgetown.edu

REFERENCES

1. Soukhova N, Soldin OP, Soldin SJ. Isotope dilution tandem mass spectrometric method for T4/T3. Clin Chim Acta 2004;343:185-90.
2. Gu J, Soldin OP, Soldin SJ. Simultaneous quantification of free triiodothyronine and free thyroxine by isotope dilution tandem mass spectrometry. Clin Biochem 2007;40:1386-91.
3. Soldin O, de Ridder S, Vinks A, et al. Levothyroxine pharmacokinetics in pregnancy. 79th Annual Meeting of the American Thyroid Association; October 1–5, 2008; Chicago, IL.
4. Soldin OP, Mendu DM, Soldin SJ. Development of a method for the simultaneous measurement of stable isotope C13- and C12-thyroxine in human serum or plasma. Thyroid 2008;18:S84-5.

PREDICTION OF PLACENTAL DRUG TRANSFER USING THE HUMAN PLACENTAL PERFUSION MODEL

Janine R Hutson

BSc, MSc, student in the MD/PhD Program and the Collaborative Program in Biomedical Toxicology, University of Toronto, Canada

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ABSTRACT

The placental perfusion model and a newly developed complementary computational model are reviewed. Examples are provided, where the computational model can be applied to adjust drug pharmacokinetic data obtained from the perfusion model to more closely resemble the *in vivo* placental transfer of therapeutic agents. After modelling the data, placental perfusion experiments can be used to predict placental drug transfer and can be useful for clinical assessment of the risks and benefits of drug therapy in pregnancy.

**Key Words:** *Placental perfusion, computational model, pharmacokinetics, pregnancy*

Introduction

In the basic science laboratory we have many models to look at the transfer of drugs across the placenta. However, one of the challenges of medical research is the translation of findings from the basic science laboratory into clinical practice. I will present an evaluation of the placental perfusion model and also a computational model, developed to adjust data obtained from the perfusion model to more closely resemble the *in vivo* placental transfer of therapeutic agents. After modelling the data, placental perfusion experiments can be used to predict placental drug transfer and can be useful for clinical assessment of the risks and benefits of drug therapy in pregnancy.

Background

The placenta separates the maternal and fetal circulations and performs many functions that support the maintenance of pregnancy and the normal development of the fetus. The cotyledon is the functional vascular unit of the placenta and each cotyledon contains highly branched villi suspended in the intervillous space. Maternal blood fills this intervillous space and is supplied by spiral arteries and carried away by uterine veins. Because there is virtually no basement membrane between the fetal endothelial cell and the syncytiotrophoblast, only 5 micrometres separate the fetal from the maternal blood. It is here that the rate-limiting step of drug transfer across the placenta occurs. Figure 1 shows a schematic of this interface, with the large middle rectangle representing the syncytiotrophoblast.

FIG. 1   Schematic of Fetal-Maternal Blood Interface in the Placenta[a]
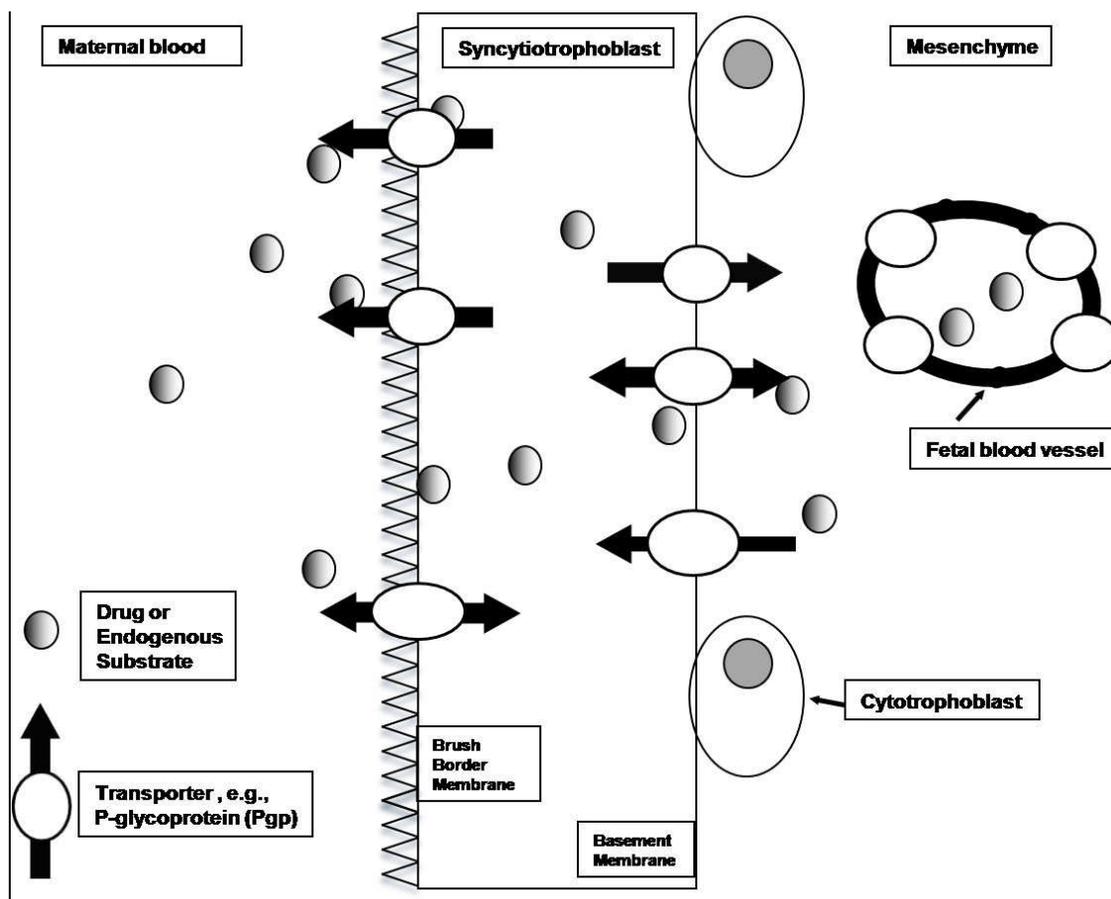
[a]Adapted from Hutson JR, et al. Placenta. 2010;31(5):351-7.[1]

The syncytiotrophoblast has many membrane transport proteins that can efflux or facilitate transfer of drugs across the placenta. For example, P-glycoprotein (Pgp) on the maternal brush border membrane can prevent drugs from crossing the placenta by effluxing the drug back into the maternal circulation.

Various factors can influence placental transfer, including the physicochemical properties of a drug. These include the pK_a—*in vivo*, fetal blood pH is ~7.35 and maternal blood is ~7.4, which can lead to ion trapping of basic drugs due to the slightly more acidic fetal blood; molecular weight—drugs larger than 500 or 600 Da cannot cross the placenta unless transport is facilitated by a transport protein; and the higher the drug lipid solubility, the more drug will be transferred. Non-

placental pharmacokinetic properties will also influence drug transfer, that is, protein binding in the maternal and the fetal circulations, suggested as the most important factor that can influence steady state distribution of drug across the placenta, and maternal and fetal drug distribution and elimination. Placental pharmacokinetic properties include drug transport proteins, placental drug metabolism, and binding to placental tissue, all of which can influence the rate or duration of transfer.

To study drug transfer across the placenta, various models are available (*see* Table 1), as it is often unethical to study this transfer directly in humans. The placental perfusion model should theoretically be best able to predict how drugs are transferred over time in humans.

TABLE 1   Models of Placental Drug Transfer

Type	Example	Notes
In vivo	Animal	The placenta is the most species-specific mammalian organ, therefore findings from animal studies cannot be generalized to humans with certainty.
	Termination of pregnancy	In older studies, the drug was given to the woman before termination of pregnancy. Such studies are generally no longer conducted for ethical reasons.
	Umbilical cord blood	The majority of human data originates from umbilical cord blood taken at the time of delivery. Almost always, only one time-point is collected, therefore no information is obtained about the rate of drug transfer or whether the maternal and fetal units are at steady state.
In vitro	Trophoblast cultures and tissue preparations	Membrane vesicles, placental explants, or trophoblast cell lines are used to determine whether a drug is a substrate for a placental drug transporter. This model is useful to determine the mechanism of transport, but does not provide information on the amount of drug transferred.
Computer Models	Physiologically based pharmacokinetic models (PBPK)	A computer model to estimate placental drug transfer may be developed, but it is difficult to determine how well it can be generalized to the human *in vivo*. Especially in pregnant women, caution needs to be exercised when attempting to generalize the results.
Ex vivo	Placental perfusion	This is the only experimental method that can be used to study human placental transfer of substances in organized placental tissue.

FIG. 2   A Schematic of the Placental Perfusion Model[b]
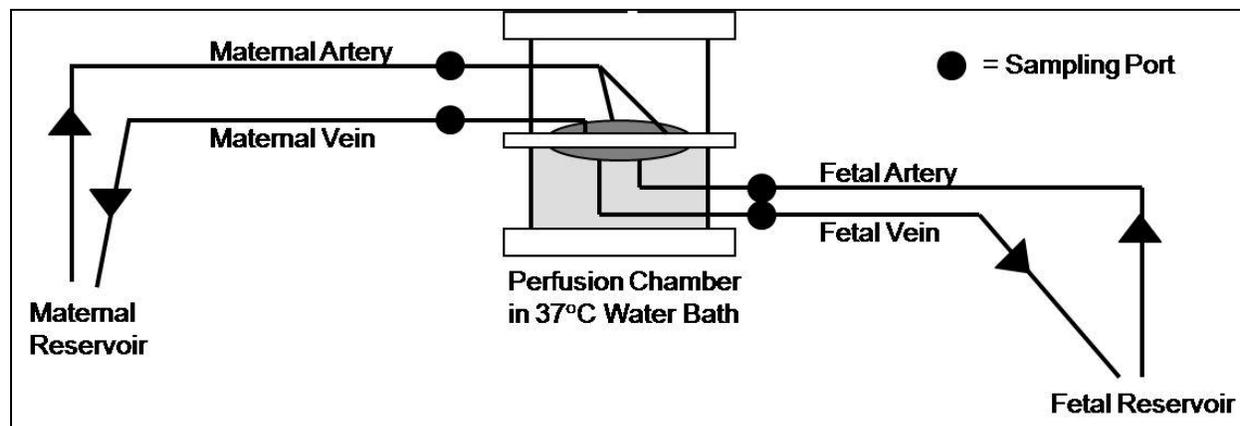
[a] Diagram adapted from Hutson JR, et al. Clin Pharmacol Ther. 2011;90(1):67-76.[3]

The Placental Perfusion Model

Perfusions are usually performed on placentae collected after caesarean section. The following procedure is performed in our laboratory. After tissue collection, a fetal vein/artery pair that supplies one cotyledon is found, usually around the periphery of the placenta. The fetal vein and artery are cannulated and the lobule is clamped into a plexiglass chamber, the maternal side facing up. The maternal and fetal circulations are maintained, as shown in Figure 2, and the chamber is kept at physiological temperature, 37°C.

The maternal and fetal circulations are independently controlled using roller-pumps, the physiological state being mimicked insofar as possible. The model can be open or closed. In the open model, the perfusate is not recycled, with fresh perfusate being constantly supplied to the placenta. In a closed model, the same perfusate is recirculated and used throughout the experiment.

Prior to our study, to our knowledge there had been no systematic evaluation of how well the perfusion model predicted fetal drug exposure. Only reviews of specific drug classes, such as antivirals, had been performed. Before the perfusion model can be used routinely to predict placental drug transfer in preclinical evaluation, careful validation of this model is needed.[2]

Evaluation of the Placental Perfusion Model

Our study, recently published in *Clinical Pharmacology and Therapeutics*,[3] had 3 primary objectives:

- To systematically evaluate the placental perfusion model in predicting placental drug transfer by comparing it to *in vivo* data.
- To construct a pharmacokinetic model that best allows prediction of the *in vivo* maternal-fetal drug distribution at steady state.
- To provide recommendations to improve the reliability of the predictions provided by the perfusion model.

To evaluate how well the perfusion model predicts *in vivo* drug transfer, comparisons were made between fetal to maternal drug concentration ratios (F:M) from perfusion experiments and cord blood to maternal blood drug concentration ratios (C:M) at the time of delivery.

We performed a systematic search for papers evaluating placental transfer of therapeutic drugs using the perfusion model. Many drug classes have been investigated using the placental perfusion model. Most frequently studied have been antivirals and anaesthetic agents, with reports also for analgesics, antidepressants, antiepileptics, antimicrobials, antipsychotics, asthma medications, cardiac medications, chemotherapeutics, diabetic agents, endocrine agents, H_2-blockers, immunologic agents, and tocolytics. For our purposes, drugs were identified from the papers that met our inclusion criteria.[3] A subsequent search was performed, on each identified drug that was evaluated by the perfusion model, to locate papers reporting *in vivo* data, i.e., human cord blood and maternal blood concentrations at the time of delivery. F:M ratios from perfusion experiments were compared to C:M ratios, both qualitatively and quantitatively. From 1732 papers returned from the search for human placental perfusion, 147 full text articles were assessed for eligibility, resulting in 70 drugs to be compared qualitatively and 26 drugs quantitatively.

The 70 drugs compared qualitatively were classified as having limited transfer (F:M < 0.1), transfer (F:M = 0.1 to 1.0), or fetal accumulation (FM > 1.0). Forty-nine drugs showed placental transfer in both placental perfusion experiments and *in vivo*, and 9 drugs showed limited transfer in both placental perfusion and *in vivo*. It was found that any drug that showed limited transfer in the perfusion model, also had limited transfer *in vivo*. Of the 12 drugs that showed discrepancies, 5 had an F:M > 1.0 observed *in vivo*, but not in the model, and in 7, steady state was reached neither in perfusion nor *in vivo*.

Twenty-six drugs could be compared quantitatively (Figure 3). Of note, when accumulation in the fetal circulation was observed *in vivo* (C:M > 1), the perfusion model did not predict a F:M > 1 for all examples.

FIG. 3   Quantitative Comparison for 26 Drugs Using Original Data from Perfusion Experiments and Measurements[b]
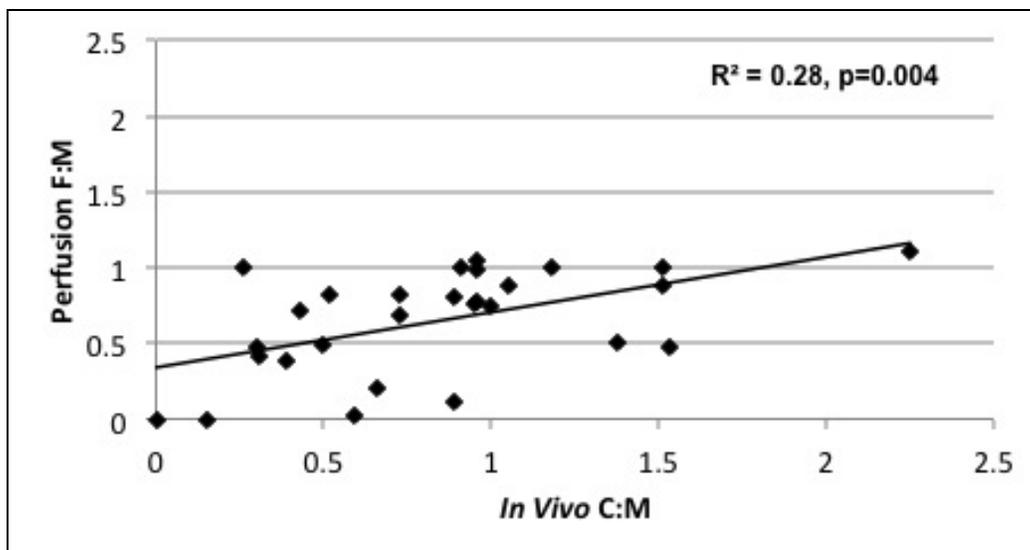
[b] From Hutson JR, et al. Clin Pharmacol Ther. 2011;90(1):67-76.[3]

Adjusting the Placental Perfusion Results to Better Predict *In Vivo* Transfer

Where the placental perfusion model is excellent for investigating placental pharmacokinetics, it cannot incorporate maternal or fetal pharmacokinetic factors. These *in vivo* factors include maternal and fetal protein binding: the F:M ratio of albumin increases from 0.28 in the first trimester to 1.20 at term, and the F:M ratio of α_1- acid glycoprotein (AAG) increases from 0.09 in the first trimester to 0.37 at term. We therefore proposed a calculation model to adjust

the perfusion results to better predict *in vivo* findings.

Our equation takes into account fetal and maternal protein binding, the pK_a of the drug in question, the difference in blood pH between fetus and mother, and the drug clearance in the fetus and in both mother to fetus and fetus to mother. Examples of the use of our equation to better predict *in vivo* disposition with the placental perfusion model are summarized below. Additional examples are published in our paper.[3]

$$F:M = \frac{\% \text{ unbound}_M}{\% \text{ unbound}_F} \times \frac{1+10^{pK_a - pH_F}}{1+10^{pK_a - pH_M}} \times \frac{CL_{MF}}{CL_{FM} + CL_F}$$

Valproic Acid
Two studies from closed perfusion experiments published steady state F:M ratios of 0.90 and 0.85.[4,5] Five *in vivo* studies showed fetal accumulation, with a weighted mean C:M ratio of

Diazepam
Myllynen *et al*. reported a steady state F:M ratio of 0.55, using a closed perfusion model.[11] Thirteen *in vivo* studies provided a weighted mean C:M ratio of 1.27 (n = 255).[12-24] Taking this experimental ratio and *in vitro* protein binding values (maternal unbound drug = 3.24%, fetal unbound drug = 1.50%),[25] the adjusted F:M is 1.2. Again the equation better estimates the observed *in vivo* findings.

Propranolol
Here is an example where the perfusion experiment overestimates the *in vivo* drug transfer. Schneider *et al*. reported a steady state

1.51 (n = 37).[6-10] Using our equation and taking *in vitro* protein binding values from the literature (maternal unbound drug = 15%, fetal unbound drug = 9.1%),[10] we arrive at an adjusted F:M ratio of 1.67, thus better estimating the *in vivo* value. F:M ratio of 1.0, using a closed perfusion model.[26]

However, Erkkola *et al*. measured the *in vivo* C:M to be 0.26 ± 0.62 in a sample of 8 patients.[27] Adjusting the perfusion results using our equation and *in vivo* protein binding data (maternal unbound drug = 21%, fetal unbound drug = 39%),[28] the adjusted F:M is 0.6 and is much closer to that observed *in vivo*.

We then calculated the F:M ratios for the 26 drugs for which we could perform quantitative analysis using our equation and replotted the data (Figure 4). This gave a better correlation between the two parameters and supports the use of the equation as an accurate way of determining placental drug transfer from perfusion experiments

FIG. 4 Quantitative Comparison for 26 Drugs after Adjusting the Placental Perfusion Results[b]
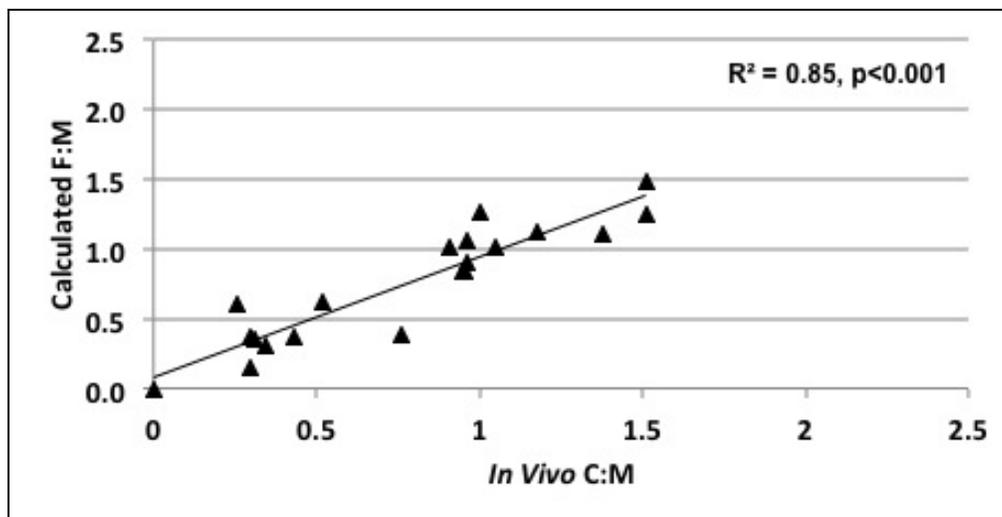
[b] From Hutson JR, et al. Clin Pharmacol Ther. 2011;90(1):67-76.[3]

Open vs. Closed Placental Perfusion Configuration

Perfusion model results and interpretations need to be considered with caution, as there are differences between the open and closed configurations. Normally, the open configuration, because of the constant supply of new perfusate containing the same drug concentration, underestimates the *in vivo* steady state C:M ratio or placental drug transfer. The results are calculated using only initial maternal drug concentration and the drug does not distribute between the maternal-placental-fetal compartments as it does *in vivo*. The open configuration is useful for calculating clearance calculations and not drug distribution. Caution should be exercised in comparing results to *in vivo* findings, as shown with alfentanil.

Alfentanil

Alfentanil is a weak base with a pK_a of 6.5, and is bound to AAG. The drug was perfused in open configuration with no protein in the perfusate.[29] At steady-state, the F:M ratio was 0.22. *In vivo* cord measurements gave a C:M ratio ranging from 0.29 to 0.35 in 4 studies (n = 45),[30-33] prompting the authors to note that their perfusion results closely estimated the *in vivo* findings. However, one study also reported a C:M ratio of about 1.0 for free drug levels (n = 31), which is what the perfusion results would be estimating, given there was no protein added to the perfusates.[30] Using our equation with the free drug data from the perfusion, the adjusted perfusion F:M ratio is 0.37: a ratio that represents total (free + bound) drug and is much closer to the *in vivo* observation.

Some investigators will add protein to either the maternal or the fetal perfusate, or to both, to more closely represent *in vivo* conditions. Albumin is commonly added to the perfusate; human plasma has also been used. The following example for bupivacaine is of a closed perfusion experiment with added protein.

Bupivacaine

In the two studies by Johnson *et al*., 2% human serum albumin was added to both perfusates.[34-35] The findings were then F:M ratios of 0.81 and 0.74. By using human plasma on the maternal side and 4% human serum albumin on the fetal side, the F:M ratio was lower at 0.51 and 0.40, respectively.[13,14] *In vivo* observations from 3 studies (n = 51) gave a free C:M ratio of 0.73.[36-38] This closely resembles the perfusion findings where albumin was added to both sides, and represents the free drug equilibrating between the two circulations. With data from 16 studies (n = 232), a weighted mean C:M for total (bound + free) drug was calculated to be 0.30.[36,38-52] Our calculated F:M ratio, using clearance from the open placental perfusions, pK_a and protein binding data, is 0.28. By using the equation to estimate *in vivo* drug disposition, the addition of human plasma to the perfusate could be avoided. Furthermore, the results would be more accurate, and this proves to be a more practical approach to these experiments.

Limitations of the Model

When placental perfusions show limited transfer, our equation model cannot be applied. As mentioned earlier, 9 drugs from the literature search showed limited perfusion in both placental perfusion experiments and *in vivo*. In such cases, the agreement in results between the two experimental methods obviates the need to adjust the *in vitro* results using our equation. Indinavir is one such drug.

Indinavir

Two perfusion experiments were performed in open configuration with no protein added. At steady state, the F:M ratio was 0.04 and 0.06.[53,54] This matched well with what was observed *in vivo* in 2 studies (n = 25), where the C:M ratio ranged from below the limit of detection to 0.08.[55,56] If data from the perfusion model were used in the equation, the result would come to F:M = 0.26, an overestimation of the ratio.

Recommendations

As a result of reading and reviewing the papers that have been published on the subject of placental perfusion and *in vivo* fetal and maternal drug levels, we arrived at some suggestions for future studies. The details of our recommendations are published elsewhere,[3] but the key messages are:

- Publication of perfusion results should report: the absolute drug concentrations, not only the F:M ratios (to facilitate secondary analyses); placental tissue binding, to

provide a fuller picture of the drug concentrations in the maternal, fetal and placental units; and the pH of maternal and fetal perfusates, so that it is clear whether physiological values are being mimicked.

- *In vitro* measurements of protein binding, conducted alongside the perfusion experiments, would enhance interpretation of perfusion results and could be used together with our equation.
- Authors need to state or show whether steady state was achieved, which would be useful for secondary analyses.

Comparison of *in vivo* timing to perfusion timing needs to be viewed with caution. The time to reach steady state *in vivo* can be very rapid, compared to perfusion experiments, as shown in Figure 5. This can be explained by the time it takes to circulate all the maternal blood in the body through the action of the heart pumping, i.e., about 1 minute. In the placental perfusion model, it takes approximately 25 minutes to pump the complete volume of maternal perfusate.

FIG. 5 *In Vivo* vs. *In Vitro* F:M of Morphine[b]
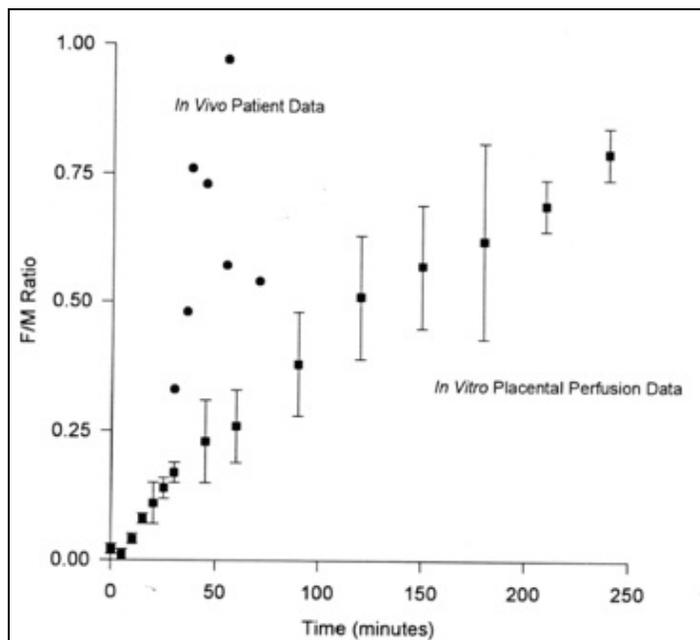
[b]From Hutson JR, et al. Clin Pharmacol Ther. 2011;90(1):67-76.[3]

SUMMARY

A systematic evaluation of the placental perfusion model shows that it is a suitable model to predict placental drug transfer. Using perfusion data together with data from *in vitro* protein binding experiments in maternal and cord blood would enhance interpretation of results from the placental perfusion model. The placental perfusion model, used appropriately, can be applied to help guide decisions regarding the benefits and risks of new medications that may be required during pregnancy.

Corresponding Author: j.hutson@utoronto.ca

REFERENCES

1. Hutson JR, Koren G, Matthews SG. Placental P-glycoprotein and breast cancer resistance protein: influence of polymorphisms on fetal drug exposure and physiology. Placenta. 2010;31(5):351-7.
2. Ala-Kokko TI, Myllynen P, Vähäkangas K. Ex vivo perfusion of the human placental cotyledon: implications for anesthetic pharmacology. Int J Obstet Pharmacol. 2000;9(1):26-38.
3. Hutson JR, Garcia-Bournissen F, Davis A, Koren G. The human placental perfusion model: a systematic review and development of a model to predict in vivo transfer of therapeutic drugs. Clin Pharmacol Ther. 2011;90(1):67-76.
4. Barzago MM, Bortolotti A, Stellari FF, Diomede L, Algeri M, Efrati S, Salmona M, Bonati M. Placental transfer of valproic acid after liposome encapsulation during in vitro human placenta perfusion. J Pharmacol Exp Ther. 1996;277(1):79-86.
5. Fowler DW, Eadie MJ, Dickinson RG. Transplacental transfer and biotransformation studies of valproic acid and its glucuronide(s) in the perfused human placenta. J Pharmacol Exp Ther. 1989;249(1):318-23.
6. Dickinson RG, Harland RC, Lynn RK, Smith WB, Gerber N. Transmission of valproic acid (Depakene) across the placenta: half-life of the drug in mother and baby. J Pediatr. 1979;94:832-5.
7. Nau H, Rating D, Koch S, Hauser I, Helge H. Valproic acid and its metabolites: placental transfer, neonatal pharmacokinetics, transfer via mother's milk and clinical status in neonates of epileptic mothers. J Pharmacol Exp Ther. 1981;219:768-77.
8. Takeda A, Okada H, Tanaka H, Izumi M, Ishikawa S, Noro T. Protein binding of four antiepileptic drugs in maternal and umbilical cord serum. Epilepsy Res. 1992;13:147-51.
9. Ishizaki T, Yokochi K, Chiba K, Tabuchi T, Wagatsuma T. Placental transfer of anticonvulsants (phenobarbital, phenytoin, valproic acid) and the elimination from neonates. Pediatr Pharmacol (New York); 1982;1:291-303.
10. Froescher W, Gugler R, Niesen M, Hoffmann F. Protein binding of valproic acid in maternal and umbilical cord serum. Epilepsia. 1984;25(2):244-9.
11. Myllynen P, Vähäkangas K. An examination of whether human placental perfusion allows accurate prediction of placental drug transport: studies with diazepam. J Pharmacol Toxicol Methods. 2002;48(3):131-8.
12. Moore RG, McBride WG. The disposition kinetics of diazepam in pregnant women at parturition. Eur J Clin Pharmacol. 1978;13:275-84.
13. Ridd MJ, Brown KF, Nation RL, Collier CB. The disposition and placental transfer of diazepam in cesarean section. Clin Pharmacol Ther. 1989;45:506-12.
14. Cavanah D, Condo CS. Diazepam-- A pilot study of drug concentrations in maternal blood, amniotic fluid and cord blood. Curr Ther Res Clin Exp. 1964;6:122-6.
15. Cree JE, Meyer J, Hailey DM. Diazepam in labour: its metabolism and effect on the clinical condition and thermogenesis of the newborn. Br Med J. 1973;4:251-5.
16. Kanto J, Erkkola R, Sellman R. Accumulation of diazepam and N-demethyldiazepam in the fetal blood during the labour. Ann Clin Res. 1973;5:375-9.
17. Mandelli M, Morselli PL, Nordio S, Pardi G, Principi N, Sereni F, Tognoni G. Placental transfer to diazepam and its disposition in the

e541

newborn. Clin Pharmacol Ther. 1975;17:564-72.

18. Bakke OM, Haram K, Lygre T, Wallem G. Comparison of the placental transfer of thiopental and diazepam in caesarean section. Eur J Clin Pharmacol. 1981;21:221-7.

19. Erkkola R, Kangas L, Pekkarinen A. The transfer of diazepam across the placenta during labour. Acta Obstet Gynecol Scand. 1973;52:167-70.

20. Owen JR, Irani SF, Blair AW. Effect of diazepam administered to mothers during labour on temperature regulation of neonate. Arch Dis Child. 1972;47:107-10.

21. Haram K, Bakke OM. Diazepam as an induction agent for caesarean section: a clinical and pharmacokinetic study of fetal drug exposure. Br J Obstet Gynaecol. 1980;87:506-12.

22. Kanto J, Scheinin M. Placental and blood-CSF transfer of orally administered diazepam in the same person. Pharmacol Toxicol. 1987;61:72-4.

23. Haram K, Bakke OM, Johannessen KH, Lund T. Transplacental passage of diazepam during labor: influence of uterine contractions. Clin Pharmacol Ther. 1978;24:590-9.

24. Desilva JA, D'Arconte L, Kaplan J. The determination of blood levels and the placental transfer of diazepam in humans. Curr Ther Res Clin Exp. 1964;6:115-21.

25. Ridd MJ, Brown KF, Nation RL, Collier CB. The disposition and placental transfer of diazepam in cesarean section. Clin Pharmacol Ther. 1989;45(5):506-12.

26. Schneider H, Proegler M. Placental transfer of beta-adrenergic antagonists studied in an in vitro perfusion system of human placental tissue. Am J Obstet Gynecol. 1988;159(1):42-7.

27. Erkkola R, Lammintausta R, Liukko P, Anttila M. Transfer of propranolol and sotalol across the human placenta. Their effect on maternal and fetal plasma renin activity. Acta Obstet Gynecol Scand. 1982;61(1):31-4.

28. Belpaire FM, Bogaert MG, Rosseneu M. Binding of beta-adrenoceptor blocking drugs to human serum albumin, to alpha 1-acid glycoprotein and to human serum. Eur J Clin Pharmacol. 1982;22(3):253-6.

29. Zakowski MI, Ham AA, Grant GJ. Transfer and uptake of alfentanil in the human placenta during in vitro perfusion. Anesth Analg. 1994;79(6):1089-93.

30. Gepts E, Heytens L, Camu F. Pharmacokinetics and placental transfer of intravenous and epidural alfentanil in parturient women. Anesth Analg.1986;65:1155-60.

31. Meuldermans W, Woestenborghs R, Noorduin H, Camu F, van Steenberge A, Heykants J. Protein binding of the analgesics alfentanil and sufentanil in maternal and neonatal plasma. Eur J Clin Pharmacol. 1986;30:217-9.

32. Heytens L, Cammu H, Camu F. Extradural analgesia during labour using alfentanil. Br J Anaesth. 1987;59:331-7.

33. Cartwright DP, Dann WL, Hutchinson A. Placental transfer of alfentanil at caesarean section. Eur J Anaesthesiol. 1989;6:103-9.

34. Johnson RF, Herman N, Arney TL, Gonzalez H, Johnson HV, Downing JW. Bupivacaine transfer across the human term placenta. A study using the dual perfused human placental model. Anesthesiology. 1995;82(2):459-68.

35. Johnson RF, Cahana A, Olenick M, Herman N, Paschall RL, Minzter B, Ramasubramanian R, Gonzalez H, Downing JW. A comparison of the placental transfer of ropivacaine versus bupivacaine. Anesth Analg. 1999;89(3):703-8.

36. Ala-Kokko TI, Alahuhta S, Jouppila P, Korpi K, Westerling P, Vahakangas K. Feto-maternal distribution of ropivacaine and bupivacaine after epidural administration for cesarean section. Int J Obstet Anesth. 1997;6:147-52.

37. Datta S, Camann W, Bader A, VanderBurgh L. Clinical effects and maternal and fetal plasma concentrations of epidural ropivacaine versus bupivacaine for cesarean section. Anesthesiology. 1995;82:1346-52.

38. Irestedt L, Ekblom A, Olofsson C, Dahlstrom AC, Emanuelsson BM. Pharmacokinetics and clinical effect during continuous epidural infusion with ropivacaine 2.5 mg/ml or bupivacaine 2.5 mg/ml for labour pain relief. Acta Anaesthesiol Scand. 1998;42:890-6.

39. Decocq G, Brazier M, Hary L, Hubau C, Fortaine MR, Gondry J, Andréjak M. Serum bupivacaine concentrations and transplacental transfer following repeated epidural administrations in term parturients during labour. Fundam Clin Pharmacol. 1997;11:365-70.

40. de Barros Duarte L, Moises EC, Carvalho Cavalli R, Lanchote VL, Duarte G, Pereira da Cunha S. Placental transfer of bupivacaine enantiomers in normal pregnant women receiving epidural anesthesia for cesarean section. Eur J Clin Pharmacol. 2007;63:523-6.

41. Papini O, Mathes AC, Cunha SP, Lanchote VL. Stereoselectivity in the placental transfer and kinetic disposition of racemic bupivacaine administered to parturients with or without a vasoconstrictor. Chirality. 2004;16:65-71.

42. Kuhnert BR, Zuspan KJ, Kuhnert PM, Syracuse CD, Brown DE. Bupivacaine disposition in mother, fetus, and neonate after spinal

anesthesia for cesarean section. Anesth Analg. 1987;66:407-12.

43. Thomas J, Long G, Moore G, Morgan D. Plasma protein binding and placental transfer of bupivacaine. Clin Pharmacol Ther. 1976;19:426-34.

44. Thomas J, Climie CR, Mather LE. The maternal plasma levels and placental transfer of bupivacaine following epidural analgesia. Br J Anaesth.1969;41:1035-40.

45. Scanlon JW, Ostheimer GW, Lurie AO, Brown wu JR, Weiss JB, Alper MH. Neurobehavioral responses and drug concentrations in newborns after maternal epidural anesthesia with bupivacaine. Anesthesiology. 1976;45:400-5.

46. Magno R, Berlin A, Karlsson K, Kjellmer I. Anesthesia for cesarean section IV: placental transfer and neonatal elimination of bupivacaine following epidural analgesia for elective cesarean section. Acta Anaesthesiol Scand. 1976;20:141-6.

47. McGuinness GA, Merkow AJ, Kennedy RL, Erenberg A. Epidural anesthesia with bupivacaine for Cesarean section: neonatal blood levels and neurobehavioral responses. Anesthesiology. 1978;49:270-3.

48. Beazley JM, Taylor G, Reynolds F. Placental transfer of bupivacaine after paracervical block. Obstet. Gynecol. 1972;39:2-6.

49. Reynolds F, Taylor G. Maternal and neonatal blood concentrations of bupivacaine: a comparison with lignocaine during continuous extradural analgesia. Anaesthesia. 1970;25:14-23.

50. Bader AM, Tsen LC, Camann WR, Nephew E, Datta S. Clinical effects and maternal and fetal plasma concentrations of 0.5% epidural levobupivacaine versus bupivacaine for cesarean delivery. Anesthesiology. 1999;90:1596-1601.

51. Datta S, Camann W, Bader A, VanderBurgh L. Clinical effects and maternal and fetal plasma concentrations of epidural ropivacaine versus bupivacaine for cesarean section. Anesthesiology. 1995;82:1346-52.

52. Loftus JR, Holbrook RH, Cohen SE. Fetal heart rate after epidural lidocaine and bupivacaine for elective cesarean section. Anesthesiology. 1991;75:406-12.

53. Sudhakaran S, Ghabrial H, Nation RL, Kong DC, Gude NM, Angus PW, Rayner CR. Differential bidirectional transfer of indinavir in the isolated perfused human placenta. Antimicrob Agents Chemother. 2005;49(3):1023-8.

54. Sudhakaran S, Rayner CR, Li J, Kong DC, Gude NM, Nation RL. Inhibition of placental P-glycoprotein: impact on indinavir transfer to the foetus. Br J Clin Pharmacol. 2008;65(5):667-73.

55. Mirochnick M, Dorenbaum A, Holland D, Cunningham-Schrader B, Cunningham C, Gelber R, Mofenson L, Culnane M, Connor J, Sullivan JL. Concentrations of protease inhibitors in cord blood after in utero exposure. Pediatr Infect Dis J. 2002;21:835-8.

56. Chappuy H, Tréluyer JM, Rey E, Dimet J, Fouché M, Firtion G, Pns G, Mandelbrot L. Maternal-fetal transfer and amniotic fluid accumulation of protease inhibitors in pregnant women who are infected with human immunodeficiency virus. Am J Obstet Gynecol. 2004;191:558-62.