

Exploration of the biochemical differences between high and low dose methadone clients on stable maintenance therapy.

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Abstract

There is large variability in the dose of methadone required to prevent withdrawal symptoms in chronic, stable methadone users. The difference in dose between low-dose and high-dose patients may vary >50 fold, and could be as low as < 5-10 mg/day, or greater than >300 mg/day. Our Objective was to identify factors which account for the difference in biochemical response of patients to low- and high-dose administration of methadone. We hypothesized that differences in high dose vs. low dose methadone clients are due to lower number of human μ -opioid receptors (hMORs) in high dose maintenance therapy patients than in those on lower doses, and/or desensitization down-stream from the opioid receptor that manifests as an attenuated cyclic AMP (cAMP) response to opioid agonists. We also hypothesized that concurrent drug use as well as P-glycoprotein levels may influence dosing requirements.

Using white blood cells as a model, we measured hMOR expression, *in vivo* cAMP levels, cAMP levels in response to exposure to increasing levels of methadone, P-GP expression and the presence of other drugs.

Our findings indicated that hMOR numbers on lymphocytes, granulocytes and monocytes did not vary for controls, low-dose, and high-dose methadone-treated patients. Baseline levels of cAMP in white blood cells were higher in controls than in low-dose methadone patients, and significantly lower in high-dose patients than either controls or low-dose patients. Increasing concentrations of methadone exposure for control leucocytes resulted in a dose-related reduction in cAMP. In contrast, increasing doses of methadone treatment had no

effect on cAMP levels in white cells of either low- or high-dose methadone patients. P-glycoprotein levels did not correlate with dose requirements. Concurrent drug use was detected in a high percentage of patients.

In conclusion, the dose of methadone required to prevent withdrawal symptoms in high-dose and low-dose methadone patients is not related to changes in hMOR number. In contrast, baseline cAMP levels were significantly lower in high-dose patients than in low-dose patients. Chronic treatment also abolished the methadone dose-related reduction in cAMP in-vitro in lymphocytes, indicating desensitization. Concurrent drug use may play some part in dosing requirements; however P-glycoprotein levels appeared not to. It is possible that mechanisms of the hMOR signal transduction cascade are responsible for these dosing discrepancies as related to of methadone-treated patients, however, more research is required to determine exact mechanisms

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List of Abbreviations

Abbreviations	Definition
AC	Adenylyl Cyclase
ACN	Acetonitrile
ATP	Adenosine Triphosphate
BG	Background
Bio-REB	Biomedical Research Ethics Board
cAMP	cyclic Adenosine 3', 5'-cyclic MonoPhosphate
CD45	A protein tyrosine phosphatase located in hematopoietic cells
CHO	Chinese Hamster Ovary cells
CNS	Central Nervous System
CREB	cAMP response element-binding
CV	Coefficient of Variation
DAMGO	[D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin acetate
DOA	Drugs of Abuse
DRM	detergent -resistant membranes
EDDP	2-ethylidene-1,5-dimethy-3,3-diphenylpyrrolidine
EDTA	Ethylenediaminetetraacetic acid
ES	Electro Spray
F	Bioavailability (Fraction of oral drug absorbed)

f_u	Free unbound fraction
FA	Formic Acid
FC	Flow Cytometry/Flow cytometer
FITC	Fluorescein isothiocyanate
G	Guanine
GAP	GTPase activating proteins
GC/MS	Gas Chromatography - Mass Spectrometry
GDP	Guanine Diphosphate
Gi/o	G-Inhibitory
GPCR	G (guanine nucleotide-binding) Protein Coupled Receptor
GRK	G-protein receptor kinase
Gs	G-stimulatory
GTP	Guanine Triphosphate
hMOR	human Mu Opioid Receptor
HRP	Horseradish Peroxidase
iNOS	Inducible nitric oxide synthase
Kg	Kilogram
KOR	kappa Opioid receptor
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LIS	Laboratory Information System

LLD	Lower Limit of Detection
LOD	Limit of Detection
LOQ	Limit of Quantitation
LSD	Lysergic Acid Diethylamide
MAPK	Mitogen-activated protein kinase
MMT	Methadone Maintenance Treatment
MOR	Mu Opioid Receptor (μ Opioid Receptor)
MS	Mass Spectrometry
MS/MS	Tandem Mass spectrometry
NMDA	N-Methy-D-aspartate
NO	Nitric Oxide
NSB	Non Specific Binding
OD	Optical Density
OIH	Opiate Induced Hyperalgesia
PBS	Phosphate Buffered Saline
PD	Pharmacodynamics
PDE	Phosphodiesterase Enzymes
P-GP	P-Glycoprotein
PK	Pharmacokinetics
PKA	Protein Kinase A
PKC	Protein kinase C
pNpp	p-Nitrophenyl Phosphate
QC	Quality Control

R	Right (<i>rectus</i>) enantiomer
RGS	Regulator of G-Protein
RNA	Ribonucleic acid
S	Left (<i>sinister</i>) enantiomer
SAMHSA	Substance Abuse and Mental Health Service Administration
SB	Specific Binding
SD	Standard Deviation
SPE	Solid Phase Extraction
SRM	Single Reaction Monitoring
TA	Total Activity
TB	Total Binding
T_{max}	Time to maximum concentration
TMB	3,3',5, 5' Tetramethyl-benzidine
UPLC	Ultra Pressure Liquid Chromatography
V	Volt
V_d	Volume of Distribution
WBC	White Blood Cells
WHO	World Health Organization

Chapter 1.

Introduction

A frequent challenge to methadone program clinicians is monitoring, adjusting and confirming appropriate methadone dosing for all of their treatment patients. Understanding dosing requirements is a difficult task. Many patients are motivated to obtain, either for use or for distribution, extra quantities of drug. Saskatchewan methadone physicians have described cohorts of patients who fall outside of normal dosing ranges, either requiring much lower dosing or much higher dosing. In many cases, both of these groups of patients have clinical presentation to support their claims. The intent of this research is to try and determine mechanisms responsible for this dosing discrepancy.

1.1 Methadone:

Methadone, a synthetic human μ -opioid receptor agonist (hMOR), is used in the management of pain as well as a maintenance treatment drug for opioid dependent patients. It is used as an opioid withdrawal medication because it differs from morphine in pharmacokinetic (PK) properties with higher bioavailability, a much longer half-life, a faster onset of action, is metabolized by liver cytochrome P450 and is associated with much less withdrawal. Methadone typically causes less adverse effects than traditional opiates partly due to the fact that its metabolites are not pharmacologically active (1-4).

After many years of performing analytical toxicological testing, and in particular performing drugs of abuse screening on “drug treatment” patients, a subject of inquiry became evident. Addictions clinicians wanted to know why certain patients exhibited very good clinical progress on what is considered to be normal methadone dosing, while others did very well on much lower doses and yet others required very high methadone doses. Methadone dosing is still an issue of debate and controversy among clinicians in methadone maintenance treatment (MMT) programs (5). In 1998 the National Institutes of Health proposed a recommended guideline for the proper methadone dose to be at least 60 mg/day, yet a recent review of methadone dosing in 2010 suggests that 14% of MMT patients receive < 40 mg/day(5). While most patients do well on between 60 and 100 mg/day, a subset of patients requires significantly higher dosing. Other researches have determined that methadone doses in the range of 120 –

150 mg/day are more effective in reducing heroin self-administration in some opiate dependent patients (6). In a 2002 study, 22% of the 1400 MMT patients in a Colorado facility were on doses exceeding 100 mg/day with a few cases as high as 300 mg/day (7).

High dose methadone treatment was followed for 152 weeks with an outcome described as having lower rates of illicit drug use and high retention rates. In this study, 144 patients were successfully treated with a mean methadone dose of 285 mg/day, much higher than control subjects who were maintained on a mean of 64 mg/day (8). This literature evidence seems to support the statements by local addiction clinicians that although many patients thrive on what has been described as “normal” dosing, a subset of patients remains that require either significantly lower or higher doses.

It has always been difficult to optimize individual therapeutic dosing regimens. Traditional approaches normally include starting naive patients on a very low dose (approximately 20 mg) daily and increasing gradually over time until withdrawal symptoms are satisfied. Typically this has been accomplished with doses between 60 and 100 mg/day; however, some patients require as much as 350 mg/day and some need as little as 2.5 mg/day (9). Most recent research suggests that the concentration vs. response relationship is quite variable (8;10;11). Serum methadone levels vary significantly with dose, so that therapeutic monitoring in general, may not be reliable for obtaining optimal dose. Serum methadone levels may be of practical value in obtaining dosing level to serum concentration relationships for an individual patient. Coefficients of

variation for plasma levels are unacceptably large and poor concentration response is exhibited in most patients (11).

The adverse effects and toxicity of methadone are similar to morphine and include respiratory depression, nausea, vomiting, dizziness, mental clouding, dysphoria, constipation, urinary retention and hypotension(5). Long-term treatment will result in tolerance to analgesic, euphoric and sedative effects, with a lowering of toxicity.

The risk of methadone overdose tends to be in the early stages of treatment, since for a non tolerant adult a dose of 40 – 60 mg can be toxic (12). After no signs of intoxication, and with indications that the signs of withdrawal are still present, a patient may be dosed higher by up to 10 mg/day. Generally 60 mg would not be exceeded in the first 7 days and 100 mg would not be exceeded in the first 14 days (13). The fact that some patients do surprisingly well on lower doses, such as 10 – 30 mg/day, and other patients require very high doses, 200 – 350 mg/day, speaks to the peculiarities of methadone and indicates why it is important for clinicians to optimize dosing on a patient by patient basis. The reasons for this variability are most probably attributed to a combination of pharmacokinetic/pharmacodynamics (PK/PD) parameters, especially in patients who have high tendencies to use other illicit drugs while on treatment (14). In methadone maintenance programs under the “harm reduction” model, it is common to see concurrent use of other opioid drugs by patients. In addition to co-medication, genetic polymorphisms and other factors (environmental,

biological) may contribute significantly to PK variability and to variation in response.

1.1.1 Methadone structure and chirality

Methadone contains an asymmetrical carbon atom, which allows it to exist in two enantiomeric forms (Figure 1.1). Both forms (R, S) have identical composition, however, different spatial arrangements mean that they are mirror images of one another. Methadone activates μ -opioid receptors at low concentrations in a stereo-specific manner in rat locus coeruleus (15). The R form possesses most of the pharmacological activity in human methadone patients (16). Original preparations contained only the enantiomerically pure (R) methadone, but based on its expense, racemic mixtures are the standard preparation administered today. Several studies indicate that the PK parameters suggested for the different enantiomers, show high variability, with coefficients of variation as high as 70%. The stereoselective differences in the PKs of methadone may be important for PK/PD modeling; however, it is unlikely to be of significant consequence in the therapeutic monitoring of compliance with drug treatment patients (17). If therapeutic drug monitoring was a practical approach to dosing requirements, then a measurement of each enantiomeric isomer would become much more important. To a large extent (R) methadone prevents the occurrence of opioid withdrawal symptoms, while the (S) form is ineffective. The (R) form has a lower intrinsic clearance and receptor binding compared to the (S)

form. Stereoselective differences may play an important part in PK/PD modeling and therefore it follows that with racemic methadone dosing, stereochemistry plays a significant role in dosing (15). Most treatment centers utilize racemic methadone simply based on cost and availability factors.

Methadone

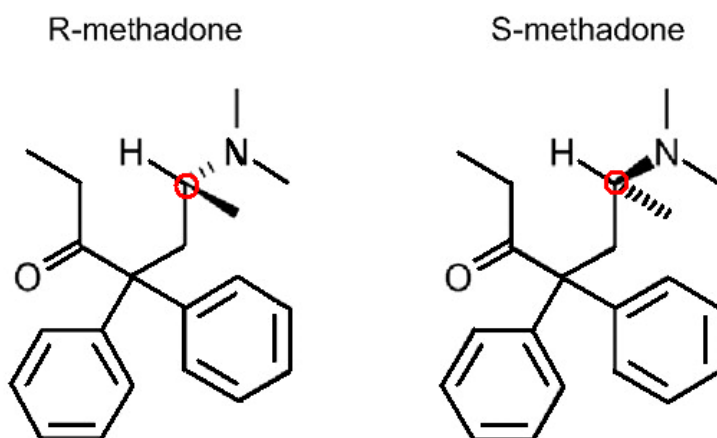


Figure 1.1 Structure of Methadone Enantiomers; (R) and (S)- 6-Dimethylamino-4,4-diphenyl-3-heptanone (C₂₁H₂₇NO; MW = 309.4 g/mol).

The red circle indicates the chiral carbon, which creates the difference in symmetry between enantiomers.

1.1.2 Methadone pharmacokinetics

Methadone total body clearance is approximately 0.095L/minute with a wide inter-individual variability (0.02 – 2 L/minute). Its elimination half-life ($t_{1/2}$) is approximately 22 hours with a very wide range reported from 5 – 130 hours. The

more pharmacologically active (R) enantiomer has a mean $t_{1/2}$ reported to be approximately 40 hours (12).

Methadone is a lipophilic drug that can be administered by a variety of routes. It is rapidly absorbed and can be detected in blood 15 – 45 minutes after oral administration. Plasma concentrations (T_{max}) peak at 2.5 – 4.4 hours after dosing and seem to be independent of dose. Oral doses are subject to first-pass effect in the liver and GI tract. The average oral bioavailability is approximately 80 – 90 %, but can range from 36 – 100% (18).

In humans, high volumes of distribution (V_d) have been reported and exceed actual physiological volumes. The mean apparent volume of distribution in humans is approximately 4.5 L/kg with a range as large as 1.7 – 13 L/kg (19). Methadone distributes to brain, gut, kidney, liver muscle and lung tissue (1). Tissue binding and the large volume of distribution despite extensive plasma protein binding suggest a higher affinity for tissues relative to plasma proteins.

Methadone undergoes hepatic metabolism predominantly to an inactive form of the drug, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), by N-demethylation and spontaneous cyclisation. Approximately eight other urinary metabolites have been identified, all of which have very little or no pharmacological effect. Urinary excretion of methadone and EDDP accounts for up to 60% of an oral dose. Since methadone is basic ($pK_a = 9.2$), changes in urinary filtrate pH have an important influence on the elimination of methadone. At urinary pH values above 6, renal clearance accounts for only 4% of the total drug elimination, while at pH values lower than 6, elimination of unchanged drug

can increase to 30% of the total dose (19). Until recently, the isoforms of P450 (CYP) enzymes thought to be predominantly responsible for the metabolism of methadone were CYP3A4 and CYP2D6. More recently it has been shown that CYP2D6 plays a much lesser role and that CYP2B6 is more involved than originally thought (20).

Methadone is highly bound by plasma proteins such as albumin and lipoproteins, but more specifically, it is found predominantly associated with α_1 -acid glycoprotein. Mean free fractions (f_u) of drug are reported to be between 2 and 14%, with a high degree of variability and slight differences between enantiomeric forms (14). Inconsistent data exists in the literature about stereoselective binding. Most sources suggest a very low free fraction of circulating methadone of approximately 3% (19). This extent of protein binding will likely be responsible for some of the PK variability of methadone. Only free methadone is available for pharmacological effect and thus measurement of total methadone levels for therapeutic monitoring will not truly accurately reflect drug-effect status. Since it is an acute-phase reactant protein, marked changes in α_1 -acid glycoprotein levels due to other conditions (illness, stress etc.) may significantly alter methadone PK values (21). In the case of oral methadone maintenance treatment (MMT), these alterations related to fluctuating α_1 -acid glycoprotein levels may be a major consideration and are difficult to determine. Furthermore, plasma protein binding must be considered a potential factor responsible for some of the inter-individual variation in clearance (17).

Methadone can be considered to be a restrictive clearance or low extraction ratio drug for which clearance is strongly dependent on the fraction of protein binding.

Table 1.1 Table of P450 enzyme involvement including which isoform and its function & significance

Enzyme Isoform	Function / Significance
CYP3A4	<ul style="list-style-type: none"> • Main isoform involved in metabolism • Activity among individuals varies 40 fold • Involved in the metabolism of approximately half of the drugs used today • Inhibitors include grape fruit juice, anti-retroviral drugs, diltiazem, erythromycin and ciprofloxacin • Inducers include Phenobarbital, carbamazepine, phenytoin and St John's Wort
CYP2B6	<ul style="list-style-type: none"> • Important contributor to methadone metabolism • Stereoselectivity to (S-) enantiomer • Wide inter-individual variability in expression • Inhibitors include ticlopidine, orphenidrine & sertraline • Inducers include anticonvulsants & rifampicin
CYP2D6	<ul style="list-style-type: none"> • Minor contribution to methadone metabolism • Stereoselectivity to (R-) enantiomer • Largest phenotypical variability among the CYPs, largely due to genetic polymorphism.
CYP2C9, CYP219	<ul style="list-style-type: none"> • Very minor involvement
CYP1A2	<ul style="list-style-type: none"> • No known involvement

Adapted with permission from: Yonfang, Li et al 2008

1.1.3 Methadone pharmacodynamics

Methadone elicits its pharmacodynamic properties predominantly by binding to the human μ -opioid receptor (hMOR) as well as to the δ - and κ -opioid receptors. Although methadone is a synthetic opioid agonist, it displays morphine like properties. The affinity constant to hMOR is 3.51 nM for methadone, which suggests slightly less affinity than morphine (22). Activation of hMOR via ligand (methadone) binding produces analgesia, respiratory depression, physiological dependence and tolerance. Methadone does exhibit a lower potential for abuse than morphine, which enhances its potential for drug maintenance therapy programs, but does induce some dependence (21). Methadone differs from morphine in that it displays a non-competitive antagonist activity at the N-methyl-D-aspartate (NMDA) receptor. This receptor is well known for its ligand binding of dextromethorphan, a common cough suppressant. This receptor does play a role in pain transmission and both enantiomeric forms of methadone have some binding affinities to this receptor. This may partially explain why both enantiomers display some antinociceptive effect (R form has most of the effect at hMOR) (17).

Methadone is also a strong inhibitor of serotonin and norepinephrine uptake. Genetic polymorphisms in the gene encoding the NMDA receptor have been described, without significant evidence of a relationship to methadone (13). Genetic polymorphisms in the gene encoding hMOR have been well described. More than 20 variants that produce amino acid changes in the receptor have been identified and some variants exhibit altered affinity to various substrates. The overall effect on methadone binding is unclear with little consensus, although

some studies have shown a decrease in opioid effect and increased opioid dosage requirements (23).

1.1.4 Inter-individual variability

Methadone PK varies significantly between individuals, and probably PD parameters also vary as illustrated by the lack of consensus of descriptions of these parameters in the literature. This variability appears to be both environmentally and genetically influenced (22) and may be largely attributed to CYP enzyme activities. CYP3A4 is mainly responsible for the N-demethylation of methadone to inactive metabolites, and CYP2B6, another enzyme involved in metabolism, are present at up to a 30 fold variability in the liver and intestinal mucosa (24). Some more remotely involved enzymes such as CYP2D6 display a more than 100 fold variation between subjects (20). CYP2C19, CYP2C9 and CYP1A2 are all variable between individuals. Existence of genetically determined poor, extensive and ultra-rapid metabolizer phenotypes have been described based on the expression levels of these phenotypically different enzymes. Thus methadone, like many other drugs, displays a wide dose-plasma concentration relationship, since it is metabolized by CYP3A4 as well as these other polymorphic enzymes (18).

Due to the nature of methadone drug treatment, a high percentage of patients are likely to use other drugs, both illegal and prescription. As most drugs are substrates (inducers, inhibitors) of these isoenzymes, drug-drug interactions involving methadone commonly occur. This scenario is especially true in “harm

reduction” models, which are predominantly used in Western Canada (25). Harm reduction programs, as the name suggests, are not intended to treat all patients until they are weaned completely off all other drugs. Rather, the program’s intent is to monitor and control to some degree other drug use so that there is an overall lessening of harm to the patient as well as to the society they live in. The principle suggests that less crime (e.g. prostitution, theft etc.) will result if methadone patients have less of a need for illegal drugs and are not required to be completely free of other drugs. Thus, the likelihood of at least some concurrent drug use is high. Co-medications are able to introduce a further variability by either inhibition or induction of methadone clearance (enzyme activity and bioavailability). Multi-drug use will complicate normal metabolic processes and clearance. Cessation of a CYP active drug can also have severe consequences. When a potent inducer such as carbamazepine is discontinued, the patient now becomes a relatively slower metabolizer (due to loss of enzyme induction) and what was previously an adequate dose of methadone becomes excessive (2).

Some studies have shown that patients on MMT with controlled administration of drug exhibit up to a 5-fold variation in trough plasma levels. Significant differences in dose-plasma level relationships are not limited to compliant patients. Based on very high inter-individual variability of methadone blood concentrations for a given dosage, a theoretical dosage of 55 – 920 mg/day would be required to provide a blood concentration of 250 µg/L of (R) methadone for a theoretical 70 Kg patient without any co-medication (26).

Another possible variable, which contributes to methadone response, is the expression of p-glycoprotein. Sequence variations of the gene have been characterized, potentially leading to polymorphisms in the expressed protein (27). Thus, individuals may have significantly lower levels of expression in the duodenum with resultant variation of drug levels in the plasma. As well, lower p-glycoprotein expression levels at the blood brain barrier may lead to higher brain levels of drug. More recent work suggests less of an influence by p-glycoprotein, but the inconsistency of the associated literature implies it should be considered (28).

PK/PD parameters of methadone during pregnancy and lactation have been studied in the context of female MMT patients maintaining their treatment while pregnant and/or breastfeeding. The effectiveness of a particular methadone dose will vary during pregnancy, but overall there is higher clearance of methadone during pregnancy. Several reasons may account for this observation, such as hormonal induction of intestinal and hepatic CYP enzymes, metabolism in placental tissue and variation of methadone transporters (29). Alterations in methadone clearance may create opioid withdrawal symptoms during pregnancy, which will be mirrored by the fetus (30). The factors affecting methadone concentrations in fetal circulation during pregnancy and how they relate to fetal outcome are poorly understood. Most neonates born to MMT patients display some type of opioid withdrawal (30).

Few studies have examined the exposure of infants to methadone through breast milk (31). Measured concentrations of methadone in breast milk are low

and remain stable over time. Methadone doses of 27 – 180 mg/day produce milk concentrations of 25 – 260 ng/mL, in turn delivering approximately 0.05 mg of methadone based on an average daily intake of 500 mL (32). Even after correcting for lower clearance in neonates, the relative infant dose would be very small. Since methadone offers important therapeutic effects to the mother in dealing with opiate dependency, the benefit of methadone to the mother far outweighs the risk to the breastfeeding neonate (31).

Since methadone is quite often used for very long term, it is likely that patients may remain under treatment until they are elderly. Altered PK/PD parameters of elderly patients have been well described. Generally elderly patients have reduced levels of clearance, often requiring a reduced or adjusted dosing regimen to maintain levels within the therapeutic range without creating toxicity (33).

Although serum methadone levels are often of minimal value, they can be used in special cases to confirm requests for increased dosage or to identify patients who may require split doses. The clinical presentation of a patient should always override serum methadone level values (2). Definitive toxic serum levels of methadone remain undetermined with concentrations ranging from 60 to 4,500 ng/mL in observed fatalities (34).

1.2 μ-Opioid receptor

Opioid receptors (hMOR – human mu opioid receptors) are G-protein coupled receptors responsible for most of the physiological action of opioids. G-protein coupled receptors consist of seven trans-membrane spanning domains with an N-terminus extra cellular domain and a C-terminus intracellular domain (Figure 1.2). When a ligand such as a drug, hormone or neurotransmitter, interacts with a heptahelical receptor on the surface of the cell, the ligand either stabilizes or induces a conformation in the receptor that activates a heterotrimeric G-protein (35).

1.2.1 The role of G-proteins

G proteins, named for their interaction with guanine nucleotides GTP and GDP, most commonly consist of 3 discrete protein subunits - α , β and γ . Guanine nucleotides bind to the α subunit, which has enzymatic activity catalyzing the conversion of GTP to GDP. The β , γ subunits remain associated as $\beta\gamma$ complex. All 3 subunits are anchored to the membrane through a fatty acyl chain covalently linked to an amino acid residue by prenylation. G-proteins appear to be freely diffusible in the plane of the membrane. In the resting state, the G-protein exists as an unattached $\alpha\beta\gamma$ trimer, with GDP occupying the binding site of the α subunit. When a receptor is occupied by an agonist molecule, a conformational change occurs, involving the cytoplasmic domain of the receptor

causing it to acquire high affinity for $\alpha\beta\gamma$. Association of $\alpha\beta\gamma$ with the receptor causes a conformational change in $\alpha\beta\gamma$ that has less affinity for GDP and which is replaced with GTP [GDP/GTP exchange]. This exchange causes dissociation of the G – protein trimer, releasing α -GTP and $\beta\gamma$ subunits; these are the “active” forms of the G-protein, which diffuse along the membrane and can associate with various enzymes and ion channels, causing their activation or inactivation. The process is terminated with hydrolysis of GTP to GDP via the GTPase activity of the α -subunit. The resulting α -GDP dissociates from the effector and reunites with the α -subunit, completing the cycle. Targets for G – proteins include the adenylyl cyclase or cAMP system and the phospholipase system.

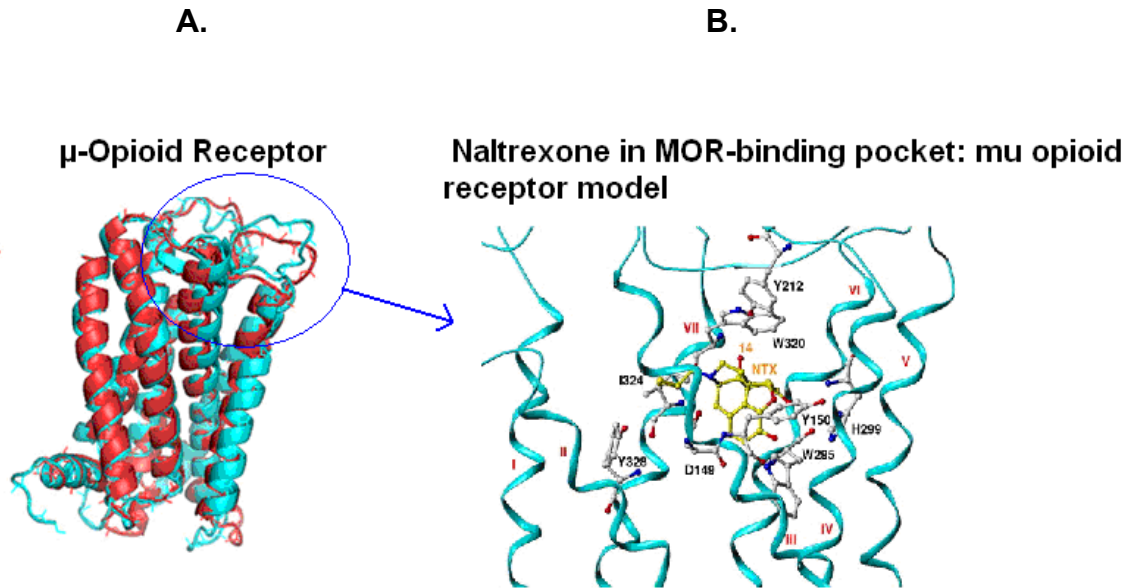


Figure 1.2

X-ray crystallographic ribbon models of A) the human μ -opioid receptor – a G-protein coupled receptor consisting of a seven transmembrane spanning domain and B) the binding pocket of the μ -opioid receptor with modeled bound naltrexone.

Adapted with permission from (36)

Opioid receptors send cellular signals indirectly via a variety of G-proteins that, once activated, directly modulate channel activity or adenylyl cyclase (AC) that produces second messengers such as cyclic adenosine 5-monophosphate (cAMP) (37). Eventually this leads to reduced excitability along the neuronal cell

membranes involved in pain pathways. A reduction of cAMP production leads to a suppression of Na⁺ and Ca⁺ channels and results in analgesia.

Neurotransmission of pain is lessened by a lowered neuronal excitability, reduced action duration and lowered neurotransmitter release. These G proteins are inhibitory in nature and inhibit AC activity as well as regulating the activity of mitogen-activated protein kinase (MAPK) and Ca⁺ and K⁺ ion channels. Opioids may also activate G-proteins, which have stimulatory properties in some tissues and stimulate AC to raise the cAMP levels in cells (38). Isoform-specific and differential regulation of various classes of effectors by the opioid receptors are interwoven into a complicated signaling network, which mediates opioid action (39).

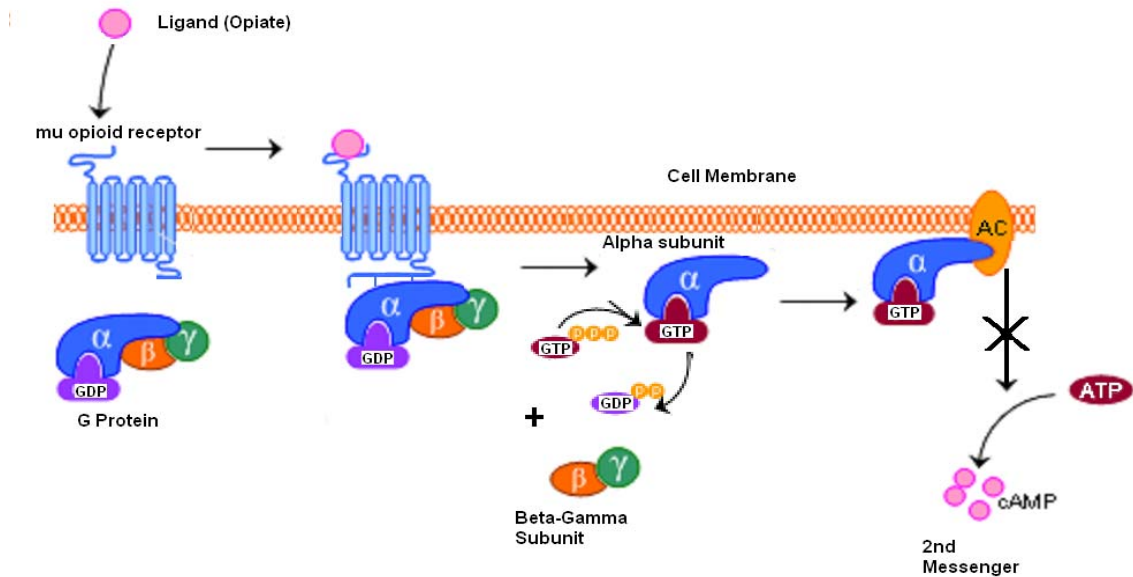


Figure 1.3

A simplified schematic of activation of down stream signaling by human mu opioid receptors - See 1.2.2 below

Adapted with permission from (40)

1.2.2 Receptor binding

Ligand binding to the receptor creates a conformational change in the cytosolic portion of the receptor. This in turn activates a trimeric G protein, which causes a conformational change in the α -subunit. This allows GDP to dissociate and be replaced by cytoplasm-abundant GTP. The GTP binding causes the trimeric G-protein to dissociate into separate α and β / γ components. The active

α - subunit goes on to bind to other proteins or enzymes such as AC. This inhibits AC, which now reduces the production of cAMP, an important downstream signaling molecule. Neurotransmission of pain is lessened by a lowered neuronal excitability, reduced action duration and lowered neurotransmitter release.

Eventually the α -subunit hydrolyzes its bound GTP back to GDP, which inactivates the subunit. Another protein called the regulator of G-protein signaling (RGS), which inactivates the G-protein by combining with the β / γ complex, may influence this step. As long as the receptor remains stimulated it can continue to activate G- proteins. Upon prolonged stimulation, the receptor will become inactive even if its ligand remains bound. In this case, a G-protein receptor kinase (GRK) phosphorylates the cytosolic portions of the receptor. Once the receptor has been phosphorylated, it binds with great affinity to an arrestin protein (β -arrestin), which then inactivates the receptor by preventing it from binding to G-protein. Arrestins act as adapter proteins and recruit the receptor to clathrin coated pits, from where the receptors are endocytosed. The receptors may now be degraded in lysosomes or recycled back to the cell membrane and re-activated (41).

1.3 Tolerance and dependence

Physical *dependence* is a property of many drugs, not just drugs that have a high abuse potential. Physical dependence suggests that if drug use is suddenly discontinued there is a predictable physiological response. The body

will make changes to adapt to the drug leading to unpleasant withdrawal sensation upon cessation (42-44). Someone who is physically dependent on a drug requires that drug in order to function normally. Cross dependence is the partial or complete ability of one drug to suppress the manifestations of physical dependence produced by another drug.

Drug *addiction* is not necessarily just a physical phenomenon, but also a psychological phenomena (for which there may be an unknown biochemical cause) consisting of loss of control, continued use despite adverse consequences and the preoccupation and obsession with obtaining and using more drug. The WHO defines addiction as “a behavioral pattern of drug use, characterized by overwhelming involvement with compulsive use of the drug, securing of the supply and the likelihood of relapse after withdrawal (42).

Drug *tolerance* may be defined as a state of progressively decreased responsiveness to a drug resulting in a larger dose of the drug needed to achieve the effect originally obtained by the smaller dose (45). Tolerance to the desired action of the drug and the side effects may differ. Opioid tolerance is characterized by a reduced response to opioid drugs such as morphine or codeine, which are commonly used as pain medications, but are commonly abused based on their euphoric effects. Profound tolerance may develop, especially if the drug is chronically administered. A large range of neuroadaptations develop in response to chronic administration of these drugs, which are thought to be a significant contributor to tolerance and dependence (46). A very “short term” tolerance may develop within minutes of administration,

which is thought to involve receptor desensitization and internalization. A more substantial “long term” tolerance will emerge after days to weeks of opioid use. This tolerance results from mechanisms of adaptation at the levels of the receptor, cellular signaling and the synaptic network. Neuroadaptations are considered to be critical to bring about the major symptoms of opioid addiction - tolerance and withdrawal (47).

A focus on the opioid drugs and their specific receptor types in relation to drug tolerance and dependence shows this to be a very complex process, including receptor dimerization processes which are not yet fully understood.

1.3.1 Desensitization

Desensitization at the receptor is considered one of the major mechanisms of the development of drug tolerance (48). It is observed when intracellular regulatory proteins and/or enzymes are activated in such a way that they “decouple” the receptor from the G protein or produce a “switch” in coupling to a “nonanalgesic” G protein. Uncoupling of receptors is associated with phosphorylation by GRK and subsequent binding by arrestins. This leaves the G protein uncoupled from the receptor and inactive. It also prevents the G protein from any further interaction with a receptor (specifically if it is bound to either GRK or β -arrestin). The receptor may in turn be internalized and either destroyed or returned to the cell surface with restored activity, where different opioid agonists may be associated with different mechanisms (48). It is possible that the

varying effects on GRKs or protein kinase C (PKC) will depend on the specific opiate agonist. This has been shown by PKC inhibitors, seemingly providing inhibition to morphine tolerance but not inhibiting tolerance to all opioids (49). Morphine induction of PKC could cause the direct phosphorylation of MOR or phosphorylation and activity enhancement of other components involved in desensitization (50). Desensitization can be caused by such factors including 1) β -arrestin mediated receptor internalization; 2) Down-regulation of opioid receptors; 3) Uncoupling of opioid receptors from G proteins; 4) Increased production of nitric oxide via inducible nitric oxide synthase (iNOS); and 5) Signaling via G proteins.

1.3.2 Internalization

A second mechanism believed to contribute to the development of drug tolerance is internalization of the receptor from the cell membrane (51). Once it is internalized, the receptor can no longer function and is essentially down-regulated. Down-regulation is defined as a disappearance or reduction of total cell surface and functional intracellular receptors. Once again the disparity in the literature suggests that this mechanism may indeed decrease tolerance by removing desensitized receptors from the membrane and recycling new receptors to the membrane (52). More complexity than clarity is often the case with opioid – related cellular mechanisms.

Internalization of MOR has been observed in neuronal cells after treatment with etorphine, a high-affinity MOR agonist (53), with up to 50% undergoing

internalization within minutes of exposure. Yet, a more recent study suggests that treatment of cells with morphine does not result in a significant down-regulation of MOR, but tolerance was still observed to increase linearly with infusion dose (48). Thus, it can be speculated that although internalization seems to play a role in tolerance, it is not a mandatory requirement. Tolerance is likely a multi-factor process involving varying degrees of both desensitization and internalization or down-regulation, which may be opioid agonist dependent.

1.3.3 Signal regulation by G-proteins

G proteins are an important consideration in the understanding of the molecular basis of opioid dependence. Although eight or nine distinct G proteins have been identified, it is unclear which of them primarily carry the signals (54). Opioid receptors display differences in their specificities towards these G proteins. Many combinations of the G – α β γ heterotrimers are theoretically available for signal transduction. The coupling specificity to G proteins may be partly governed by the types of tissue and cells being examined. The same opioid receptor subtypes in various tissues and cells exhibit different preferences for G proteins. This suggests that receptor – G protein coupling may rely on the specific cellular environment (54).

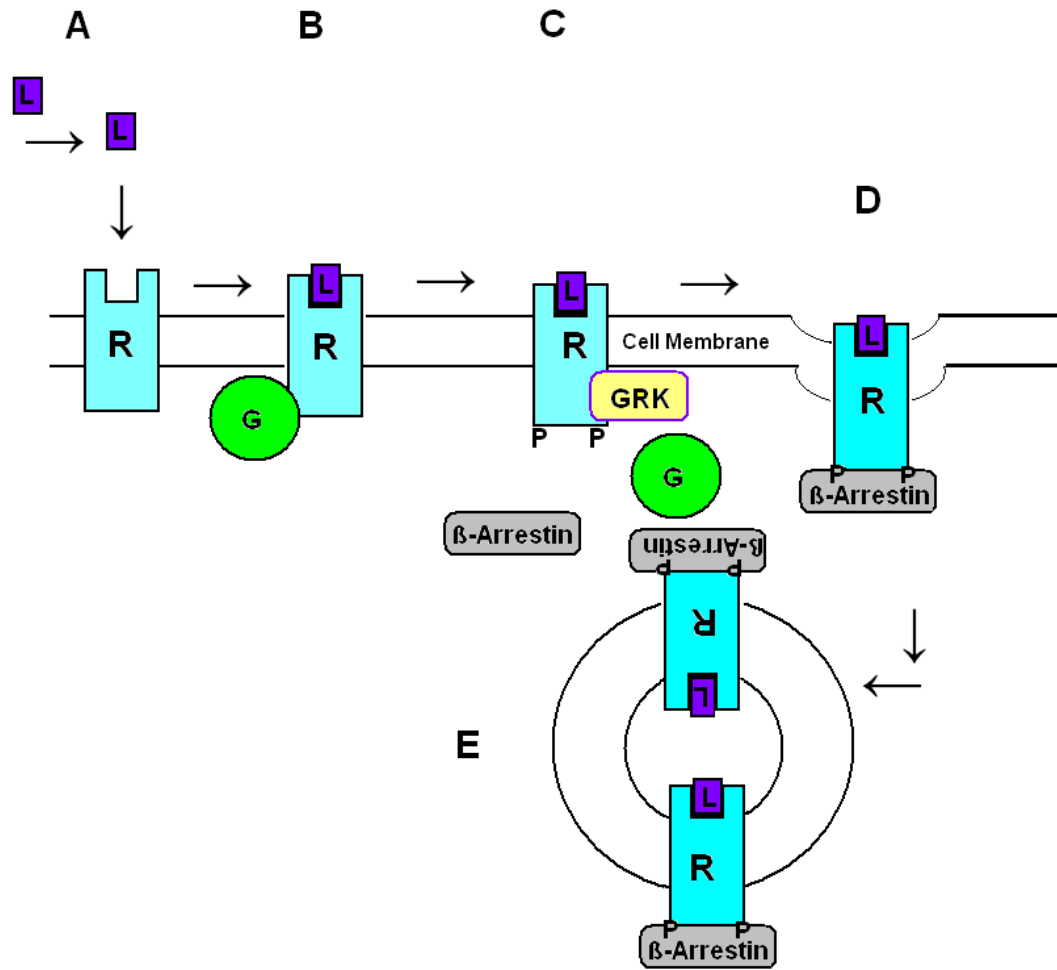


Figure 1.4 Desensitization Mechanisms of hMOR

Desensitization mechanisms of hMOR can affect the efficacy of many drugs. A) Binding of an opiate agonist (L) to the receptor (R) leads to B) Coupling of the receptor to the G-protein (G) and activation. In C) G-protein receptor kinases can phosphorylate (P) receptors, which then leads to binding of β -arrestins and other accessory proteins. This, in turn, results in uncoupling of the G-proteins and to D) & E) internalization and either recycling or degradation of the receptor.

Adapted with permission from (55)

GRK proteins, which subsequently phosphorylate MOR and enable its binding with β -arrestin, play a significant role in the development of tolerance. GRK proteins appear to be more involved in both the electrophysiological and behavioral tolerance of high efficacy opioids such as fentanyl than in morphine tolerance, a lower efficacy agonist. Specific inhibition of PKC has also been studied to determine its role in tolerance (56). Tolerance to some opioid agonists was completely reversed by the administration of PKC inhibitors, but not all agonists display this reversal. An explanation for this discrepancy could be that different opioids induce particular conformational changes of the receptor. The G protein coupled receptors could acquire different conformations when activated by different ligands. This phenomenon has been labeled functional selectivity. Specific agonists appear to stabilize distinct conformations that allow the receptor to couple to the appropriate G protein to elicit downstream responses controlled by different desensitization mechanisms. It is possible that different mechanisms control tolerances to opioids of different efficacies and that both PKC and GRK proteins play a role (57).

1.3.4 RGS proteins

Important regulatory molecules in signal activation by the receptor are regulators of G protein signaling (RGS) proteins. These proteins serve as GTPase activating proteins (GAP) for G proteins and thus can modulate the duration of G protein signals (54). RGS proteins play a role in modulating opioid receptor – G protein interactions. and coupling specificity, with specific

correlations between RGS protein levels and the development of acute opioid tolerance (58).

A primary function of RGS proteins is to regulate negatively G proteins via GAP to accelerate GTP hydrolysis and, hence, facilitate the switch of the G α subunit from a GTP active state to a GDP inactive state. RGS proteins effectively halt the signaling event and return the receptor/G protein back to a “ligand receptive” state (58). Over twenty different RGS proteins have been identified and categorized based upon their structure. They play a relatively selective role in regulating opioid receptors. RGS19 seems to be most involved in opioid receptor internalization and recycling. The functionality of RGS proteins is related to their ability to interact with G proteins and opioid receptors. The critical role they play in receptor desensitization and internalization/recycling is the basis for their contribution to tolerance.

1.3.5 Hyperalgesia and antinociceptive tolerance

Treatment of both severe acute pain and chronic pain is accomplished routinely with opiates. In addition to the desired analgesic actions, there is evidence that opiate administration can paradoxically lead to hyperalgesia (59;60). Hyperalgesia is an enhanced pain response to a noxious substance. Opioids may actually increase sensitivity to pain and may aggravate existing pain. The mechanisms by which chronic opiate exposure induces hyperalgesia and the relationship of this state to antinociceptive tolerance remain unclear. Data suggests that analgesic tolerance and sustained opiate induced

hyperalgesia and corresponding reduction in sensory thresholds are related and are a consequence of prolonged activation of MOR (61). The underlying mechanism linking tolerance and hyperalgesia is thought to be the NMDA receptor (60).

The NMDA receptor is a glutamate receptor responsible for synaptic plasticity and memory function. *N*-methyl *D*-aspartate (NMDA) is a selective NMDA receptor agonist, which binds to this receptor but not other glutamate receptors. Some opioids such as methadone are partial NMDA agonists. MOR activation increases the NMDA receptor-mediated glutamate response by intracellular protein kinase. The NMDA system has also been implicated in acute tolerance to morphine but not to other selective MOR agonists. NO (nitric oxide), which is formed by NMDA activation, diffuses to adjacent nerve terminals to modulate neurotransmitter release. It also acts at several levels to develop hyperexcitability, which results in hyperalgesia (60).

1.3.6 Endogenous morphine considerations

Animal tissues and human cells have been shown to produce small amounts of endogenous morphine as well as other opioid ligands (endorphin, enkephalin), which act as neurotransmitters (62). They are produced by the pituitary gland and the hypothalamus and they resemble the opiates in their abilities to produce analgesia and a feeling of well-being. In various stressful situations, the levels of naturally occurring opiates do rise dramatically. Since we have described tolerance as a common phenomenon, what would the role of

tolerance be in the naturally occurring opiate system? Some researchers suggest that the phenomenon of opioid tolerance and rebound is an explanation of how we control down regulating occurrences (62). When down-regulation is initiated, the concentration of endogenous opioids rises to overcome competitively the initial stimulatory molecules. The effector cells would become tolerant to endogenous morphine, so that the morphine induced down regulation would be terminated. Tolerance would set in once down-regulation was resumed.

Researchers have also speculated that addiction emerges from tolerance if the concentrations of endogenous opiates do not return to their previous or pre-stimulation low levels. Tolerance may be an evolutionary designed phenomenon required to mediate critical life sustaining activities, which are required to be repetitive in nature.

Drugs, as substances of abuse, may take control of these processes and create urges and desires, which have no benefit to the host (62).

1.3.7 Shared mechanisms (other receptors)

Studies suggest that the kappa opioid receptor (KOR) mediates inhibition of morphine tolerance (33;53;63;64). Recent studies have confirmed that KOR during repeated morphine treatment suppresses the development of analgesic tolerance to morphine (65). GRK proteins, PKA and PKC also play a role in receptor desensitization (66). PKC activity in the brain increases when tolerance has developed. As well, various PKC inhibitors block the development of morphine tolerance (67;68). Mechanisms governing KOR-mediated inhibition of

morphine tolerance may result from a decrease in PKC activation and prevention of MOR desensitization. Stimulation of KOR during repeated morphine treatments suppresses the analgesic tolerance by inhibition of MOR desensitization and/or acceleration of MOR recycling (65). Thus, other receptors (including both KOR and DOR) may influence tolerance and dependence normally associated primarily with MOR.

1.3.8 Post receptor adaptations

Other research suggests that post-opioid receptor adaptations may functionally alter signaling processes. These mechanisms involve covalent modification of signaling molecules and altered associations among them (69). Multiple considerations suggest that additional signal transduction modifications contribute to the development of tolerance. Supportive evidence shows that morphine (a tolerance producing drug) results in little or no receptor internalization and that morphine tolerance is frequently not accompanied by receptor down-regulation (70).

As described previously, GPCR (G protein coupled receptor) signaling results from the signaling activity of both the $G\alpha$ and the $G\beta\gamma$ subunits of the heterotrimer. The same effector (AC) can be regulated differentially by these two subunits altering their respective signaling capacities based on drug exposure. In opioid naïve tissue, opioid receptors signal predominantly through the $G\alpha$ subunit, but with sustained exposure to morphine, inhibitory opioid signaling is

replaced with more frequent excitatory signaling. AC activity now instead of being inhibited becomes enhanced. This may result in opioid tolerance, since the sustained generation of $G\alpha$ – inhibitory would be mitigated by the emergence of $G\beta\gamma$ – stimulatory (69). Chronic morphine-induced changes in AC may be related to functionality of segmented synthesis of AC isoforms, which are stimulated by $G\beta\gamma$. Signaling via $G\beta\gamma$ is very complicated and may in fact be counterproductive to the $G\alpha$ mediated effects. cAMP production may be regulated in opposite directions by these two competing subunits. Some of the $G\beta\gamma$ regulated pathways during chronic opioid treatment can link opioid receptors to gene transcription, which can then lead to modified cellular responses (54).

The molecular basis of opioid dependency involves receptor coupling to G proteins. Since eight or nine distinct G proteins have been described, and their coupling specificity is governed at least in part by the type of tissue and cells involved, there are many factors, which control signaling. As well, specific regulatory molecules (e.g. RGS proteins) may be present in different types of cellular environments (49).

1.3.9 Membrane rafts and receptor localization

Membrane rafts and caveolae influence cell functions including intracellular sorting of proteins and lipids, establishment of cell polarity, the function of vesicular transport processes and fine tuning of cell signaling processes specifically on the cell membrane (71). G Proteins, kinases, beta arrestin and phosphatases are located in membrane rafts and/or caveolae and significant evidence suggests they are involved in the functional regulation of signaling components. KOR and MOR localize mainly in membrane raft domains and normally internalize through clathrin-dependent pathways (72;73)

Membrane rafts are planar domains of cell membranes enriched in specific lipid and proteins. High content of glycosphingolipids and cholesterol in the outer leaflet of the lipid bilayer gives these regions gel-like properties and organization. Caveolae have been identified by electron microscopy as 50 – 100 nm diameter flask-shaped invaginations at or near the plasma membrane. Specific proteins (Caveolin-1, -2 and -3) are responsible for their shape and structure. Caveolae are presumed to function as non-planar membrane rafts in areas of invagination. Several methods of microscopy as well as co-immunoprecipitation of GPCRs and caveolin have been used to determine receptor localization in caveolae (74).

Mechanisms responsible for how GPCRs locate to membrane rafts include the transmembrane regions of GPCRs interacting with the lipid components of the rafts. The affinity for some GPCRs can be modulated by

cholesterol. This is probably not a general phenomenon, since cholesterol does not affect all GPCR agonist affinities. Alpha helices may also play a role as with receptor activation mechanisms. Intracellular loops and carboxyl-terminal tails may also be involved through fatty acylation and protein-protein interactions (72;74).

Most research suggests that rafts are important for GPCRs at all stages of their life cycles (exocytic, plasma membrane and endocytic). Membrane rafts at the cell surface may be responsible for receptor stability by providing a stable, resistant environment to internalization and thus sustained specific signaling. The precise shuttling inside and outside of membrane rafts remains unclear although several paradigms exist (73;75)

The fact that the main function of most GPCRs is to elicit and modulate cell signaling responses and second messenger signaling, membrane rafts must elicit some effect on the signaling cascade. One view is that membrane rafts are “stations” where GPCRs meet specific signaling molecules enabling selective and efficient G protein coupling. This is a significant consideration since receptor activation outside versus inside membrane rafts creates different signaling pathways. The same receptor may also be differentially located in different cell types implying that compartmentalization may be cell specific. Protein distribution in membrane rafts may also be dependent on other factors such as age, concurrent drug use and diseased state suggesting different signaling efficiencies in normal vs. diseased conditions. The overall view is that recruitment of GPCRs into membrane rafts/caveolae affects the stages of their lifecycle by

regulating intracellular trafficking and signaling properties. GPCR domain compartmentalization appears to depend on receptor cell type, metabolic state, growth stage and differentiation, which affects its regulation (71).

Comparison of rat neuronal cells, which exhibit no significant caveolae, and CHO (Chinese hamster ovary) cells, which are enriched in caveolae, suggests that segregating proteins in caveolae vs. non-caveolae rafts has quite different functional consequences. In both systems, cholesterol was found to be important for organizing opioid receptors and G proteins signaling molecules in membrane rafts, and agonist treatment did not affect the association of hMOR with membrane rafts. Membrane rafts sustained hMOR mediated G protein activation in neuronal cells but inhibited it in CHO cells (75).

1.3.10 Opioid receptor genetics

Drug – induced long-term functional alterations of cell signaling presumably also involves changes or modifications to gene expression. Several opioid-induced signals converge at the level of transcription factors. Research has shown several transcription factors, including the cAMP-response element DNA binding protein (CREB), members of the MAPK cascade and the nuclear factor (NK)- κ B (54). Opiate dependence would seemingly require adjustments in transcriptional activities.

Another influential factor in opioid dependence, tolerance and hyperalgesia may be related to the activities of p-glycoprotein drug transporters.

These proteins are a family of ATP-dependent drug efflux pumps for xenobiotic compounds with broad substrate specificity. They are responsible for decreased drug accumulation in multidrug-resistant cells and often mediate the development of resistance to anticancer drugs. This protein family also functions as a transporter at the blood-brain barrier.

Studies have shown that analgesic tolerance and physical dependence accompany the development of opiate induced hyperalgesia (OIH) and that they require P-glycoprotein activity to achieve maximal expression. Multiple morphine response –related traits also require P-glycoprotein (PGP) activity and genetic variations in PGP account for modifications to morphine levels. Recent reviews discuss the fact that genetic variants of PGP expression control the clinical effects of opioids and other drugs differently (76). Gene expression array studies may be an important tool to discover information on GPG modulation of opiate hyperalgesia, tolerance and dependence.

Much work has been done to identify specific genetic haplotypes that confer genetic individual differences in addiction vulnerability. Genetic markers linked to or associated with vulnerability to substance abuse are beginning to emerge supporting polygenic inheritance of substance abuse vulnerability. These classic genetic studies document a strong, complex genetic contribution to abuse of multiple addictive substances. The variations could act via: 1) Metabolism or bio-distribution, 2) Drug rewarding properties, 3) Traits manifested by the addict, and 4) Psychiatric co-morbidities (76). Understanding opiate drug tolerance and dependence is a very complex task with as yet many unanswered questions.

There are many contributions at the cellular or molecular level, which require further investigation. The cellular result of hMOR activation is generally understood, however, the complete understanding of neurotransmitter systems and intracellular signaling variations in acute and chronic opioid exposure are not as well known. The key is to understand which of the molecular level opioid actions contribute to the production of tolerance and dependence. These adaptations give rise to persistent changes in behavior in animals and humans exposed to long term opiate use (4).

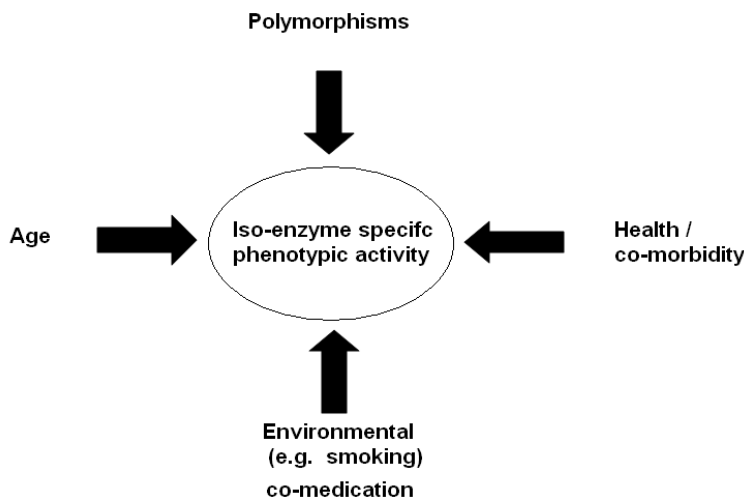


Figure 1.5 Determinants in drug metabolism [adapted with permission from (76)]. Genetic influences as well as health, environmental influence and age factors all play a role in methadone metabolism

1.4 Downstream signalling

G Protein mediated cell signaling is a widely used mechanism for transmembrane signal transduction. There are four major types of G proteins (77). Gi/o and Gs are primarily involved in a decrease or increase in adenylyl cyclase (AC) activity, respectively. In normal opioid initiated pain management, ligand binding initiates G protein coupling that provides an inhibition of AC activity, which in turn reduces the level of cyclic adenosine 5-monophosphate (cAMP) and regulates the activity of mitogen-activated protein kinase (MAPK) and Ca⁺ and K⁺ ion channels. Eventually this leads to decreasing excitability along the cell membranes of neurons in the pain pathways (49;77-79). The mechanism is very similar in methadone maintenance treatment (MMT) where methadone is used as a replacement drug for opiate-dependent patients. Methadone binds to μ -opioid receptors, similar to morphine or some of the other more potent opiates. As previously stated its major advantages are that it displays much less associated dependence than morphine and has a much longer half-life so that its effect is prolonged and does not produce as much of a euphoric affect as do the true opiates.

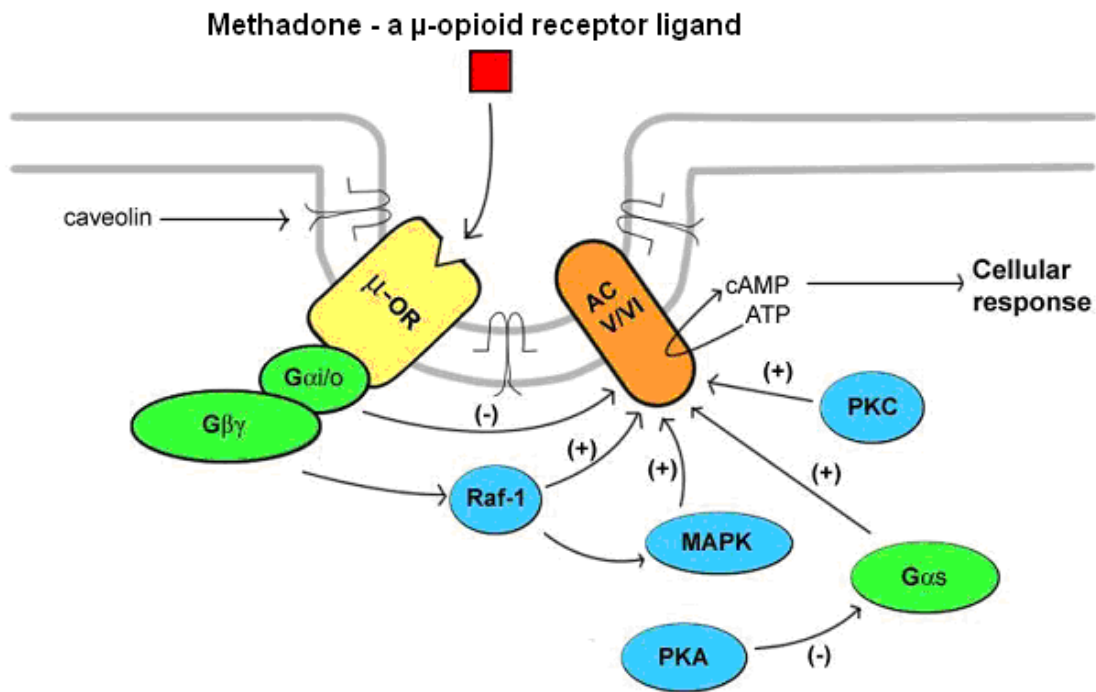


Figure 1.6 Methadone activation of $G\alpha_{i/o}$ is a negative effector of AC, which in turn reduces conversion of ATP to cAMP and lessens downstream signaling events to control withdrawal symptoms in MMT patients.

Adapted with permission from (80)

As discussed the AC family of enzymes are influenced by GPCRs such as hMOR and in turn convert ATP to cAMP and inorganic pyrophosphate. Negative feed back is provided by cAMP phosphodiesterase enzymes (PDE), which control levels via degradation (81). When cAMP is produced, it binds to protein kinases within the cell and initiates phosphorylation events, which regulate transcription factors as well as target enzymes.

(See Figure 1.7)

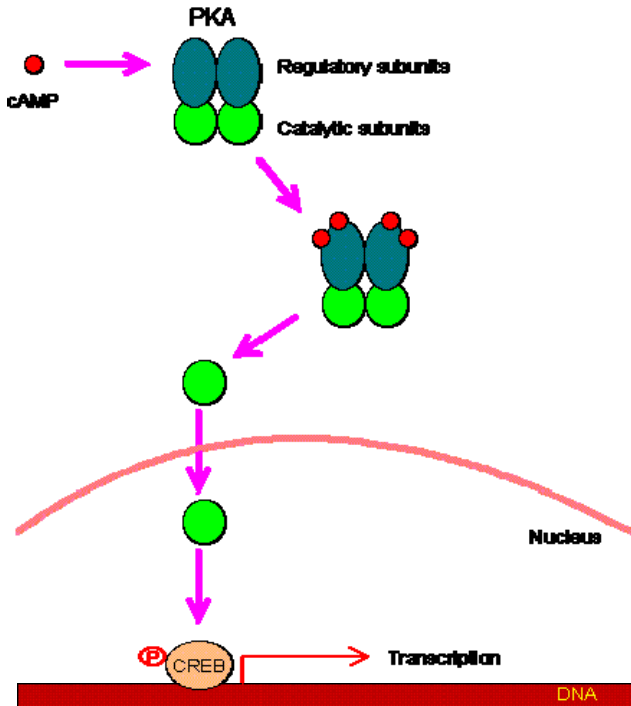


Figure 1.7 A diagram depicting what happens when cAMP is produced. It binds to protein kinases within the cell and initiates phosphorylation events, which regulate transcription factors (i.e. CREB) as well as target enzymes. Adapted with permission from (82)

The signaling pathway is more complex, with a variety of proteins, enzymes and other molecular effector compounds involved at all levels. Monitoring cAMP levels provides a useful method to follow the influence of GPCRs and the accumulation of this second messenger in intact cells (83).

1.4.1 cAMP has many functions

One of the key symptoms during opioid withdrawal is a state of sensitized pain. Although the cAMP system is known to have multiple effects on central neuron function, its mechanism mediating behavioural opioid dependence and withdrawal is not clearly understood. Morphine withdrawal, for instance, enhances the hyperpolarization-activated current in neurons by increased intracellular cAMP (83). In morphine-dependent rats *in vivo*, blocking the cAMP pathway significantly reduces withdrawal-induced pain sensitization (83). Chronic morphine use, with resultant tolerance and dependence, may induce adaptive changes in the regulation of transmitter release at synapses and with a compensatory increase in AC activity and cAMP concentration (84). These adaptations mediated through the AC cascade are not universal at opioid-sensitive synapses and most are observed during acute withdrawal. During morphine withdrawal, cAMP concentrations may actually “overshoot” pre-morphine use- levels (85) indicative of a cellular level of adaptation, by the receptor initiated signalling cascade.

1.4.2 cAMP as an important marker for signaling

A number of factors may alter cAMP levels including: 1) β -arrestin mediated receptor *internalization*, 2) *down-regulation* of opioid receptors, 3) Uncoupling of opioid receptors from G proteins (*desensitization*), 4) increased

production of nitric oxide via inducible nitric oxide synthase (iNOS), 5) other variations in signaling via G proteins (G- $\beta\gamma$ vs. G- α), and 6) variations in isoforms of various other cell signaling molecules involved in signal transduction.

By measuring cAMP levels in methadone patients, we immediately acquire information about the μ -opioid receptor-signaling cascade. This information together with knowledge of specific dosing requirements, receptor expression and possibly levels of G-glycoprotein expression may allow us to make conclusions about why dosing requirements are so variable. Understanding the variability in PK/PD factors affecting dose requirements provides a general knowledge that variability will exist (86). The fact that there are two major cohorts of MMT patients that require very low or very high dosing compared to the majority is perplexing. The study of these patients at the cellular level will shed light on whether neuro-adaptive changes are the cause of dosing variation.

1.5 P-Glycoprotein influence

P-Glycoprotein is an integral membrane protein that serves as an energy-dependent transport protein of diverse substrates (87). The importance of this protein in drug resistance has been well described (88) and it has been shown to be one of several transporters at the blood brain barrier. P-Glycoprotein 170 (P-GP) is a multi drug transport pump. It mediates the efflux of many therapeutic

reagents and has been implicated in the treatment failure of many infectious diseases, cancers and other medical conditions (27). P-GP is a 170 kDa trans-membrane glycoprotein, which includes 10-15 kDa of N-terminal glycosylation. The x-ray structure of P-GP reveals an internal cavity with a separation of the two nucleotide binding domains. Two additional P-GP structures with cyclic peptide inhibitors demonstrate distinct drug binding sites in the internal cavity capable of stereo-selectivity that is based on hydrophobic and aromatic interactions (89). Substrate and ATP binding are simultaneous, followed by ATP hydrolysis, which shifts the substrate into a position to be released and secreted from the cell, concurrent with release of the inorganic phosphate. ADP is released and a new molecule of ATP binds to the secondary ATP-binding site (90). Hydrolysis and release of ADP and a phosphate molecule resets the protein.

P-GP expression originally was thought to be within the brain, liver, pancreas, kidney, gut and adrenal gland. More sensitive methods soon revealed the presence of P-GP on lymphocytes (91). Successful attempts to measure P-GP expression in naïve volunteer blood cells were accomplished in the early 1990's, although the physiological role is still not completely understood (87). A conclusion of work done in 2002 was that the determination of levels of P-GP expression was a more reproducible and accurate approach to clinical investigation than determining the functional activity of this transporter on a specific substrate (27). Therefore, an assay to detect P-GP expression on white blood cells of methadone maintenance individuals should provide information about the role of multi-drug resistant proteins in methadone therapy.

Methadone is a substrate for the human P-Glycoprotein transporter (28) with very weak stereo-selective transport of the R and S enantiomers (28). This supports our previous work which suggested there is very little difference in enantiomeric form of methadone in relationship to metabolism and dosing requirements (92).

Although P-GP is thought to be involved in the transport of cytokines such as IL-2 and IFN- in peripheral blood lymphocytes, the function of P-GP on these cell populations is still largely unknown. Relating P-glycoprotein expression on white cells to overall effect in methadone treatment may provide at least some insight into the discordant dosing levels observed in high and low dose patients.

P-glycoprotein is often expressed at low levels and therefore its measurement has proven to be a considerable technical challenge. Multicenter studies have demonstrated lack of agreement between laboratories, with consensus opinion that flow cytometry is a promising way forward (93). Other studies used immunoassay methods with micro titer plates pre-coated with an antibody specific to P-GP.

Since methadone is a substrate for CYP3A4 it may be necessary to consider the combination effect of P-GP and CYP3A4. Since these entities are distributed extensively throughout the human body, but the main location for interaction is the intestine and liver, it may be important to consider the bioavailability of methadone with variable P-GP levels (94). The absorption of drugs from the intestine is an important factor in determining their bioavailability. P-GPs functions to pump out drugs from the enterocytes into the lumen –

decreasing their bioavailability. P-GP also performs this action at the blood brain barrier, thus reducing the delivered amount of drug to neuronal cells. Thus P-GP plays an important role in drug absorption and disposition (in the case of the blood brain barrier), acting as a biological barrier (94).

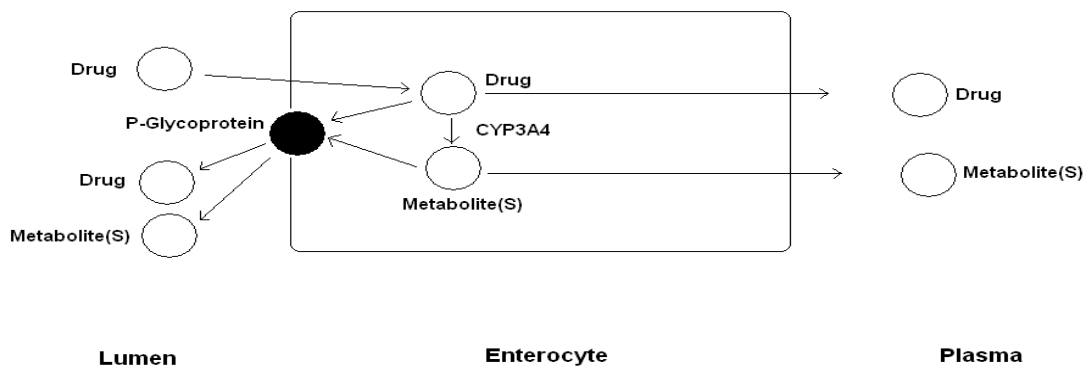


Figure 1.8 Simplified schematic representation of drug pumped out from intestinal cells into the lumen of the gut

Adapted with permission from (94)

1.6 Concurrent drug use

In one study from the UK no significant reductions in the prevalence of use of any of the main illicit drugs were observed during an examination of changing patterns of illicit and non-prescribed substance use among ongoing clients of a methadone maintenance treatment service (95). This theme is consistent in the literature and suggests that a significant factor in MMT success revolves around concurrent illicit and prescription drug use while on methadone treatment. Saskatchewan currently uses the “Harm Reduction” model of methadone treatment. The methadone assisted recovery program in Saskatchewan can be an important harm reduction strategy to prevent the transmission of HIV and other infectious blood-borne pathogens. Furthermore, this form of treatment has potential to assist those using opioids to reduce illicit use, needle sharing, and criminal activity associated with opioid use. As well, there is strong evidence to support improved outcomes when methadone treatment for opioid dependence includes addiction counseling (96).

1.6.1 Understanding drug tolerance, dependence and addiction – as both a physical and psychological condition

Drug addiction although considered a chronic relapsing neurobiological disease is also related to multiple pathways of severe psychological distress (97). Large-scale surveys indicate that addiction cannot be described as a chronic

relapsing condition for the general population; however, it is so for psychiatric patients. Mood and anxiety disorders have been associated with an increased prevalence of drug use disorders. The mechanisms underlying the association with mental disorders and harmful drug use are still unclear, however, they may relate to receptor activity. Some research has suggested that higher exposure to opportunity to obtain illicit drugs among individuals with pre-existing mental disorders may contribute to the co-existence of drug dependence and other mental disorders (98). There appear to be several motives for drug use including social stress and pressures. Most drug addicts use their primary substance of choice in response to unpleasant emotions, urges and temptations and social pressure (99). Vulnerability to opioid substance abuse and dependence is behaviorally complex. It is a function of biological, psychological and environmental interactions and influences (100). Opioid abuse, as well as drug abuse in general, is dependent upon social factors and behavioral disorders that often coexist with psychiatric illness as well as co-morbid medical conditions.

The use of pharmacotherapies such as methadone, buprenorphine or other drug-based treatments must be used in a coordinated medication assisted treatment programs in order to enhance public health issues. Integrating these medication based treatment programs with other social, medical and community services provides the best platform for promoting recovery from opioid dependence (100).

1.7 Peripheral white blood cells as a model?

The site of μ -opioid receptor action is most significantly on neuronal cells in the brain and central nervous system (CNS). The obvious disadvantage to this is that *in vivo* experimentation with human patients does not allow sampling spinal fluid or brain tissue. As early as 1988 research suggested that μ -opioid receptors were present on cells of the immune system (101;101), and more recently it was shown that μ -opioid receptors were present on human white blood cells subtypes such as lymphocytes, monocytes and granulocytes (102). Human μ opioid receptors (hMORs) are detectable on WBC using polyclonal antibodies and flow cytometry (103). Different studies (103), (104) have suggested measuring WBC levels of opioid receptors mimics the overall effect of certain pathological conditions on neuronal expression of hMOR and therefore can be used as a relevant assessment of receptor behavior in neuronal cells under these conditions. This provides a convenient, practical way to assess drug dosing and its effect on opioid receptor function in human patients. However, no unambiguous proof exists that the cellular behavior in white blood cells of hMOR is identical to that in neuronal cells. This data, however, with consideration of these factors, should provide suggestive evidence of the behavior of certain signaling events related to methadone dosing. This model provides a practical way to involve MMT patients in dosing studies and measurements of *in vivo* signaling under different dosing regimens.

Defining the preexisting level of P-GP expression and activity in peripheral lymphocytes of transplant candidates may be a prerequisite to understanding how P-GP function may be modulated by immunosuppressive therapy or alter the therapeutic response (105). Thus, other researchers have looked at the correlation of P-glycoprotein levels versus activity on peripheral blood cells. The level of P-GP expression on lymphocytes of transplant candidates did not always correlate with the level of P-GP activity observed (105).

1.7.1 Drawbacks of using human leucocytes

Although collection of peripheral blood is a relatively non-invasive process and provides minimal risk or discomfort to the patient, it does provide a sample, which is not in direct involvement of the receptor function being studied. The effect of opioids is primarily on neuronal cells. By extrapolating the data from blood cells to neuronal cells may not be without challenge. As well, the expression of hMOR on WBC is significantly lower than of neuronal cells so that measurement is a more difficult task (106).

Preliminary measurement of cAMP levels from WBCs of MMT patients revealed that this sample type provided relevant data about cAMP levels compared to methadone dose. This not only suggested a reliable model but was also suggestive of some kind of correlation between neuronal cell receptor function and levels of cAMP in blood cells. There is very little opportunity to experiment with human opioid receptors under *in vivo* conditions. The study of endogenous opioid receptors using *in vivo* models has produced some

interesting results that normally would not have been anticipated in vitro. Unfortunately, almost all of these studies are limited to rat, guinea pig or mouse (107). *In vivo* studies are essential to provide more relevant insight into the mechanisms underlying opioid receptor regulation. Therefore, the model of WBC receptor function mimicking neuronal cell function may hold great promise in providing suggestive knowledge of tolerance, dependence and appropriate methadone dosing in human patients.

1.8 Hypothesis & objectives

Using a white blood cell model of neuronal receptors to assess practically several factors in the peripheral blood of both “low” dose and “high” dose methadone treatment subjects it may be possible to detect specific differences. Understanding discrepancies may contribute information necessary to make a better prediction of expected dosing levels. The objectives of this study were to: 1) validate methods for the measurement of μ -opioid receptor expression and P-Glycoprotein expression on white blood cells using flow cytometry, 2) reliably measure cAMP levels as an indicator of down stream signalling in white blood cells of methadone treated patients, 3) develop and validate a reliable method using ultra performance liquid chromatography coupled to tandem mass spectrometry to easily identify and quantitate a comprehensive list of more than 40 drugs / drug metabolites in blood of drug dependent individuals.

These objectives are designed to provide information relevant to our hypotheses that: 1) Differences in dosing requirements may be due to differences in human μ -opioid receptor expression and/or P-glycoprotein expression in human subjects, 2) There may be differences between subjects in downstream signalling, which manifests as an attenuated cyclic AMP response and 3) Variations in concurrent drug use may influence methadone dosing requirements.

By comparing all of the acquired data, we hope to determine any significant relationships between these parameters and the necessity to maintain some methadone subjects on “low” and “high” methadone dosing. Our overall objectives are: 1) To gain an understanding of extreme methadone dosing requirements in methadone maintenance subjects and 2) To determine practically measured chemical/biochemical markers, which may be used to predict more accurately, individual methadone dosing requirements.

Chapter 2.

Methods and Analysis

2.1 Experimental

All experimental analytical work was performed at the Saskatchewan Disease Control Laboratory and the Pasqua Hospital in Regina, Saskatchewan, Canada for which I wish to acknowledge gratefully the foresight and understanding of management and staff at these facilities for placing value on educational enhancement and methodological research and validation. Ethics approval (Bio# 05-150) was obtained for this research on a yearly basis from the University of Saskatchewan Biomedical Research Ethics Board (Bio-REB)

2.1.1 Daily schedule

An overall approach was developed to collect and analyze samples appropriately. Several challenges arose when trying to acquire samples in a timely matter for analysis of viable samples, required for isolates and flow cytometry. Limitation in flow cytometer availability (1 h per day) was a significant factor. We created a theoretical schedule, which allowed us to collect from 2 – 3 samples /day and yet complete all necessary analysis before the white cells

became less viable. Early on in this investigation we performed cell viability analysis by flow cytometry to ensure our sample collection, dextran sedimentation process and cell labeling produced reliable information.

2.1.2 Blood collection and leukocyte isolation

Patients were selected at random based upon their methadone dose, their arrival at the clinic (no appointment format) and as well their willingness to participate. An attempt was made to collect from both genders equally and over as wide an age range as practically possible. The intent of the research was explained to patients prior to their signing a consent form and each was remunerated an amount of twenty dollars per sample.

Blood collection would take place early each day of analysis so that samples could be processed same day. Blood was collected by venipuncture with an attempt to acquire 5-8 mL of whole blood from each patient, in 2 X 5mL mL EDTA vacutainer tube. An aliquot of 200 μ L was used for DOA screening. From most of the remaining blood (approximately 4 mL), WBC (section 2.1.4) were then isolated and used for measurement of cAMP, receptor number and p-glycoprotein. Although a small amount of blood was retained whenever possible, it was found to be of limited value based on the strict time requirements and necessity to perform experiments on viable cells.

2.1.3 Preparation of Cells for further Laboratory Analysis

Since the methadone clinic was remotely located, samples were immediately transported using appropriate transport containers (as required by “Transportation of Dangerous Goods” regulations). In the laboratory, a 200 μ L aliquot of whole blood was refrigerated for LC-MS/MS analysis at a convenient time. The white cell isolation process was then begun in the laboratory, which took approximately 90 minutes to perform. Immediately following white cell isolation an aliquot of white cell suspension was prepared for cAMP analysis as described later. Once suspensions were ready, they were frozen at -20° C for analysis at a more convenient time. Simultaneously, a sample of white cells was aliquotted for analysis of receptor expression by flow cytometry. These samples were prepared (as described later) with tubes placed as much as possible in a chilled container. The flow cytometry analysis was done at another facility so suspensions were stored in a chilled container on ice since cell viability was essential. Once these preparations were ready, they were immediately transported following appropriate transportation regulations and analyzed.

A further aliquot of white cell suspension was stored at -20° C for analysis of P-glycoprotein levels at a more convenient time. Any unused white cells were then placed in storage at -20° C. Preparation of cells for analysis was accomplished within 3 hours of arriving back at the laboratory since the flow

cytometry experiments required travel to an off-site facility and had to be performed on fresh, viable cells.

2.1.4 WBC isolation

Leukocyte Separation from Blood Samples

Dextrose was purchased from Becton Dickinson (Sparks, MD, USA), while dextran, sodium chloride, citric acid and sodium citrate·2H₂O were all purchased from Sigma-Aldrich (Oakville, ON, Canada).

ACD stock reagent was prepared by dissolving 2.25 g anhydrous dextrose, 2.51 g sodium citrate 2H₂O and 0.73g anhydrous citric acid in 100 mL of 0.9% sodium chloride. A 6% dextran solution was prepared by dissolving 6 g dextran in 100 mL 0.9% NaCl. 0.9% NaCl was prepared by dissolving 9 g NaCl in 1000 mL of de-ionized water. 5% NaCl was prepared by dissolving 5 g NaCl in 100 mL water and 5% dextrose was prepared by dissolving 5 g dextrose in 100 mL 0.9% NaCl.

Sedimentation was performed using a dextran sedimentation method. We prepared sufficient volume of ACD/Dextran/Dextrose sedimentation solution as to be able to dilute blood sample with equal volume of solution. This combination reagent was prepared fresh each day and we used the following table to make the amount of solution required dependent upon how many samples we had.

Table 2.1 Volumes required to make working ACD/Dextran/Dextrose sedimentation solution

ACD	1.5	4.5	mL
Dextran	4.0	15.0	mL
Dextrose	3.5	10.5	mL
	10 mL	30 mL	

Approximately 4 mL of fresh whole blood was pipetted into a 10 mL graduated cylinder with the exact volume being recorded. It was important for high yield of white cells that all reagents be equilibrated at room temperature specifically since they were stored at 4° C. At room temperature, we added equal volume of ACD/Dextran/Dextrose sedimentation solution to blood sample in a graduated cylinder. The cylinders were mixed by gentle inversion and allowed to sediment for 30-45 minutes. Yield of WBC would begin to decrease if sedimentation was allowed to continue past 45 minutes. The volume of blood was recorded.

After sufficient time, the opaque supernatant was removed (plastic Pasteur pipettes) and equal amounts transferred equal to two 16 x 100 mm glass

round-bottom tubes for centrifugation (2000 rpm; 4 °C; 10 min), after which the supernatant was discarded. To the pellets in each tube we added NaCl (0.8 mL; 0.9%), and mixed well to suspend cells. After 90 seconds, we added 0.58 mL of 5% NaCl and mixed to restore isotonicity. Pellets were recovered by centrifugation at 2000 rpm for 5 minutes and supernatant was discarded.

This entire process from the point of adding 0.8 mL of 0.9% NaCl was repeated once more. If the pellets remained contaminated with red blood cells, we added 1.5 mL distilled water and mixed well. After 90 seconds, we added 0.36 of 5% NaCl to restore isotonicity. Pellets were then recovered by centrifugation at 2000 rpm for 5 minutes and supernatant was discarded. Pellets were then stored at -70° C or assayed immediately dependent upon the specific test requirements. Each pellet represented approximately 2 mL of whole blood collected (exact volume recorded)

2.2 cAMP measurement

For determination of cAMP levels in cell lysates, a commercial immunoassay kit from Assay Designs, a division of Enzo Life Sciences was used (Product # **ADI-901-163**). Immunoassay kits were purchased from MJS Biolynx Inc. Brockville, Ontario, Canada).

The cyclic AMP Complete Enzyme-Linked Immunosorbent Assay (ELISA) kit is a competitive immunoassay for the quantitative determination of cyclic AMP in cells and tissue treated with 0.1M HCl, in addition to culture supernatants, saliva, and serum. The optional acetylated assay format provides an approximate 10 fold increase in sensitivity and is ideal for samples with extremely low levels of cAMP. If expected levels of cAMP are unknown, the investigator may evaluate a few samples in the non-acetylated format in order to determine if higher sensitivity is required (108).

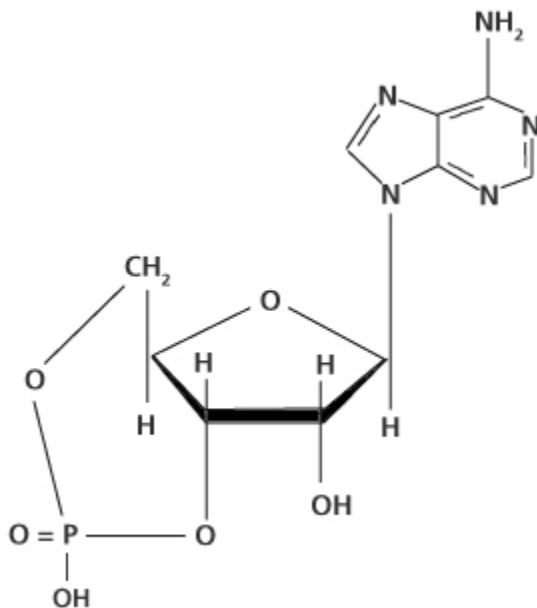


Figure 2.1 Structure of cAMP Molecule

2.2.1 Assay procedure

For each run, we generated a 96-well layout sheet and referred to it to determine the locations of unused wells. We removed wells that were not needed for the assay and returned them, with the desiccant, to the mylar bag. We stored unused wells at 4°C.

Note: If the acetylated format of the assay is to be run, all standards, samples, and the diluents for the NSB and Bo wells must be acetylated as per the instructions in the reagent preparation section. Preparation of standards must be performed within 30 minutes of use if the acetylated format is being run. We found that for our purposes we acquired sufficient sensitivity when measuring cAMP in cell lysates using the non-acetylated protocol.

Since we were using samples prepared in 0.1M HCl, it was necessary to pipet 50 µL of neutralizing reagent into each well except the Total Activity (TA) and Blank wells. To each well we then pipetted 100 µL of the appropriate standard diluent (Assay Buffer 2, 0.1M HCl, or non-conditioned culture media) into the NSB (non-specific binding) and Bo (0 pmol/mL standard) wells. Next, we added 50 µL of standard diluent to the NSB wells. 100 µL of Standards #1 through #5 were then pipetted into the bottom of the appropriate wells. This was followed by the addition of 100 µL of the samples to the bottom of the appropriate wells. 50 µL of the blue conjugate was pipetted into each well except the TA and Blank wells, followed by 50 µL of the yellow antibody into each well except the Blank, TA, and NSB wells.

Note: At this point we performed a quick check to ensure that every well used was a green in color except the NSB wells which should be blue. The Blank and TA wells were empty at this point and had no color.

The plates were then sealed and incubated on a plate shaker (~500 rpm) at room temperature for two hours. The plates were then manually washed by emptying the contents of the wells and washing with 400 μ L of wash buffer. This was repeated 2 more times for a total of 3 washes. After the final wash the plates were emptied by aspiration and firmly tapped on a lint free paper to remove any remaining wash buffer.

The final steps involved pipetting 5 μ L of blue conjugate into the TA wells, after which, 200 μ L of the substrate solution was pipetted into each well. The plates were then incubated for one hour at room temperature without shaking. Finally 50 μ L stop solution was pipetted into each well.

After blanking the plate reader against the substrate blank, we read optical densities at 405 nm. **Note:** If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

The procedure can be summarized as follows:

1) Standards and samples are added to wells coated with a GxR IgG antibody. A blue solution of cAMP conjugated to alkaline phosphatase is then added, followed by a yellow solution of rabbit polyclonal antibody to cAMP. 2) During a simultaneous incubation at room temperature the antibody binds, in a competitive manner, to the cAMP in the sample or conjugate. The plate is washed, leaving

only bound cAMP. 3) pNpp substrate solution is added. The substrate generates a yellow color when catalyzed by the alkaline phosphatase on the cAMP conjugate. 4) Stop solution is added. The yellow color is read at 405nm. The amount of signal is indirectly proportional to the amount of cAMP in the sample.

Measurement of cAMP levels from WBC isolates were performed after an incubation period with increasing concentrations of methadone. Maximal activation of cAMP production can be consistently achieved using forskolin (a known adenylyl cyclase activator) (109). Enhancement of adenylyl cyclase by forskolin was successful with a 30 minute incubation at room temperature. On three separate occasions, using three different whole blood samples, white cells where isolated using the dextran sedimentation method. Incubation of intact cells with 30 uL of 20 uM forskolin produced the following increase in cAMP concentration:

Approximately 10^6 cells/ mL of lysate

Concentration cAMP (pmol/mL)

	Pre incubation	Post incubation	increase
1.	1.2	11.1	X 9.25
2.	0.65	5.2	X 8
3.	0.69	7.0	X 10.1

2.2.2 Further assessment of white cell viability

To assess white cell viability and the cAMP second messenger signaling pathway, we treated white cell isolates with epinephrine and measured cAMP levels. 200 uL of cell isolates were incubated with 50 uL of 20 uM epinephrine. To test the viability of our experiments we utilized epinephrine and evaluated the β -adrenergic receptor pathway, by measuring cAMP levels.

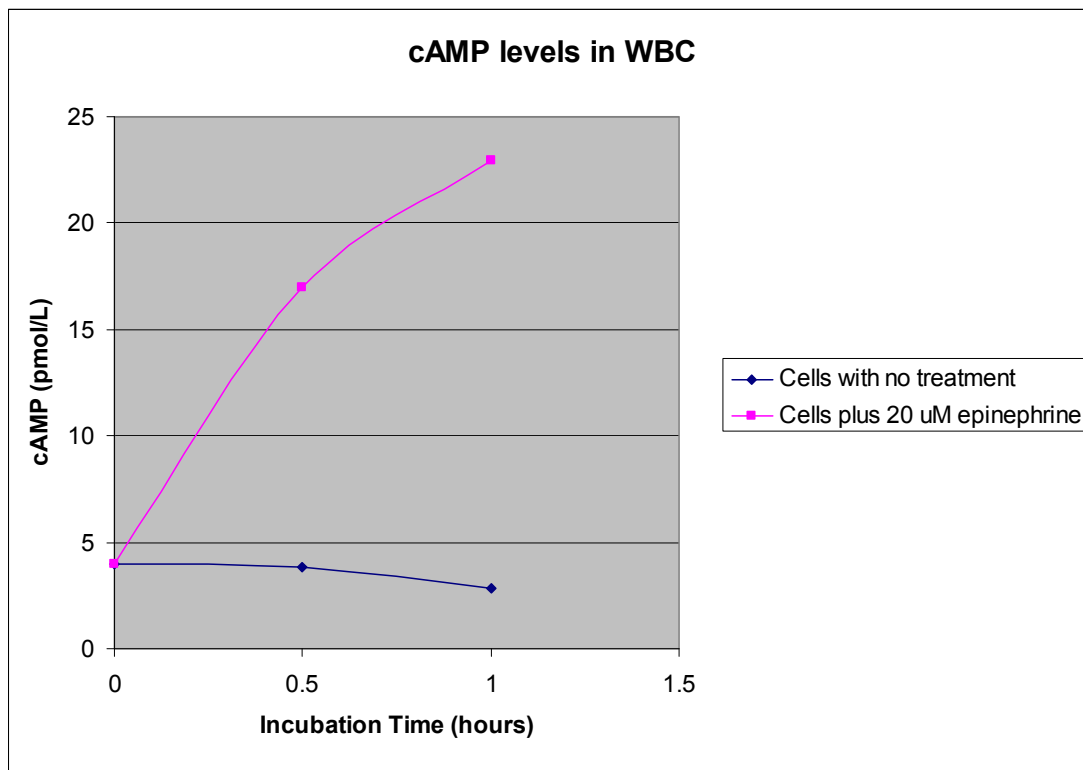


Figure 2.2 Plot of cAMP level as a function of incubation time with epinephrine

The resulting increases of cAMP levels in cells treated with epinephrine were suggestive that the white cell isolates were still viable. Along with flow cytometric evidence of the same, we concluded that the cells should be receptive to treatment with methadone for dose response curves and subsequent measurement of cAMP production. We also performed brief experiments to ascertain that frozen white cell samples were suitable for measurement of cAMP with no degradation.

Table 2.2 cAMP values from frozen cell lysates

Assessment of frozen White Cell Stability for cAMP Measurement

Storage at -20	2 hr post isolation	1 day post isolation	4 days post isolation
	cAMP pmol/mL		
	4.7	4.8	4.7
	5.2	5.5	5.2
	5.2	4.6	5.2
	5.4	4.4	5.4
	5.5	5.3	5.5
	5.2	4.9	5.2
	4.8	5.4	
	5.5		
	4.6		
	4.4		
Mean	5.05	5.0	5.2
SD	0.395	0.422	0.276
%CV	7.82	8.46	5.30

Thus, incubation with hMOR agonist must be performed on the same day as collection of blood samples; however, analysis of cAMP can be performed at a later date on frozen, lysed cell preparations.

When the activity of adenylyl cyclase becomes inhibited, less or no cAMP is being made. Since cAMP is constantly degraded by phosphodiesterase, the levels begin to fall. This should allow us to measure the specific dose-response of cAMP for each patient. Work was performed to optimize experimental parameters so that reliable dose-response curves could be acquired for each patient.

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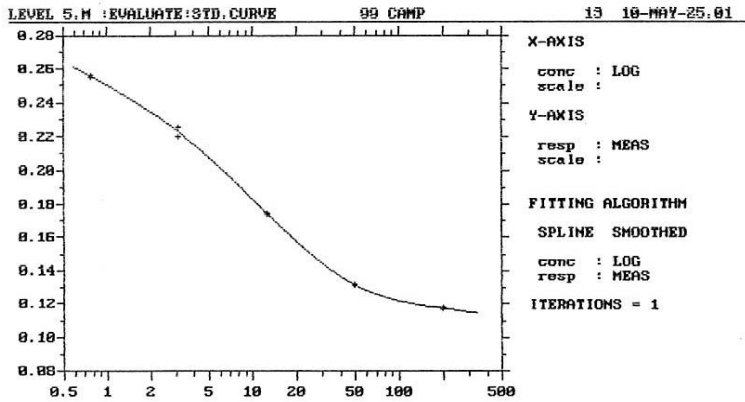
TOTAL = 0.263
BLANK = 0.104 ( 0 )

B/T = 0.40 ED-20 = 43.79 ED-50 = 10.77 ED-80 = 2.853

X-AXIS (CONCENTRATION) = LOG SLOPE = 0.0372
Y-AXIS (RESPONSE) = MEAS INTERCEPT = 0.533
FITTING ALGORITHM = SPLINE SMOOTHED VARIANCE RATIO = 0

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STD	CONC	CALC.CONC	%DIFF	RESPONSE	%cvRESP	%cvCONC
1.1	0.780	0.799	2.47	0.255		
1.2	0.780	0.761	-2.41	0.256		
AVG.	0.780	0.780	0.00	0.256	***** 0.27	3.45
2.1	3.120	3.449	10.5	0.220		
2.2	3.120	2.815	-9.78	0.226		
AVG.	3.120	3.120	0.00	0.223	***** 1.90	14.3
3.1	12.50	12.67	1.36	0.174		
3.2	12.50	12.33	-1.35	0.175		
AVG.	12.50	12.50	0.00	0.174	***** 0.41	1.92
4.1	50.00	48.80	-2.41	0.132		
4.2	50.00	51.23	2.47	0.131		
AVG.	50.00	50.00	0.00	0.132	***** 0.54	3.45
5.1	200.0	221.1	10.6	0.117		
5.2	200.0	179.5	-10.3	0.118		
AVG.	200.0	200.0	0.00	0.117	***** 0.60	14.7



99 CAMP

13 10-MAY-25.01

Figure 2.3 Typical cAMP Standard Curve. Standard curve plot of concentration vs. O.D for cAMP assay

2.3 Flow Cytometry*

*Isolate white cells as before (2.1.4)

Reagents and materials

32 μM [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin acetate salt (DAMGO), Forskolin and Epinephrine were purchased from Sigma-Aldrich Oakville, ON, Canada Naloxone –FITC was purchased from Invitrogen (Burlington, ON, Canada). PBS buffer (phosphate buffered saline) was acquired from the Media Preparation Facility at the Saskatchewan Disease Control Laboratory (Regina, Canada).

Reagent Prep

32 μM [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin acetate salt (DAMGO) MW: 513.59, was prepared by dissolving 1 mg of pure chemical in 5 mL PBS buffer. This solution was subsequently diluted 1/12 by addition of 5 mL DAMGO solution in 55 mL PBS buffer and stored in a screw top glass tube.

8 μM Naloxone Fluorescein in PBS buffer was prepared by weighing out approximately 2 mg in a screw top glass tube. The exact weight was recorded and 100 mL PBS buffer for every 2.0 mg was added to yield a 25.3 μM naloxone solution. A further 1/3 dilution was made to provide a Naloxone solution of 8 μM .

Method

One pellet of white blood cells acquired in the isolation process was suspended in 3 mL PBS buffer. Five 200 μ L aliquots of this homogenous suspension were pipetted into five separate 12 X 75 polystyrene flow cytometry tubes. Background (BG), total binding (TB) in duplicate, and specific binding (SB) in duplicate. To the BG and TB tubes, we added 30 μ L PBS buffer. To the SB tubes, we added 30 μ L 32 μ M DAMGO. The tubes were then vortexed thoroughly for 15 seconds. These tubes were incubated at room temperature in the dark for 30 minutes with a quick vortex approximately every ten minutes. These tubes were then Centrifuged at 2000 rpm for 3 minutes after which, the supernatant decanted. Next, the pellet was re-suspended in 2 ml of PBS buffers After 30 seconds of vigorous vortexing the tubes were centrifuged at 2000 rpm for 3 minutes and the supernatant was decanted off.

This wash step was repeated once more and then the pellet was reconstituted in 200 μ L of PBS buffer. We then added 30 μ L of Naloxone-FITC solution to all TB & SB tubes. Next we added 30 μ L of PBS buffer to BG tube. We then vortexed all tubes to mix well. These tubes were incubated at room temperature in the dark for 30 minutes with a quick vortex approximately every ten minutes. These tubes were then centrifuged at 2000 rpm for 3 minutes after which, the supernatant decanted. We repeated the above wash step three times. Finally we re-suspended in 0.5 mL PBS buffer for flow cytometry analysis.

Samples had to be kept cool on ice for the approximately 20 minute drives to the Pasqua Hospital for flow cytometric analysis. Scheduling with this facility was crucial so that there would be allotted time to perform our analysis. Sometimes clinical samples of medical emergency would arrive that would exclude us from the arranged schedule or prevent us from having any time for that particular day. Staff at the facility was very accommodating, but understandably, clinical samples from hospital patients were given priority.

Analysis

Earlier work with University of Regina honors student Adam Clay had resulted in a workable flow cytometry method for the detection and measurement of relative expression of the human μ -opioid receptor on the surface of leukocytes (110). Initial work revealed that high dose methadone maintenance patients had expression of receptors at a high enough level for analysis by this method. Further work in this study suggests that expression of μ -opioid receptors is adequate for measurement by this technique on chronically dosed subject, regardless of the dose. Naïve subjects, however, did not express levels that could be reliably measured using this technique. This data is displayed later in the thesis.

Isolated leukocytes, as described above, were introduced to the flow cytometer in appropriate dilutions, which provided approximately 1×10^6 cells/mL. The instrumentation was an automated FC 500 from Beckman Coulter, which utilized CXP version 2.2 controlling software. Optimized dot plot resolution was achieved on the first couple of experimental attempts and then specific method files were created with appropriate parameters for subsequent analysis. Voltages and gains were adjusted for forward and side scatter to isolate the 3 leukocyte populations of interest (monocytes, granulocytes and lymphocytes).

Cells were labeled with a fluorescently coupled opioid receptor ligand – naloxone. This naloxone-FITC (fluorescein isothiocyanate) was incubated with cells in buffer alone to determine total fluorescence. Some of the binding of this label would be contributed to non μ -opioid receptors. Cells not treated with naloxone-FITC, but all other components were used to determine background fluorescence levels. Specific μ -opioid receptor ligand DAMGO ([D-Ala², N-MePhe⁴, Gly-ol]-enkephalin) was added in excess to cell isolates and incubated for 20 minutes in the dark at room temperature to samples for specific binding fluorescence. DAMGO has preferential affinity over the naloxone-FITC for the μ -opioid receptor.

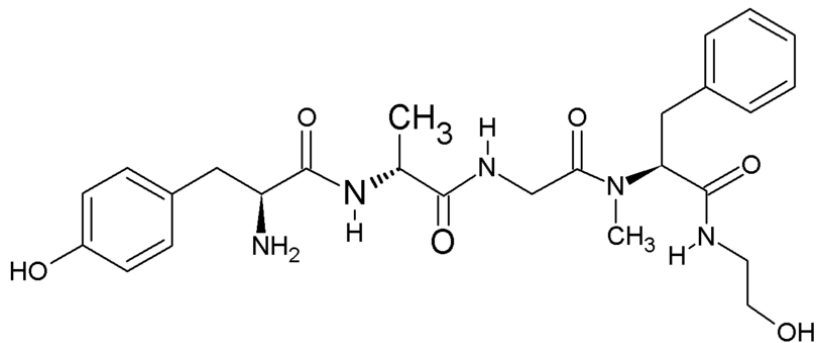


Figure 2.4 Chemical structure of DAMGO

Thus competitive binding resulted in only non-specific binding in these samples by the fluorescent label-compound.

Percent specific labeling was calculated by the following equation:

$$\frac{[(TF - BG) - (NSF - BG)]}{(TF - BG)} \times 100\%$$

Where: TF = Total Fluorescence

BG = Background fluorescence

NSF = Non-specific fluorescence

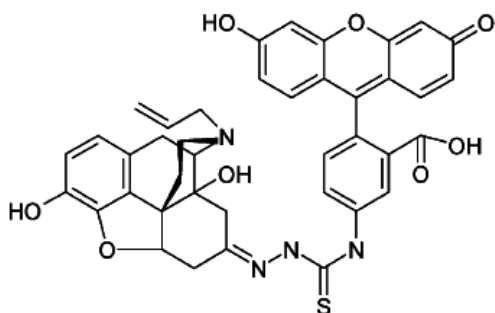


Figure 2.5 Chemical Structure of Naloxone-FITC. This was the fluorophore used in labelling hMOR for analysis by flow cytometry.

Using CD45, a leukocyte marker, we were able to create representative histograms of regions within the flow cytometer dot plot for the 3 sub populations. They were defined as granulocytes (red), monocytes (blue) and lymphocytes (yellow) based on forward and side scatter. Histograms showing regions of viable and non viable cells based on morphology gave the following data from our dextran sedimentation method.

Table 2.3 Table of viability of white blood cell types after isolation by dextran sedimentation (n =4)

Leukocyte Subpopulation	Dextran Sedimentation - Cell Viability by Flow Cytometry
Granulocytes	81 ± 3 %
Leukocytes	91 ± 2 %
Monocytes	95 ± 5 %
Average	90 ± 6 %

In the analysis of μ -opioid receptors all fluorophores were excited using a 20 mW argon laser, excitation wavelength = 488nm. Emission intensities were detected at 510 – 540 nm for FITC. Raw data was analyzed using the CXP cytometer software.

Negative Control (background fluorescence)

Institution: RQHR

Run Date: 12-May-11, 13:54:41

Protocol: Prov Neg Cont.PRO

Sample ID: Neg Control

Runtime Results

User ID: Pathology

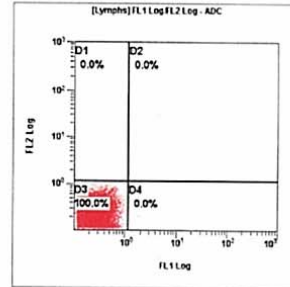
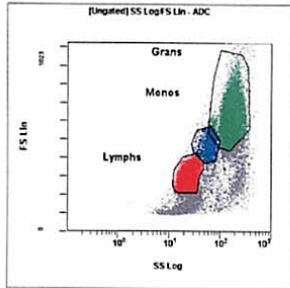
Analysis Date: 12-May-2011, 13:55:37

Acquisition Time/Events: 54.4s / 82048 (MANUAL)

Log Files: Prov Neg Cont.PRO, 05-May-2011, 14:39:53

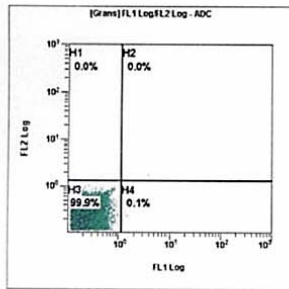
Tube ID: NoRead

Listmode File: Neg Control May 12 00005522 2011-05-12 1355 166.LMD

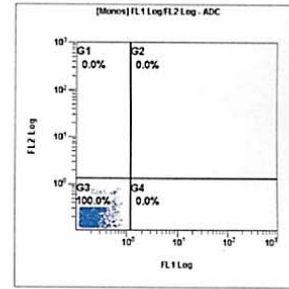


[Ungated] SS Log/FS Lin						
Region	Number	%Total	%Gated	X-Mean	X-Min	X-Ma
ALL	82048	100.00	100.00	105	###	###
Grans	35819	43.66	43.66	166	###	###
Lymphs	28302	34.49	34.49	23.3	###	###
Monos	5865	7.15	7.15	61.2	###	###

[Lymphs] FL1 Log/FL2 Log						
Region	Number	%Total	%Gated	X-Mean	X-Min	X-Ma
ALL	28302	34.49	100.00	0.282	###	###
D1	0	0.00	0.00	0	###	###
D2	0	0.00	0.00	0	###	###
D3	28301	34.49	100.00	0.282	###	###
D4	1	0.00	0.00	1.28	###	###



[Grans] FL1 Log/FL2 Log						
Region	Number	%Total	%Gated	X-Mean	X-Min	X-Ma
ALL	35819	43.66	100.00	0.255	###	###
G1	1	0.00	0.00	0.464	###	###
G2	2	0.00	0.01	10.1	###	###
G3	35785	43.61	99.91	0.253	###	###
G4	31	0.04	0.09	1.79	###	###



[Monos] FL1 Log/FL2 Log						
Region	Number	%Total	%Gated	X-Mean	X-Min	X-Ma
ALL	5865	7.15	100.00	0.246	###	###
G1	0	0.00	0.00	0	###	###
G2	0	0.00	0.00	0	###	###
G3	5865	7.15	100.00	0.246	###	###
G4	0	0.00	0.00	0	###	###

Figure 2.6 Flow cytometry data for a negative control showing background fluorescence

Total Fluorescence

Institution: RQHR

Protocol: Prov Nal-FITC.PRO

Runtime Results

Analysis Date: 12-May-2011, 13:56:46

Log Files: Prov Nal-FITC.PRO, 05-May-2011, 14:50:32

Listmode File: 25 S May 12 00005523 2011-05-12 1356 167.LMD

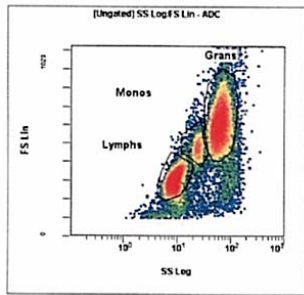
Run Date: 12-May-11, 13:56:04

Sample ID: 25 Total

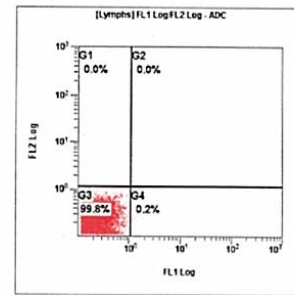
User ID: Pathology

Acquisition Time/Events: 40.8s / 44288 (MANUAL)

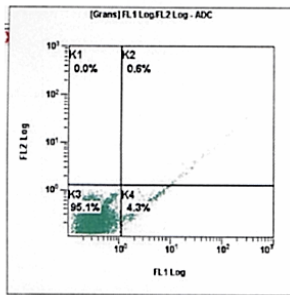
Tube ID: NoRead



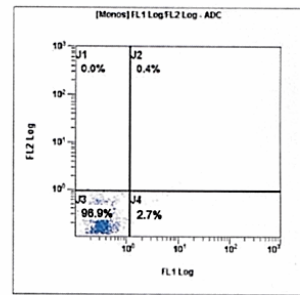
Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	44288	100.00	100.00	45.7	###	###
Lymphs	21930	49.52	49.52	67.3	###	###
Monos	14781	33.37	33.37	9.47	###	###
Grans	2540	5.74	5.74	25	###	###



Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	14781	33.37	100.00	0.261	###	###
G1	0	0.00	0.00	0	###	###
G2	0	0.00	0.00	0	###	###
G3	14750	33.30	99.79	0.258	###	###
G4	31	0.07	0.21	1.72	###	###



Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	21930	49.52	100.00	0.68	###	###
K1	0	0.00	0.00	0	###	###
K2	138	0.31	0.63	33.2	###	###
K3	20853	47.08	95.09	0.352	###	###
K4	939	2.12	4.28	3.2	###	###



Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	2540	5.74	100.00	0.408	###	###
J1	1	0.00	0.04	0.177	###	###
J2	9	0.02	0.35	11.8	###	###
J3	2461	5.62	97.95	0.34	###	###
J4	69	0.09	1.65	2.04	###	###

Figure 2.7 Flow cytometry data for an MMT subject showing total fluorescence (specific and non-specific labelling)

Specific fluorescent Labeling (non-DAMGO)

Institution: RQHR

Run Date: 12-May-11, 13:58:38

Protocol: Prov Nal-FITC.PRO

Sample ID: 25 Spec.

Runtime Results

User ID: Pathology

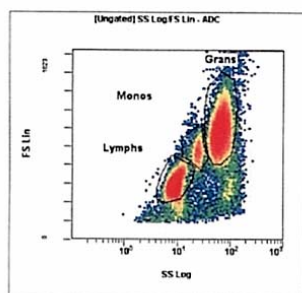
Analysis Date: 12-May-2011, 13:59:38

Acquisition Time/Events: 58.7s / 59008 (MANUAL)

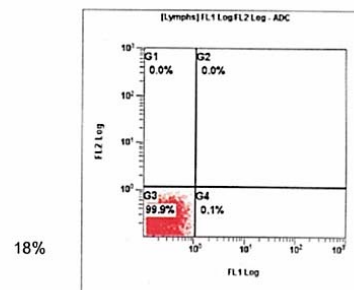
Log Files: Prov Nal-FITC.PRO, 12-May-2011, 13:58:10

Tube ID: NoRead

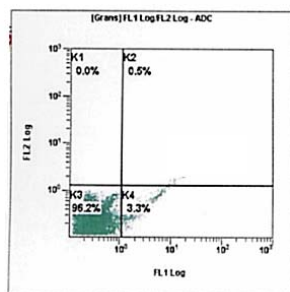
Listmode File: 25 T May 12 00005525 2011-05-12 1359 169.LMD



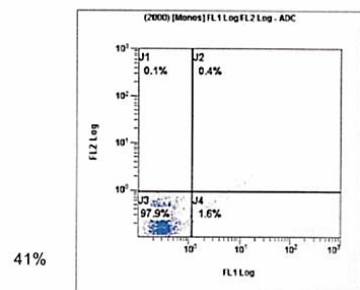
Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	59008	100.00	100.00	47.7	###	###
Grans	29166	49.43	49.43	69.3	###	###
Lymphs	19238	32.60	32.60	9.49	###	###
Monos	3212	5.44	5.44	25.1	###	###



Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	19238	32.60	100.00	0.263	###	###
G1	0	0.00	0.00	0	###	###
G2	0	0.00	0.00	0	###	###
G3	19216	32.57	99.89	0.261	###	###
G4	22	0.04	0.11	1.77	###	###



Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	29166	49.43	100.00	0.553	###	###
K1	0	0.00	0.00	0	###	###
K2	140	0.24	0.48	28.2	###	###
K3	28067	47.56	96.23	0.327	###	###
K4	959	1.63	3.29	3.13	###	###



Region	Number	%Total	%Gated	X-Mean	X-Min	X-Max
ALL	3212	5.44	100.00	0.388	###	###
J1	2	0.00	0.06	0.291	###	###
J2	14	0.02	0.44	8.68	###	###
J3	3146	5.33	97.95	0.317	###	###
J4	50	0.08	1.56	2.52	###	###

Figure 2.8 Flow cytometry data for an MMT subject showing non-specific fluorescence (non-DAMGO)

2.4 LC-MS/MS Analysis of Drugs and Metabolites in Blood Samples

(Published Procedure)(111)

(a)Abstract

Primary objective: To replace immunoassay screening for drugs of abuse (DOA) with a cost effective tandem mass spectrometry method.

Secondary objective: To substantially expand the drugs of abuse assay menu.

Design and methods: The requirement was to perform high throughput DOA screening for 200 urine specimens/day for 40 drugs/metabolites. The total analysis time had to be < five minutes. We used UPLC chromatography, small particle size LC columns and fast scanning tandem mass spectrometry. Urine samples were hydrolyzed enzymatically, diluted and injected with isotopically labeled internal standards. The data produced was transferred by exporting reports as text files to an LIMS system followed by auto certification of the results.

Results: 40 different drugs were separated by UPLCTM (ultra high-pressure liquid chromatography) with a run time of 5.2 minutes. Detection limits were below our cut-off values. Individual drug species instead of drug classes were identified; correlation with GC/MS was excellent. A high throughput, robust assay with acceptable accuracy, precision and specificity was developed. The procedure can also be used as a quantitative method with simple modifications.

Conclusions: An improved, high throughput, cost-effective method for drugs of abuse screening has been implemented. GC/MS confirmations were reduced or eliminated. The new procedure is a viable alternative to our previous immunoassay method. Acceptable turn around times, an expanded menu, simplified sample preparation and analytical reliability make this method a desirable option in the clinical laboratory setting.

(b) Introduction

Commercial immunoassay systems, which were originally developed in the 1950's, have evolved over time to provide extensive test menus in the area of drugs of abuse screening (112;112). Today's immunoassays provide forensic laboratories with qualitative screens for many different drugs. Previously NIDA (National Institute for Drugs of Abuse) and currently SAMHSA (The Substance Abuse and Mental Health Service Administration) set proposed cut-off concentrations for drugs of abuse levels in urine based on detection of drugs and/or families of drugs by immunoassay techniques (113).

Screening methods for drugs of abuse include immunoassays, which are calibrated at established cut-off levels. These cut-off values are not synonymous with assay detection limits, but are higher than the detection limit to ensure reliability. Immunoassays lack drug specificity, positive screens must therefore be confirmed by an alternate, more definitive confirmatory test (generally GC/MS) (114).

Substantial and variable cross-reactivity exist to each species within a class of drugs such as in a specific amphetamine/methamphetamine assay (115), where sometimes more than a ten-fold difference in concentration is required to create a “positive” result. In measuring the benzodiazepines (116) further complications arise because not only different parent species cross react differently, but the metabolites and conjugated forms of the drug display varying degrees of cross reactivity as well.

With the employment of tandem mass spectrometry, much more specific results can be obtained as to exactly which drug(s) is present (117). For the clinician this provides a much better understanding of drug usage. As well, as previously discussed, custom test “menus” could be developed to suit the needs of individual screening laboratories based on the demands of individual geographical or social regions.

“Rapid” liquid chromatography coupled with tandem mass spectrometry is a promising approach to replacing immunoassay techniques in the quest for more reliable, high through put drugs of abuse screening (118). Many forensic laboratories have incorporated liquid chromatography – tandem mass spectrometry in drug screening and identification (119-121), however, to date there is no comprehensive approach to replacing immunoassay systems which many drugs of abuse screening facilities currently use (122).

In the method described in this paper 40 drugs were analyzed using tandem mass spectrometry using labeled internal standards for every class or

family of drugs. Rapid chromatography and short run-times made it possible to analyze at least 200 urines in a 24- hour period(123).

(c) Materials and Methods

Chemicals and Reagents: HPLC grade acetonitrile and methanol, as well as reagent grade formic acid were obtained from Fisher Scientific (Ottawa, ON, Canada). Steam-distilled water was purchased from Arctic Glacier Inc. (Regina, SK, Canada). 96-well V-bottomed plates were purchased from Sarstedt Ind. (St. Leonard, QC, Canada), and the plates were sealed with common aluminum foil. All pipetting was done using adjustable Gilson and/or Rainin pipettes, Mandel Scientific (Guelph, ON, Canada). Certified drug-free urine as well as Urine Toxicology controls C3, C4, S2E, and S1 were purchased from Bio-Rad Laboratories (Montreal, QC, Canada). Beta-glucuronidase, type H-1; from Helix pomatia was purchased from Sigma-Aldrich (Oakville, On. Canada) All *Cerilliant* (Round Rock, Texas, USA) analytical drug reference standards both labeled and unlabeled were obtained from the Canadian supplier Diagnostix (Thermo Fisher), (Mississauga, On. Canada). - See Table 1. Ritalinic acid was purchased directly from the pharmaceutical company (Novartis, Basel, Switzerland).

Instrumentation: Chromatography (UPLC) was performed using a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, USA), which included a

micro-titer well-plate auto-sampler. Chromatographic separation was achieved using an Agilent Zorbax™ Eclipse XDB-C18 (Agilent, USA) rapid resolution column (4.6 x 50 mm, 1.8 μm) with a 0.2 μm stainless steel frit guard assembly (Waters Acquity UPLC™ part # 205000303). Mobile phase **A** was water with 0.1 % formic acid (FA) and mobile phase **B** was acetonitrile (ACN) with 0.1 % formic acid. Flow rate through the chromatographic system was 0.6 mL/min. A gradient method was used starting with a 90% aqueous composition at time 0, and incorporating a linear decrease to 10 % aqueous composition at time 4.0 min. We then used a steep ramp to 2 % aqueous at time 4.2 followed by a return to starting conditions at time 4.7 min. Injection volume was 15 uL with a strong solvent wash of 50:50; ACN: H2O with 0.1 % FA and a weak solvent wash of 5:95; ACN: H2O with 0.1 % FA. The seal wash was 5:95; ACN: H2O with no FA. MS/MS detection was performed on a Waters Premier XE triple quadrupole mass spectrometer (Waters Corp., MicroMass UK Limited). Selected Reaction Monitoring (SRM) analysis was done using the electrospray source in positive ion mode. (See Tables)

Chromatogram displaying 10 labeled internal standards from 10 SRM function windows, as well as 9 drugs/metabolites detected in a urine specimen from a drug dependant patient.

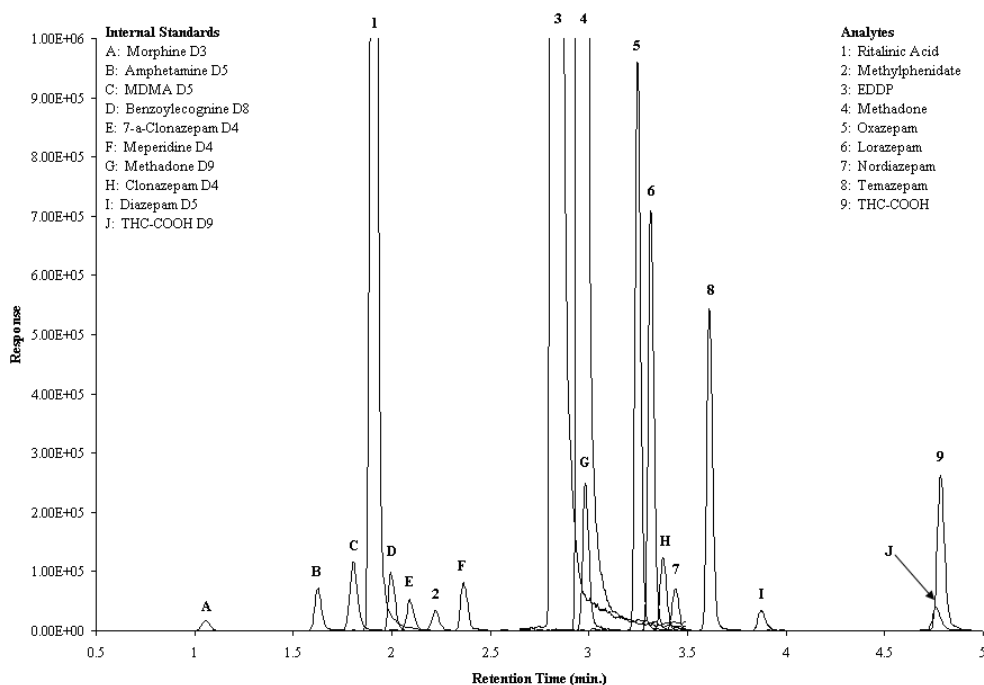


Figure 2.9 LC-MS/MS chromatogram displaying 10 labelled internal standards from 10 SRM function windows, as well as 9 drugs/metabolites detected in urine.

Standards and QC Material: Stock drug standards of either 1.0 mg/mL or 0.1 mg/mL were purchased as solutions in methanol. Further dilutions were made using methanol to obtain 1.0-ug/mL concentrations. All standards were stored at -20 degrees C. Labeled standards were acquired for each species, with the exception of ritalinic acid, which was not available commercially.

Combined intermediate standards of varying concentration were prepared and stored at -20 degrees. C. Fresh working standards were prepared weekly in certified drug free urine at 2 concentrations. The first standard was prepared to be at or near the cut-off value for each drug and the second to be at 2 times the

cut-off value. Limited 3- point calibration curves were deemed appropriate, as this is a screening procedure to provide evidence of drug levels above the established cut-off level. Values greater than the cut-off value are simply reported as positive for that particular species. Extrapolated values were never reported quantitatively. Each analytical batch required running a standard curve for each drug species using certified urine blank, a standard at the cut-off level for each drug in duplicate and a standard at 2 times the cut-off level. All standards were processed as samples with labeled internal analogs added. In cases of significant consequences or in medico-legal situations, subsequent quantitative analyses were performed using similar analytical techniques with alterations as listed:

1. A multi-point standard curve.
2. Utilization of a labeled standard for each drug/metabolite identified
3. Incorporation of at least one qualifier transition for each species
4. Acceptable relative abundance ratios between transitions (+/- 20%) (124)

Recovery: To assess recovery from matrix, blank urine was spiked with each drug at 2 levels (n = 10). The first level was at 80 % of our proposed cut-off values, the second level was twice that of our proposed cut-off. The spike levels were chosen to focus on the expected levels of interest near and above our anticipated cut-off points. (Table 3.)

Precision and Carry over: Carryover was assessed by injecting samples spiked with high levels of each drug (20 X cut-off), followed by 3 blank urines. For

instance, if the cut-off value is 500 ng/mL, as in the case of amphetamine, a urine blank was spiked at 10,000 ng/mL. Three blank samples were analysed immediately following this sample and assessed to ensure they exhibited a value of no more than 20% of the cut-off for this compound (100 ng/mL). Inter- and Intra-assay precision was established by spiking urines at 80 % of cut-off as well as 120 % of cut-off. For inter-assay precision, 10 samples of each level, for each drug were injected on 5 separate days and % CV's calculated. For intra-assay precision, 20 replicates of each level of spiked urine were run re-pipetted and analysed in the same manner.

(Table 3.)

Quality Control: For day-to-day quality control, an in-house QC material in urine was prepared at the cut-off level for each drug. As well, two levels of commercially available “drugs of abuse” QC material were analyzed, which contained 14 commonly found drugs of abuse. These controls also contain morphine-glucuronide, so that monitoring of our hydrolysis process is accomplished by calculating daily recoveries of free morphine. Quantitative values were plotted on a Levy-Jennings Chart. (Figure 3.)

Sample Preparation: In order to develop a high throughput method, a simplified preparation process was developed in-house to handle up to 250 specimens/day. A 500-uL aliquot of urine was placed in a small glass tube to which 50 uL of beta

glucuronidase solution was added ([121](#)). The tubes were then vortexed briefly and placed in a water bath at 65 deg. C. for 60 minutes. After removal from the water bath, 20 uL of supernatant was pipetted from each tube into a numbered location on a 96 well micro-titer plate. Standards and control were treated similarly so that the dilution factor from addition of the enzymatic solution was accounted for. Once all of the samples, controls and standards were added to the plate, 180 uL of water: methanol - 80:20 containing labeled internal standards was added. The plate was mixed for 30 seconds and analysed. When LSD testing was required, we performed a basic solvent extraction into chlorobutane, evaporated the solvent to dryness and reconstituted in mobile phase. This allowed reliable detection of less than 0.1 ng/mL LSD. Two external commercial control levels were carried through this same process. Matrix effects play a substantial role in quantitative analysis and must be considered for each analyte. Matrix effects are the alteration of ionization efficiencies due to the presence of co-eluting species ([125](#)).

For further validation, the effects of ion suppression were measured, by running serial dilutions of all standards in urine matrix and monitoring the response (normalized for dilution) versus dilution (see Figure 2.). Inter and intra-assay precision at different levels was determined. Accuracy was measured by comparing recoveries to external QC material. Both LOQ (limit of quantitation) and LOD (limit of detection) were established for all analytes as well as determining linear ranges for standard curves.

Table 2.4 Complete List of drug compounds including internal standards along with their mass spectrometry parameters.

Drug Name	R.T. (min)	Function	Mass Transition (Quant.)	Cone (V)	Collision Energy (V)	Dwell (sec)	Cut-Off (ng/mL)
Function 1: SRM of 3 mass pairs, Time 0.8 to 1.40							
Morphine	1.11	1	286.2 > 164.9	45	36	0.1	100
Hydromorphone	1.21	1	286.1 > 185.0	45	33	0.1	100
<i>Morphine D3</i>	1.1	1	289.2 > 164.9	45	36	0.1	NA
Function 2: SRM of 7 mass pairs, Time 1.40 to 1.90							
Amphetamine	1.68	2	136.0 > 118.9	12	10	0.01	500
<i>Amphetamine D5</i>	1.68	2	141.0 > 124.0	12	10	0.01	NA
Pseudoephedrine	1.5	2	166.2 > 148.0	12	10	0.02	500
MDA	1.74	2	180.0 > 163.0	18	15	0.01	500
Codeine	1.49	2	300.2 > 165.0	35	33	0.02	150
Oxycodone	1.65	2	316.3 > 241.2	30	25	0.01	100
6-MAM		2	328.2 > 211.0	45	30	0.01	25
Function 3: SRM of 7 mass pairs, Time 1.65 to 2.10							
Methamphetamine	1.8	3	150.1 > 119.0	15	12	0.01	500
MDMA	1.85	3	194.1 > 163.0	20	16	0.01	500
<i>MDMA D5</i>	1.84	3	199.1 > 165.1	20	16	0.01	NA
MDEA	2.01	3	208.1 > 163.1	25	12	0.01	500
Ritalinic Acid	1.98	3	220.5 > 84.0	25	22	0.01	500
Norfentanyl	2	3	233.0 > 84.3	25	18	0.01	50
Hydrocodone	1.75	3	300.3 > 199.1	45	30	0.01	100
Function 4: SRM of 3 mass pairs, Time 1.85 to 2.30							
Ketamine	1.99	4	238.0 > 179.0	40	20	0.01	100
BE	2.08	4	290.2 > 167.9	30	25	0.01	150
BE D8	2.08	4	298.2 > 171.0	30	25	0.01	NA
Function 5: SRM of 6 mass pairs, Time 1.85 to 2.30							
Methylphenidate	2.26	5	234.1 > 83.8	25	22	0.02	100
Normeperidine	2.37	5	234.2 > 160.0	30	22	0.02	200
7-amino-Clonazepam	2.16	5	286.2 > 222.1	40	25	0.02	100
<i>7-amino-Clonazepam D4</i>	2.16	5	290.1 > 226.1	40	25	0.02	NA
LSD	2.35	5	324.3 > 223.1			0.02	0.5*

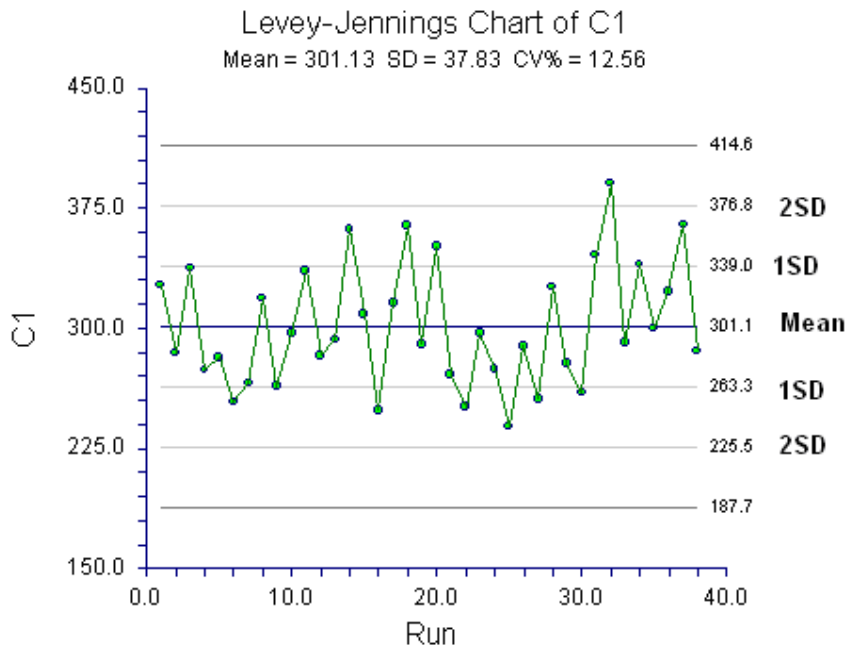
Cocaine	2.39	5	304.2 > 182.0	30	25	0.02	150
Function 6: SRM of 7 mass pairs, Time 2.20 to 2.90							
PCP	2.62	6	244.2 > 158.9	20	14	0.005	25
Meperidine	2.4	6	248.1 > 220.1	35	26	0.005	200
<i>Meperidine D4</i>	2.4	6	252.2 > 224.1	35	26	0.005	NA
Diphenhydramine	2.71	6	256.0 > 167.0	20	15	0.005	100
7-amino-Flunitrazepam	2.42	6	284.1 > 135.1	45	28	0.005	100
Fentanyl	2.67	6	337.1 > 187.8	40	26	0.005	25
Flurazepam	2.68	6	388.2 > 315.1	33	22	0.005	100
Function 7: SRM of 5 mass pairs, Time 2.75 to 3.50							
EDDP	2.88	7	278.2 > 234.1	40	25	0.008	100
Oxazepam	3.31	7	28.0 > 241.0	25	22	0.008	100
Methadone	3	7	310.3 > 265.1	25	20	0.008	100
<i>Methadone D9</i>	3	7	319.4 > 268.2	25	20	0.008	NA
a-Hydroxy-Alprazolam	3.12	7	325.1 > 297.1	50	28	0.008	100
Function 8: SRM of 5 mass pairs, Time 3.20 to 3.60							
Alprazolam	3.34	8	309.2 > 281.1	45	33	0.005	100
Clonazepam	3.44	8	316.2 > 270.1	40	25	0.005	100
Clonazepam D4	3.44	8	320.2 > 274.1	40	25	0.005	NA
Lorazepam	3.38	8	320.9 > 229.1	40	30	0.005	100
Triazolam	3.4	8	344.2 > 309.1	40	28	0.005	100
Function 9: SRM of 6 mass pairs, Time 3.3.5 to 4.50							
Nordiazepam	3.49	9	271.1 > 140.0	42	24	0.01	100
Diazepam	3.96	9	285.1 > 154.0	42	30	0.01	100
Des-alkyl-Flurazepam	3.56	9	289.1 > 139.9	42	28	0.01	100
<i>Diazepam D5</i>	3.96	9	290.1 > 154.0	42	30	0.01	NA
Temazepam	3.68	9	301.0 > 255.1	25	20	0.01	100
Flunitrazepam	3.64	9	314.0 > 268.1	40	30	0.01	100
Function 10: SRM of 2 mass pairs, Time 4.48 to 5.20							
THC-COOH	4.85	10	345.3 > 327.2	40	18	0.12	15
THC-COOH D9	4.85	10	354.3 > 308/.2	40	18	0.12	NA

* Extra sample preparation

LOQ was determined by using minimum criteria of < 20% CV (interassay precision) and a signal to noise ratio of > 10. LOD was determined using a minimum criterion of signal to noise ratio of > 5:1, determined by analysis of blank compound enriched with increasing amounts of pure drug standard. Linearity from zero to twice the cut-off value for each drug was determined by constructing calibration curves (n=5) for each drug at 1:3, 1:2, 2:3, 1:1, 3:2 and 2:1 of the cut-off values. The best fit for the calibration curves was determined by applying both linear and quadratic equations and trying different types of weighting. For screening purposes, 1 X blank urine, 2 X the cut-off standard and 1 X twice the cut-off standard were used to construct a 3-point calibration curve with extra weighting at the cut-off (crucial) value.

Examples of daily QC charts

Nordiazepam Level 1 June 2009



Nordiazepam Level 2 June 2009

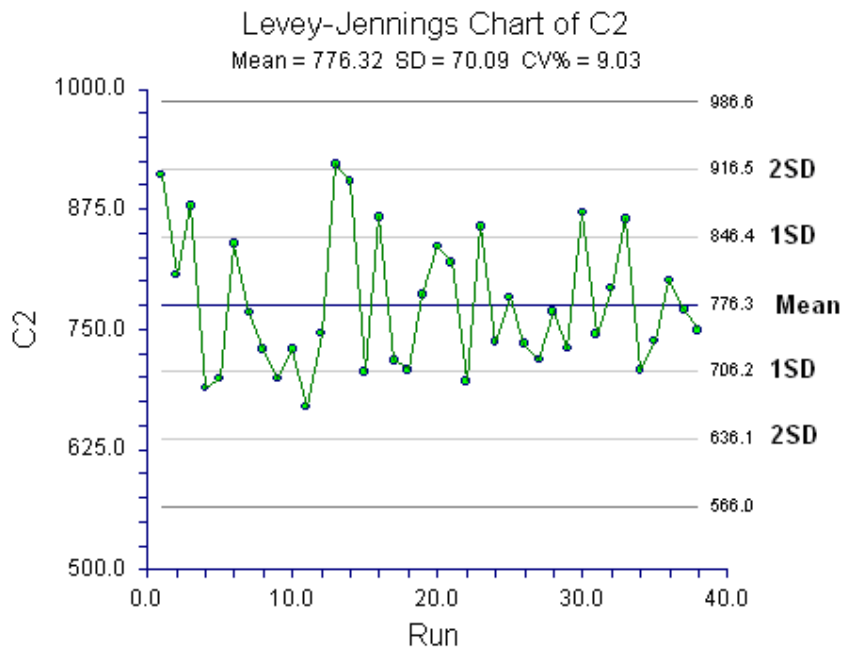


Figure 2.10 Example of Levi-Jennings QC charts plotted on a daily basis for each analytical run. Each drug is plotted at 2 different levels

With the introduction of automated 96-well extraction plates, high-throughput approaches to biological sample preparation are now possible (123). When using a urine matrix, ion suppression could be adequately controlled and monitored using a dilution approach that gave reliable results.

Table 2.5 LC-MS/MS data - correlation to other screening and confirmatory methods. Data based on n > 2000 urine samples from drug treatment clients screened

Positive for Amphetamines by CEDIA*	Confirmed Positive by GC/MS**	Screened Positive by LC/MS/MS***	Correlation	Identification
169	151 – species identified	151 – species identified	100% correlation+ (GC/MS to LC/MS/MS)	+Same species identified above the cut-off
	18 samples negative	18 samples negative	100% correlation +	+same samples negative
Positive for Opiates by CEDIA*	Confirmed Positive by GC/MS**	Screened Positive by LC/MS/MS***		
203	200 – species identified	201 – species identified	99.5% correlation + (GC/MS to LC/MS/MS)	+Same species identified above the cut-off
	3 samples negative	2 samples negative		
Positive for THC by CEDIA*	Confirmed Positive by GC/MS**	Screened Positive by LC/MS/MS***		
227	227	227	100% correlation + (GC/MS to	+ THC-COOH identified above 15

			LC/MS/MS)	ng/mL
Positive for Cocaine by CEDIA*	Confirmed Positive by GC/MS**	Screened Positive by LC/MS/MS***		
96	96	96	100% correlation + (GC/MS to LC/MS/MS)	+Positive for Cocaine and/or BE above 150 ng/mL

•*Greater than 1000 ng/mL (Amphetamines), 300 ng/mL (Opiates), 50 ng/mL (THC), 300 ng/mL cocaine

•** Greater than 500 ng/mL (Amphetamines), 150 ng/mL (Opiates), 15 ng/mL THC-COOH (THC) and 150 ng/mL cocaine or benzoylecgonine (cocaine) - Species identified

•*** Greater than 500 ng/mL(Amphetamines), 100 ng/mL (Opiates), 20 ng/mL THC-COOH, 150 ng/mL cocaine or benzoylecgonine (cocaine) – Species identified – 100% correlation (GC/MS)

Analytical Optimization: Injection of each compound was performed to optimize dwell times so that a minimum of 25 scans across each chromatographic peak were obtained. Since the entire run is comprised of 10 different experimental windows (different SRM functions – see Figure 1.), there exists slight overlap of SRM functions between individual experiments. Considering this overlap of SRM functions (potentially 2 X number of SRM scans), an effort was made to obtain a minimum of 20 points across each peak for any particular overlap period. This provided adequate sensitivity for all compounds at an acceptable LOQ. More points across the peak resulted in better integration and reproducibility; hence better analytical precision, especially at the lower end of quantitation. A method file was created to analyze the 40 drugs, which included all of the individually optimized parameters for each compound.

Analyzer and source parameters, which are not compound specific, were also developed (Table. 2.6)

Table 2.6 Waters Quattro Premiere XE Tune Parameters

Source (ES+)	Settings	Read backs (where applicable)
Capillary (kV)	3.3	3.24
Cone voltage	Compound Specific	Variable
Extractor (V)	5.0	5.01
Radio Frequency Lens (V)	0.5	
Source Temp. (Deg. C)	120	119
Desolvation Temp. (Deg.C)	400	398
Cone gas flow (L/Hr)	100	100
Desolvation Gas Flow (L/Hr)	800	798
Analyser		
Low Mass Q1 Resolution	15.0	
High Mass Q1 Resolution	15.0	
Ion Energy 1	0.8	
Entrance	-2	-27
Collision energy	Compound Specific	Variable
Exit	0.2	-29
Low Mass Q2 Resolution	15.0	
High Mass Q2 Resolution	15.0	
Ion Energy 2	0.8	
Multiplier (V)	665	-663
Collision cell Gas Flow (mL/m)	0.11	

Quantitation: Optimization of quantitation parameters were developed using Waters Quanlynx software. Quantitation parameters for each SRM transition were individually assigned based on peak width, retention time, and other general peak characteristics. Smoothing parameters were incorporated with a maximum number of smoothing iterations of two and in most cases the number of iterations was set at one, so that insignificant smoothing biases were created. Limit of quantitation (LOQ) for most compounds was set at 30 % of Cut-off value

or less. This allowed integration, quantitation and archiving of numeric data even when values were below the cut-off. Results were being reported as negative if below our established cut-off points (standard 1).

Table 2.7 Method validation parameters for each drug

*Calibration concentrations (ng/mL)

- A: 0, 0.5, 1.0, 2.0
- B: 0, 25, 50, 75
- C: 0, 150, 300, 450
- D: 0, 200, 400, 600
- E: 0, 500, 1000, 1500

Compound (LSD requires extra sample prep)	Cut-Off Value (ng/mL)	%CV Intra-assay Precision n = 15	%CV Inter-assay Precision (n = 6) X 5	S/N	Spiked Recovery in blank patient Urine (%) n=10	% Accuracy to Biorad Urine QC material n=10
7-amino-Clonazepam *(D)	100	12	14.7	29	97.18	
7-amino-Flunitrazepam *(D)	100	16.2	18.4	97	95.2	
a-Hydroxy-Alprazolam *(D)	100	8.6	13.9	176	97.3	91.8
Alprazolam *(D)	100	14.6	18.4	345	77.6	
Amphetamine *(E)	500	5.4	12.9	1804	88.8	95.2
BE *(B)	150	9	13.2	573	87.3	87.9
Clonazepam *(D)	100	16.4	17.6	545	82.8	
Cocaine *(C)	150	12.2	12.5	8832	102.2	
Codeine *(C)	100	9.3	13.4	165	95.3	82
Des-alkyl-Flurazepam *(D)	100	10.6	11.2	577	86.4	
Diazepam *(D)	100	6.9	9.7	475	95.7	
Diphenhydramine *(D)	100	13.4	13.8	6986	96.5	
EDDP*(D)	100	7	7.1	452	113	
Fentanyl *(B)	25	6.7	8.6	147	86.4	
Flunitrazepam *(D)	100	7.7	8.2	464	107.2	
Flurazepam *(D)	100	4.9	9.6	1488	99.7	
Hydrocodone *(C)	100	9.5	9.5	266	94.7	
Hydromorphone *(C)	100	6.1	9.4	1740	92	
Ketamine *(D)	100	8	10.5	379	101.5	
Lorazepam *(D)	100	17.7	19.6	261	80.1	
LSD *(A)	0.5*	11.1*		197*	83.8*	89.3*
MDA *(E)	500	4.1	8.0	1155	104.2	97.1

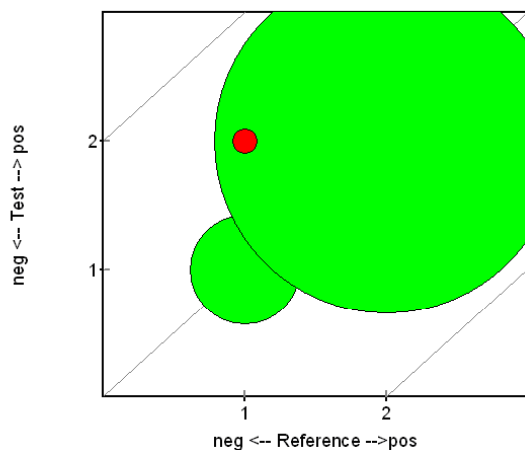
MDEA *(E)	500	7.6	8.8	192	83.4	96.7
MDMA *(E)	500	8.1	7.9	87	90.2	84.2
Meperidine *(E)	100	12.2	13.1	502	85.8	
Methadone *(C)	100	7.5	7.9	746	114.1	107
Methamphetamine *(E)	500	8.5	8.8	221	87	86.1
Methylphenidate *C	100	15.2	18.9	263	88.2	
Morphine *(C)	100	4.9	10.0	294	92.7	
Nordiazepam *(D)	100	10.3	10.3	733	95.8	113.6
Norfentanyl *(B)	25	9.8	15.1	289	107.6	
Normeperidine *(D)	100	14.8	15.8	131	80.1	
Oxazepam *(D)	100	8.2	11.3	236	83.1	
Oxycodone *(C)	100	9.3	13.0	104	105.3	
PCP *(B)	25	14.5	18.7	56	82.5	
Pseudoephedrine *(E)	500	5.6	10.3	595	101.2	
Ritalinic Acid *(E)	500	11.1	16.5	260	118.1	
Temazepam *(D)	100	8.6	8.2	315	97.1	
THC-COOH *(B)	20	15	18.8	19	117.2	115
Triazolam *(D)	100	17.8	18.0	203	113.1	

Method Validation Results (At cut-off values for each drug)

Qualitative Method Comparison

Ref. Method: GC MS

Test Method: LC MS MS



Statistical Analysis

(Comparison of Test Method to a Gold Standard)

Agreement	99.5% (97.5 to 99.9%)
Sensitivity	100.0% (98.1 to 100.0%)
Specificity	95.2% (77.3 to 99.2%)
95% confidence intervals calculated by the "Score" method.	
Positive Agreement	100.0%
Negative Agreement	95.2%
Prevalence	100.0%
Predictive Value Positive	100.0%
Predictive Value Negative	--
Prevalence input by the user.	

Statistical Summary

	Negative Reference	Positive Reference	Total
Negative Test	20	--	20
Positive Test	1	199	200
Total	21	199	220

Number excluded or missing: 1

Legend:

	Reference	Test
1	Negative (N)	Negative (N)
2	Positive (P)	Positive (P)

Figure 2.11 Qualitative analysis of “positive” opiate results, which are generated based on SAMSHA guidelines: Comparison to analysis by GC/MS

Laboratory Information System (LIS) Interface: In order to handle the significant amount of data generated for each urine specimen in a timely manner with error-free data transfer, it was necessary to incorporate an interface directly to our laboratory information data system. Our in-house IT staff accomplished this task

by developing a text file transfer using the “export to LIS” feature in the Mass Lynx software. This allowed for the transfer of three crucial bits of information: the sample identification number, the test name e.g. morphine, codeine etc., and the numeric result e.g. 500 ng/mL. Specific analytes were reported as - **Positive** (equal to or greater than the established cut-off value) or **Negative** (less than the established cut-off value). Only positive test results were reported along with a comment stating that all other results were negative. (Figure 2.)

Data Review: Post-run data review was performed every morning on QuanLynx software from data acquired the night before. Every individual integrated peak was reviewed to ensure reliable data had been generated. All internal standard peaks and response ratios were reviewed. While this may seem to be a labor-intensive task, it has become a routine part of the process and is normally accomplished in 1.5 – 2 hours each morning. Daily records are kept of column pressure, retention times and integrated areas of internal standards to assess method performance. Quality Control results of QC material were plotted daily on Levi-Jennings charts for each 96-well plate (Figure 3.). As soon as data files have been saved, they were up-loaded to our LIS system. If the LIS did not receive internal standard data it was assumed the sample has not been run. This prevented potential reporting of erroneous results where no data was acquired on a particular sample. We were able to process 200 – 225 specimens in this manner each morning. This process still allowed time each workday for instrument maintenance, other low-volume analyses and/or the performance of “priority” analyses, or Stat samples.

Final Drug Screen Report



Saskatchewan
Ministry of
Health

Saskatchewan Disease Control Laboratory

Toxicology Result

3211 Albert Street
Regina, Saskatchewan
S4S 5W6

Patient Name:		Accession #:	
PHN:		Requisition #:	
Date of Birth:		Collection date:	08 May 2009 15:00
Sex:	Female	Received date:	09 May 2009 12:12
		Reported date:	12 May 2009 12:01
DR.		Ordering Physician:	
		Date of Onset:	
	MEDICAL CLINIC	In / Out patient:	Out Patient
		Specimen Type:	Urine
REGINA, SK		Diagnosis:	
		Medication:	
		Hospital ID:	

Results

Test Name	Result
Drug screen (MS/MS) panel	
Drug Screen	Completed
Drugs of abuse screen completed. Sample negative for all drugs tested except those listed below.	
Methylphenidate	Positive
Ritalinic Acid	Positive
Methadone	Positive
EDDP	Positive
Oxazepam	Positive
Lorazepam	Positive
Nordiazepam	Positive
Temazepam	Positive
THC-COOH	Positive

Report Comments

Standard Urine Drug Screen Detects:

7-amino-clonazepam, 7-amino-flunitrazepam, alpha-hydroxy-alprazolam, alprazolam, amphetamine, benzoylecognine, clonazepam, cocaine, codeine, des-alkyl-flurazepam, diazepam, diphenhydramine, EDDP, fentanyl, flunitrazepam, flurazepam, hydrocodone, hydromorphone, ketamine, lorazepam, MDA, MDEA, MDMA, meperidine, methadone, methamphetamine, methylphenidate, morphine, nordiazepam, norfentanyl, normeperidine, oxazepam, oxyodone, PCP, pseudoephedrine, ritalinic acid, temazepam, THC-COOH (cannabinoids), triazolam.

Figure 2.11 Drug screen (Toxicology) report based on data transferred from instrument to LIS and converted from numeric to qualitative information based on cut-off values

(d) Results and Discussion

Thousands of clinical urine specimens from drug treatment centers have been analyzed using the above described method. Results indicated that this is a reliable, high throughput method, which may replace traditional immuno-assay techniques in drug screening laboratories. Comparison to confirmatory methods (namely GC/MS) revealed a very high level of correlation. (Table 2.) Specificity was markedly better than immunoassay. Even when only one SRM transition is used along with a chromatographic retention time, the tandem mass spectrometry method compares very well with GC/MS. Precision at or above cut-off levels was acceptable when spikes and recovery experiments were done. Analysis of external QC material and comparisons to GC/MS suggest very acceptable accuracy. (Table. 3)

All analytes produced relatively strong signals (S/N) at the cut-off levels, yet some analytes yielded significantly higher CV's than others. This may be in part due to compound specific ionisation variability in the source. This is worthy of further investigation but was not addressed in this work. Imprecision of these compounds may also be improved by using a labeled internal standard for every compound, as is our practice in confirmatory testing. For screening purposes, this is less practical, since it begins to reduce SRM sensitivity by introducing many more transitions.

Chromatographic robustness was verified by confirming that the percent coefficient of variation for retention times from column to column was consistently around 1%. This was true when internal standard retention times were analyzed even across several column changes as well as daily mobile- phase changes over a four-month period. (Table 5.)

An Agilent column (as discussed in *Methods and Materials*) was found to be more robust and develop much less backpressure. Pressures rarely exceed 5,000 PSI even after a couple of weeks of use. Initial backpressure with a brand new Agilent column is approximately 2000 PSI with increase over time to 5000 PSI, before any decrease in chromatographic resolution is noticeable. The Waters UPLC columns developed much higher backpressure and only lasted about 1- 2 weeks under strenuous use. The Agilent columns last on average 3 – 4 weeks under similar conditions.

The simplified sample preparation process allowed high sample throughput, and was compatible with routine and safe laboratory practice. No toxic extraction processes or sample clean up were required. Isotopically labeled internal standards were utilized, which are not present in urine samples of drug users. Specimens were enzymatically hydrolyzed since significant levels of several drug species such as morphine, oxazepam and lorazepam exist in urine as conjugates (126). Overall analysis time after hydrolysis was 5 minutes/sample. This achieved the goals of relatively high sample throughput. The fact that no chemical derivatizations were required eliminated the concerns relating to

variable derivatization efficiencies, as well as the time and labor associated with such tasks.

Ion suppression is a common phenomenon in tandem mass spectrometry and must be evaluated and minimized for each drug of interest (127). This required some effort, since laborious, time-consuming sample preparation is not a viable option if high throughput and cost considerations are to be met. To remove or minimize matrix effects, modifications to the sample extraction methodology and/or improved chromatographic separation were required (125). Whether by solid phase extraction (SPE), liquid-liquid extraction, dilution and precipitation or fast flow, on line extraction with or without column switching, all analytes needed to be monitored for extraction efficiency by spikes and recoveries (128). The post column infusion method as defined by Bofiglio et al. was used. This involves the infusion of a constant amount of each analyte separately while injecting urine blanks. Regions of signal reduction indicate ion suppression. While this method is not truly quantitative, areas of ion suppression and their magnitude can be detected [108;109].

In our case simple dilution of urinary supernatant did not eliminate matrix effects, so careful consideration was given to this phenomenon. Drug standards were introduced to the source through a tee junction along with mobile phase. Urine blanks were diluted exactly as was done in our sample preparation process.

Areas of signal decrease were monitored to detect major suppression effects for any of the drugs. The possibility exists that other drugs, which are not included in this panel, may interfere with this method. The use of two transitions, the

monitoring of ion abundance ratios and the use of chromatographic retention times are accepted criteria for quantitative tandem mass spectrometry, and provides much higher specificity and selectivity than immunoassays. As indicated in Table 2., even as a screening procedure, this method shows high correlation to GC/MS confirmation.

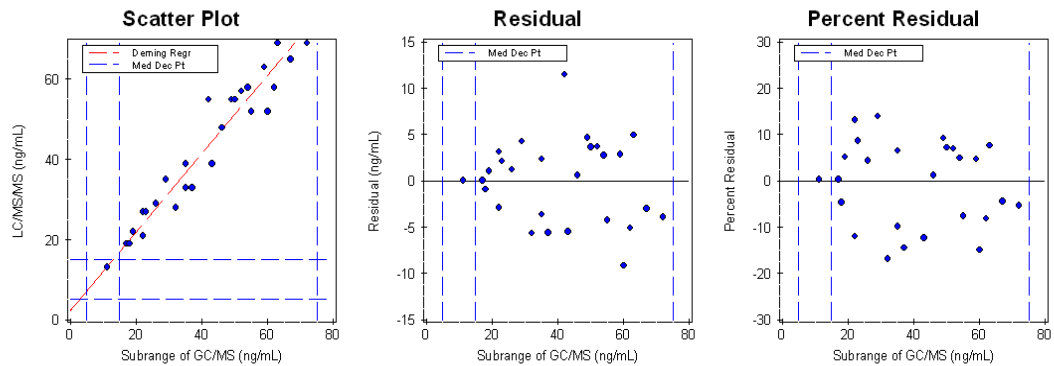
Confirmatory quantitative testing using more rigorous analytical criteria is easily accommodated for individual samples by the addition of multi-point standard curves, isotopically labeled internal standard for each compound, the use of a second qualifier ion and relative abundance ratio limits (119;124). Separate MS methods have been set up for this purpose, with a limited number of specific drugs in each method (e.g. opiates, benzodiazepines, etc.). This allows adequate sensitivity even with additional labeled internal standards and qualifier mass transitions.

Quantitative comparison to GC/MS

Alternate (Quantitative) Method Comparison

X Method: GC/MS

Y Method: LC/MS/MS



Regression Analysis

	Deming	Regular
Slope:	0.981 (0.875 to 1.087)	0.948 (0.842 to 1.053)
Intercept:	2.27 (-2.42 to 6.97)	3.62 (-1.03 to 8.28)
Std Err Est:	4.60	4.56

95% Confidence Intervals are shown in parentheses

Medical Decision Point Analysis

Calculated by Deming Regression (Disparate Scales)

X Method MDP	Y Method Pred. MDP	95% Conf. Limits	
		Low	High
5	7.2	3.1	11.3
15	17.0	13.8	20.2
75	75.8	71.9	79.8

Supporting Statistics

Corr Coef (R): 0.9655 SubRange Bounds: 5.0 to 75.3 Scatter Plot Bounds: None
 X Mean ± SD: 40.74 ± 17.51 Points (Plotted/Total): 27/27
 Y Mean ± SD: 42.23 ± 17.19 Outliers: None

Figure 2.13 Quantitative comparison of LC-MS/MS data to GC/MS data for analysis of THC-COOH in urine specimens

Financial considerations: Previous to the development of a tandem mass spectrometry methodology for “drugs of abuse” screening, the annual budget was \$250,000 to \$300,000 on immunoassay reagents alone. This cost did not include staffing, overhead or the added cost of confirmation by GC/MS. Our current cost of providing immuno-assay testing for barbiturates and ethanol (or possibly ethyl glucuronide) is approximately \$25,000 / year. Annual costs to run the UPLC-MS/MS system are approximately \$60,000 to \$70,000. This includes reagents, control material, standards, service contract, chromatographic columns and other expendables as well as the beta-glucuronidase required for sample hydrolysis.

This produced a yearly savings of between \$160,000 and \$210,000 and allowed cost recovery of the instrumentation within a 2- 3-years as well as providing improved testing capabilities.

In summary, our method validation indicated that this is a highly specific, reliable and robust analytical technique in the challenging endeavor of detecting drug use for clinical reasons and may well meet or exceed the requirements for reliability and accuracy even for medical-legal purposes.

2.5 Measurement of total P-glycoprotein (P-GP) levels in white cell isolates

P-GP was measured using a commercial immunoassay kit (**Catalog No. CSB-E11709h**) purchased from CUSABIO BIOTECH CO., Ltd. (Hubei Province, P.R.China). This immunoassay kit allows for the in vitro quantitative determination of human P-GP concentrations in serum, plasma and other biological fluids.

Principle of the Assay

The microtiter plate provided in this kit has been pre-coated with an antibody specific to P-GP. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for P-GP and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3',5, 5' tetramethylbenzidine) substrate solution is added to each well. Only those wells that contain P-GP, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm \pm 2 nm. The concentration of P-GP in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Detection Range

1.56 ng/ml-100 ng/ml.

The standard curve concentrations used for the analyses were 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.12 ng/ml, 1.56 ng/ml.

Specificity

This assay recognizes recombinant and natural human P-GP. No significant cross-reactivity or interference was observed.

Sensitivity

The minimum detectable level of human P-GP was described as typically less than 0.39 ng/mL. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.

Assay Procedure

Note: It was important to bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

We added 100µl of Standard, Blank, or Sample to each well. We then covered the plate with a tight seal cover (adhesive strips). The plate was allowed to incubate for 2 hours at 37° C. After incubation, we removed the liquid from each well, without a wash step. Next we added 100µl of Biotin-antibody working solution to each well and incubated for another 1 hour at 37°C.

Note: Biotin-antibody working solution may appear cloudy. For this reason we warmed to room temperature and mixed gently until solution appeared uniform.

The next step was to aspirate each well and wash, repeating the process three times for a total of three washes. We washed by filling each well with approximately 350µl of wash buffer using a squirt bottle, multi-channel pipette, manifold dispenser or auto-washer. Complete removal of liquid at each step is essential to good performance. After the last wash, we remove any remaining wash buffer by aspirating or decanting and then inverted the plate and blotted it against clean paper towels. Next we added 100µl of HRP-avidin working solution to each well and after covering the microtiter plate with a new adhesive strip, we incubated for 1 hour at 37° C. Immediately following this incubation we repeated the aspiration and wash step three times and then at this point we added 90µl of TMB Substrate to each well. The plate was then incubated in the dark for 30 minutes at 37 °C - keeping the plate away from drafts and other temperature fluctuations. Finally, we added 50µl of Stop Solution to each well. To ensure the color change appeared uniform, we gently tapped the plate to facilitate thorough mixing. We determined the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

Calculation of Results

We averaged the duplicate readings for each standard, control, and sample and subtracted the average zero standard optical density. We then created a standard curve by reducing the data using computer software capable

of generating a four parameter logistic (4-PL) curve-fit. As an alternative, a standard curve could be constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best fit curve through the points on the graph. The data may be linearized by plotting the log of the P-GP concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

All analyses of optical densities were performed on Wallac Victor-2 automated plate reader (Perkin Elmer Life Sciences, Turku, Finland) using Muti-Calc software. Standards, samples and controls were all run in duplicate.

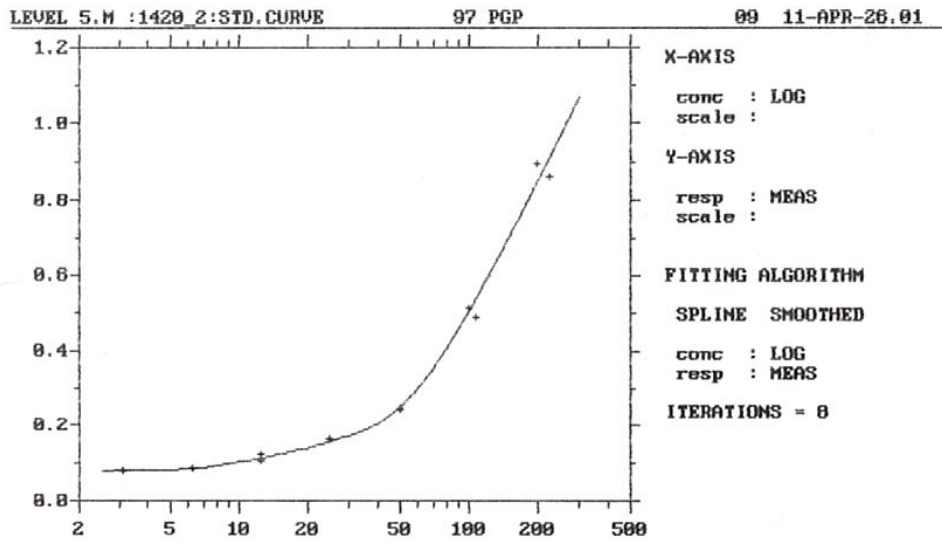


Figure 2.14 P-GP Standard Curve GP based on a plot of concentration vs. O.D.

Chapter 3

Results

3.1 Methadone maintenance subjects

Early on in this research, consultation was undertaken with Dr. W. Wildenboer, an addictions clinician here in Regina. Discussion with Dr. Wildenboer provided insight into the treatment process for her patients. She agreed to allow access to her clinic for collection of samples for this study, since she understands fully the challenges of methadone dosing. A copy of a letter of agreement with Dr. Wildenboer is attached in the appendix as appendix 1.0. The format for patients to receive counselling and treatment is a non-scheduled come and go arrangement. This made it difficult for us to predict if and when subjects would be available for specimen collection. The process, however, did allow for a random sampling of subjects based on who showed up on any particular day. This may have actually removed some of the possible sampling bias by us or by the clinician. The only requirements were that the subjects had been on chronic dosing for at least 6 months and they revealed age, gender and dose to us for the study. All other information was kept confidential. Originally, we were going to request a sample of urine for drug detection, however, modifications to the developed drug screening technique allowed this testing to be performed on whole blood.

3.2 Methadone maintenance subject information:

A total of 38 samples of blood were successfully obtained from individual subjects who agreed to provide blood samples for this study. An attempt was made to obtain roughly the same amount of samples from both genders, with the final number being 19 female and 19 male participants. The age range was from 20 to 66 years old. The mean age was 43 years and the median age was 42 years. A strict requirement was that all subjects were on chronic dosing for at least the past 6 months.

The dosing range was from 0.5 mg/day to 220 mg/day. The mean daily dose was 81 mg and the median daily dose was 50 mg. By our categorization of “**Low**” dose as less than 50 mg/day – we had 16 subjects in this group. By our categorization of “**High**” dose as ≥ 90 mg/day – we had 16 subjects in this group. By our above categorizations we had 6 subjects on “**Normal**” dosing

Although a few samples were collected earlier on to verify process and methods, the collection and analysis of the blood samples used for this research was performed between July, 2010 and December, 2011. Some frozen samples were re-analyzed in early 2012.

Blood samples were collected in standard K₂ EDTA Becton Dickinson Vacutainers©, with appropriate expiry dates. Samples were drawn with 21 gauge standard needles, 21 gauge butterfly needles or 23 gauge butterfly needles dependent upon how difficult the collection was based on each individual subject.

Many of the subjects had very poor quality veins from a history of intravenous drug use, so collection was frequently very challenging. All blood collections were performed by a trained phlebotomist (certified Medical Laboratory Technologist), with as little discomfort to the subject as possible.

Naïve subjects (n = 6) were volunteers from the Saskatchewan Disease Control Laboratory. All of their information remains confidential, however, they did all testify to being non-drug users. (2 males, 4 females)

3.3 Measurement of receptor numbers on leukocytes

A total of 38 blood samples were collected from different methadone maintenance patients. The first 6 samples were used in the process of method development and verification of analytical capabilities. Some measurements of receptor numbers were unsuccessful for both known and unknown reasons. Since cell viability was crucial a second attempt or repeat analysis of any particular sample was impossible. Thus, the number of samples successfully measured varied slightly for all of the analytical measurements. The number of samples for any particular data set is identified.

Analytical Data for μ -Opioid Receptor Number Data on White Blood Cells

Expression of μ -opioid receptors was measured by flow cytometry on leucocytes as described previously. Cells had to be analyzed within a few hours of collection to ensure their viability. Percent specific labelling relates directly to the number of receptors expressed. Percent specific labeling was calculated using the previously described equation.

Table 3.1 Receptor expression data. White blood cells were isolated from each subject's blood and analyzed by flow cytometry for expression of μ -opioid receptors. The level of expression correlates directly to the percent specific labelling.

Subject Number	Dose (mg/day)	hMOR Receptor Expression	hMOR Receptor Expression	hMOR Receptor Expression
37	0.5	20	49	15
16	1.5	33	44	14
21	3	21	51	19
33	10	31	43	10
32	15	31	46	12
29	20	25	46	9
30	20	19	50	17
34	20	24	45	20
36	25	33	58	17
24	30	22	42	20
28	30	31	41	19
31	30	31	42	21
38	50	20	56	15
15	54	32	35	18
17	70	21	45	19
11	90	19	44	20
19	90	28	40	20
18	95	26	57	21
25	100	23	41	18
13	120	33	40	15
22	135	27	48	18
20	145	28	44	17
23	145	24	44	20
27	160	26	33	22
10	185	27	47	17
12	195	31	40	30
26	195	24	41	15
9	220	16	45	17

3.3.1 μ -Opioid receptor expression levels

There was little correlation between receptor expression and dose (Figures 3.1 – 3.3).

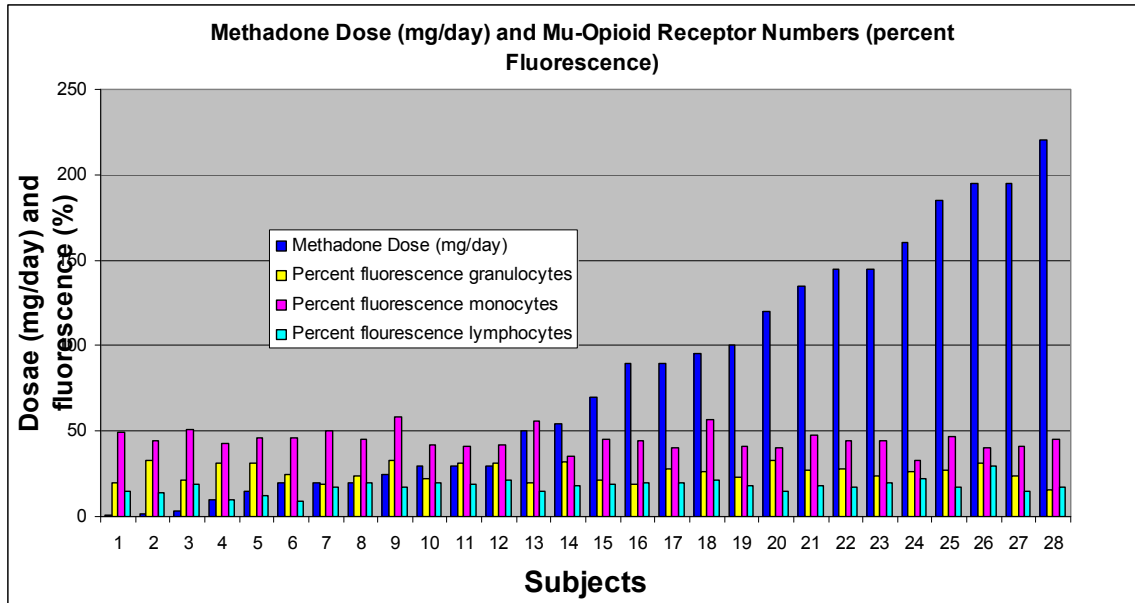


Figure 3.1 Receptor expression (as measured by percent fluorescence) in leukocytes as a function of methadone dose (all subjects measured – n=28)

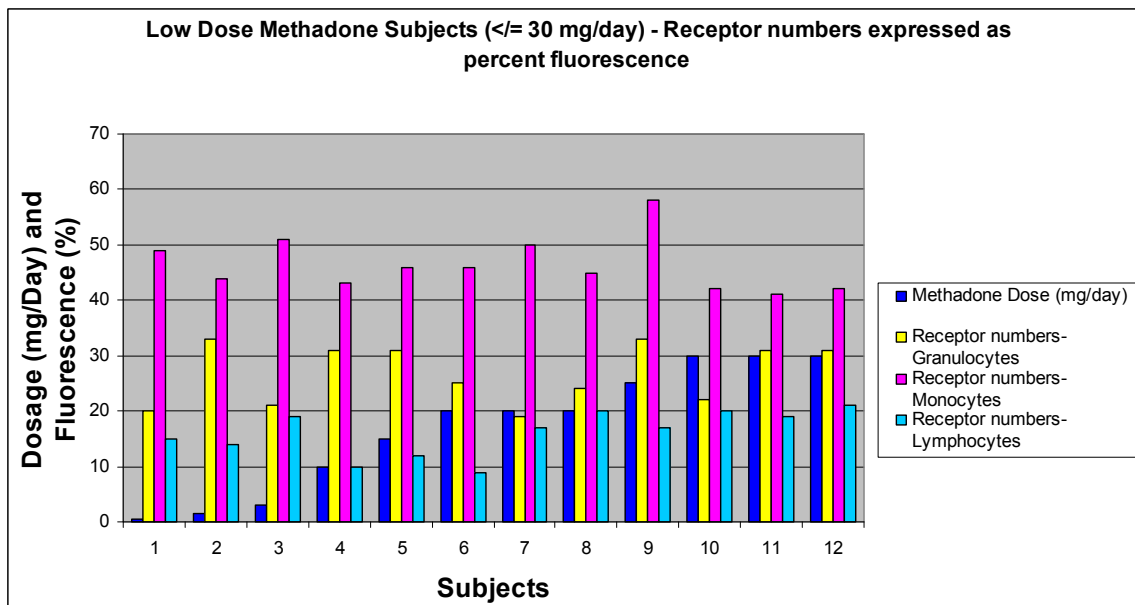


Figure 3.2 Receptor expression (as measured by percent fluorescence) in leukocytes as a function of methadone dose (“low” dose subjects – n= 12)

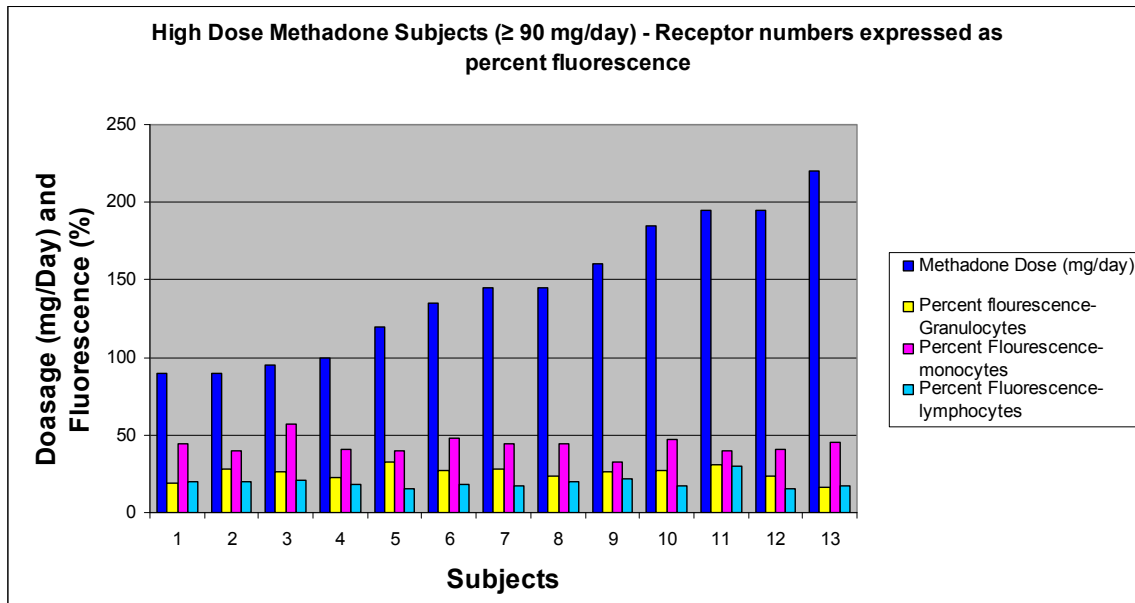


Figure 3.3 Receptor expression (as measured by percent fluorescence) in leukocytes as a function of methadone dose (“high” dose subjects – n= 13)

As displayed in Figures 3.1 – 3.3, increased methadone dose did not correlate with increased expression of μ -opioid receptors on white blood cells or any of the three white blood cell types. However, chronic dosing, regardless of level, did appear to enhance μ -opioid expression on leucocytes to detectable flow cytometry levels.

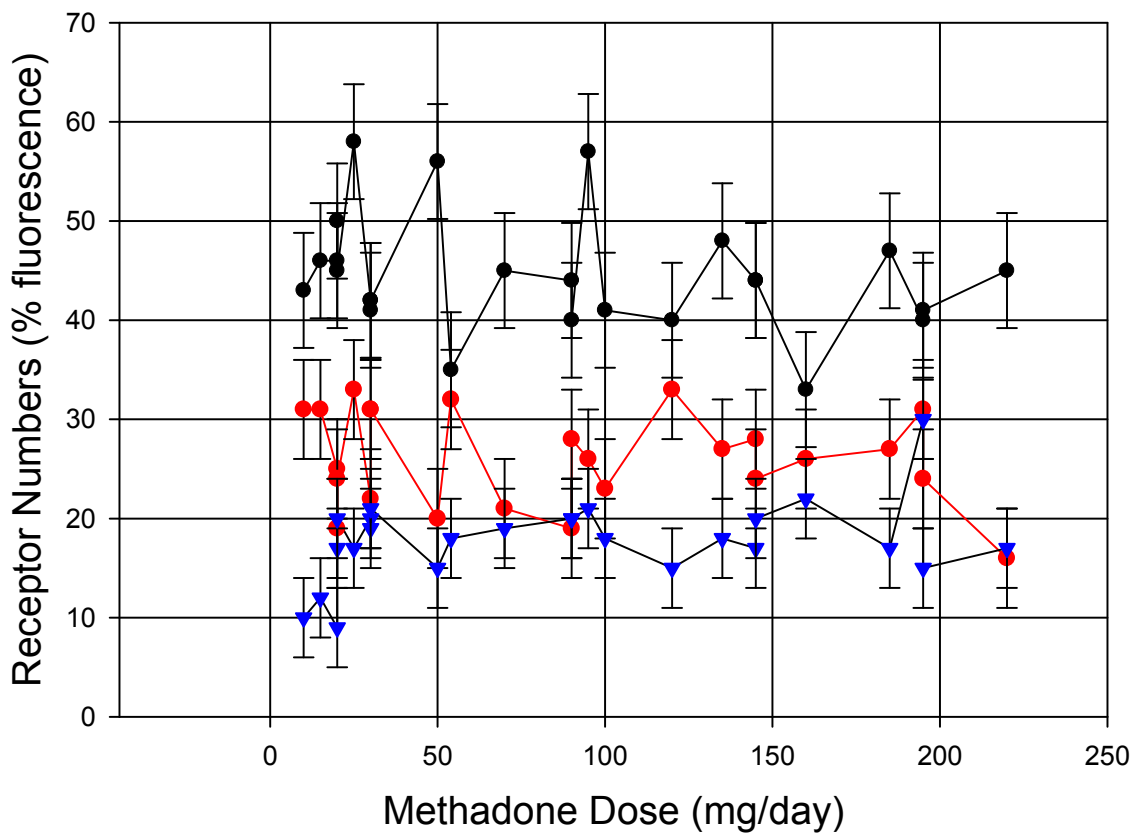


Figure 3.4 Plot of μ -opioid receptor numbers on white blood cells expressed as percent fluorescence versus methadone dose (mg/day). —●— Indicates percent fluorescence for granulocytes, —●— indicates percent fluorescence for monocytes and —▼— indicates percent fluorescence for lymphocytes

Statistical analysis was accomplished using *Sigma Plot* and performing correlation between group means using the t test with a level of significance $\alpha = 0.05$

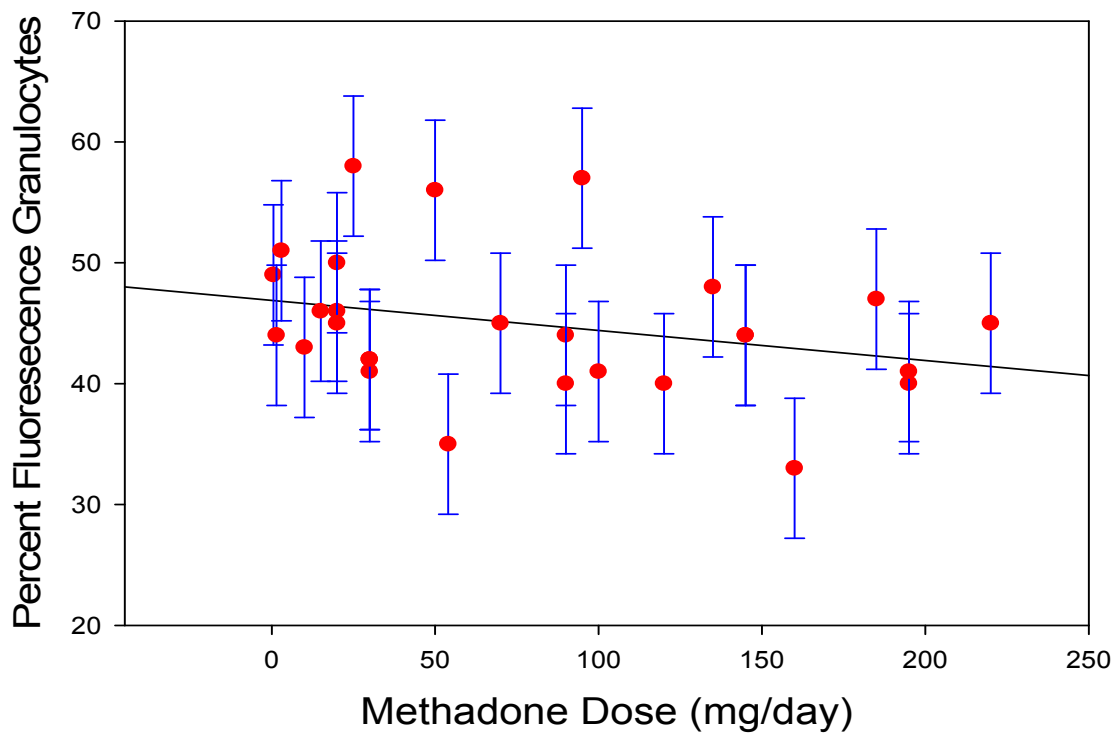


Figure 3.5 Plot of μ -Opioid receptor expression as percent fluorescence on granulocytes versus methadone dose (mg/day). Statistical analysis reveals no significant relationship between dose and receptor expression

Statistical Correlation: t test

Using Sigma Plot software to analyze this data set, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups. (CI = 99%, $\alpha = 0.05$, $P = 0.73$)

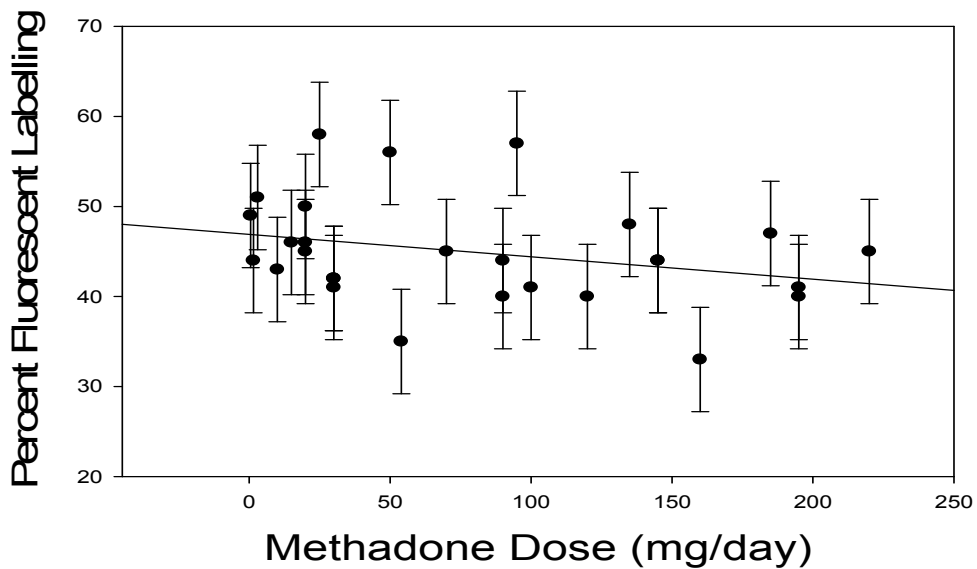


Figure 3.6 Plot of μ -Opioid receptor expression as percent fluorescence on monocytes versus methadone dose (mg/day).

Statistical Correlation: t test

Using Sigma Plot software to analyze this data set, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (CI = 99%, $\alpha = 0.05$, $P = 0.093$)

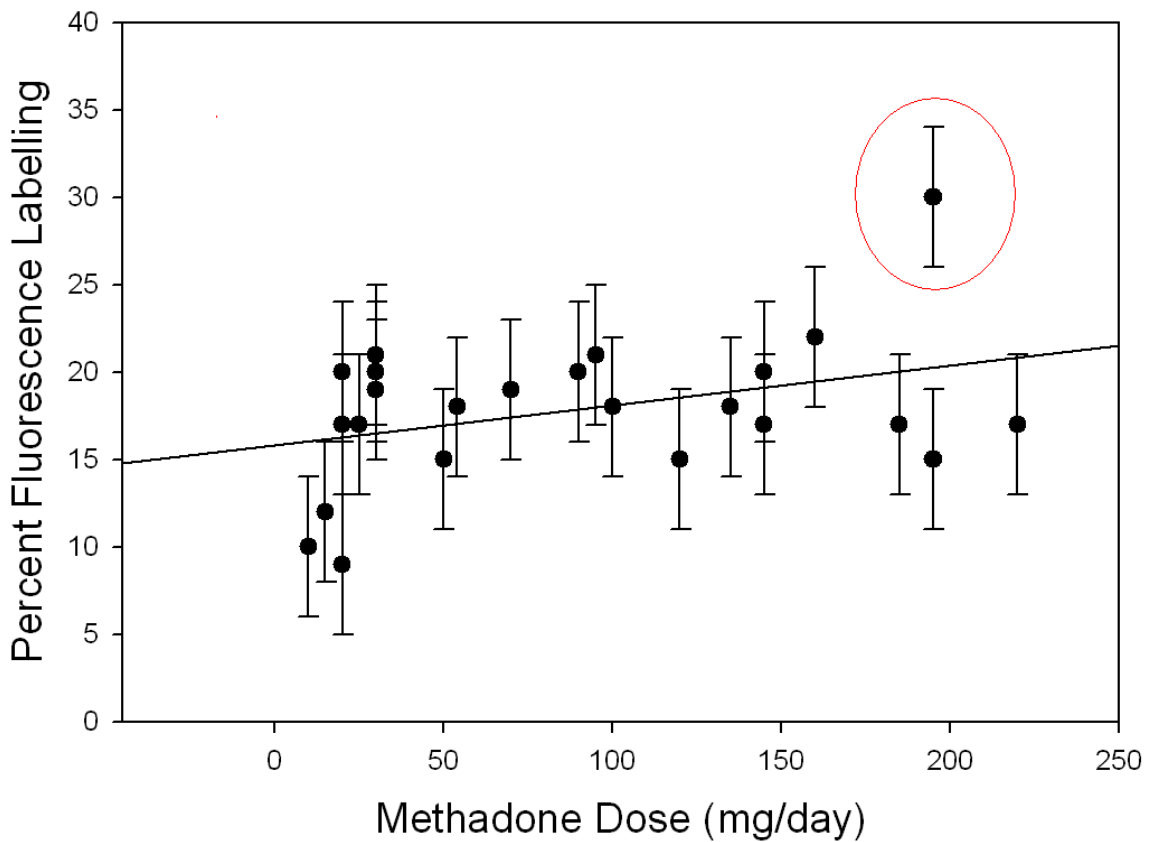


Figure 3.7 Plot of μ -Opioid receptor expression as percent fluorescence on lymphocytes vs. methadone dose (mg/day). The data point circled in red is a suspected outlier. The t-test indicated a slight difference in the mean of the 2

groups; however, both Mann-Whitney Rank Sum Test and Wilcoxon Signed Rank Test indicated no difference. If we look at the plot in Figure 3.7, it seems apparent that we may have an outlier. Removing this outlier clearly indicated by all 3 statistical methods that this data has no statistical trend.

Lymphocyte data with all points included

Statistical Correlation: t test

Using Sigma Plot software to analyze this data set, the difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups

Lymphocyte data with "high" value excluded

Statistical Correlation: t test

Using Sigma Plot software to analyze this data set, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (CI = 99%, $\alpha = 0.05$, $P = 0.064$)

From our flow cytometry data we would have to conclude that the difference in dosing requirements for our methadone subjects was not simply due to differences in the number of μ -opioid receptors as related to their presence on white blood cells. There was no significant difference in receptor numbers based on dosing.

Apparent Information obtained from receptor expression data

Evident from our receptor expression data displays the fact that chronically dosed subjects (> 6 months) expressed measureable levels of receptors on white blood cells. This is suggestive that methadone treatment induces the expression of hMOR, since we were unable to reliably measure receptor expression on naïve subjects using our flow cytometry technique.

Receptor down regulation / internalization alone does not explain dose requirements for individuals, since we established no significant difference in receptor expression between low and high dose subjects

3.4 cAMP in “vivo” estimations and dose response curves

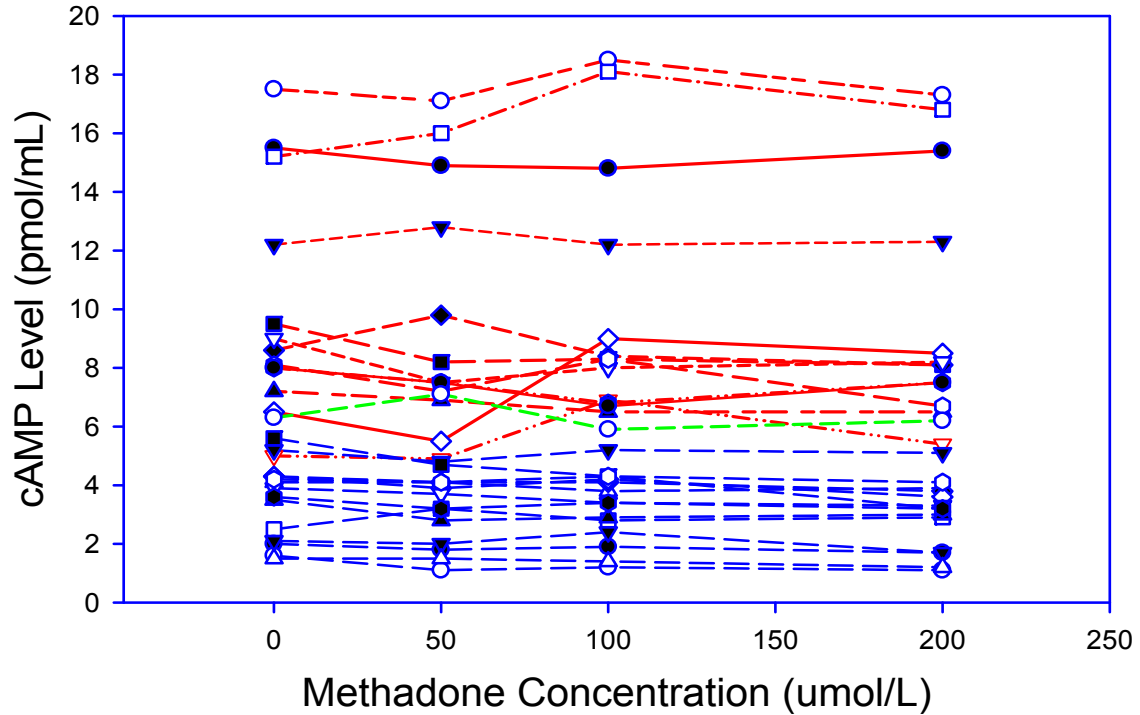


Figure 3.8 Plot of cAMP levels measured from white blood cells vs.

methadone dose of all low and high dose subjects (mg/day)

Red = “Low” dose

Blue = “high” dose

Green = “High” dose subject with highest cAMP values

Time “0” represents in-Vivo concentrations of cAMP from cell lysates

Incubation with increasing concentrations of methadone appear to have very little

dose – response effect.

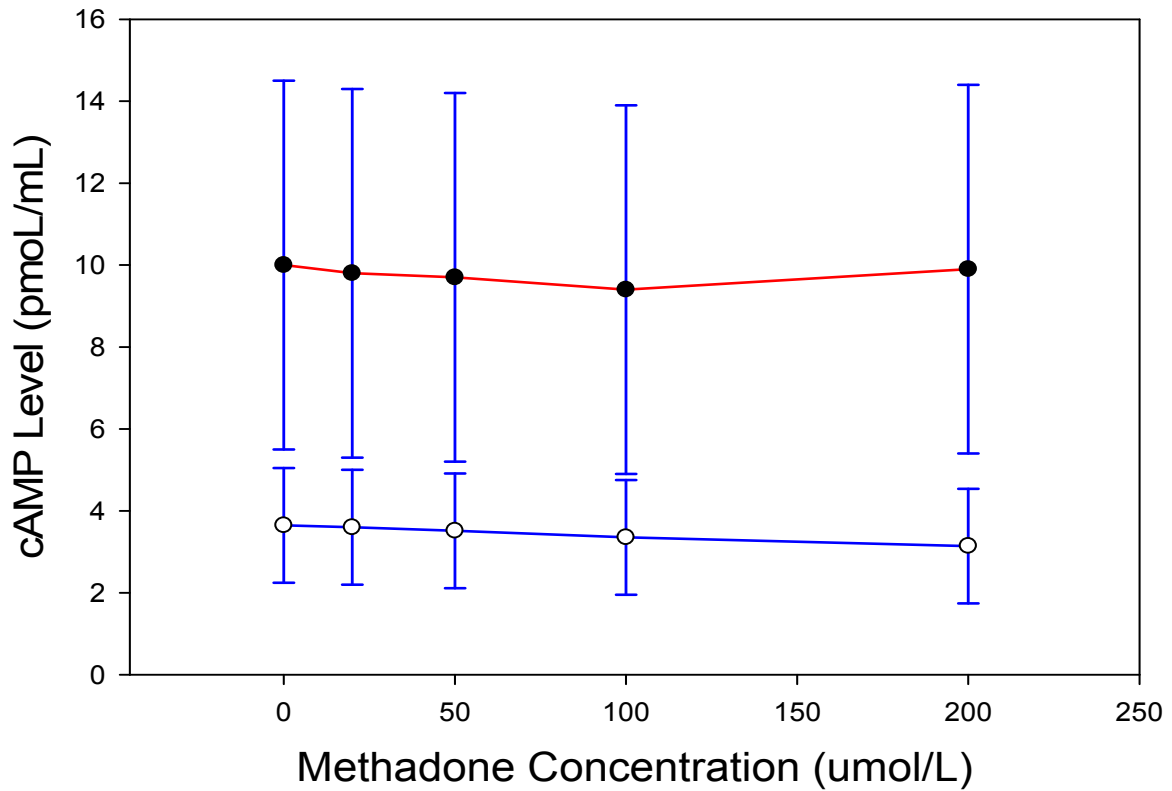




Figure 3.9 Dose response curves displaying the mean \pm 2 standard deviation of cAMP levels for both high and low dose methadone subjects.  indicates cAMP levels from low dose patients and  indicates cAMP levels from high dose patients

Mean levels of our defined "low" and "high" dose groups of methadone maintenance subjects appear to have no over-lap and clearly display significantly different in-Vivo amounts of cAMP .

Statistical Correlation: t test

Using Sigma Plot software to analyze this data set, the difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups ($P = <0.001$, $\alpha = 0.05$)

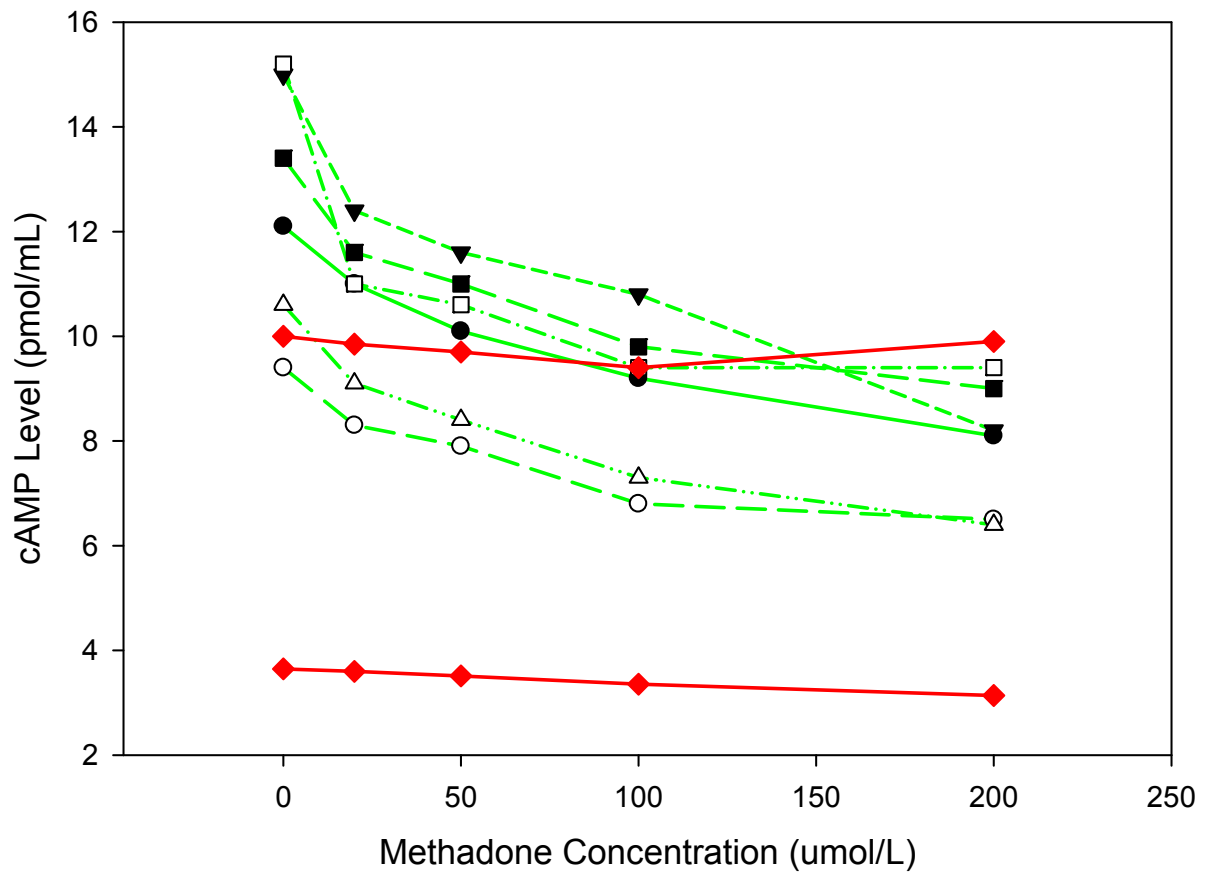


Figure 3.10 Plot of dose response curves displaying difference in both slope and level of cAMP in Naïve subjects. (green = naïve subjects, red = high and low dose users)

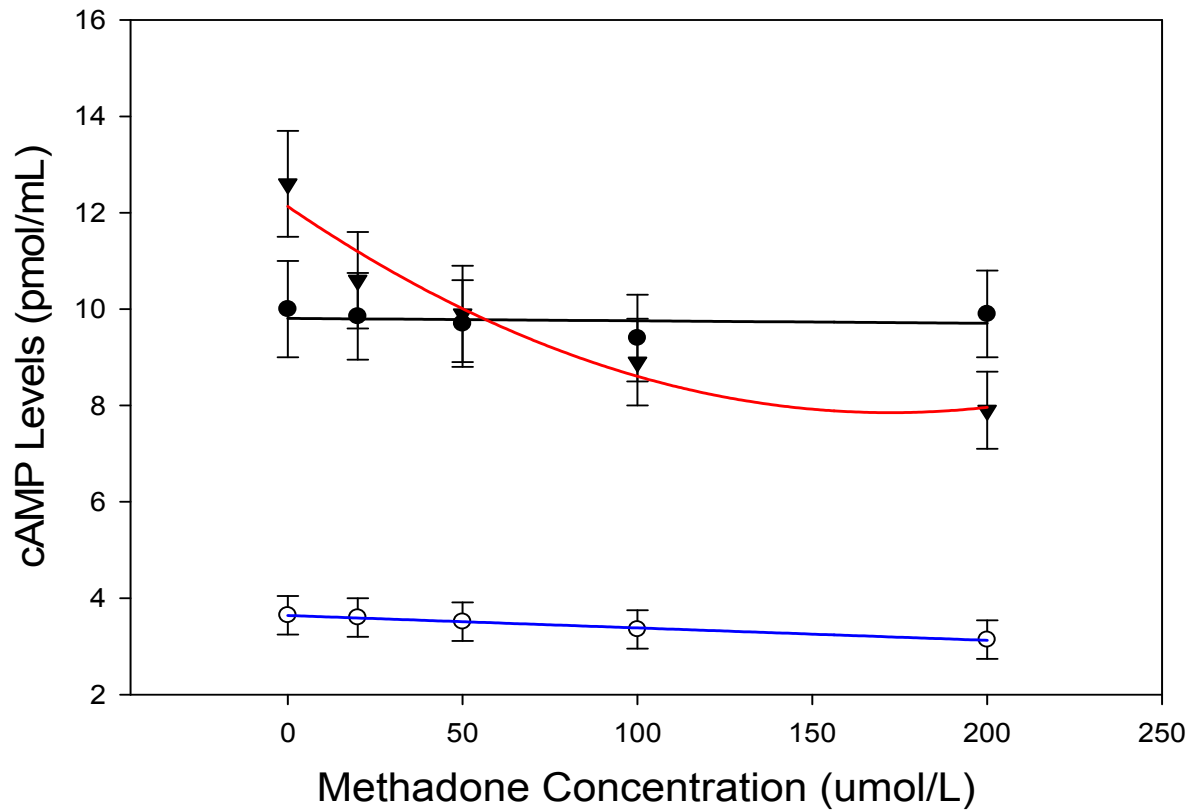


Figure 3.11 Plot of “mean” cAMP levels of naïve users vs. both low dose subjects and high dose subjects (shown as dose response curves)

Mean “low” Dose n = 16, displays no significant difference (99% CI = 9.30 – 10.22, $\alpha = 0.05$, $P = < 0.01$)

Mean “high” Dose n=16, displays no significant difference (99% CI =3.01 – 3.87, $\alpha = 0.05$, $P = < 0.01$)

Mean Naïve user n=6, displays significant dose response relationship as described by: $Y = 0.0001 x^2 - 0.0497x + 12.131$, $R^2 = 0.9469$

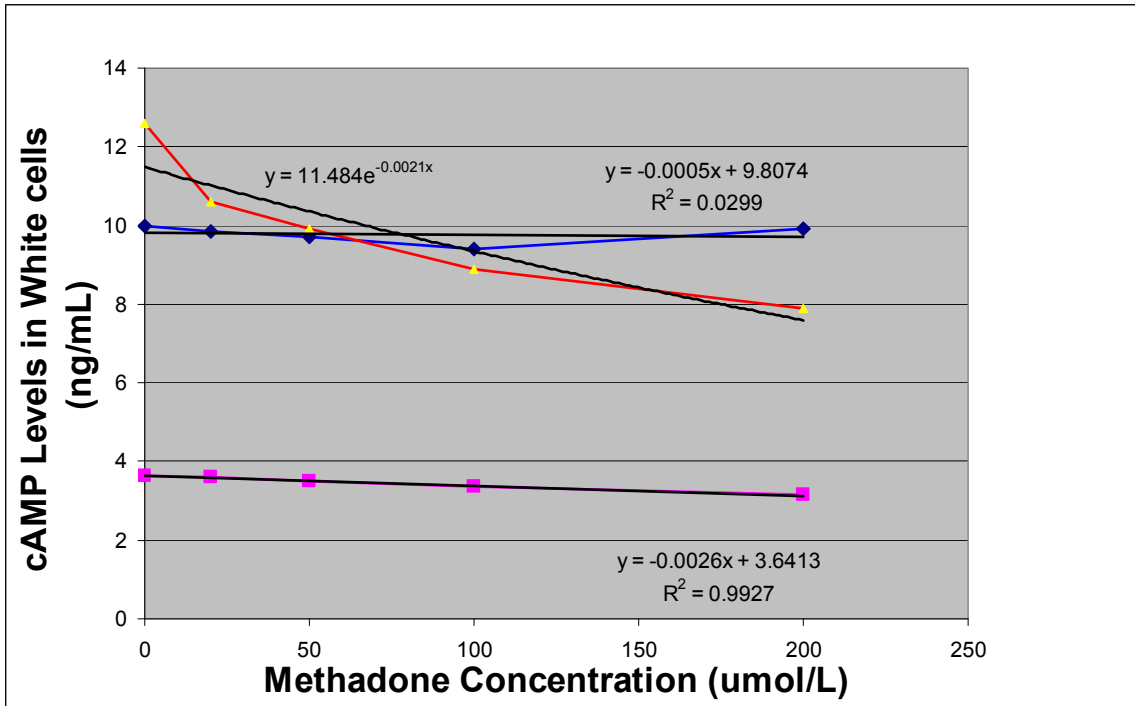


Figure 3.12 plots of dose response curves and baseline levels of cAMP in naïve subjects versus low and high chronically dosed subjects.

- indicates mean of naïve subjects
- indicates mean of low dose subjects
- indicates mean of high dose subjects

Statistical Significance of Naïve Subject Dose Response Data

Figures 3.10 and 3.11 clearly display that the difference in dose – response curves in naïve users as compared to chronic users both at low and high dose. Neither “low” dose or “high” dose subjects display a significant trend

across the increasing dose (as described previously), while the naive users display an exponential response to increased methadone dose.

In Vivo cAMP levels as represented by our values at time = 0, appear to indicate that there is a direct relationship to dose and baseline cAMP.

Apparent Information obtained from cAMP level and dose response data

Figure 3.9 clearly illustrates that chronic methadone dosing at any level appears to create a state of tolerance or desensitization to increased methadone dose. Dose response curves display a tendency to remain “flat” or to display little or no response to increased methadone. It also displays that there is a significant difference in mean *in vivo* cAMP levels between low and high dosed subjects

Naive subjects display logarithmic change in cAMP levels when exposed to increasing methadone dose

3.5 P-GP levels measured by immunoassay in Leukocytes

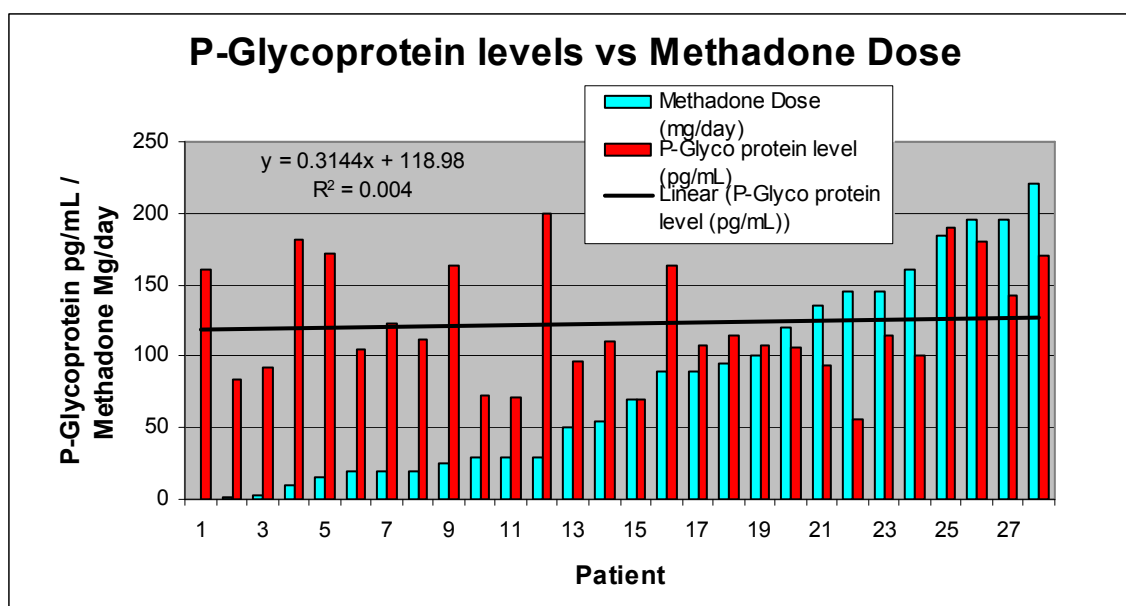


Figure 3.13 Experimental data comparing P-GP levels (pmol/mL) vs methadone dose in all subjects $n = 28$. White cell lysates from blood samples ($n=28$) were analyzed for total P-GP levels. There appeared to be no correlation of P-GP level based on methadone dosing requirement.

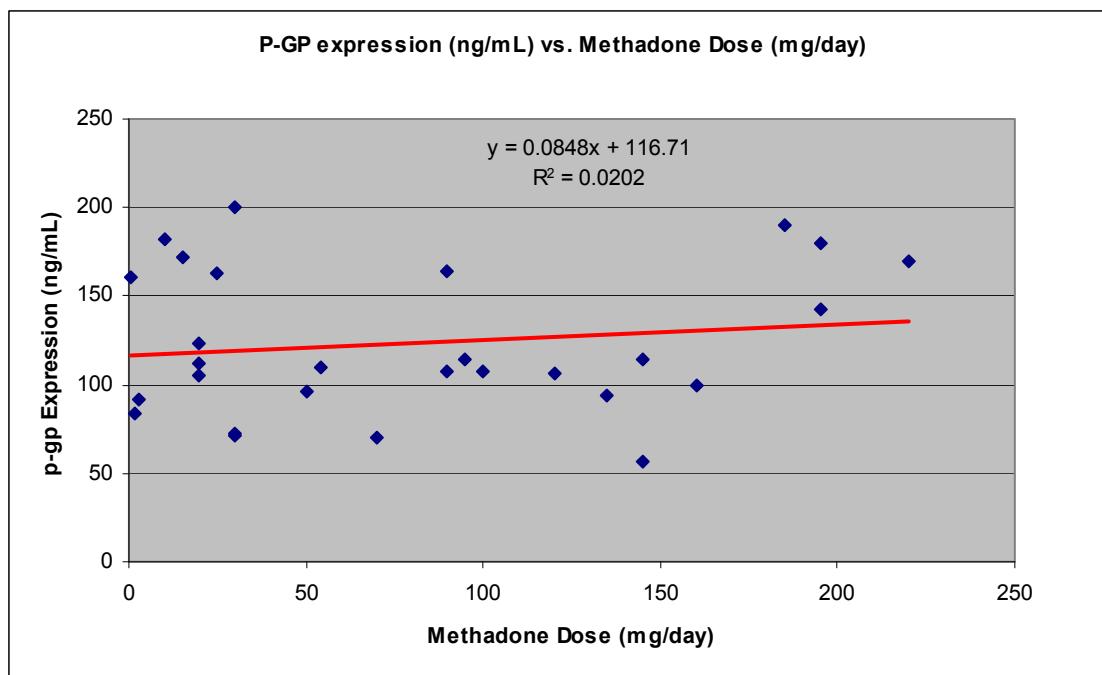


Figure 3.14 Plot of P-GP expression vs. methadone dose in all subjects n = 28

Statistical Correlation: t test

Using Sigma Plot software to analyze this data set, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (CI = 99%, $\alpha = 0.05$, $P = 0.413$)

Apparent Information obtained from measuring levels of P-GP in white blood cells

The data suggests no significant relationship between P-glycoprotein levels expressed on peripheral white blood cells and methadone dosing requirements. Both figures 3.14 and 3.15 illustrate a lack of correlation between dose of methadone and expression of p-glycoprotein in white blood cells

Both co-medication and P-glycoprotein have been shown to play a role in the evolution of methadone analgesic effect (27;129). It is possible that P-glycoprotein may play a role in methadone dosing requirements; however, it appears not just to be a case of P-glycoprotein expression levels specifically in white cells.

We must consider that P-glycoprotein genetic variants are more likely to be associated with methadone dose required or concurrent use of P-glycoprotein inhibitors may be more significant.

3.6 Concurrent drugs of abuse usage

The majority of methadone patients, specifically on the “Harm Reduction” model, concomitantly use other drugs during their treatment. By assessing concurrent drugs use with a broad spectrum screen, predications may be possible about multi-drug contribution to dosing requirements. Some of the more “desperate” drug use belonged to the high dose group, with both gabapetin and diphenhydramine detected. (Table 3.2)

Table 3.2 Drugs and/or metabolites detected by LC-MS/MS screen

	Methadone Dose		
	Low (< 50 mg/day)	High (\pm 90 mg/day)	Normal (50 – 80 mg/day)
Drugs/Metabolites Detected	n=14	n=14	n=4
Methadone	13	14	4
EDDP	6	13	4
THC	2	4	3
Cocaine	-	-	1
Benzoyllecognine	-	-	1
Morphine	3	1	-
Codeine	1	-	-
Gabapentin	-	2	-
Diphenhydramine	-	3	-
Ritalinic Acid	1	1	-
Fentanyl	1	1	-
Oxazepam	5	3	1
Lorazepam	2	3	-
Nordiazepam	6	10	2
Temazepam	2	6	1
Diazepam	4	9	1
Alprazolam	1	-	-
Clonazepam	-	1	-
7-amino-clonazepam	-	1	-

Many benzodiazepine metabolites were detected. However, most of these compounds are both metabolites and available as drug preparations making it difficult to assess which drugs were ingested.

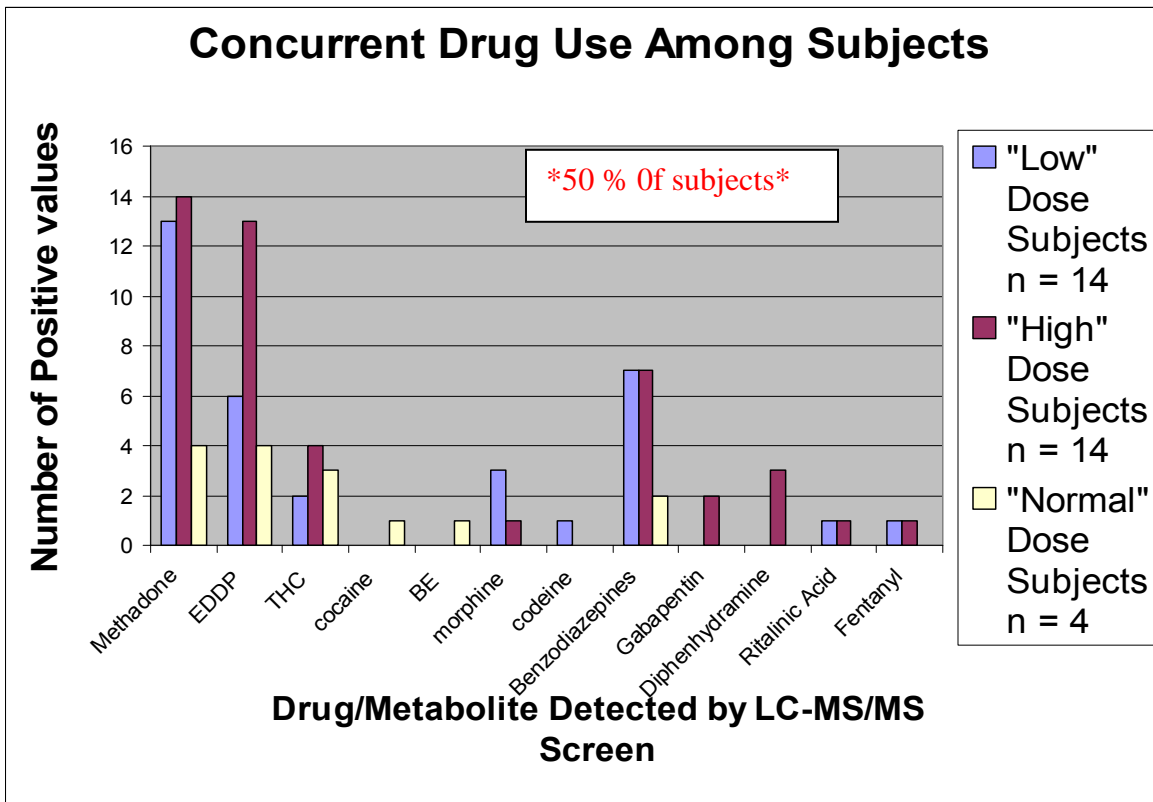


Figure 3.15 Data comparing concurrent drug detection in all methadone subjects

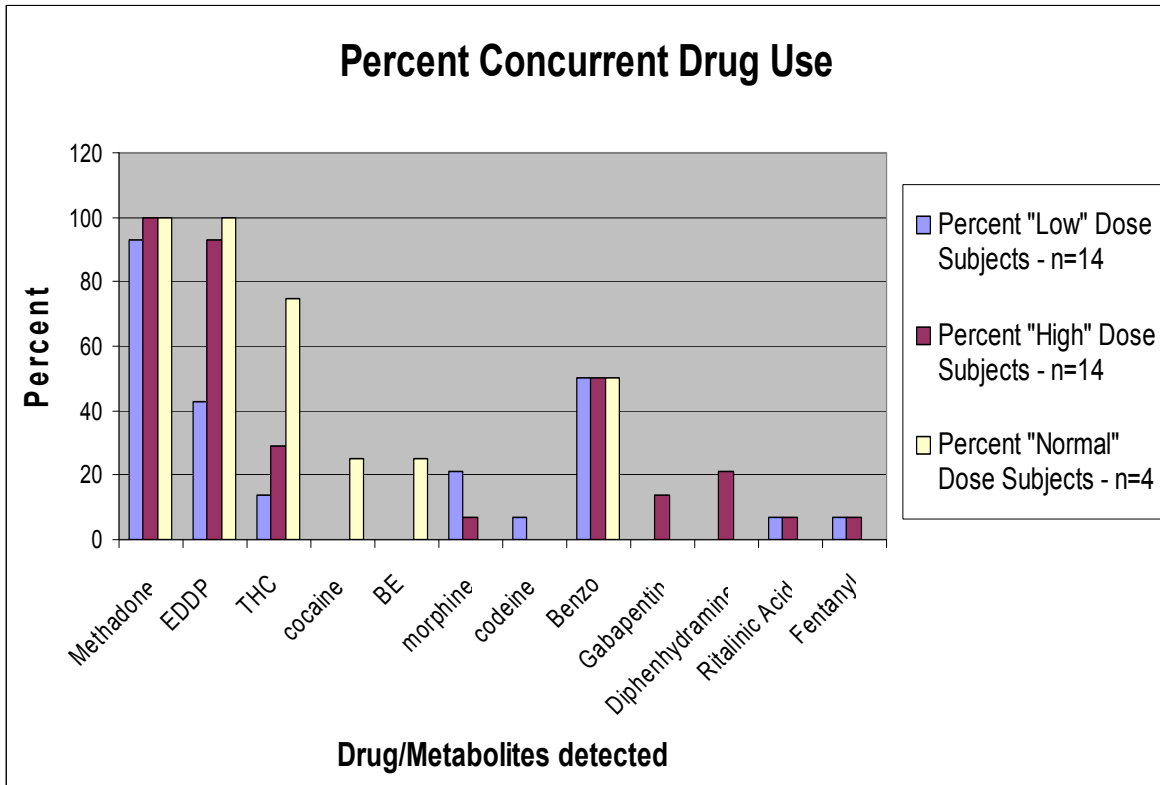


Figure 3.16 Data comparing concurrent drug detection in all methadone subjects expressed as a percentage

Chapter 4

Discussion and Conclusions

4.1 Methadone treatment and concurrent drug use.

Most researchers would agree that individuals who receive methadone maintenance therapy have a better outcome probability than those that had either in-patient detoxification or no treatment at all (95). Benefits of such programs include improvements on many levels – other drug use, injecting and sharing needles, infectious disease transfer, physical and psychological health and social functioning and behavior (11).

Methadone maintenance treatment programs are common drug-treatment programs in North America that can involve complex situations for clinicians to determine specifically when patients are involved in concurrent illegal drug use and/or being treated for other disorders with prescription drugs (34). The common use or abuse of other drugs creates a state of increased likelihood of variability in patient response to methadone.

PD or PK drug interactions must be considered when utilizing chronic methadone therapy. Drug-drug interactions can alter methadone levels in a significant fashion. Some drug-drug interactions occur without any significant clinical effect; however, there is potential for medical consequence. Drugs that inhibit CYP3A4 may lead to methadone toxicity, while drugs that induce CYP3A4 may lead to lowered plasma levels of methadone and possibly opioid or methadone withdrawal in extreme cases. This is exactly the opposite effect desired. Part of methadone's wide variability of action can be attributed to varying

CYP3A4 activity. Since methadone is primarily metabolized by CYP3A4, inhibitor and inducers of this cytochrome enzyme can affect levels (130).

Our data also clearly shows a high subject usage of benzodiazepines, which was by far the most frequently detected drug. Our drug screening methodology allowed us to detect benzodiazepine use with a high degree of reliability, since parent drug and in most cases major metabolites were detected. This is an important consideration because the benzodiazepine family of drugs displays extensive metabolism with many active metabolites (131). Fully 50 % of all subjects were positive for at least one benzodiazepine or metabolite – many of them for several metabolites.

One study identified diazepam as the most commonly detected concomitant drug (44 per cent of cases), followed by alcohol (33 per cent), morphine (28 per cent) and Temazepam® (21 per cent). Fifty-four per cent of all index cases involved at least one benzodiazepine and, overall, more than 80 per cent of methadone-related deaths involved one or more other drugs. The general trend from that study was a decrease in people testing positive for benzodiazepines (129).

Our data correlates well with a recent study from Switzerland, in which the benzodiazepine usage rate amongst methadone patients was 51.5% (132), with a worldwide range of 20 – 70% for MMT patients. They also quote a range worldwide of 20 – 70% for patients who are on methadone maintenance treatment. Of the 101 methadone patients in the Swiss study, 52 (51.5%) were regular users of benzodiazepines and 48 of those received their benzodiazepines

by medical prescription. There is a lack of evidence-based recommendations for benzodiazepine prescription to MMT patients, so physicians often find themselves in a dilemma. Not prescribing risks the likelihood of patients that are benzodiazepine abusers, not tolerating cessation and dropping out of the program. On the other hand, prescribing means a risk of continued dependence (132). There is also the risk of new dependence upon new concurrent drugs being used.

There is clinical evidence of significant methadone drug interactions among MMT patients (133), making it imperative that clinicians diligently check past history before prescribing medications. Certain combinations of methadone and other psychotropic or opioid medications may affect treatment outcomes or precipitate withdrawal (19). Evidence from this work suggests clinicians should be aware that a high percentage of methadone patients will be using other drugs concomitantly. A recent study from Ontario, Canada revealed that 18.4% of patients on methadone maintenance treatment in that province were prescribed other opioids for more than 7 days duration (134). This would suggest that many patients receiving methadone therapy receive overlapping prescriptions for other opioids, often for extended periods. Polypharmacy and legitimate prescriptions contribute to other drug use as well as street drug acquisitions. General recommendations of most methadone programs are to prescribe appropriate doses of methadone with a minimum use of concomitant benzodiazepine prescriptions (135).

Other drugs detected in MMT subjects included Gabapentin, which has recently been identified in Saskatchewan, by addiction clinicians, as a commonly abused drug. It was only identified in two high dose patients and without prescription drug history we are unable to ascertain if use was legitimate or illegal. Morphine was identified in 3 low dose subjects; however, all three levels were very low, possibly indicating codeine usage (morphine being a major metabolite of codeine). Cocaine metabolite was detected in one normal dose patient, while ritalinic acid, a major metabolite of methylphenidate, was detected in one high dose and one low dose subject. Methylphenidate is a very commonly abused drug in Saskatchewan (136), but may also be a result of legitimate prescription. The same could be said for fentanyl, which was detected in one high and one low dose subject. Diphenhydramine, a common over the counter drug with significant abuse potential was detected in three high dose patients.

Our low dose subjects (n=14) were positive for a total of 26 drugs/metabolites and the high dose group (n=14) for a total of 45, other than methadone or EDDP (major methadone metabolite), suggesting a preponderance of higher dose patients to be associated with more frequent illicit drug use.

These data are consistent with previous work showing that higher dosed methadone MMT patients have a higher rate of other drug use (92). The fact that more benzodiazepine metabolites were detected in the high dose subjects is suggestive of higher dosing of individual benzodiazepines, but lack of access to

patient information as outlined by our ethics approval precluded us from demonstrating this more definitively.

It is clear from our results that concomitant drug use exists among MMT patients. As discussed below, there is likely to be a contributing effect of this drug use to overall methadone dosing requirements.

Most drugs are substrates, either inducers or inhibitors of the major P450 .isoenzymes CYP3A4, CYP2B6 and CYP2D6 as is methadone. Therefore MMT patients can readily have drug-drug interactions. Drugs such as rifampin or carbamazepine are classical CYP3A4 inducers which could potentially enhance methadone metabolism leading to under dosing and withdrawal symptoms. On the other hand a drug such as fluconazole, which inhibits several CYP enzymes, may increase parent methadone blood concentrations.

Since there is a high incidence of positive HIV status associated with drug users, antiretroviral drugs used in treating HIV positive individuals must be carefully dosed and monitored because they are known to be responsible for drug-drug interactions involving methadone (137).

In some cases it is not only the initiation of a specific drug treatment that creates an issue, but rather at the discontinuation of interacting agents. When a potent inducer is discontinued the patient may become a slower metabolizer as the CYP3A4 pathway also back to its normal metabolic rate (138).

Other mechanisms may involve inhibition or induction of P-glycoprotein, which is responsible for active transport of substrates such as methadone. Co-administration of hypericum causes a decrease in plasma methadone

concentration associated with increased withdrawal symptoms (139). Other drugs can increase the bioavailability of methadone by inhibition of P-glycoprotein activity and result in an overall increase of methadone effect.

Drugs such as other opioids or narcotics may interact with methadone via their effect on μ -opioid receptors. Buprenorphine, a partial agonist or mixed agonists-antagonist analgesics such as nalorphine, can displace methadone from receptors and should not be used in patients undergoing MMT (18).

4.20 Mu opioid receptor numbers, regulation and responsiveness

In an attempt to use a non-invasive model for assessing μ -opioid expression in MMT individuals we analysed receptor expression on three different white blood cell types. Since neuronal cells are not readily available from methadone maintenance subjects, this peripheral blood model is the basis for our scientific assessment of dosing variance. Measurement of μ -opioid receptors on white blood cells was feasible for all dosing groups, but reliable, reproducible receptor number data from naïve subjects was below detection limits. Cell culture studies have shown that methadone treatment of human lymphocytic cell line increases hMOR expression (140). Increased receptor expression on leukocytes was essential for detection by flow cytometry. Thus, it would seem that a more sensitive method of receptor number detection is required to perform this task on Naïve subject cells. Other studies have shown that there may be differential expression of hMOR splice variants hMOR-1A and

hMOR-10 mRNA in peripheral white blood cells of opioid addicts ([141](#)). Currently only a single gene for this receptor has been identified; however, alternative splicing is responsible for production of several human MOR variants.

Our analysis of both low dose and high dose cohorts of MMT subjects revealed no statistical difference in levels of hMOR expressed on 3 types of white blood cells. Flow cytometry is limited to measuring hMOR through specific binding and subsequent fluorescence, and so only detected total hMOR as % fluorescence, thus only reflecting plasma membrane receptor expression but not internalized receptors, which may have the potential to return to the cell membrane and resume activity. Plasma membrane receptors for the most part represent active receptors, yet there may be significant numbers of internally housed receptors, which would be unaccounted for by our model.

Receptor expression on WBC plasma membranes was a function of chronic dosing with methadone, regardless of dose. There are studies that suggest expression of receptors in peripheral blood lymphocytes parallels and may represent expression in the brain ([142](#)). Up-regulation of receptor numbers in MMT patients does not seem dose dependent. What are the implications of up-regulation in opioid addiction? These answers have not yet been revealed, however, there may be usefulness in looking at WBCs as a reflection of neuronal receptor expression status. In our study receptor down regulation of high dose patients did not seem apparent, which would suggest other mechanisms of desensitization such as possibly receptor phosphorylation or binding by β -arrestins and decoupling with subsequent down stream signalling effects and/or

receptor internalization or sequestering (44). According to our data this did not appear to correlate more with high vs. low dose. These combined regulatory processes may still contribute to the phenomena of tolerance and dependence. Other factors to consider include the major key accessory proteins - regulators of G protein signalling (RGS), which dictate timing and duration of G protein cycles (143). RGS proteins control the lifetime of active G α and G $\beta\gamma$ signalling molecules and thereby regulate downstream effects of G protein mediated signalling.

4.3 Downstream signaling and variations in cAMP levels

The μ -opioid receptor mediates or acts as a transducer for the action of most clinically important analgesics and opioids including methadone. This includes analgesia as well as tolerance and dependence (144). Recent studies have shown that there can be increased MOR signalling that was not associated with increased hMOR or G protein expression (145). One such mechanism is through scaffold proteins such as RanBPM, a hMOR interacting protein. Other hMOR receptor modulating proteins likely exist that affect or change responses in signalling despite constant receptor expression and binding characteristics. Other mechanisms may involve multiple receptors being tethered into complexes having signalling proteins that access shared AC (146). Receptors may be able to diffuse along the membrane to access free available G proteins, or be

corralled together in a micro domain (lipid rafts) by scaffolding proteins or dimerization (147).

RGS proteins share a conserved 120 amino acid domain; however, they vary extensively in size and architecture. They are expressed in brain regions rich in μ -opioid receptors and participate in the manifestation of clinically relevant opioid behaviours (143). Thus RGS proteins may play a significant role in downstream signalling and subsequent variation in cAMP levels.

Biochemically, both tolerance and dependence are found to be associated with an up-regulation of the second messenger cAMP against removal of the agonist (148). cAMP is a very important signalling molecule and for this reason we measured baseline levels in all patients as well as levels at increasing doses of methadone. Chronically treated MMT patients appeared to display *in vivo* baseline cAMP levels with a strong correlation to methadone dose. Higher dosed subjects in our study displayed lower levels of cAMP than their lower dosed counterparts. All of these patients also displayed tolerance to exposure to higher levels of methadone unlike naïve subjects. White blood cell isolates from all patients were incubated for 60 min with increasing doses of methadone. Naïve users displayed an overall higher baseline level of cAMP than chronically treated MMT patients. As well, naïve users displayed an obvious response to increased levels of methadone, while MMT patients did not. These results are in excellent agreement with much of the literature, demonstrating that prolonged exposure to opioids causes an up regulation of cAMP, and upon removal of the ligand gives rise to moderate levels of tolerance (83;149).

cAMP production as a result of AC induction or inhibition may also be related to AC splice variants. Marked differences have been reported in the agonist inhibition of different AC isoenzymes (150). At least nine isoenzymes have been cloned and shown to differ in their sensitivity characteristics to the G subunits (α and $\beta\gamma$) and PKC. Persistent action of activated inhibitory $G\alpha$ subunits with their respective AC isoforms provide the signals for induction of tolerance and dependence (151). We observed a statistically significant correlation of decreasing cAMP levels *in vivo* with increasing chronic methadone dose in our subjects. Thus, it appears that the higher dosed patients required a lower baseline level of cAMP in order to prevent withdrawal symptoms. This points in the direction of downstream signalling as a major factor in controlling withdrawal from opioid drugs.

As illustrated in Figure 3.13, specific relationships between chronic dosing levels and cAMP levels exists. Also a dose response to methadone appears to be dependent upon naive versus chronically dosed status. A large range of changes or neuroadaptations develop in chronic opioid users or patients who have been on chronic methadone therapy. Our data clearly shows a relationship between endogenous cAMP levels and methadone dose. Adaptations causing cellular tolerance are certainly more complex than this; however, the data does suggest receptor desensitization and alterations in downstream signalling as a major potential for dose requirements.

Recent research has revealed that cAMP-mediated signaling relies on an intricate network of multiple signaling pathways (152). In such a system, a tight spatial control of signal propagation allows for the signal to be transduced along defined branches of the network, depending on the specific extracellular stimulus. More recent literature suggests that compartmentalisation is a mechanism which allows individual extracellular cues to mediate specific cellular events (153). Once again, however, most of the particular events of the cAMP/AC/PKA signalling network components remain unknown (153).

4.40 P-Glycoprotein and methadone dosing

This study sought to also understand if P-glycoprotein expression played a significant role in methadone PK/PD processes, which could affect dosing requirements. Although we could not measure this influence on PK or PD parameters directly, we attempted to measure P-glycoprotein expression with the assumption that increased expression may represent increased likelihood of potential for PK/PD influence. As well as at the blood brain barrier, P-GP is a transmembrane protein expressed in various tissues including the intestine. Here it functions as an efflux pump to excrete drugs from the intracellular to the extracellular lumen (91). Since we know methadone is a substrate for P-GP, pumping of this drug from the intestine to the lumen may limit the amount of absorption. In a recent study, methadone pharmacogenetics were explored in

relation to polymorphisms in the MRD1 gene which encode the drug transporter P-GP, showing no evidence of genotype significance for the methadone concentrations studied (154).

Here, the measurement of P-GP in white blood cells was an attempt to determine expression level variation from subject to subject. Large interindividual differences in the amount of P-GP expressed in the intestine exists and it is possible that some of this difference is associated with genetic polymorphisms (154). Several studies indicate contradictory results about whether or not genetic variants influence both expression and function of P-GP (155). This study showed no statistical link between P-GP expression in white blood cells and methadone dose. Therefore we would have to conclude that extreme differences in methadone dosing requirements among MMT patients is not linked to P-GP expression in leukocytes or the latter does not reflect levels in other organs systems such as the intestines or blood brain barrier. The effect of P-GP at the blood brain barrier would influence physiological effects of drug (105). If we assume that P-GP levels detected in peripheral white blood cells are indicative of P-GP expression at these other sites, there appears to be very little correlation between P-GP expression to methadone dosing requirements and absorption of drug in the gut or transfer across the blood brain barrier.

There is significant literature investigating the effect of drug transporter polymorphisms associated with pharmacokinetic characteristics for many different drugs (156-158). Many drugs display marked variability between patients following standard dosing. The extent to which the polymorphisms of

drug transporters such as P-Glycoprotein play a role in this variability is currently being studied. Most studies conclude that knowledge of genotypes may be useful to adjust optimal dosing of drugs in patients (157). Since this work did not explicitly address the issue, this represents a potential future work research direction for methadone dosing requirements.

4.5 Conclusions and summary

High levels of methadone variability in response and its relatively narrow therapeutic index are related to metabolism, drug transport and MOR interaction (18). All of these phenomena are complex and require consideration by clinicians in order to personalize methadone administration so that it is safe and effective. Genetic factors are not the only cause of inter-individual variability and it is important to include other factors such as co medication, state of health and environmental and biological factors.

This study focussed only on the white blood cell model to assess specific key factors including μ -opioid receptor expression, cAMP levels and response curves, P-glycoprotein expression and concurrent drug use.

A total of 71 drug / metabolites other than methadone were identified in our 28 high and low dose methadone subjects, 26 in these low dose patients and 45 in the high dose patients, with a mean of 1.86 other drugs in low dose patients and a mean of 3.21 other drugs in high dose patients. This correlates well with other literature and the fact that higher dose patients tend to exhibit more drug use (92).

Opioid tolerance, a complex process of neuroadaptations must be considered in all dosing schemes. It is essential to consider patients' opioid-dependence and tolerance prior to initiating methadone treatment. Tolerance and adaptations to methadone treatment are difficult parameters to measure. In our model we determined no significant difference in receptor expression between high and low dose patients. Chronic exposure appeared to up-regulate expression in white blood cells; however, this up-regulation was not dose dependent. No significant difference existed in receptor expression in 3 types of white blood cells amongst low versus high dosed patients.

P-GP involvement in drug efflux out of cells was also considered. Methadone, a known P-GP substrate may or may not be significantly transported out of cells at various organs. An overall correlation of P-GP expression to methadone dose requirements was not demonstrated, nor a correlation of P-GP expression in white blood cells to dose requirements. Thus, there is a more complex association than just expression levels of P-GP and methadone dosing requirements.

The evidence pointed to downstream signalling variations in methadone patients that may control or influence dosing needs. In contrast to the apparent absence of P-GP effect on methadone dose we determined a correlation between cAMP levels in white blood cells and methadone dosing levels. Naïve individuals displayed the highest cAMP levels, as well as a definite response effect to increased methadone exposure. Chronically dosed methadone subjects,

regardless of dosage, displayed no significant response to increased methadone exposure and the highest dosed subjects displayed the lowest levels of cAMP.

It is likely those patients in MMT programs who require higher doses of methadone require lower levels of cAMP signalling in order to achieve desired control of withdrawal and cravings. We can hypothesize that the lower levels of cAMP are merely a result of higher drug doses, but then we would have to ignore the clinical presentations which support these individuals' claims. Most clinicians base their decisions on dosing adjustments with the aid of several factors, including the single most important factor, clinical presentation. Withdrawal symptoms or reliable history of medically significant symptoms provide important evidence for dosing adjustments. Thus, in general, higher dosed subjects appear to have a clinical need for higher dosing and legitimately require higher methadone doses to control withdrawal symptoms. Thus there may be a yet unexplained link between differences in downstream signalling and methadone dose requirements.

The challenge going forward is to unravel some of the details of these signalling events and establish links between individual GCPRs such as the hMOR.

A key feature of the cAMP signalling pathway is the very high degree of diversity and unique regulatory mechanisms of its multiple components. Many GPCRs signal via the $G\alpha_i$ pathway, which inhibits AC activity ([153](#)). There may be significant localized control within membrane domains to tune specific

signalling events. The challenge ahead is to provide a more detailed map of cAMP signalling associated with methadone induced hMOR - GPCR signalling pathways.

Studies directly involving human MMT patients and analysis of hMOR-initiated cell signalling are uncommon based on the unavailability of human neuronal cells. Thus, animal models remain a significant focus. By using this peripheral white blood cell model for receptor expression and signalling cascades it may be more practical to effectively and safely study opioid-induced effects in humans, especially the mechanisms of methadone action in MMT patients.

4.6 Future work

There are many directions that could be taken to further understand methadone dosing requirements. There may be a requirement to compare complex neuronal systems and peripheral cell models to better understand the mechanistic basis for methadone tolerance and opioid dependence. As well, further study of OR signalling is merited since it is the engine for neuronal adaptations that account for dosing adjustments. We still have yet to understand how many of these receptor adaptations develop *in vivo*, with limited opportunity to involve MMT patients. Despite the identification of several adaptations to chronic opioid agonist treatment, the molecular mechanism underlying these processes has yet to be definitively established. There are many phenomena

associated with adaptations to chronic methadone administration and they continue to create difficulties for clinicians who need to establish appropriate dosing.

The logical step forward from this work is to investigate the downstream signaling pathways, explore cAMP production (AC activity), and protein kinases using their various inhibitors. The specific agents able to regulate cyclic AMP levels are either adenylyl cyclase, which induces cAMP production, or phosphodiesterases, which break down cAMP.

Another area of focus would be inhibition or induction of protein kinases. Protein kinase control may involve several mechanisms, including protein conformational changes, or phosphorylation (or some other post-translational modification) of the protein kinase that results in increased or decreased activity.

One of the leading challenges is to provide a more detailed map of cAMP signalling associated with methadone induced GPCR signalling pathways. In this regard, future work could aim to identify local domains for which cAMP signals may affect a specific function. These strategies could help achieve valid therapeutic approaches to methadone dosing, if a biochemical marker(s) could be used to estimate signalling efficiencies.

Methadone PK/PD is also affected by genetic polymorphisms of drug transporters. This line of research may also provide a plausible explanation for the variation we observe amongst individuals in the dosing requirements for many drugs, including methadone. Further evaluation of these phenomena is definitely warranted.

The most valuable studies in the future will likely analyze multiple phenomena which could be coalesced into a single model to help explain tolerance, dependence and appropriate dosing requirements for methadone.

Reference List

1. Garrido M, Troconiz I F. Methadone: a review of its pharmacokinetic/pharmacodynamic properties. *Journal of Pharmacology and Toxicology* 1999;42:61-6.
2. Gagajewski A, Apple FS. Methadone-related deaths in Hennepin County Minnesota. *Journal of Forensic Sciences* 2003;48:1-4.
3. Jansson L J, Choo R, Velez M L, Lowe R, Huestis M A. Methadone Maintenance and long Term Lactation. *Breastfeeding Medicine* 2007;3:34-7.
4. Kreek M J. Methadone-related opioid agonist pharmacotherapy for heroin addiction. History, recent molecular and neurochemical research and future in mainstream medicine. *Annals of the New York Academy of Science* 2000;909:186-216.
5. Fareed A, Casarella J, Amar R, Vayalapalli S, Drexler K. Methadone maintenance dosing guideline for opioid dependence, a literature review. *Journal of Addictive Diseases* 2010;29:1-14.
6. Fareed A, Casarella J, Roberts M, Sleboda M, Amar R, Vayalapalli S, Drexler K. High dose versus moderate dose methadone maintenance: is there a better outcome? *Journal of Addictive Diseases* 2009;28:399-405.
7. Krantz MJ, Lewkowicz L, Hays H, Woodroffe MA, Robertson AD, Mehler PS. Torsade de pointes associated with very-high-dose methadone. *Annals of Internal Medicine* 2002;137:501-4.
8. Maxwell S, Shinderman MS. Optimizing long-term response to methadone maintenance treatment: a 152-week follow-up using higher-dose methadone. *Journal of Addictive Diseases* 2002;21:1-12.
9. Eap C B, Buclin T, Baumann P. Interindividual variability of the Clinical Pharmacokinetics of Methadone. *Clinical Pharmacokinetics* 2002;41:1153-93.
10. Dorsey JS. Serum methadone levels and optimal dosing in methadone maintained patients. 2003. Washington, DC. 2003.
Ref Type: Conference Proceeding
11. Leavitt SB, Shinderman M, Maxwell S, Eap CB, Paris P. When "enough" is not enough: new perspectives on optimal methadone maintenance dose. *Mt. Sinai Journal of Medicine* 2000;67:404-11.
12. Auret K Mea. Pharmacokinetics and Pharmacodynamics of Methadone Enantiomers in Hospice Patients with Cancer Pain. *Therapeutic Drug Monitoring* 2010;28:359-66.

13. Lugo R A, Satterfield K L, Kern S E. Pharmacokinetics of Methadone. *Journal of Pain and Palliative Care Pharmacotherapy* 2005;19:13-24.
14. Boulton D W P, Arnaud P MP, Devane C L. Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clinical Pharmacology and Therapeutics* 2001;70:48-57.
15. Matsui Aya and Williams John T. Activation of μ -opioid receptors and block of Kir3 potassium channels and NMDA receptor conductance by l- and d-methadone in rat locus coeruleus. *British Journal of Pharmacology* 2010;161:1403-13.
16. Foster D J, Somoqyi A A, Dyer K R, White J M, Bochner F. Steady -state pharmacokinetics of (R)- and (S)-methadone in methadone maintenance patients. *British Journal of Clinical Pharmacology* 2000;50:427-40.
17. Foster D J R, Somoqyi A A, White J M, Bochner F. Population pharmacokinetics of (R)-, (S)- and rac-methadone in methadone maintenance patients. *British Journal of Clinical Pharmacology* 2004;57:742-55.
18. Li Y, Kantelip J P, Gerritsen-van Schieveen P, Davani S. Interindividual Variability of Methadone Response: impact of genetic polymorphism. *Molecular Diagnostic Therapy* 2008;12:109-24.
19. Ferrari A, Coccia C P, Bertolini A, Sternieri E. Methadone - metabolism, pharmacokinetics and interactions. *Pharmacological Research* 2004;50:551-9.
20. Kharasch E D M^{Pea}. Methadone Pharmacokinetics Are Independent of Cytochrome P4503A (CYP3A) Activity and Gastrointestinal Drug Transport. *Anesthesiology* 2009;110:660-72.
21. Lotsch J MP. Pharmacokinetic - Pharmacodynamic Modeling of Opioids. *Journal of Pain and Symptom Management* 2005;29:S90-S103.
22. Hanna J, Foster D J, Salter A, Somoqyi A A, White J M, Bochner F. Within - and between - subject variability in methadone pharmacokinetics and pharmacodynamics in methadone maintenance subjects. *British Journal of Clinical Pharmacology* 2005;60:404-13.
23. Waldhoer M, Bartlett S E, Whistler J L. Opioid Receptors. *Annual Reviews in Biochemistry* 2004;73:953-90.
24. Garrido M, Troconiz I F. Methadone: a review of its pharmacokinetic/pharmacodynamic properties. *Journal of Pharmacology and Toxicology* 1999;42:61-6.

25. College of Physicians and Surgeons of Saskatchewan Saskatchewan Health. Saskatchewan Methadone Guidelines for the Treatment of Opioid Addiction. 2008. Saskatoon, Canada.
26. Byrne A J. Ten patients prescribed high dose methadone maintenance in general practice. *Medical Journal of Australia* 1996;165:239.
27. Meadon E R, Hoggard P G, Khoo S H, Back D J. Determination of P-Glycoprotein and MRP1 expression and function in peripheral blood mononuclear cells in vivo. *Journal of Immunological Methods* 2002;262:159-65.
28. Crettol S, Déglon JJ, Besson J, Croquette-Krokar M, Hämmig R, Gothuey I et al. ABCB1 and cytochrome P450 genotypes and phenotypes: influence on methadone plasma levels and response to treatment. *Clinical Pharmacology and Therapeutics* 2006;80:668-81.
29. Wolff K, Boys A, Rostami-Hodjegan A, Hay A, Raistrick D. Changes to methadone clearance during pregnancy. *European Journal of Clinical Pharmacology* 2004;61:763-8.
30. Glatstein M M Mea. Methadone exposure during lactation. *Canadian Family Physician* 2008;54:1689-90.
31. Jansson L M, Choo R, Velez M L, Harrow C, Schroeder J R, Shakleya D M, Huestis M A. Methadone Maintenance and Breastfeeding in the Neonatal Period. *Pediatrics* 2008;121:106-14.
32. Jansson L M M, Velez M M, Harrow C R-B. Methadone Maintenance and Lactation: A Review of the literature and Current Management Guidelines. *Journal of Human Lactation* 2004;20:62-70.
33. Yang F, Tong X, McCarver DG, Hines RN, Beard DA. Population -Based Analysis of Methadone Distribution and Metabolism Using an Age-Dependent Physiological Based Pharmacokinetic Model. *Journal of Pharmacokinetics and Pharmacodynamics* 2006;33:485-518.
34. Mikolaenko I, Robinson A, Davis GG. A review of methadone deaths in Jefferson County, Alabama. *Journal of Forensic Medicine and Pathology* 2002;23:299-304.
35. Cabrera-Vera T M, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE. Insights into G Protein Structure, Function and Regulation. *Endocrine Reviews* 2003;24:765-81.
36. Guo Li a, Lindsey C.K., Aschenbach a, Hengjun He b, Dana E., Selley b, Yan Zhang. 14-O-Heterocyclic-substituted naltrexone derivatives as non-peptide mu

- opioid receptor selective antagonists: Design, synthesis, and biological studies. *Bioorganic & Medicinal Chemistry Letters* 2009;19:1825-9.
37. Gintzler A R, Chakrabarti S. Opioid Tolerance and the Emergence of new Opioid Receptor-Coupled Signaling. *Molecular Neurobiology* 2001;21:21-33.
 38. Hull L C, Llorente J, Gabra BH, Smith FL, Kelly E, Bailey C et al. The Effect of Protein Kinase C and G Protein-Coupled Receptor Kinase Inhibition on Tolerance Induced by μ -Opioid Agonists of Different Efficacy. *The Journal of Pharmacology and Experimental Therapeutics* 2010;332:1127-35.
 39. He L, Kim J A, Whistler J L. Biomarkers of morphine tolerance and dependence are prevented by morphine-induced endocytosis of a mutant μ -opioid receptor. *The FASEB Journal* 2009;23:4327-33.
 40. Morris Andrew J, Malbon Craig C. Physiological Regulation of G Protein-Linked Signaling. *Physiological Reviews* 1999;79:1373-430.
 41. Whistler hL, Chuang H H, Chu P. Functional Dissociation of μ Opioid Receptor Signaling and Endocytosis: Implications for the Biology of Opiate Tolerance and Addiction. *Neuron* 1999;23:737-46.
 42. Christie M J. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *British Journal of Pharmacology* 2008;154:384-96.
 43. Gintzler A R, Chakrabarti S. The Ambiguities of Opioid Tolerance Mechanisms: Barriers to Pain Therapeutics or New Pain Therapeutic Possibilities. *Perspectives in Pharmacology* 2008;325:709-13.
 44. DuPen A, Shen D, Ersek M. Mechanisms of Opioid-Induced Tolerance and Hyperalgesia. *Pain Management Nursing* 2007;8:113-21.
 45. Joseph E K, Reichling D B, Levine J D. Shared Mechanisms for Opioid Tolerance and a Transition to Chronic pain. *The Journal of Neuroscience* 2010;30:4660-6.
 46. Ingram S L. Cellular and molecular mechanisms of opioid action. *Progress in Brain Research* 2000;129:483-92.
 47. Anand K J, Willson DF, Berger J, Harrison R, Meert K L, Zimmerman J et al. Tolerance and Withdrawal From prolonged Opioid Use in Critically ill Children. *Pediatrics* 2010;125:1208-25.
 48. Connor M, Osborne P B, Christie M J. μ -Opioid receptor desensitization: Is morphine different? *British Journal of Pharmacology* 2004;143:685-96.
 49. Terman G W, Jin W, Cheong YP, Lowe J, Caron MG, Lefkowitz RJ, Chavkin C. G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. *British Journal of Pharmacology* 2004;141:55-64.

50. Ueda H, Inoue M, Matsumoto T. Protein kinase C-Mediated Inhibition of μ -Opioid receptor Internalization and Its Involvement in the Development of Acute Tolerance to Peripheral μ -Agonist Analgesia. *The Journal of Neuroscience* 2001;21:2967-73.
51. Schwartz R P, Jaffe J H, Highfiel D A, Callamna J M, O'Grady K E. A randomized controlled trial of interm methadone maintenance: 10 month follow-up. *Drug and Alcohol Dependence* 2007;86:30-6.
52. Christie M J. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *British Journal of Pharmacology* 2008;154:384-96.
53. Narita M, Suzuki M, Niikura K, Nakamura A, Miyatake M, Yajima Y, Suzuki T. μ -Opioid Receptor Internalization-dependent and -Independent Mechanisms of the Development of Tolerance to μ -Opioid Receptor Agonists: Comparison between Etorphine and Morphine. *Neuroscience* 2006;138:609-19.
54. Tso P H, Wong Y H. Molecular basis of Opioid Dependence: Role of signal Regulation By G-Proteins. *Clinical and Experimental Pharmacology and Physiology* 2003;30:307-16.
55. A.Claing, S.A.Laporte, M.G.Caron, R.J.Lefkowitz. Endocytosis of G protein-coupled receptors roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Progress in Neurobiology* 2002;66:61-79.
56. Bailey C P, Smith FL, Kelly E, Dewey WL, Henderson G. How important is protein kinase C in μ -opioid receptor desensitization and morphine tolerance? *TRENDS in Pharmacological Sciences* 2006;27:558-65.
57. Terman G W et al. G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. *British Journal of Pharmacology* 2004;141:55-64.
58. Xie G X, Pierce Palmer P. RGS Proteins: New Players in the Field of Opioid Signaling and Tolerance Mechanisms. *Anesthesia and Analgesia* 2005;100:1034-42.
59. DuPen A, Shen D, Ersek M. Mechanisms of Opioid-Induced Tolerance and Hyperalgesia. *Pain Management Nursing* 2007;8:113-21.
60. Bekhit M H M. Opioid-Induced Hyperalgesia and Tolerance. *American Journal of Therapeutics* 2010;17:498-510.
61. Gardell L R, King T, Ossipov M H, Rice KC, Lai J, Vanderah TW, Porreca F. Opioid receptor-mediated hyperalgesia and antinociceptive tolerance induced by sustained opiate delivery. *Neuroscience Letters* 2005;396:44-9.

62. Stefano G B, Kream R M, Esch T. Revisiting tolerance from the endogenous morphine perspective. *Medical Science Monitor* 2009;15:RA189-RA198.
63. Waldhoer M, Bartlett S E, Whistler J L. Opioid Receptors. *Annual Reviews in Biochemistry* 2004;73:953-90.
64. Tokuyama S, Nagae R, Mashida E, Hamabe W. Involvement of kappa opioid receptors in formalin-induced inhibition of analgesic tolerance to morphine in mice. *Journal of Pharmacy and Pharmacology* 2007;59:1109-15.
65. Hamabe W, Yamane H, Harada S, Tokuyama S. Involvement of kappa opioid receptors in the inhibition of receptor desensitization and PKC activation induced by repeated morphine treatment. *Pharmacy and Pharmacology* 2008;60:1183-8.
66. Mao J, Sung B, Ji RR, Lim G. Chronic Morphine Induces Downregulation of Spinal Glutamate Transporters: Implications in Morphine Tolerance and Abnormal Pain Sensitivity. *The Journal of Neuroscience* 2002;22:8312-23.
67. Smith FL, Jayed RR, Smith PA, Dewey WL, Gabra BH. PKC and PKA inhibitors reinstate morphine-induced behaviours in morphine tolerant mice. *Pharmacological Research* 2006;54:474-80.
68. Ueda H, Inoue M, Matsumoto T. Protein Kinase C-mediated inhibition of mu-opioid receptor internalization and its involvement in the development of acute tolerance to peripheral mu-agonist analgesia. *Journal of Neuroscience* 2001;21:2967-73.
69. Gintzler A R, Chakrabarti S. Post-opioid receptor adaptations to chronic morphine; Altered functionality and associations of signaling molecules. *Life Sciences* 2006;79:717-22.
70. Stafford K, Gomes A B, Shen J, Yoburn B C. μ -Opioid receptor downregulation contributes to opioid tolerance in vivo. *Pharmacology, Biochemistry and Behavior* 2001;69:233-7.
71. Chini B, Parenti M. G-Protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? *Journal of Molecular Endocrinology* 2004;32:325-38.
72. Qiu Y, Wang Y, Law PY, Chen HZ, Loh HH. Cholesterol regulates micro-opioid receptor-induced beta-arrestin 2 translocation to membrane lipid rafts. *Molecular Pharmacology* 2011;80:210-8.
73. Huang P, Xu W, Yoon Si, Chen C, Chong PL, Liu-Chen L Y. Cholesterol reduction by methyl-beta-cyclodextrin attenuates the delta opioid receptor-mediated signaling in neuronal cells but enhances it in non-neuronal cells. *Biochemical Pharmacology* 2007;73:534-49.

74. Levitt ES, Clark MJ, Jenkins PM, Martens JR, Traynor JR. Differential effect of membrane cholesterol removal on mu- and delta-opioid receptors: a parallel comparison of acute and chronic signaling to adenylyl cyclase. *Journal of Biological Chemistry* 2009;284:22108-22.
75. Huang P, Xu W, Yoon Si, Chen C, Chong PL, Unterwald EM, Liu-Chen LY. Agonist treatment did not affect association of mu opioid receptors with lipid rafts and cholesterol reduction had opposite effects on the receptor-mediated signaling in rat brain and CHO cells. *Brain Research* 2007;1184:46-56.
76. Liang D Y, Liao G, Lighthall GK, Peltz G, Clark DJ. Genetic variants of the P-glycoprotein gene *Abcb 1b* modulate opioid-induced hyperalgesia, tolerance and dependence. *Pharmacogenetics and Genomics* 2006;16:825-35.
77. Allegaert K, van den Anker J N, Naulaers G, de Hoon J. Determinants of drug metabolism in early neonatal life. *Current Clinical Pharmacology* 2007;2:23-9.
78. Stafford K, Gomes A B, Shen J, Yoburn B C. μ -Opioid receptor downregulation contributes to opioid tolerance in vivo. *Pharmacology, Biochemistry and Behavior* 2001;69:233-7.
79. van Hermert F, Lazova M D, Snaar-Jagaska B E, Schmidt T. Mobility of G proteins in heterogeneous and polarized during chemotaxis. *Journal of Cell Science* 2010;123:2922-30.
80. Suresh S M, Anand K J S. Opioid tolerance in neonates: a state-of-the-art review. *Paediatric Anaesthesia* 2001;11:511-21.
81. Kim K S, Lee K W, Im J Y, Yoo JY, Kim SW, Lee JK et al. Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. *Proceedings of the National Academy of Science U S A* 2005;103:3908-13.
82. Williams Christine. cAMP detection methods in HTS: selecting the best from the rest. *Nature Reviews Drug Discovery* 2004;3:125-35.
83. Bie B, Peng Y, Zhang Y, Pan Z Z. cAMP-Mediated Mechanisms for Pain Sensitization during Opioid Withdrawal. *The Journal of Neuroscience* 2005;25:3824-32.
84. Gupta A, Mulder J, Gomes I, Rozenfeld R, Bushlin I, Ong E et al. Increased Abundance of Opioid Receptor Heteromers After Chronic Morphine Administration. *Science Signaling* 2010;3:1-7.
85. Williams J T, Christie MJ, Manzoni O. Cellular and Synaptic Adaptations Mediating Opioid Dependence. *Physiological Reviews* 2001;81:299-343.

86. Berger A C, Whistler J L. How to Design an Opioid Drug That Causes Reduced Tolerance and Dependence. *Annals of Neurology* 2010;67:559-69.
87. Klimeki Walter T, Futscher Bernard W, Grogan Thomas M, Dalton William S. P-Glycoprotein Expression and Function in Circulating Blood Cells From Normal Volunteers. *Blood* 1994;83:2451-8.
88. Mercadante S, Eldorado A. Delivery of opioid analgesics to the brain: role of blood-brain barrier. *Gene Therapy and Molecular Biology* 2009;13:82-90.
89. Aller S G, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R et al. Structure of P-glycoprotein Reveals a Molecular Basis for Poly-Specific Drug Binding. *Science* 2009;323:1718-22.
90. Giraud C, Manceau S, Treyluyer J-M. ABC transporters in human lymphocytes: expression, activity and role, modulating factors and consequences for antiretroviral therapies. *Expert Opinion in Drug Metabolism and Toxicology* 2011;6:571-89.
91. Pilarski Linda, Paine D, McElhaney JE, Cass CE, Belch AR. Multi drug Transporter P-Glycoprotein 170 as a Differentiation Antigen on Normal Human Lymphocytes and Thymocytes: Modulation With Differentiation Stage and During Aging. *American Journal of Hematology* 1995.
92. Lehotay DC, George S, Etter M L, Graybiel K, Eichhorst J C, Fern B. Free and bound enantiomers of methadone and its metabolite, EDDP in methadone maintenance treatment:relationship to dosage? *Clinical Biochemistry* 2005;38:1088-94.
93. Pallis M&D-GE. Flow Cytometric Measurement of Functional and Phenotypic P-Glycoprotein. In: R.D.Blumenthal , ed. **Totowa, NJ: © Humana Press Inc.**, 2006:167-81.
94. Chen, Jiezhong and Raymond, Kenneth. The role of CYP3A4 and p-glycoprotein in food-drug and herb-drug interactions. *Australian Pharmacist* 25[9], 732737. 2006.
Ref Type: Magazine Article
95. Best Deal. Use of non-prescribed methadone and other illicit drugs during methadone maintenance treatment. *Drug and Alcohol Review* 2000;19:9-16.
96. Saskatchewan Ministry of Health. Methadone Assisted Recovery Guidelines for Saskatchewan Addiction Counsellors. 1-35. 2004.
97. Pickard Hanna. The Purpose in Chronic Addiction. *AJOB Neuroscience* 2012;3:40-9.

98. Liang Wenbin, Lenton Simon, Allsop Steve, Chikrizhs Tanya. Does Availability of Illicit Drugs Mediate the Association between Mental Illness and Substance Abuse? *Substance Use and Misuse* 2011;46:1304-8.
99. Hartwell Karen J, Back Sudie E, McRae-Clark Aimee L, Shaftman Stephanie R, Brady Kathleen T. Motives for using: A comparison of prescription opioid, marijuana and cocaine dependent individuals. *Addictive Behaviors* 2012;37:373-8.
100. Kresina Thomas F, Lubran Robert. Improving Public Health Through Access to and Utilization of Medication Assisted Treatment. *International Journal of Environmental Research and Public Health* 2011;8:4102-17.
101. Sibinga N E S, Goldstein A. Opioid peptides and opioid receptors in cells of the immune system. *Annual Reviews in Immunology* 1988;6:219-249.
102. McCarthy, Szabo I, NJ, Pintar JE, Rogers TJ. Expression of functional mu-opioid receptors during T cell development. *Addiction* 2001;88:257-63.
103. Beck M, Mirmohammadsadegh A, Franz B, Blanke J, Hengge UR. Opioid receptors on white blood cells: effect on HIV infection and methadone treatment. *Pain* 2002;98:187-94.
104. Mombeini T, Roushanzamir F, Jorjani M, Pourpak Z, Gaskari S A, Mehr S E, Dehpour A R. Alteration in specific opioid-receptor labeling on peripheral blood leukocytes of bile duct-ligated rat. *Pathophysiology* 2006;13:111-7.
105. Vasquez E M, Petrenko Y, Jacobssen V, Sifontis NM, Testa G, Sankary H, and Benedetti E. An Assessment of P-Glycoprotein Expression and Activity in Peripheral Blood Lymphocytes of Transplant Patients. *Transplantation Proceedings* 37, 175-177. 2005.
106. Wang H Y, Frankfurt M, Burns L H. High-Affinity Naloxone Binding to Filamin A Prevents Mu Opioid Receptor-Gs Coupling Underlying Opioid Tolerance and Dependence. *Public Library of Science* 2008;3:1-10.
107. Wang Y, Van Bockstaele E J, Liu-Chen L Y. In vivo trafficking of endogenous opioid receptors. *Life Sciences* 2008;83:693-9.
108. Assay Designs, a division of ENZO Life Sciences. *Cyclic AMP Complete Manual*. 4-2-2008. Ann Arbor, MI, USA.
109. Zhao H., Loh HH, Law PY. Adenylyl Cyclase superactivation induced by long term treatment with opioid agonist is dependent on receptor localized within lipid rafts and is independent of receptor internalization. *Molecular Pharmacology* 2006;69:1421-32.

110. Clay, Adam T. Detection of the Human μ -Opioid Receptor In Leukocytes Isolated From Patients Enrolled in Methadone Maintenance Treatment. 1-42. 12-7-2009. University of Regina.
111. Eichhorst JC, Etter ML, Rousseaux N, Lehotay DC. Drugs of abuse testing by tandem mass spectrometry: a rapid, simple method to replace immunoassays. *Clinical Biochemistry* 2009;42:1531-42.
112. Wineker, R. Immunoassays Continue to Improve, Expand and Evolve. American Association for Clinical Chemistry. 1-9. 2008. Washington, D.C. Toxicology News.
113. Smink BE, Mathijssen MP, Lusthof KJ, de Gier JJ, Egberts AC, Uges DR. Comparison of urine and oral fluid as matrices for screening of thirty-three benzodiazepines and benzodiazepine-like substances using immunoassay and LC-MS(-MS). *Journal of Analytical Toxicology* 2006;30:478-85.
114. Pil K, Verstraete A. Current developments in drug testing in oral fluid. *Therapeutic Drug Monitoring* 2008;30:196-202.
115. Mortier KA, Dams R, Lambert WE, De Letter EA, Van Calenbergh S, De Leenheer AP. Determination of paramethoxyamphetamine and other amphetamine-related designer drugs by liquid chromatography/sonic spray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry* 2002;16:865-70.
116. Kintz P, Villain M, Concheiro M, Cirimele V. Screening and confirmatory method for benzodiazepines and hypnotics in oral fluid by LC-MS/MS. *Forensic Science International* 2005;150:213-20.
117. Fitzgerald RL, Rivera JD, Herold DA. Broad spectrum drug identification directly from urine, using liquid chromatography-tandem mass spectrometry. *Clinical Chemistry* 1999;45:t-34.
118. Allen KR, Azad R, Field HP, Blake DK. Replacement of immunoassay by LC tandem mass spectrometry for the routine measurement of drugs of abuse in oral fluid. *Annals of Clinical Biochemistry* 2005;42:277-84.
119. Maralikova B, Weinmann W. Confirmatory analysis for drugs of abuse in plasma and urine by high-performance liquid chromatography-tandem mass spectrometry with respect to criteria for compound identification. *Journal of Chromatography B Analytical Technology & Biomedical Life Science* 2004;811:21-30.
120. Tatsuno M, Nishikawa M, Katagi M, Tsuchihashi H. Simultaneous determination of illicit drugs in human urine by liquid chromatography-mass spectrometry. *Journal of Analytical Toxicology* 1996;20:281-6.

121. Weinmann W, Vogt S, Goerke R, Muller C, Bromberger A. Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS. *Forensic Science International* 2000;113:381-7.
122. Nordgren HK, Beck O. Multicomponent screening for drugs of abuse: direct analysis of urine by LC-MS-MS. *Therapeutic Drug Monitoring* 2004;26:90-7.
123. Jemal M. High-throughput quantitative bioanalysis by LC/MS/MS. *Biomedical Chromatography* 2000;14:422-9.
124. Sauvage FL, Gaulier JM, Lachatre G, Marquet P. Pitfalls and prevention strategies for liquid chromatography-tandem mass spectrometry in the selected reaction-monitoring mode for drug analysis. *Clinical Chemistry* 2008;54:1519-27.
125. Taylor PJ. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clinical Biochemistry* 2005;38:328-34.
126. Maurer HH. Current role of liquid chromatography-mass spectrometry in clinical and forensic toxicology. *Analytical & Bioanalytical Chemistry* 2007;388:1315-25.
127. Dams R, Huestis MA, Lambert WE, Murphy CM. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *Journal of American Society for Mass Spectrometry* 2003;14:1290-4.
128. Mallet CR, Lu Z, Mazzeo JR. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Communications in Mass Spectrometry* 2004;18:49-58.
129. Oliver Phillips, Forrest Robert, and Keen Jenny. Does the combined use of heroin or methadone and other substances increase the risk of overdose? Drug related death publications . 2-1-2007. London, UK, National Treatment Agency for Substance Misuse.
Ref Type: Magazine Article
130. Armstrong S C M, Wynn G H M, Sandson N B M. Pharmacokinetic Drug Interactions of Synthetic Opiate Analgesics. *Psychosomatics* 2009;2-169.
131. Concheiro M, De CA, Quintela O, Cruz A, Lopez-Rivadulla M. Determination of illicit drugs and their metabolites in human urine by liquid chromatography tandem mass spectrometry including relative ion intensity criterion. *Journal of Analytical Toxicology* 2007;31:573-80.
132. Meilera Aeal. Benzodiazepine use in a methadone maintenance programme: patient characteristics and the physician's dilemma. *Swiss Archives for Neurology and Psychiatry* 2005;6:156-64.

133. Lee H Y, Li J H, Wu L T, Wu J S, Yen C F, Tang H P. Survey of methadone - drug interactions among patients of methadone maintenance treatment program in Taiwan. *Substance Abuse and Treatment Prevention Policy* 2012;7:Epub ahead of print.
134. Kurdyak Peal. Use of other opioids during methadone therapy: a population based study. *Addiction* 2012;107:776-80.
135. Aeschbach Jachmann C et al. Office-based treatment in opioid dependence: a critical survey of prescription practices for opioid maintenance medications and concomitant benzodiazepines in Vienna, Austria. *European Addiction Research* 2008;14:206-12.
136. Eichhorst J, Etter M, Lepage J, Lehotay DC. Urinary screening for methylphenidate (Ritalin) abuse: a comparison of liquid chromatography-tandem mass spectrometry, gas chromatography-mass spectrometry, and immunoassay methods. *Clinical Biochemistry* 2004;37:175-83.
137. Jung BH, Rezk NL, Bridges AS, Corbett AH, Kashuba AD. Simultaneous determination of 17 antiretroviral drugs in human plasma for quantitative analysis with liquid chromatography-tandem mass spectrometry. *Biomedical Chromatography* 2007;21:1095-104.
138. Kharasch E D, Hoffer C, Whittingtin D, Walker A, Bedynek P S. Methadone Pharmacokinetics Are Independent of Cytochrome P4503A (CYP3A) Activity and Gastrointestinal Drug Transport. *Anesthesiology* 2009;110:660-72.
139. Kreek M J, Garfield JW, Gutjahr CL, et al. Rifampicin - induced methadone withdrawal. *New England Journal of Medicine* 1976;297:1104-6.
140. Suzuki Shunji et al. Morphine Upregulates *mu* Opioid Receptors of Human and Monkey Lymphocytes. *Biochemical and Biophysical Research Communications* 2000;279:621-8.
141. Vousooghi Nasim et al. Expression of mu opioid receptor splice variants mRNA in human blood lymphocytes: A peripheral marker of opioid addiction studies. *International Immunopharmacology* 2009;9:1016-20.
142. Kwak Y T, Koo MS, Choi CH, Sunwoo I. Change of dopamine receptor mRNA expression in lymphocyte of schizophrenic patients. *BMC Medical Genetics* 2001;2:3.
143. Talbot J N, Roman D L, Clark M J, Roof R A, Tesmer J J, Neubig R R, Traynor J R. Differential modulation of mu-opioid receptor signaling to adenylyl cyclase by regulators of G protein signaling proteins 4 or 8 and 7 in permeabilised C6 cells is G α subtype dependent. *Journal of Neurochemistry* 2010;112:1026-34.

144. Jordan B, Devi L A. Molecular mechanisms of opioid receptor signal transduction. *British Journal of Anaesthesia* 1998;81:12-9.
145. Talbot J N, Skifter DA, Bianchi E, Monaghan DT, Toews ML, Murrin LC. Regulation of mu opioid receptor internalization by scaffold protein PanBPM. *Neuroscience Letters* 2009;466:154-8.
146. Levitt Erica S, Purington Lauren C, Traynor John R. $G_{i/o}$ - Coupled Receptors Compete for Signalling to Adenylyl Cyclase in SH-SY5Y Cells and Reduce Opioid-Mediated cAMP Overshoot. *Molecular Pharmacology* 2011;79:461-71.
147. Xu W, Yoon Si, Huang P, Wang Y, Chen C, Chong PL, Liu-Chen LY. Localization of the kappa opioid receptor in lipid rafts. *Journal of Pharmacology and Experimental Therapeutics* 2006;317:1295-306.
148. Wang Zaijie, Bilsky EJ, Wang D, Porreca F, Sadée W. 3-Isobutyl-1-methylxanthine inhibits basal μ -opioid receptor phosphorylation and reverses acute morphine tolerance and dependence in mice. *European Journal of Pharmacology* 1999;371:1-9.
149. Bekhit M H M. Opioid-Induced Hyperalgesia and Tolerance. *American Journal of Therapeutics* 2010;17:498-510.
150. Steiner Debora, Avidor-Reiss T, Schallmach E, Butovsky E, Lev N, Vogel Z. Regulation of adenylyl cyclase type VIII splice variants by acute and chronic-coupled receptor activation. *Biochemistry Journal* 2005;386:341-8.
151. Ammer Hermann, Christ Thomas C. Identity of adenylyl cyclase isoform determines the G protein mediating chronic opioid-induced adenylyl cyclase supersensitivity. *Journal of Neurochemistry* 2002;83:818-27.
152. Zaccolo M. cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. *British Journal of Pharmacology* 2009;158:50-60.
153. Zaccolo M. Spatial control of cAMP signalling in health and disease. *Current Opinion in Pharmacology* 2011;11:649-55.
154. Buchard Anders et al. Postmortem Blood Concentrations of R- and S-enantiomers of Methadone and EDDP in Drug Users: Influence of Co-medication and P-glycoprotein Genotype. *Journal of Forensic Sciences* 2010;55:457-63.
155. Chinn L W, Koetz D L. ABCB1 pharmacogenetics: progress, pitfalls, and promise. *Clinical Pharmacology & Therapeutics* 2007;81:265-9.

156. Kolesar J, Brundage R C, Pomplun M, Alberti D, Holen K, Traynor A et al. Population pharmacokinetics of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine©) in cancer patients. *Cancer Chemotherapy and Pharmacology* 2011;67:393-400.
157. Akbas S H, Bilgen T, Keser I, Tuncer M, Yucetin L, Tosun O et al. The effect of MDR1 (ABCB1) polymorphism on the pharmacokinetics of tacrolimus in Turkish renal transplant recipients. *Transplantation Proceedings* 2006;38:1290-2.
158. Kim K A, Park P W, Park J Y. Effect of ABCB1 (MDR1) haplotypes derived from G2677T/C3435T on the pharmacokinetics of amlodipine in healthy subjects. *British Journal of Clinical Pharmacology* 2007;63:53-8.

Appendix

Research proposal
Collaboration with other organizations

Letter of Collaboration

Dr. Wilhelmina H. Wildenboer
Regina Rehab and Family Medical Clinic
5950 Rochdale Blvd.
Regina, Saskatchewan
(306) 545-3700, Fax (306) 545-8105

October 9, 2009

Dr. Denis C. Lehotay
Saskatchewan Control Disease Lab
Regina, Saskatchewan
S4S 5W6

Re: "Molecular determinants of methadone tolerance."

Dear Denis,

I am excited about participating with you and your colleague, Dr. Tanya E. S. Dahms from the University of Regina, in the proposed research project on Opioid receptor binding and downstream signalling mechanisms in Methadone maintenance patients. I will inform patients about their opportunity to participate by providing a single collection of blood as well as a urine specimen. As discussed all of the patient information will be de-identified except for age, gender, length of time on methadone therapy and daily dosage.

I am optimistic that some of this research data will contribute to the challenging task of optimising methadone dosage in a safe, timely and effective manner. This will provide an opportunity to enhance the treatment of drug dependant individuals by understanding dosage requirements better and subsequently benefit both patients and clinicians.

I look forward to working with you in the future and wish you and Dr. Dahms success in the granting competition.

Sincerely,

Dr. W. Wildenboer

Appendix 1.0 Letter of Co-operation with Dr. Wildenboer, a certified addictions clinician in Regina.



Certificate of Re-Approval

PRINCIPAL INVESTIGATOR: Denis C. Lehotay; DEPARTMENT; Bio #: 05-150; INSTITUTION (S) WHERE RESEARCH WILL BE CARRIED OUT: Provincial Health Laboratory, Regina, SK; SUB-INVESTIGATOR(S): Eleanor J. Fernandez; FUNDER(S): UNFUNDED; TITLE: Relationship Between Dosage and Free Methadone Levels in Patients on Methadone Maintenance; RE-APPROVED ON: 13-Nov-2011; EXPIRY DATE: 14-Nov-2012

Full Board Meeting []
Delegated Review [x]

CERTIFICATION

The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This re-approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face meeting). Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This re-approval and the views of this REB have been documented in writing.

XXXXXXX

Jim Blackburn, Pharm.D., Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
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Saskatoon, SK S7N 4J8
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Appendix 2.0 Letter of ethics approval for the collection and analysis of blood samples from MMT subjects for the designated purposes of this study