The Effects of Elements of Cardiometabolic Syndrome on Metformin Disposition: Obesity and Hyperlipidemia

by

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Abstract

The influences of obesity, Roux-en-Y gastric bypass bariatric (RYGB) surgery and hyperlipidemia on metformin pharmacokinetics (PK) were investigated. A sensitive and novel HPLC-UV reverse phase assay method was developed and utilized. Detection and mass characterization for metformin metabolites were performed. In a non-blind single dose PK study, 16 post RYGB obese patients and 16 sex and body mass index (BMI) matched control subjects were administered 1000 mg metformin tablet orally, with plasma and urinary metformin levels being measured thereafter. Comparing PK parameters of metformin in surgically untreated obese subjects with healthy subjects in literature, it showed that obesity is associated with a lower metformin clearance, volume of distribution and bioavailability. RYGB surgery seemed to cause a normalization of these parameters. Because the effect of hyperlipidemia (HL) on metformin PK and its PK-dependent transporters (OCT-1, OCT-2 and MATE-1) was unknown, the poloxamer 407 (P407) rodent model of HL was used for assessment. HL was found to not influence the PK of metformin, neither was gene nor transporter protein expression affected. In literature, the ability of metformin to be metabolized is unclear. Although most studies indicated little if any metabolism, a few recent studies suggested substantial metabolism in rats given supratherapeutic doses. Examination of urine samples from rats and human suggested some peaks

that might be metabolites of metformin. Using rat hepatocytes there appeared to be chromatographic evidence of metabolite(s). Upon incubation of 100 μg/mL metformin for 30 min with isolated rat microsomes 1 mg protein/mL, we could qualitatively detect the presence of metabolites, one of which coeluted with structurally-similar guanylurea. Fractional separation and mass characterization revealed the presence of mixture of two main masses: one with m/z ratio of 103 which matched guanylurea m/z ratio retention time on HPLC run. The other was with 104.9 m/z ratio which is presumed reduced guanylurea. In conclusion, obesity may affect metformin PK. RYGB has a normalizing effect on metformin PK parameters. P407 model of HL has no effect on metformin related protein transporters PK parameters. Metformin seems to be metabolized only at very high concentrations and supratherapeutic doses.

Preface

The research project, of which this thesis is a part of, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Absorption of drugs post-bariatric surgery-Metformin", number Pro00004553, March 2009. The post-bariatric surgery was in research collaboration led by professor Raj Padwal at Faculty of Medicine and Dentistry, University of Alberta. In methodology section 2.2.2, the post-bariatric surgery study was published as Padwal RS, Gabr RQ, Sharma AM, Langkaas LA, Birch DW, Karmali S, Brocks DR, "Effect of gastric bypass surgery on the absorption and bioavailability of metformin. Diabetes Care journal, 2011, vol. 34, issue 6, 1295-300. I was responsible for the data collection and analysis as well as the manuscript composition. The method of metformin analysis in methodology section 2.2.1 was published as Gabr RQ, Padwal RS, Brocks DR, "Determination of metformin in human plasma and urine by high-performance liquid chromatography using small sample volume and conventional octadecyl silane column. Journal of Pharmacy and Pharmaceutical Sciences 2010, vol. 13,486–494 and it is an original work by Gabr RQ. The rest of work has not been previously published.

To my parents Laila and Qassem

To my lovely children Maryam and Hussain

To my brother Tariq and my twin sister Randa

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List of abbreviations and symbols

ABC	ATP-Binding cassette
ASCVD	Atherosclerotic Cardiovascular Disease
AUCp.o.	Area under the concentration versus time curve for oral route
AUMC	Area under the concentration versus first moment time curve
BMI	Body Mass Index
BCRP	Breast Cancer Resistance Protein
CAR	Constitutive Androsterone Receptor
CMS	Cardiometabolic Syndrome
CETP	Cholesteryl ester transfer protein
CLt	Total Clearance
CL	Clearance
CL/F	Oral Clearance
CLr	Renal Clearance
CLnr	Non Renal Clearance
Cmax	Maximum plasma drug concentration
CRP	C reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
CYP450	Cytochrome P450
C18	Octadecylsilane column
dL	Deci Litre
DME	Dulbecco’s modified eagle's
DMII	Type two diabetes mellitus
F	Oral bioavailability

FDA	Food and Drug Administration authority
HCL	Hydrochloride salt
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNF-4α	Hepatic nuclear transcription factor 4α
HL	Hyperlipidemia/Hyperlipidemic
HPLC	High performance liquid Chromatography
h	Hour
HER-2	Human epidermal growth factor receptor 2
ip	Intraperitoneal
iv	Intravenous
IS	Internal standard
KCL	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Kg	Kilogram
LC/MS	Liquid Chromatography/Mass Spectrometery
LDL	Low density lipoprotein
LKB1	Liver kinase B1
LLQ	Lower limit of quantification
Log P	Logarithm value of octanol/water partition coefficient
LP	Lipoproteins
LPL	Lipoprotein lipase
LXR- α	liver X receptor α
L	Liter
µL	Micro liter
mL	Milli liter

µM	Micro Mole
mg	Milligram
µg	Micogram
min	Minutes
MATE	Multidrug and toxin extrusion protein transporter
MDR	Multiple drug resistance protein
MRP	multidrug resistance gene associated proteins
mRNA	Messenger RNA
MS	Mass spectrometer
MTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate terra sodium
NCEP	National Cholesterol Education Program Adult Treatment Panel III
NL	Normolipidemic
NMR	Nuclear Magnetic Resonance analysis
ng	Nanogram
Nrf-2	Nuclear factor erythroid related factor 2
OAT	Organic anion transporter protein
OATP	Organic anion transporter polypeptide
OCT	Organic cationic transporters
OC	Organic cations
ºC	Degree Celsius
PK	Pharmacokinetics/ Pharmacokinetic
pKa	Acid dissociation constant
pH	negative log of hydrogen cation activity in an aqueous solution
PPARGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
PPAR-α	peroxisome proliferator-activated receptor α

PXR	Pregnane X receptor
P407	Poloxamer 407
P-gp	P-glycoprotein
POMC	pro-opiomelanocortin signaling
r^2	Correlation coefficient
RT	Room temperature
Rpm	Rotations per minute
SDS	Sodium Dodecyl Sulphate
SD	Standard deviation
SLC	Solute carrier family
SHP	Small heterodimer partner
SP-1	Specificity protein 1
SREBP-1	Sterol regulatory element binding protein
t ½	Terminal elimination phase half-life
TEA	tetraethylammonium
TG	Triglyceride
TC	Total cholesterol
Tmax	Time to reach Cmax
TBW	Total Body Weight
TNF-α	Tumour necrosis factor α
UV	Ultra-violet
Vd	Volume of distribution
Vdss	Volume of distribution at steady state
vs	Versus
VLDL	Very low density lipoprotein
WHO	World Health Organization

α	Level of significance
λz	Elimination rate constant

1. Introduction

1.1. Cardiometabolic syndrome

Many patients within the Canadian healthcare system are living with one or more chronic diseases (1). Cardiovascular disease (CVD), chronic obstructive pulmonary disease, cancer and type II diabetes mellitus (T2DM) are the most common causes of hospitalization and premature death in Canada constituting almost three quarters of all deaths (2). Knowing more about the risk factors and indicators for chronic diseases may help public health efforts addressing this growing concern.

Cardiometabolic syndrome (CMS) which is known as metabolic syndrome, syndrome X and metabolic syndrome X, is defined as a multiplex risk factor which combines several risk correlates mostly of metabolic origin. Atherogenic dyslipidemia along with hyperglycemia and abdominal obesity are the main metabolic risks that are commonly used to define CMS (3). CMS is highly associated with a number of chronic diseases such as atherosclerotic cardiovascular disease (ASCVD) (4), T2DM (5), cancer (6) and chronic kidney disease (7). For example, the risk for ASCVD development is approximately doubled in a patient with CMS compared with a patient without the syndrome (8). Moreover, in patients who do not have T2DM, the likelihood of developing the disease is increased approximately 5-folds (9). In Canada, 14.9% of Canadian adults were

reported to have CMS according to recruited data from a cross-sectional survey conducted by statistics Canada in 2007-2009. The importance of CMS for public health was demonstrated by its significant association with chronic diseases as mentioned above in relation to the general population, which renders it a relevant risk factor in the development of chronic diseases (10).

Obesity and low physical activity are thought to be the underlying causes behind CMS (11, 12) in addition to socioeconomic status (13) and metabolic susceptibility. Susceptibility factors include adipose tissue disorders which are typically manifested as abdominal obesity, genetic and racial factors, aging, and endocrine disorders (3) (14). Clinical criteria that aid to diagnose CMS are determined by the World Health Organization (WHO), US National Cholesterol Education Program Adult Treatment Panel III (NCEP), and International Diabetes Federation (IDF). The used criteria for CMS diagnosis have been conditioned in part by views of the pathogenesis of the syndrome. For example, WHO has recognized insulin resistance as the dominant cause of the CMS. Accordingly, clinical indicators of insulin resistance were required for the diagnosis (15). However, with the growing evidence for the critical role of abdominal obesity, NCEP has replaced insulin resistance with increased waist circumference (abdominal obesity) as the main clinical criteria (16). Both NCEP and IDF have determined the same set of the clinical criteria for CMS diagnosis (17). The level of hyperglycemia was set to be

100-110 mg/dL Triglycerides (TG) should be equal to or higher than 150 mg/dL while high density lipoprotein (HDL) should be less than 40 and 50 mg/dL in men and women respectively. As for body weight, weight circumference should be ≥102 and 88 cm in men and women respectively. As for WHO, BMI was used as a different body weight caliber than waist circumference. According to WHO, BMI should be equal to or more than 30 kg/m^2 in CMS patients to diagnose obesity. In addition, insulin resistance and microalbuminurea were other clinical signs used by WHO to define CMS (15). The most common symptoms shared between different health organizations definitions for CMS are obesity, HL and hyperglycemia.

Metformin, is a first-line drug for T2DM and is one of the most commonly prescribed drugs worldwide especially for the obese and overweight (18). Metformin mechanism of action is mainly due to activation of AMPK enzyme Treatment with metformin is associated with weight loss, lowering plasma TG and low density lipoprotein (LDL) aside from its main effect on hyperglycemia making it the drug of choice in treatment of CMS patients. Metformin mechanism of action is mainly due to activation of AMP activated protein kinase (AMPK) enzyme which lead to most of its pharmacological actions (19, 20). Despite the discovery of its potential benefits in lowering blood glucose in 1950's, it has been rediscovered for other promising therapeutic indications. Recent studies suggested that it possessed anticancer (21, 22), cardio-protective effects (23) and therapeutic

effect on polycystic ovary syndrome (24) . To date, little is known about the impact of obesity and HL, the two arms of CMS, on the pharmacokinetic (PK) of metformin.

1.2. Obesity

Over the past 40 years, obesity has become major public health concern in most societies. By definition, obesity is a metabolic disorder which is evident when abnormal or excessive accumulation of adipose tissue takes place with adverse medical consequences (25). BMI, an approved indirect measure of anthropometrics incorporating body weight and height, classifies people as overweight (pre-obese) if their BMIs range from 25 to 29.9 kg/m^2, and obese if equal or greater than 30 kg/m^2(26). Obesity has been recognized as an epidemic health issue since 1997 (27). Its prevalence has increased by 400% in the last two decades and continues to rise. Severe or morbid obesity (Class III or BMI $\geq$ 40 kg/m^2) is the fastest growing subgroup of the disorder (28).

Obesity is associated with various types of serious medical comorbidities such as hypertension, insulin resistance and T2DM, HL, atherosclerosis and other cardiovascular diseases, many of which necessitate pharmacological intervention (25). At least 44% of diabetes cases and 23% of cases of ischemic heart disease have been attributable to obesity (25). Obesity is also associated with osteoarthritis

largely due to the increased weight load on the load-bearing joints (29). Sleep apnea is another disorder associated with excessive body mass (25). To illustrate the effect of obesity on overall mortality, it was found that an increase in BMI of 5 kg/m^2 is associated with a 30% higher mortality risk (30). Severe obesity, in particular, is associated with a 13-18 fold increased risk of T2DM compared to normal weight individuals (31). It also shortens life expectancy by 8-13 years (32) and dramatically reduces quality of life. In addition to the major health and socioeconomic implications, obesity poses challenges to the practicing physician in ensuring the optimal therapeutic dosing of drugs (25).

Physiological characteristics in the obese differ from non-obese or lean subjects. Observed differences were in regional blood flow, hormonal release, cardiac output, fat versus (vs) non-fat tissue mass (33), and expression of pro-inflammatory cytokines (34, 35). These pathophysiological changes can lead to alterations in the PK of medications used for the treatment of obesity-related comorbidities. This may necessitate adjustments to the dosage regimen. This is a highly relevant concern as obese individuals typically receive multidrug therapy. A population-based study conducted within general practices in the United Kingdom found that 30 - 46% of obese patients received therapy with either central nervous system acting drugs, antimicrobials, cardiovascular drugs or agents for musculoskeletal disease over an 18-month period (36). The HL and insulin

resistance which are usually associated with obesity are predisposing factors for observed comorbidities along with the chronic inflammatory condition.

1.2.1. Effect of obesity on pharmacokinetics of drugs

Most focus on the effects of obesity on PK has been placed on the optimal body descriptor to be used as a dose scalar (37-39). Such measures include total body, ideal and lean body weight, body surface area and BMI. However, this mostly empirical approach by nature mostly focuses on the partitioning of drug into the tissues and calculation of Vd. The Vd is of most practical use in determining initial dosing requirements (e.g., a loading dose) and of limited use for estimating chronic dosing regimens. Drug CL is not expected to be directly associated with anthropometrics of body mass, yet it is the main parameter to consider when examining the influence of disease states on drug exposure with repeated doses. From the relatively limited data that have been published, it is difficult to make generalized conclusions regarding the effects of obesity on drug disposition (37-40). We can more closely examine the relationship between drug PK and obesity by looking at the determinants of drug PK and resultant concentrations in the body.

1.2.1.1. Absorption

In many cases, the rate or amount of orally absorbed drug does not appear to significantly differ between obese and non-obese subjects. Examples include cyclosporine A (41), dexfenfluramine (42), midazolam (43), and propranolol (44).

For other routes of administration, there are some data focused on extravascular routes of drug absorption in the obese population. For example the subcutaneous absorption of a low-molecular-weight heparin, enoxaparin, was examined in obese and lean volunteers (45). Antifactor Xa and anti-factor IIa activity levels were used as surrogates or activity markers for enoxaparin after once-daily subcutaneous administration, and after a single intravenous (iv) infusion. The rate of enoxaparin absorption after subcutaneous administration was slower in the obese volunteers, with median time to reach maximum activity level being 1 h longer than lean subjects for both anti-factor Xa and anti-factor IIa. However, the extent of absorption appeared to be complete in both groups.

Absorptive mechanisms for some drugs are transporter dependent. In this case, any change in the level of expression or function of transporters may affect the process of absorption. Drug transporter proteins are present in localized segments of the small intestine and colonic regions of the gastrointestinal tract. The possible impact

of obesity on intestinal transporter function has presently not been well characterized.

1.2.1.2. Distribution

Drug distribution depends both upon the physicochemical properties of the drug as well as tissue makeup and quantity. The Vd, which reflects the ability of the drug to leave the blood and enter the tissues can be influenced by increases in adiposity or extra fat in tissues. Drug plasma protein binding can also influence Vd. The ability of a lipophilic drug to penetrate into excess tissue stores of adipose is expectedly higher than a hydrophilic drug. As a consequence, the Vd of lipophilic drugs may increase in the obese state. For example, highly lipophilic drugs such as diazepam, verapamil, and sufentanil exhibit a total body weight (TBW) normalized Vd ratio between the obese and non-obese of more than one (1.3 -1.9). This indicates that the extra adipose tissue in obese subjects increases the Vd by preferential uptake of those lipophilic drugs (33). Thus, for moderate to highly lipophilic drugs, TBW is the preferred parameter to account for Vd in obese individual (38). In contrast, for hydrophilic drugs with low octanol-water partition coefficient (log P) the Vd based on total body mass should expectedly decrease because as a proportion of the total body mass, there is proportionately less tissue space available for the drug to penetrate. In some cases, drugs with a higher log P

were found to have a reduced Vd/TBW ratio (e.g. cyclosporine A and methylprednisolone). Other factors aside from oil to water partition coefficient clearly control penetration into adipose tissue. Such factors may include molecular size and chemical structure.

Protein binding is the most crucial determinant of Vd, with the major binding proteins being albumin, α1-acid glycoprotein and lipoproteins (LP). It has been reported that serum albumin is unaltered in both moderate and morbid obesity (33). In line with this, it has been seen that the plasma protein binding of weakly acidic drugs such as thiopental and phenytoin, which bind primarily to albumin, appears unaltered by obesity. In obesity serum levels of pro-inflammatory cytokines are significantly elevated. One of the acute phase reactant proteins, orosomucoid protein, was shown to be positively correlated with mRNA expression of TNF-α, IL-6, and adiponectin in obese mice as well as with BMI. This is consistent with higher serum concentrations of α1-acid glycoprotein, which are known to rise in inflammation. This can have implications on basic drugs which have high affinity to bind with α1-acid glycoprotein such as clindamycin and propranolol (46). LP, which can bind lipophilic drugs and increase or decrease drug uptake into specific tissues (47), are often increased in obese patients (48).

1.2.1.3. Metabolism

Some pathophysiological changes associated with obesity may possibly change drug metabolism and hepatic drug transport (49). In morbidly obese people, fatty liver infiltration resembles alcoholic hepatitis and may induce liver damage (25). However, changes in liver function enzymes are not routinely seen in obesity (37, 50). In situations where fatty liver infiltration compromises hepatic function, concentrations of drug-binding plasma proteins may decrease, which depending on the drug, might increase Vd and possibly CL of the total (bound+unbound) drug.

The hepatic cytochrome P450 (CYP450) family of enzymes are important in phase I oxidative drug metabolism. Hepatic CYP2E1, which mediates endogenous metabolism of fatty acids, ketones, and ethanol, plays a role in the development of non-alcoholic fatty liver disease. Chronic exposure to large amounts of fatty acids and ketones, which are elevated in obese subjects, can induce CYP2E1, leading to free radical formation, lipid peroxidation and consequently liver injury (51). Thus, expectedly, morbid obesity can increase CYP2E1 activity (33). Inhalation anesthetics such as methoxyflurane and halothane are substrates of CYP2E1 and were found to have higher CL in obese individuals as compared to non-obese controls (52). Changes to CYP activity in the obese appears to be isozyme-specific,

with increases seen in CYP2E1 but observed decreases in CYP3A4. However the effect on other CYP isoenzymes are less clear (52).

With respect to phase II metabolism, obesity appears to decrease the conjugative pathways of glucuronidation. The expression levels of uridine diphosphate-glucuronosyltransferases 1A1, 1A6, 2B1, and CYP2C11 mRNA in liver were lower (30–50%) in Zucker compared with lean Sprague-Dawley rats (53). In addition hepatic glutathione S-transferases and quinone reductase mRNA were lower by 70 and 30% in obese Zucker rats respectively (53).

On the other hand, the CL of the benzodiazepines oxazepam and lorazepam, each of which is excreted primarily as glucuronyl conjugates, were enhanced in obese individuals compared to lean subjects. However, when normalized to body weight no difference was seen in CL (54). Acetaminophen, which has sulfation and glucuronidation as primary elimination pathways, was found to have higher CL in obese volunteers than in the normal weight controls but when corrected for body weight, no desirable change was observed (55). These studies raise a salient point, in that conclusions may differ depending on how the PK data is reported, be it normalized to body mass or not. In some cases, greater obesity-related CL may simply reflect a higher organ mass rather than an intrinsically higher capacity to clear a given drug.

1.2.1.4. Excretion

Obesity may affect CLr by changing either the rates of glomerular filtration (GFR) or tubular secretion. Chronic obesity and hypertension can cause renal injury which leads to a progressive lowering in GFR, greater arterial pressure and escalation of cardiovascular morbidity and mortality (50, 55). GFR is normally estimated by calculating the creatinine CL, which could be used to predict drug elimination and dosing requirements (37, 56). Alterations in non-body weight normalized creatinine CL have been observed in obese patients vs. lean patients. Because serum creatinine is dependent on muscle mass, and in obesity the muscle mass to total body mass ratio decreases, when using the Cockroft-Gault equation to estimate creatinine CL one should ideally use ideal body weight rather than the TBW (57).

Henegar et al. reported an altered renal excretion in the early stages of obesity as a result of increased kidney weight, Bowman's capsule size, renal blood flow and GFR in dogs (25, 58); thus an increased renal CL (CLr) of drugs subject to GFR can be anticipated. On the other hand, it was reported that GFR and perfusion of renal tissue appears similar in obese and lean subjects, provided they were normotensive and did not have microalbuminurea (59). In contrast, another investigation found that non-body weight normalized GFR was higher in obese

subjects; when normalized to lean weight, however, no difference was seen (60). Tubular reabsorption, which is highly dependent on urine pH and drug pKa, is expected to be less affected by obesity (50). However, visceral adipose tissue may physically compress the kidney, and this has been associated with increased intra-renal pressure and tubular reabsorption (61).

1.2.1.5. Drug transport proteins

Inflammation, present in obesity, is itself associated with changes in drug transporter expression. Inflammation in rats can lead to a suppression of P-glycoprotein (P-gp) in liver and various gastrointestinal segments (62, 63). Human hepatocytes exposed to the cytokines tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6) have likewise been seen to cause decreases in a number of the non-energy consuming transporters encoded by solute carrier (SLC) genes, including the organic anion transporting polypeptide (OATP)1B1/1B3/2B1, OCT-1, organic anion transporters 2 (OAT-2) and sodium taurocholate co-transporting polypeptide (64). These decreases followed a dose response curve, with larger declines occurring in the presence of greater concentrations of each cytokine. The same study found that the expression of bile salt export pump was diminished by TNF-α. Multiple drug resistance gene (MDR-1; coding for P-gp), multidrug resistance gene associated proteins (MRP-2 and 4), and breast cancer resistance

protein (BCRP) were unaffected. In contrast, IL-6 caused decreases in MDR-1, MRP-2 and 4, and BCRP (64). In the liver of rats exposed to bacterial lipopolysaccharide, findings similar to those in human hepatocytes were seen (65). Some of transporter substrates may represent medicinal agents that are commonly administered to obese subjects. For example, OATP2B1 plays a role in the intestinal absorption of several statins such as atorvastatin which is used to treat HL (66, 67). Although it has not been well studied, it is possible that in obesity there would be an inflammatory-related alteration in the expressions of protein transporters, with implications on drug PK. Some evidences showed a trend of down-regulation of transporter proteins in obesity. Hepatic OATP-2 and MRP-2 messenger RNA (mRNA) and protein levels were markedly reduced in obese Zucker rats (68). In obese/obese mice which represents a dual model for obesity and diabetes, renal Mrp-3, Oatp1a1, Oat-2 showed significant reductions with mRNA expression levels in line with hepatic Oatp1a1 gene and protein expression (69).

Metformin is commonly prescribed to obese population. Moreover, its PK parameters are highly affected by protein transporters especially CL which is mainly through tubular secretion. Little is known about the possible effects of obesity on transporters of metformin such as kidney OCT-1, OCT-2, MATE-1 and MATE2-K. Interestingly, in genetically engineered diabetic model of mice, which

exhibited deficient leptin receptor signaling and excessive weight gain, a reduced expression of Oct-2 mRNA was shown (70). To date, there is no available data on the effect of obesity either experimentally or clinically on metformin related transporters or on the CL of metformin which is exclusively transporters dependant.

The primary target in obesity treatment is induction and sustained maintenance of weight loss. However, successful weight reduction by conservative measures is particularly difficult in obese patients. In recent years there has been a substantial increase in the use of bariatric surgical procedures to reduce the body mass of morbidly obese patients. These procedures are highly effective in causing a large reduction in body mass (71). Because of the anatomical and physiological alterations that this approach may incur on the gastrointestinal system, there is a possibility of altered PK of orally administered medications. Other changes in drug PK beyond the gastrointestinal system modification are also possible.

1.3. Roux-en-Y gastric bypass surgery

Bariatric, or weight loss surgery, is recommended and considered the most suitable treatment option for morbidly obese patients in whom other dietary or medical weight loss modalities have failed despite its intensive nature (72). In 2008, about 220,000 bariatric surgeries were performed in the USA and Canada. More recently

it was estimated that close to 350,000 bariatric operations were performed worldwide, with 63% occurring in the USA and Canada (73). This rise in the conduct of these procedures is related to the rapidly increasing population of patients presenting with morbid obesity (74), and RYGB is considered the most effective way in which to alleviate many of the other serious comorbidities that obese patients are afflicted with, notably diabetes, hypertension, and dyslipidemia which also comprises CMS (25, 75, 76).

The surgical procedures used are typically either purely restrictive (gastric banding, gastroplasty), malabsorptive (jejunoileal bypass), or combined restrictive and malabsorptive (gastric bypass; biliopancreatic diversion) in nature (77). Laparoscopic RYGB, a procedure that circumvents the upper gut, is the most commonly performed bariatric procedure, accounting for nearly 47% of all procedures (78). RYGB surgery comprises about 80% of bariatric surgeries performed by American surgeons (79) and it represents the gold standard in the United States for weight loss in morbid obesity . It results in malabsorption in which the stomach capacity is reduced by 95%. The proximal portion of the stomach is reattached to a more central part of the small intestine, bypassing the duodenum and 50 to 70 cm of the jejunum (Figure 1) (80).

In Europe, the use of gastric bypass has nearly quadrupled from 11.1 to 39.0% of total bariatric surgeries performed since 2008, while the use of gastric banding had

decreased from 63.7 to 43.2% (81). RYGB has been documented to achieve up to 69% long-term weight loss (82, 83). The laparoscopic RYGB approach is associated with a more rapid recovery, less pulmonary complications and wound infections, and less postoperative pain compared to open procedures (72).

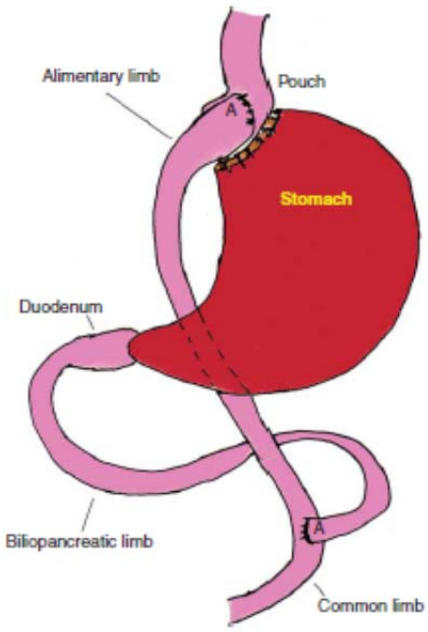


Figure 1: Schematic diagram of Roux-en-Y gastric bypass. The proximal stomach is separated from the distal stomach using surgical staples to form a small, restrictive gastric pouch (20-30 mL capacity). The mid-jejunum is transected (site A) and the distal section is connected to the pouch. The length of intestine leading from the duodenum is reconnected at the jejunal-ileal region. The reconnected pouch forms an alimentary limb which bypasses immediate contact with biliopancreatic secretions; the outcome resembles a Y shape configuration (hence the name). The length of small intestine distal to site at which the two limbs join is termed the common limb; this is where biliopancreatic secretions eventually mix with ingested food and most absorption occurs. The alimentary limb is typically 100 cm in length and the biliopancreatic limb is usually from 30 to 50 cm in length (73).

1.3.1. Effect of Roux-en-Y gastric bypass surgery on pharmacokinetics of drugs

RYGB surgery causes a number of anatomical and physiological alterations that may potentially influence the F of orally administered drugs. Indeed, bioavailability has been found to be lower for a number of drugs. Antimicrobial drugs such as azithromycin (84), nitrofurantoin and amoxicillin (85); the immunosuppressive agents cyclosporine A (86), tacrolimus , sirolimus and mycophenolic acid (87); the replacement hormone levothyroxine (88); and the -adrenergic blocker, talinolol (89) are reported to have their F affected by RVGB. The circulating concentrations of the anticonvulsants phenytoin and phenobarbital (90), the anticancer drug tamoxifen (91), and antifungal isavuconazole (92) were also reported to have lower F after RYGB.

In some cases, no significant changes were observed in oral drug absorption after RYGB. For example, no sustained alterations were seen in the maximum plasma concentration reached (Cmax), time at which maximum plasma concentration takes place (tmax), or area under concentration time curve after oral administration (AUCp.o.) of several antidepressants such as sertraline, venlafaxine, duloxetine, citalopram, and escitalopram (72). Each of these assessments was conducted in patients before and after surgery, with the drug PK being studied at different times

(1, 6, and 12 months) after surgery (72, 93). Although there was a temporary decrease in oral absorption at 1 month in post-RYGB individuals, absorption returned back to pre-RYGB level at 6 and 12 months, which shows that there could be time dependent changes in absorption after RYGB (25). These changes could be attributed to intestinal restoration of the absorptive surface area that gradually developed within the months following the surgery. Nevertheless, the authors suggested that transient increases in dosing requirements might be warranted in some patients after the surgery. With respect to the lipid-lowering drugs such as atorvastatin, RYGB surgery did not cause any change in its F (94). Recently, some antivirals were also investigated after RYGB. Tenofovir, emtricitabine, and darunavir/ritonavir PK parameters were not affected in a HIV patients who underwent RYGB (95) and the F of linezolid was not impaired by RYGB either (96).

It is difficult to see a clear pattern of RYGB on PK. Some of the studied drugs are substrates for drug transporter proteins. Examples include azithromycin (OATP, P-gp and MRP-2), nitrofurantoin (BCRP), and the cyclosporine A (P-gp) (97). These transporters can influence not only oral absorption and excretion, but also drug metabolism.

P-gp is expressed along the intestinal wall and acts to diminish the absorption of many pharmaceuticals (98). However, the expression of P-gp increases as one

moves from the proximal to the distal small intestine (99). Consequently, bypassing the proximal small intestine may increase the relative influence that P-gp plays in reducing cyclosporine A absorption as well as its plasma concentration (77).

Anatomical changes after RYGB alone might lead to a change in drug absorption. Bile salts and the extent of exposure to duodenal mucosa might affect absorption of highly lipophilic drugs such as azithromycin (84), cyclosporine A (86) and tacrolimus (87). In bypassing a large absorptive area of proximal small intestine, and reducing direct exposure of bile salts with drug at this region, a combination of reduced solubility and loss of mucosa for optimal absorption will take place which could explain the observed decrease in F after RYGB of lipophilic drugs such as azithromycin and tacrolimus.

Recently, it was reported that the tmax of a series of well absorbed compounds such as caffeine, tolbutamide, omeprazole, and midazolam were shortened after RYBG, suggesting an increase in the rate of absorption. However there was no change in changes in other PK parameters (100, 101). In contrast, the tmax of ethanol was longer and Cmax was reduced post-surgery for both RYGB procedures (102), suggesting a delay in absorption.

Based on the limited numbers of studies completed thus far, it is not possible to make comprehensive conclusion of the effect of RYGB surgery on drug pharmacokinetics. Only a few formal studies have been performed while a fair proportion of the available literature relies upon case reports.

1.4. Hyperlipidemia

As stated above, HL is one of the metabolic disorders used to define CMS. HL is a metabolic disorder characterized by abnormal elevation of serum lipids total cholesterol (TC) and TG. In Canada over 30% of all deaths are associated with cardiovascular diseases (103) where HL constitutes a major risk factor for the development and prognosis of CVD. It is a direct cause of atherosclerosis which itself is associated with major life-threatening cardiovascular illnesses such as myocardial infarction and stroke (104, 105). The etiological factors for HL development include genetic predisposition, sedentary life style, poor nutrition, metabolic abnormality such as T2DM and obesity and chronic intake of some drugs like cyclosporine A (106). Diabetic dyslipidemia, another form of HL, is a well-recognized and modifiable risk factor that must be identified and treated aggressively in order to reduce the associated cardiovascular risk in diabetic population. Dyslipidemia is an imminent feature of the CMS that accelerates atherosclerosis. High plasma concentrations of TG rich and ABLP, small dense

LDL, and depressed HDL are the main features for dyslipidemia diagnosis (107-110).

The difference between HL and dyslipidemia is the level of HDL; while it is elevated in HL, it is suppressed in dyslipidemia (111). Usually animal models imitate the condition wherein all LP are elevated. Lipoproteins exist in several forms and are classified based on size and density. They range from chylomicrons (largest size with lowest density) to very low, low, and high density (smallest size with highest density) LP (112). All LP are unique in that they are embedded with specialized apoproteins within their outer core. These proteins not only have important structural and enzymatic functions, but also act as ligands for the cellular endocytotic uptake by tissues from the blood.

1.4.1. Effects of hyperlipidemia on pharmacokinetics of drugs

The impact of HL on the disposition of lipophilic drugs in particular has been extensively studied. Lipophilic drugs have a natural propensity for encapsulation into the serum LP already elevated in HL. Lipophilic drugs are thus engulfed with and carried through the blood by plasma LP which represents an endogenous drug delivery system for hydrophobic moieties or lipid-based formulations (112).

Association of these lipophilic drugs within LP might have some implications on their PK properties as well as on their pharmacological and toxicological activities.

Cyclosporine A (106, 113, 114), amiodarone (115), halofantrine (116) and amphotericin B (117) are good examples to illustrate the impact of HL on lipophilic drug PK. For a highly hydrophilic drug like metformin, there is no binding to plasma LP. However, the possibility of HL induced molecular modulation of the transporters genes and protein remains with subsequent modulation of drug metabolism, distribution and excretion.

Elevated plasma LP are associated with attenuated expression of some genes and proteins. The expression of CYP450 enzymes in liver and other tissues are altered as well (47). The P407 induced HL model showed 1.94 fold lower total CYP content compared to normolipidemic (NL) Sprague-Dawley rats (118, 119). CYP2C11 and CYP3A1/2 isoenzymes were downregulated in the P407 model (118). Such down regulation is not restricted to the P407 rat model but was also documented in the obese Zucker rat model; CYP1A1, 1A6, 2B1, glutathione-S transferase, and quinine reductase were also inhibited in the obese Zucker rat model (53, 118). The down-regulation of CYP3A in obese Zucker rat is thought to be due to the lower constitutive androstane receptor (CAR) levels (120), while that of CYP 3A1/2 and CYP2C11 in P407 Sprague-Dawley rat is suggested to be due to decreased expression of CAR and pregnane X receptor (PXR) transcription factors (47). Thus at the molecular level, HL can interfere with transcriptional factors and consequently can interfere with drug metabolizing enzymes and

transport protein expression. Amiodarone metabolism was found to be lower in HL rats with respect to NL controls (118).

Hyperlipidemia could affect the renal and biliary excretion of medications, contributing to the net CL of the lipophilic drugs. However, the exact clinical contribution of each effect is not clearly reported in the literature. HL has been shown to decrease the glomerular filtration rate (121). In addition, the increase of bound drug to LP would potentially decrease the drug filtered passively by the glomerulus. HL has also shown an inhibitory effect on kidney P-gp activity. This could possibly result in an increase of CL of drugs transported via kidney P-gp such as cyclosporine (122).

1.4.2. Animal models used to assess the effects of hyperlipidemia

Experimentally, HL could be achieved in vivo using a selection of available animal models. These include the use of high fat diet (123, 124), genetic manipulation (125, 126) and treating animals with chemical compounds such as triton (127) and P407 (128) (129).

The P407-induced model of HL has been used to study the effect of HL on several drugs including cyclosporine A (106), amiodarone (130), nifedipine (131), halofantrine (116), and carbamazepine (132). In most of these studies, PK assessments were conducted within 24-36 h from the time of P407 i.p. injection

where large increases in plasma LP occur postdose. The P407 HL model provides a convenient and robust screening tool for the influence of LP on drug PK and PD aspects. However, the changes in LP levels occur over a short period of time, therefore, chronic changes to drug PK associated with HL may not be evident. Moreover, LP concentrations at this time (24-36 h) are significantly higher than are seen in humans while at later time points such as 108 h, LP elevations are not excessively high and are more comparable to what is present in humans with HL. The P407 HL model at 108 h post dose therefore perhaps offers an extended time window for more chronic actions of elevated lipoproteins than that present at peak LP levels at 36 h after P407 dosing (129).

1.4.3. Mechanism of P407 induced hyperlipidemia

P407 increases serum LP by inhibiting lipoprotein lipase (LPL), which facilitates the hydrolysis of TG (128). After 3 h of P407 i.p. injection in rats the LPL enzyme activity decreased by 95% with respect to saline treated rats (128). P407 also indirectly stimulates 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) which is assigned for cholesterol biosynthesis (133, 134). Cholesteryl ester transfer protein (CETP) is another enzyme for which activity is elevated upon P407 administration. When CETP activity is increased, a large number of small dense atherogenic LDL particles are produced (111). Concurrently, activity of

hepatic lipase involved in the metabolism of TG, was also significantly inhibited in P407 treated rats (135).

The P407 model of HL is reversible in nature (129). Within 2-3 days from the time of injection, the plasma or serum visually changes from clear to a deep milky white then back to a normal clear appearance over a period of days. The return to clear plasma indicates that LP levels are subsiding. The plasma concentrations of lipids have been followed after dosing of rats with a single dose of P407 of 0.4 g/kg, concentrations of TC, TG and HDL were determined to be subsiding within 12-24 h after dose administration. However, LDL was still rising and the other lipid levels were still substantially higher than baseline levels up to 48 h after dosing with the agent (132).

1.5. Metformin

The primary treatment goal of patients with diabetes is to control their hyperglycemia so as to reduce the occurrence of diabetes-related complications such as retinopathy, nephropathy and neuropathy. One of the most widely and effectively used oral medications for treatment of T2DM is metformin. Metformin was first introduced in the treatment of T2DM in 1957 to Europe and in 1995 was approved in US. In Canada, metformin was introduced in 1972. Since publication of the results of the United Kingdom prospective diabetes study in 1998,

metformin has become the most widely prescribed oral agent for the treatment of T2DM. Metformin is the most commonly prescribed antidiabetic in the world where more than 48 million prescriptions were filled in 2010 for its generic formulations in the USA (136, 137).

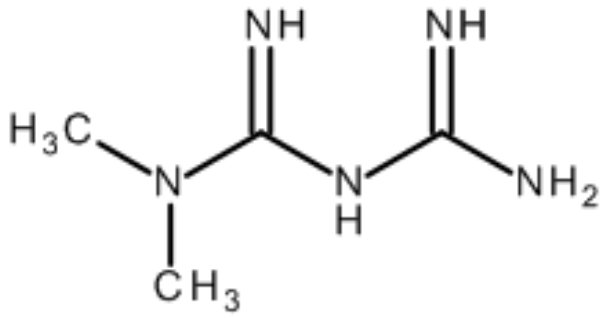
Administration of metformin has shown a 36% reduction in all-cause mortality, a 42% reduction in diabetes-related mortality and a 32% reduction in diabetes-related end points (138). At 2006, metformin was recommended by the International Diabetes Federation, the European Association for the Study of Diabetes and American Diabetes Association to be administered as the first-line treatment in all newly diagnosed patients, regardless of age (139, 140). Currently, metformin is the only oral antidiabetic approved for use in children (age > 10 years) with T2DM in Europe and USA (141).

1.5.1. Origin and physicochemical properties

Chemically metformin is a guanidine derivative that was extracted from *Galega officinalis*, the French lilac flower (142). It was then synthesized and found to reduce blood sugar in the 1920s. As new discoveries of insulin and other antidiabetic drugs were developed, metformin was largely forgotten until the 1940's. Interest in metformin returned then after several reports that showed it

could reduce blood sugar levels in rabbits, and in 1957, French physician Jean Sterne first published a clinical trial of metformin as a treatment for diabetes (136).

Chemically, it is a small molecular weight compound with 5 nitrogen containing groups and lacks heterocycles (Figure 2) which renders it a highly hydrophilic base. It has an acid dissociation constant value (pKa) of 12.4 (143) and, therefore, exists predominantly as the hydrophilic cationic species at physiological pH values (>99.9%). This high pKa makes it a stronger base than most other basic drugs with less than 0.01% unionized in blood. In addition, lipid solubility of the unionized species is slight as shown by its low log P value which is 1.43 (144). These physicochemical properties indicate a very low lipophilicity and, consequently, efficient diffusion of metformin through cell membranes must be facilitated. The dose of metformin is quoted as the HCl salt (molecular weight 165.63) but all concentrations in biological fluids are expressed as the free base (molecular weight 129.16).



N,N-Dimethylimidodicarbonimidic diamide

Figure 2: Chemical name and structure of metformin.

1.5.2. Pharmacological effects and mechanism of action

Metformin is known for its pleiotropic effects. Besides lowering blood glucose, metformin helps in weight reduction, lowering of plasma lipids and prevention of some vascular complications (145). As the prevalence of obesity continues to increase, the use of metformin is expected to increase. Metformin is also used for other indications such as polycystic ovary syndrome (146). Moreover, treatment with metformin significantly associated with reduced cancer risk (147). In a study of more than 10,000 diabetic patients being treated with metformin or other sulfonylureas, those that were treated with metformin had a decreased risk of cancer-related mortality with respect to those patients on sulfonylureas (147). In a second study using a cohort, it was observed that patients treated with metformin had a lower incidence of cancer when compared to patients on other treatments (148).

Metformin works by suppressing excessive hepatic glucose production, through a reduction in gluconeogenesis (20). In addition its pleotropic actions include increasing glucose uptake, stimulation of insulin signaling, lowering in fatty acid and triglyceride synthesis and increasing the β-oxidation of fatty acids. Metformin also increases glucose uptake and utilization in peripheral tissues and reduces intestinal glucose absorption. Metformin does not stimulate endogenous insulin

secretion therefore, hypoglycemia or hyperinsulinemia do not develop during treatment (19).

The molecular mechanism underlying metformin action appears to be complex. However, most data suggest that metformin activates adenosine monophosphate (AMP) activated protein kinase enzyme (AMPK) in the liver by phosphorylation, which in turn may lead to diverse downstream effects, including inhibition of glucose and lipid synthesis (149, 150). The molecular components liver kinase B1 (LKB1) and AMP have been shown to play a role in the phosphorylation of AMPK in the presence of metformin (150). AMPK is a heterotrimeric protein consisting of a catalytic α- subunit and two regulatory subunits, β and γ. AMPK is activated by an increase in the intracellular AMP/ATP ratio resulting from an imbalance between ATP production and consumption. Activation of AMPK involves AMP binding to regulatory sites on the γ -subunits. This causes conformational changes that allosterically activate the enzyme and hinder dephosphorylating of threonine amino acid within the activation loop of the catalytic α-subunit (150). AMPK direct phosphorylation could be achieved by upstream kinases such as tumour suppressor STK11 (serine/threonine kinase 11) with the aid of LKB1 (151).

However, a recent in a study using liver-specific AMPK and LKB1-knockout mice has shown that inhibition of hepatic glucose production by metformin is maintained, suggesting that metformin may also inhibit hepatic gluconeogenesis in

a LKB1- and AMPK-independent manners (152). The role of AMPK in gluconeogenesis inhibition could still be considered, as it has been demonstrated that metformin inhibits hepatic gluconeogenesis through AMPK-dependent regulation of the orphan nuclear receptor small heterodimer partner (SHP) (153). Therefore, a reduction in gluconeogenesis could happen through both AMPK dependent and an AMPK-independent manner. Recently, metformin was found to suppress gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase (154) and also by decreasing intracellular cyclic AMP (155).

The direct key target of metformin is the inhibition of complex I of mitochondrial respiratory chain which activates AMPK by increasing the cellular AMP to adenosine triphosphate (ATP) ratio (156-159). AMPK is a major cellular regulator of lipid metabolism in addition to glucose metabolism. The activated AMPK inhibits HMG-CoA reductase, mammalian target of rapamycin (MTOR), acetyl-CoA carboxylase-1 (ACAC-1), acetlyl CoA carboxylase-2 (ACAC-2), malonyl CoA decarboxylase (MCAD), glycerol-3-phosphate acyl transferase (GPAT), and carbohydrate response element binding protein (CREBP) (149, 160). The activation of AMPK by metformin also suppresses the expression of sterol regulatory element binding protein-1 (SREBP-1), a key lipogenic transcription factor implicated in the pathogenesis of insulin resistance and dyslipidemia (Figure 3) (161).

Another action of phosphorylated AMPK is that it also increases peroxisome proliferator-activated receptor γ coactivator-1 (PPARGC-1) expression, consequently, downstream activation of mitochondrial biogenesis takes place (162). Collectively, activated AMPK results in an increased glucose uptake in skeletal muscle through enhancing the glucose transporter 4 (GLUT-4) translocation activity (163). The overall pharmacological effect of AMPK activation in the liver includes the stimulation of fatty acid oxidation, inhibition of cholesterol, and TG synthesis. A systemic increase in insulin sensitivity owing to improved insulin binding to insulin receptors also takes place (Figure 3) (157).

Another study showed that metformin induced AMPK/liver X receptor α (LXRα) phosphorylation, followed by pro-opiomelanocortin (POMC) suppression in rat pituitary cells. It is another new discovery for metformin's extrahepatic central action. The production of adrenocorticotropic hormone (ACTH)/cortisol is reduced through AMPK/LXRα phosphorylation in the pituitaries (164).

Metformin has also been evaluated for its potential of tumour suppression activity (22). Population studies showed that treatment with metformin is associated with a significant reduction of neoplasia in multiple cancer types, cancer of the breast and prostate, in particular (19, 21). Metformin may also inhibit the growth of cancer cells. The mechanisms underlying this anticancer effect are poorly elucidated and may involve multiple pathways (150) (22). The cell cycle arrest in metformin

treated breast cancer cells seems to involve the activation of AMPK (165, 166). Animal studies showed that metformin suppresses human epidermal growth factor receptor 2 (HER-2) onco-protein, that play a role in the overexpression of breast cancer onco-protein which occur through the inhibition of the MTOR effector in human breast carcinoma cells (167).

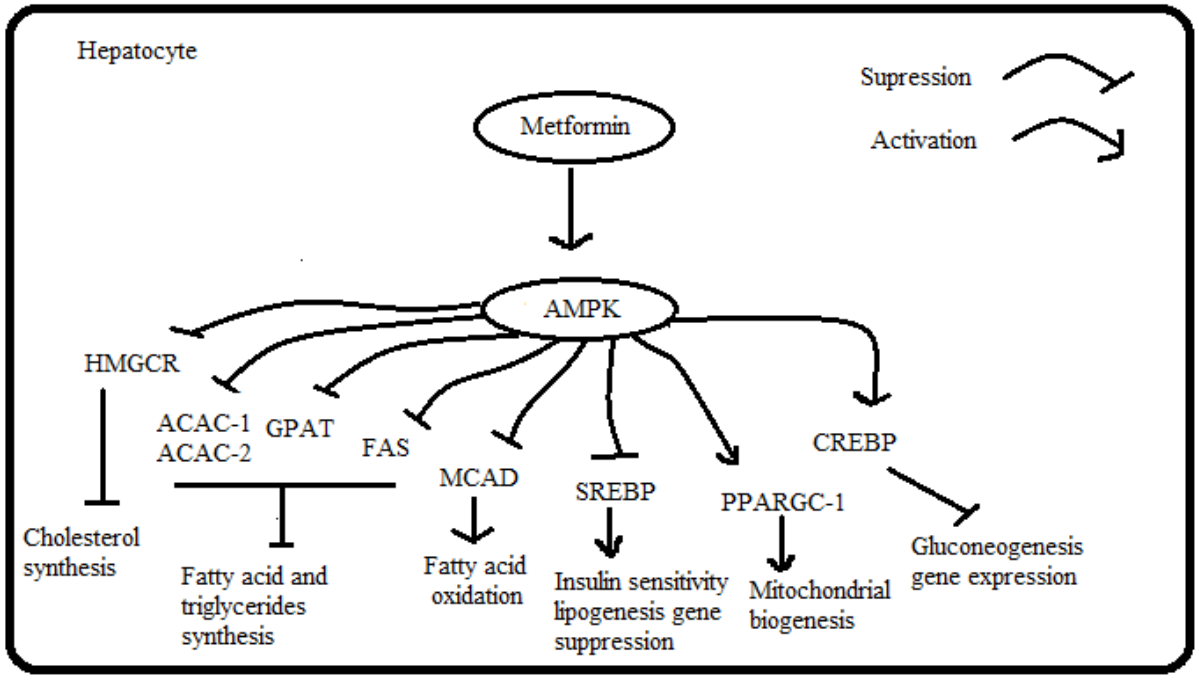


Figure 3 : A diagram showing molecular bases for the pleotropic actions of metformin.AMPK= AMP activated protein kinase, HMGCR= 3-Hydroxy methyl gluteryl CoA reductase, ACAC-1= Acetyl CoA carboxylase 1, ACAC-2= Acetyl CoA carboxylase 2, GPAT= Glycerol-3-phosphate acyl transferase, FAS= Fatty acid synthase, MCAD= Malonyl CoA decarboxylase, SREBP= Sterol regulatory element binding protein, PPARGC-1= peroxisome proliferator activated receptor γ coactivator-1, CREBP= Carbohydrate response element binding protein.

1.5.3. Metformin is a transportable substrate

Most clinically used drugs are administered orally, from which approximately 40% are cations or weak bases at physiological pH (168). For ADME, drugs need to be taken up into, and effluxed from, various cell types in the body by various mechanisms. Transport of drug molecules across cell membranes could be through simple diffusion or facilitated by specific type of transmembrane proteins or transporters. Families of membrane transporters have been recognized to play an important role in the transport of organic cations (OC) across the plasma membrane. Metformin is a highly polar/ionized character and is also basic in nature. Therefore, its fate in the body is governed by special type of transporter proteins. Collectively, three families of membrane proteins have been assigned to play a role in metformin disposition by facilitating its movement across cell membranes. These include members of the SLC family 22 that refers to the OCT family, the SLC family 47 which includes MATE (153), and the SLC family 29 which includes plasma membrane monoamine transporter (PMAT) (169).

1.5.3.1. Classification and isolation

Recently, five distinctive families of ATP independent transporters, to which a variety of polyspecific uptake transporters belong to, were identified. These families are the H^+/oligopeptide co-transporter family solute carrier (SLC)15 (170),

the organic anion transporter family SLC01(171, 172), the organic cation/ anion/ zwitterion transporter family SLC22 (173, 174), the SLC47 H^+/drug antiporters (175), and the SLC29 family (176).

OCT that are responsible for facilitating OC movement across membranes belong to the last three families, SLC22A family, SLC47A family (177), and SLC29A family. Most of plasma membrane transporters are oligospecific, being specialized for translocation of specific substrates/nutritional compounds. In contrast, polyspecific transporters accept compounds with different sizes and molecular structures. OCT are polyspecific type of transporters. The SLC22A or OCT family includes the following transporter proteins OCT-1 (SLC22A1), OCT-2 (SLC22A2), and OCT-3 (SLC22A3) with the electroneutral organic cation/carnitine transporters (OCTN1-3) (173).

In 1994, the first member of the SLC22 transporter family was isolated and identified by expression cloning from rat kidney and was named rat OCT-1 (rOCT-1) (178). In that study, rOCT-1 had functional characteristics similar to the OC transport process in the basolateral membrane of renal proximal tubules and hepatocytes. In 1997, OCT-1 was cloned from human (179, 180), rabbit (181) and mouse (182). SLC22A2, the second member of the OCT family, was isolated by homology screening from rat kidney (183) and designated rOCT-2. It was later cloned from human (179), pig (184), mice (185) and rabbit (186). The proton

cation antiporters MATE-1/SLC47A1 and MATE2-K/SLC47A2, which is kidney-specific homologue, were cloned from human (175, 187). MATE-1 was also found in mice and rats (188, 189) while MATE2-K has been only detected in mice (175). PMAT transporter was first cloned and isolated from human intestine in 2007(176).

1.5.3.2 Tissue distribution

Human OCT-2 (hOCT-2) was found to be expressed at the basolateral side in the proximal tubule (190). It is to be noted that different OCT isoforms have species and tissue specific distribution. For example, OCT-2 is the main OCT of human kidney (Table 1) (179) while in rat kidney, both OCT-1 and OCT-2 are expressed and both are involved in the excretion of metformin (Figure 4). OCT-1 is also the main isoform found (179) in human liver and was also detected in the small intestine of both human and rats (177).

MATE-1 in human is expressed in the kidney, liver, muscle and heart (175). Both MATE-1 and MATE2-K are localized in the brush-border membrane of proximal tubules in the kidney, and mediate the transport of cationic drugs with an oppositely directed H^+ gradient as a driving force (187). In mice, only Mate-1 is expressed in the kidney while Mate-2 is expressed in testis (175). In rats only MATE-1 is expressed in the brush-border membrane of proximal tubules in the

kidney (191). By using a polyclonal antibody against PMAT, it was proven that the PMAT protein was expressed in human small intestine and concentrated on the tips of the mucosal epithelial layer (Table 1). It was also found to be expressed in the podocytes of the epithelial cells of the glomeruli in Madin-Darby canine kidney cells with unknown function.

Table 1: Species differences in tissue distribution of OCT, MATE and PMAT transporters.

Transporter protein type	Tissue distribution in human	Tissue distribution in rat
OCT-1	Liver > small intestine	Liver, kidney> small intestine
OCT-2	Kidney	Kidney
MATE-1	Kidney > liver > skeletal muscle	Kidney, placenta > pancreas
MATE2-K	Kidney	Testis
PMAT	Small intestine	Not determined

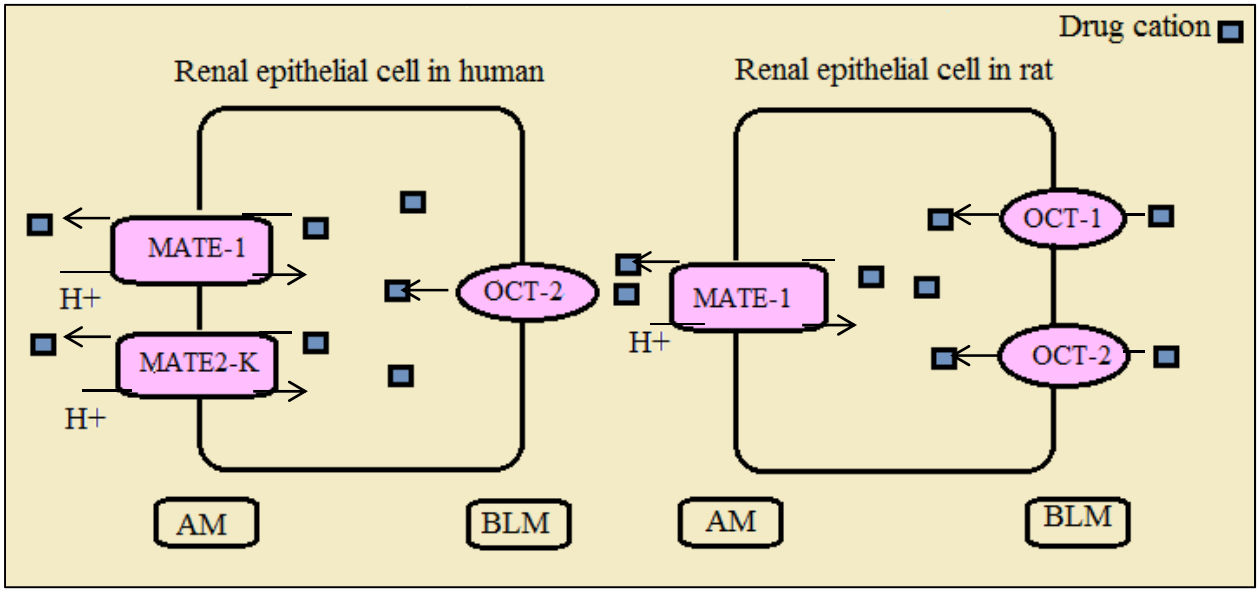


Figure 4: Organic cation transport systems in the proximal tubular epithelial cells of human and rat kidney. BLM= Basolateral membrane, AM= Apical membrane.

1.5.3.3. Physiological function

The nature of OC transport by any of the two OCT subtypes (OCT-1and OCT-2) is electrogenic, independent of Na^+, and reversible with respect to direction (173). Since OCT are polyspecific, they can interact with several structurally different compounds. It is to be noted that interaction of a substrate with a particular isoform of OCT does not necessarily predict the interaction with other OCT. OCT affinity for different substrates is species and isoform specific. The driving force is supplied only by the electrochemical gradient of the transported organic cations (192, 193). MATE transporters are electroneutral transporters that operate independently of a sodium gradient, but use an oppositely directed proton gradient as driving force; translocation of OC across the plasma membrane is bidirectional (194).

To investigate the physiological function of OCTs, knockout mice models were utilized for Slc22a1 and Slc22a2. The Oct-1 and Oct-2 deficient mice were viable and display no obvious phenotypic abnormality which indicates that they are not life determinant (195-200). A significant contribution to the regulation of OC was confirmed recently in mice in which the Oct-3 gene was disrupted (201-203). However, Oct-1 knockout mice showed dramatically reduced hepatic uptake of tetraethylammonium (TEA) and metformin (196, 198). In Oct-1 and Oct-2 double knockout mice, the renal secretion of TEA was abolished and the plasma levels of

TEA were substantially increased (197). Considering the differences in tissue distribution between mice and humans, a combined deficiency of Oct-1 and Oct-2 better reflects the effect of OCT-2 deficiency on kidney function in human (195).

These knockout animal studies emphasize the role of Oct in the hepatic uptake and renal elimination and tissue distribution of substrates. In addition, many neurotransmitters are transported across membranes by OCT such as acetylcholine, histamine, dopamine, serotonin and norepinephrine (204).

PK characterization of Mate-1 knock out mice was carried out with metformin. After iv injection, renal and hepatic metformin concentrations were markedly increased in the Mate-1 knock out mice compared to wild-type mice. In addition, plasma metformin levels were increased, whereas urinary metformin excretion was significantly decreased. These data indicates a crucial role of mate-1 in the CLr of metformin. However, the Mate-1 protein seems to be not crucial for maintaining life as mice were viable without any abnormalities (205).

1.5.3.4. Organic cations substrates

OC substrates include model OC (e.g., TEA, decynium 22), clinically important therapeutic drugs other than metformin like procainamide, cisplatin, citalopram, cimetidine, quinine, vecuronium, endogenous compounds such as choline, histamine, dopamine, norepinephrine and toxic cationic substances like MPP^+

(177, 179, 180, 206). These OC are mostly positively charged at physiological pH and cannot freely pass through the cellular membrane or be reabsorbed from the lumen into the interstitium indicating that the transportation is bidirectional. There are some evidences that hOCT-1 could represent a part of this transport system (207, 208). The efflux across the basolateral membrane into the interstitium may be mediated by the same OCT.

1.5.3.5. Regulation of OCT-1, OCT-2 and MATE-1 expression and function

OCT-1, OCT-2 and MATE-1 regulation has been reported. Some data concerning transcriptional and posttranscriptional regulation of human OCT-1 have been studied. For example, the hepatic nuclear transcription factor HNF-4α interacts with the promoter of SLC22A1 gene and activates transcription of hepatic OCT-1 protein (209, 210). Protein kinase A (PKA) activity and calcium-calmodulin stimulated pathway are involved in posttranscriptional regulation of OCT-1 (211). Nies *et al* found that OCT-1 expression was reduced during cholestasis (212). Also down-regulation of OCT-2 mRNA and protein has also been observed in rat kidneys pretreated with a cholestatic dose of ethinyl estradiol (213). Streptozotocin induced diabetes was shown to down regulate rOCT-1 and rOCT-2 proteins in kidneys with suppression being reversed by insulin (214). Ramipril administration also reversed the down-regulation indicating a possible role of

angiotensin II signaling in rOCT-1 regulation (215). Regarding OCT-2 levels in human, a low expression in liver vs kidney is correlated with methylation of the OCT-2 promoter in liver (216). In addition, other several signaling pathways involving protein kinase C (PKC), protein kinase A (PKA), phosphatidylinositol-3-kinase (PI3K), and calmodulin participate in posttranscriptional regulation of hOCT-2 (177, 210).

As for rMATE-1, the first study was published to clarify the transcriptional mechanism of the MATE-1 gene in 2007 (217). Specificity protein 1 (SP-1) has been implicated in modulating mRNA levels of rMATE-1(217) Activating enhancer binding protein 2 (AP-2) has been shown to have a repressive effect on rMATE-1 gene expression (217, 218).

Growth factors, cytokines, neurotransmitters, polypeptide hormones, infections and stress could affect the AP-2, consequently affecting gene transcription (219). Therefore, the general transcription factor SP-1 and transcriptional regulator AP-2 could coordinately play a role in the transcriptional regulation of the SLC47A1 gene. Expression of mate-1 and mate-2 in murine liver seems to be regulated independently of some nuclear factors, such as aryl hydrocarbon receptor (AhR), CAR, nuclear factor erythroid-2-related factor 2 (Nrf-2), peroxisome proliferator-activated receptor alpha (PPARα), and PXR (220) while data is still unavailable

regarding regulation of rMATE-1 expression in kidney through these transcriptional regulators.

1.5.4. Pharmacokinetics of metformin in healthy subjects

1.5.4.1. Absorption

PMAT may be considered as the major transporter responsible for the uptake of metformin from the gastrointestinal tract. It is localized on the luminal side of enterocytes (Figure 5) (169). OCT-1 and OCT-3 are also expressed in the small intestine but at low levels (221, 222). OCT-3 is also localized in the apical or brush border membrane of enterocytes (223) and may therefore, in part, act as an influx carrier of metformin into enterocytes. By contrast, OCT-1 is localized to the basolateral membranes of enterocytes and may transport metformin into the interstitial space (223). Genetic variants of OCT-1 and OCT-3 have been detected, many of which show lesser ability to transport metformin into model cells (224, 225). Recently, two additional metformin transporters were identified in Caco-2 cell lines that contribute to apical uptake and accumulation of metformin inside the cells but in vivo data are still unavailable for confirmation (226).

The Cmax of metformin occurs approximately 3 h after dosing in healthy subjects (227). The peak plasma concentrations range from 1.0 to 1.6 mg/L after a 0.5 g

dose, increasing to about 3 mg/L after a 1.5 g dose (227). The plasma concentrations decrease rapidly after a single oral dose and, as is the case after iv doses, the urinary recovery can be followed for a longer time than the plasma concentrations due to greater sensitivity (higher concentrations). Terminal half-life ($t_{1/2}$) calculated using plasma concentration time data, ranges between 1.5 to 4.5 h (227-229). The variability in $t_{1/2}$ could be attributed to different experimental conditions used in the different studies. The multiphasic behavior of plasma concentrations with respect to time course may be due to metformin being taken up by erythrocytes and cleared slowly (227, 230). It was found that the value of metformin blood/plasma concentration ratio is time-dependent rather than concentration-dependent (227, 230) and there was no difference in area under the curve between plasma and erythrocytes (230). Mean values of metformin blood to plasma ratio were 1.37, 1.25, and 1.33 for initial blood concentrations of 1, 5, and 20 mg/mL (231).

The gastrointestinal absorption of metformin from standard release tablets is incomplete, and as is the case for most drugs, the F shows intrasubject as well as intersubject variability. There is incomplete F ranging between 29 and 50% (227, 228). Absorption ceases at about 6–10 h after administration irrespective of the amount of metformin that has been absorbed up to this time. This is about the time taken for the passage of drugs through the stomach and small intestine (232). As

absorption from the stomach is negligible, it is confined very largely to the small intestine with negligible absorption from the large intestine. This conclusion is supported by the administration of metformin solutions containing a gamma emitter, which shows that the plasma concentrations of metformin begin to decline when the drug starts to arrive the large intestine (233). Collectively, metformin absorption is the rate-limiting step in drug disposition because absorption is saturable and mostly transporter-dependent, which dictates an inverse relationship between F and dosage (227, 234).

1.5.4.2. Distribution

Metformin is not bound to plasma proteins (227). The Vd was measured to range from 63 L (229) to 276 L (227) after iv administration to humans. Interestingly, the apparent Vd after oral administration (Vd/F) was estimated during multiple dosing. When 2 g of metformin was administered daily, either as immediate-release or sustained-release tablets, Vd/F was approximately 648 L (235). As 50% is approximately absorbed, the actual Vd during multiple dosage is about 300 L. This large Vd indicates a considerable tissue uptake of metformin. One of the contributing reasons behind that large Vd is the nearly 100% unbound fraction in plasma.

Peak concentrations of metformin in the jejunum are up to about 500 μg/g of tissue. Although it may be difficult to wash out all the extracellular drug within the brush border, it does appear that the concentrations within the small intestine are much higher than in other tissues or in plasma (236).

With respect to transporter distribution, OCT-3 and PMAT are known uptake transporters of metformin in the small intestine. OCT-1 is believed to act as efflux transporter of metformin in enterocytes which is expressed in the basolateral membrane of the intestinal cell. In liver, the diminished hepatic uptake of metformin in Oct-1 knockout mice indicates that Oct-1 is the major uptake transporter (198, 237). Both OCT-1 and OCT-3 are present in highest levels on the sinusoidal membrane of hepatocytes (212, 225) and thus are located in a position for uptake of metformin from blood into hepatocytes (Figure 5). They could also transport metformin in the reverse direction from liver to blood. Both transporters are also present, at lower levels, in the cell membrane of cholangiocytes which are epithelial lining cells of bile ducts (212) where their function is unknown.

The variation in the hepatic expression of OCT-1 may be relevant in the clinical response to metformin because its major effect may be in the liver (237). The large intersubject variation in the hepatic levels of OCT-1 was detected by both the variation in the transporter protein (83-fold) and also in the corresponding mRNA (113-fold). OCT-1 genotypes had a significant effect on metformin PK such as

elevated AUC, higher Cmax, and lower Vd/F in individuals carrying a reduced function OCT-1 allele (237). Until now, the intersubject differences in the expression of OCT-3 appear wide judging from the 27-fold intersubject variation in the mRNA expression (212).

MATE-1 has been proposed to mediate the transport of metformin into bile canaliculus as it is expressed the bile ducts and is a carrier of metformin (Figure 5) (194). However, metformin is not present in faeces after iv administration but it is present after oral dosage. The biliary excretion of metformin is therefore negligible in man (19).

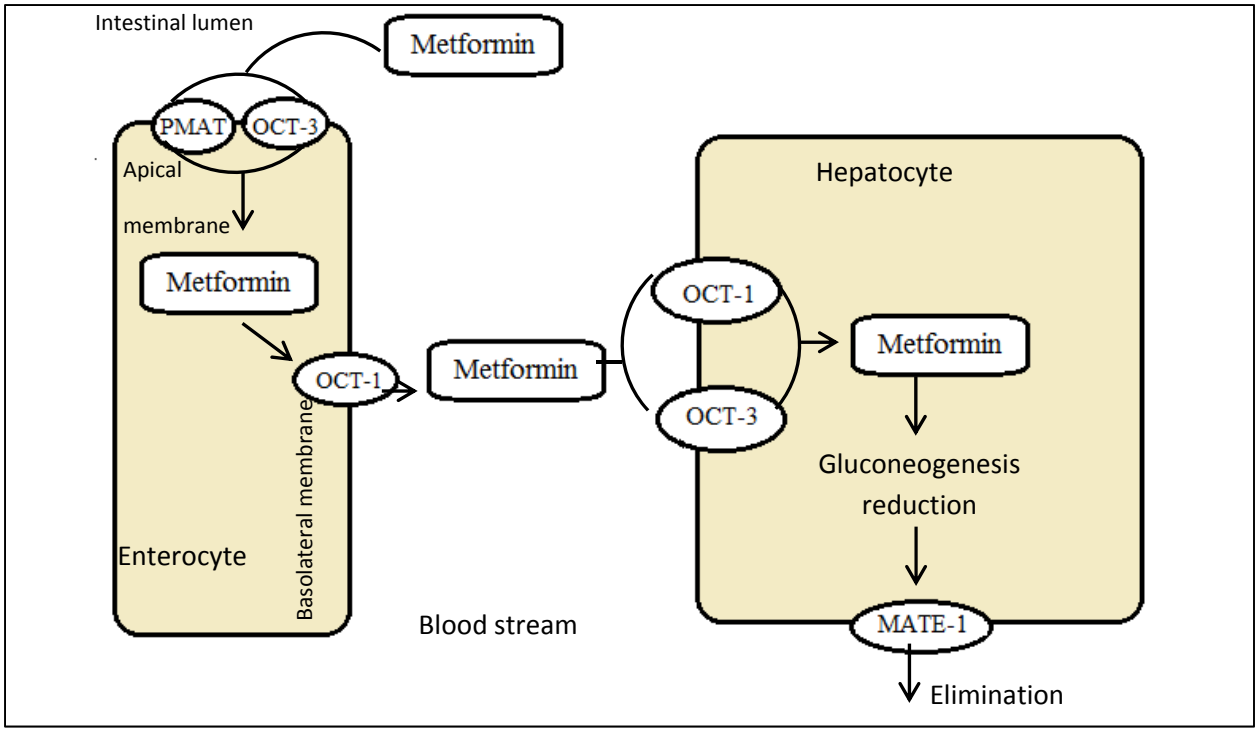


Figure 5: Major known transporters involved in the absorption and hepatic uptake of metformin. PMAT = plasma membrane monoamine transporter, OCT-1 = organic cation transporter-1, OCT-3 = organic cation transporter-3, MATE-1 = multidrug and toxin extrusion transporter protein.

1.5.4.3. Metabolism and excretion

Excretion of unchanged drug in urine through the kidneys is the major mode of elimination of metformin. To date no metabolites of metformin have been identified in urine (227, 228, 238). Pentikainen *et al* (228) administered ^{14}C-labelled metformin iv and found 100% recovery of unchanged drug in urine. On the other hand, Tucker *et al* and Sirtori *et al* could not account for 20%-23% of the drug respectively, using chromatographically based assays of unlabelled drug (227, 229) and no intact drug was detected in the faeces after iv dosage (227, 229). Thus, it is still possible that small proportions of doses of metformin may be metabolized (144). It is of note that in both Tucker's and Sirtori's studies, urine samples were collected over 72 h, which is long enough to collect all of metformin excreted. Both Tucker *et al* and Sirtori *et al* administered 1500 and 850 mg metformin orally, respectively, while Pentikainen *et al* used relatively lower dose (500 mg) orally which may explain the 100% recovery of the entire dose absorbed.

The CLr of metformin represents the major mode of elimination of metformin (19, 144, 227, 229). The estimated population metformin CL is 500-519 mL/min in subjects with good renal function (207, 227-229, 233, 234). Metformin possesses special characteristics that render it an excellent candidate for high CLr. Relatively low molecular weight and zero affinity to bind to plasma proteins make it readily

filtered at the glomerulus. In addition, as discussed above, metformin is a well-known substrate of several transporters highly expressed in kidney due to high polarity and low lipid solubility of metformin.

The movement of metformin as cationic substrate across cell membrane is achieved by the coordinated activities of OCT and MATE. In the kidney, the first step for the secretion or the uptake of metformin from circulation into renal epithelial cells is primarily facilitated by OCT-2 (223), expressed predominantly at the basolateral membrane in the renal tubules and transports metformin inside the proximal tubular lining cells. The uptake process is solely conducted by OCT-2 in human (191, 239). Renal excretion of metformin from the tubular cell into the lumen is mediated through two H+/OC antiporters MATE-1 and MATE2-K (205, 240, 241) which are expressed in the apical membrane of the renal proximal tubule cells in human (Figure 6). PMAT has been detected in the podocytes in the glomerulus (242). However, its function in podocytes is not known and no in vivo data available for its role in metformin excretion.

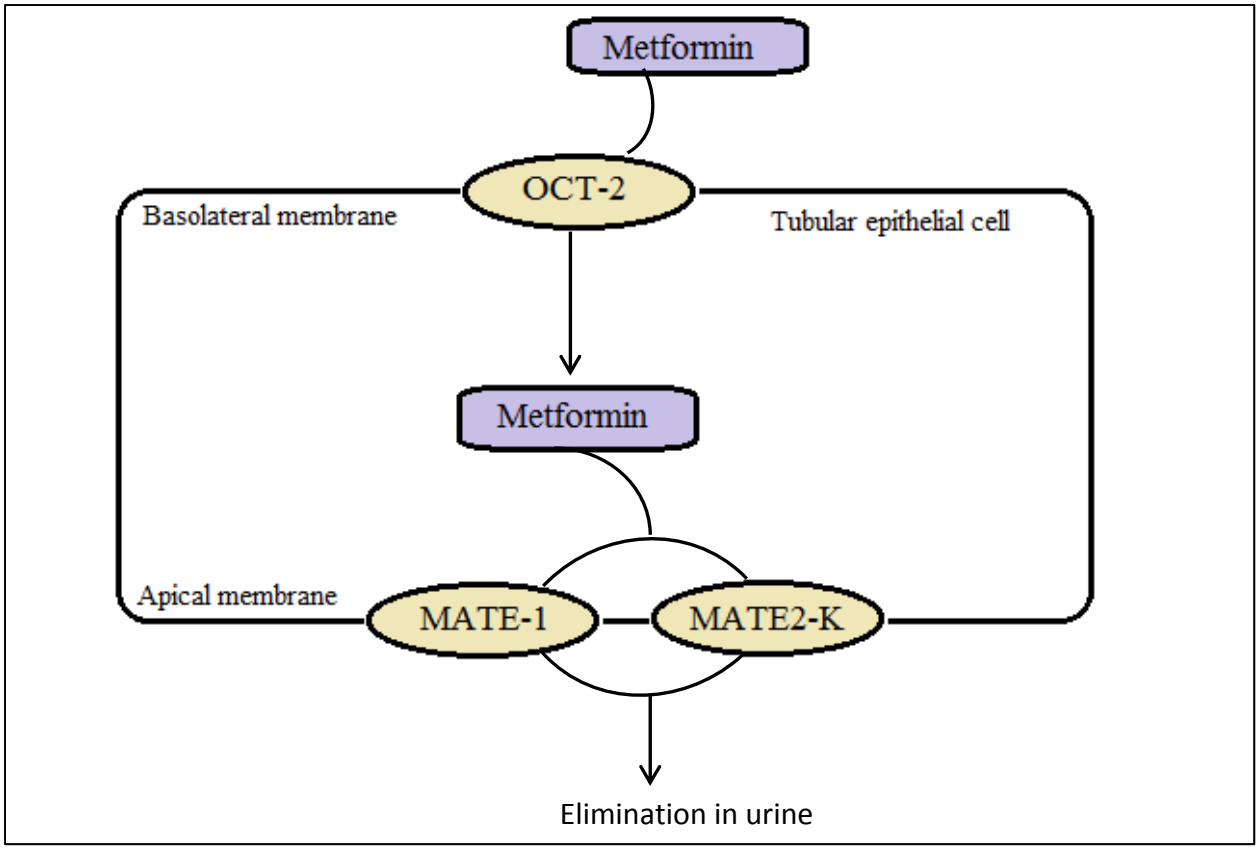


Figure 6: Major known transporters involved in the renal clearance of metformin in human. OCT-2= organic cation transporter 2, MATE-1= multidrug and toxin extrusion transporter protein 1, MATE2K= multidrug and toxin extrusion transporter protein 2-K.

1.5.5. Pharmacokinetics of metformin in rats

1.5.5.1. Absorption

In rats, after oral administration of 100 mg/kg metformin, Cmax was reported to be 6.5-8.8 μg/mL (243, 244). Terminal half-life calculated using plasma data ranged from 5.8- 6.2 h (244). The F in those studies was conducted at high dose levels such as 100 mg/kg ranged from 37- 39 % (244) which is considered comparable to F of human. The same mechanism of facilitated absorption applies in rat intestine (144).

1.5.5.2. Distribution

The large Vd of metformin is confirmed by studies in mice and rats. After a single oral dose to mice, concentrations up to seven times the serum concentrations are found in the kidneys, adrenal glands, pancreas and liver, with lesser amounts in lung, muscle and spleen suggesting relatively high tissue binding (238, 245). The high concentrations in kidney are not necessarily due to uptake in kidney tissue and may be due, in part, to high concentrations of metformin in the urinary tract which represents the main route of elimination. In rats, Vd at steady state (Vdss) was determined after 100 mg/kg iv administration to male Sprague–Dawley rats. It ranged from 0.625 to 0.826 L/kg (244) with the lowest value to be 0.444 L/kg

(243). The intestine appears to have the highest concentration of metformin which was shown in distribution study carried out in mice (246), followed by the kidney. Liver came in the third place after kidneys in metformin accumulation (246).

1.5.5.3 Metabolism and excretion

Recently, Choi *et al* tried to prove that metformin undergoes substantial hepatic and gastrointestinal metabolism with about 65% in rats. They found that the percentages of metformin that disappeared per gram of liver, stomach, small intestine, large intestine, after a 30-min incubation of 10 μg/mL of metformin with 9000 *g* supernatant (S9) fraction of tissue homogenates, were 20.5, 10.4, 5.28, 4.68 %, respectively. In addition they found that after intragastric and intra-duodenal instillation of metformin in rats, at a high dose of 100 mg/kg, the AUC values were significantly lower of than after intra-portal administration. The gastrointestinal first-pass effect of metformin was estimated to be approximately 54% of oral dose in rats. They concluded that at a dose of 100 mg/kg, the F was low (30%) mainly due to considerable hepatic and gastrointestinal metabolism in rats. However, they did not separate and isolate the presumed metabolites and identify their chemical structure. In addition, they performed another in vivo study in which metformin at a dose of 100 mg/kg was administered intravenously to rats. The rats were pretreated non-specific CYP inhibitors such as SKF-525 with sulfaphenazole,

quinine, and troleandomycin which are major inhibitors of CYP2C11, 2D1, and 3A1/2, respectively. Metformin CLnr was found to be lower compared to that of controls suggesting that CYP enzymes are involved in its elimination (247). It must be noted that the drug dose level used in their in vivo study was 100mg/kg, which is 4-6 times higher than what is used in human (30 mg/kg).

Choi *et al* reported CLnr to be between 8.6 and 10.2 mL/min/kg claiming that there is a considerable gastrointestinal and hepatic CL (244). By using high dose level for rats such as 100 mg/kg iv, they explained that CLnr was probably due to metabolism via hepatic CYP2C11, 2D1, and 3A1/2 in rats (247).

With respect to kidney transporters in rats, rOCT-1 and rOCT-2 are the main proteins involved in the process of metformin uptake from glomerular filtrate, while in human, it is mainly performed by OCT-2 only. Another difference between rat and human is detected in process of metformin efflux where rMATE-1 is the only predominantly transporter expressed at the apical membranes of the epithelial cells in the proximal tubules while MATE2-K transporter is not detected in rats (contrary to human; Figure 7). OCT-3 mRNA (225) was found to be expressed in kidney while OCT-1 was detected on the apical and subapical domain side of both the proximal and the distal tubules in the kidney (207).

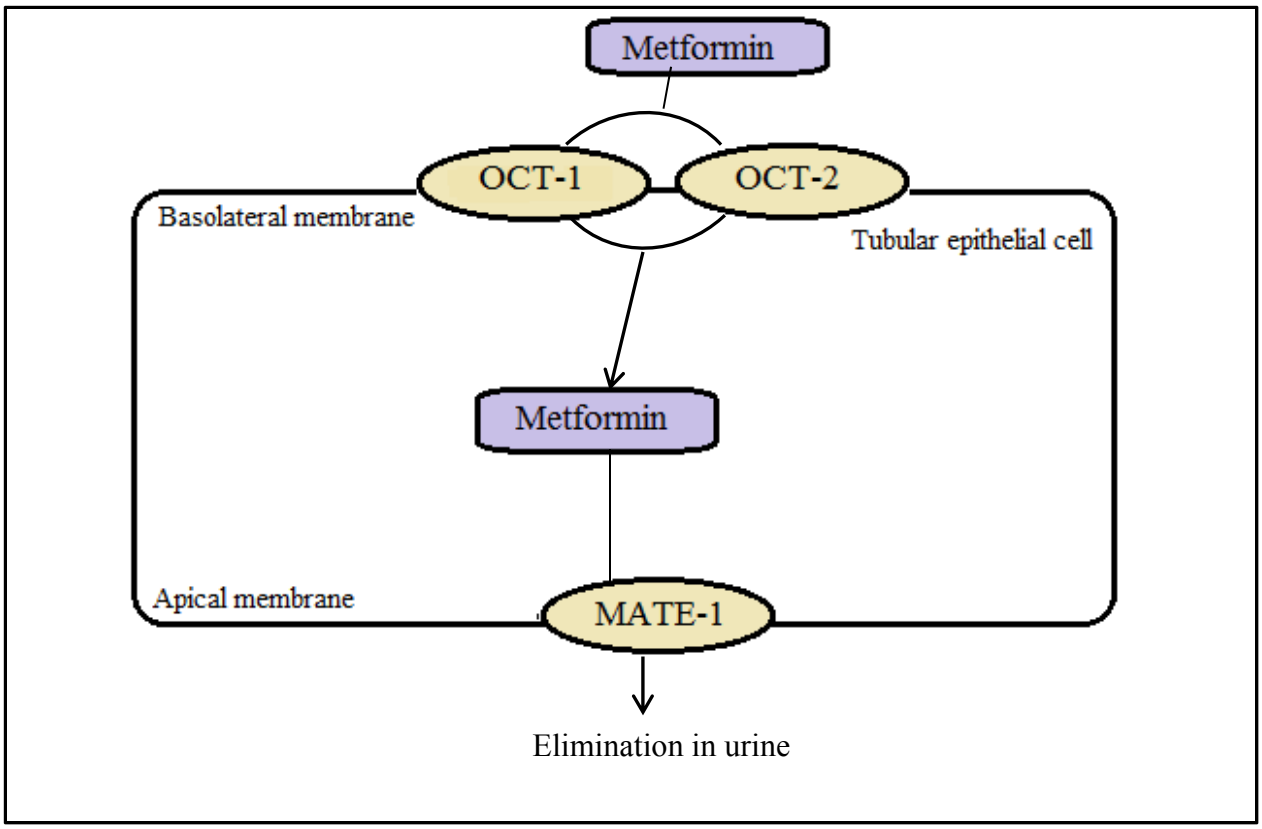


Figure 7: Major known transporters involved in the renal clearance of metformin in rat. OCT-1= organic cation transporter 1, OCT-2= organic cation transporter 2, and MATE-1= multidrug and toxin extrusion transporter protein 1.

1.5.6. Methods of metformin assay

Many methods are available for quantitation of metformin in biological samples. For its separation from endogenous components most of these methods rely on liquid or gas chromatographic assays (Table 2) (248-269). Others involving capillary electrophoresis have also been reported (270). For sample preparation from biological fluids, extraction and clean-up of the sample is a critical step in bioanalysis to remove interfering substances, while selectively ensuring minimal analyte loss. The extraction of metformin from biological matrices is complicated by the highly polar nature of the molecule. This factor, in addition to the widespread clinical use, may explain the relative abundance of different methods for analyzing metformin in biological specimens. Many of the current methods utilize various techniques for sample cleanup, such as simple protein precipitation, solid phase extraction, ultrafiltration with column switching, chemical derivatization and liquid-liquid extraction. As for both ion-pair liquid–liquid (258, 270) and ion-pair solid phase extraction (264), they have been used to overcome clean up plasma samples, which is difficult to achieve owing to the high polarity of metformin (271).

Use of other solid-phase extraction (256) or ultrafiltration with column switching (265) has also been incorporated into some methods, although they do not always

yield optimal sensitivity (Table 2), and may necessitate the use of certain devices which can add elements of expense and time into sample preparation.

Chemical derivatization of metformin has been used in gas chromatography (250, 260) and HPLC methods (263), which can add complexity into the method with little advantage in terms of sensitivity. Recently, liquid chromatography–mass spectrometry (LC/MS) methods following plasma deproteination have been reported for metformin (255, 266, 269) and these techniques certainly enhance selectivity and sensitivity. They have a disadvantage however in that the instrumentation is quite expensive is not as accessible as conventional HPLC in many laboratories, particularly in clinical laboratories. Organic liquid-liquid extraction is a simple and effective method of affording sample cleanup for most analytes. Unfortunately, in the case of metformin, this approach is challenging due to drug's polar characteristics. There is a need for a method that successfully measures metformin in small volumes (0.1 mL) with high sensitivity. A liquid-liquid extraction technique was first described by Amini *et al* (249). However, normal phase mode was used rather than conventional reverse phase C18 column for separation.

Table 2: Comparisons of some published assay methods for metformin determination in human matrices.

Volume of specimen (mL)	Validated LLQ (ng/mL)	Type of human matrix	Sample preparation	Analytical column	Reference
0.5	30	Plasma	Protein ppt and dichloromethane wash	Phenyl	(261)
0.1 and 0.5	10	Plasma	Protein ppt and dichloromethane wash	Silica	(253)
1 serum NS urine	50 serum 2000 urine	Serum & urine	Ion pair SPE	C18 or F5HS	(264)
0.1	15.6	Plasma	Liquid-liquid	Silica	(249)
0.5	200	Plasma & urine	Protein ppt	CX	(251)
NS	10	NS	Protein ppt	Silica	(262)
0.3	100	Plasma	Ultrafiltration	CX	(265)
0.25	60	Plasma	Protein ppt	Cyano	(257)
0.1	250	Plasma	IPE	Silica	(270)
0.5	50	Plasma	SPE C8	Phenyl	(256)
1	10	Plasma & urine	IPE	C18	(258)
0.1	7.8	Plasma & urine	Liquid-liquid	C18	Current method

SPE=solid phase extraction, protein ppt= protein precipitation, IPE= ion pair extraction, LLE= liquid-liquid extraction, F5HS= pentaflorohypersil, CX= cation exchange. NS denotes not specified or unclear

1.5.7. Metformin adverse effects and lactic acidosis

The most common adverse effects of metformin are diarrhea, cramps, nausea, vomiting, and flatulence. Metformin has more gastrointestinal side effects than most other antidiabetic drugs (272). Recently, metformin has also been reported to decrease the blood levels of thyroid-stimulating hormone in people with hypothyroidism (273). The most serious potential adverse effect that accompanies metformin use is lactic acidosis. It is rare and most of the cases seems to be related to comorbidity conditions, such as kidney function, rather than to the metformin itself (274).

Lactic acidosis occurs when a patient blood pH is less than 7.35 and plasma lactate concentrations are more than 45 mg/dL or 5.0 mM with electrolyte disturbances characterized by increased anion gap (224). The incidence of lactic acidosis during treatment with metformin is of great clinical concern as mortality in reported cases has ranged from 8% to 50% (275). Lactic acidosis could lead to other less life threatening symptoms such as abdominal or stomach discomfort, decreased appetite, diarrhea, fast or shallow breathing, a general feeling of discomfort, muscle pain or cramping, unusual sleepiness, and tiredness or weakness occur with lactic acidosis incidence (276). Once lactic acidosis is diagnosed, an immediate treatment or hospitalization is required (276). Therefore, it is commonly stated that metformin should not be prescribed for patients with defined high risk factors such

as renal impairment where plasma creatinine values 1.5 mg/dL (132 mM) for men and 1.4 mg/dL (124 mM) for women. High risk groups include those patients with liver disease, including alcoholic liver disease, as demonstrated by abnormal liver function tests and cardiac or respiratory insufficiency that is likely to cause central hypoxia or reduced peripheral perfusion.

There is a considerable debate about metformin being the direct cause of lactic acidosis. A recent estimation of the incidence of lactic acidosis is 3.3 cases per 100,000 patient years of treatment with metformin (277). Surprisingly, lactic acidosis was found to develop during treatment with the other major group of oral anti-hyperglycaemic drugs, the sulfonylureas, where the incidence of lactic acidosis was estimated as 4.8 per 100,000 patient years (277). It has also been reported in diabetic patients not treated with metformin, under conditions in which there is a considerable tissue hypo-perfusion or hypoxia (277). There was no case of lactic acidosis recorded in clinical trials on metformin (276). These trials included studies with over more than 70,000 patients on chronic metformin treatment. It is worth mentioning that patient selection criteria, to exclude those with risk factors for lactic acidosis and good patient care, may have contributed to the absence of this adverse effect in these clinical trials. Recently, LaLau *et al* suggested that metformin is rarely the sole cause of lactic acidosis. There are many factors can be defined with contrasting prognoses other than metformin. Moreover,

they found that the prognosis of severe lactic acidosis is better in metformin treated patients than in non-metformin users (278).

Conversely, Graham *et al* (144) stated that high concentrations of metformin can promote lactic acidosis development, and proposed more than one rationale. Acute overdoses taken with suicidal intent have caused lactic acidosis (279-282). Furthermore, plasma lactate begins to increase when plasma metformin concentrations are greater than about 20 mg/L (150 mM) in rats (198). Additionally, it was recorded that plasma concentrations of metformin of 20–107 mg/L (150–820 mM) in 24 of 49 patients were associated with lactic acidosis (283). It appears that most patients can take metformin safely for prolonged periods while in a very small proportion of treated patients, lactic acidosis and renal impairment develop over a short time (284). In many cases, the lactic acidosis has followed excessive vomiting and/or diarrhoea (285). Graham *et al* suggested that dehydration might have caused acute renal failure, reduced CLr of metformin, and consequently increased plasma concentrations of metformin when its dosage was continued (144). This may exacerbate the acidosis especially for a drug like metformin with high Vd and negligible protein binding. Considering the PK rat studies performed by Choi *et al*, high metformin concentrations resulted in saturation of tubular secretion mechanisms and switching on CYP metabolism. The

presumed formed metformin metabolite could play a role in initiating lactic acidosis. Knowledge in this regard is incomplete.

In summary, metformin is the most commonly used drug for treatment of T2DM. Because of metformin's newly discovered promising effects, an even greater increase in the number of prescriptions filled for it is anticipated. CMS clusters three metabolic disorders: obesity, HL, and hyperglycemia. Metformin represent a typical drug of choice for CMS and first line drug for T2DM treatment as well. Despite that a substantial number of obese patients are on metformin treatment, there are no available studies on the effect of obesity on metformin PK parameters. Most of controlled PK studies included healthy non-obese vs diabetic volunteers. The most effective treatment of obesity as well as CMS is RYGB. However, data regarding the effect of RYGB on metformin PK is not available. HL is one of the metabolic disorders used to define CMS. The effect of HL on drug PK parameters was extensively studied especially on lipophilic drugs. However, there is a lack in information regarding HL effect on metformin PK parameters and related protein transporters.

1.6. Rationale

Metformin is a highly polar drug with (pKa >12). Therefore, it exists predominantly as the hydrophilic cationic species at physiological pH values

(>99.9%). The behaviour of metformin during absorption, tissue uptake, distribution and elimination is largely governed by the role of transporters (19, 144). Absorption of metformin from the small intestine is facilitated through PMAT and OCT-3 transporters (169). Biodistribution across organs and tissues is achieved though OCTs (137). In addition, metformin elimination is assigned to be through tubular secretion where OCT and MATE transporters are involved (19). Despite the epidemic prevalence of both obesity and T2DM, to date, little is known about the effect of obesity on metformin PK. The effect of obesity on some protein transporters has to date been poorly studied. Some animal studies showed a suppressive effect of obesity on the expression of protein transporters such as hepatic OATP-2 and MRP-2 in Zucker rats (68). Renal Mrp-3, Oatp1a1, and Oat-2 were also inhibited in genetically engineered obese mice (69). Obesity is considered as a low grade of inflammation. In literature, pro inflammatory cytokines such as TNF-α and IL-6 have been associated with suppression of various protein transporters such as OATP1B1/1B3/2B1, OCT-1 and OAT-2 (64). Moreover, in a diabetic model of mice, which exhibited deficient leptin receptor signaling and excessive weight gain, a reduced expression of Oct-2 mRNA was evident (70). These observations suggest that obesity is associated with a trend towards transporter suppression. We anticipated that obesity could decrease the expression of metformin related transporters and consequently lowering Vd, CL

and F. To date, there is no clinical PK assessments were carried on obese subjects to test for the effect of obesity on metformin Vd, CL, and F which are highly governed by transporters.

With respect to the most effective and invasive treatment option for morbid obesity to sustain long-term weight loss with improved glycaemic control, bariatric surgeries are considered, RYGB in particular (286). RYGB constitutes about 80% of bariatric surgeries performed by American surgeons (Figure 1) (79). Due to the anatomical changes occurs after the RYGB surgery, it is associated with some nutritional deficiencies (287). This logically raises questions about the impact of this procedure on metformin absorption. Recently RYGB was shown to decrease other PK parameters like oral clearance (CL/F) and Vd/F, central and peripheral, in case of oral administered morphine (288).

Approximately 15% of patients undergoing bariatric surgery have T2DM, and one-quarter of these patients still require oral antihyperglycemic treatment immediately after surgery (71). Furthermore, the large disparity between the demand for and the availability to perform RYGB surgery has resulted in efforts to prioritize high-risk populations, particularly those with T2DM, for bariatric procedures (289-291). Therefore, it is likely that the number of patients with diabetes who require metformin treatment and receiving surgery will increase markedly in the future.

Metformin possesses several characteristics rendering it a good candidate for malabsorption after RYGB surgery. The drug primarily is absorbed in the upper small intestine (144, 292) which is bypassed in the surgery and has a relatively low and variable oral F that ranges between 30 to 50% (227, 228, 231, 247). Accordingly, examining metformin PK after gastric bypass surgery is of high clinical relevance.

HL usually clusters with obesity as a metabolic disorder and used to define CMS (17). HL is known to influence biochemical functions, transcriptional factors or protein expression with consequences on drug elimination and/or distribution (47, 116, 120, 293). Moreover, some rat studies showed evidence of the association of HL with down-regulation of mRNA of protein transporters such as MRP-2 and OATP-2 (53). Metformin CL is mainly through tubular secretion which is transporter-dependent. Consequently any change in the level of expression of elimination transporters will be detected in CL. No studies have been performed to test for the effect of HL on metformin urinary excretion transporters and PK parameters despite the fact that metformin is usually described for patients with CMS (3).

Metformin is up to 100% excreted unchanged in the urine (228, 238). Therefore, its absolute F which is defined as the fraction of the parent compound that reaches the systemic circulation can be measured using timed urine collections without

requiring iv administration. However, recently, some in vivo rat studies showed that metformin undergoes substantial gastrointestinal and hepatic metabolism using high dose level (100 mg/kg) iv compared to that used clinically in people (30 mg/kg) (243, 244, 247, 294). Meanwhile, in another in vivo study by the same group, CLnr, which represent an estimate for metformin elimination through metabolism, was lower in the presence of some specific CYP450 enzyme inhibitors like sulfaphenazole, quinine, and troleandomycin for CYP2C11, 2D1, and 3A1/2, respectively. Consequently, they suggested that metformin is metabolized through CYP2C11, 2D1, and 3A1/2 (247). Moreover, in their in vitro studies, Choi *et al* found that percentages of metformin disappeared per gram of liver, stomach, small intestine, large intestine homogenates after 30-min incubation were 20.5, 10.4, 5.28, 4.68 % respectively suggesting substantial gastrointestinal metabolism. However, a very high concentration of metformin was used (10,000 ng/mL). Because isolation or identification of the presumed metabolite(s) was not undertaken, the knowledge is incomplete regarding metformin metabolism. It is possible that metformin could be metabolized more at high doses/concentrations due to saturation of transport mechanism.

With respect to metformin analysis, many of the developed assay methods utilized varying techniques for sample cleanup which is a difficult process owing to the problem of high polarity of metformin. Current methods utilized many steps and

techniques for sample preparation such as simple protein precipitation, solid phase extraction, ultrafiltration, and chemical derivatization which consumed more time and add complexity (256-258, 261, 263, 264). Normal phase mode is the usual chromatography that is mostly used to retain metformin, as a polar compound. To the best of our knowledge, there is no validated reverse phase chromatography utilizing 0.1 mL of either plasma or urine and octadecylsilane column for separation of metformin based on liquid-liquid extraction technique.

1.7. Hypotheses

1- Obesity can decrease renal transporter expression, with consequences on metformin CL, Vd and F compared to lean subjects.

2- RYGB decreases metformin F.

3- Short and/or longer term experimental HL causes reduction in metformin CL due to suppression of transporters involved in its elimination (e.g. rat OCT-1, OCT-2 and MATE-1).

4- Metformin undergoes metabolism at very high concentration levels. Furthermore, the nature of the metabolite can be elucidated using suitable chromatorgraphic techniques.

1.8. Objectives

This thesis incorporates the following specific aims, namely to:

1. Develop and validate a sensitive reverse phase mode HPLC-UV assay for the determination of metformin in both human plasma and urine matrices.

2. Examine the effect of obesity on a single-dose PK study using a standard oral release preparation of metformin.

3. Examine the effect of RYGB on a single-dose PK study using a standard oral release preparation of metformin.

4. Test the effect of short term (36 h) and long term (108 h) experimental HL on metformin PK parameters.

5. Examine the effect of experimental short and long term HL on gene and protein expression of metformin urinary excretion transporters rOCT-1, rOCT-2 and rMATE-1 in rat kidneys.

6. Test for metabolism of metformin using isolated rat hepatocytes and microsomal incubation studies.

7. Isolate metformin metabolite/s by means of fractional isolation then apply mass characterization of metformin metabolite/s by means of mass spectroscopy.

2. Methodology

2.1. Materials and reagents

Metformin HCL (97% pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ranitidine injection USP (Sandoz, Quebec, Canada) was used as a source of ranitidine HCL for internal standard (IS) and purchased from the University of Alberta Hospitals (Edmonton, Alberta, Canada). Acetonotrile, 1-butanol and water (all HPLC-grade) were purchased from Caledon Laboratories Ltd (Georgetown, Ontario, Canada). Sodium dodecyl sulphate (SDS) was purchased from Anachemia (Mississauga, Ontario, Canada). Analytical grade 1-butanol and KH2PO4 were purchased from Caledon Laboratories Ltd (Georgetown, Ontario, Canada). Nicotinamide adenine dinucleotide phosphate tetrasodium (NADPH), sodium chloride, sodium bromide, P407, NaOH, fetal calf serum, collagenase, sodium dithionate, thiobarbituric acid, diethyldithiocarbamic acid, butylated hydroxytoluene, ethylene diamine tetraacetic acid (EDTA), trypsin inhibitor, Percoll gradient, collagen, HEPES sodium salt, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol (PEG) 400 (analytical grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Potassium dihydrogen orthophosphate (KH_2PO4), dipotassium hydrogen orthophosphate, KCl, $MgCl_2 \cdot 6H_2O$, sucrose, and $CaCl_2 \cdot 2H_2O$ (all analytical grades) were obtained from BDH (Toronto, ON, Canada). Isoflurane USP was purchased from Halocarbon Products Corporation (River Edge, NJ, USA).

Penicillin-streptomycin, insulin, dexamethasone phosphate, DME media, and trypsin were obtained from GIBCO, Invitrogen Corporation (Carlsbad, CA, USA). Heparin sodium injection, 1000 U/mL and 10000 U/mL, were obtained from Leo Pharma Inc. (Thornhill, ON, Canada). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR Green SuperMix, and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA). Real-time PCR primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) according to previously published sequences.

2.2. Methods

2.2.1. Development of a reverse phase HPLC-UV assay of metformin in both human plasma and urine using octadecylsilane column

2.2.1.1. Instrumentation and chromatographic conditions

The chromatographic system consisted of a Waters (Milford, MA, USA) 600E multi-solvent delivery system pump, auto sampler with variable injection valve (Waters 717) and UV–visible tunable absorbance detector (Waters 486). The chromatograms were recorded using EZStart software (Scientific Software, Pleasanton, CA, USA) in a computer system for data collection and processing. Separation was performed on a 250 mm×4.6 mm internal diameter, 5 μm particle

size Alltima C18-column (Alltech, Deerfield Il, USA). The mobile phase consisted of acetonitrile-potassium dihydrogen phosphate buffer of 25 mM concentration with adjusted pH to 6.5 (34:66, v/v) and 3 mM SDS. It was prepared daily and degassed by filtering it under vacuum through a 0.045 μm nylon filter. The flow rate of the mobile phase through the analytical column was 0.7 ml/min, at room temperature (RT). The detection wavelength was set at 236 nm.

2.2.1.2. Standard and stock solutions

A stock solution of 5 mg/mL metformin was prepared by dissolving 100 mg in 20 mL of water. Working solutions were prepared freshly on the day of experiment from the stock by successive dilutions with water. Calibration samples of plasma (0.1 mL) were prepared containing metformin equivalent to 10, 30, 100, 500, 1000, 2000 and 5000 ng/mL. For urine, 0.1 mL samples containing metformin equivalent to 2, 20, 100, 500, 1000, and 2000 μg/mL were prepared. These samples were used for generation of standard curves.

An IS stock solution of 25 mg/mL was further diluted in water to prepare the working solution of 25 and 250 μg/mL for plasma and urine analysis respectively. All stock solutions were kept at -20°C until use.

2.2.1.3. Extraction procedure

The extraction procedure was similar to that described by Amini *et al*. (249), with some modification. To 100 µL matrix was added 20 µL of IS (25 µg/mL) solution in case of plasma. In case of urine, 30 µL of IS (250 µg/mL) was added to diluted matrix by 100 fold. An 80 µL volume of 10 M NaOH was added to alkalinize the media. Analytes were then extracted using a 3 mL mixture of 1-butanol: hexane (50:50 v/v). The tubes were vortex-mixed for 30 s for extraction and centrifuged at 3000 g for 3 min. The supernatant (organic layer) was transferred into clean glass tubes and acidified with 500 µL of 0.2% acetic acid, vortex-mixed for 60 s and centrifuged at 3000 g for 3 min. The supernatant was removed by aspiration using a pipette attached to a vacuum flask, and the remaining aqueous layer was evaporated to dryness in vacuo. The residues were reconstituted with 200 µL HPLC water. The injection volume into the chromatographic system ranged from 60-70 µL.

2.2.1.4. Recovery

The recoveries were determined with metformin concentrations of 100 and 400 ng/mL of plasma, and 100, 2000 µg/mL of urine using four replicates for each concentration. The extraction efficiency was determined by comparing the

extracted peak heights of analyte in samples to the peak heights of the same amounts of analyte directly injected to the instrument, without extraction.

2.2.1.5. Calibration, accuracy and validation

Full inter- and intra-day validation assessment was undertaken in human heparinized plasma samples. After validation was established in plasma, for urine a partial, one day, validation was undertaken. Samples were processed by adding IS and known amounts of metformin to 100 μL human matrix providing a concentration range of 10-1000 ng/mL in case of plasma and 2-2000 μg/mL in case of urine. The ratios of metformin to IS peak height were calculated and plotted vs nominal metformin concentrations to construct calibration curves. Data for calibration curves was weighted by a factor of 1/concentration due to the wide range of concentration used.

Intraday, accuracy and precision of the assay were determined using a range of concentrations of metformin in both matrices. The concentrations were selected at 10, 30, 100, 500 and 1000 ng/mL and at 2, 20, 100, 500 and 2000 μg /mL for human plasma and urine respectively. The urinary concentrations were selected to be higher because the drug is excreted extensively in urine. Each concentration had a replicate of five samples. Regarding human plasma samples, to permit the assessment of interday accuracy and precision, the assay was repeated on three

separate days. For each daily run, a set of calibration samples separate from the validation samples were prepared to permit quantification of the peak height ratios of metformin to IS. Precision was assessed by percentage coefficient of variation (CV%) while accuracy was represented by determining mean intra- or inter-day percentage error. The equations are as the following:

CV% intraday = (100×SD)/ (mean measured concentration)

CV% interday = (CV% run_1+CV% run $_2$+CV% run $_3$)/3

Mean % error intraday = 100× (measured concentration-expected concentration)/ (expected concentration)

Mean % error interday=(error% run_1+error % run_2+error% run_3)/3

2.2.2. Determination of the effect of obesity and RYGB on the single-dose pharmacokinetics of a standard release preparation of metformin in obese patients.

This clinical study received research ethics approval from the University of Alberta Research Ethics Board. Participants were recruited from the weight wise program which is a comprehensive region-wide obesity initiative established in 2005 and designed to deliver integrated, accessible, patient-focused, evidence-based care to adult and pediatric patients within Edmonton and surrounding area (total population of over 1 million). Blood samples were performed in the clinical

investigation unit (CIU), University of Alberta Hospital. The CIU is a joint venture between Alberta Health Services and the University of Alberta. Phase 1 studies are routinely performed in the CIU, which is an 8-bed unit located within the university hospital and staffed by administrators, nurses, lab specialists, pharmacists and dieticians experienced in PK studies. Standardized study meals, protocol-drive blood sampling and sample storage are all available on-site.

2.2.2.1. Study Design

This is non-blinded, cross sectional, controlled PK study. The PK parameters of a single dose of metformin between two groups of participants: post-RYGB and their matched BMI control obese subjects were evaluated.

2.2.2.2. Selection and number of participants

Thirty two consenting participants were enrolled in this study, sixteen in each group. They were randomly recruited 1 post-RYGB patient, 1 sex, age-matched (within 5 years) and BMI-matched (within 2 kg/m^2) control patient. To ensure that only clinically stable patients with successful operations are enrolled, surgical patients must be $\geq$ 3 months post-surgery and must not have experienced any major post-operative gastrointestinal complications, such as an anastomotic leak, outlet

obstruction or persistent vomiting. Eligible patients were asked to participate and informed consents were obtained.

2.2.2.3. Inclusion and exclusion criteria

Inclusion criteria were: 1- male and female, 2- (18 – 60) years old, 3- three months to one year post-RYGB surgery subjects and obese subjects who were recruited as controls, and 4- able to provide written informed consent. Exclusion criteria were: 1- undergone or undergoing revision of a previous bariatric procedure, 2. any major post-operative gastrointestinal complications, such as an anastomotic leak, outlet obstruction or persistent vomiting, 3- currently on metformin therapy, 4- any contraindications to metformin therapy such as allergy to the drug, chronic metabolic acidosis, history of lactic acidosis, liver failure or baseline liver enzymes higher than 3 fold above the upper limit of normal, congestive heart failure, renal failure (GFR< 60 ml/min), alcoholism, acute illness, fatty liver disease, 5- pregnant or nursing and not taking furosemide or nifedipine 6- diabetic patients

2.2.2.4. Dosing and risks

Glucophage is a standard release preparation of metformin. All study participants in both groups were administered a single dose of 2 tablets, 500 mg, following the collection of fasting blood samples. Risks were minimized because participants

with contraindications to metformin therapy were excluded. In addition, as this is was a single dose PK study, the risks associated with chronic metformin therapy were deemed negligible. Metformin does not cause hypoglycemia and has been used safely in non-diabetic patients. The major serious adverse effect of chronic administration is lactic acidosis (0.01% incidence), which typically occurs in the setting of renal failure. This is not an expected adverse effect of single dose therapy. Patients were warned that they should not receive iodinated contrast media for a radiological procedure within 48 h of ingesting metformin because this may increase the risk of lactic acidosis.

2.2.2.5. Detailed visit schedule

There were 3 visits. In visit 1 and after consent, participants underwent a baseline assessment, consisting of a history (sociodemographic variables, obesity-related comorbid conditions, alcohol/smoking status, medications), physical examination (including weight and BMI) and laboratory investigations (liver enzymes and creatinine) and confirm eligibility by reviewing inclusion/exclusion criteria. Height was measured at the first visit, without shoes, in centimetres to the nearest 0.5 centimetres. BMI is defined as the participant weight in kilograms divided by the participant height in meters squared (kg/m^2) and was calculated to the nearest one-

tenth unit. The height in meters recorded at visit 1 was used for all calculations. BMI is calculated using the following formula:

BMI (kg/m^2) = weight (kg) /(height (m) at Screening Visit)^2

In visit 2 (metformin day 1) following an overnight fast and prior to the start of blood collection, participants were asked to empty their bladder and the time was recorded starting as of, or as close to dosing as possible. The recorded time was the start of a 24 h urine sample collection. The participants voided all urine into a collection cup and pour it into a collection container for the next 24 h. Standardized 24 h urine collections were performed from time 0 to 24 to measure 24-hr metformin urinary excretion. By the aid of an iv catheter, fasting samples were collected prior to time zero for the determination of lipids. After the administration of 2 metformin tablets each 500 mg at time zero, blood samples were collected in lithium coated tubes at 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h for the analysis of metformin levels. Thereafter, blood samples were centrifuged for 30 minutes at RT 3000 g directly following the draw. Then, participants were discharged from the unit and instructed to return the following day for blood sampling 24 h post-drug administration. A standardized meal 2 and 6 h after drug administration, and a standardized snack at 4 h after were provided while the vitals and adverse events were monitored. In visit 3 (metformin day 2), the collection of the 24 h post drug administration blood sample for metformin level was performed.

The participant completed the 24 h urine sample collection and emptied their bladder into the collection container 24 h after the start time of collection.

2.2.2.6. Study procedures

Laboratory tests were analyzed, using venous blood samples collected from participants at all study visits. The screening/baseline visit laboratory tests include liver enzymes (ALP, ALT, AST, GGT) and cholesterol, TG, HDL and LDL. Timed blood collections were performed at visits 2 and 3 in the CIU. Laboratory tests to include fasting glucose, TC, TG, HDL and LDL. An iv catheter was inserted in the forearm. Fasting samples were collected for the determination of metformin. Participants then ingested 2 metformin tablets (500 mg), which is Time 0. Blood samples were again obtained at 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h after ingestion of the metformin tablets for the analysis of metformin levels. Next day, visit 3, participants returned to the CIU and a blood sample was collected at 24 h after metformin administration for the determination of metformin plasma level.

2.2.2.7. Drug assay

Each sample was stored at -80°C and assayed by a validated assay described above.

2.2.3. Determination of the effect of experimental short term and long term hyperlipidemia on metformin pharmacokinetics in rats

All study protocols involving were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Sprague–Dawley rats (Charles River, Quebec, Canada) with body weight ranging from 280 to 380 gm were recruited. All rats were housed in temperature controlled rooms with 12 h light per day. The animals were fed a standard rodent chow containing 4.5% fat (Lab Diet® 5001, PMI nutrition LLC, Brentwood, USA). Free access to food and water was permitted prior to the experiments.

2.2.3.1. Induction of hyperlipidemia

The HL rats were injected 1 g/kg intraperitoneal (ip) doses of P407 (0.13 g/mL solution in normal saline) as previously described (116, 295). To ensure the proper injection of P407, the animals were lightly anesthetised using isoflurane, and then allowed to recover. After 24 hrs, a 200 µL blood sample was withdrawn from the tail vein and spun to make sure that milky white plasma was present. Absence of such white plasma indicated that the dose had improperly been delivered (i.e. intramuscularly or subcutaneously). The NL control rats were injected normal saline in equivalent amounts. Dosing of metformin was performed at either 36 h to be considered as short term HL or at 108 h to be accounted for long term HL.

2.2.3.2. Surgical procedures

The day before the PK experiment, the jugular veins of the rats were catheterized with a cannula under isoflurane anesthesia (Surgivet, Waukesha, WI, USA). Each cannula was made by inserting 2-3 cm of silastic tubing (Laboratory tubing, 508-003, Dow Corning Corporation, Midland, MI, USA) over the tip of approximately 5 cm length of polyethylene tubing (Intramedic®,Clay Adams, Sparks, MD, USA) with about 0.5 cm of overlap. The Silastic tubing end was inserted into the right jugular vein and the polyethylene end was pulled out through the dorsal side of the neck. The exposed areas were closed using a surgical suture. The cannula was flushed with 100 u/mL heparin in saline and the end of the cannula was capped with a 23 G stainless steel needle, which was crimped using pliers. After recovery, each rat was allowed to have free access to water but not food. The morning after, each rat was transferred to a metabolic cage and after adaptation for approximately 30 min with free allowance to water, they were dosed iv with the metformin. Food was reintroduced ad libitum 2 h after the dose was administered.

2.2.3.3. Dosing and sample collection

The study used a total of 20 male Sprague–Dawley rats. The rats were allocated into three groups. The protocol included saline-treated NL rats, and rats exposed to P407 at 2 different periods: short term, high level HL at 36 h postdose and longer

term, subsided level HL at 108 h postdose. Each rat was housed in a metabolic cage where urine could be collected during the 24 h after metformin injection. The NL control group included 6 rats, whereas HL groups contained 6 rats for the short term HL and 8 rats for the long term HL.

Metformin at clinical weight-equivalent dose level of 30 mg/kg was injected starting at approximately 36 or 108 h after the ip doses of P407 or saline. The metformin injectable solution was made using sterile saline for injection to provide a final concentration of 12.5 mg/mL. Each iv dose was injected over 60 s via the jugular vein cannula, followed immediately by injection of 0.5 mL of 0.9% NaCL for injection. Serial blood samples (0.15-0.25 mL) were collected at at 0.08, 0.33, 0.65, 1, 2, 3, 4, 6, 8, 12 and 24 h post-dose for iv dosing into polypropylene micro-centrifuge tubes. Heparin in normal saline (100 U/ml) was used to flush the cannula after each collection of blood. Plasma was separated by centrifugation of the blood at 2500 g for 3 min. The samples were kept at -20°C until assayed for metformin. Urine was collected at the following periods: 0-3, 3-6, 6-12, 12-24 h and kept in -20°C until assayed for metformin within one month of the collection.

2.2.4. Effect of relative short and long term hyperlipidemia on gene and protein expression of metformin-related urinary excretion transporters

In this study, 12 male Sprague–Dawley rats (Charles River, Quebec, Canada) were used in the study. The mean±SD of their body weights were 310 ±55 g. As mentioned previously all the rats were housed in temperature controlled rooms with 12 h light per day. The animals were fed a standard rodent chow containing 4.5% fat (Lab Diet1 5001, PMI nutrition LLC, Brentwood, USA). Free access to food and water was permitted during experimentation.

2.2.4.1. Induction of hyperlipidemia

As mentioned before, the HL rats were injected with 1 g/kg ip doses of P407 (0.13 g/mL) solution in normal saline. To ensure the proper injection of P407, the animals were lightly anesthetised using isoflurane, and then allowed to recover. After 24 h, a 200 μL blood sample was withdrawn from the tail vein and centrifuged at RT to make sure that milky white plasma is separated. The NL control group were injected normal saline in equivalent amounts. Kidney collection was performed at either 36 h (n=4) to be considered as short term HL or at 108 h (n=4) to be accounted for long term HL. At those time points, the rats were killed by cervical dislocation under anesthesia using isoflurane and kidneys were excised and directly frozen in liquid nitrogen and kept at – 80 ºC freezer.

2.2.4.2. Extraction of mRNA and cDNA synthesis

Total RNA was isolated from the frozen kidney tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. First strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions.

2.2.4.3. Quantification by real time PCR

Quantitative analysis of mRNA expression was performed by real-time polymerase chain reaction (RT-PCR) by subjecting the resulting cDNA to PCR amplification in 96-well optical reaction plates using the ABI Prism 7500 System (Applied Biosystems). The 25 µl reaction mix contained 0.1 µl of 10 µM forward primer, 0.1 µl of 10 µM reverse primer, 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water and 1.25 µl of cDNA sample. Sense and anti-sense primer sequences for rOCT-1, rOCT-2, rMATE-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are illustrated (Table 3). The thermocycling conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Melting curve or dissociation stage was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

Table 3: Sequences of primers used for RT-PCR analysis

Gene	Sense primer	Anti-sense primer
rOCT-1 (296)	TCC TGC TGA CCT GAA GAT GCT	GAA CAG GTC GGC AAA CGA TAG
rOCT-2 (297)	GCT TGG GTA GAA TGG GCA TC	GTG AGG TTG GTT TGT GTG GG
rMATE-1 (217)	CAC ACT GGC AAT TGC GGT TA	CTT CAA GTT CTG GCT CCC GT
GAPDH (298)	GGC CAA GGT CAT CCA TGA	TCA GTG TAG CCC AGG AGG

2.2.4.4. Real time PCR Data Analysis

The real-time PCR data were analyzed using the relative gene expression (i.e., ΔΔ CT) method, as described in Applied Biosystems User Bulletin No. 2 and explained by Livak and Schmittgen (Livak and Schmittgen, 2001). The data are presented as the fold change in gene expression normalized to the endogenous reference gene GAPDH and relative to a calibrator. NL was used as the calibrator to measure the change in gene expression caused by HL.

2.2.4.5. Protein extraction and western blot analysis

2.2.4.5.1. Preparation of crude kidney membranes

Rat kidney biopsies (200 mg) were homogenized in 1 mL of homogenizing buffer (250 mM sucrose, 10 mM HEPES, and 10 mM Tris-HCL, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using electric tissue grinder. Differential centrifugation was used to obtain a crude membrane fraction. In brief, the kidney homogenates were centrifuged at 9000 *g* for 10 min at 4°C. Then, the supernatant was spun at 33,000 *g* for 60 min at 4°C. Finally, the resulting pellet was suspended in phosphate-buffered saline containing 0.1 mM PMSF. Samples were then sonicated on ice for 10 s to ensure homogeneity (299). Protein

concentrations were determined, using BSA as a reference protein. Aliquots were stored at -80°C until analysis.

2.2.4.5.2. Assay of total protein

The Lowry assay method was used, with quantitation being based on comparison of the concentration of an unknown sample with serial standard solutions of BSA(300). In order to assay the concentration of protein in microsomal preparations of kidney tissues, the following solutions were prepared:

Reagent A contained 1mL of $CuSO_4$ 1% in distilled water, 1mL of sodium and potassium tartarate 2% in distilled water, and 20 mL of Na_2CO_3 anhydrous 10% in 0.5 M NaOH. Reagent B contained 1:10 diluted solution of Folin-phenol reagent in distilled water. Working standard solutions of BSA were prepared at the concentrations of 500, 400, 300, 200, 100, and 0 µg/mL of BSA in distilled water from the stock solution of 500 µg/mL (50 mg/100 H_2O). To a number of clean test tubes containing 10 µL of microsomal preparation and 240 µL of distilled water (unknown concentration of protein) or 250 µL of each standard solution, 250 µL of reagent A were added and the tubes were incubated at RT for 10 minutes. In the next step and under continuous vortex mixing, 750 µL of reagent B was added to each of the test tubes and samples incubated at 50 °C for 10 more minutes. At the

last step 200µl of each mixture were transferred to a well in the ELISA plate and analyzed using an ELISA reader at 600 nm.

2.2.4.5.3. Western blot analysis

Briefly, 50 µg protein from each rat kidney was diluted in 2X loading buffer, boiled for 5 min at 100°C, separated by 7% SDS Polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane at 120 V for 1.5 h. Protein blots were blocked for 24 h at 4°C in blocking buffer containing 5% skim milk powder and 0.05% (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM KCl, 25 mM Tris–base). After blocking, the blots were washed three times with wash buffer (0.1% Tween-20in Tris-buffered saline). Membranes were incubated with the following primary antibodies: polyclonal rabbit OCT-1 (1:1000), polyclonal rabbit OCT-2 (1:1000), polyclonal rabbit MATE-1(1:1000) overnight at 4 °C and polyclonal rabbit β-actin (1:1000) after striping of the original membranes. The primary antibody solutions were removed and blots were rinsed three times with a wash buffer, followed by incubation with horseradish peroxidase conjugated with goat anti- rabbit secondary antibody (1/1000 dilution) for 2 h at RTand washed as previously described. Immunoactive proteins were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE

Healthcare Life Sciences, Piscataway, NJ). Optical density of different protein bands were quantified and normalized relative to the signals obtained from same membrane after striping for bands of β-actin using ImageJ software [National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij].

2.2.5. Detection of metformin metabolism

2.2.5.1. Comparing chromatograms of human and rat urine

Assay of metformin was conducted for rat urine samples (n=18) which were injected 30 mg/kg metformin iv. Urine samples were collected over 24 h then 100 µl urine was extracted as described before. The same procedure was utilized with human urine where urine samples from 32 subjects pretreated with 1000 mg metformin orally were used. All chromatograms were examined for any different peaks other than metformin and IS peaks.

2.2.5.2. Isolated rat hepatocytes

The isolation of hepatocytes followed a two-step liver perfusion procedure (301) with minor modifications (302). All study protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Sprague-Dawley rats (300-400 g) were used for isolation of hepatocytes. These rats were housed in cages and fed with water and food ad libitum.

2.2.5.2.1. Preparation of primary cultures

24-well plastic culture plates (VWR International: Mississauga, Ontario, Canada) were pre-coated with collagen for seeding the cells. After viability assessments, the cells were added in an appropriate quantity of DME media supplemented with 10% NL tar serum and antibiotics (penicillin/streptomycin, 1%). 0.3 million cells were added per well (300µL of cell suspension) and the plates were incubated for 6 h at 37°C in a humidifier with 95% O_2 and 5% CO_2. After 6 h, media containing the dead cells was removed and cells were washed twice with 1X PBS and then incubated with metformin in media at concentration of 1000 at 0, 12 and 24 duration time points.

2.2.5.2.2. Drug solutions

a. Stock solution: A 2.5 µg/µL concentration was prepared by dissolving 10 mg metformin in 4 mL of double distilled water (DDW).

b. Treatment (working) solutions: Metformin for use in co-incubation with hepatocytes: 10 µL of metformin stock solution (2.5 µg/µL) in DDW was added in 25 mL media to provide a final concentration of 1000 ng/mL.

2.2.5.2.3. Media preparation

DME media was supplemented with 10% NL rat serum, 1% penicillin/streptomycin antibiotic, 0.0063 mg/mL insulin, and 1M dexamethasone. The pH was adjusted to 7.4 using 1 M HCL or 10 M NaOH.

2.2.5.2.4. Hepatocytes-metformin incubations

2.2.5.2.4.1. Incubation of metformin with β –glucuronidase and sulphatase phase II enzymes.

Metformin was co-incubated in the media mentioned before with hepatocytes at a concentration of 1000 ng/mL alone or with either β–glucuronidase at (1200 Roy unit/mL) sulphatase at 150 (Fishman unit /mL) for each of the following time points 0, 12 and 24 hr (n=6 wells for each time point) at 37 ºC (303). At the various time points from 0-24 h after drug treatment, experiments were terminated by addition of 0.2 mL autoclaved H_2O to burst the cells. To each well, 30 µL of I.S were added then all 500 µL were collected and transferred test tube and kept at -20ºC until analyzed for the quantitative determination of metformin and qualitative detection of metformin presumed metabolite. Of note, Roy and Fishman are units to describe enzyme activity. A one Fishman unit is the enzyme activity that increases the rate of release of 1 µg 2-hydroxy-5-nitrophenyl sulfate in 1 h at +38°C. Meanwhile, one Roy unit is the enzyme activity that increases the rate of

release of 1 µg phenolphthalein from phenolphthalein-β-glucuronide in 1 h at +38°C.

2.2.5.3. Qualitative determination of metformin metabolism using isolated rat microsomes

2.2.5.3.1. Preparation of microsomal protein

Preparation of Microsomes was carried out as previously described (304). The microsomal protein concentrations were determined by Lowry method using BSA as a standard (300).

2.2.5.3.2. Microsomal incubation study

The microsomal solutions were diluted with cold sucrose to reach (1 mg protein/mL). Incubation was performed in incubation buffer at 37 °C in a shaking water bath (90 rpm) for three test tubes. One contained microsomal proteins only. The second one contained microsomal protein with (100 µg/mL) metformin. The third one was 100 µg/mL metformin alone with the media. Each tube had five replicates. The total volume of microsomal incubates was 1mL/tube of incubation buffer (3 mM magnesium chloride hexahydrate dissolved in 100 mM potassium phosphate buffer, pH 7.4). The reaction was initiated by the addition of 1 mM NADPH (final concentration 2 mM) for 30 minutes at 37 °C after which the

reaction was terminated by the addition of 300 µL ice-cold acidified acetonitrile. Aliquots of 100 µL were subjected to extraction method as in 1.3 for metformin and analysis by a modified HPLC method.

2.2.6. Fractional separation and mass characterization for the presumed metformin metabolite/s

2.2.6.1. Separation and collection of metabolite

The purpose of these experiments was to separate and identify any presumed predominate metformin metabolite after exposure of drug to rat microsomal proteins. A fractional separation was performed to isolate the chromatographic peak of interest, using several HPLC injections. In brief, an aliquot from microsomal incubation media was extracted by the same extraction procedure in section 1.3. A reverse phase chromatography was performed with a modification in the mobile phase. The mobile phase in this experiment consists of acetonitrile and ammonium hydroxide to adjust the pH to 6.5 in a ratio of (17:83) without adding SDS or using buffer, as the collected fractions were used for mass spectral analysis. All the other chromatographic conditions were the same. A solution of guanylurea and biuret solution of (1000 ng/mL each) were injected to test the matching elution times and m/z with any presumed metabolite peaks.

2.2.6.2. Mass spectrum characterization

Due to structural similarities between metformin, guanylurea and biuret, we expected that the isolated unknown metabolite m/z ratio and its fragmentation pattern might match with any of these compounds. The mass analysis was carried out using a single quadrupole system (Waters Micromass ZQ™ 4000 spectrometer coupled to a Waters 2795 pump, Milford, MA, USA) equipped with electron spray ionization (ESI) source. The mass detection was through a direct injection of analyte solutions into the detector at a concentration of 2 µg/mL for each analyte. Each of the analyte peaks was detected using selective ion recorder (SIR) in positive ion mode. The other parameter settings were gas source temperature of 140°C, capillary voltage of 3.45 kV and cone voltages set at 15 V for analytes. The injection volume was 10 µL. The gas flow of desolvation and cone gas flow were set at 550 and 120 L/h, respectively.

Using the apparatus, these conditions were found to be optimal for ionization of metformin, guanylurea, biuret and the unknown metabolite to and establishment of the m/z values of molecular ions. The concentrations of all injected analytes were 2µg/mL except for the isolated metabolites which were pooled from several HPLC runs as mentioned before and vacuum concentrated at low temperature.

2.2.7. Metformin stability study

To account for the different values of metformin renal recoveries, a stability test for metformin in urine was conducted. A 50 μg/mL sample of metformin was prepared in either human or rat urine and incubated at room temperature for 0, 3, 6, 9 and 24 h before assay of metformin (n=4-6). At the end of each time point, IS was added for each tube and extraction was performed according to the method in section 1.

2.2.8. Data analysis

All compiled data were expressed as mean±SD unless otherwise indicated. Regarding the clinical PK study, paper-based standardized case report forms were populated, and data were entered into a Microsoft Excel spreadsheet using double-data entry. Analyses were performed using Microsoft Excel (version 2008; Microsoft), InStat (version 3.1a; GraphPad Software, San Diego, CA), and SAS (version 9.2; SAS Institute, Cary, NC). Non-compartmental methods were used to calculate the PK parameters. Both Cmax and Tmax were determined directly from the data.

The terminal elimination rate constant (λz) was calculated by subjecting the plasma concentrations in the terminal phase to linear regression analysis. The t½

was calculated by dividing 0.693 by λz. The AUC_{0}-$_{24}$ of metformin (up to 24 h after dose) was calculated using the linear trapezoidal rule log-linear trapezoidal rule. The AUC0–∞ of metformin concentrations was calculated by adding to the $AUC_{0–24}$ h the last measured concentration divided by λz. The concentration at time 0 h after iv dosing was estimated by back extrapolation of the log-linear regression line using the first three measured plasma concentrations to time 0. The total CL was calculated as the quotient of dose to $AUC_{0-\infty}$ and the steady state volume of distribution (Vdss) as CL×AUMC/AUC, where AUMC is the area under the first moment plasma concentration vs. time curve, from time of dosing to infinity. The mean residence time (MRT) was determined as the quotient of AUMC to AUC. Due to the assumption that metformin is completely excreted unchanged in the urine, urinary recovery is indicative of the cumulative amount of systemically available drug. This allows estimation of F, calculated by dividing urinary recovery over total oral dose.

The oral F was estimated as follows:

F= dose given (mg)/ total amount recovered from urine (mg)

CLr was estimated by multiplying the fraction of the dose excreted in urine to the CLt. Between-group differences in the arithmetic means of continuous baseline variables were analyzed using a paired t test (if the normality assumption passed)

or a Mann-Whitney test (if the normality assumption failed). A Fisher exact test was used for binary variables. The level of significance in all statistical testing was set at $\alpha = 0.05$.

For the rat PK and hepatocytes incubation studies, single factor ANOVA was used to assess the significance of differences between groups.

Lean healthy subjects data from Tucker *et al* was utilized as controls to compare the effect of obesity on metformin PK. Statistical analysis was carried out between both groups using independent two sample t-test for unequal sample sizes.

3. Results

3.1. Development of an HPLC-UV assay for the determination of metformin in human plasma and urine

The chromatographic retention times were 9.5 min for metformin and 13.5 min for IS. The method provided specificity, with baseline resolution of IS and metformin with a lack of interfering peaks from endogenous components in plasma or urine (Figure 8). The peaks were symmetrical. The average recoveries were 93.7 and 88.5% with 100 and 1000 ng/mL metformin HCL in plasma, respectively. In urine, recovery was 83.0% and 89.4 for 100 and 2000 μg/mL, respectively. The average extraction recoveries for IS were 83.3 and 82.7% in plasma and urine respectively. There were excellent linear relationships (r^2>0.999) noted between the peak height ratios and concentrations over metformin HCL ranges of 10–5000 ng/mL plasma, and 2-2000 μg/mL of urine. Based on regression analysis of the concentration vs. peak height ratios of metformin to IS, in plasma and urine average slopes of 0.00041 and 0.0274 were observed, respectively. Corresponding intercepts were 0.00594 and 0.6385 respectively (Figure 9 a and b). The mean r^2 for plasma and urine standard curves were 0.9999 and 0.9972 respectively. The CV of intra-and inter-day assessments for both matrices were less than 19% (Tables 4 and 5). Mean inter-day error in human plasma was less than 5.2% (Table 4). In urine, mean percentage error was up to 9% (Table 5). Based on the inter- and intra-day CV% and mean error, it could be determined that the lower limits of quantitation (LLQ)

of metformin base were 7.8 ng/mL and 1.6 µg/mL based on 0.1 mL of human plasma and urine respectively (Tables 4 and 5).

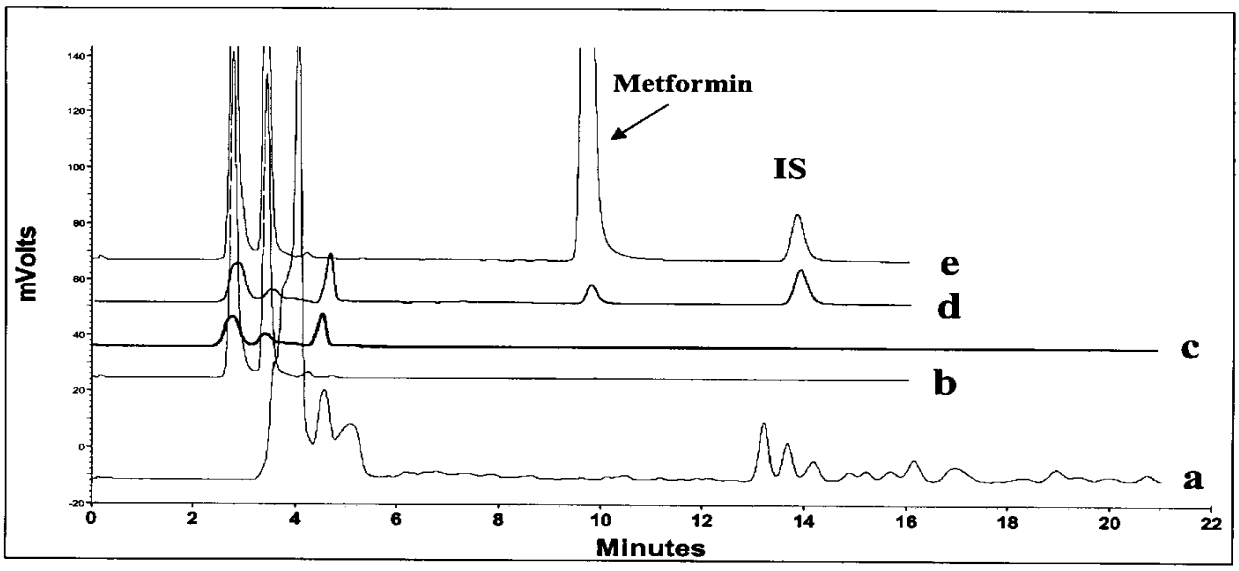


Figure 8: HPLC-UV chromatograms obtained for blank human plasma extracted by protein precipitation method (a), blank human urine (b), blank human plasma (c), human plasma sample after metformin administration (d) and human urine sample collected during 24 after metformin administration (e). In contrast to chromatogram a, chromatograms b through e were extracted using the described liquid-liquid extraction technique.

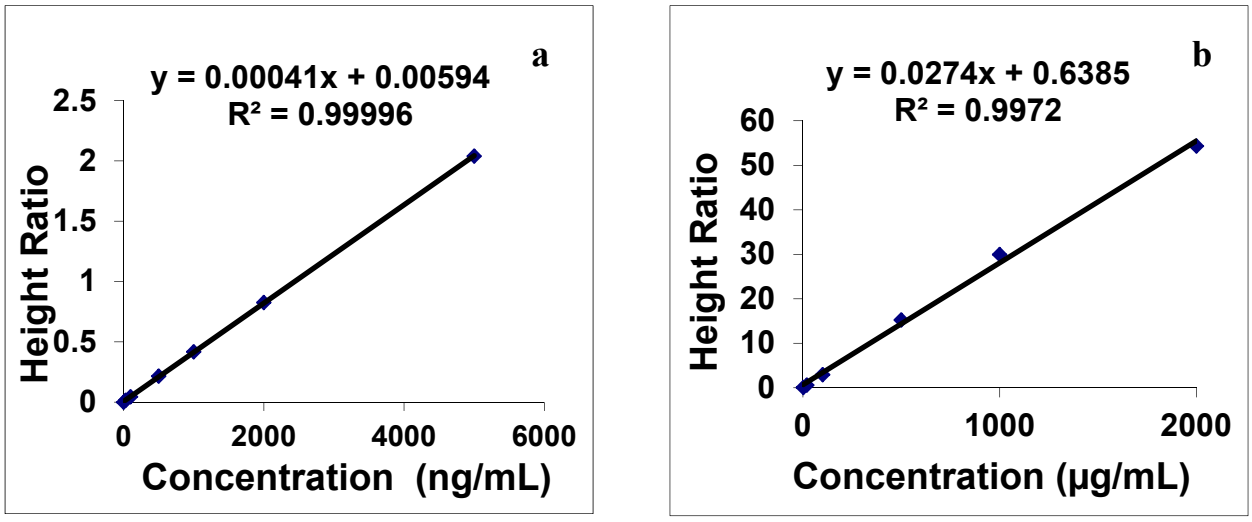


Figure 9: Representative calibration curves of metformin in human plasma (a) and urine (b).

Table 4: Validation data for the assay of metformin in human plasma. For each nominal concentration five analytical replicates were prepared. Analysis was carried out for the entire set of concentrations on 3 separate days, n=3.

Nominal concentration (ng/mL)	Intraday			Interday		
	Mean ±SD ng/mL (CV%)			Mean ±SD, ng/mL	CV%	Error %
10	7.46±0.51 (6.81)	10.4±1.11 (10.7)	10.6±1.78 (16.8)	9.50±1.77	18.6	-5.01
30	29.3±4.12 (14.1)	27.3±1.30 (4.76)	31.2±0.62 (1.98)	29.3±1.95	6.64	-2.37
100	97.2±5.06 (5.21)	95.5±10.4 (10.9)	97.9±1.86 (1.90)	96.9±1.26	1.31	-3.14
500	492±3.44 (0.70)	493±6.67 (1.35)	522±34.4 (6.58)	502±17.4	3.46	0.46
1000	975±13.10 (1.34)	982±40.3 (4.10)	1096±39.7 (3.63)	1017±67.8	6.66	1.79

Table 5: One day validation data for the assay of metformin in human urine. For Each nominal concentration five analytical replicates were prepared.

Nominal concentration (µg/mL)	Mean ±SD, µg/mL	CV%	%Error
2	2.09±0.04	1.95	4.56
20	20.07±0.19	0.93	0.34
100	105±2.09	2.00	4.72
500	543±5.12	0.94	8.66
2000	2047±89.30	4.36	2.34

3.2. Stability study for metformin in urine

To account for the different values of metformin renal recoveries, a stability test for metformin in urine was conducted. There was no significant change in peak height ratios of metformin/IS at 3, 6, 9 and 24 hr with regard to control. Metformin has no instability issue in either human or rat urine (Figure 10).

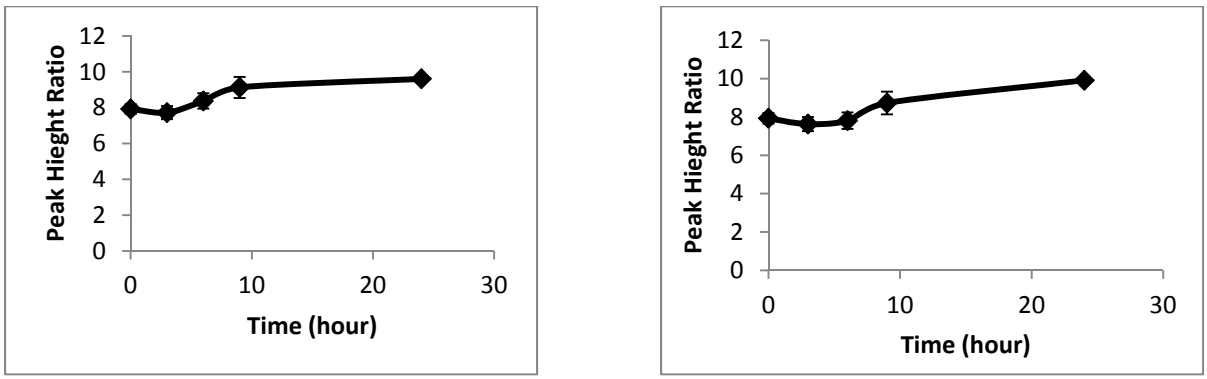


Figure 10: A 50 µg/mL concentration of metformin was prepared in human or rat urine and incubated for 0, 3, 6, 9 and 24 h. Extraction and metformin assay were performed after the addition of 30 µL of IS solution (5 µg/mL). Peak height ratios of metformin/ IS did not change significantly. Values are expressed as mean ± SD.

3.3. Determination the effect of Roux-en-Y gastric bypass surgery on a single-dose pharmacokinetics of a standard release preparation of metformin

Baseline characteristic of the patients such as mean age, BMI and weight did not change between both groups despite a 10.4 kg higher weight in the obese control group compared to surgically manipulated subjects (114.6 vs 104.0 kg; P = 0.3) (Table 6). Fasting glucose level was significantly higher in obese controls than the RYGB subjects (5.1 vs. 4.4 mmol/L; P =0.0006) although the glycated haemoglobin (HbA1c) levels were the same between groups. LDL and TC were significantly higher in control obese subjects compared with gastric bypass subjects (3.0 vs 2.4 mmol/L; P =0.03) and (3.96 vs 4.78 mmol/L; P =0.02) respectively. Regarding AST and creatinine clearance, no significant change was observed suggesting no liver or kidney dysfunctions (Table 6).

As for metformin PK parameters, gastric bypass-subjects demonstrated a 50% higher F compared with control obese subjects (41.8 vs 27.8%) contrary to our hypothesis. The difference in F remained statistically significant after normalization for baseline body weight (Table 7). Interestingly, the weight-normalized Vd and weight-normalized CLr were 40 and 43% higher (P, 0.05) in gastric bypass subjects than control obese subjects, respectively (Table 7). It is critical to note that about ninety-eight percent of renal excretion took place within the first 24 h. The $t_{1/2}$ as well as both Cmax and Tmax did not show any significant

change between groups. Compared to before surgery, post-surgical plasma lipid and LDL levels were significantly lower (Figure 11). In addition, operated subjects showed a 21% increase in the AUC0–∞ compared with obese subjects (13.7 vs. 11.4 μg.h/mL) but this increase was statistically insignificant (Figure 12). Most of the measured PK parameters in post-surgery seemed to be corrected with respect to their obese controls.

Table 6: Baseline characteristics of the obese control and post-surgery subjects recruited in the clinical study.

Variable	**Bypass n=16 Mean or No. (SD or %)**	**Controls n=16 Mean (SD or %)**	**p-value**
Age (years)	44.4 (10.0)	43.5 (11.7)	0.82
Sex	F (82)	F (82)	n/a**
BMI (kg/m^2)	38.0 (7.9)	40.5 (6.9)	0.36
Weight (kg)	104.0 (29.0)	114.6 (26.1)	0.30
Creatinine (μmol/L)	62.9 (9.8)	65.2 (11.8)	0.56
HbA1C (%)	5.5 (0.3)	5.6 (0.6)	0.28
Fasting glucose (mmol/L)	4.4 (0.4)	5.1 (0.6)	0.0006
AST (U/L)	22.6 (5.4)	25.3 (6.6)	0.22
Total cholesterol (mmol/L)	3.96 (0.69)	4.78 (1.08)	0.02
Triglyceride (mmol/L)	1.0 (0.33)	1.5 (0.88)	0.29***
HDL cholesterol (mmol/L)	1.1 (0.25)	1.1 (0.38)	0.74
LDL cholesterol (mmol/L)	2.4 (0.53)	3.0 (1.02)	0.03
Hypertension	8 (50)	7 (43.8)	1.0
Type 2 Diabetes	1 (6.3)*	0 (0)	1.0
Dyslipidemia	1 (6.3)	4 (0.25)	0.33
Hypothyroidism	3 (0.38)	2 (0.13)	1.0
Sleep Apnea	7 (43.8)	4 (0.25)	0.46
Gastrointestinal Reflux	1 (6.3)	4 (0.25)	0.33

*diabetes in remission post-RYGB

** subjects were sex matched

*** Mann-Whitney U-statistic

HDL=high-density lipoprotein; LDL=low-density lipoprotein; mmol=millimoles; U=units

Table 7: Effect of RYGB on metformin PK parameters after oral single dose administration (1000 mg)

	Obese subjects	Gastric bypass subjects
F %	27.81±10.40	41.80±16.20*
Urinary recovery (0-24h) (mg/kg)	2.0±0.78	3.10±1.20*
AUC_{0-24h} (µg.h/L)	11.10±3.60	13.4±5.70
AUC_{0-inf} (µg.h/L)	11.40±3.60	13.71±6.0
t½ (h)	4.0±0.87	3.90±0.74
CLr (mL/min)	336.7± 131.0	461.30±199.20*
CLr (mL/min/kg)	3.0±1.01	4.30±1.60*
Vd (L/kg)	1.0±0.40	1.40±0.40*
Cmax (µg/L)	1.80±0.61	2.01±0.86
Tmax (h)	3.0 (1.5–3.0) †	3.0 (1.5–3.0) †

Numbers are means±SD. †Numbers are medians (range). Mann-Whitney U statistic was used.

(*) $P < 0.05$, compared to control.

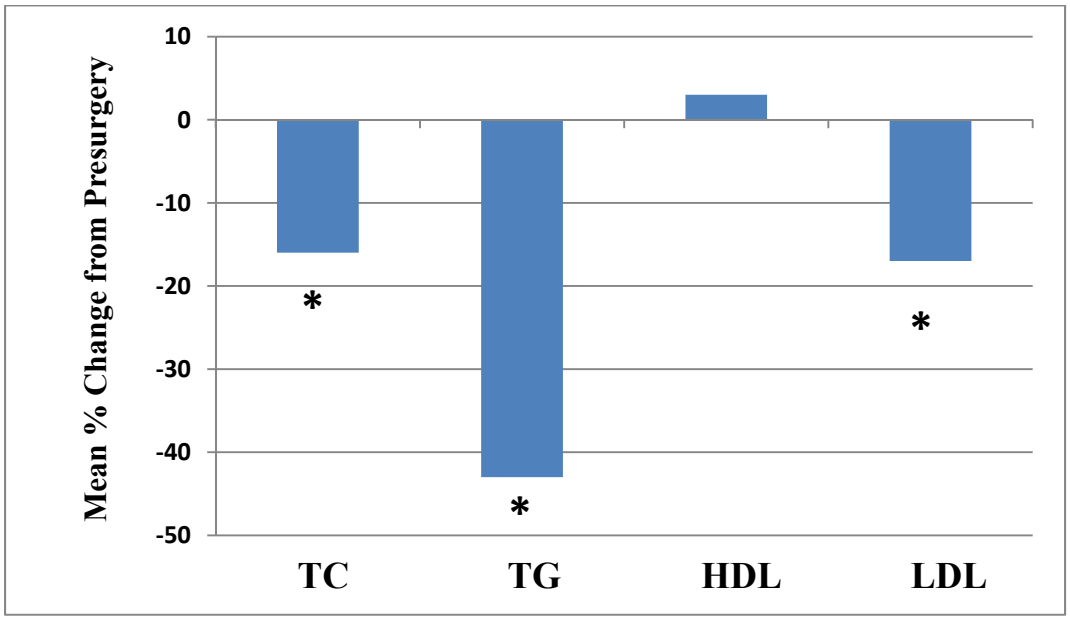


Figure 11: Changes in lipid profiles in post RYGB subjects who were administered 1000 mg metformin orally. Compared to before surgery, surgery resulted in significant reductions in plasma lipid profiles. (*) P < 0.05, compared to control.

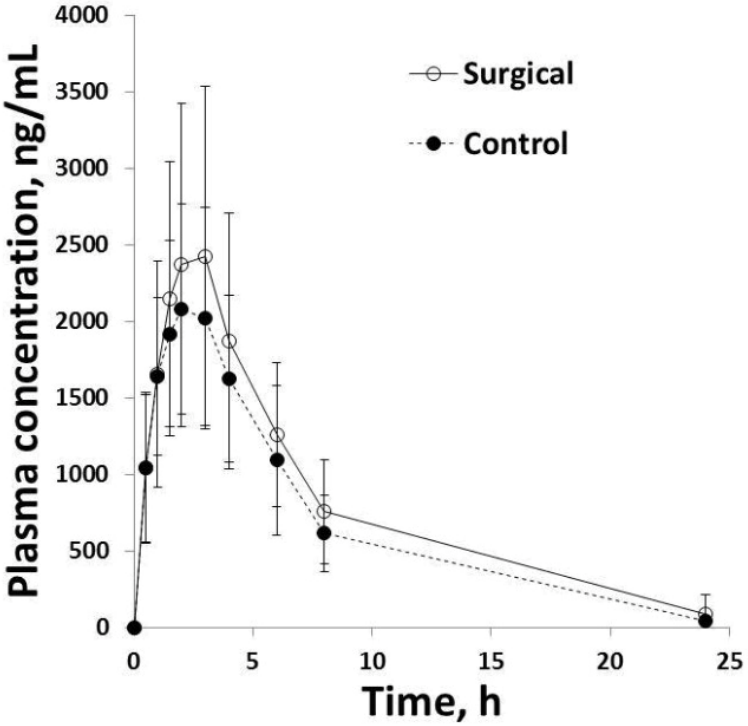


Figure 12: Plasma metformin concentration-time profile in control obese subjects (closed circle) and RYGB subjects (opened circle) after 1000 mg metformin oral administration.

3.4. Determination the effect of experimental short term and long term hyperlipidemia on metformin pharmacokinetic parameters in rats.

In order to determine the effect of short term exposure and longer term exposure to elevated plasma lipoproteins on the PK of metformin, a 30 mg/kg iv dose of metformin was given to rats pretreated with P407 (1 g/kg). For short term exposure, the PK study was carried out after 36 h of HL induction while for long term exposure; rats were left for 108 h before the PK study started.

After iv administration of 30 mg/kg of metformin, the plasma concentrations for both HL and NL rats declined bi-exponentially, and a 2-compartmental model fit well to the plasma concentration vs. time profiles. The exposure for elevated plasma LP for short or longer periods was found not to cause any significant change in metformin PK (Table 8). Both HL groups showed equivalent plasma concentrations, CL and Vd to NL rats for both duration treatments 36 h and 108 h (Figure 13, Table 8). When comparing the values of CLnr to the CLt, it was found that from 92 to 100% of the metformin was eliminated unchanged via urinary excretion.

Table 8: The PK parameters (mean ±S.D.) of metformin in normal and hyperlipidemic rats after iv administration of 30 mg/kg calculated as base.

	NL	HL 36	HL 108
n	6	6	8
$AUC_{0-\infty}$ (µg·h/L)	16.92± 5.12	13.75±4.01	13.83±2.67
F%	92.17±7.09	92.15±15.00	101.85±3.24
t½ (h)	1.83±0.42	1.69±0.40	1.88±0.48
CLt (mL/min/kg)	32.23±9.33	37.13±7.92	34.81±5.62
CLr (mL/min/kg)	29.71±10.42	33.85±8.78	36.80±5.71
CLnr (mL/min/kg)	2.64±2.59	3.28±6.25	0.06±0.14
Vdss (L/kg)	1.47±0.56	2.39±1.03	1.53±0.59
Vd_{β} (L/kg)	4.85±1.81	5.61±1.52	5.78±2.15
MRT (h)	0.85±0.23	1.14 ±0.48	0.73±0.24

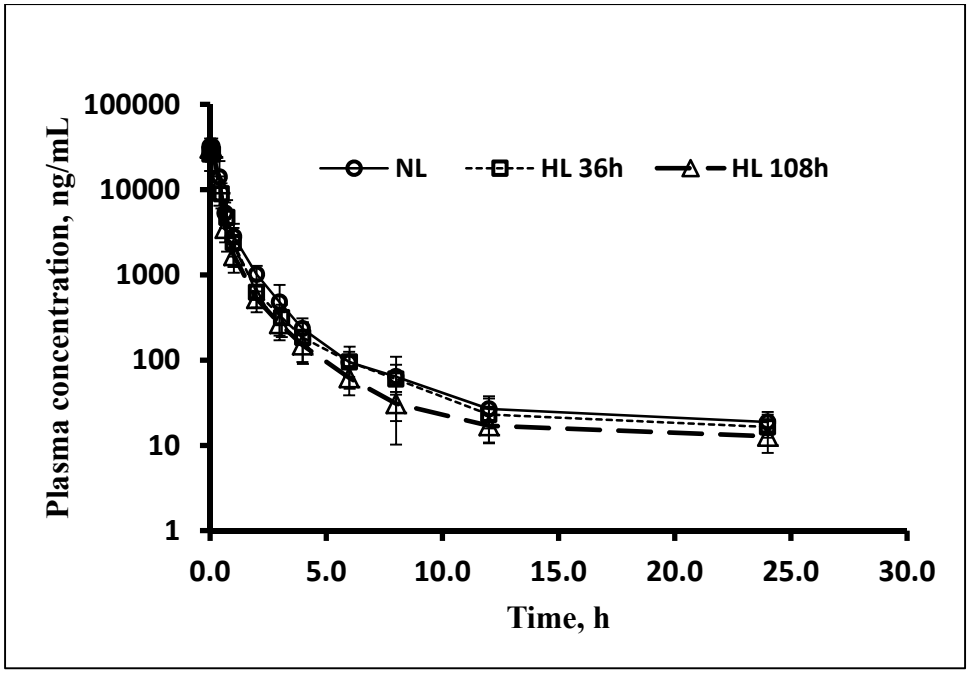


Figure 13: Plasma concentrations vs. time plots of metformin after administration of 30 mg/kg as iv doses in normolipidemic rats (n=6) circles, hyperlipidemic rats injected with P407 for 36 h (n=6) squares and hyperlipidemic rats injected with P407 for 108 h (n=8). Values are plotted as means ±SD.

3.5. Effect of short term and long term hyperlipidemia on gene and protein expression of metformin- related urinary excretory transporters

3.5.1. Expression of rOCT-1, rOCT-2 and rMATE-1 transporters in rat kidney

Elevated plasma lipoproteins for both experimental periods did not cause any significant change in renal expression of rOCT-1, rOCT-2 and rMATE-1. In the short term HL, the percentage changes in fold of mRNA induction of rOCT-1 and rOCT-2 were 84 and 225% respectively, however they were not significant, with P=0.07 and 0.09 for rOCT-1 and rOCT-2 respectively (n=4/group) (Figure 14a and 14b). The CV% were 32 and 67% for rOCT-1 and rOCT-2 HL groups respectively which could be attributed to the small number of rats used (n=3/group). As for the rMATE-1 gene expression, the CV% was 24% for the HL group. Although the mean increase was 43% it was statistically insignificant (Figure 14c).

In longer term HL, elevated plasma lipoproteins caused a significant decrease in mRNA levels by 48% and 66% for rOCT-1 and rMATE-1 respectively ($P<0.05$) (n=4/group) (Figure 15a and 15c). The %CV was about 30% for both test groups. However, HL did not change the mRNA expression of rOCT-2 where the test group had high variability (CV%=57) (Figure 15b).

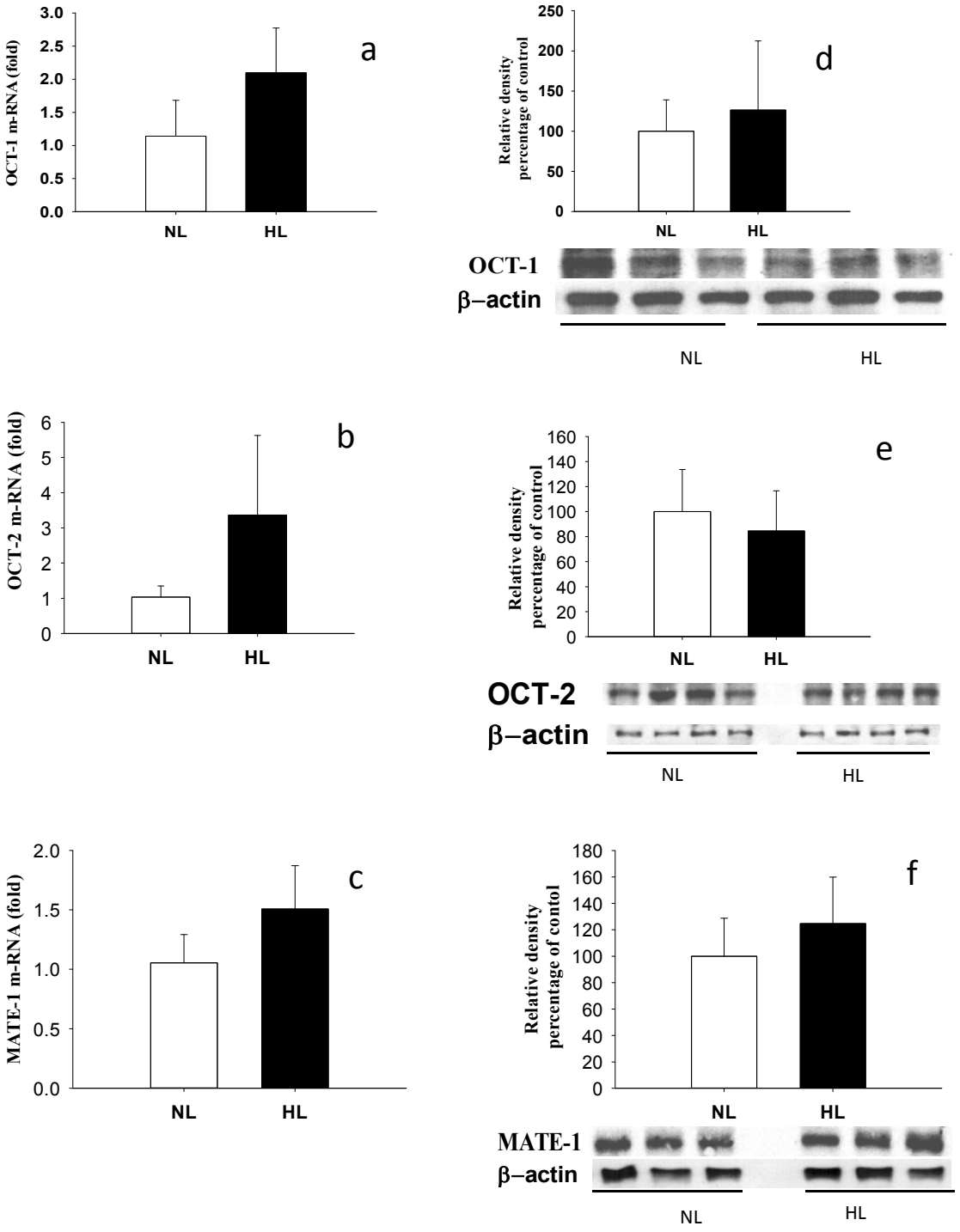


Figure 14: Effect of hyperlipidemia after 36 h ip injection of P 407 on the mRNA of rOCT-1(a), rOCT-2 (b) and rMATE-1(c) (left panel) and protein expression (right panel) of rOCT-1 (d), rOCT-2 (e) and rMATE-1(f) transporters in rat kidney tissues. Values presented are means ± SD; n=3 or 4 per group.

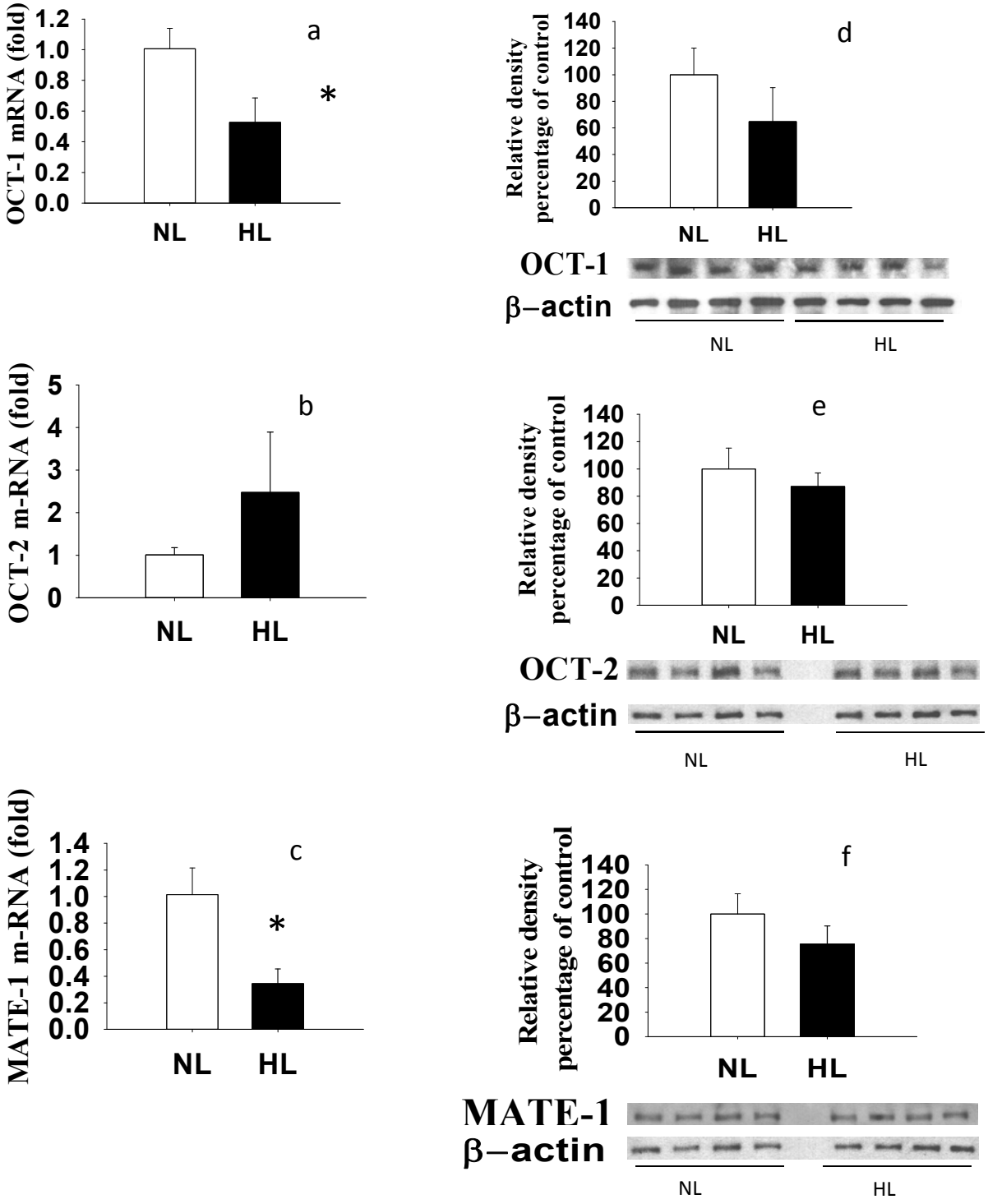


Figure 15: Effect of hyperlipidemia after 108 h ip injection of P 407 on the mRNA of rOCT-1(a), rOCT-2 (b) and rMATE-1(c) (left panel) and protein expression (right panel) of rOCT-1 (d), rOCT-2 (e) and rMATE-1(f) transporters in rat kidney tissues. Values presented are means ± SD; n= 4 per group.

3.5.2. Effect of HL on protein expression of rOCT-1, rOCT-2 and rMATE-1 transporters in rat kidney

In short term HL, the elevation of LP did not cause any change in the expression of metformin transporter proteins. This result goes in accordance with the gene expression results (n=3/group) (Figure 14d, 14e, 14f). As for longer term HL, despite the observed significant difference in gene expression of rOCT-1 and rMATE-1, protein expression did not change at this more extended time point (n=4/group) (Figure 15d, 15f). The protein expression of rOCT-2 was likewise unaffected by HL (n=4/group) where only a 13% differential in the band optical density was observed (Figure 15e).

3.6. Detection of metformin metabolism

3.6.1. Comparing chromatograms of human and rat urine

Prior to the onset of any concerted attempts to isolate metabolites, chromatograms of extracted rat urine were carefully compared to that of drug-free rat urine from rats given 30 mg/kg metformin orally. There were at least 3 small peaks apparent that eluted before that of metformin at 6.9, 7.8 and 8.6 min, which were not seen in the blank urine. These peaks were likewise seen in chromatograms of extracted human urine from the RYGB study (Figure 16). It was believed that these peaks might represent metformin presumed metabolites, and this prompted further

investigation. Interestingly, the solvent front peak in blank chromatogram at 4.8 min was also enlarged by more than 8% in extracted chromatograms of either human or rat urine, suggesting perhaps more metformin-associated material. For the clinical study, a 100 fold dilution of 100 μL urine was performed before extraction while for the purpose of metabolite (s) detection; extraction was performed directly on 200 μL of either human urine or rat urine. Both blank and treated urine samples were similarly processed.

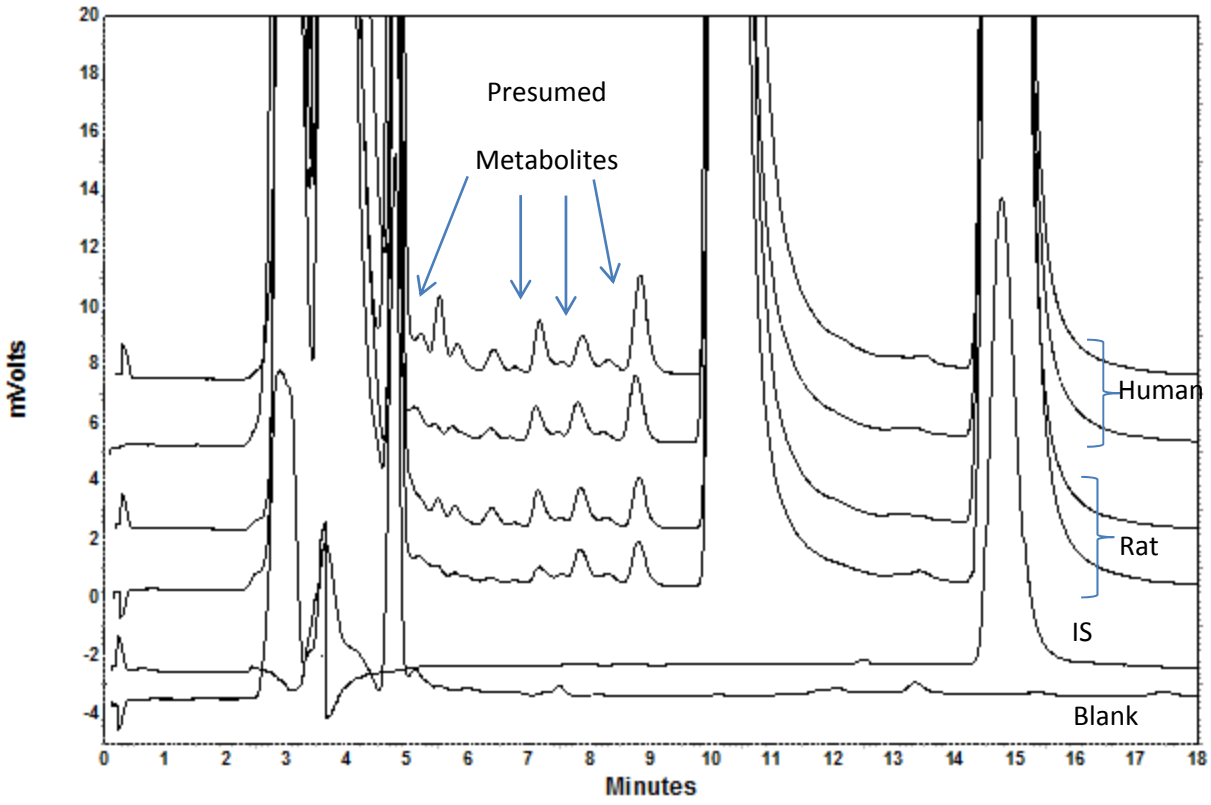


Figure 16: HPLC-UV five chromatograms were obtained from extracted urine samples. Two chromatograms illustrate extracted urine from subjects administered metformin orally 1000 mg and another two chromatograms illustrate extracted urine samples of 2 rats administered 30 mg metformin iv respectively. There are unidentified small peaks detected to be eluted before metformin peak. No unknown peak was detected in blank or in IS run.

3.6.2. Chromatograms of metformin incubated hepatocytes

In the wells that contained the metformin at 0 h time, there was no appearance of unusual peaks other than metformin main peak at 11.6 minute. However, an obvious small peak appeared with longer incubation times of 12 and 24 h, eluting just before metformin (at 10.7 min). In addition, the solvent front peak at 4.8 min in the blank was noticeably higher after incubation with metformin at longer time points which may account for the presumed metabolite eluted at the same retention time of the front peak (Figure 17).

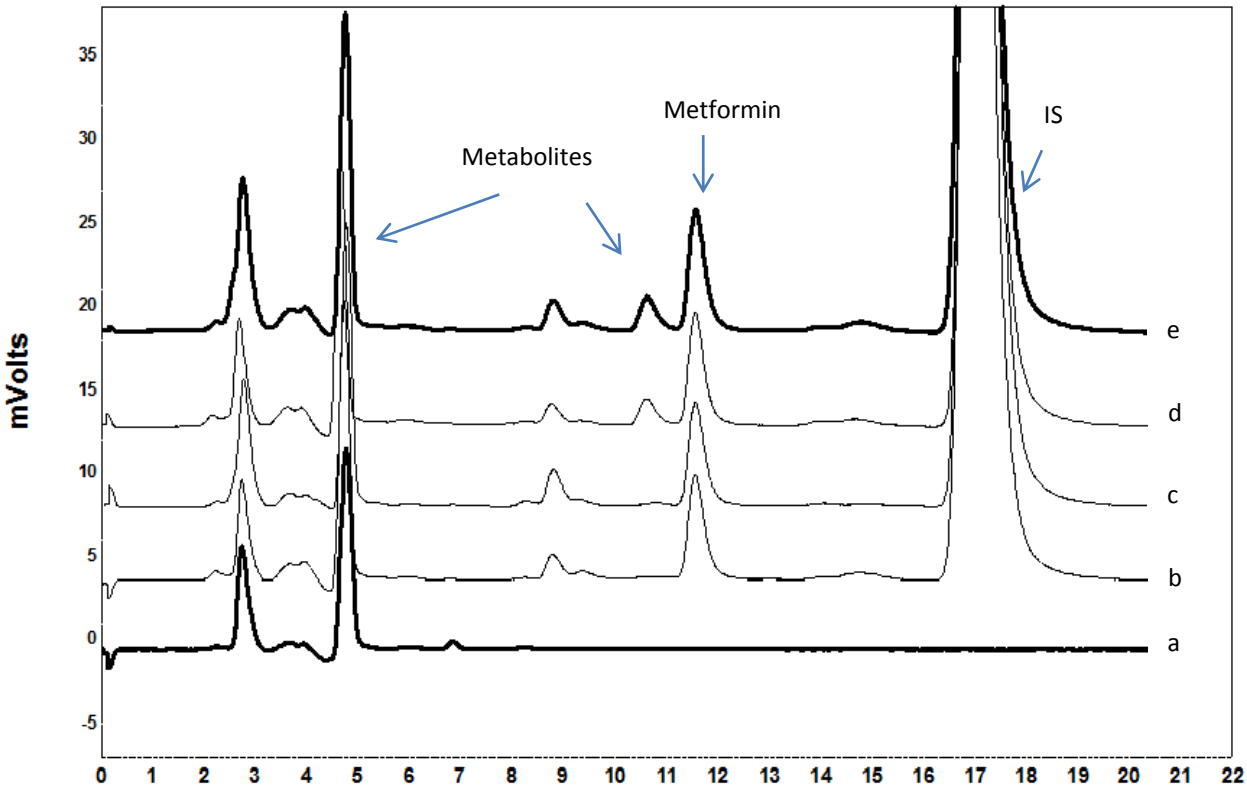


Figure 17: Effect of hepatocytes on metformin metabolism. Hepatocytes were incubated with 1000 ng/mL metformin at different time points. Extraction for individual wells was performed and HPLC-UV chromatograms were obtained for: blank incubation media for 24 h (a), spiked media with 1000 ng/m/L metformin (b), incubation media with hepatocytes at 0 time point (c), 12 h time point (d) and 24 h time point (e). There is an increase in the front peak at 4.8 min and a distinctive growing peak which was eluted at 10.7 min that appeared clearly at longer periods of incubations. Each chromatogram is a representative for 6 replicates except for blank.

3.6.3. The effect of phase I and phase II metabolism on metformin using primary rat hepatocytes

Incubation of metformin with hepatocytes at a concentration of 1000 ng/mL for 12 and 24 h time points did not yield any significant change in metformin concentrations (C12 and C24) (Figure 18). There are be 3 different scenarios possible in the hepatocyte experiment. Firstly, if metformin undergoes metabolism by phase I enzymes, concentrations of metformin at C12 and C24 should be decreased due to the formed metabolite/s. Upon incubation with β-glucuronidase at either 12 or 24 h (G12, G24) or with sulphatase at 12 and 24 h (S12, S24), no change would be expected because no conjugates were formed with respect to time zero (C_0). The second possibility is when metformin undergoes phase II metabolism only and conjugates are produced. In this event, the concentrations at C12 and C24 should be significantly less than the corresponding time points with the phase II hydrolyzing enzymes (G12, G24, S12, S24). The third and last possibility is when metformin has not been metabolized. At this case, no expected change would be detected throughout the different time points either with or without incubation with the hydrolyzing enzymes, nor should any new peaks appear in the chromatograms. Our results showed that metformin concentrations did not change during different time points of incubation with or without adding

the phase II hydrolyzing enzymes. This suggests that metformin did not undergo metabolism under the used experimental conditions (Figure 18). On the other hand, new peaks appeared in the chromatogram with extended incubation times. It must be recognized that the UV max of the new peak(s) appearing at 12 and 24 h were not known or evaluable. This could have an impact on assessing whether the metformin peak should have noticeably decreased or not.

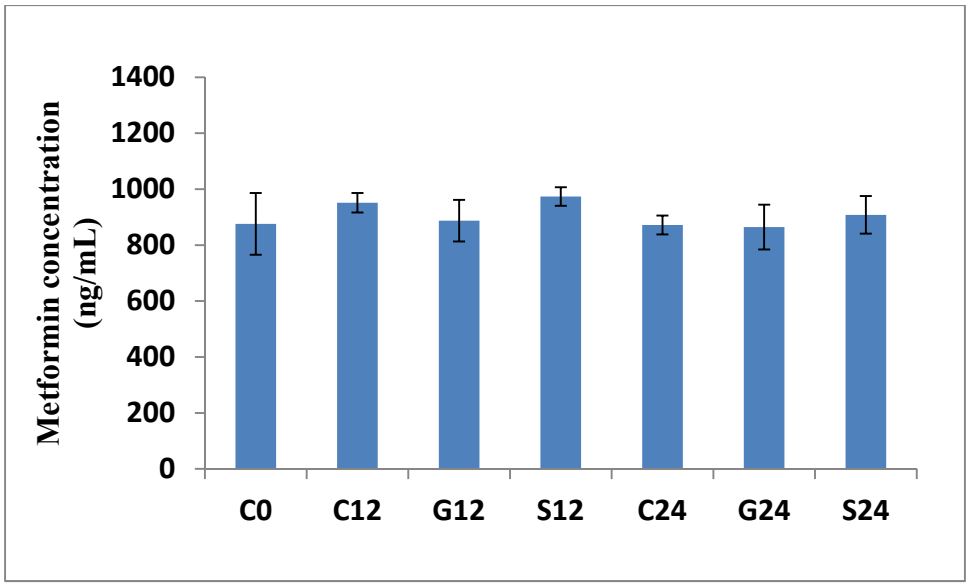


Figure 18: Effect of hepatocytes on metformin metabolism. Hepatocytes were incubated with 1000 ng/mL metformin at (0, 12 and 24) h time points alone or with β- glucuronidase (1200 Roy) or sulphatase enzyme (150 Fishman).A the end of each time point the incubation was stopped, cells were burst by the addition of 0.2 mL autoclaved H_2O to wells. The content of each well was subjected for extraction to determine the change in metformin concentrations. There was no significant change in metformin concentrations at different time points with or without the hydrolyzing enzymes. C0, C12 and C24 are hepatocytes with metformin at 0, 12 and 24 h respectively.G12 and G24 are hepatocytes incubated with metformin and β-glucuronidase at 12 and 24 h. S12 and S24 are hepatocytes incubated with metformin and sulphatase at 12 and 24 h.

3.6.4. Effect of metformin incubation with microsomal protein on metformin chromatography

Upon the incubation of 100 μg/mL metformin for 30 min with isolated rat microsomes 1 mg protein/mL, 2 obvious new peaks at 4.8 and 8.6 min were detected in the chromatogram of extracted sample which are not present in the chromatogram of the blank microsomes or drug injected alone (Figure 19). This is qualitative evidence for metabolic products having been produced during the incubation period by phase I metabolic enzymes such as CYP. The incubation media were then extracted as outlined in the developed HPLC assay. A chromatographic run was performed using a modified mobile phase; SDS and buffer free, to facilitate later fractional separation and mass characterization by mass spectroscopy. Metformin was not retained on the column due to free SDS mobile phase used (Figure 20 b).

Using UV detection and the HPLC column with the modified mobile phase, it was observed that a presumed metabolite peak eluted at nearly the same retention time (4.8 min) (Figure 20 c) as in the run with the original mobile phase in the hepatocyte incubation study (Figure 17). A pure injection of biuret solution yielded a peak (Figure 20 d) that did not match that of the metabolite peak. However, a pure injection of guanylurea yielded a peak with the same retention time as that of

the presumed metabolite peak (Figure 20 e). Fractional separation was then performed to collect that peak of interest.

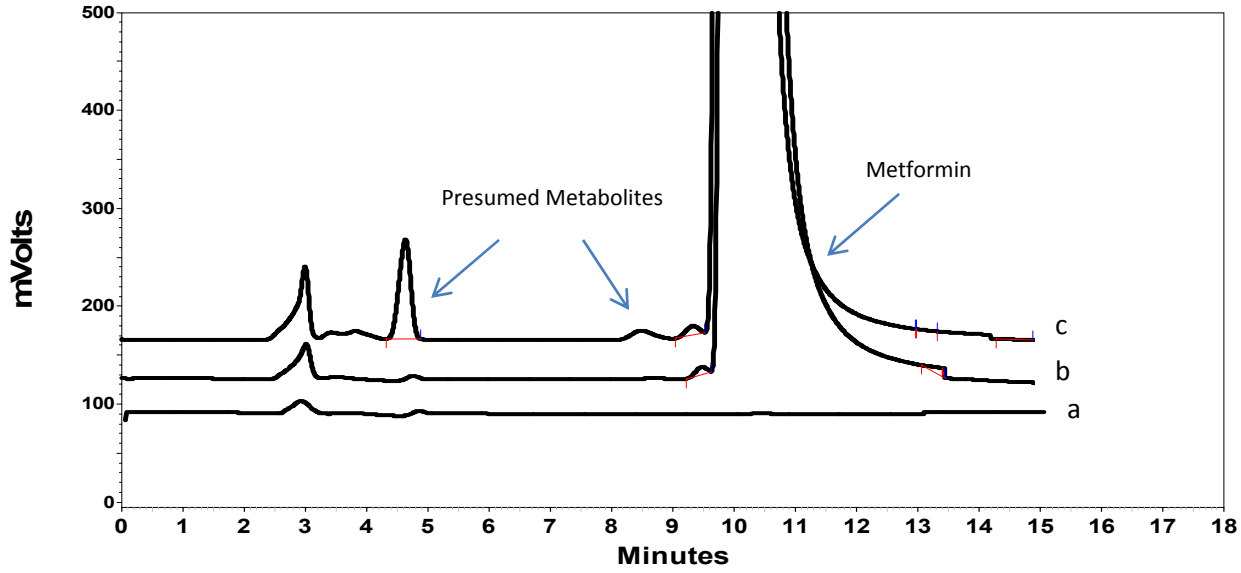


Figure 19: Three chromatograms representing microsomal incubation study. Microsomes (1 mg protein/mL) were incubated for 30 minutes at 37 °C in a shaking water bath with (100 µg/mL) metformin (c) or without (a). Chromatogram (b) is only metformin (100 µg/mL) acting as positive control.

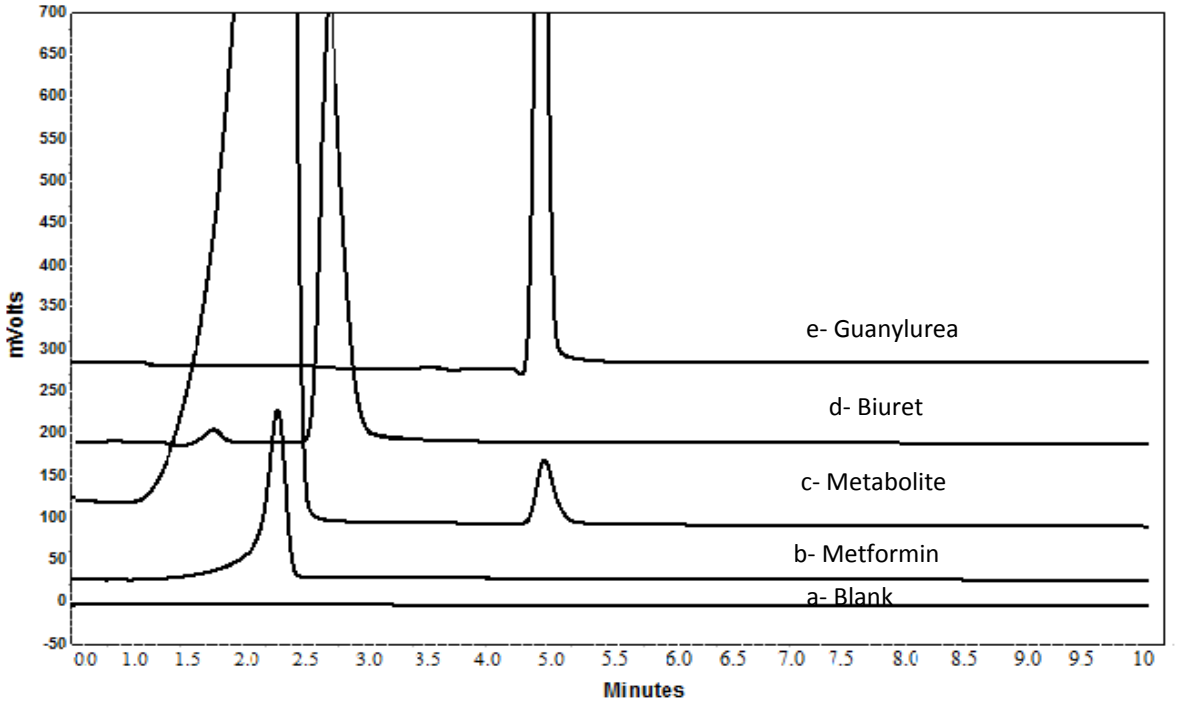


Figure 20: Five chromatograms showing the run for the extracted of microsomal incubation media using modified mobile phase without SDS to facilitate mass characterization. Fractional separation of metabolite that appears at 4.8 min was performed for several runs. Biuret (1000ng/mL) peak does not match with the metabolite peak while guanyl urea (1000 ng/mL) was eluted with the same retention time as metabolite peak. Extracted microsomal media which contains both metformin (100 µg/mL) and metabolite (c). Metformin (50 ng/mL) (b), guanylurea (e) and biuret (d) solutions were directly injected on the HPLC system. Blank is the extracted microsomal media without metformin (a).

3.7. Spectral analysis of the isolated metabolites

A mass spectral analysis of the isolated eluted peak of interest from the HPLC run was performed using the conditions mentioned in section 2.2.6.2. It revealed the presence of a mixture of two main m/z ratios. One of the values was m/z ratio of 103 and the other was 104.9 m/z ratio, both of which were lower than metformin with 129.9 m/z ratio. A pure injection of biuret gave a m/z ratio of 104 and its fragmentation pattern was somewhat similar to that of metabolite, perhaps due to to structural similarities. Guanylurea gave a m/z ratio of 103, which matched that of the largest m/z ratio in the collected microsomal incubation fraction (Figure 21).

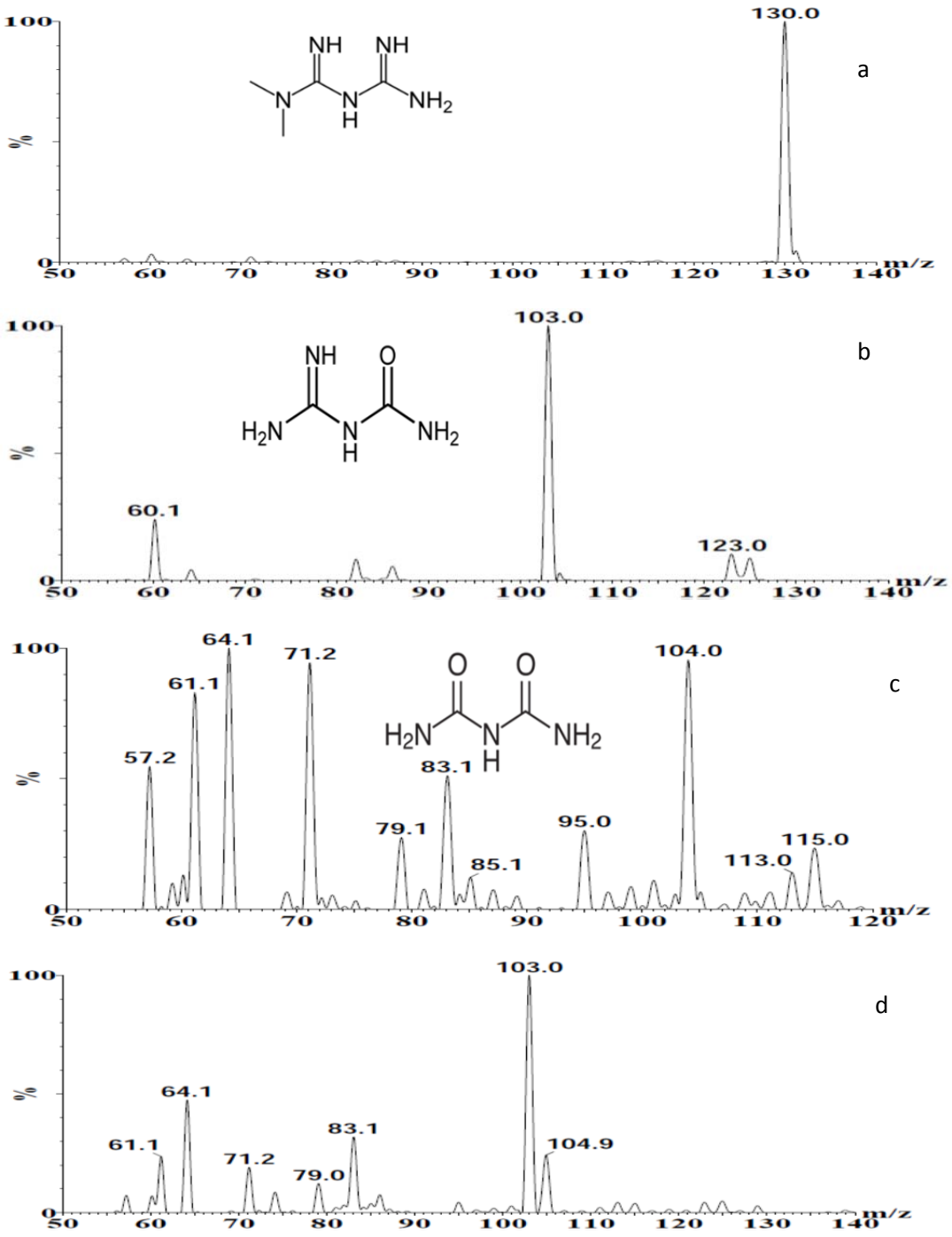


Figure 21: Mass spectra and chemical structures for metformin (a) guanyl urea (b) and biuret (c). Mass spectrum of metabolite mixture is (d). All compounds were dissolved in DDW with 1% formic acid. The m/z ratio of metabolite did not match with that of the analysed compounds except for guanyl urea. The fragmentation pattern of biuret was similar to that of metabolites.

4. Discussion

4.1. Development of an HPLC-UV assay for the determination of metformin in human plasma and urine

Several of the pre-existing methods cited for assay of metformin are presented in table 2. To the best of our knowledge, there has been no HPLC method developed to determine metformin concentration in both human plasma and urine for volume sizes as small as 0.1 mL, using a liquid–liquid extraction procedure, and with a column specifically designed for reverse phase chromatography such as octadecylsilane column.

The retention time of metformin was examined using C18, C8, and phenyl columns with mobile phases that consisted of a mixture of phosphate buffer solutions and acetonitrile. Metformin retention was very close to the column dead volume and no separation could be achieved from endogenous components using silica columns and mobile phase composition with the least percentage of acetonitrile in mobile phase composition. The polar nature of metformin inhibits its ability to interact with the hydrophobic chains of C8, C18, and phenyl moiety. To overcome this problem, we tried manipulating the mobile phase composition by incorporating different concentrations of SDS. SDS was first used in metformin separation within tablet formulations at 5mM concentration (259). It is a well-known anionic surfactant usually used as a pseudo-stationary phase. At concentrations above the critical micelle concentration (CMC), SDS forms

micelles which provide a pseudo-stationary phase that can effectively separate compounds based on partitioning between the aqueous mobile phase and the hydrophobic interior of the SDS micelles (305). Accordingly, metformin could be retained through its partitioning with SDS micelles; the more SDS added to mobile phase, the longer the retention time of metformin on C18 column. A concentration of 3 mM was found to be appropriate for the separation and both metformin and IS, and showed acceptable peak symmetry and suitable retention times (Figure 8d and 8e).

Regarding extraction of metformin from biological matrices, protein precipitation followed by an organic solvent wash has been used successfully for plasma sample preparation for metformin only by Ahmini *et al* (249). Protein precipitation was utilized by several other assays as a method of sample purification (251, 253, 261) . When protein precipitation was tested in developing our method, it was ineffective in removing all of the endogenous substances (Figure 8a). A disadvantage of protein precipitation is a low grade of sensitivity due to the sample dilution that it entails. Alkaline conditions have been used to extract metformin into a suitable organic solvent (249). For the present method workup, the extractability of metformin from plasma was tested in dichloromethane, hexane, TBME and 1-butanol/n-hexane (50:50 % v/v) mixture using recovery experiment. Both the TBME and 1-butanol/hexane mixtures were found to be most effective in

removing endogenous substances, while 1-butanol and hexane provided the most optimal recovery. Both evaporation of extracting solvent and back-extraction into 0.2% acetic acid were tested. Back extraction of metformin into an acidic aqueous medium yielded a satisfactory outcome in terms of both selectivity and removal of interferences to obtain a clean chromatogram.

The use of the structurally related biguanide compound phenformin, which had been used as internal standard in other assays (248, 256), proved to be not suitable within the chromatographic conditions employed, due to its delayed elution time. In those methods phenformin retention times were two to three-fold longer than that for metformin, thereby prolonging the total run time for the assay. In method workup, atenolol (253) and propranolol (261) were tried but were not suitable using the chromatographic conditions used. Ranitidine was used as reported by Amini *et al*. (249) and with the analytical conditions used here, it was found to offer a high and a reproducible recovery with reasonable retention time.

Based on regression analysis of the nominal concentrations vs. peak height ratios of metformin to IS in plasma and urine, the square of the sample correlation coefficient (r^2) were 0.999 and 0.9972 respectively (Figure 9a and 9b) which indicates an excellent linearity between the observed data and the nominal values. The CV%, which is a measure of precision, for intra-and inter-day assessments in human plasma were less than 17% and 19% respectively for the LLQ (Table 4 and

5). In urine, the CV% was found not to exceed 4.5% which were found to comply with the FDA guidance for bioanalytical method validation. It states that the precision determined at each concentration level should not exceed 15% of the coefficient of variation except for the LLQ, where it should not exceed 20% of the CV (306) which is the case here. As for accuracy, mean inter-day error in human plasma was less than 5% (Table 4) while in urine mean percentage error was up to 9% (Table 5) which comply with FDA guidelines for accuracy (307). Based on the inter- and intra-day CV% and mean error, it could be determined that the LLQ of metformin base were 7.8 ng/mL and 1.6 μg/mL based on 0.1 mL of human plasma and urine respectively (Tables 4 and 5). The LLQ reached is the lowest limit to be published for metformin by means of HPLC method.

4.2. The effect of obesity on a single-dose pharmacokinetics of metformin

The established assay method was employed to determine metformin plasma and urine concentrations in post-RYGB subjects who were administered 1000 single dose metformin. The corresponding control group included 16 obese subjects who were BMI and sex matched with the operated patients. Healthy lean subjects were not recruited in our study. We compared metformin PK parameters of obese subjects in our study with healthy and lean controls from Tucker *et al* (227). The average basic characteristics for the lean controls are listed in table 9.

Table 9: Baseline characteristics of the healthy lean controls extracted from Tucker *et al* (227) and obese subjects recruited in the clinical study. Numbers are mean±SD.

Parameters	**Lean subjects n=4**	**Obese subjects, current study n=16**
Age (years)	32.5 (3)	44.4 (10.0)
Sex (%)	M (100)	M (18)
BMI (kg/m^2)	21.8 (3.2)	38.0 (7.9)
Weight (kg)	70.3 (8.7)	104.0 (29.0)
Creatinine CL (mL/min)	135.8 (33.4)	143.0 (37.1)

It appears that obesity is associated with a reduction in metformin PK. Metformin CLr was determined to be 337 mL/min in our clinical study while it was reported to be 519 mL/min in lean subjects (P value = 0.060). However, the standard deviation in the healthy group was more than 50% which render the test not significant. Interestingly, the difference turned to be significant after normalization to body weight (Table 10). Additionally, Vd normalized to body weight in obese subjects is lower than that in the healthy subjects by more than 70% (1L/kg vs 3.97L/kg) (P value > 0.05) (Table 10). The AUC p.o. of metformin in obese subjects is relatively the same as that in healthy subjects (11.1 µg.h/L vs 12.27µg.h/L) for the same dose. For F, it was significantly lower in obese subjects

compared to control. The Cmax was relatively lower than normal lean subjects (1.8 vs 3.0 µg/L) likely due to dose difference between both studies which was 1500 mg in Tucker *et al* (227). This difference could be also attributed to different F and absorption rate from small intestine between obese and non-obese. Moreover tmax is relatively longer in obese subject than in lean subjects while terminal t1/2 was shorter in obese with respect to control (Table 10). Collectively, obesity had a substantial effect on metformin PK

Table 10: Effect of obesity on metformin PK parameters. A single dose of 1000 mg metformin was orally administered to obese subjects. Lean subject data was extracted from Tucker *et al* (227). Values are mean±SD. †median (range). (*) $P < 0.05$, compared to control. Independent two sample student t test for unequal sample sizes was used.

	Lean subjects	**Obese subjects**
F %	50.0±0.21	27.81±10.40*
24 h Urinary recovery (mg/kg)	8.18±1.79	2.0±0.78*
$AUC_{0\text{-}24h}$ (µg·h/L)	12.27±4.35	11.10±3.60
$AUC_{0\text{-}inf}$ (µg·h/L)	NA	11.40±3.60
t½ (h)	5.98±1.49	4.0±0.87*
CLr (mL/min)	519±278	336.7± 131.0
CLr (mL/min/kg)	7.16±2.83	3.0±1.01*
Vd (L/kg)	3.97±2.68	1.0±0.40*
Cmax (µg/L)	3.1±0.93	1.80±0.61*
Tmax (h)	1.5±0.40	3.0 (1.5–3.0) †

Obesity possesses a number of pathophysiological changes that may potentially modulate PK aspects and could likely be beyond the possible changes in metformin transporters. To our knowledge, this study is the first one to assess metformin PK in obese subjects (308).

Adipose tissue possesses characteristics of an endocrine organ and can secrete not only hormones such as adipokines but also pro-inflammatory cytokines (34). Adipokines include leptin and adiponectin. Leptin is involved in regulation of the hypothalamic hunger response, energy consumption, sodium reabsorption and blood pressure (309, 310) while adiponectin is involved in oxidation of fatty acids , glucose synthesis, and has anti-inflammatory properties (310). Unlike leptin where plasma concentrations tend to be higher in obesity due to leptin resistance, adiponectin levels tend to decline. Along with an increase in adipocytes in fat tissue in obesity, an infiltration of macrophages also takes place. Macrophages represent key cells in initiating and propagating the immune response due to their ability to secrete a number of pro-inflammatory cytokines. These include TNF-α, IL-6 and C-reactive protein (CRP) (34) (35). In obese mice, up to 40% of adipose tissue may be composed of macrophages (311, 312). It is reported in literature that plasma levels of IL-6, CRP and TNF-α are elevated in obese patients with respect to non-obese (313, 314). Because adipose can secrete significant amounts of cytokines, obesity is often considered to be a low grade and chronic form of inflammatory disease.

There has been little attention paid to the impact of adipokines in combination with pro-inflammatory cytokines, on altering the expression of transporters that determine aspects of metformin disposition in obesity. Pro-inflammatory

cytokines, alone, are already known to be correlated with a decrease in the expression of certain drug metabolizing enzymes and drug transport proteins (315, 316). Inflammation in rats can lead to a suppression of P-gp in liver and various gastrointestinal segments (62, 63). TNF-α and IL-6 have been shown to cause inhibition in a number of non-energy consuming transporters encoded by SLC gene family such as OATP-1B1/1B3/2B1, OCT-1and OAT-2 (64).

The essential factors that could affect metformin CL are transporter related factors. Metformin CL is solely dependent on the function and the expression of OCT-2 and MATE-1 in human. In addition, Vd also is dependent on the function and the expression of the SLC family of OCT. The relative decrease in metformin CL, Vd, and F in obese patients provide suggestive evidence of metformin dependent transporter suppression. Pro-inflammatory cytokines such as but not limited to TNF-α and IL-6 could further modulate the expression level of metformin related transporters. Transcriptional regulators of OCT and MATE such as AP-2 and SP-1 may be affected by the increased amounts of TNF-α in obese. More studies are in need to prove and to explore how obesity as an inflammatory condition, affects metformin related transporters protein expression.

4.3. The effect of Roux-en-Y gastric bypass surgery on the single-dose pharmacokinetics of metformin

Oral F of metformin between 16 obese subjects and BMI and sex matched post-RYGB subjects was compared. Contrary to our hypothesis, metformin oral F in post-RYGB subjects was increased significantly by 50%. To our knowledge, this is the first controlled study of metformin PK to be performed in post-RYGB subjects.

Metformin is commonly thought to be not metabolized. In the literature, two studies showed that the absorbed amount is 100% excreted in the urine with nearly the entire dose being eliminated within 24 h (228, 234). Most studies have shown a human recovery of metformin in urine ranging from 80 to 100%. Therefore, it was felt that a 24-h recovery of drug in urine could reliably be used to estimate F without the need for iv administration. Accordingly, oral F was estimated from the ratio of the dose recovered from urine to the total dose given. Because metformin has an intrinsically low F and appears to be maximally absorbed in the proximal small intestine given its large overall surface area per unit length (317), we hypothesized that F would be diminished significantly following RYGB. To explain the unexpected increase in F, one has to consider the physiological and anatomical changes that took place after RYGB surgery.

RYGB is known to delay gastric emptying and increase intestinal transit time, (318, 319) which may increase the overall absorption of metformin by increasing the duration of exposure of the drug to small intestinal mucosa. The absorption of metformin is permeability rate-limited and the drug is almost exclusively absorbed in the small intestine (227, 234, 292). Drug that reaches the colon is not absorbed and is excreted into the feces. Indeed approximately 30% of the drug is recovered in the feces after oral dosing suggesting lack of absorption (292). Because metformin has a limited window for absorption, and bioavailability is incomplete, prolonging intestinal transit time increases absorption (233). Similarly, delaying gastric emptying may decrease the rate at which the drug enters the small bowel, thus preventing saturation of absorptive mechanisms and increasing overall absorption (318). Slow-release formulations of metformin act in an analogous manner where drug is physically retained in the stomach and is being released gradually into the upper small bowel, resulting in sustained and prolonged steady-state metformin levels (320).

Another important change that took place after RYGB is the newly created 20-30 cc gastric pouch which is largely devoid of acid producing cells and acid secretion is virtually absent (321). This alkaline environment may enhance the disintegration, dissolution of metformin, as a basic drug, and this may lead to increased absorption. However, metformin absorption may not be enhanced by

administering it as a solution (322). Conversely, because metformin has a pKa of 12.4 to 13.8, it is almost completely ionized at all ranges of intestinal pH (169, 323). This suggests that the increase in intestinal pH is unlikely to play an effective role in increasing F post-bypass. In addition, the extent of absorption from the stomach is generally small due to its relatively small effective surface area.

With regard to the mode of metformin absorption, the in vitro modelling through monolayers of Caco-2 cell lines suggested that OCT and/or PMAT mediated transport may not be the rate-limiting step in metformin absorption and paracellular route, between cells, contributes by 90% in metformin transfer (323). However, Caco-2 cells absorption cellular models could not be considered a perfect imitating model for the intestine. The expression of OCT-2 makes those cells different to normal human enterocytes (223).

Metformin is a known substrate for OCT-1, OCT-2, PMAT and MATE-1 protein transporters (19, 144). The behavior of such positively charged compound in the body regarding absorption from the small intestine, uptake by different tissues and elimination through the kidney is highly dependent and facilitated through protein transporters. In human, the intestinal absorption of metformin is primarily mediated by PMAT, which is expressed on the luminal side of the enterocytes (169). However, there is no current in vivo data on the role of PMAT in the

disposition and pharmacological effect of metformin. OCT-3 is also expressed in the brush boarder of the enterocytes and may contribute to metformin uptake (223). Additionally, OCT-1, which is expressed on the basolateral membrane of the enterocytes, may facilitate transfer of metformin into the interstitial space (223) (Figure 5).

We speculate that up-regulation of metformin gene and protein transporters took place post-RY gastric bypass due, but not limited to, biochemical changes after the surgery. If metformin transporters were up regulated, this will lead to the increase of metformin Vd and CL which were significantly increased. Consequently absorption and availability in systemic circulation also will be increased which can account for the significant increase in F. However, the AUC was not increased, which is expected and could be explained by the same adopted explanation of transporters up-regulation. Oral AUC is a parameter dependent on both F and CL according to the equation:

$$AUC_{0-\infty} = \frac{Dose \times F}{CL}$$

Both F and CL were affected. F and CL were increased significantly by 50% and 43% respectively. This led to a counter balanced effect on the AUCleading to no significant change. A significant rise in oral CL in post-surgical patients is consistent with transporter up-regulation. The uptake of metformin from the circulation into renal epithelial cells is primarily facilitated in human by OCT-2

(239) which is expressed predominantly at the basolateral membrane in the distal renal tubules. Renal excretion of metformin in human is also mediated via MATE-1 and MATE2-K (205, 240, 241). Accordingly, an up-regulation of OCT-2, MATE-1 or MATE2-K protein is expected to increase CLr, the main pathway for metformin elimination.

The biochemical changes that took place after RYGB could be related to changes in adipokines and cytokines levels associated with obesity. Recently, RYGB was observed to cause a relatively rapid decrease in some pro-inflammatory cytokines such as TNF soluble receptor and CRP concomitantly with an increase in adiponectin. These changes are stable and do not change with time as weight loss progresses (324).

In the same study (324), leptin concentrations declined within 3 weeks when weight loss was less than 9%, and progressively dropped up to 6 months after surgery. On the other hand, IL-6 levels did not differ between post-surgery patients and weight-matched controls and were still above those seen in a lean healthy population (324). All patients' control and treatment groups were still considered obese, mean BMI 36.8, at the time of the evaluation, although they had experienced substantial weight loss of around 40 kg. It seems that after the surgery, there is a healing process associated with the surgery itself, alongside changes in

nutritional uptake, long term changes associated with the weight loss, reduction in inflammation and normalization of adipokines (324).

The major limitation of this study was that a single dose was administered to fasting subjects. Fasting subjects were recruited to minimize the effect of food on metformin absorption. It is shown that food decreases metformin absorption by approximately 25% (322) . In clinical practice, metformin is administered with food to limit gastrointestinal side effects. While single dose studies are easier to perform and are typically used to gain initial insight into an area, they have the drawback of not being able to assess steady state levels. This should not be a problem as metformin accumulation in the body is very low due to its short $t_{1/2}$. A single dose study is only a drawback when the main target would be set for testing metformin PD effects which requires longer time than single dose studies. A cross over design would have perhaps provided a better experimental design used with recruiting a third group of healthy lean volunteers to act as control for the obese subjects. However, those arrangements could not be achieved due to financial (funding) considerations.

4.4. Effect of P407 induced hyperlipidemia on PK parameters, gene and protein expression of metformin kidney transporters

In the clinical study RYGB patients, plasma lipid profiles were significantly reduced after the surgery (Figure 11). This important finding may shed light on the

role that HL might have in the secretion of metformin by rOCT-1, rOCT-2, and rMATE-1 kidney transporters. In addition, HL as a metabolic disorder is linked to obesity. Plasma LP were reported to be elevated in obese subjects (325, 326). HL is known to influence biochemical functions or protein expressions with consequences on elimination and/or distribution of both LP-bound and unbound drugs alike. In experimental HL, it was shown that the expressions of a number of hepatic drug metabolizing enzymes and transport proteins are down-regulated. These include those of the CYP enzymes CYP3A1/2 and CYP2C11; the uridine diphosphate glucuronosyltransferases 1A1, 1A6 and 2B1; MRP-2 and OATP-2 (53, 118, 293). There have been no studies performed to test for the effect of HL on the transporters involved in metformin transport until now. According to the plasma lipid profiles pre and post-surgery in concomitant with the increased CL, we hypothesized that HL may play a role in down-regulation of SLC22 and SLC47 family genes that encode for metformin renal transportation.

To test for this hypothesis, we adopted an animal model for induction of HL through injecting P407 ip in rats. Several studies have used the P407 model of HL for the study of HL on PK and pharmacodynamics of a variety of drugs (106, 131) (327, 328) (132) (329) (130, 295). In most of these assessments, the sampling of blood was performed after dosing at 24 or 36 h from the time of P407 i.p injection. At those time points, large increases occur in serum lipids. This provides for a

convenient screening tool for the influence of LP on drug behavior. On the other hand, pathophysiological changes normally would occur over a more extended period of time. Therefore longer term changes associated with HL as a chronic disorder might not be present at the time of assessment particularly at 36 h especially where plasma LP concentrations are far higher (129) than would be found in a human population.

To hopefully allow for a consideration fo the effects of short term high level exposure to LP, and perhaps a more chronic (albeit still short term) exposure to elevated LP concentration, we performed an assessment of HL effects at 2 different time points after P407 dosing, namely 36 and 108 h. The 108 h time point was selected according to study performed by Chaudhary *et al* (129) . By 108 h, serum concentrations of LP and lipids are still higher than normal but not excessively so compared to what would be expected in HL patients. Although levels would be lower than those at 36 h but the duration of HL at 108 h is more sustained. This may perhaps provide greater opportunity for LP to affect the molecular factors, transcriptional regulators for metformin gene and/or protein kidney transporters, than the acute situation might.

A comparable dose of metformin to the human dose was chosen which (30 mg/kg) and injected as iv bolus. Short term exposure to elevated LP did not cause any change in the PK parameters of metformin especially CLr (Table 8) (Figure 13).

This finding was in line with a lack of change in gene and protein expression data of SLC transporters (Figure 14). At 108 h, similar to 36 h, there was no discernable change in metformin PK. Despite the significant decrease present in mRNA levels of rOCT-1 and rMATE-1, no changes were observed for their respective proteins (Figure 15).

This could be caused by the minimal difference between treatment and control group cycle thresholds (Ct). The two to three Ct decrease in mRNA of the HL group compared to their controls was not enough to be translated to its corresponding protein. As it was shown in (Figure 14d and 14f), the expression of both rOCT-1 and rMATE-1 proteins was not decreased. The unchanged CLr of metformin in 108 h PK study confirmed that further. It is known that mRNA levels and their corresponding protein levels do not always correlate (330), and indeed they did not for our data. The discrepancy between mRNA and protein could be attributed to the differences in stability between the gene and protein or in the rate of translation of each protein (331).

Transcriptional regulation is a complex process which involves more than one nuclear receptor. As was mentioned in the introduction, it seems that transcriptional factors that modulate the expression of metformin transporters such as SP-1, AP-2 and HNF4-α were not affected with HL. Another possibility that could help understanding the unchanged levels of transporter proteins is that P407

HL model did not provide an enough chronic exposure (>108 h) of elevated LP which could be achieved under multiple dosing of P407.

From this experiment, HL by itself does not seem to be the direct cause behind the speculated transporter down-regulation in obese patients. Other underling factors may be responsible for the observed decrease of metformin CL, Vd, and F in the obese subjects with respect to the healthy subjects. The P407 HL model is not linked with changes in inflammatory markers. Neither TNF-α nor IL-6 plasma concentrations are altered (129, 332). It seems that HL by itself, albeit short-lived, is not directly able to alter metformin PK or to modify expression of the cationic SLC transporter. Further studies are using alternate obese rat models are warranted.

4.5. Does metformin undergo metabolism?

Metformin is thought to be largely secreted unchanged in urine (19, 144, 228, 333-335). However, Choi *et al* provided evidence that metformin undergoes hepatic and gastrointestinal metabolism at considerable levels in rat in vivo studies (231, 247). As indicated above, the dose of metformin used in their studies is 3 times more than the human clinical dose. Decline of metformin was up to 21% after incubation with 1 gm S9 fractions of several organs supported the ability of the drug to be metabolized. However, the used metformin concentrations in their in vitro studies were 10 times higher than metformin plasma concentration at steady

state. It was reported that metformin concentration in liver is only 5 times higher than plasma concentration (246).

In addition, they found that after intra-gastric and intra-duodenal administration of metformin at a high dose of 100 mg/kg in rats, the AUC of metformin was significantly lower than after intra-portal administration. They suggested that gastrointestinal first-pass effect of metformin could be approximately 56.3% of the oral dose in rats. They concluded that at a dose of 100 mg/kg, the F was low (30%) mainly due to considerable gastrointestinal first-pass effect where hepatic and gastrointestinal first-pass effects were approximately consuming 11 and 54% of dose, respectively, in rats (336). The calculated unabsorbed percentage of metformin was 4.4% which is against what was found previously (227, 229). This low percentage could be due to the use of methanol in the process of metformin extraction from faeces which hinders metformin solubility and may underestimate its measurement. It was not stated if fecal samples were used for standard curves, which may also have influenced the interpretation of the results. Moreover, the F value of 30% could be attributed to a lack of absorption of the drug rather than first pass effect. Basically, with these high doses used in the experiments, saturation of the absorption mechanism could have happened and lack of absorption for high dose as well. It must be recognized that they at no time reported evidence of a metabolite in any of their analyses.

They continued supporting their finding by referring to their previous work when they tried an array of enzyme inhibitors and inducers on metformin metabolism in vivo. A dose of 100 mg/kg was administered intravenously to rats and the CLnr of metformin was compared with that of controls. They showed that in rats pretreated with dexamethasone (used as a CYP450 inducer), the CLnr was significantly 57% faster than the controls while in rats pretreated with SKF-525-A, sulfaphenazole, quinine, and troleandomycin as CYP450 enzyme inhibitors, the CLnr was significantly slower with respect to controls. Accordingly, it was concluded that metformin was metabolized mainly via CYP2C11, 2D1 and 3A1/2 in rats (247). In truth, it is probable that metformin is metabolized but it is not that considerable in clinical reality given the high degree of urinary recovery.

The used doses in Choi *et al* in vivo studies ranged from 100 to 200 mg/kg iv which is 4-5 times higher than the one used clinically (30 mg/kg). These dose levels could achieve supratherapeutic plasma concentrations of metformin which could saturate the active renal secretion mechanism which is the main route of elimination. Consequently, metformin may have been directed to the fate of metabolic pathways by the liver. Indeed metformin given through oral route at a dose level of 200 mg/kg is considered as a non-observable adverse effect level (NOAEL) (337). Additionally, it is recognized that a dose level of 150-200 mg/kg

metformin given orally for rats is 4-5 higher than the maximum daily dose used in human (3 g, or about 42 mg/kg in a 70 kg man)(158).

The use of S9 fractions and incubation the drug at this high concentration might not be relevant to the cellular environment in vivo but also may ignore the role of uptake by transporters. In our animal PK studies, in the three animal groups, the percentages of metformin urine recoveries were 95, 96 and 100% (Table 8) which indicates metformin may undergo very minimal metabolism at the usual therapeutic dose level used in humans. Moreover, in our clinical study performed on obese and RYGB patients, more than 84% was recovered of metformin dose after oral administration in urine. Another evidence to be added is the insignificant difference between CLr and CLt of metformin in our rat PK study (Table 8). A claim of a low F of 30% could simply be attributed to the saturation of all absorption mechanism of metformin, causing a higher remainder as being unabsorbed. As mentioned above, metformin is a drug with limited window of absorption.

This being said, metformin does appear to have some a minimal measure of metabolism in rats and humans. We noticed some unknown detectable peaks in the urine of both human subjects pretreated with 1000 mg metformin (~15 mg/kg) and rats treated with a dose (30 mg/kg) near that of the maximum recommended human daily dose (Figure 16). These peaks were absent in in urine of humans not given

metformin. The ratio of both peaks to the parent drug was very small <3%, although it is not a definitive comparison because those components might have different UV maximum absorption wavelengths than the 236 nm specified for metformin.

For the incubation of metformin with hepatocytes, the concentration of metformin was chosen to be clinically relevant to that seen in human plasma, where the average concentration at steady state ranges from 1000 to 1500 ng/mL. There were two unknown evident peaks at longer periods of incubation (Figure 17). However, upon the assay of metformin in the wells, there was only an insignificant decrease in metformin concentrations, indicating that regardless of UV maxima, the amount of metabolites formed is relatively very small with respect to the parent drug. The addition of β–glucuronidase at (1200 Roy unit/mL) or sulphatase at 150 (Fishman U /mL) (303) in the hepatocytes incubation experiment at each of the following time points 12 and 24 h was performed in order to aid in the hydrolysis of possible metabolite conjugates formed in case metformin undergoes phase II metabolism. There was no significant change in metformin concentrations between wells with and without conjugate-cleaving enzymes suggesting no metabolism (Figure 18).

Most studies involving hepatocytes were performed to explore the mechanism of action of metformin and its signaling pathways downstream cell membrane, but not for detection of formed metabolites (338-341). Thus, very high concentrations

of metformin far above the plasma concentrations at steady state were used. Liver concentrations of metformin are only 2 to 5 times higher than those of plasma concentrations (246). To our knowledge, our study is the only one in which hepatocytes are incubated with metformin for the purpose of testing its metabolism. More importantly there are no reports of identified metformin metabolites in the literature. The microsomal study showed confirmatory evidence that some metabolites are formed, based on the appearance of unidentified peaks in the chromatograms.

Metformin is metabolized but its metabolism is minimal or too small to be clinically significant, and likewise it is difficult to identify metabolites in humans for the same reason. The use of 100 μg/mL of metformin as used in our microsomal study is 100 times higher than the plasma concentration at steady state. In addition, there is direct exposure of the drug to the metabolizing enzymes with no limitations on the permeability factors or transport proteins which are pivotal in the transport of metformin which suffer from saturation at high doses.

Many researchers have tried to look for metformin metabolism by comparing the amount of the dose given to the amount of dose recovered from urine. In this case, iv route of administration is the only route to be used for that purpose. Some showed a urine recovery near 100% in urine and claimed virtually that it is not metabolized (228, 335). Sertori *et al* showed metformin recovery in urine was only

38% after oral administration (229). They explained that this small recovery is possibly not as a consequence of low oral F as similar low recovery was found after oral administration of the metformin solution used for the iv studies, but because of binding to the intestinal wall and high percentage that escaped absorption. They concluded that metformin is not metabolized (229). This is a reasonable conclusion because the F is about 50%; a 12% loss of drug is relatively small and could be attributed to acceptable range of analytical errors. However, Tucker *et al* found that after the 1.5 g oral dose, the mean total recovery of unchanged metformin was about 80% of the dose, of which 50% appeared in the urine and 30% in the faeces (227). They could not account for about 20% of the dose and suggested that the unaccounted for amount must have been subjected to pre-systemic metabolism. When they incubated urine samples with a glucuronidase-sulphatase mixture, no change in metformin concentrations were detected (227). It indicates that conjugates were not formed which is in accordance with what we found (Figure 18). It is possible that bacteria in the lower gastrointestinal tract may have metabolized a portion of the unabsorbed metformin. Karttunen *et al*, found that 24-h after single doses, 40% of the given dose was recovered in urine from rapid release formulation while the value for sustained release formulation was 25% (342). Wang *et al*, showed that when metformin was iv injected at a dose of 5 mg/kg in mice, 55% to 70% of the dose was recovered in

the urine up to 60 min after i.v. administration. One h, however, is not enough time to clear all metformin systemically present. They could have recovered more of the drug if they collected urine for a reasonable amount of time based on the terminal phase $t_{1/2}$. There was only a minimal insignificant difference in both CLr and CLt which strongly suggests an underestimation of the urine recovery (198).

In our rat study, there was no significant difference between CLr and CLt (Table 8). The stability of metformin in urine was investigated to exclude the possibility of degradation in urine which may account for the loss of dose recovery seen in some studies. As seen in (Figure 10), there was no effect of time on urinary metformin concentration. Metformin stability was confirmed in literature (336). Metformin (5 µg/mL) was incubated in several solutions such as rat plasma, urine, 9000g suprnatant fractions of liver homogenates and gastric juices (pH of 2.5 and 4.5) respectively, and various buffer solutions with pH ranging from 1 to 13 for 48h in a shaking water bath of 37 ºC. It was found that the concentrations did not changed except for in pH 13 buffer, a pH of which is really not physiological.

4.6. Spectral analysis of the isolated metabolites

Isolation and structural elucidation of metformin metabolite/s have not been investigated. Even Choi *et al* who concluded that metformin is metabolized by CYP2C11, 2D1 and 3A1/2 in rats according to competitive inhibition studies,

could not show any direct evidence of a metformin metabolite (247). To the best of our knowledge, this is the first approach to characterize metformin metabolites masses by the aid of LC/MS. However characterization is limited to a description of the molecular weight distribution. Unfortunately, NMR spectroscopy is quite insensitive and requires microgram to milligram amounts of material for successful identification which is practically unattainable from in vivo specimens. Our method in identifying the metabolites depends on determination of chromatographic elution times of possible metabolite structures coupled with the mass to charge ratio for the presumed metabolite.

In literature, Trautwein and Kümmerer found that guanylurea is a product of anaerobic degradation of metformin (343). A scan for molecular ions was carried out up to 500 m/z. The metabolite solution revealed a mixture of two monoisotopic masses for two compounds: 103 and 105 m/z. According to structural similarities between metformin and biuret we thought that biuret could be the unknown metabolite therefore its m/z ratio will match with that of metabolite solution. Our results revealed that guanylurea (103 m/z) matched with one of the molecular ions while biuret (104 m/z) did not match with the metabolites molecular ions (Figure 21). A UV scan for guanylurea was performed to detect the maximum absorption peak but it was unusually flat and clear maxima could be identified.

The mass spectral data suggests the formation of guanylurea by a double dealkylation of the branched amine in metformin, followed by oxidative deamination. Further reduction of one of the double bond could explain the presence of a compound of 105 m/z ratio (Figure 21d). There would be two possibilities in the reduced product: either the formation of hydroxyl group that resulted from reduction of the carbonyl group or the formation of primary amine from the reduction of imine group. Due to the fact that the nitrogen atom is intrinsically more nucleophile and a stronger base than the oxygen atom, a preferential reduction of the imide group could occur leading to the formation of compound b (Figure 22). In the condition where ESI mode is used, the expected m/z ratio would equal 105, which coincidentally matched the measured unknown compound m/z ratio.

When the eluted fraction of presumed metabolite was injected into HPLC system with UV detection, it yielded a peak with the same retention time as of guanylurea solution injected under the same chromatographic conditions. In contrast the retention time of biuret did not match with the metabolite peak which ruled it out as the presumed metabolite (Figure 20).

It is worth mentioning that hepatic intrinsic CL may be better expressed by using hepatocytes (or other methodology that preserves the transporter/enzyme architecture of the liver) rather than microsomes, so that transporter effects are

taken into account. Despite that, the amounts of the formed metabolites could be relatively small in normal dose levels, but it could be increasingly large under certain conditions at which plasma metformin concentrations rise such as in urinary dysfunction. This could raise the possibility of the potential role of metformin metabolite in inducing lactic acidosis.

A possible link between the metabolites formed at high dose levels and lactic acidosis is worthy of investigation

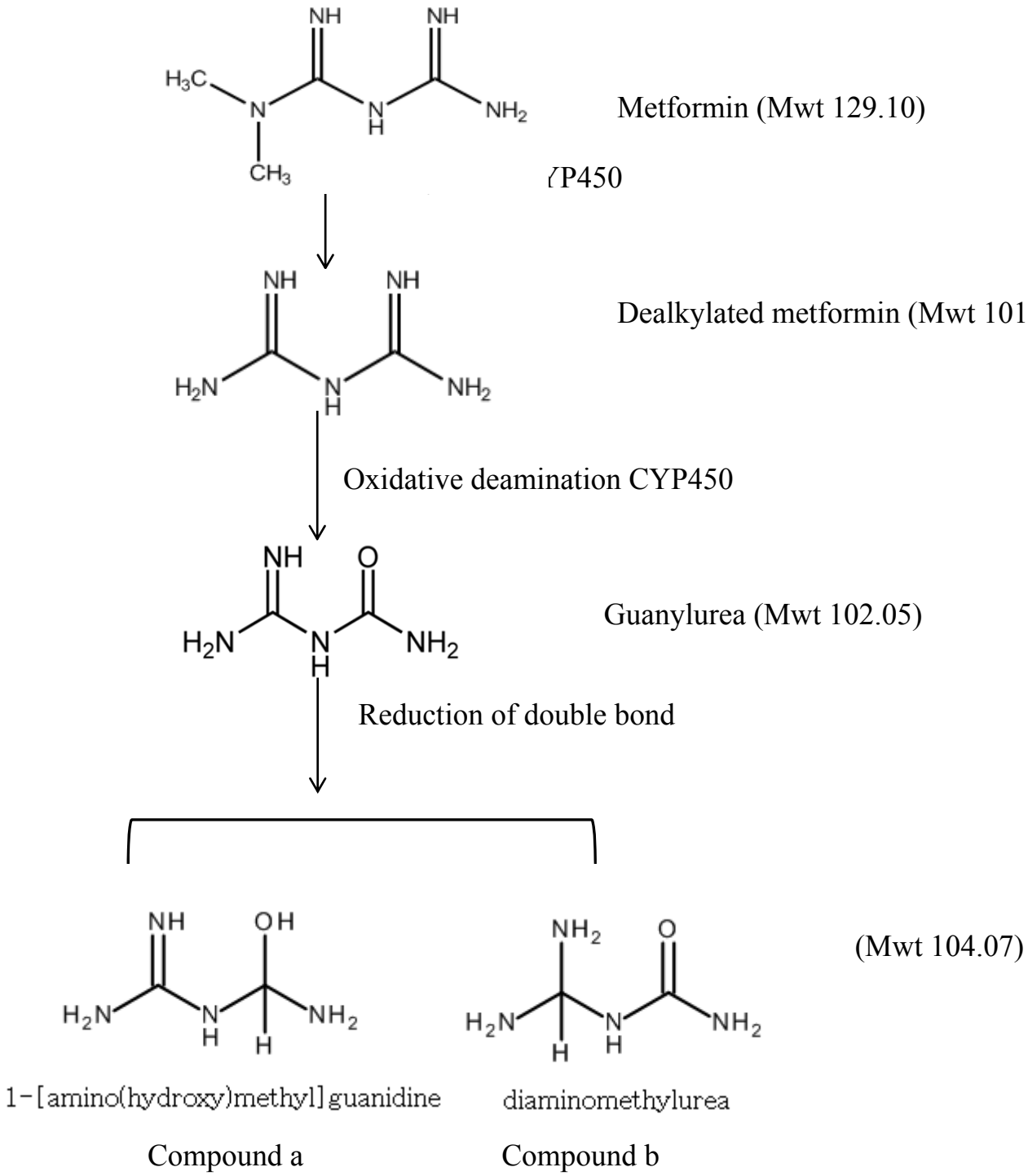


Figure 22: Flow chart illustrates a suggested pathway of metformin metabolism

5. Conclusion

Metformin is a highly polar drug which makes it difficult to be extracted and retained in a reverse phase chromatography mode. In our studies, we have developed a simple and rapid HPLC assay for metformin using liquid-liquid extraction technique and octadecylsilane column with a reverse phase chromatography mode. The method was the first to be validated for the determination of metformin in both human plasma and urine based on 0.1 mL sample volume and with a LLQ of 7.8 ng/mL calculated as a base. The method showed high caliber of sensitivity and validity to be sufficient for use in PK studies including both human and rats.

Utilizing this method to explore the effect of obesity and RYGB on PK parameters of metformin, we have found that obesity causes a significant decrease in metformin CL, Vd and F in patients treated with a single oral dose of 1000 mg metformin. This is the first study to explore the effect of obesity on metformin PK. It expanded the understanding of the effect of obesity on metformin biodistribution and elimination, which is especially clinically relevant as the number of obese patients who are on metformin treatment is increasing continually (344) .

The rate and extent of absorption of metformin between post-RYGB subjects and matched obese controls were compared. Contrary to our hypothesis, the metformin oral F in RYGB subjects was significantly increased. We observed an increase in

the F of metformin, which is large enough to be potentially clinically relevant, particularly with chronic dosing and especially in the presence of renal dysfunction. AUCp.o. of metformin did not change because the increase in oral F effect was abolished by the increase in metformin CL in the RYGB group owing to the dependency of AUC parameter on CL. In addition to the increase in oral F, higher metformin Vd and CLr values in the post-surgical patients were evident with respect to obese subjects and those values were nearly normalized when compared with lean subjects from Tucker's study. It seems that factors other than diminished small bowel length influence metformin F which might be due to transporter up-regulation. This has potential implications for metformin dosing after bypass and for medications with similar physicochemical properties. More study is needed to delineate potential mechanisms underlying these changes.

An understanding of the impact of RYGB on metformin PK is needed to allow for recommendations regarding dosing in this population. There might be a need for dose adjustment after the surgery especially for high risk population who suffer from kidney dysfunction. One cannot just focus on the immediate impact of the changes that occur in gastrointestinal physiology and anatomy caused by RYGB because they are time dependent (72). Hence, on a longer-term basis the patients will not only have altered gastrointestinal physiology with the possibility of altered drug absorption, but also acquired changes will take place in neuroendocrine

hormone-levels as a result of rapid and substantial, and hopefully sustained weight loss.

When we compared the LP levels between 2 groups, we found that obese control subjects had higher level of TC and LDL which were significantly lower in post-RYGB subjects. In addition, the LP levels were decreased significantly in post-surgical patients. In addition, the possible role of HL in manipulating or suppressing some protein transporters was evident in literature. There is incomplete knowledge regarding the effect of HL on metformin PK and transporters. Therefore, we were compelled to examine the possibility of HL to have a suppressive effect on gene and/or protein expression of metformin related transporters. The HL rat models used in the experiments utilized relatively short term exposure to very high LP concentrations of 36 h time duration, followed by a longer term exposure of declining yet still high LP levels of 108 h duration. There was no noticeable effect of HL on PK parameters and it was consistent with a lack of change in gene and protein expression of metformin related transporters.

However, in our hepatocytes incubation study, there was a visible unidentified peak at 12 and 24 h of incubation suggesting formation of metabolite. Moreover, when a microsomal incubation study was performed using 100 µg/mL metformin concentration, we obtained a qualitative evidence for the presence of metabolites. Thereafter, separation and characterization of metabolite by means of HPLC and

mass spectroscopy, respectively, were performed. The spectrum revealed a mixture of two compounds with 103 and 105 m/z ratios suggesting that the presence of guanylurea as a metabolite.

6. Future directions

The P407 induced HL model could not be considered as a chronic model even if it has 108 h duration. It is also does not mimic the inflammatory condition exists in obesity. To investigate role of HL associated with obesity on the level of expression of metformin related transporters as well as PK aspects of metformin, valid obese rat models like genetically engineered rats or high fat diet fed rats showing obvious signs of CMS such as hyperglycemia, weight gain and HL are recommended. Those animal models can provide a comprehensive model that comprises the effect of elevated pro-inflammatory cytokines and HL as well. Looking at the transporter level of expression using obese rat models in the gastrointestinal track and kidney vs lean rats could help also in confirming molecular modifications that lead to decreased metformin PK parameters in obesity.

It was a limitation of our study to administer single dose to fasting subjects. In clinical practice, multiple doses of metformin typically are administered with food to decrease its gastrointestinal side effects. Thus, additional study examining steady-state levels in non-fasting subjects would be required to more closely mimic clinical practice. Multiple dosing of metformin is only required for testing the change in PD parameters, such as glycemic index, that takes longer period of time to occur.

We also could not rule out the role of secondary metabolic changes occurring after RYGB such as adiponectin, leptin, TNFα and other hormones such as glucagon like peptide-1 in modulating metformin related transporter gene and protein expression. Further studies are in need in this regard.

To investigate for the effect of RYGB on metformin related transporter suppression, rat models of RYGB could be used with the ability of assessing transporter protein expression in the small intestine (PMAT and OCT-3) and in the kidney (OCT-1, OCT-2 and MATE-1) (345).

A worthy objective of future study would be to collect relatively large amounts of metformin metabolites so that NMR analysis could be used to identify their chemical structures. Meanwhile, as long as these compounds are produced at high metformin concentrations, it is important to assess their toxicological effects and test for any correlation between those compounds and metformin most critical adverse effect lactic acidosis. Another hepatocytes incubation study could be performed with guanylurea to test for a possible link between guanylurea cellular concentration as metformin metabolite and the formation of lactic acid.

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