Université de Montréal

DÉCONJUGAISON DES SUBSTRATS GLUCURONIDÉS À L'AIDE D'ENZYMES IMMOBILISÉS SUR UNE COLONNE

DECONJUGATION OF GLUCURONIDE SUBSTRATES ON AN IMMOBILIZED ENZYME COLUMN

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DECONJUGATION OF GLUCURONIDE SUBSTRATES
ON AN IMMOBILIZED ENZYME COLUMN

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SUMMARY

The purpose of this study was to investigate the activity of an immobilized β-gucuronidase HPLC stationary phase column and its applicability to the on-line deconjugation of substrates.

A β-glucuronidase column was coupled to a C_8 (or C_{18}) column which is used to separate substrates and products eluted from the β-glucuronidase column. The activity, efficiency and reproducibility of the enzyme column was followed by measurement of percent deconjugation or percent conversion of glucuronide substrates to aglycones (parent compounds). Parameters investigated were: the substrate concentrations, pH (4 to 6), temperature (r.t., 37°C) and the enzyme/substrate contact time using flow rates of 0.1 to 1.0 ml/min (15 minutes to 1.5 minutes). 4-methylumbelliferyl-β-D-glucuronide, p-acetaminophen-β-D-glucuronide,3'-azido-3'-deoxythymidine-β-D-glucuronide, phenyl-β-D-glucuronide,chloramphenicol-β-D-glucuronide,estradiol-17-β-D-glucuronide and morphine-β-D-glucuronide were the substrates tested using this column.

At a substrate concentration of 2.0 mM, a flow rate of 0.1 ml/min and at room temperature, five of the glucuronides were completely hydrolyzed. With the same experimental conditions, the hydrolysis of 3'-azido-3'-deoxythymidine-β-D-glucuronide and morphine-β-D-glucuronide was only 20% and 11% respectively. Ninety percent conversion was however observed when the concentration of 3'-azido-3'-deoxythymidine-β-D-glucuronide was lowered to 0.039 mM at 0.1

ml/min (70% conversion at 1.0 ml/min). The activity of the immobilized enzymes were compared to that of free enzymes in solutions under equivalent conditions (enzyme and substrate concentrations, pH, reaction time, temperature). Identical activities were observed with the exception of the hydrolysis of 3'-azido-3'-deoxythymidine-β-D-glucuronide and morphine-β-D-glucuronide. For the substrate 3'-azido-3'-deoxythymidine-β-D-glucuronide, the immobilized enzymes were slower at higher substrate/enzyme ratios while the opposite results were observed for morphine-β-D-glucuronide where a decrease in substrate concentration resulted in a decrease in the extent of hydrolysis. When the effect of pH change (pH 5.0, 6.0 and 4.0) on the activity of the β-glucuronidase column was examined, the optimum activity was observed at pH 5.0. P-acetaminophen-β-D-glucuronide (0.1 mM) was the substrate used in the experiment. The following results were obtained for this study: 46% deconjugation (pH 4.0), >98% (pH 5.0), 92% (pH 6.0).

An on-line HPLC method has been developed for the deconjugation of glucuronides. The deconjugation of glucuronides is much easier with the immobilized β-glucuronidase column. The results of these tests demonstrates that the β-glucuronidase enzyme column has equivalent enzymatic activity for most substrates when compared to free non-immobilized β-glucuronidase in solution.

RÉSUMÉ

L'enzyme β-glucuronidase est une hydrolase acide provenant de souches intracellulaires de lysosomes, qui a été identifié chez plusieurs espèces, au niveau de plusieurs tissus dont principalement le foie.

Une colonne immobilisée avec des enzymes β-glucuronidases a été développée afin de déconjuguer des substrats β-glucuronidés. L'objectif de cette recherche était de déterminer l'activité de ces enzymes immobilisées sur un gel de silice, et d'appliquer cette colonne enzymatique de β-glucuronidases sur un système "en série" de chromatographie liquide à haute performance (HPLC). La méthode d'analyse à développer devait être simple, facile à utiliser et reproductible. Il était également important d'établir une méthode de HPLC similaire aux essais biologiques *in vitro*.

Les β-glucuronidases immobilisées sur la colonne provenaient de souches bactériennes d'E. coli K12. Les enzymes d' E. coli sont très stables, à l'intérieur d'un écart de pH se situant entre 5 à 7.5, et sont résistantes aux inactivations thermiques allant jusqu'à 50°C [55]. Disponibles commercialement, ces β-glucuronidases d'E. coli sont idéales afin d'être immobilisées de façon covalente sur une colonne.

L'activité spécifique des enzymes immobilisées sur la colonne fut évaluée et validée par la déconjugaison de sept substrats β-glucuronidés. Les sept substrats (avec leurs alcools respectifs) utilisés étaient les suivants: (**1**) 4-

méthylumbélliferyl-β-D-glucuronide (méthylumbélliferone); (**2**) p-acétaminophène-β-D-glucuronide (acétaminophène); (**3**) 3'-azido-3'-déoxythymidine-β-D-glucuronide (Zidovudine); (**4**) phényl-β-D-glucuronide (phénol); (**5**) chloramphénicol-β-D-glucuronide (chloramphénicol); (**6**) estradiol-17-β-D-glucuronide (estradiol); (**7**) morphine-3-β-D-glucuronide (morphine).

Une méthode analytique a été développée sur un système de HPLC pour chacun des substrats glucuronidés avec des colonnes analytiques de phase inverse (C_8 et C_{18}) et différentes longueurs d'ondes de détection UV (254 à 280nm). Afin d'obtenir une méthode de déconjugaison et de détection rapide, la colonne de β-glucuronidases était disposée en série avant la colonne de phase inverse. Les colonnes de phase inverse (C_8 et C_{18}) étaient utilisées afin de séparer les substrats et les produits élués par la colonne de β-glucuronidases. Il était très important de séparer la colonne de β-glucuronidases de la colonne de phase inverse après injection d'un substrat glucuronidé, puisque l'emploi fréquent de solvants organiques peut entraîner la destruction des enzymes immobilisées sur la colonne. La colonne d'enzymes était rééquilibrée après chaque injection (indépendamment de la colonne de phase inverse) avec la phase mobile (0.01 M ammonium acétate à pH 5.0) en utilisant 2 pompes, une valve de changement (SV), et 2 injecteurs (Inj.1A et inj.1B). Une fois les deux colonnes équilibrées, les substrats glucuronidés étaient transférés d'une colonne à l'autre du système de HPLC à un débit de 0.1 ml/min pour une durée de 30 minutes. Après 30 minutes (temps requis pour que

le contact s'établisse entre les enzymes et les substrats glucuronidés) les valves étaient changées afin de permettre l'analyse des produits sur la colonne analytique.

Les expériences suivantes ont permis d'évaluer l'activité de cette colonne de β-glucuronidases:

Validation de l'activité de la colonne de β-glucuronidases (BG-IMER).

Les solutions initiales étaient préparées par dissolution des substrats glucuronidés dans le solvant ammonium acétate [0.01M,pH 5.0] (phase mobile B). Différentes concentrations des substrats étaient injectées dans la colonne de BG-IMER avec la phase mobile B sur le système "en série" de HPLC, et ceci en fonction de la solubilité de ces sept glucuronides [0.003 à 2.0 mM]. Des contrôles (sans et avec substrats) étaient directement injectés avec et sans la colonne de β-glucuronidases afin de valider le système de HPLC.

Comparaison de l'activité des enzymes libres et immobilisées.

Cette deuxième expérience comparait l'activité des enzymes non-immobilisées de β-glucuronidases en solution avec les enzymes qui étaient immobilisées sur la colonne. Les concentrations des substrats glucuronidés utilisés étaient proportionnelles avec les concentrations des substrats injectés sur la colonne de β-glucuronidases. Les unités des enzymes libres étaient les mêmes que ceux des enzymes immobilisées sur la colonne. Le temps d'incubation était de 30 minutes à la température de la pièce. Les résultats indiquèrent que 5 des 7

substrats glucuronidés furent déconjugués à plus de 98%. L'AZT-g et la morphine-g furent respectivement hydrolysés à seulement 20 et 11% respectivement. Une augmentation de la déconjugaison fut cependant observée en relation avec des concentrations décroissantes d'AZT-g. Les pourcentages de déconjugaison étaient de 20, 89, et 98% à des concentrations respectives de 2.0, 0.04 et 0.02 mM. Aucune déconjugaison ne fut observée pour la morphine-g suite à une diminution de sa concentration de 0.04 à 0.02 mM.

Effet de la variation du débit sur le pourcentage d'hydrolyse.

L'effet du débit de la phase mobile au travers de la colonne d'enzymes de β-glucuronidases a été étudié avec deux substrats, soit le p-acétaminophène-β-D-glucuronide (APAP-g) et le 3'-azido-3'-déoxythymidine-β-D-glucuronide (AZT-g) à des concentrations respectives de 0.14 et 0.04 mM. Ces substrats ont été choisis parce qu'un des substrats fut hydrolysé très facilement (APAP-g) et l'autre pas (AZT-g). La colonne d'enzymes β-glucuronidases était utilisée à la température de la pièce avec un débit pour la phase mobile B de 0.1 à 1.0 ml/min avec un temps de passage à travers la colonne de 15 minutes à 1.5 ml/min (1 volume de colonne). Les résultats de cette expérience ont démontré que le pourcentage d' hydrolyse du substrat APAP-g est demeuré constant (>98%) pour l'intervalle de débit étudié. Au contraire, le pourcentage d'hydrolyse du substrat AZT-g était de plus de 98% à un débit allant jusqu'à 0.4 ml/min, mais de seulement 70% pour un débit de 1 ml/min.

Effet d'élution des substrats sur la colonne BG-IMER.

Dans cette expérience, les deux mêmes substrats, l' APAP-g et l'AZT-g étaient utilisés afin d'évaluer à 5, 10 et 15 minutes, le pourcentage de déconjugaison des substrats glucuronidés injectés. Deux débit différents étaient utilisés lors de cette procédure. Le premier débit était de 0.3 ml/min appliqué (substrats injectés sur la colonne de β-glucuronidases en série avec la colonne analytique de phase inverse) pour des durées de 5, 10, 20 et 30 minutes. Le deuxième débit était de 0.1 ml/min utilisé pour des durées de 15 et 30 minutes. Aucune différence ne fut observée sur les chromatogrammes du substrat APAP-g pour des débits allant de 0.1 à 0.3 ml/min. L'analyse de la surface des pics démontrait le même résultat peu importe le débit utilisé. En ce qui concerne l'AZT-g, les résultats indiquèrent une meilleure hydrolyse à un débit de 0.1 ml/min (75% en série pour 15 ou 30 minutes) plutôt qu'à un débit de 0.3 ml/min (33% en série pour 10 ou 30 minutes et 26% en 5 minutes). Aucun changement au niveau de la surface des pics ne fut décelé en fonction des différents débits ou des durées de contact utilisées.

L'effet du pH sur l'activité de la colonne.

Jusqu'à présent, la colonne de β-glucuronidases a toujours été utilisée avec la phase mobile, soit de l'ammonium acétate (0.01M) à un pH de 5.0. Il était cependant important d' étudier l'activité de cette colonne enzymatique avec différentes valeurs de pH. La colonne de β-glucuronidases a donc été équilibrée à un pH de 6.0 et par la suite à un pH de 4.0. Les deux substrats glucuronidés,

l' APAP-g et l'AZT-g furent utilisés avec le système de HPLC en série à un débit de 0.1 ml/min pendant 30 minutes. Les pourcentages d'hydrolyse de l' APAP-g obtenus à des valeurs de pH de 5.0, 6.0 et de 4.0 étaient respectivement de 98, 92 et 42%. L'exposition de la colonne à un pH de 4.0 entraîna une diminution substantielle du pourcentage d'hydrolyse. De plus, la ré-exposition de cette colonne à un pH de 5.0, suite à l'analyse au pH 4.0, résulta en une hydrolyse de 12% seulement de l'AZT-g, indiquant une perte d'activité importante des enzymes immobilisées sur la colonne. Le même résultat fut observé avec le substrat APAP-g (concentration de 1.0mM). Lorsque la colonne de β-glucuronidases était utilisée à un pH de 5.0 une hydrolyse de plus de 98% fut observée. Suite à la mise en contact de la colonne de β-glucuronidases avec un pH de 4.0, seulement 20% d'hydrolyse était apparente.

L'effet du changement de la température sur l'activité de la colonne de β-glucuronidases.

La colonne utilisée dans cette expérience était celle partiellement désactivée obtenue lors de l'expérience précédente. Le substrat utilisé dans cette étude était l' APAP-g et la colonne était utilisée à la température de la pièce (24°C) et à 37°C. Les résultats indiquèrent qu' à la température de la pièce, l'hydrolyse de l'APAP-g diminua de 54 à 15% suite à l'augmentation des concentrations de ce substrat. A 37°C l'hydrolyse de l'APAP-g était cependant de plus de 98% lorsque les concentrations utilisées variaient entre 0.02 et 0.5 mM. L'hydrolyse du substrat diminua rapidement à 19% lorsque la concentration de l'APAP-g

injectée fut de 1.2 mM en série au travers de la colonne de β-glucuronidases.

Cette étude a démontré que la colonne de β-glucuronidases facilite le processus d'hydrolyse des substrats glucuronidés comparativement aux essais *in vitro* utilisés fréquemment. Cette colonne enzymatique de β-glucuronidases s'avère d'une utilité prometteuse pour les études de pharmacocinétique (détermination du pourcentage total de déconjugaison des substrats glucuronidés) et de métabolisme (identification de métabolites).

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LIST OF ABBREVIATIONS AND DEFINITIONS

APAP-g.....p-acetaminophen-β-D-glucuronide

AZT-g.....3'-azido-3'-deoxythymidine-ß-D- glucuronide

A°.....Angstrom

α.....alpha

BG-IMER.....β-glucuronidase-immobilized enzyme reactor

β-glucuronidase.....Beta-D-glucuronosohydrolase

C.....centigrade

CSP.....chiral stationary phase

C_8..... .....carbon 8 silica support packing

C_{18}.....carbon 18 silica support packing

CYP.....cytochrome p450

E.coli.....Escherichia coli

ex. .....example

etc.....etcetera

g.....grams

γ.....gamma

GI.....gastrointestinal

HCL.....hydrochloric acid

HPLC.....high pressure liquid chromatography

HSA.....human serum albumin

IAM.....Immobilized artificial membrane

i.d. ..... internal diameter

LD_{50}.....the lethal dose of a compound that causes death to 50 % of the animals

$LC\text{-}NH_2$.....liquid chromatography-amino column

ml.....milliliter

mm.....millimeters

ml/min.....milliliters per minute

min.....minutes

M.....molar

mM.....millimolar

N/A.....not available

nm.....nanometers

NADH.....nicotinamide adenine dinucleotide

N.....Normality= number of equivalent weights of solute per liter of solution

O.....oxygen

%.....percent

r.t.....room temperature

SV.....switching valve

S.....sulphur

UDP.....uridine diphosphate

UDPGA.....uridine diphosphoglucuronic acid

UDPGT.....uridine diphosphoglucuronosyltransferase

UDPGT-IMER.....uridine diphosphoglucuronosyl-
transferase-immobilized enzyme reactor

ul.....microliters

UV.....ultraviolet (detection)

U/ml.....units per milliliter

v/v/v.....volume /volume / volume

ZDV.....Zidovudine

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DEDICATION

I would like to dedicate my thesis to a few special people in my life:

I would like to dedicate my thesis.....

- to my best friend and loving soul mate, my husband ***Luciano Di Marco***, with his love, support and encouragement has made my studies possible.
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CHAPTER I

INTRODUCTION

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1.0 Glucuronides

1.1 General Information

One of the important purposes of drug metabolism or biotransformation reactions is to convert foreign compounds (i.e. xenobiotics) into a form which is more water soluble and readily excreted by the organism [1]. One of the predominant biotransformation reactions is glucuronidation of primary metabolites by uridine diphosphate glucuronyltransferase (UDPGT) in the presence of glucuronic acid [2]. This membrane bound enzyme is located predominantly in the microsomal fraction of the endoplasmic reticulum of many tissues, particularly the liver [3]. The activity of UDPGT has also been detected in other organs and tissues such as kidney, bladder, blood, brain, eye, lung, heart [4]. UDPGT is believed to be a heterogenic enzyme existing in more than one isoform [5]. These enzymes are involved in the conjugation of various exogenous (ex. drugs) and endogenous (ex. bilirubin) aglycones or alcohols to form water soluble glucuronides [6]. Glucuronides are more water soluble than the parent structures due to the addition of such hydrophilic moieties as carbohydrates, sulphates or glucuronic acids. These molecules are then ionized at physical pH which makes urinary excretion of these molecules possible. One of the ways of achieving water soluble glucuronides is by conjugation with,

for example, glucuronic acid in a phase II reaction. In order for conjugation to occur there must be a functional group (such as thiols, hydroxyls, amino groups ect.) in the molecule which can react with the conjugating agent.

As early as 1855 researchers have established that in various animal species certain compounds were excreted in the urine as glucuronide substrates, conjugated from D-glucuronic acid [7]. Glucuronides are not only excreted in urine but in the bile as well. In the kidney, glucuronides are often excreted by tubular secretion but some, such as glucuronides of menthol or phenols are also excreted by glomerular filtration. Some compounds such as chloramphenicol and morphine are excreted via the bile as glucuronide metabolites [7].

1.2 Mechanism of Glucuronide Formation

Metabolic transformations which compounds can undergo in the animal body can be conveniently classified into two distinct types: Phase I processes involving oxidation, reduction and hydrolysis and Phase II processes involving conjugation or glucuronidation reactions [8]. Phase I reactions comprises the production of pharmacological active or inactive metabolites, while Phase II reactions usually ends the pharmacological or toxic effects of the compounds undergoing metabolism, and results in a more water soluble compound which is easily excreted [6].

The mechanism of glucuronidation or formation of the major types of glucuronides is a two step process involving :

1) the synthesis of the coenzyme donor, uridine diphosphate alpha-D-glucuronic acid (UDPGA)

2) the transfer of the glucuronic acid moiety from UDPGA to a foreign compound or metabolite. This then leads to uridine diphosphate (UDP) formation upon the addition of one of a multitude of closely related physical and catalytic enzymes of UDPGT [8].

Drug (aglycone) $\xrightarrow[\text{Conjugation}]{\text{UDPGT +UDPGA}}$ β-glucuronide (urine, bile)

1.3 Conjugation

Conjugation is often preceded by Phase I metabolic reactions such as hydrolysis or oxidation of which cytochrome P450 plays a major role. Compounds that already have a group available for conjugation when they enter the body, can however be readily conjugated. The conjugation reactions are named "Phase II" processes and they consist mainly in glucuronidation, sulfation, acetylation, O and S methylation, amino acid and glutathion conjugation. In these reactions, a compound or a metabolite is combined with an endogenous molecule to give a product known as a conjugate [9]. The products of principle conjugation reactions are generally inactive or much less active than the parent compounds. Conjugation occurs in most living species except in insects and in cats [6]. Within particular orders of animals, there may be pronounced species variations in the formation of some glucuronides.

There are a number of reasons why conjugation reactions are important in biochemical pharmacology and toxicology. Conjugation is not only an inevitable process that leads to excretion but its importance in drug interactions is well known. Also, saturation in the formation of metabolite conjugates may occur, and this may lead be due to genetic and species differences [6]. One of the important roles of conjugation reactions is the detoxication function. In most cases, conjugation leads to compounds that are usually less toxic and more rapidly excreted from the body. Products conjugated by glucuronic acid differ from one species to another resulting in variable toxicity levels of metabolite formation. For instance, toxicity levels of glucuronidated compounds such as paracetamol is much less in the rabbit (LD_{50}=1200 mg/kg) than in the cat (LD_{50} 250 mg/kg) [6].

Conjugation reactions may become important when the conjugate becomes more active than the parent compound. This is known as "metabolic activation" of the parent drug [6].

Conjugation reactions are very important in pharmacokinetic studies. They may have an impact on the disposition, excretion and interactions of drugs [6]. Some of these pharmacokinetic implications involve the contribution to total body clearance, saturable conjugation reactions leading to non-linear Michaelis-Menten kinetics, and the biliary excretion of conjugates. Conjugation reactions may also influence the route and the rate of administration. For example, if conjugation of a drug administered orally undergoes conjugation in the gut and becomes toxic, the intravenous route for administration will be preferred.

Saturation is a frequent problem in conjugation reactions. When saturation in the formation of the conjugated drug occurs, the elimination of the drug and its metabolite is no longer described by a first order process but by Michaelis-Menten kinetics [10]. This non-linearity in the elimination makes it very difficult to manipulate dosage regimens in order to obtain desired pl;asma levels [11].

1.4 Deconjugation

Humans and many other species possesses enzymes that are able to break to break down conjugated molecules.Such enzymes are β-glucuronidases, sulfatases, O- and N-deacetylases [6]. Morphine, for instance, is an analgesic that undergoes conjugation on its two hydroxyl groups. One of the conjugates, morphine-3-glucuronide, possesses very little pharmacological activity but may undergo hydrolysis by β-glucuronidase enzymes. Morphine-6-glucuronide, the other conjugated metabolite, is more potent than its parent compound and may also be deconjugated by β-glucuronidase enzymes [6].

The products of conjugation reactions are usually acidic and more water soluble. These metabolites are eliminated by urinary and biliary excretion. For example, salicylic acid is almost completely metabolized and conjugated to three metabolites (salicyluric acid, ester and phenyl glucuronides) which are eliminated in the urine [12]. The overall total elimination of the glucuronides are important to take into account clinically if they contribute to the pharmacological effect or toxicity.

Highly soluble and ionized conjugates of small drug molecules are usually excreted in the urine while larger drug molecules possessing a lipophilic or hydrophobic portion favors biliary excretion. Once, glucuronic acid conjugation occurs (glucuronidation) a compound eliminated in the bile may be hydrolyzed by the gut microflora [13]. Metabolic activities include the hydrolysis of glucuronides by β-glucuronidase enzymes and reabsorption of these hydrolyzed molecules from the gut. Chloramphenicol is an example of a drug metabolized by glucuronic acid conjugation through its primary alcohol group. The glucuronide is excreted in the bile but is deconjuated by β-glucuronidases in the gut flora yielding free chloramphenicol [14].

Unfortunately, some molecules eliminated in the bile cause localized toxicity to the gastrointestinal tract. This was the case with indomethacin, an anti-inflammatory drug for which the conjugate is hydrolyzed back to its parent form in the gut flora by bacterial β-glucuronidases [6,15]. The indomethacin liberated is ulcerogenic in the small intestine when the glucuronide is excreted in the bile. This acidic drug must therefore be absorbed from the stomach after oral absorption in such minute amounts as to avoid causing toxicity [6,16]. The fact that the glucuronide undergoes hepatic elimination means that not only the metabolic pathway but also the excretion pathway of the compound is dose dependent. Thus, at low doses, bile and urine account for half the excreted material while at high doses excretion is performed almost entirely by the bile. The β-glucuronidase enzymes and their importance in drug activity, pharmacokinetics and disposition in biological systems leads us to the next

part of this chapter and the enzymes used in this study.

2.0 β–Glucuronidase Enzymes

2.1 General Information and principal distribution

β-glucuronidase (EC 3.2.1.31; β-D-Glucuronosohydrolase), first identified in 1908 by Röhman [17], is an acid hydrolase found in subcellular distributions of the lysosomal and microsomal fractions of mammalian tissues, albeit, the enzyme may be localized in variable amounts by either fractions in different species [18]. After homogenization, 5 % of the liver β-glucuronidase is found in the cytosol or supernatant fraction [18]. For instance, high β-glucuronidase activity has been reported in rat (Wistar) liver and reported to have a dual intracellular localization in which 75-85 % were localized in lysosomes and 15-25 % in microsomes (endoplasmic reticulum) as compared to low β-glucuronidase activity which showed an absence of the enzyme in the microsomal fraction but also a reduction of the enzyme in the lysosomal fraction [19]. In mice, 30-50 % of the total high β-glucuronidase activity is present in the microsomal fraction and the rest in the lysosomal fraction. With reference to catalytic properties and the stability of the enzyme in solution, microsomal and lysosomal β-glucuronidase in the rat appears to be identical. The lysosomal or microsomal enzyme cannot be distinguished by gel filtration chromatography by the two-dimensional electrophoresis technique.

As early as 1932, β-glucuronidase enzymes were shown to be active on glucuronides of the flavone type in mammals [12]. It was found to hydrolyze aryl, alkyl, alicyclic and acyl β-glucuronides (β -D-glucopyranosiduronic acids) to free glucuronic acid yielding various alcohols [12]. β-glucuronidases can be found in vertebrates, insects, molusks and certain bacteria (such as E. coli, anaerobes, etc...) [12]. Enzyme levels generally vary considerably between different mammalian species especially between body fluids in certain tissues. High activities of the enzymes can be predominantly found in the liver, kidney, spleen, epididymis and cancer tissues [12]. Very few β-glucuronidase enzymes are found in the blood and brain and therefore, their accurate measurement is not possible [12]. The urine of rats has been found to contain high levels of the β-glucuronidase enzymes and large quantities have been discovered in the preputial (clitoral) gland of the female rat [20]. Pigs have very low levels of this enzyme [12]. Human and mouse tissues have been found to contain β-glucuronidase enzymes in an intermediate quantity compared to pigs and rats. Enzyme variability within species and tissues, makes it difficult to extrapolate the effects of drugs subject to deconjugation from one animal to another.

The β-glucuronidase enzymes are predominantly stable at high temperatures (the enzyme purified from mouse kidney has been found to be stable at 55°C) [21] but at low temperatures a slow decrease in activity of highly purified β-glucuronidase enzymes was observed in frozen samples isolated from rats (upon the addition of 50 % glycerol at neutral pH, the enzymes were found to be stable when stored at -20°C) [22].

Specific stabilizers are sometimes used in preparations of mammalian β-glucuronidase enzymes in order to increase their stability. Such stabilizers are albumin (0.01 %) [12] and chitosan as well as heat inactivated enzymes and certain diamines [12]. In the past deoxyribonucleic has been used as a stabilizer in enzyme preparations but was found less efficient to protect the enzymes because it caused an alkaline shift in the optimum pH at which the enzymes are stable[12]. Chloroform has been used as a preservative of bacerial β-glucuronidases (from E. coli) during incubations because it was found not to inhibit the activity of the enzymes [23].

The activity of β-glucuronidases recovered from urine samples has been used as a marker in cytological examinations to identify patients at risk of presenting urinary tract malignancies. Results confirmed that urine β-glucuronidase activity was a sensitive test for the identification of both transitional cell and renal cell carcinoma compared to the conventional urine cytology tests [24,25]. It has also been reported that urinary endogenous β-glucuronidases are not only indicators for urinary tract malignancy but elevated amounts of the enzymes have been associated with various renal, ureteral, and bladder diseases [25].

Interestingly, β-glucuronidase enzymes have been found in cancer cell tissues. The enzymes have been demonstrated to convert relatively non-toxic prodrugs concentrated in tumor lesions to active compounds [26]. Drug manufacturers have used this advantageous "targeting" method by attaching a glucuronic acid molecule to a drug structure. This enables the drug to be more

stable, hydrophilic and reduces its accumulation in normal cells. Another currently used strategy is to administer the β-glucuronidase enzymes bound to a monoclonal antibody in order to increase the concentration of this enzyme in tumors so that these prodrugs are easily activated [27].

2.2 Structure of β-glucuronidase enzymes

2.2.1 Amino acid and carbohydrate composition

The amino acid composition of the β-glucuronidase enzymes were evaluated from highly purified preparations of rat liver lysosomal fractions. High concentrations (20 %) of aspartic and glutamic acid with only a trace of cysteine [28, 29] and a low content of sulphur containing amino acids were found [30,31]. The amino acid composition detected from mouse liver lysosomal fractions were found to contain high levels of arginine and glutamic acid [31]. Carbohydrate composition of β-glucuronidase in mouse liver lysosomal fractions and rat preputial gland showed the presence of mannose, glucosamine, galactose and glucose sugars [31]. Fucose and sialic acid sugars were not detected in the mouse β-glucuronidase enzymes although fucose (but not sialic acid) was detected in β-glucuronidases isolated from rat preputial gland [31].

2.2.2 Different molecular forms of β-glucuronidases

The β-glucuronidase enzyme is a tetrameric glycoprotein composed of four subunits [32] with a total molecular weight in the range of 280,000 to

300,000 and a molecular weight per subunit in the neighborhood of 75,000 [32,33]. Evidence that the enzyme is a glycoprotein was produced when precursor proteins from many lysosomal enzymes isolated from rat liver were discovered to be glycosylated due to the transport of the β-glucuronidase molecules through the Golgi system or apparatus [22,29,34]

Five isoforms of the β-glucuronidase enzyme were found in lysosomal fractions isolated from Sprague-Dawley male rats [32]. They were found to be electrophoretically homogenous [32,35]. When SDS-polyacrylamide gel electrophoresis was used to separate each of the 5 isoforms , three main bands or subunits were isolated in order of increasing mobility: α, β and γ [32,35]. Isoform I contained only the α subunit and isoenzyme V mainly the β subunit while the other ones possessed different proportions of the three main bands. The following tetrameric structures were identified:

- isoform I: αααα
- isoform II: αααβ
- isoform III: αββ
- isoform IV: αβββ
- isoform V: ββββ

The γ band was thought to be an artifact produced from the β subunit through purification procedures where trypsin was employed [35]. The enzyme was digested with trypsin to demonstrate wether subunits were identical with respect to their amino acid sequence. The results of the tryptic digest yielded 57 (ninhydrin) positions out of the theoretical 61 indicating that the peptide mapping

of the subunits were identical [36].

2.2.3 Molecular genetics of mammalian β-glucuronidases

Genetic variants have been extensively investigated using mice β-glucuronidase enzymes [37,38]. Four genes coding for the β-glucuronidase enzymes have been described so far. Three of the genes *Gus, Gur* and *Gut* are located at close proximity to each other on chromosome 5.The fourth gene, *Eg*, is situated on chromosome 8 [37-42].

2.3 Excretion of β-glucuronidase enzymes

In mammals, β-glucuronidase enzymes are found and excreted in the urine and bile. The primary source of urinary β-glucuronidases is thought to arise from the renal lysosomes [43]. These enzymes are found in abundant amounts in the cells of the urinary tract especially in the epithelial of the proximal and distal tubules [43]. Very minimal amounts of the enzymes can be found in red blood cells but have been detected in white blood cells [44]. In the plasma, β-glucuronidase is too large of a molecule to be filtered through the glomeruli and therefore, it is mainly cleared by the liver instead of the kidney [43].The enzyme can also be detected in variable amounts in the serum, gastric juice, amniotic fluid, cervical mucus and cerebral spinal fluid [45].

Biliary excretion of a compound is influenced by a number of factors such as its physiochemical characteristics (molecular weight, polarity) and biological

factors of the host (species, sex, genetic factors, etc...) [46]. The source of β-glucuronidases in the bile may be endogenous or exogenous [45]. The activity of β-glucuronidases in the bile has been shown in a few studies to be affected greatly by pH, bile salts and direct bilirubin diglucuronide[47]. The pH associated with the optimum activity of mammalian β-glucuronidase enzymes in the bile was observed to range from 4.7 to 5.2 [47]. The pH of normal hepatic bile is 8.1 (as observed in rats) [45] and the enzyme activity of β-glucuronidases at this pH was reported only to be 15%. Therefore, at pH of 8.1, the decrease of β-glucuronidase activity in the bile limits the formation of excessive water insoluble unconjugated bilirubin. However, if the bile duct becomes infected or obstructed, lowering the pH of the bile can increase the activity of the β-glucuronidase enzymes [45]. Conjugated bilirubin, a natural substrate for β-glucuronidase enzymes in the bile, has been reported to be a noncompetitive inhibitor with other substrates [47]. The activity of biliary β-glucuronidases in *in vitro* assays can be increased by removing bile acids and conjugated bilirubin in the bile by tetrahexylammonium chloride in ethyl acetate [48]. The intake of a high fat diet may increase bile acid excretion especially in the feces [49]. This is because conjugates in the form of bile acids may be secreted into the duodenum which are then degraded by the β-glucuronidase enzymes found in the intestinal bacteria (E. coli) in the large intestine and then transformed into deconjugated bile acids.The effect of bile salts on the activity of the β-glucuronidase enzymes were studied using such bile salts as glycocholate, taurodeoxycholate, glycodeoxycholate, etc... [45]. In rats, glycocholate and taurodeoxycholate barely

affected the activity of β-glucuronidases while the activity decreased in the presence of glycodeoxycholate. However, the more the bile was diluted, the higher the enzymatic activity of the β-glucuronidase enzymes tended to be. This suggested that the inhibitory effect on the enzymatic activity of β-glucuronidase enzymes may be due to certain bile salts and/or other factors in the bile [50].

2.4 Regulation of β-glucuronidase enzymes

Increase in β-glucuronidase activity has been detected in various clinical conditions such as acute tubular necrosis, active pyelonephritis, cystitis as well as early renal transplant rejection [45].

The elimination half-life of β-glucuronidase in the bile has been measured by *in vivo* radioactive labeling of the enzyme in rats. Its value was determined to be 5.1 days [51].

2.4.1 Inhibitors of β-glucuronidase enzymes

Inhibitors of β-glucuronidase enzymes have been studied and standardized. The source and amounts of many of the enzyme's inhibitors in human urine are varied and unpredictable. The degree of inhibition may vary from individuals and from day to day within the same individual. Much of the work performed on urinary inhibitors was not to quantify or to identify them but to eliminate as much as possible their inhibitory activity in the urine. Dialysis is used as a convenient and efficient method to eliminate much of the inhibitors from the urine. The body manufactures a powerful, competitive and highly

specific inhibitor of β-glucuronidase called the glucaro-(1→4)-lactone (saccharolactone) [49]. This inhibitor can be employed to establish the presence of β-glucuronidase in impure preparations. Although glucaro-(1→4)-lactone specifically inhibits β-glucuronidases in the liver, it was found to be nonspecific for glucuronide synthesis which utilizes UDPGT and UDPGA. This makes the application of glucaro-(1→4)-lactone a biomedical reagent used in synergy with β-glucuronidases in the identification of unknown glucuronide conjugated compounds. Other factors that have been found to decrease or stop the activity of the β-glucuronidase enzymes are reagents such as concentrations of 4 to 8 M urea and 3 M quanidine chloride as well as 10% 6N HCL [53]. Sodium dodecyl sulfate (SDS) (0.1% concentration) has been found to dissociate the β-glucuronidase tetramer to a monomer in *in vitro* assays [30,35]. The activity of the enzymes have been found to be terminated by such cocktail solutions as 1M glycine / NaOH pH 10.5 [27]. Heat has also been used to inactivate the enzymes. This was accomplished by placing tubes containing the reaction mixtures in a water bath of 71°C for up to 60 min [54]. Bacterial enzyme activity can also be inhibited with the use of ammonium ion solutions containing 0.1% ammonium chloride (decreases activity of the enzyme by 20%) and 1% sodium chloride [23].

Substances such as acidic mucopolysaccharides, heparin, hyaluronic acid, chodroitin sulfate as well as acid ion exchange resins and anionic Teepol XL detergents were found to cause noncompetitive inhibition (>98%). These substances resulted with an inactivation of the β-glucuronidase enzymes [12].

2.4.2 Inducers of β-glucuronidase enzymes

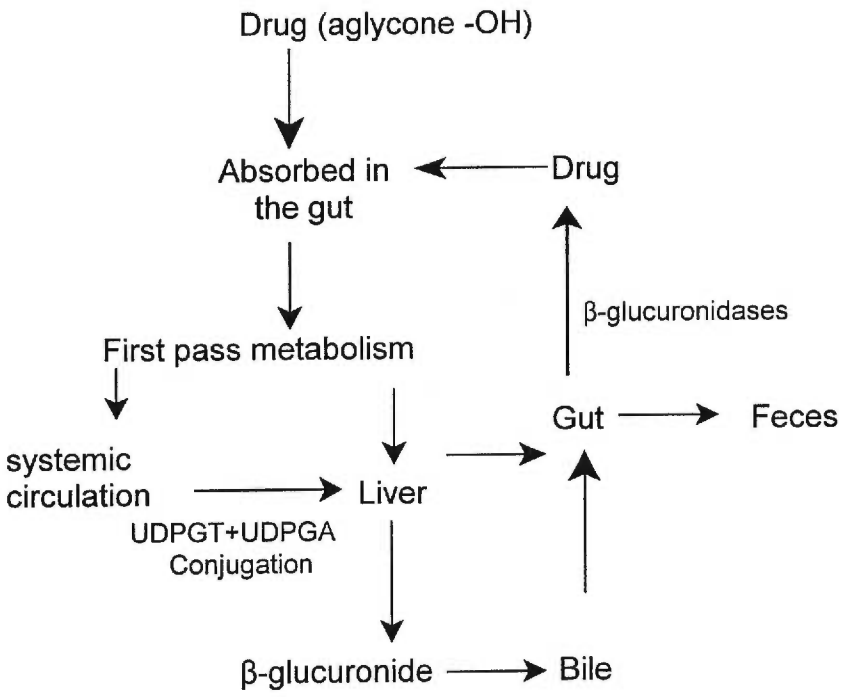
The regulation of β-glucuronidase enzymes has been studied extensively with respect to its induction by androgens in mouse kidney .Gonadotropin, an androgen stimulant, was found to specifically induce the enzymatic activity of β-glucuronidase enzymes in male mouse kidneys [34]. Investigation of the mechanism of the intracellular transport and distribution of the enzyme has been facilitated by the specific induction of this enzyme in the mouse (male) kidney by the administration of androgen.

β-glucuronidase activity in E. coli has been reported to be induced by a variety of β-glucuronides. Methyl glucuronide has been mentioned as one of the most effective inducers [55].

2.5 Mechanism of Deconjugation

β-glucuronidase has been isolated from various sources and the enzymes tested with various β-glucuronide substrates. Under normal circumstances, β-glucuronidases liberate aglycones and glucuronic acid [12]. The route of drug excretion is largely influenced by molecular weight. That is, drugs with low molecular weight are (<300 MW) usually excreted in the urine, while higher molecular weight drugs are excreted into the bile and then into the intestine. Once in the intestine the conjugate can either be excreted into the feces or can be deconjugated by β-glucuronidase enzymes. β-glucuronidase enzymes, therefore play an important part in the enterohepatic circulation of

drugs. The hydrolysis of conjugated drugs in the gut may also be mediated by β-glucuronidases produced by E. coli or bacterial anaerobes [7]. If the conjugates are readily hydrolyzed, the aglycones (alcohols) can undergo reabsorption. It may be responsible for a new pharmacological effect and the drug will again eventually undergo conjugation in the liver and be re-excreted in the bile. The following is a representation of the schematic diagram of the enterohepatic circulation of drugs [9]:



Theoretically, the enterohepatic circulation can make a significant contribution to the activity and toxicity of a drug by prolonging its elimination half-life and pharmacological effect. Examples of drugs that undergo enterohepatic circulation are chloramphenicol, morphine, erythromycin and

digitoxin [46]. Bile acids are also subject to enterohepatic circulation. In the rat, the pool of bile acids undergo 10 complete enterohepatic circulations per day while in humans two enterohepatic circulations of the bile acid pool occurs with each meal [46,56].

The enterohepatic circulation of a drug is influenced by various factors. The rate and extent of excretion of the compound in the bile is influenced by its physicochemical characteristics (molecular weight, polarity etc...) and by biological factors specific to the host such as enzymes responsible for drug metabolism [46].

2.6 In Vitro Studies with free β–Glucuronidase enzymes

β-glucuronides can be identified because they readily hydrolyze in the presence of β–glucuronidase enzymes. During pharmacokinetic and drug metabolism studies, it is frequently necessary to deconjugate glucuronide metabolites in various fluids such as urine, in order to calculate the free and total concentration of the parent drug. Unfortunately, these studies are often tedious and time consuming to perform. They involve lengthy incubations of the substrate in free enzyme solution, extraction of the deconjugated substrate and preparation of the solution for injection into the HPLC column which consists in part in the precipitation of proteins.

Let us look at the example of Zidovudine (ZDV). Extensive research was performed on Zidovudine (ZDV) to determine its main metabolite which is a β-

glucuronide. *In vitro* procedures were developed involving the main metabolite in which they incubations with 3000 U of β–glucuronidase free enzymes (purchased from Sigma) at 37°C for 2 hours [53]. This reaction was done after a glucuronidation reaction of the parent compound, ZDV, with UDP-glucuronosyltransferase and UDP-glucuronic acid. A control study was also simultaneously performed without the addition of the β–glucuronidases to estimate the nonenzymatic hydrolysis of the glucuronide. Another similar study was conducted on ZDV involving first the isolation of the metabolite from pooled urine samples obtained from monkeys and humans. The metabolite of ZDV was isolated and purified from the collection of urine, over a period of 18 hours, from an AIDS patient as well as from monkeys (given ZDV in order to perform clinical and toxicological studies) [56]. The metabolite of ZDV was isolated by numerous extraction steps which included SPE (solid phase extraction) columns. Once the metabolite was isolated from the pooled urine samples, β–glucuronidase enzymes were added and incubated at 37°C. Parallel incubations were conducted in the presence of a β–glucuronidase inhibitor, saccharolactone. Quantitative hydrolysis of the isolated metabolite of ZDV was observed. In the presence of the specific enzyme inhibitor the hydrolysis of the metabolite to the parent compound, ZDV, was completely prevented. The enzymatic characterization of the metabolite of ZDV provided strong evidence that the metabolite was a glucuronic acid conjugate of ZDV. The linkage was in the β configuration (also confirmed by NMR), since the hydrolytic activity of the β-

glucuronidase enzymes is specific for β-glucuronides. Further use of the specific inhibitor (saccharolactone) of the β–glucuronidase enzymes showed enzyme dependent hydrolysis [56].

β–glucuronidases may be used to increase the sensitivity of certain conjugated compounds. For example, β–glucuronidase enzymes isolated from E. coli were used in an *in vitro* assay to increase the detection (sensitivity) of urinary benzodiazepines using fluorescent polarization assays without reducing the specificity of the method [57]. The detection of conjugated benzodiazepines such as oxazepam was reduced in immunoreactivity assays resulting in false-negative results. Screening of urinary benzodiazepines has become routine practice to diagnose chemical dependency and therefore, a highly sensitive method of detection was important to detect the presence of these psychotic drugs in the urine. The addition of β–glucuronidases in the assay greatly increased (10 fold) the limit of detection of benzodiazepines. The study further investigated the addition of β–glucuronidases as a routine assay in the analysis of benzodiazepines. Results obtained from this investigation showed that the enzyme did not alter the specificity or did not produce possible artifacts of the benzodiazepines but simply increased the sensitivity of the compounds extracted from urine samples.

An assay was developed for epirubicin, an anthracycline antibiotic and antitumor derivative of doxorubicin, in which the β-glucuronidase enzymes were added in order to separate glucuronide metabolites epirubicin (EPI) and of

epirubicinol (EOL) otherwise not separated on the HPLC chromatogram [58]. Incubation of the enzyme with human plasma samples were performed at 37°C for 4 hours. A control plasma sample (without the addition of β-glucuronidase enzymes) was injected on a HPLC system and showed a major peak eluting at 4.3 min on the chromatogram. Upon the addition of the β-glucuronidase enzymes to control samples, this major peak of retention time 4.33 min disappeared while the EPI and EOL glucuronide peaks (hardly detected before the addition of the enzyme) increased by as much as 50% [58]. This demonstrated that the peak on the HPLC chromatogram that disappeared contained both glucuronides, EPI and EOL and that the addition of β-glucuronidase enzymes increased the detection of EPI and EOL glucuronide metabolites.

These examples demonstrate that many *in vitro* studies employ regularly the β-glucuronidase enzymes for enzymatic identification as well as to increase the sensitivity and separations of the glucuronic acid conjugates. We propose that these examples of *in vitro* assays may be replaced by an on-line system in which an immobilized β–glucuronidase column would be used to identify the glucuronides, calculate the total parent drug and increase peak sensitivity of conjugated drugs in HPLC assays.

3.0 Immobilization of Enzymes

Drugs constantly interact with proteins and enzymes as well as other biopolymers such as receptors in the biological systems in order to produce a pharmacological effect. Therefore, it is routinely necessary to isolate the major interactions of substrates and enzymes and initially examine these interactions by developing *in vitro* assays. These experiments are often complicated, costly, and very long and tedious to perform. In the last few years there has been an increasing demand in many laboratories for the development of fast and efficient *in vitro* assays utilizing stable enzymes for routine biological studies. These demands have focused more and more onto immobilizing enzymes onto HPLC column supports employing various techniques. These, for example, may consist of on-line chromatography applying an immobilized enzyme column reactor with a regular reversed phase column. This kind of chromatography must be a reproducible reflection of the enzyme/substrate interaction that would otherwise be done in routine biochemical assays.

Immobilization of enzymes onto column supports evolved following the successfully immobilization of proteins for enantiomeric resolutions of racemic compounds. These protein columns (for example, human serum albumin {HSA}) were useful to monitor protein/ligand interactions which would also be used to identify specific and nonspecific interactions between two ligands [59].The immobilization of enzymes often stabilizes the enzyme by entrapment in insoluble supports resulting in an easy separation of the products [60]. One

advantage of using immobilized enzymes onto column supports is the facilitation of the quantification and identification of metabolites such as glucuronides because of its direct injection procedure. However, chromatographic analysis methods must model and correlate biological processes. Biochemical analysis of direct separations of very low concentrations of drugs and their metabolites from biological matrices are not straightforward [61]. Isolation by multi-step extraction and purification procedures of metabolites from biological fluids for drug metabolism studies often involves difficult and unstable manipulations. Usually a sufficiently large quantity of metabolites are needed to be isolated before they are used with reactive enzymes. Some enzymes such as UDP-glucuronosyltransferases (UDPGT) are often labile and unstable after solubilization and purification [62]. These enzymes are tested for stability and specific activities with known standard substrates before they are used. Other times, the metabolites are not stable in the enzymatic reaction medium used by the method. Substrate/enzyme reactions have to be suppressed by various methods (chemical, temperature, etc...) and immediately injected onto a chromatographic system to obtain reliable analytical results. The chromatographic system usually involves a highly sensitive, standard operating HPLC method to analyze *in vitro* substrate/ enzyme reactions.

Various methods of immobilization of enzymes have been investigated. Some of these methods include covalent binding, adsorption onto supports by ionic or hydrophobic interactions, entrapment of microsomes into alginate beads or trapping enzymes in hydrophobic cavities in immobilized artificial membrane

(IAM) supports. [63,64]. HPLC columns with IAM supports have been developed to model a number of biological processes [61]. Some of these involve the prediction of the transport of the solutes across biological membrane barriers such as human skin and liposomal membranes found in the (GI) tract [64]. Many innovative drug candidates are evaluated for oral drug absorption employing animal models requiring extensive cost and effort. HPLC methods are much more simpler to develop than animal or cell culture model techniques (Caco-2 cells) to predict oral drug absorption [64]. IAM columns have been shown to model oral drug absorption and delivery through the intestinal mucosa of the GI tract [63,64]. The major absorption barrier to oral drugs is the cell membrane in the GI tract in which drugs are absorbed across the intestinal mucosa by a passive diffusion mechanism. Unfortunately, many factors (examples: enzymatic, bacterial and chemical degradation, poor dissolution and precipitation of the drug at the absorption site, etc...) also contribute besides the intestinal membrane barriers, to the reaching of drug into the circulating blood system limiting the usefulness of IAM predictions to the initial start of oral drug transport [63,64]. The IAM columns have also been used as supports for numerous enzymes including trypsin and α-chymotrypsin, NADH oxidoreductase and active forms of cytochrome P450 proteins.

Many immobilized biopolymer columns utilizing experimental HPLC techniques have been successfully developed as research tools for the identification of metabolites of drugs in biological fluids. For instance, glucuronide/sulphate metabolites of benzene have been determined in urine

using an HPLC with on-line pre-column performing enzymatic hydrolysis [65]. The pre-column enzyme reactor contained beta-glucosidase immobilized on a LC-NH_2 beaded packing material. The conjugates were hydrolyzed and phenol was produced in the enzyme reactor column by direct injection of the urine sample into the HPLC system. Endogenous urinary components were then separated from phenol by switching from the reactor column on-line to a reversed phase column. This chromatographic method provided a simple and rapid procedure for urinary phenol determination.

Chiral separations of racemic molecules have been performed due to the enzymatic activity of the immobilized enzyme on a support packing. The stereochemical resolution of N– and O- derivatized amino acids have been achieved on the enzymatic activity of trypsin immobilized on a silica support column [66]. The procedure involved HPLC experiments using a chiral stationary phase (CSP) column and an immobilized trypsin column on the same HPLC system. Identification of the compounds eluted from the trypsin-CSP column were collected and analyzed on a reversed phase or chiral column.

This revolution in immobilized enzyme reactor columns has encouraged another column to be produced, the β-glucuronidase enzyme column. This type of column has never been developed before for the deconjugation of glucuronide substrates. In this study, β-glucuronidases from E. coli K12 (EC. 3.2.1.31) were covalently bonded on a silica support packing [67]. It has been reported that the E. coli K12 β-glucuronidase enzymes are very stable with a

broad pH range of optimum activity of 5 to 7.5, and are resistant to thermal inactivation at 50°C [55]. The E. coli β-glucuronidase enzymes have been reported to loose half of their activity at pH 4.3 [55]. (Other studies have, however, reported conflicting results. Hence, some researchers have reported this enzyme to have an optimum activity at pH of 7.3, with a 26% activity loss when pH was decreased to 6.5, and an 80% activity loss when pH was 5.0)[24,70]. The enzyme is commercially available (Boerhinger Mannheim) and given these factors, is an interesting enzyme to immobilize into column support packings.

4.0 Objective of this study

The objective of this study was to investigate the activity of the immobilized β-glucuronidase enzymes to a HPLC stationary phase packing, and its applicability to the on-line deconjugation of glucuronides for drug metabolism and pharmacokinetic studies. It was important to develop a fast, simple efficient and validated method for analyzing deconjugated glucuronides. The purpose of this immobilized enzyme column would be to reproduce or be comparable to *in vitro* biomedical assays that would simulate biological processes. The validation of this immobilized β-glucuronidase enzyme column by an on-line HPLC system, would open the way for other enzyme reactor columns of this type to be developed in the future. It was important to examine the activity of the column through a battery of experimental tests involving various known substrates that have been demonstrated to deconjugate to their aglycones dervatives by β-

glucuronidase enzymes in *in vitro* assays. These battery of tests would include the effect of substrate/enzyme contact time at various flow rates and substrate concentrations. The pH associated with the optimum enzyme activity in the column would also be important to establish because it greatly influences the HPLC method developments.

The development of a validated, standard HPLC method was essential to establish before the activity of the enzyme in the column may be tested. This would include first, establishing a HPLC reversed phase method (without the enzyme column) for analyzing the eluted products. This would involve injecting the glucuronide substrates and the alcohol as control standards. Blank solutions (without standard controls) would be injected as negative control standards in the method. Secondly, a reproducible method (i.e. which may be automatized) involving the β-glucuronidase column connected on-line to a reversed phase column would be developed. This method would then allow switching of the lines so that the enzyme column would be independently washing and equilibrating after each on-line injection. The reversed phase column would then be used to analyze the eluted products with a gradient HPLC solvent system.

CHAPTER II

On-line deconjugation of glucuronides using an immobilized enzyme reactor based upon β-glucuronidase

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1.0 ABSTRACT

An immobilized enzyme reactor based upon β -glucuronidase {BG-IMER} has been developed for the on-line deconjugation of substrates. The activity of the BG-IMER and its applicability to on-line deconjugation was investigated. The BG-IMER was coupled to a reversed-phase column (C_8 or C_{18}) and the latter column was used to separate substrates and products eluted from the β-glucuronidase reactor. The activity of the BG-IMER was followed by measurement of percent deconjugation and the parameters investigated were: substrate concentration, pH (4 to 6), temperature (r.t., 37°C), enzyme/substrate contact time using flow rates of 0.1 to 1.0 ml/min (15 min to 1.5 min). The glucuronides used in the evaluation of the BG-IMER were: 4-methylumbelliferyl-β-D-glucuronide, p-acetaminophen-β-D-glucuronide, 3'-azido-3'-deoxythymidine-β-D-glucuronide, phenyl-β-D-glucuronide, chloramphenicol-β-D-glucuronide, estradiol-17-β-D-glucuronide and morphine-β-D-glucuronide. The development of on-line HPLC deconjugation of glucuronide substrates using the BG-IMER will facilitate the identification of metabolites and quantification of aglycones in metabolic and pharmacokinetic studies.

Keywords: β-glucuronidase, glucuronides, immobilization, on-line deconjugation

2.0 INTRODUCTION

β-glucuronidase enzymes are widely distributed in mammalian tissues with particularly high concentrations in the liver [1]. These enzymes catalyze the hydrolysis of β-glucuronide conjugates to yield aglycones and free glucuronic acid [2]. β-glucuronidase plays an important role in the enterohepatic circulation of drugs [3] and the hydrolysis of β-glucuronides can contribute significantly to the overall biological activity or toxicity of a xenobiotic.

Since the glucuronidation of a parent drug or its metabolite(s) may play a role in the overall pharmacological activity of the compound, it is often necessary to measure both free {non-conjugated} and total {conjugated plus non-conjugated} drug and metabolite concentrations. Total drug concentrations are usually determined by off-line hydrolysis of the conjugates. This is a multi-step process involving incubation with β-glucuronidase, extraction from the biological matrix and analysis by a suitable chromatographic method. These procedures can be time consuming, expensive and may yield unreproducible results because of the instability of the enzymes in the assays [4,5].

One approach to the reduction of the time and expense involved in the deconjugation of glucuronides is the development of immobilized β-glucuronidase reactors for on-line conversion of these conjugates. The feasibility of this approach has been previously demonstrated by the covalent immobilization of solubilized rat liver uridine glucuronosyltransferase (UDPGT)

on a cyanogen bromide activated agarose matrix [6] and on Sepharose beads [7,8] as well as by entrapment into alginate beads in the presence of polyethyleneimine [9]. These immobilizations produced viable enzymes for *in vitro* studies. However, these supports could not be used in an on-line HPLC format.

An HPLC compatible immobilized enzyme reactor {IMER} containing UDPGT has also been reported [10]. In this study, nonsolubilized rat liver microsomes were noncovalently immobilized on an immobilized artificial membrane (IAM) HPLC chromatographic support. The resulting UDPGT-IMER was coupled to a second IAM column and used on-line to study the synthesis of the glucuronides of 4-nitrophenol and 4-methylumbelliferone.

This manuscript reports the development of an IMER containing immobilized β-glucuronidase {BG-IMER} and its application in an on-line HPLC system. The activity of the BG-IMER was compared to non-immobilized β-glucuronidase in the deconjugation of a series of glucuronides. In addition, the activity and stability of the BG-IMER was investigated at various pHs and temperatures.

3.0 EXPERIMENTAL

3.1 Materials

Glucuronide substrates and (respective alcohols): (**1**) 4-methylumbelliferyl-β-D-

glucuronide (methylumbelliferone);(**2**) p-acetaminophen-β-D-glucuronide (acetaminophen); (**3**) 3'-azido-3'-deoxythymidine-β-D-glucuronide (Zidovudine); (**4**) phenyl-β-D-glucuronide (phenol);(**5**) chloramphenicol-β-D-glucuronide (chloramphenicol); (**6**) estradiol-17-β-D-glucuronide (estradiol); (**7**) morphine-3-β-D-glucuronide (morphine). Compounds **1-6** were purchased from Sigma (Oakville, ON., Canada) while compound **7** was purchased from RBI (Natick, MA USA). The structures of these compounds are presented in Figure 1.

Chromatographic equipment: A Spectra-Physics HPLC system purchased from Thermo Separation Products (San Jose, CA, USA) was used for this project. The system consisted of: a UV Spectra 100 variable wavelength detector and a Chromjet integrator linked up to a computer integration system; a single solvent (p1000) and a binary gradient (p2000) pump. A Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 100 ul loop was used for injection. A second Rheodyne injector (7125) and a Rheodyne switching valve (7010) were used for on-line connection between 2 systems to be run on-line and independently from each other. Column temperature was controlled with a Flatron Systems TC-50 column temperature controller (ASTEC, Whippany, NJ, USA.).

β-glucuronidase column: The β-glucuronidase column was synthesized according to previously published procedures [11]. A 200 A°, 5 µm epoxide silica support packed in a 4.6 mm x 50 mm stainless steel column was used in the

synthesis (Kromasil 200, Eka Chemical, AB, Bohus Sweden). The β-glucuronidase immobilized on the support was isolated from *Escherichia coli* K12, Rnase negative and was purchased from Boehringer Mannheim (Laval, Canada). The enzyme had a specific activity of 10 U/ml at 25°C (20 U/ml at 37°C) with 4-nitro-phenyl-β-D-glucuronide as substrate (pH 7.0) and 910 U were immobilized on the stationary phase. When not in use, the column was stored at room temperature in a solution of ammonium sulphate [1.0 M]: sodium azide [0.01 M]: potassium phosphate buffer [0.05 M, pH 7.0] (v/v/v).

Reversed-phase columns: Octylsilane (C_8) and an octadecylsilane (C_{18}) stationary phases were used for the on-line experiments. Both columns were 250 mm x 4.6 mm i.d., stainless steel columns packed with 100 A° pore size and 5μm particle size silica purchased from Regis (Morton Grove, IL, USA). Column filters were also purchased from Regis.

Mobile phases : Mobile phase A: Consisted of HPLC grade acetonitrile (Fisher Scientific, Ottawa, ON, Canada). The solvent was filtered and degassed under nitrogen for 15 min. *Mobile phase B:* consisted of ammonium acetate buffer [0.01 M, pH 5.0]. The buffer was made by dissolving 0.8 g HPLC grade ammonium acetate (Fisher Scientific) per L of Millipore water (Millipore, Bedford, MA, USA) and the pH was adjusted with acetic acid. The solution was filtered through a Millipore filtering apparatus using a 47 mm, 0.2 μm nylon filter purchased from WSC Inc. (Ottawa, Canada) and degassed under nitrogen for

30 min.

Coupled HPLC system: A schematic diagram of the coupled HPLC system is presented in Figure 2. The following experimental steps were followed:

1) Preparation of the system for on-line injection : Stop pump 2; place inj.IB to inject position; switch SV to B position; start pump 1 with mobile phase B through the on-line system at a flow rate of 0.1 ml/min; place inj.IA into load position to inject an aliquot of the substrate.

2) Injection of the substrate: 100 ul of the substrate was loaded into inj.IA and the valve switched to the inject position at a flow rate of 0.1 ml/min for 30 min; after 30 min the SV valve was switched back to position A (flow of 0.3 ml/min), inj.IB was changed to the load position and pump 2 was turned back on at the initial flow rate of 0.3 ml/min and mobile phase B. The analytes were concentrated on the reversed-phase column bed. The conjugated and deconjugated peaks were then separated by a HPLC gradient system.

3) Analysis of the substrate: Substrates were analyzed using either the C_{18} or C_8 stationary phases, at ambient temperature or 37°C. Mobile phases A and B were used at different gradients for each substrate. The samples were detected at UV wavelengths of 280 nm (substrate **1**, **5, 7**); 254 nm (**2, 4**) ;270 nm (**3**); and 230 nm (**6**).

β-Glucuronidase solution: The β-glucuronidase was isolated from *Escherichia coli* K12, Rnase negative (Boehringer Mannheim) and was purchased as a

solution with a specific activity of 10 U/ml at 25°C (20 U/ml at 37°C) using 4-nitrophenyl-β-D-glucuronide as substrate (pH 7.0). The strength of the solution in units of protein/ml of suspension is at least 200 units of enzyme protein/ml (suspension) at 37°C and 100 units of enzyme protein/ml at room temperature.

3.2 Methods

Validation of the activity of the column: Stock solutions of the glucuronide substrates were prepared by dissolving the glucuronides in ammonium acetate buffer [0.01 M, pH 5.0]. Depending on the solubility of the glucuronide, different concentrations of the substrates were achieved (range 0.003 to 2.0 mM). The solutions were injected onto the BG-IMER and the mobile phase B was passed through the BG-IMER for 30 min at a flow rate of 0.1 ml/min. The eluent from the enzyme column was passed directly on to a reversed-phase analytical column. Blanks were used as negative controls and respective alcohols of the glucuronide substrates as positive controls for retention time verification. The controls were injected on-line through the enzyme column and off-line directly onto the analytical column.

Comparison of the activity of free and immobilized enzymes: Concentrations of the substrates which were proportional to the substrate concentration injected into the BG-IMER were spiked into 250 ul of the enzyme solutions. The quantity

of non-immobilized enzyme was 910 U which was equal to the units of enzyme immobilized on the BG-IMER. Incubations were carried out for 30 min at room temperature. The reaction solutions were then centrifuged for 10 min at 12,000 xg and 100 ul of the supernatant was immediately injected into HPLC for analysis or stored at -80 °C until analyzed. HPLC gradients were modified from the HPLC gradients performed on immobilized enzyme column to separate the endogenous protein peaks extracted from the free enzyme and peaks from the substrates. Blanks and alcohols (respective to their glucuronide metabolites) were used as negative and positive controls.

Effect of flow rate on enzyme activity: The effect of flow rate through the BG-IMER column was undertaken using p-acetaminophen-β-D-glucuronide (APAP-g), and 3'-azido-3'-deoxythymidine-β-D-glucuronide (AZT-g) at concentrations of 0.17 mM and 0.04 mM, respectively. After injection onto the BG-IMER, one column volume (1.5 ml) was passed through the BG-IMER and onto the analytical column. The enzyme column was used at room temperature at flow rates of from 0.1 to 1.0 ml/min at 0.1 ml/min increments producing substrate/enzyme contact times of from 15 to 1.5 min.

Effect of pH on the activity of the BG-IMER: The effect of pH on the activity of the BG-IMER was investigated using 0.01 M ammonium acetate buffers at pH

4.0, 5.0 and 6.0. APAP-g [1.0 mM] and AZT-g [0.04 mM] were the test substrates.

Effect of temperature on the activity of the BG-IMER: A partially pH deactivated BG-IMER was used at ambient temperature and 37°C to determine the effect of temperature on the hydrolytic activity of the BG-IMER. APAP-g was used as the substrate at concentration of 0.27 mM.

4.0 RESULTS AND DISCUSSION

In the coupled LC system, the glucuronide substrates were injected onto the BG-IMER in an ammonium acetate buffer and the resulting substrate/product mixture transferred to a reversed-phase analytical column. The solutes from the BG-IMER were concentrated at the head of the analytical column and then analyzed using ammonium acetate: acetonitrile gradients. Representative chromatograms obtained from the chromatography of AZT-g on the coupled BG-IMER/analytical column system are presented in Figure 3. The BG-IMER column was used for 8 weeks (0.01 M ammonium acetate pH 5.0) without significant changes in the activity.

The enzymatic activity of the BG-IMER was evaluated using 7 glucuronides. The results from this experiment are presented in Table I and demonstrate that at an average substrate concentration of 2.00 mM, >98%

hydrolysis was achieved for 5 of the 7 glucuronide substrates, while with AZT-g and morphine-g the extent of hydrolysis reached only 20% and 11%, respectively.

When the concentration of AZT-g was decreased from 2.00 mM to 0.04 mM and then to 0.02 mM, the extent of hydrolysis increased from 20% to 76-89% to 98%, Table I. This suggests that the low level of hydrolysis of ATZ-g observed at the higher concentration was due to saturation of the glucuronidase isoforms metabolizing AZT-g. The opposite results were obtained for morphine-g, where a decrease in substrate concentration from 2.00 mM to 0.04 mM and then to 0.02 mM resulted in a decrease in the extent of hydrolysis from 11% to <2% to no observable hydrolysis, Table I. This suggests that low-affinity glucuronidase isoforms are responsible for the hydrolysis of morphine-g. This is consistent with the observation that morphine-g is relatively stable in the intestine of the dog [12].

Comparison of the activity of the free and the immobilized enzyme

The enzymatic activity of the BG-IMER was compared to that of an equivalent amount of free β-glucuronidase using the 7 glucuronide substrates. All of the glucuronides were hydrolyzed >98% by the non-immobilized enzyme, Table 1. The hydrolytic activities of the immobilized and non-immobilized enzymes were equivalent except with respect to the hydrolysis of AZT-g and

morphine-g. The β-glucuronidase immobilized on the support and used in the enzyme incubations was isolated from *Escherichia coli* K12 and as such is a mixture of a number of β-glucuronidase isoforms as well as other enzymes. The results suggest that the immobilization process may be isoform selective. Thus, the glucuronidase subtypes primarily responsible for the hydrolysis of AZT-g and morphine-g are found in reduced concentrations on the BG-IMER relative to the other glucuronidase isoforms. A second possibility is that the immobilization process sterically hinders the active site of the AZT-g and morphine-g glucuronidase isoforms thereby reducing the observed activity. Both of these possibilities will be investigated through a further study of the structure-activity relationships on the BG-IMER and by the immobilization of single isoforms of UDPG-glucuronosyltransferase and β-glucuronidase, if the latter are available.

Effect of flow rate on the extent of substrate hydrolysis

The effect of the flow rate through the BG-IMER on the extent of hydrolysis was investigated using p-acetaminophen-β-D-glucuronide (APAP-g) and AZT-g at concentrations of 0.18 mM and 0.04 mM, respectively. These substrates were chosen to differentiate between a substrate which appeared to be extensively hydrolyzed under all experimental conditions (APAP-g) and one whose hydrolysis was shown to be problematic (AZT-g), Table 1. The lower concentration of AZT-g was chosen because the previous experiments had

demonstrated a >98% conversion at this concentration.

The flow rates ranged from 0.1 ml/min to 1.0 ml/min and the experiment was carried out through one column volume. Thus, the substrate was in contact with the immobilized enzyme on the BG-IMER from 15 min (0.1 ml/min) to 1.5 min (1.0 ml/min). The hydrolysis of APAP-g remained constant (>98%) throughout the range of flow rates, Figure 4. The deconjugation of AZT-g remained >98% up to a flow rate of 0.4 ml/ml and then began to decrease to 70% at a flow rate of 1.0 ml/min. The results indicate that for glucuronides which are easily hydrolyzed on the BG-IMER, flow rates of 1.0 ml/min or more can be used in the development of optimized analytical methods. For more difficult substrates such as AZT-g, lower flow rates may be necessary to achieve quantitative conversions.

Effect of pH on BG-IMER activity

The optimum enzymatic activity of β-glucuronidases occurs between pH 5.0 and 7.5 [13]. Thus, the activity of the BG-IMER relative to the hydrolysis of APAP-g was determined at pH 4.0, 5.0 and 6.0. Optimum activity was observed at pH 5.0 with a >98% deconjugation of APAP-g. Lower levels of conversion were observed for pH 6.0 and pH 4.0, 92% and 46%, respectively.

The exposure to pH 4.0 resulted in a reduction in the enzymatic activity of the BG-IMER. For one of the BG-IMERs tested, the conversion of APAP-g

>98% at pH 5.0 before exposure to the pH 4.0 buffer and only 20% after exposure to the lower pH. Similarly, at pH 5.0, a 90% hydrolysis of AZT-g was observed before exposure to pH 4.0 and only a 12% conversion after. The effect of exposing the BG-IMER to pH 4.0 was irreversible.

Effect of temperature on BG-IMER activity

APAP-g was passed through a BG-IMER which had been previously exposed to a pH 4.0 buffer. The experiment was carried out at ambient temperature and with a pH 5.0 buffer. Under similar chromatographic conditions, >98% of the APAP-g had been hydrolyzed. However, after exposure to the pH 4.0 buffer, the extent of conversion had dropped to 30%. When the temperature of the BG-IMER was raised to 37°C, a >98% hydrolysis of the APAP-g was again obtained. The results indicate that temperature can be used to increase the enzymatic activity of the BG-IMER.

5.0 CONCLUSION

An on-line HPLC method has been developed for the deconjugation of glucuronides. The activity and stability of the BG-IMER was tested through various assays using different substrates. The results of these tests demonstrates that the BG-IMER has equivalent enzymatic activity for most substrates when compared to free β-glucuronidase solution. Its use is however

much easier in the deconjugation of glucuronides. We are currently studying the application of this β-glucuronidase column for biological samples.

6.0 ACKNOWLEDGMENTS

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7.0 REFERENCES

[1] I. Shollhammer, D.S. Poll and M.H.Bickel, Enzyme, 20 (1975) 269.

[2] G.A.Levvy and J.Chonchie, in G.J.Dutton (Editor), Glucuronic Acid, Academic Press, New York, 1966, 301.

[3] G.J. Mulder, M.W.H. Coughtrie and B.Burchell, in G.J. Mulder (Editor), Glucuronidation, Conjugation Reactions in Drug Metabolism, Taylor and Francis, New York,1990.

[4] R.L. Smith and R.T.William, in G.J.Dutton (Editor), Glucuronic Acid, Academic Press, New York, 1966, 457.

[5] R. Kalizan and I.W.Wainer, J.Pharm. Pharmacol., 45 (1993) 367.

[6] I.W.Wainer and X.Zhang, Tetrahedron Lett., 34, 30 (1993) 4731.

[7] I.W,Wainer and W.Chui, Anal. Biochem., 201 (1992) 237.

[8] R. Gillissen, J.H.N. Meerman and G.J.Mulder, Biochem. Pharmacol., 43

(1992) 2661.

[9] I. Parikh, D.W. MacGlashan and C.Fenselau, J.Med.Chem.,19 (1976) 296.

[10] I.W. Wainer and T. Alebic-Kolbah, Chromatographia, 37 (1993) 608.

[11] E. Domenici, C.Bertucci, P.Salvadori, G.Felix, I.Cahagni, S.Motellier and I.W. Wainer, Chromatographia, 29 (1990) 170.

[12] K.J.Ho, L.C.Ho and O.R.Kruger, J.Lab.Clin.Med.,June (1970) 916.

[13] R. A. Jefferson, S.M. Burgess and D. Hirsh, Proc. Natl. Acad. Sci. USA., 83 (1986) 8447.

8.0 Legend of Figures

Figure 1 : Structures of the glucuronides used in this study

Figure 2: Diagram of the On-line HPLC Injection System

Figure 3 : Chromatograms representing the deconjugation of AZT-g in immobilized enzyme; (a) Blank (mobile phase B); (b) AZT-g deconjugated at 0.04 mM; (c) AZT-g deconjugated at 0.4 mM; (d) Stock Zidovudine (AZT)

Figure 4: A graph representing the results from flow rate change on substrate (AZT-g) /enzyme contact (0.1 -1.0 ml/min)

Table I: Results on validation of the activity of the enzyme: comparing deconjugation of substrates on immobilized and non-immobilized enzyme

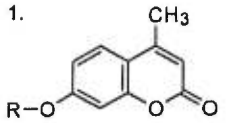
% Hyd. = % Hydrolysis
> 98 % refers to 100 % deconjugation with < 2.0 % error
N/A = Not available (substrate precipitated at 1.2-2mM and therefore could only be injected at 0.5 mM)

Concentrations		2.0-1.2 mM	0.07-0.04 mM	0.02 mM
		% Hyd.	% Hyd.	% Hyd.
Substrate	enzyme type			
1	Immobilized	98	>98	>98
	non-immobilized	>98	>98	>98
2	Immobilized	>98	>98	>98
	non-immobilized	>98	>98	>98
3	Immobilized	20	76-89	98
	non-immobilized	>98	>98	>98
4	Immobilized	>98	>98	>98
	non-immobilized	>98	>98	>98
5	Immobilized	>98	>98	>98
	non-immobilized	>98	>98	>98
6	Immobilized	N/A	>98	>98
	non-immobilized	>98	>98	>98
7	Immobilized	11	< 2	0 %
	non-immobilized	>98	>98	>98

Refer to "Experimental/Materials"and Figure 1 for references to substrate numbers

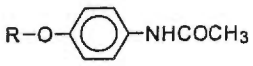
Figure 1

1.



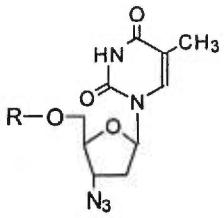
Methylumbelliferone

2.



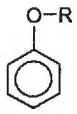
Acetaminophen

3.



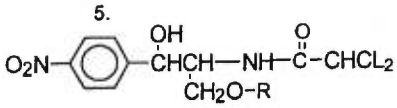
AZT

4.



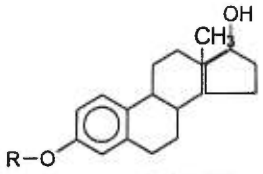
Phenol

5.



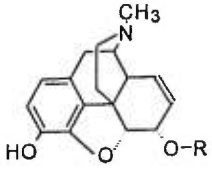
Chloramphenicol

6.

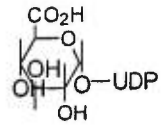


Estradiol

7.

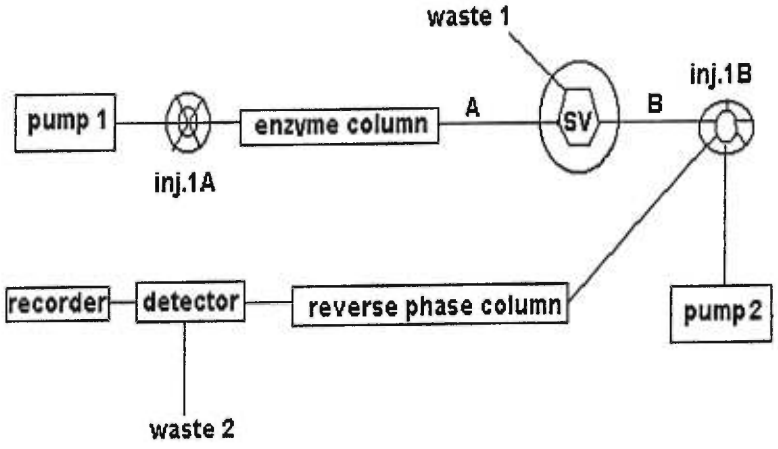


Morphine



R= UDP-glucuronic Acid

Figure 2



1A: Rheodyne injector A
1B: Rheodyne injector B
SV: Switching valve

Figure 3.

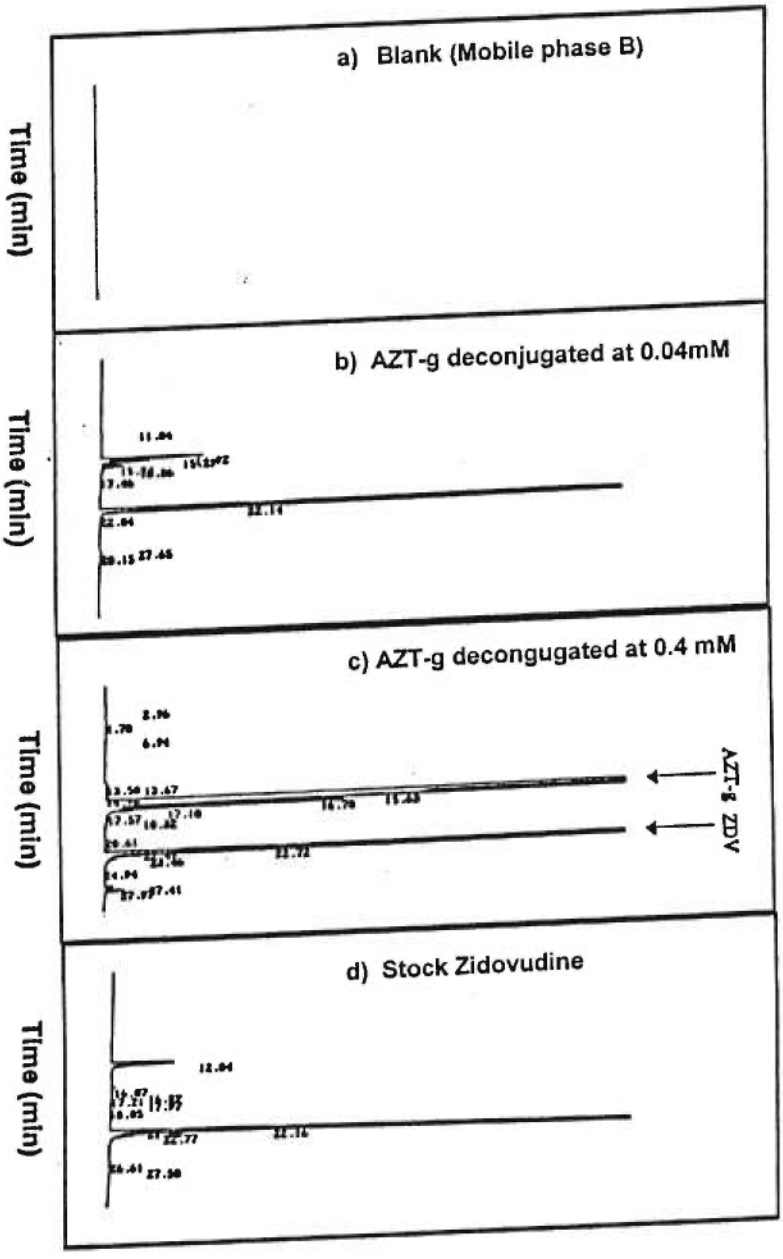
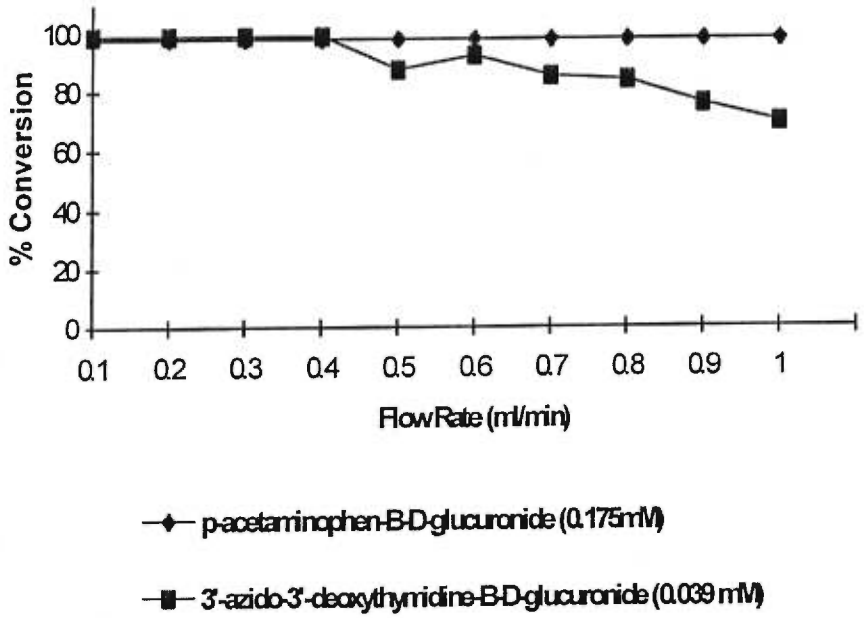

a) Blank (Mobile phase B)
Time (min)
b) AZT-g deconjugated at 0.04mM
Time (min)
c) AZT-g decongugated at 0.4 mM
Time (min)
AZT-g
ZDV
d) Stock Zidovudine
Time (min)

Figure 4


% Conversion
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Flow Rate (ml/min)
p-acetaminophen-B-D-glucuronide (0.175mM)
3'-azido-3'-deoxythymidine-B-D-glucuronide (0.039 mM)

CHAPTER III
DECONJUGATION OF GLUCURONIDE SUBSTRATES ON AN IMMOBILIZED ENZYME COLUMN
(Supplementary results to Chapter II)

CHAPTER III

DECONJUGATION OF GLUCURONIDE SUBSTRATES ON AN IMMOBILIZED ENZYME COLUMN (Supplementary results to Chapter II)

1.0 CONSEQUENCES OF IMMOBILIZATION ON THE ACTIVITY OF THE ENZYME

1.1 Comparison of the activity of the free and the immobilized enzyme

1.1.1 Objective

To compare the enzyme (immobilized) column to the free (non-immobilzed) enzyme solution.

1.1.2 Procedure

The maximum concentration of 2.0 mM (stock solutions dissolved in 0.01 M ammonium acetate pH 5.0 buffer solutions) from each of the 7 substrates (reflecting in proportion to the same concentration of substrates injected into the immobilized enzyme column) were spiked into 250 ul non-immobilized β-glucuronidase enzyme suspensions purchased from Boerhinger (Non-immobilized enzyme equal to approximately the same units of enzyme immobilized on the column). Incubation was at room temperature for 30 minutes. The substrate/enzyme suspensions were then centrifuged for 10

minutes at 11,818 xg and 100 ul of the supernatant was immediately injected into HPLC for analysis or placed at -80°C until used. HPLC gradients were modified from the HPLC gradients performed on immobilized enzyme column to separate the endogenous protein peaks extracted from the free enzyme and peaks from the substrates (refer to Appendices Section I for analytical HPLC methods). Blanks and alcohol compounds (respective to their glucuronide metabolites) were used as negative and positive controls. The immobilized β-glucuronidase column was used at room temperature. Substrates were injected on-line (on-line procedure as described in Chapter II "Experimental section,coupled HPLC system"). After injection of the substrate, the mobile phase was passed through the enzyme column for 30 minutes at a flow rate of 0.1 ml/min. The eluent from the enzyme column was passed directly onto a reverse phase analytical column. Controls standards were injected off-line without the enzyme column.

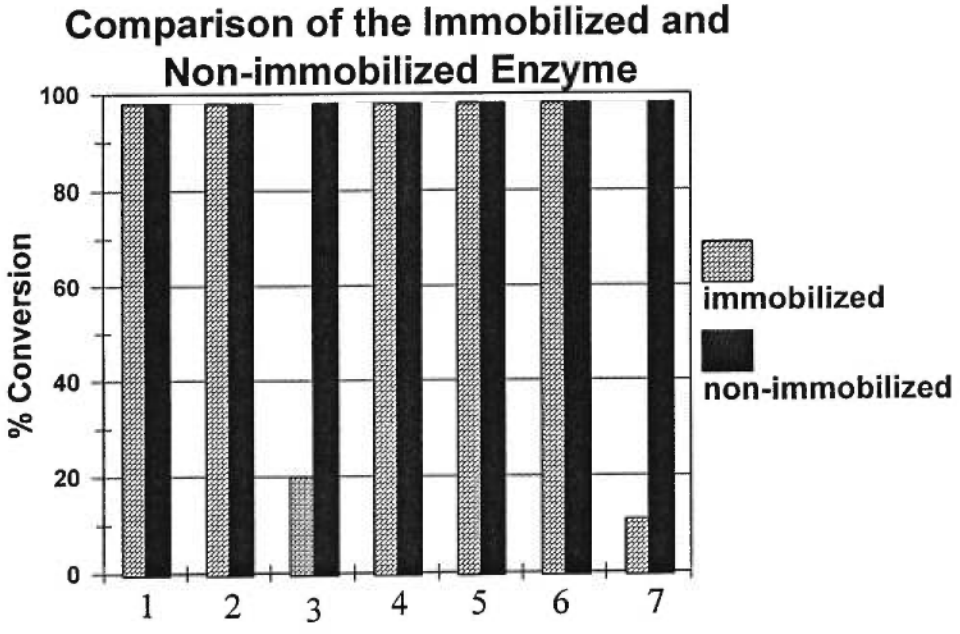
1.1.3 Results

HPLC chromatograms from the substrates and free enzyme suspensions (E.coli) purchased from Boerhinger were found to contain many endogenous peaks. Attempts to clean the suspensions with various precipitating solvents (acetonitrile,1N HCL etc.) were not successful. Centrifugation was the method chosen to precipitate the proteins in the suspensions and produce fairly resolved chromatograms.

Results of this study are presented in Figure 1. Substrates tested resulted

with a >98% conversion when spiked into the β-glucuronidase enzyme suspensions. While the results obtained from injecting the 7 substrates directly into the immobilized column were the following: out of the 7 glucuronides substrates tested, at an average concentration of 2.0 mM, (estradiol-17-β-D-glucuronide was injected at 0.5 mM because of precipitation problems at 2.0 mM), 5 glucuronide substrates resulted with >98% hydrolysis while 3'-azido-3'-deoxythymidine-β-D-glucuronide hydrolyzed 20.0 % and morphine-3-β-D-glucuronide 10.9%.

Figure 1. Consequences on the activity of the enzyme immobilized in the column and non-immobilized in solution.



Substrate concentration at @ 2.0 mM

(immobilized enzyme, substrate 6 injected at 0.5 mM)

1. 4-methylumbelliferyl-β-D-glucuronide
2. p-acetaminophen-β-D-glucuronide
3. 3'-azido-3'-deoxythymidine-β-D-glucuronide
4. phenyl-β-D-glucuronide
5. chloramphenicol-β-D-glucuronide
6. estradiol-17-β-D-glucuronide
7. morphine-3- β-D-glucuronide

2.0 ENZYME KINETICS

2.1 Flow Rate and contact time of substrates on-line through the β-glucuronidase column

2.1.1 Objective

A time course study was performed at various flow rates on-line through the β-glucuronidase column. The objective of this experiment was to study the effect of contact time of substrates on the enzyme in the column at flow rates equivalent to one column volume.

2.1.2 Procedure

Two substrates were chosen for this study, p-acetaminophen-β-D-glucuronide, and 3'-azido-3'-deoxythymidine-β-D-glucuronide .at an average concentration of 0.17 mM. Flow rate and contact time were equivalent to one column volume (1.5 ml). That is, 100 ul of the substrates (dissolved in 0.01M

ammonium acetate pH 5.0 buffer) were injected at a flow rate of 0.3 ml/min for 5 minute contact time, while at 0.05 ml/min the substrate was in contact with the enzyme in the column for 30 minutes (equivalent to 1.5 ml column volume). The following flow rates vs contact times were performed :

Flow Rate (ml/min)	Contact time (min)
0.3	5
0.2	7.5
0.1	15
0.05	30

2.1.3 Results

The results of this study are presented in Table I.

Results obtained using p-acetaminophen-β-D-glucuronide and 3'-azido-3'-deoxythymidine-β-D-glucuronide as substrates, showed no significant difference on the extent of reaction to various flow rates at a column volume of 1.5 ml.

Table I. Flow rate and contact time of substrates on-line through the β-glucuronidase column.

p-acetaminophen-β-D-glucuronide (0.175 mM)

Flow Rate (ml/min)	Time (min)	% Conversion
0.3	5	>98
0.2	7.5	>98
0.1	15	>98
0.05	30	>98

3'-azido-3'-deoxythymidine-β-D-glucuronide (0.039 mM)

Flow Rate (ml/min)	Time (min)	% Conversion
0.3	5	98
0.2	7.5	98
0.1	15	98
0.05	30	98

2.2 *Duration of Substrate Elution on Substrate Deconjugation*
(Effect of substrate elution on the β-glucuronidase column)

2.2.1 Part A

2.2.1 A Objective

A flow rate time course study of p-acetaminophen-β-D-glucuronide and 3'-azido-3'-deoxythymidine-β-D-glucuronide was performed on-line through the enzyme column to observe the effect of substrate elution on the β-glucuronidase column. In other words, to observe if after a certain time (5,10 or 15 minutes) any of the major peaks (conjugated or deconjugated peaks) have bonded to the enzymes in the column.

2.2.1 B Procedure:

A concentration of substrate was injected online at 2 different flow rates and stopped at various time points :

- 0.3ml/minute --- injected online at 5,10, 20 and 30 minute
- 0.1ml/minute --- injected online at 15 and 30 minutes

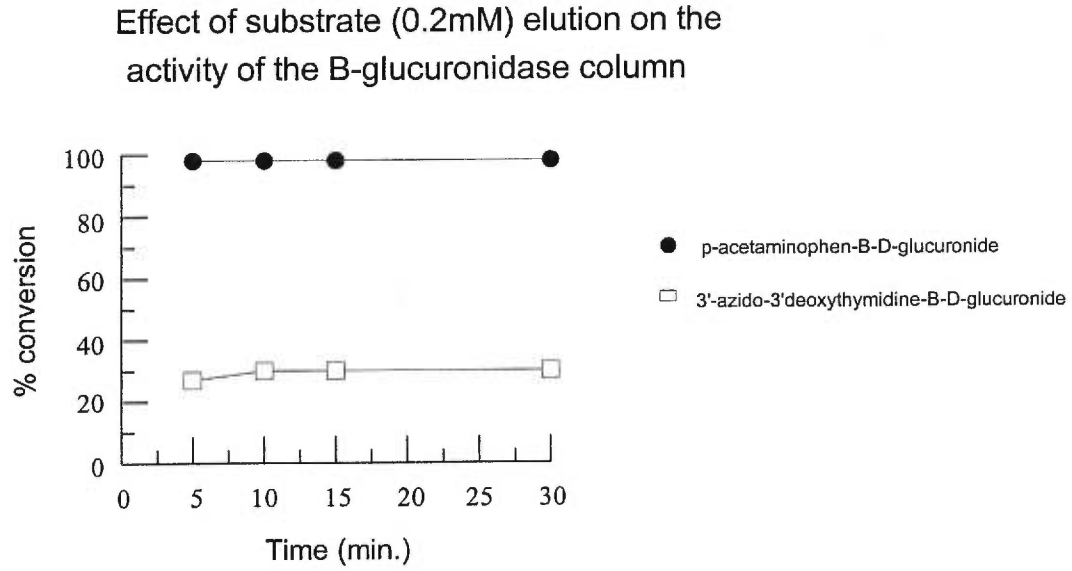
After each injection time point the valve was switched and the deconjugation was monitored on the reversed phase column.

2.2.1C Results

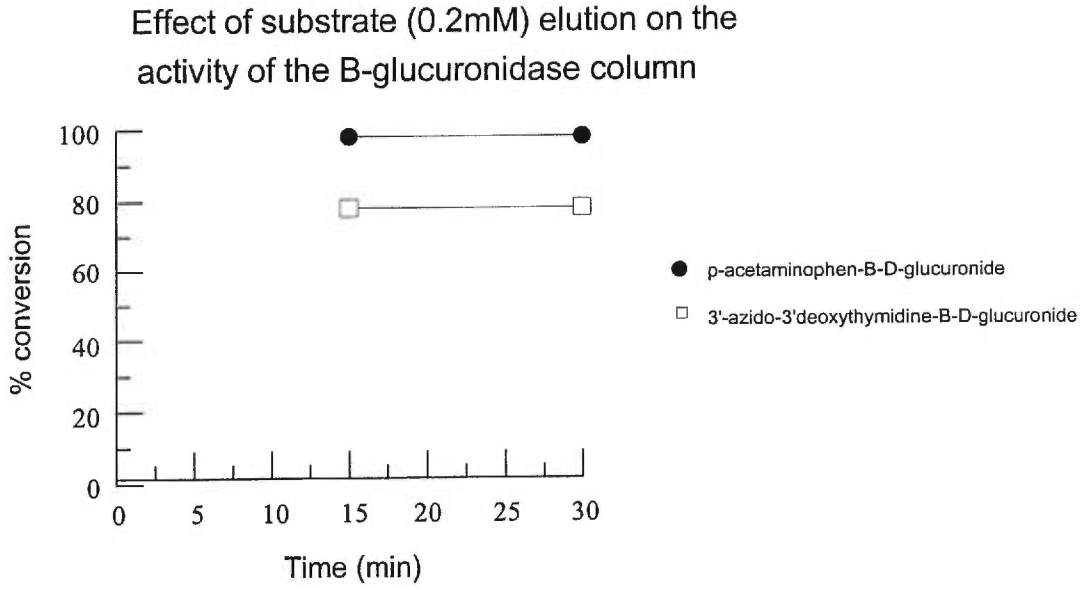
The results from this experiment are presented in Figure 2. Results show that the deconjugation (% conversion) of p-acetaminophen-β-D-glucuronide at flow rates of 0.1 or 0.3 ml/min (switched online at various time points) remained the same. The area of the alcohol peaks in the chromatograms remained consistent throughout the changes, demonstrating that no bonding of the conjugated or the deconjugated compound to the enzyme in the column (switched on-line at various time points) had occurred. The next substrate tested, 3'-azido-3'-deoxythymidine-β-D-glucuronide showed an improved hydrolysis at 0.1 ml/min (75% on-line switching at 15 or 30 minutes) than at 0.3 ml/min (33% at 10 to 30 minutes and 26% at 5 minutes). Regardless at what time point the on-line system was switched, peak areas were constant throughout the injections.

Figure 2. Effect of substrate (0.2mM) elution at flow rates of 0.1 and 0.3 ml/min on the activity of the B-glucuronidase enzyme column

a) At flow rate of 0.3 ml/min



b) At flow rate of 0.1 ml/min



2.2.2 Part B

2.2.2 B Objective

A flow rate time course study of 4-methylumbelliferyl-β-D-glucuronide was performed to compare the percent of deconjugation at two flow rates, 0.1 and 0.7 ml/min.

2.2.2 B Procedure

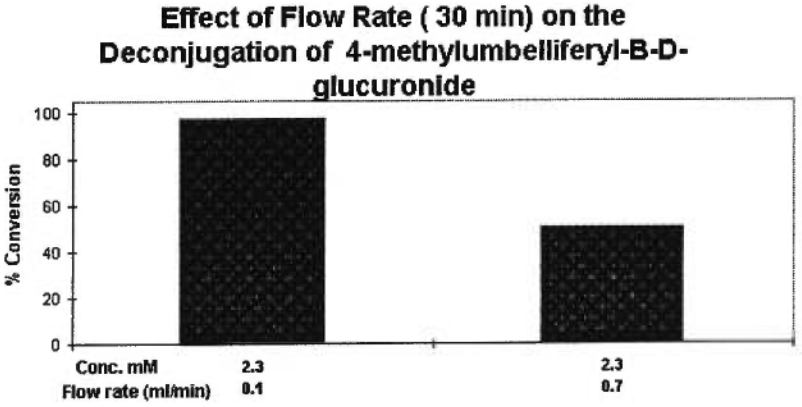
A concentration of 2.3 mM of the substrate was injected on-line into the β-glucuronidase column at flow rates of 0.1 ml/min and 0.7 ml/min for 30 minutes.

2.2.2 C Results

Results are presented in Figure 3. These results demonstrate that changing the flow rate from 0.1 to 0.7 ml/min decreased the hydrolysis of the substrate, 4-methylumbelliferyl-β-D-glucuronide by 50%.

Effect of substrate elution on the β-glucuronidase column demonstrates that increasing the flow rate for some substrates has an effect on the % conversion due to the amount of contact time of substrate and enzyme immobilized on the column.

Figure 3. Effect of flow rate change (0.1 and 0.7 ml/min) by the β-glucuronidase column



3.0 Effect of pH Change on the Activity of the Column

3.1 Objective

To study the effects of pH on the deconjugation of glucuronide substrates by the β-glucuronidase column.

3.2 *Procedure*

The β-glucuronidase column was studied at room temperature using 0.01 M ammonium actetate buffer at pH 5.0, 6.0 and 4.0. The substrates chosen for this experiment were p-acetaminophen-β-D-glucuronide (APAP-g concentration of 1.0 mM) and 3'-azido-3'-deoxythymidine-β-D-glucuronide (AZT-g concentration of 0.04 mM). The same concentrations of substrates were injected throughout the three pH changes.

3.3 *Results*

Results of this experiment are presented in Figure 4 and Figure 5.

In Figure 4, the observed optimum activity of the β-glucuronidase enzymes in the column was at pH 5.0. In Figure 5, the effect of exposure to pH 4.0 on the activity of the column before and after the column was tested at pH 5.0 was demonstrated. These results show that there is a substantial decrease in hydrolysis for both substrates. APAP-g, % deconjugation decreased from 98% to 20% and AZT-g deconjugation decreased from 90% to 12% after exposing the column to a pH of 4.0.

The effect of exposing the β-glucuronidase column to pH 4.0 caused an irreversible decrease in enzymatic activity even after washing the column with the column storage solution (refer to column storage solution Chapter II "Experimental" section β-glucuronidase solution).

Figure 4. **Effect of pH change (pH 4, 5, 6) on the activity of the β-glucuronidase column**

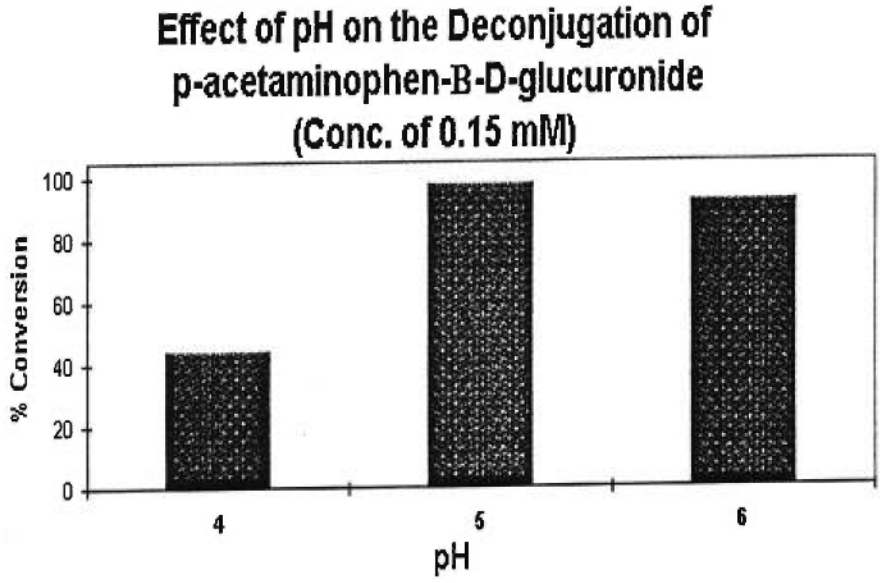
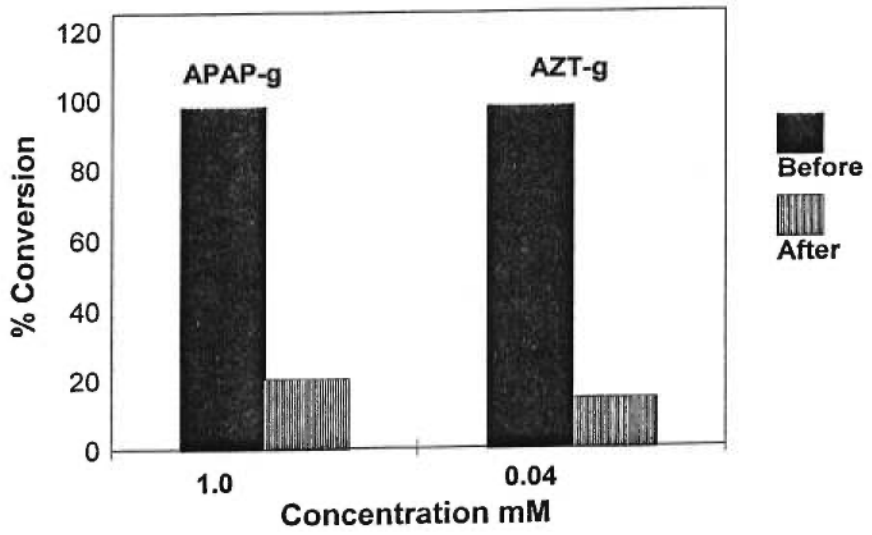


Figure 5. Effect of exposing the β-glucuronidase column to pH 4.0



4.0 Effect of Temperature Change on the Activity of the β-glucuronidase column

4.1 Objective

To study the effects of temperature (r.t., 37°C) on the deconjugation of the glucuronide substrates by a partially deactivated β-glucuronidase column (after experimenting with a change in pH).

4.2 *Procedure*

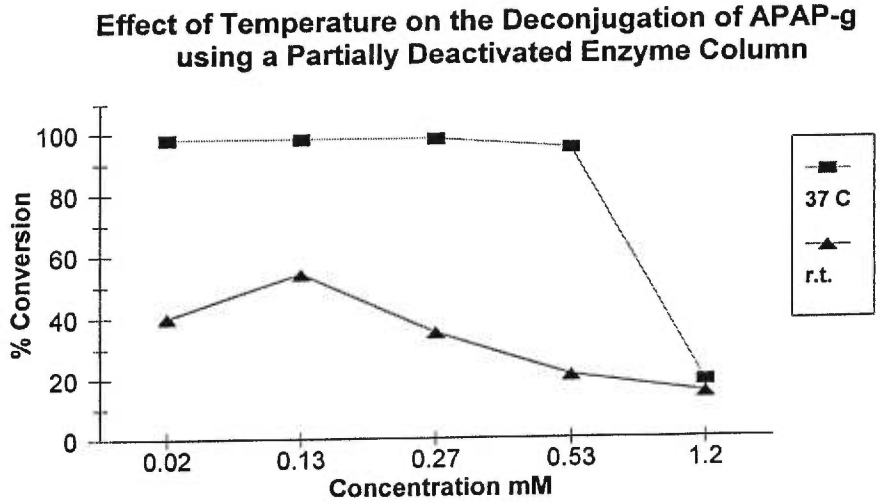
APAP-g was the substrate chosen for this experiment. The β-glucuronidase column was partially deactivated because it was easier to observe if the activity of the column increased by placing the column at 37°C from the decreased results obtained at room temperature. The concentrations of APAP-g that were injected into the column were identical at both temperatures.

4.3 *Results*

Results of this experiment are presented in Figure 6.

Results demonstrate that at room temperature (r.t.), the hydrolysis of APAP-g decreased from 54% to 15% when the concentration of APAP-g was greater than 0.13 mM (to 1.2 mM). At 37°C, the hydrolysis of APAP-g was greater than 98% for concentrations of substrates between 0.02 mM to 0.5 mM. The percent of hydrolysis rapidly decreased to 19% when a concentration of 1.2 mM was injected on-line through the immobilized enzyme column.

Figure 6. **Effect of temperature change (r.t. and 37°C) on the activity of the β-glucuronidase enzyme column**



GENERAL DISCUSSION

GENERAL DISCUSSION

An on-line HPLC method utilizing an immobilized β-glucuronidase column was developed for the deconjugation of glucuronides. This novel method for substrate deconjugation is considerably faster than using the β-glucuronidase solution because it eliminates the multi-step preparation of the samples that is necessary before injecting the sample to a chromatographic system. For instance, a typical glucuronide substrate and enzyme reaction in solution may involve the following steps:

1. Developing and validating an *in vitro* assay in order to establish sufficient time for the substrate and enzyme to react in solution. According to various studies, validating enzymatic reactions in solutions involves first ensuring the specific activity of the enzyme with known standard substrates. The specific activity can be measured by using substrates such as biosynthetic phenolphthalein-mono-β-glucuronide (possesses a high affinity for β-glucuronidase enzyme). β-glucuronidase can be determined using the method of Fishman [67] in which specific activity is expressed as nmol of substrate hydrolyzed per min per mg of protein.
2. Selecting incubation temperatures of the reaction mixture must be taken into account in developing the assay method. Studies have shown that the incubation of β-glucuronidase enzyme isolated from various species and tissues have been incubated with glucuronides at various temperatures (from r.t. to 55°C) according to stability studies performed

on the enzyme. For instance, mammalian β-glucuronidase enzymes were found not to be stable at temperatures above 50°C [23] while others reported the enzyme isolated from mouse kidney tissue to be stable at tempertures up to 55°C [22]. The length of incubation of the reaction mixture is also a factor that must be considered in the assay development. β-glucuronidase enzymes have been reported to be incubated with glucuronides from 0.5 to 24 hours depending on the substrates and species of enzymes used [24,68]. It has been demonstrated in *in vitro* assays that when the concentration of substrate in the enzymatic reaction increases so does the amount of time needed for hydrolysis [69].

3. Choosing the appropriate solvents to stop the enzymatic reaction is also a factor in the assay. The inhibitors chosen (see Chapter I "Introduction, "Inhibitors of β-glucuronidases) may sometimes influence the HPLC analysis. Saccharolactone has been found to be unstable [23,70] and unpredictable in enzymatic reactions and often 0.01 % acetic acid or 10 % of 6N HCL are used.

4. Finally, an extraction or purification method for the products in the reaction mixture must also be developed before they can be injected into a HPLC column. This often involves increasing the concentrations of substrate used in the reactions to compensate for low extraction recoveries or higher sensitive HPLC detection methods. Errors are increased when long extraction assays are involved. A HPLC method

to analyze the products of the enzymatic reaction must then be developed.

These assay preparations are eliminated when the BG-IMER column is employed to deconjugate glucuronide substrates. The advantages of using the BG-IMER column are:

1. The solubilized substrates are directly injected into the BG-IMER column.
2. The concentration of substrate injected into the enzyme column may be decreased substantially compared to *in vitro* assays.
3. Inhibitors were never considered in the on-line HPLC procedure because the β-glucuronidase enzymes are covalently immobilized into the silica support packing. The enzyme in the column became active when there was contact with the glucuronide substrate.

One crucial component of this research was the development of an on-line HPLC system in order to deconjugate glucuronides efficiently. This was important to establish and validate before injecting glucuronides on the BG-IMER column. Various factors have influenced the method development of the HPLC system. Firstly, it was important to safeguard the enzyme column from the other column (reversed phase) which one generally employs an organic solvent (acetonitrile) in its gradient system to analyze the products. An HPLC system that would continually equilibrate the BG-IMER column independently from the reversed phase column was needed. This was accomplished by using 2 pumps, a switching valve, and a Rheodyne injector (inj.1B) to separate the two columns

after an on-line injection of a studied sample. The system would then be switched on-line so that the sample containing the glucuronide substrates would pass through the BG-IMER column, then concentrate on the packing bed of the reversed phase column so that the deconjugated substrate be analyzed with a gradient system. The valve would then be switched in order for the solvent to be pumped through the immobilized enzyme column to the waste recipient. The injection valve would be switched so that the deconjugated product would be analyzed using the reversed phase column. The comparative injection of blanks and aglycone substrates (respective deconjugated substrates of each glucuronide used) with and without the BG-IMER column validated the system. Second, HPLC methods were developed on a reverse phase column for each of the substrates used in the study when analyzing the glucuronide substrates and their products obtained from the enzyme column. Samples of glucuronide substrates were prepared in initial buffer solutions (0.01 M ammonium actetate pH 5.0), were filtered, and then were injected into the on-line HPLC system. Thirdly, the pH of the buffer used in the mobile phase had to be considered in the overall development of these HPLC procedures. The optimum pH at which the BG-IMER column is active has to be known precisely to get the most activity from the enzymes in the column. Unfortunately, in developing the method at various pH buffers, the enzyme column became partially deactivated when the ammonium acetate solution of pH 4.0 was used to equilibrate the enzyme column. When the enzyme column was used again with an optimum pH buffer of 5.0, complete deconjugation of substrates were no longer observed. It has

been reported in some studies that poly-D-lysine (25%,a solution of 1mg/ml) may be used to activate the β-glucuronidase enzyme when it was no longer active in assay [30]. For example, a 40% lost in enzyme activity was corrected when 30 ug/ml of poly-D-lysine was included into a reaction mixture [30]. However, it was also reported that although enzyme stored in an acetate buffer (pH 5.0) lost activity rapidly, it could not be reactivated by adding polylysine solution to the mixture [30]. This may serve as a problem for the BG-IMER column since our study involves using ammonium acetate (0.01M at pH 5.0) as our mobile phase to be pumped through the β-glucuronidase column. Leaving the column in this buffer may,therfore, deactivate the enzyme over a certain period of time. Finally, since the immobilized enzyme column was to be used on a HPLC system with various glucuronide substrates, it was important to check the activity of the BG-IMER column at different pH solutions in the method development of the HPLC procedure. It was especially important to check its activity at lower pH buffer solutions because some substrates require lowering the pH of the buffers in order to acquire better peak resolutions and separations on HPLC analytical systems.

The BG-IMER column is stored in a cocktail solution of ammonium sulphate, sodium azide and potassium phosphate buffer of pH 7.0. Saturated ammonium sulphate is often used to precipitate β-glucuronidase enzymes from the tissue medium, and maintain enzyme activity in suspension. The concentration of ammonium used in these storage solution is usually 1.0 M.

Unfortunately, using this extremely high quantity of salt may cause problems on pump seals in the HPLC system if the seals are not washed thoroughly with water after using the storage solution. Glycerol (50%) has been used in many studies for long term storage without loss of activity of the enzyme in suspension [20].The storage solution may then be modified by reducing the quantity of ammonium sulphate and adding glycerol to the solution. Sodium azide was used in our solution to prevent bacterial growth in the aqueous buffer solution [28].

The BG-IMER was successful in deconjugating 72% of the substrates used in this study. Unfortunately it could not deconjugate all of them. At high concentrations of AZT-g, only 20% deconjugation was observed. When the concentration of AZT-g was decreased to 0.02 mM, the percent of hydrolysis of the conjugate increased to 98%. The opposite results were obtained when morphine-g was injected into the BG-IMER column. At higher concentrations, only 11 % of the substrate deconjugated while at 0.02 mM no deconjugation was observed. In comparison, more than 98% deconjugation was observed using *in vitro* assays with the same substrate concentrations and units of β-glucuronidase (E. coli) enzymes. This puzzling observation gives rise to many questions. Firstly, if the type of enzymes immobilized into the column are the same ones which are used in the *in vitro* assay (same time of enzyme/substrate contact, temperature, units of enzyme, concentrations of glucuronide substrates) why did the results differ for these two glucuronide substrates tested ? Several theories have been proposed and one of the theories is that the low level of hydrolysis observed of AZT-g at higher concentrations is due to the saturation

of the activity of the β-glucuronidase isoforms metabolizing AZT-g. Another theory is that enzymatic solutions may contain impurities from a large number of competing bacterial enzymes or strain differences in response to specific substrates [71]. This is very unlikely, as the solution of enzymes used for the immobilization on the column is the same one as the one used in the comparative *in vitro* assays. Mixtures of β-glucuronidase isoforms may also contribute to the total hydrolysis of the AZT-g and morphine-g substrates in enzyme solutions. These competing bacterial enzymes or mixtures of isoforms may not be active in the same manner when the enzyme solution is covalently immobilized into silica support packings and therefore may not react the same way to specific substrates as non-immobilized enzymes in solutions. Low affinity glucuronidase isoforms that would bind to the column in a different manner than high affinity ones, may also be responsible for decreasing the hydrolysis of morphine-g.

CONCLUSION

CONCLUSION

We have seen in this study that glucuronidation and its deconjugation process by β-glucuronidase enzymes plays an important role in drug metabolism and pharmacokinetic studies. β-glucuronidases have been demonstrated to be very useful enzymes for many *in vitro* studies. The immobilization of β-glucuronidase enzymes on a silica based HPLC support enables easy routine separations of the products [58] in solution as well as in biological samples such as urine or bile. The BG-IMER column may be used for identifying unknown metabolites and metabolites involved in conjugation-deconjugation reaction cycles of the enterohepatic circulation [72].The enzyme column may also increase the sensitivity of detection of certain metabolites (example, benzodiazepines, chapter I, Introduction, section 2.6, "*In vitro* studies with β-glucuronidase free enzymes") and may be used in pharmacokinetic studies when the total quantity of metabolite and alcohol is required to be calculated.

LIST OF REFERENCES

LIST OF REFERENCES

1. J.O. Miners and P.I.Mackenzie, Pharmacol. Ther., 51 (1991) 347.
2. C.N.Falany and T. R. Tephly, Arch. Biochem. Biophys., 227, 1, Nov (1983) 248.
3. G.Siest, B. Antoine, S.Fournel, J. Magdalou and J.Thomassin, Biochem Pharm., 36, 7, (1987) 983.
4. G.J.Dutton, in G.J.Dutton, (Editor), Glucuronic acid free and combined, Academic Press, New York, London, (1966).
5. G.J. Mulder, A.B.D. Van Doorn, Biochem. J. , 151, (1975) 131.
6. J. Caldwell. in P. Jenner, B. Testa (Editors), Concepts in Drug Metabolism, Part A : Drugs and the Pharmaceutical Sciences,(1980).
7. G.J. Mulder, M. W. H. Coughtrie and B. Burchell, in G.J. Mulder (Editor), Conjugation reactions in drug metabolism,Taylor & Francis Ltd. ,(1990).
8. S.J. Curry, in S. . Curry (Editor), Drug disposition and pharmacokinetics. Blackwell Scientific Publications, (1974).
9. G.G. Gibson, P. Skett, Introduction to drug metabolism, 2nd edition. Blackie Academic & Professional, (1994).
10. M.Rowland in D.V. Parl and R.L. Smith (Editors), Drug metabolism from microbe to man, Taylor & Francis, London, (1977).
11. J.G.Wagner in T.Teorelli, L. Dedrick and R.G. Condliffe (Editors). Pharmacology and Pharmacokinetics, Plenum Press, New York,(1977).

12. G. A. Levvy and J.Chronchie in G.J. Dutton (Editor), Glucuronic acid, Academic Press, New York, (1966) 301.

13. A. Parkinson, in C. D. Klaassen (Editor), Toxicology: The basic science of poisons, 5 th Edition, Chpt. 6,113.

14. A.J.Glazko, W.A. Dill and L. M. Wolf, J. Pharmacol. & Exper. Therap., 104 (1952) 458.

15. T.B.Vree, M. van den Biggelaar-Martea and C. P. W. G. Verwey-van Wissen, J. Chromatogr, 616 (1993) 271.

16. H. B. Hucker, A. G. Zacchel, S. V. Cox D. A. Brodie and N. H. R. Cantwell, J. Pharmacol. Exp. Ther., 153 (1966) 237.

17. A. Schapiro, W. Paul and H. Gonick, J. Urol., 100 Aug (1968) 146.

18. J.W.Owens, K.L.Gammon and P.D. Stahl, Arch. Biochem. Biophys., 166 (1975) 258.

19. B.-M. Löffler, E. Mielenz and H. Kunze, Biochem. Mol. Biol. Int.,33,2 ,May (1994) 234.

20. D. R. P. Tulsiani, R. K. Keller and O. , J. Biol. Chem., 250, 12 (1975) 4770.

21. C. Lin, M. L. Orcutt and W. H. Fishman, J.Biol.Chem., 250, 12 (1975) 4737.

22. R. T. Dean,Biochem. J., 138 (1974) 395.

23. H. J. Buehler, P.A. Katzman and E. A. Doisy, Proc. Soc. Exper. Biol. Med., 76 (1951) 672.

24. K.-J. Ho, S.-H. Kuo, Cancer, 76, 3, Aug (1995) 473.

25. K.-J. Ho, J. Urol., 154, Oct. (1995) 1335.

26. H.J. Haisma, M. vanMuijen,G. Scheffer, R.J. Scheper, H. M. Pinedo and E. Boven, Hybridoma, 14, 4, (1995) 377.

27. P. H .J. Houba, R.G.G.Lenders, E. Boven, J. W. Scheeren, H. M.Pinedo and H. J. Haisma, Biochem. Pharmac., 52, (1996) 455.

28. F . E. Brot, C. E. Bell,Jr. and W.S.Sly, Biochem., 17, 3 Feb. (1978) 385.

29. P. D. Stahl and O. Touster, J. Biol. Chem., 246 (1971) 5398.

30. B. V. Plapp and R.D. Cole, Arch. Biochem. Biophys., 116 (1966) 193.

31. M. Himeno, H. Ohhara, Y. Arakawa and K. Kato, J. Biochem.,77 (1975) 427.

32. M. Potier, R. Gianetto, Can. J. Biochem.,54 (1974) 321.

33. M. R. Dickson, M. Stewart, D. E. Hawley, and C. A. Marsh, Biochem. J., 181 (1979) 1.

34. C.A. Marsh, C-W. Lin, W H. Fishman, Biochem. J., 142 (1974) 491.

35. M. Potier, R. Gianetto, Can. J. Biochem.,51 (1973) 973.

36. R. K. Keller and O. Touster, J. Biol. Chem., 250,12, June (1975) 4765.

37. A. J. Lusis, S. Tomino and K.Paigen, J. Biol. Chem., 251, 24, Dec., (1976) 7753.

38. K. Paigen, R.T. Swank, S.Tomino and R.E. Ganschow, J. Cell Physiol., 85 (1975) 379.

39. S.Tomino and K. Paigen, J. Biol. Chem., 250, 3, Feb. (1974) 1146.

40. A. J. Lusis and K. Paigen, J. Biol. Chem., 253, 20, Oct. (1978) 7336.

41. R. T. Swank and K. Paigen, J. Mol. Biol., 77 (1973) 371.

42. S. Tomino, K. Paigen, D. R. P. Tulsiano and O. Touster, J. Biol.Chem.,250,21 (1975) 8503.

43. K.-J. Ho and L.-H C. Ho, Enzyme, 25 (1980) 361.

44. A. Schapiro W. Paul and H. Gonick, Enzym.Biol. Clin., 8 (1967)135.

45. K.-J. Ho, L.-H. C. Ho and O. R. Kruger, J.Lab.Clin. Med., June (1979) 916.

46. R.L. Smith in R.L. Smith (Editor), The excretion function of the bile, Chapman and Hall (1973).

47. Y.-C. Ho, L.-H. C. Ho, K.-J. Ho, Enzyme, 33(1985) 9.

48. K.-J. Ho, Biotech. Appl. Biochem., 16 (1992) 1.

49. T. Fujisawa and M. Mori, Lett. Appl. Microbio., 25 (1997) 95.

50. A. Nakano, D. L. Marks, P. D. Tietz, P. C. deGroen and N. F. LaRusso, Hepatology, 22 ,1, (1995) 262.

51. A. Norman and J. Sjöval.,J. Biol. Chem., 233 (1958) 872.

52. C.A. Marsh, Biochem. J., 86 (1963) 77.

53. M. Haumont, J. Magdalou, C. Lafaurie, J.-M. Ziegler,G. Siest, and J.-N. Colin, Arch. Biochem. Biophys., 28, 2, Sept. (1990) 264.

54. R. Ganschow and K. Paigen, Genetics, 59, July (1969) 379.

55. R. A. Jefferson, S.M. Burgess and D. Hirsh, Proc. Natl. Acad. Sci. USA., 83 (1986) 8447.

56. S. S. Good, C. S. Koble, R. Crouch, R.L. Johnson, J. L. Rideout and P. De Miranda, Drug Metab. Dispos., 18, 3 (1989) 321.

57. P. Simonsson, A. Lidén, S. Lindberg, Clin. Chem., 41, 6 (1995) 920.

58. I. K. Barker, S. M. Crawford and A. Fell, J. Chromatogr. B, 681 (1996) 323.

59. W.-K. Chui and I. W. Wainer, Analyt. Biochem., 201, (1992) 237.

60. M. Haumont, J. Magdalou, J.-C. Ziegler, R. Bidault, J.-P. Siest and G. Siest, Appl. Microbiol. Biotechnol., 35 (1991) 440.

61. A. Walhagen and L.-E. Edholm, J. Chromatogr., 473 (1989) 371.

62. R. A. H. J, Gilissen, J. H. N. Meerman and G. J. Mulder, Biochem. Pharmacol., 43, 12 (1992) 2661.

63. C. Pidgeon, S. Ong, H. Liu, X. Qui, M. Pidgeon, A. H. Dantzig, J. Munroe, W.J. Hornback, J. S. Kasher, L. Glunz and T. Szczerba, J. Med.Chem., 38 (1995) 590.

64. C. Pidgeon, C. Marcus and F. M. Alvarez in T.O. Baldwin and J.W. Kelly (Editors), Applications of Enzyme Biotechnology,Plenum, NY., (1992) 102.

65. J.F. Jen and M.Y. Tsai, J. Chromatogr.: B., 658, 1, Aug. (1994) 87.

66. S. Thelonan, P. Jadaud, I.W. Wainer, Chromatographia, 28, 11/12 Sept. (1989) 551.

67. E.Domenici, C. Bertucci, P. Salvadori, G. Felix, I. Cahagni, S. Motellier and I. W. Wainer, Chromatographia, 29 (1990) 170.

68. K. Kato, H. Ide, T. Shirhama and W. H. Fishman, Biochem. J., 117 (1970) 161.

69. F. M. Brunelle and R. K. Verbeeck, Biochem. Pharmacol., 46,11 (1993)

1953.

70. Y.-C Ho and K.-J. Ho, J. Urol., 134 Dec. (1985) 1227.

71. E. W. Frampton and L. Restaino, J. Appl. Bacteriol., 74 (1993) 223.

72. F. M. Brunelle and R.K.Verbeeck, Life Sciences, 60 ,22 (1997) 2013.

APPENDICES

APPENDICES

PART A HPLC Procedures

The various HPLC methods and gradient programs used for each substrate were the following:

Immobilized Enzyme Study (Online injection)

Mobile Phase A: Acetonitrile
Mobile Phase B: 0.01 M ammonium acetate pH 5.0

1. 4-methylumbelliferyl-β-D-glucuronide (Methylumbelliferone)
   Column: C_8 100 A° 5 u (4.6 mm x 250 mm)
   U V Detection: 280 nm
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100%B	0
0.5	100%B	2
0.5	25 %A/B	22
1.5	50 % A/B	30

2. p-acetaminophen-β -D-glucuronide, (Acetaminophen)
   Column: C_8 100 A° 5 u (4.6 mm x 250 mm)
   U V Detection: 254 nm
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 %B	0
0.5	100 %B	2
1.0	20 %A/B	20
1.0	50 %A/B	25

3. 3'-azido-3'-deoxythymidine-β -D-glucuronide, (AZT)
   Column: C_8 100 A° 5 u (4.6 mm x 250 mm)
   U V Detection: 270 nm

Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
1.0	100 % B	12
1.0	100 % B	20
1.0	20 % A/B	40

4. phenyl-β -D-glucuronide, (Phenol)
   Column: C_8 100 A° 5 u (4.6 mm x 250 mm)
   U V Detection: 254 nm
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
1.0	100 % B	3
1.0	30 %A/B	32
1.0	100 %A/B	40

5. chloramphenicol-β -D-glucuronide, (Chloramphenicol)
   Column: C_8 100 A° 5 u (4.6 mm x 250 mm)
   U V Detection: 280 nm
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
1.0	100 % B	3
1.0	30 %A/B	32
1.0	100 %A/B	40

6. estradiol-17-β -D-glucuronide, (Estradiol)
   Column: C_8 100 A° 5 u (4.6 mm x 250 mm)
   U V Detection: 230 nm
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
1.0	100 % B	3
1.0	60 % A/B	32

7. morphine-3- β -D-glucuronide, (Morphine Sulphate)
   Column: C_8 100 A° 5 u (4.6 mm x 250 mm)
   U V Detection: 280 nm
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
1.0	100 % B	3
1.0	30 % AB	32
1.0	60 % A/B	38
1.0	100 % A/B	47

Nonimmobilized Enzyme Study (Offline Injection,free enzyme reaction)

Note: Only the gradient changed while other HPLC conditions remain the same

Mobile Phase A: Acetonitrile
Mobile Phase B: 0.01 M ammonium acetate pH 5.0

1. 4-methylumbelliferyl-β -D-glucuronide (Methylumbelliferone)
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
0.5	100 % B	12
1.0	100 % B	15
1.0	100 % B	40
1.0	30 % A/B	45
1.0	70 % A/B	65
1.0	100 % B	72

2. p-acetaminophen-β -D-glucuronide, (Acetaminophen)
   Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
0.5	100 % B	12
1.0	100 % A/B	20
1.0	20 % A/B	40

3. 3'-azido-3'-deoxythymidine-β -D-glucuronide, (AZT) Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
0.5	100 % B	12
1.0	100 %A/B	20
1.0	20 %A/B	40

4. phenyl-β -D-glucuronide, (Phenol)

Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
0.5	100 % B	2
0.5	25 % A/B	22
1.5	50 % A/B	30

5. chloramphenicol-β -D-glucuronide, (Chloramphenicol)

Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
0.5	100 % B	12
1.0	100 % B	15
1.0	100 % B	40
1.0	55 % B	45
1.0	70 % A/B	65
1.0	100 % B	72

6. estradiol-17-β -D-glucuronide, (Estradiol)

Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
0.5	100 % B	12
1.0	100 % B	15
1.0	100 % B	40
1.0	30 % A/B	45
1.0	70 % A/B	65
1.0	100 % B	72

7. morphine-3- β -D-glucuronide, (Morphine Sulphate)

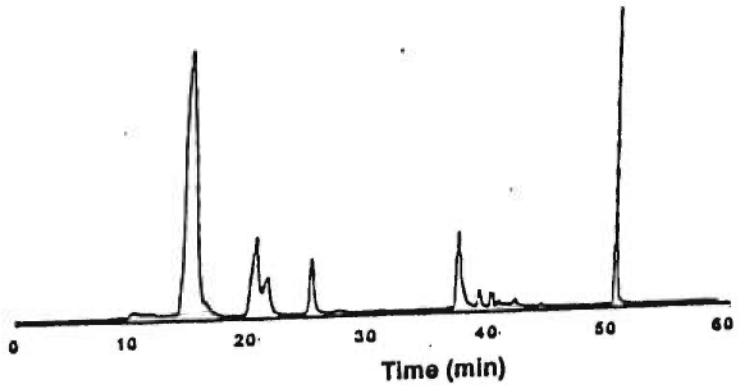
Gradient:

Flow rate: (ml/min)	Mobile phase %A/B	Time (min)
0.3	100 % B	0
0.5	100 % B	5
1.0	100 % B	15
1.0	30 % B	40
1.0	30 % A/B	45
1.0	60 % A/B	65
1.0	100 % B	72

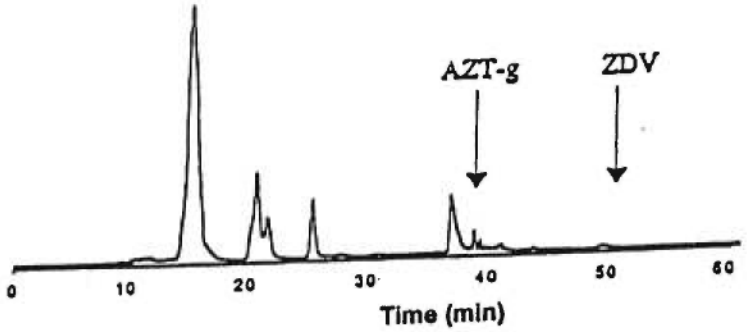
PART B Calculation of % Conversion

% conversion of glucuronides to alcohols was calculated by dividing the area of the alcohol peak by the summation of total area of the glucuronide and the alcohol peak multiplied by 100.

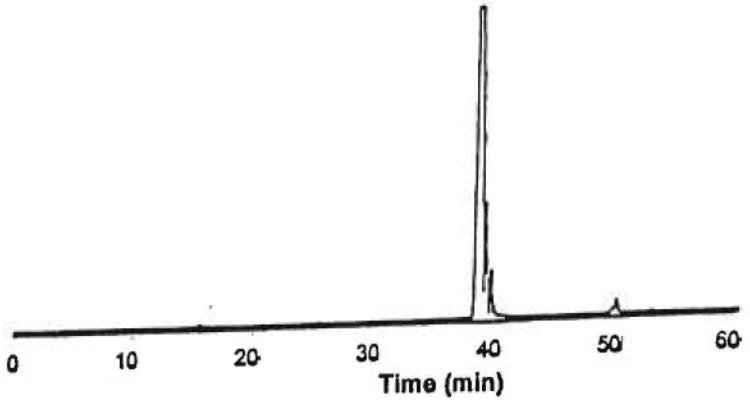
Figure 7. Chromatograms representing the deconjugation of AZT-g in non-immobilized enzyme solutions



AZT-g deconjugated in nonimmobilized enzyme solution

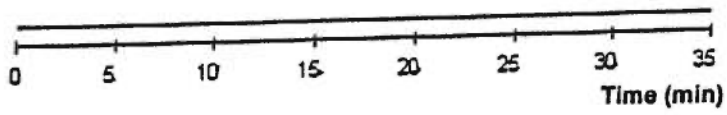


Blank nonimmobilized enzyme solution (without AZT-g)

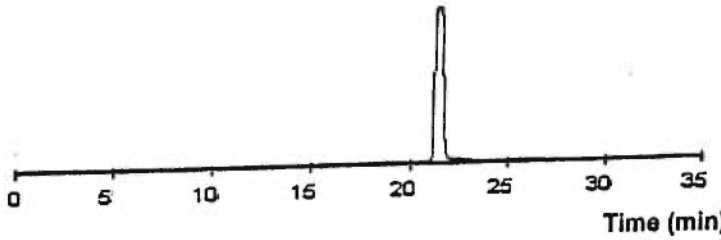


AZT-g stock solution

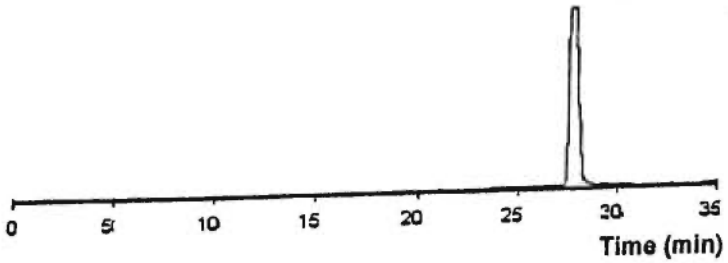
Figure 8. Chromatograms representing flow rate change (0.1 to 1.0 ml/min) on the deconjugation of AZT-g



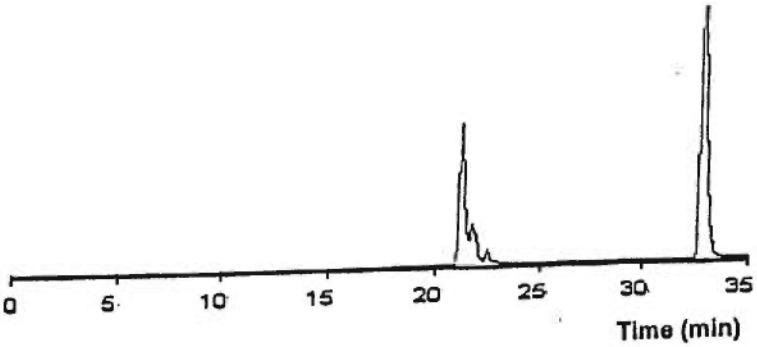
Blank (mobile phase B)



AZT-g stock solution (without the B-glucuronidase column)

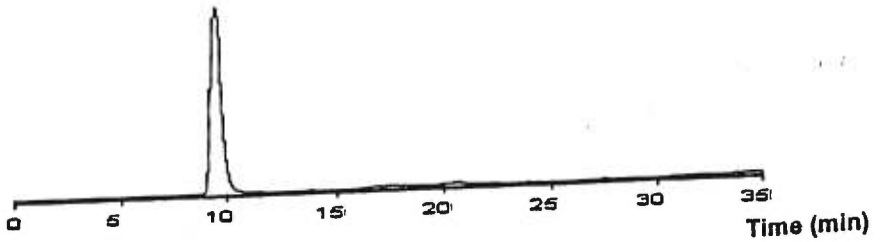


Deconjugation of AZT-g at 0.1ml/min

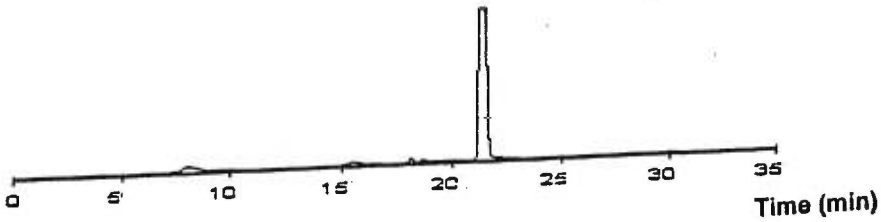


Deconjugation of AZT-g at 1.0 ml/min

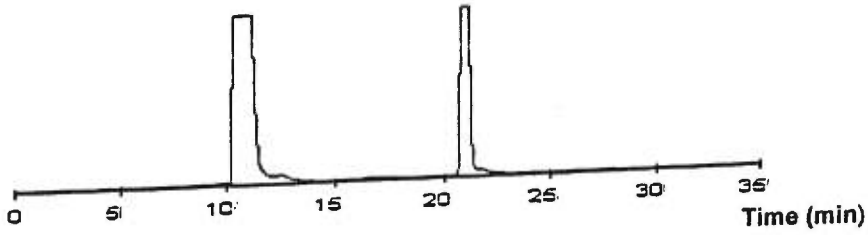
Figure 9. Chromatograms representing the effect of exposing the column to pH 4.0 on the deconjugation of APAP-g



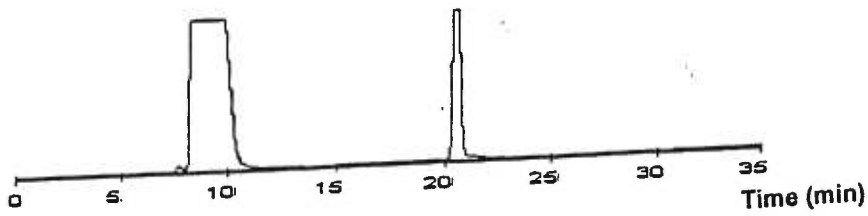
APAP-g stock solution



APAP-g injected at pH 5.0 (before injecting at pH 4.0)

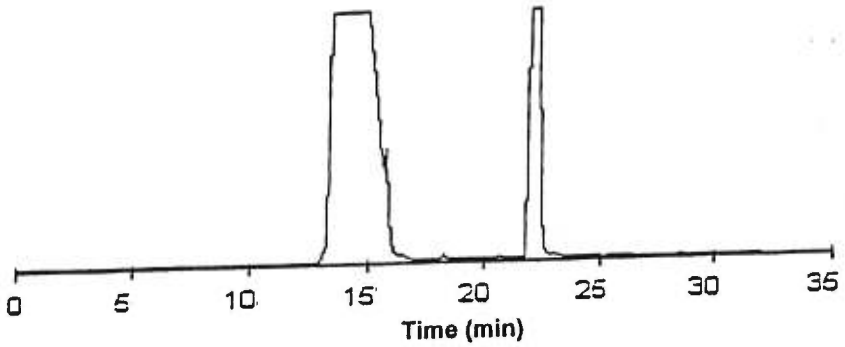


APAP-g injected at pH 4.0

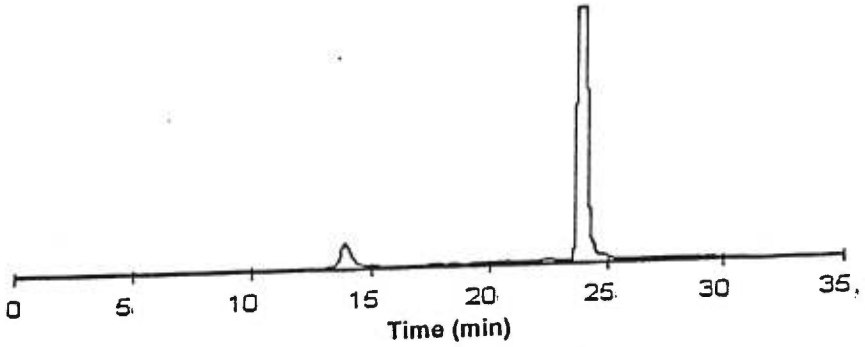


APAP-g injected at pH 5.0 (after injecting at pH 4.0)

Figure 10. Chromatograms representing the effect of temperature change on the activity of the column



APAP-g injected with enzyme column at r.t. (0.50 mM)



APAP-g injected with enzyme column at 37°C (0.5 mM)