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INVESTIGATION OF PHENYLEPHRINE SULFATION AND INHIBITION USING A NOVEL HILIC ASSAY METHOD

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INVESTIGATION OF PHENYLEPHRINE SULFATION AND INHIBITION USING A NOVEL HILIC ASSAY METHOD

A thesis submitted in partial fulfillment of the requirements for the Master's degree in Pharmaceutical sciences at Virginia Commonwealth University

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LIST OF ABBREVIATIONS

ACN	acetonitrile
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	concentration
CL	clearance
CV	co-efficient of variation
DFN	departure from normal
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
E2-3G	17 β-estradiol glucuronide
FDA	Food and drug administration
GRAS	generally regarded as safe
HIC	human intestinal cytosol
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
IC_{50}	the concentration of an inhibitor at which the enzyme activity is reduced by half
IV	Intravenous
K_i	dissociation constant of the inhibitor when bound to enzyme only
K_i'	dissociation constant of the inhibitor when bound to enzyme-substrate complex
K_m	the Michaelis-Menten dissociation constant
$K_{m\ app}$	apparent km in presence of an inhibitor

LC-MS/MS	liquid chromatography-mass spectrometry
LLOQ	lower limit of detection
LS180	human colon adenocarcinoma epithelial cell line
MAO	monoamine oxidase
MSC	Model selection criteria
4-MU	4-methyl umbelliferone
4-MUS	4-methyl umbelliferone sulfate
PAP	phosphoadenosine 5'- phosphate
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PE	phenylephrine
PES	phenylephrine-3-O-sulfate
PK	pharmacokinetic
PNP	para-nitro phenol
QC	quality control
R^2	co-efficient of determination
RP-HPLC	reverse phase high pressure liquid chromatography
S.D	standard deviation
SSQ	sum of squares
SULT	sulfotranferases
TEA	trimethylamine
TSQ	total sum of squares
V_{max}	the maximum rate achieved at saturating substrate concentrations
$V_{max\ app}$	apparent V_{max} in presence of an inhibitor

ABSTRACT

INVESTIGATION OF PHENYLEPHRINE SULFATION AND INHIBITION USING A NOVEL HILIC ASSAY METHOD

By Heta Shah

A thesis submitted in partial fulfillment of the requirements for the Master's degree in Pharmaceutical sciences at Virginia Commonwealth University

Virginia Commonwealth University, 2015
Director: Dr. Phillip M. Gerk
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Phenylephrine (PE) is the most commonly used over-the-counter nasal decongestant. The problem associated with phenylephrine is that it undergoes extensive first pass metabolism in the intestinal gut wall leading to its poor and variable oral bioavailability.

This research project aims at developing strategies in order to increase the oral bioavailability of PE by co-administration of GRAS compounds. A HILIC assay method was developed to detect the parent drug, phenylephrine (PE) and its sulfate metabolite (PES). The enzyme kinetic studies were done with phenolic dietary or GRAS compounds using LS180 human intestinal cell model, recombinant SULT enzymes and human intestinal cytosol (HIC). From the screening studies done, one inhibitor was selected in order to study the mechanism of inhibition. In conclusion the studies done *in vitro* provided a basis in order to predict *in vivo* intrinsic clearance through the sulfation pathway.

1 Introduction

1.1 Clinical significance of Phenylephrine

Phenylephrine is the most commonly used over the counter (OTC) nasal decongestant. It acts on α_1-adrenergic receptors, causes constriction of the blood vessels and prevents nasal decongestion and stuffy nose. The other available nasal decongestants that can be used are pseudoephedrine (brand name: Sudafed™), phenylpropanolamine and oxymetazoline. Pseudoephedrine is used as a precursor in manufacture of methamphetamine (1) and hence it is sold "behind the counter". Although pseudoephedrine has higher bioavailability as compared to phenylephrine it acts on both α and β receptors and is non-specific in its mode of action. This leads to both vasoconstriction and increase in mucociliary clearance due to its nonspecific activity on adrenergic receptors. In the United States, the Food and Drug Administration (FDA) issued a public health advisory (2) against the use of phenylpropanolamine (PPA) in November 2000. In this advisory, the FDA requested that all drug companies discontinue marketing products containing PPA (3). The agency estimates that PPA caused between 200 and 500 strokes per year among 18-to-49-year-old users (3). In 2005, the FDA removed PPA from over-the-counter sale (4). Because of its potential use in amphetamine manufacture, it is controlled by the Combat Methamphetamine Epidemic Act of 2005 (3). It is recommended that oxymetazoline should not be used for more than three days, as rebound congestion, or rhinitis medicamentosa, may occur (5). Because of these

reasons phenylephrine (Figure 1.1) is the most preferred alternative approach to treat nasal decongestion.

Figure 1.1 Structure of Phenylephrine (6)

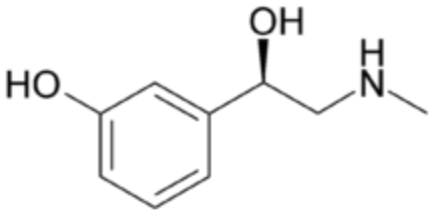


Table 1.1 Physicochemical properties of PE (7)

Physicochemical Parameter	Value
Molecular Weight	167.0 g/mol
Molecular Formula	$C_9H_{13}NO_2$
Melting Point	140-145°C
Solubility	Freely soluble in Water
LogP	-0.31
pKa (basic)	8.97

Table 1.2 Pharmacokinetic properties of PE (7)

Pharmacokinetic Parameter	Value
Bioavailability	38 ± 14%
T_{max}	1-1.3 hours
Plasma Protein Binding	95%
Volume of Distribution (Vd_{ss})	340±174 liters
Excretion	3% unchanged in urine (through IV route)
$t_{1/2}$ (half life)	2.1-3.4 hours

1.2 Low and variable oral bioavailability of PE

The problem associated with phenylephrine is that it undergoes extensive first pass metabolism in the intestinal gut wall leading to its poor and variable oral bioavailability. The metabolism pathways are influenced by route of administration. After oral ingestion, sulfate conjugation plays the most important role and the first pass metabolism (mainly conjugation within the gut-wall) decreases the amount of drug entering the systemic circulation to about 40% of the dose (8). However after I.V injection deamination through MAO (monoamine oxidase) metabolism is the pre-dominant metabolic pathway and conjugation is of minor importance (8).

Sulfated molecules are readily eliminated in bile and in urine. Sulfotransferases (also known as SULTs) are the super family of enzymes that catalyze the sulfate conjugation of various substrates such as xenobiotics, steroids, small endogenous substrates (neurotransmitters, bile acids etc). They are present in the aqueous cytosol and are called cytosolic proteins. SULT1A3 is the dominant SULT responsible for sulfation of PE (9). SULT1A3 shares seven out of ten aromatic residues in its substrate binding site with SULT1A1 but has much narrower specificity (10). The active site alters its own conformation to process molecules and it involves the enzyme automatically sensing the structural characteristics of the potential substrate and then molding itself around it (10). The co-factor involved in the sulfate conjugation reaction is PAPS (3'-phosphoadenosine-5'-phosphosulfate) which is the sulfate donor (Figure 1.2). Sulfate present in the body is loaded on ATP to form adenosine-5'-phosphosulfate (APS) (11). This APS then reacts with another molecule of ATP to form the co-factor PAPS. PAPS then donates its sulfuryl group to the substrate in presence of SULTs to form the sulfate metabolite (11). Sulfation is generally a high affinity-low capacity pathway.

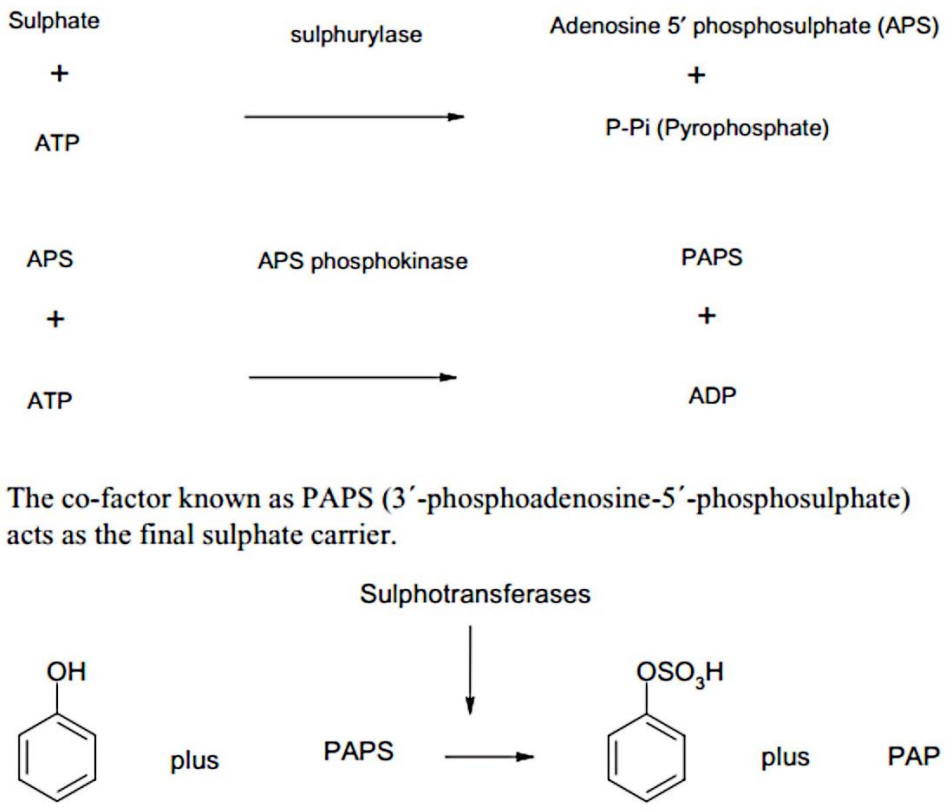


Figure 1.2 Basic reaction for Sulfotransferases (11)

A high dose of phenylephrine in order to increase the oral bioavailability of PE challenges the intestine with a very high concentration of a powerful α-adenoreceptor agonist (8). This would cause vasoconstriction of the intestinal blood vessels and prevent the transport of PE from the site of absorption to the systemic circulation (8). This research project aims at developing strategies in order to increase the oral bioavailability of PE by inhibiting the sulfation pathway. The inhibition of the sulfation pathway would help in increasing the bioavailability as well decrease the variability in fraction absorbed of PE. In order to achieve this goal it was essential to develop a bio-analytical method to detect the parent drug, phenylephrine (PE) and its sulfate metabolite phenylephrine-3-O-sulfate (PES).

1.3 Detection techniques to measure the metabolite

There are techniques available for measuring the activity of the SULT enzyme like the radiometric technique, sulfatase assay and PAPS regeneration assay. In the radiometric detection technique, the assay utilizes [^{35}S]PAPS and measures the formation of ^{35}S-conjugated substrates (12). Barium precipitation is utilized to remove the [^{35}S]PAPS and free sulfate from the reaction mixture allowing determination of the unprecipitated ^{35}S-products via scintillation counting (12). The problems associated with the use of barium precipitation assay are the sulfation of buffer components, substrate contaminants and tissue preparations as the assay does not distinguish between the sulfated products (12). The assay cannot distinguish between the sulfated products of PE and the inhibitors (GRAS or dietary compounds), thus confounding one of the goals of the present work. Also high backgrounds are generated with cytosol and cell lysate as certain SULTs are capable of sulfating free tyrosine leading to peptide sulfation (12). It also requires radiolabeled [^{35}S] PAPS which is very expensive and highly regulated.

Sulfatase assay involves use of sulfatases enzymes of the esterase class that catalyze the hydrolysis of sulfate esters. Sulfatases would cleave the sulfate metabolite and free the parent drug. But the drawback with this method is that the sulfate and phosphate esters have similar bond lengths and geometries and linear free energy relationships which suggest that their reactions in solution proceed via similar dissociative transition states (13) and hence are not completely selective. For the PAPS regeneration assay, some methods may proceed to direct quantification of the sulfated metabolite (eg, 1-naphthyl sulfate, 4-methylumbelliferyl sulfate (4MUS), acetaminophen sulfate, p-nitro phenyl sulfate (PNPS), etc). Others may use either 4MUS or PNPS as the source of sulfate, then measure the fluorescence or absorbance of the liberated products (4-MU, 1-naphthol, PNP or APAP). These would then be indirect ways to measure the rate of sulfation of a metabolite, by subtracting the baseline rate of 4MU or PNP

generation from the rate in the presence of the SULT substrate. One of the drawbacks with this method is that if the sulfation of the substrate is directly measured, we would be dependent upon one or more SULT isoforms to form PAPS from 4MUS or PNPS. Thus we may have competition between the formation of PAPS (from 4MUS or PNPS) and the formation of the sulfated metabolite of interest.

In order to overcome these shortcomings of these methods we attempted to develop an analytical method for direct detection of PES as well as help in simultaneous quantification of PE and PES. As PE and PES are highly hydrophilic compounds they were poorly retained on traditional RP-HPLC columns. Also there were matrix effects and unreliable chromatography issues seen with LC-MS/MS. Hydrophilic interaction liquid chromatography (HILIC) assay was used for direct detection of PES. It is an alternative assay method which encompasses certain aspects of reverse phase (RP-HPLC), normal phase HPLC and ion exchange chromatography. This technique is used for the separation of charged and hydrophilic compounds and involves a hydrophilic partitioning between the water enriched layer at the stationary phase and the less polar mobile phase. As PE and PES have conjugated ring structures fluorescence detection technique was used.

1.4 Strategy to improve oral bioavailability of PE

Efficacy of PE is questionable due to its low and variable bioavailability. This can be attributed to its extensive pre-systemic metabolism. If this pre-systemic metabolism is inhibited, bioavailability of PE can be improved. As mentioned earlier, more PE is conjugated mainly as sulfate by the oral route (45.7%) than that by the intravenous route (8.3%) (8). Less PE is bio-transformed to 3-hydroxymandelic acid by the oral route (24.2%) (8). This tells us that sulfation plays a major role in presystemic metabolism of PE. If the presystemic sulfation of PE can be inhibited, bioavailability of PE can be increased. The

preferred site for sulfation is the phenolic group, which is a common structural characteristic of many SULT substrate such as PE. Co-administration of phenolic GRAS (generally regarded as safe) and dietary compounds along with PE can be a suitable strategy to inhibit presystemic metabolism of PE. Several phenolic GRAS and dietary compounds were included in the study: eugenol, resveratrol, zingerone, isoeugenol, ethyl vanillin, quercetin, pterostilbene, propyl paraben, vanillin, raspberry ketone, curcumin, methyl paraben and magnolol. Many successful examples of applying dietary compounds to inhibit metabolism and finally to improve the oral bioavailability are found in the literature. Systemic exposure, metabolism, pharmacokinetics and toxicology are the aspects considered for safety of GRAS compounds and have been evaluated (14). Hence it is a safe strategy to include these phenolic GRAS compounds in the final formulation to inhibit presystemic metabolism of PE through sulfation. Since MAO inhibitors can cause hypertension, systemic MAO inhibition is not desired. The presented work involves the kinetic studies on inhibition of sulfation with phenolic compounds using recombinant SULT enzymes and human intestinal cytosol (HIC). The IC_{50} values of these compounds were determined in the human intestinal LS180 cell model and an attempt was made to determine the mechanism of inhibition in the HIC.

2 HPLC Method

2.1 Introduction

Methods are available for the determination of PE in pharmaceutical formulations by HPLC using ion-pair and hydrophilic interaction liquid chromatography (HILIC) coupled with fluorescence detection as described by Dousa et.al. (15). However these methods do not enable the simultaneous quantitation of the highly hydrophilic metabolite PES as well as PE in *in vitro* systems like LS180 cells, recombinant enzymes and human intestinal cytosol. The objective of the presented work was to develop a quick and sensitive quantitative chromatographic method with improved retention and separation of the hydrophilic PES and PE, having improved sensitivity, short run time and simultaneous detection of PE and its metabolite PES.

The sulfation of PE occurs at its phenolic group to form phenylephrine 3-O-sulfate (PES), which is the preferred structural feature of many sulfotransferases substrates. Hence it is essential to have information about PES formation vs disappearance of PE in order to study the metabolism of PE. Due to the very low lipophilicity, high polar nature of PES and its zwitterionic character it is not retained on RP-HPLC and hence its separation and quantitation are very difficult. Secondly as the mass of PES is quite low, sensitive analytical method for determination of PES is needed. In our experience, LC-MS/MS was attempted to detect PES

but the ionization and sensitivity for PES were not good, due to the highly aqueous mobile phase. Also, the chromatography was unreliable and matrix effect issues were seen.

Hydrophilic interaction liquid chromatography (HILIC) has recently become more important, particularly for the analysis of polar drugs, metabolites and biologically relevant compounds in glycomics, proteomics, metabolomics and clinical analysis (16). It is an alternative strategy to ion-exchange and normal-phase liquid chromatography. HILIC is also used for analyzing a variety of ionizable compounds. As it contains a greater portion of organic phase which has low viscosity, the generated back pressure is very low. This allows higher flow rates and smaller particle diameter size. As described in Figure 2.1, it can be regarded as a mix of reverse-phase LC (similar mobile phase, organic modifier and pH range), normal phase LC (similar stationary phase) and ion exchange chromatography (similar to type of compounds that can be analyzed). The retention mechanism of HILIC involves (i) hydrophilic partitioning between a water enriched layer at the stationary phase surface and comparatively less polar mobile phase (ii) ion-exchange between the charged analyte and the stationary phase (iii) adsorption of the analyte on the stationary phase due to hydrogen bonding [2]. As such it has overlapping characteristics with RP-HPLC, NP-LC and IC.

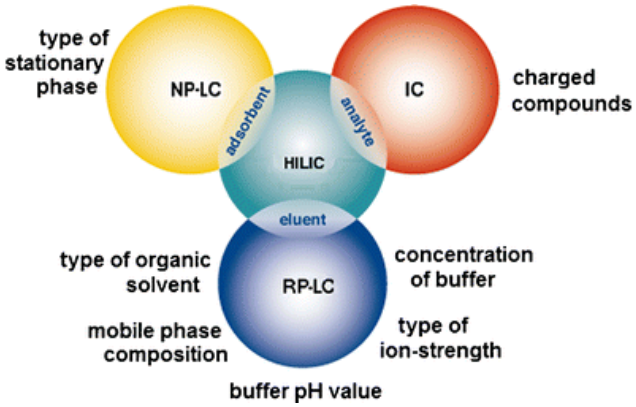


Figure 2.1 HILIC chromatography (17)

NP-LC: Normal Phase liquid chromatography, IC: Ion-exchange chromatography, NP-LC: Normal phase liquid chromatography

The main advantage of HILIC is the ability to retain polar analytes without the need of toxic, flammable, and/or expensive solvents as used in normal phase chromatography. Hence a sensitive HILIC HPLC method was developed using a zwitterionic sulphoalkylbetaine column. It contains both sulphonic acid (anionic) and quaternary ammonium (cationic), separated by a short alkyl spacer.

2.2 Method Development

2.2.1 Experimental

2.2.1.1 Chemicals and reagents

L-phenylephrine hydrochloride (USP grade), phenylephrine sulfate, β-estradiol glucuronide, (E2-3G; internal standard), 50% w/v dextrose (USP grade, Butler Schein). Methanol (Honeywell, B & J ACS/HPLC certified Solvent), acetonitrile (Macron fine chemicals), triethylamine (Fischer Scientific, HPLC grade), formic acid (Fischer Chemicals, certified ACS) and ammonium hydroxide (BDH, ACS grade) were purchased. Water was filtered through the NANOpure Diamond Ultrapure Water system from Barnstead International (Dubuque, IA, USA). All other chemicals, solvents or reagents were of analytical grade and were obtained from Sigma-Aldrich (St Louis, MO) or Fisher Scientific unless indicated above. PES was synthesized in our lab. (Zhang et al.; manuscript in preparation)

2.2.1.2 Instrumentation

Separation of analytes was performed using an EC 100X4.6 mm Nucleodur HILIC, 3µm (Macherey – Nagel,) column preceded by a phenomenex HILIC Security Guard Cartridge. The HPLC system (Waters, Milford MA) consisted of the Alliance 2695 separations module and a 2475 fluorescence detector with excitation wavelength of 268 nm and emission wavelength of 293 nm. These wavelengths were obtained from maxima observed with fluorescent scan obtained in the lab. The fluorescent intensity of PE was almost 4 times

greater than the fluorescent intensity of PES, on an equimolar basis. Data were collected and analyzed using Waters Empower 2 or Empower 3 software.

2.2.1.3 Chromatographic conditions

Preparation of buffer for mobile phase: To 250 mL of HPLC grade water, 1250 µL of TEA and 780 µL of ammonium hydroxide was added. pH was adjusted to 4.5 with formic acid.

Mobile phase A: ACN: MeOH: Buffer (72: 8: 20). This was filtered through 0.45 µM Millipore filter after mixing.

Mobile phase B: 100% ACN

Determination of PE, PES and E2-3G was performed with isocratic elution (Mobile phase A: Mobile Phase B, 90:10) at a flow rate of 0.8 mL/min. The injection volume was 25 µL. The runtime was 6.0 mins. The column temperature was maintained at 30°C and the autosampler compartment was set to 4°C**.** PE and PES were detected at the excitation wavelength of 268 nm and emission wavelength of 293 nm (as determined by fluorescence spectral scans of PE and PES in HPLC mobile phase) with retention times as follows: PES: 3.2 mins, PE : 4.3 mins and E2-3G: 2.6 mins. This model is analytically convenient since PE and PES can be readily and simultaneously quantitated by using a fluorescent detection. Due to high selectivity and sensitivity, fluorescence detection is more suitable than UV detection at 275 nm, as described in the current European Pharmacopeia and U.S Pharmacopeia (USP) methods (15). LS180 cell culture techniques were used as described in Chapter 3.

The method was intended to be developed for application to LS180 cells, recombinant enzymes and human intestinal cytosol. The samples containing PE and PES in aqueous buffer could not be injected directly on the HILIC column as they had poor retention when injected

directly because water is a strong solvent in HILIC chromatography. The nature of the sample diluent is a major issue in HILIC and can lead to severe peak distortion especially when the sample diluent contains ≥ 40% water. PE and PES, being very hydrophilic compounds were difficult to extract with an organic solvent from aqueous media. Thus the samples were deproteinized with acetonitrile and ACN/water phase separation was achieved by "sugaring out" with 50% w/v dextrose.

Conditioned DPBS was prepared by incubating confluent LS180 cells for 8 hours in DPBS followed by centrifugation for 5 mins at 4000 g at 25°C and stored at -20°C. The samples of the LS180 cells in conditioned DPBS buffer and ACN were mixed in the ratio of 1:4. The method involved the extraction of PE and PES in the ACN layer. Due to the use of an extraction technique in the sample preparation method it was necessary to include an internal standard in the method. For the selection of an internal standard, various Phase II small hydrophilic metabolites structurally similar to PE and PES were tested. The following compounds were tested as the internal standard for the method:

Table 2.1 List of compounds tested as internal standards

Analyte tested	Peak start (min)	Peak ends (min)	Peak shape
etilephrine (ET)	3.3	3.75	Broad and slightly distorted
etilephrine sulfate (ETS)	3.75	4.15	Broad and overlapping with adjacent peak
Tyrosol	3.80	4.20	Broad and overlapping with adjacent peak
Tyramine	3.64	4.20	Broad
Tyrosine	4.20	>5.0	Very broad and not completely eluted
albuterol	3.54	4.20	Very broad peak shape
1-naphthol	1.35	1.9	Negative peak, poorly retained
pyridoxine	2.15	2.80	Broad peak shape
dopamine	4.40	>5.0	Incomplete elution and broad peak shape
4-methylumbeliferone sulfate	2.55	2.95	Negative peak
4-methylumbeliferone	1.20	1.70	Peak seen in void
4-nitrosophenyl beta-glucuronide	1.30	1.70	Peak seen in the solvent front
beta estradiol	-	-	Peak seen in the solvent front
4-nitrophenyl sulfate	1.30	1.60	Peak seen in the solvent front and negative peak
beta estradiol glucuronide	2.00	2.4	Good peak shape
alpha-naphthyl sulfate	-	-	Peak seen in the solvent front
ethynyl estradiol	-	-	Peak seen in the solvent front
ellagic acid	1.25	1.75	Peak seen in the solvent front
acetaminophen glucuronide	3.85	4.22	Negative peak seen
vanillic acid	2.20	3.00	Peak split
fluorescein	-	-	Peak seen in the solvent front
Calcein	3.90	4.60	Broad peak shape

From the above studies 17 β-estradiol glucuronide (E2-3G) was selected as the internal standard for the method. E2-3G was also detected at the same excitation and emission wavelength with a retention time of 2.6 mins.

2.2.2 Extraction Efficiency

It was observed that complete extraction of PE and PES was not seen in the ACN layer. This further generated a need to have a phase separation method in order to increase the extraction efficiency of PE and PES in the ACN layer. First, "salting out" methods were attempted. Various combinations of salt solutions were used such as saturated solution of ammonium sulfate, saturated solution of ammonium sulfate (pH adjusted to 9.3 with ammonium hydroxide) and saturated solution of ammonium sulfate (pH adjusted to 9.3 with TEA). These salt solutions (4%) were added to the mixture of aqueous buffer: ACN (1:4). The extraction efficiency observed with the salting out method was less than 50% for both PE and PES. The next attempt to increase the extraction efficiency was using the "sugaring-out" method. The "sugaring- out" method is a less commonly used phase separation method which uses sugar as a mass separating agent. The use of a monomeric sugar such as dextrose to an ACN-water mixture created two phases: one that is ACN rich (top) and an aqueous phase (bottom). The uncharged but polar biomolecules such as sugars dissolve readily in water because of the stabilizing effect of hydrogen bonds between the hydroxyl group of the sugar and the polar water molecules (18). It is likely that some original hydrogen bonds in the mixture between PE, PES with water are replaced by the hydrogen bonds formed between sugar and water molecules which may force ACN molecules to separate from water molecules and form a separate phase. Sugaring out of the sample provides an advantage over the salting out technique as it does not alter pH as compared to the salting out technique. Also salting out happens at high concentrations of salt which leads to distorted peak shapes in HILIC chromatography. In this study, we tested several sugars including meso-erythritol,

maltodextrin, dextran sulfate, sucrose, sucralose, and dextrose (D-glucose), and chose dextrose for further studies since it appeared to give the highest recovery and minimal chromatographic interference.

After extraction, the large volume of ACN needed to be reduced. PE was found to be unstable during evaporation under a stream of nitrogen gas at 55°C using a Turbo-Vap, consistent with the previously published literature (19, 20) . Hence vacuum evaporation was employed in order to evaporate the ACN layer in order to concentrate the analytes, followed by reconstitution with mobile phase. These processes are described below.

2.2.3 Preparation of assay standard and QC samples

Conditioned DPBS was prepared by exposing DPBS (calcium chloride 100 mg/L, magnesium chloride 100 mg/mL, potassium chloride 200 mg/mL, potassium phosphate monobasic 200 mg/L, sodium chloride 8g/L, sodium phosphate dibasic 2.16 g/L, D-glucose 1g/L and sodium pyruvate 36mg/L in water, pH 7.4) to confluent LS180 cells for 8 hours followed by centrifugation for 5 mins at 4000 g at 25°C and stored at -20°C. Standard samples of 250 µL PE (200, 50, 5, 12.5, 3.13, 1.56, 0.781 and 0.390 µM) and standard samples of 250 µL PES (32, 8, 4, 2, 2.5, 0.5, 0.25, 0.125 and 0.0625 µM) in conditioned DPBS buffer were prepared. To the standard samples 1000 µL of ACN containing 24 µM of E2-3G was added. Dextrose solution (50%w/v) 62.5 µL was added for phase separation. The standards were vortexed for 30 seconds and then centrifuged for 5 mins at 4000g at room temperature. The ACN layer (1000 µL) was decanted and retained. Another 1000 µL ACN without the internal standard was added and the standard samples were again vortexed and centrifuged as above. ACN layers from both the extractions were pooled and evaporated using the Speed Vac (Savant Instrument Corp., Farmingdale, NY) under reduced pressure. The standard samples were then reconstituted with 200 µL of mobile phase (A:B = 9:1) and

then injected into HPLC system using the chromatographic conditions described above. Quality control (QC) samples containing PE (100, 6.25 and 0.39 µM) and PES (16, 1 and 0.0625 µM) were independently prepared in the same manner. Twenty-five microliters of the standard or QC sample was injected into the HPLC for quantitation.

The extraction efficiency of the analytes (PE and PES) and internal standard (E2-3G) into the ACN layer was checked. Minimum of two extractions were necessary to maximize the extraction of PE, PES and E2-3G. The extraction efficiency of the analytes were checked at low, medium and high concentrations of the analytes. For this study, the peak area ratios of the analyte and the internal standard of the spiked QC samples after two extractions and the analytes spiked in the mobile phase were compared, as shown in Table 2.2.

Table 2.2 Percent Extraction efficiency (mean ± S.D; %C.V) for the determination of the analytes as compared to unextracted Standards

Level	PE	PES	E2-3G
QC 1	65.6 ± 1.1	52.5 ± 1.0	32.7 ± 0.2
	(1.66%)	(1.96%)	(0.55%)
QC 2	62.5 ± 2.2	50.5 ± 1.7	33.6 ± 0.7
	(3.44%)	(3.34%)	(2.14%)
QC 3	54.3 ± 2.2	48.9 ± 1.6	37.2 ± 0.8
	(4.09%)	(3.23%)	(2.15%)

The observed absolute extraction efficiency was low but the standard deviation was low and the consistency was high. Although at least 70% of the extraction efficiency is preferred but this was acceptable because the reproducibility and the consistency was high, demonstrated by very low relative standard deviation values (all < 5%)

2.3 Validation

The validation study was performed as per the FDA bioanalytical guidelines for assay validation.

2.3.1 Specificity

The assay method was found to be specific for PE, PES and E2-3G when compared with blank. Blank was extracted in the same way as the samples with conditioned DPBS buffer and without PE, PES and E2-3G.

2.3.2 Linearity and LLOQ

Eight calibration standards with concentrations (200, 50, 25, 12.5, 3.12, 1.56, 0.78 and 0.39 µM for PE) and (32, 8, 4, 2, 2.5, 0.5, 0.25, 0.125 and 0.0625 µM for PES) were extracted in duplicate and analyzed in three independent runs. Calibration curves were fitted using the linear regression of the ratio of the peak area response of the analyte and the internal standard versus concentration. For each calibration curve, the back-calculated concentrations were required to be within ±15% of nominal concentration (DFN) except at the limit of quantification (LLOQ) where it was within ± 20%.

Table 2.3 Assay parameters for determination of analytes

Analyte	Concentration Range (µM)	Retention time[a] (min)	LLOQ (µM)	Regression coefficient[b]
PE	0.39-200	4.4±0.055	0.39	0.9997-1.0000
PES	0.063-32	3.2±0.039	0.063	0.9999-1.0000
E2-3G (internal standard)	24.0	2.7±0.029	-	-

LLOQ, Lower limit of quantification; sample volume 25µL

[a] Mean ± S.D. for four replicates. [b] Range

2.3.3 Accuracy and Precision:

The intraday accuracy and precision were evaluated by assaying three replicates of the QC samples each in two analytical runs on the same day. The interday accuracy and precision were evaluated by assaying three replicates of the QC samples each in analytical runs on 3 different days. Precision was characterized by the coefficient of variance (CV, %) whereas accuracy was expressed as deviation from the nominal value (DFN, %) as shown in Tables 2.4, 2.5, 2.6 and 2.7.

Table 2.4 Assay validation results for determination of analytes

Analyte (PES)	**Nominal Concentration (µM)**	**Intraday (n = 6)**		
		Measured concentration (µM)	**Accuracy DFN (%)**	**Precision CV (%)**
LLOQ	0.063	0.057 ± 0.0064	9.0	11
QC1	1	0.91 ± 0.020	8.7	2.2
QC2	16	16 ± 0.29	2.4	2.0
QC3	24	24 ± 0.20	0.3	0.79

Table 2.5 Assay validation results for determination of analytes

Analyte (PE)	Nominal Concentration (µM)	Intraday (n = 6)		
		Measured concentration (µM)	Accuracy DFN (%)	Precision CV (%)
LLOQ	0.39	0.46 ± 0.013	16	3.0
QC1	6.25	6.4 ± 0.20	3.1	2.8
QC2	100	103 ± 2	2.6	2.3
QC3	150	150 ± 1	0.01	0.53

Table 2.6 Assay validation results for determination of analytes

Analyte (PES)	Nominal Concentration (µM)	Interday (n = 9)		
		Measured concentration (µM)	Accuracy DFN (%)	Precision CV (%)
LLOQ	0.063	0.056±0.0071	9.0	11
QC1	1.0	0.95±0.065	4.6	6.9
QC2	16.0	16±0.30	2.6	1.8
QC3	24.0	24±0.60	1.1	2.4

Table 2.7 Assay validation results for determination of analytes

Analyte (PE)	Nominal Concentration (µM)	Interday (n = 9)		
		Measured concentration (µM)	Accuracy DFN (%)	Precision CV (%)
LLOQ	0.391	0.45±0.039	8.6	16
QC1	6.25	6.6±0.25	3.8	-5.3
QC2	100	104±3	3.2	-4.3
QC3	150	154±6	3.8	-2.5

2.3.4 Post processing stability:

The stability of the standard and spiked QC samples at the working level concentration (50 µM for PE) were determined for 18.5 hours within the injector port in order to demonstrate good stability of processed samples to facilitate long analytical sample runs. The analytes were quantified at 0 and 18.5 hours to compare the ratio of the peak areas of the analyte and the internal standard at time 0 with the ratio of the peak area of the analyte and the internal standard at 18.5 hours. These data are reported in Tables 2.8 and 2.9.

Table 2.8 Post processing storage stability (injector port stability)

PES	Nominal concentration (µM)	% PES Stability as compared to time 0 (mean ± S.D)
QC 1	1	101 ± 4.0
QC 2	16	100± 0.70
QC 3	24	94 ± 0.52

Table 2.9 Post processing storage stability (injector port stability)

PE	Nominal concentration (µM)	% PE Stability as compared to time 0 (mean ± S.D)
QC 1	6.25	103 ± 7
QC 2	100	98 ± 2
QC 3	150	94 ± 3

2.3.5 Results for validation

The retention time, LLOQ and regression coefficients for the analysis of PE and PES for n = 4 analytical runs are summarized in Table 2.3. The intraday and interday results for accuracy and precision at the LLOQ and the QC concentrations are presented in Tables 2.4, 2.5, 2.6 and 2.7 For the QC samples, both interday and intraday accuracy showed less than 15.70 % DFN and the precision was within 12.81% CV, for PE as well as PES. There was a good linear relationship ($1/Y^2$ weighted) between ratio of peak areas of the analytes and E2-3G and concentration (x) over the concentration range of 0.39 – 200 μM for PE and 0.0625 μM – 32 μM for PES with linear regression yielding $y = (0.1331 \pm 0.0006)x – (0.000491 \pm 0.000057)$ for PES and $y = (0.1555 \pm 0.0064)\ x+ (0.00547 \pm 0.00652)$ for PE. The method was found to be specific, linear, precise and accurate. The present method was applied to study the saturation of PES formation using the LS180 human intestinal cell model (HIC), recombinant SULT1A3 enzyme and human intestinal cytosol. In this study a broad substrate (PE) concentration range was used from 1 μM – 3000 μM for LS180 intestinal cells and 3.125 μM – 200 μM for recombinant enzyme and HIC. These data are presented in the subsequent chapters.

2.4 Discussion

The fluorescent spectra of PE and PES in HILIC mobile phase which is ACN rich was compared to RP-HPLC mobile phase which has high percent of aqueous content were compared. The fluorescent intensity of PE and PES in HILIC mobile phase was almost 2-fold higher as compared to RP-HPLC mobile phase. A HILIC assay method was developed and validated as the US-FDA guidelines for bioanalytical validation. The method was found to be specific, linear, accurate, precise and stable with a short run time. The developed method allowed direct quantification of PES with a LLOQ of 63 nM and had several advantages over the indirect methods used to determine PES. Although the absolute extraction efficiency was

low for the analytes, the % C.V was low (< 4%) and hence extraction was precise, reproducible and consistent. The developed method was convenient to use but at the same time its development was challenging with regards to analyte retention and separation, phase separation, and the sample preparation techniques. It was also envisioned to adapt the developed method in order to analyze PE and PES in plasma samples.

3 LS180 cell-based assays

3.1 Introduction and objectives

The LS180 is a human colon adenocarcinoma epithelial cell line. The LS180 cell line was used as a potential model to investigate the sulfation of PE. Cell lines are used as a screening tool in order to investigate drug metabolism. Sulfation activity of acetaminophen (which has a phenolic moiety) is observed with the LS180 cell line. Acetaminophen sulfation does not occur in the Caco-2 cells grown in a flask due to incomplete differentiation (21). Cell differentiation is necessary for Caco-2 cell in order to express SULT enzymes (8). The major limitations of the Caco-2 cell line are long term culturing time (3-4 weeks) and low expression of drug metabolizing enzymes. Although the *in situ* human intestinal perfusion studies gives a closer depiction on the physiological level, it suffers from several disadvantages such as complicated technology, short term viability and lack of tissue availability. The main aim behind this cell-based studies was to check the ability of the phenolic GRAS compounds to cross the intestinal cell membrane and reach the SULT enzyme in order to achieve the desired inhibition. The K_m value observed for PE sulfation was used to decide upon the substrate concentration to be used for further studies.

The objective of the presented work was to test the feasibility of using phenolic GRAS compounds to inhibit the PES formation and hence to increase the bioavailability of PE. In order to test this hypothesis *in vitro* LS180 cell model was used. The specific aims included

(i) Determine time and concentration dependent PES formation in LS180 cell model. (ii) Determine the enzyme kinetic parameters (K_m and V_{max}) using PE as the substrate. (iii) Determine the IC_{50} value for each GRAS compound used as an inhibitor for PES formation. (iv) Investigate whether the unknown metabolite seen in the chromatographic method is an MAO metabolite.

It was also observed that as the dose of the PE is increased there was depletion of PAPS and the formation of the sulfate metabolite is decreased.

3.2 LS180 Cell Culture (14)

LS180 cells were grown in DMEM with high glucose (4.5 g/L), 10% FBS, and 1% non-essential amino acid at 37 ^{o}C with 5% CO_2. The cells were fed every other day. The cell culture medium was continuously increased from 12 to 25 mL in 75 cm^2 flask to keep pace with increasing metabolic demands of the growing cells. When LS180 cells were sub-cultured, old medium was removed and the 75 cm^2 flask was filled with 5 mL fresh medium. Since trypsin changes the cell type, it was not used for cell sub-culture. Instead, cells were gently scraped by a cell scraper. In order to disperse the cells, cells were passed through a 23G ×1 needle for 6 cycles and dispensed to a new flask. Cells were sub-cultured for 6-7 days with a dilution of 1:10. Cell passage number was between 42 and 60. A new vial of LS180 cells was recovered from the liquid nitrogen about every 3 months. LS180 cells were seeded at a concentration of 1.9 x 10^5 cells/mL in the 24 well plate (14). The experiment was carried out on the 4^{th} day after plating the cells (14).

3.3 Optimization experiments

The HILIC assay method developed was used in order to study the PES formation. In order to study the PES formation it was important to determine the linearity of the PES formation with respect to time and substrate concentration of PE.

3.3.1 Time course study

In order to optimize the incubation time, the cells were incubated with DPBS buffer (calcium chloride 100 mg/L, magnesium chloride 100 mg/mL, potassium chloride 200 mg/mL, potassium phosphate monobasic 200 mg/L, sodium chloride 8g/L, sodium phosphate dibasic 2.16 g/L, D-glucose 1g/L and sodium pyruvate 36mg/L in water, pH 7.4). To each well of a 24-well plate 0.25 mL of start solution was added to each well. Time course study was done at 10 μM and 100 μM of PE in triplicates. The study was done up to 8 hours at 37°C in air incubator. The metabolic reactions were stopped using 1.0 mL ACN containing 24 μM E2-3G as the internal standard. Dextrose (50% w/v) was used for phase separation. Double extraction was done in order to increase the extraction efficiency and the second extraction was done using 1.0 mL ACN without E2-3G. The ACN layers from both the extractions were pooled and evaporated using a SpeedVac (Savant). The samples were then reconstituted with the 200 μL of mobile phase and injected into the HPLC as previously described.

3.3.1.1 Results

The results are shown below in Figures 3.1 to 3.4 and Table 3.1.

Figure 3.1 Disappearance of PE at 10 µM over 8 hours

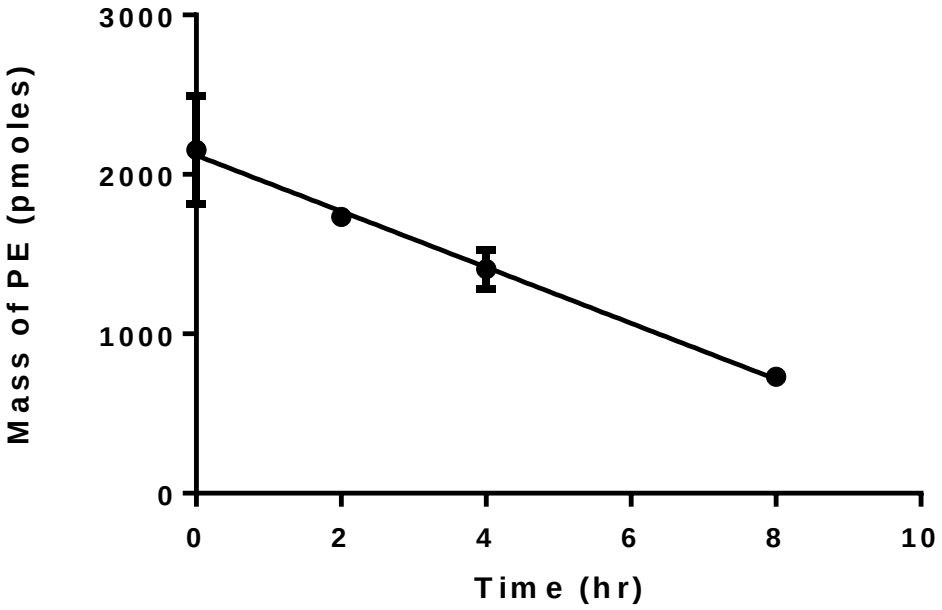


Figure 3.2 Formation of PES at PE (10 µM) over 8 hours

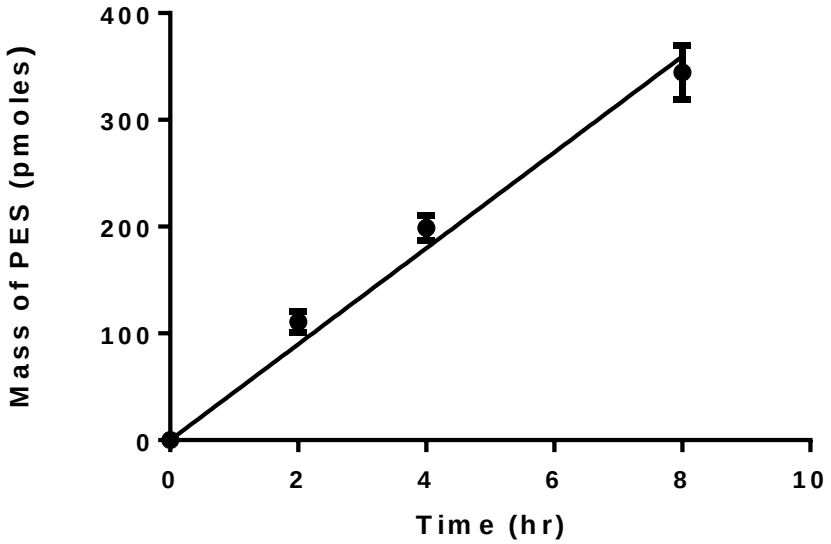


Figure 3.3 Disappearance of PE at 100 µM over 8 hours

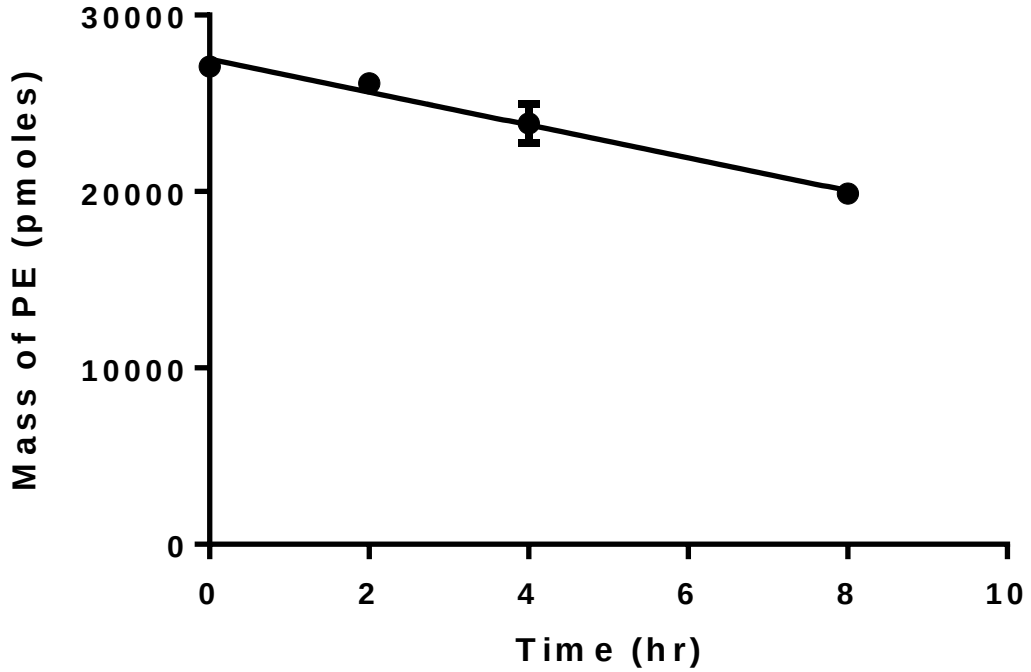
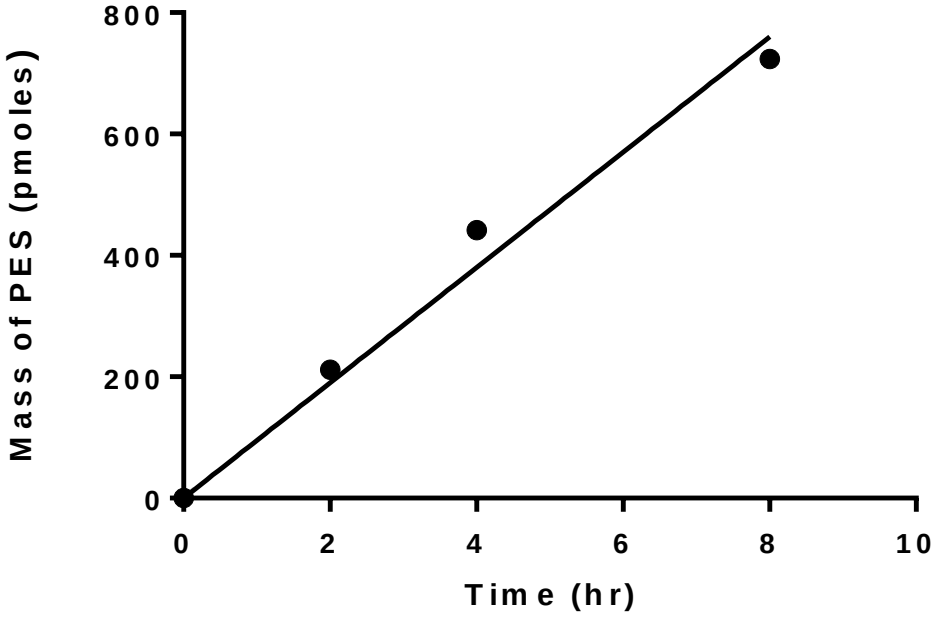


Figure 3.4 Formation of PES at PE (100 µM) over 8 hours



When PE (10 μM and 100 μM) was incubated with LS180 cells for 8 hours it was progressively sulfated over 8 hours (shown in Figures 3.2 and 3.4). The formation of PES and disappearance of PE were linear up to 8 hours with the following linear regression estimates:

Table 3.1 Linear regression estimates for the time course study

	R^2	Slope (± S.D) (pmoles/hr)
At PE (10 μM)		
Disappearance of PE	0.9215	-176 ± 16
Appearance of PES	0.9639	45 ± 1.7
At PE (100 μM)		
Disappearance of PE	0.8721	-931 ± 113
Appearance of PES	0.9842	95 ± 2.5

This study tells us about the linear range of the time course. It was observed that over 8 hours at 10 μM PE, the disappearance rate of PE was almost 4 times greater as compared to formation rate of PES. Furthermore at 100 μM PE, the disappearance rate of PE was almost 10 times greater as compared to formation rate of PES. Thus some other metabolism pathway apart from sulfation is also accountable for disappearance of PE using the LS180 cell model. Also from the data obtained, it was observed that as the concentration of the substrate is increased, the contribution of the sulfation pathway to total disappearance of PE becomes lesser and lesser. Thus PES formation appears saturable (or the cofactor PAPS may be depleted) within this range and another less saturable metabolic pathway contributes at higher concentrations of PE. Hence sulfation of PE appears to be a high affinity low capacity pathway

3.3.2 PES formation saturation study

In order to optimize the substrate concentration to be used for further studies, the cells were incubated with DPBS buffer (already defined earlier in the chapter). To each well 0.25 mL of start solution was added containing PE covering a wide concentration range of (1-3000 µM) for 8 hours (from incubation time optimization study) at 37°C in an air incubator. At the end of 8 hours, the metabolic reactions were stopped using 1.0 mL ACN containing 24 µM E2-3G as the internal standard. The samples at each concentration were prepared in triplicate and assayed by HPLC as previously described.

3.3.2.1 Results

Non-linear regression model was used to fit the data using Graph Pad Prism v5 software (GraphPad Inc., San Diego, CA). It was observed that the formation of PES is a saturable process at higher concentrations of PE as shown in Figures 3.5 and 3.6. It was also observed that the Hill model gave a better fit to the data as compared to the Michaelis-Menten model with p-value < 0.05 (for F-test). F-test takes into account the variances and the number of parameter estimates for different models. The Hill model was chosen to fit the data as shown in Table 3.2.

Figure 3.5 Formation of PES on a linear scale

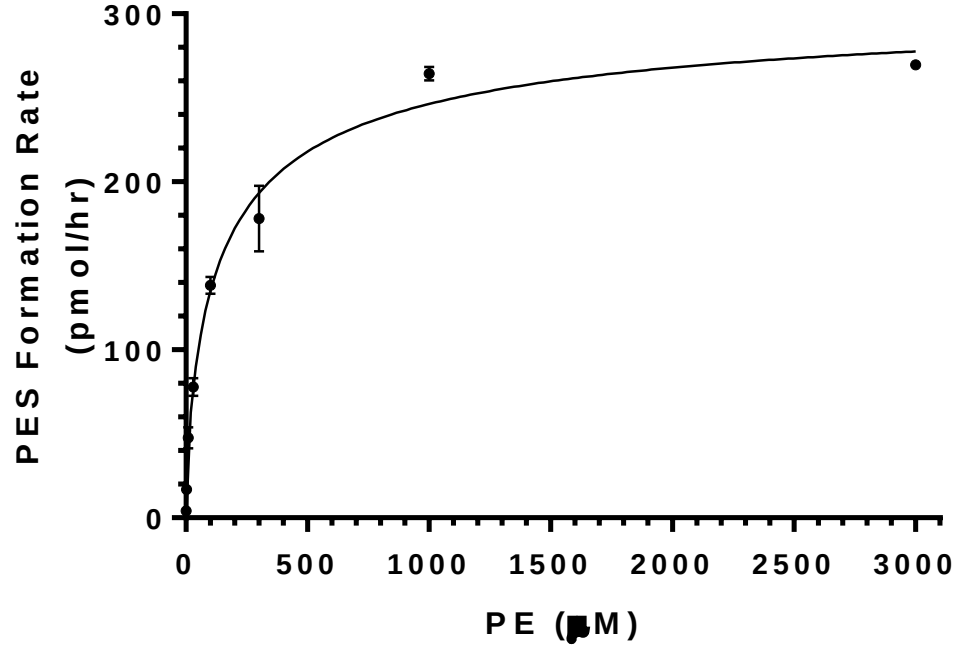


Figure 3.6 Formation of PES on a semi-log scale

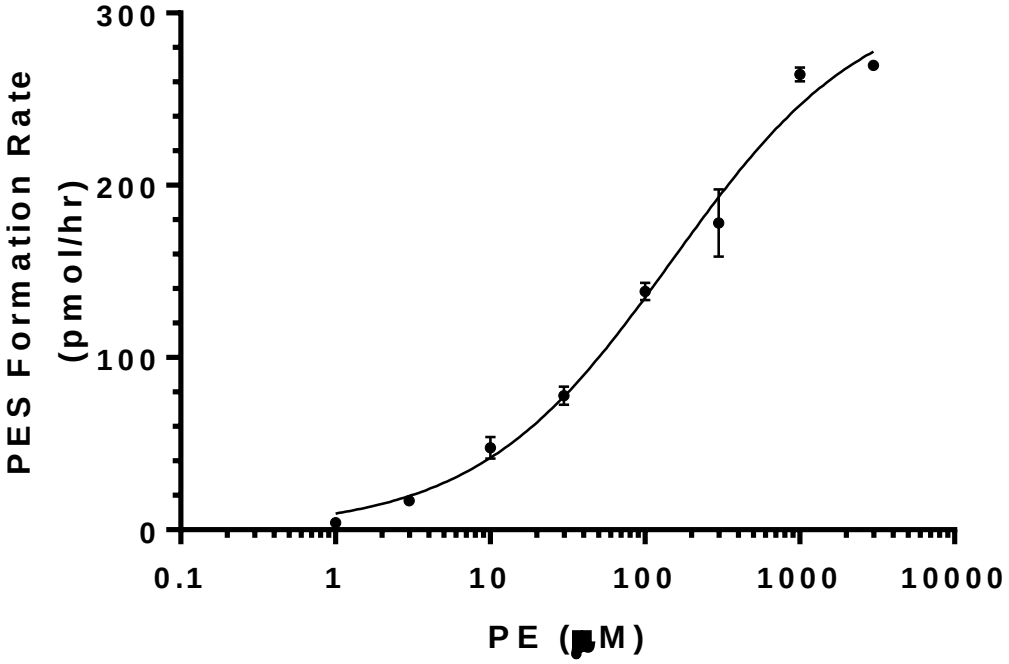


Table 3.2 Enzyme kinetic parameters using LS180 cell model

Parameter	Value
R^2	0.9727
Hill co-efficient	0.69 (95% C.I: 0.57-0.81)
K_m	149 µM (Hill model) (95% C.I: 86-213)
V_{max}	313 pmol/hr (95% C.I: 280-345)

The negative Hill co-efficient with the Hill model could be due to the lack of sulfur in the DPBS buffer. The system was falling short of sulfur as sulfur is required for the formation of PAPS. This effect was more prominent at higher concentrations of PE as PES formation was decreased and the curve shifted towards the right and its slope decreased giving rise to negative Hill co-efficient. There was a possibility that Hill co-efficient could have been 1.0 if the system had sufficient sulfur for the formation of PAPS.

The K_m value observed with this experiment for PE sulfation was used to decide upon the substrate concentration to be used for inhibition study of PES formation with the use of phenolic GRAS or dietary compounds.

3.3.3 Inhibition of PES formation using Phenolic GRAS compounds

The efficacy of PE has been questionable due to its low and variable bioavailability. This can be attributed to the extensive pre-systemic metabolism of PE. Employing strategies to inhibit the metabolism of PE can increase the oral bioavailability of PE. PE undergoes sulfate conjugation to form the sulfate metabolite PES, which is the major metabolite (45.7%) when given orally. Less PE is biotransformed to 3-hydroxymandelic acid by the oral route (24.2%) as compared to I.V route (56.9%) [2]. This tells us that sulfation is the major metabolic route *in vivo* when PE is given orally. Sulfation of PE occurs at its phenolic group which is a common feature of the SULT1 substrates. Prodrugs can be synthesized to protect the phenolic

hydroxyl group, but the stability and toxicity of these prodrugs in the gastric fluid is unknown. This would require extensive investigation. Certain phenolic GRAS compounds which have structural features similar to PE can be substrate for this SULT enzymes and inhibit the metabolism of PE through sulfation pathway. The phenolic GRAS or dietary inhibitors that were selected for the study were propyl paraben, pterostilbene, quercetin, isoeugenol, eugenol, ethyl vanillin, raspberry ketone, magnolol, resveratrol, zingerone, vanillin and curcumin.

3.3.3.1 Experimental Setup

Start solutions containing 50 µM PE as the substrate and various GRAS compounds used as inhibitors having concentrations of 50, 10, 2, 0.4 µM were prepared. Stock solutions (25mM) of various GRAS compounds in DMSO was used and final DMSO concentrations were maintained at 0.5% v/v.

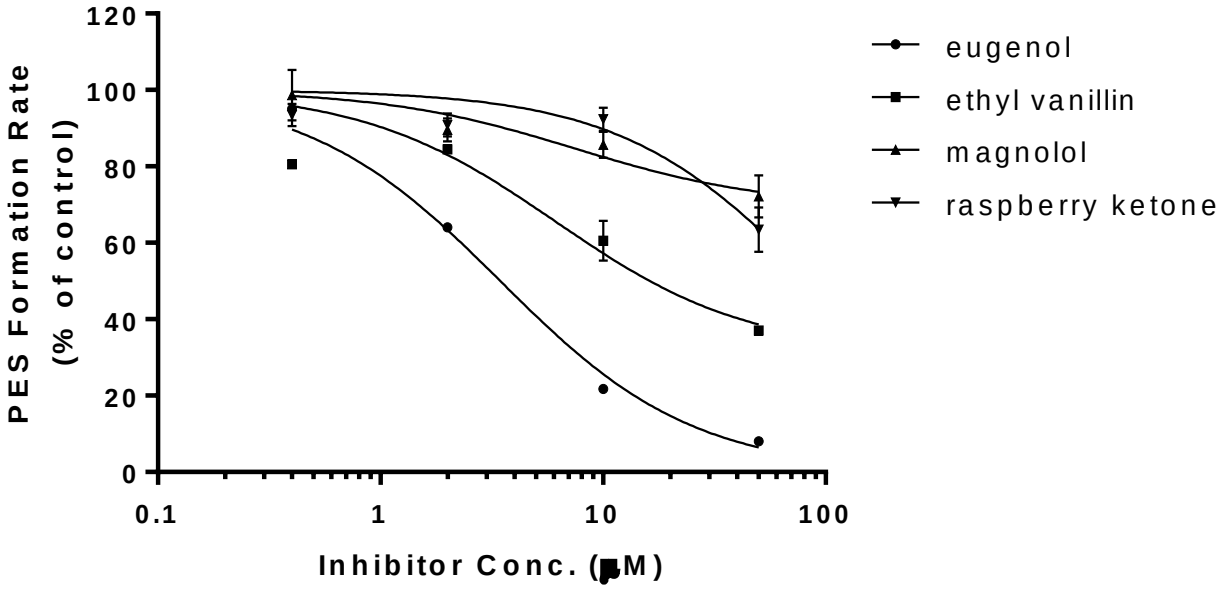
In order to study the effect of these compounds on metabolism of PE, LS180 cells were treated with 250 µL of these start solutions with various inhibitor concentrations for 8 hours (from incubation time optimization study) at 37°C with 5% CO_2. LS180 cells containing control samples with PE alone and no inhibitor were also incubated. At the end of 8 hours, the metabolic reactions were stopped using 1.0 mL ACN containing 24 µM E2-3G as the internal standard and analyzed as previously described. The samples at each concentration were prepared in triplicate.

3.3.3.2 Results

Data of inhibition of PES formation (8 hrs incubation, PE=50µM) in LS180 cells, were fitted to the equation $Y=YMAX/[1+10^{(X-IC50)}]$ fitted to the data obtained in the presence of various inhibitors. YMAX was set to the average of 6 determinations in the absence of inhibitor (444±9 pmol/hr). The model assumed: 1. no PES formation at maximal concentrations of the

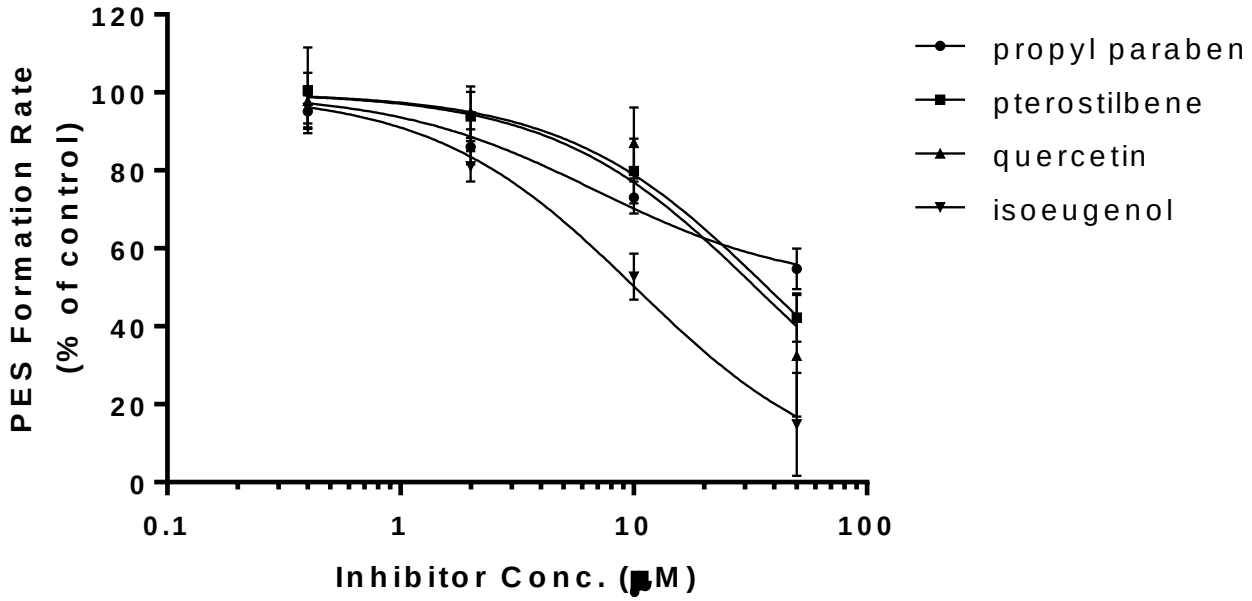
inhibitor and 2. at 0 µM concentration of the inhibitor, the PES formation was 100% (bottom = 0 and top = 100 respectively). Also it was initially assumed to be a competitive inhibition and Hill co-efficient was not included in the model. Nonlinear regression output (GraphPad Prism v5) is shown in Figures 3.7, 3.8, 3.9, 3.10 and Table 5 (below).

Figure 3.7 Inhibition of PES formation using eugenol, ethyl vanillin, magnolol and raspberry ketone



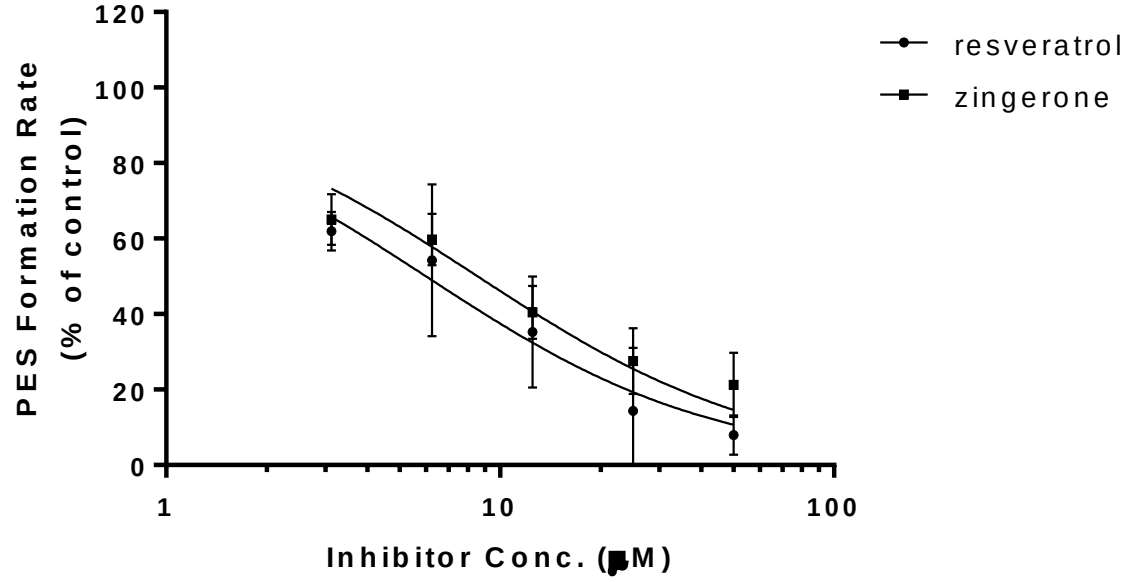
Y_{max} (control) of three determinations for Eugenol: 795 pmol/hr, Ethyl Vanillin: 795 pmol/hr, Magnolol: 550 pmol/hr, Raspberry Ketone: 795 pmol/hr

Figure 3.8 Inhibition of PES formation using propyl paraben, pterostilbene, quercetin and isoeugenol



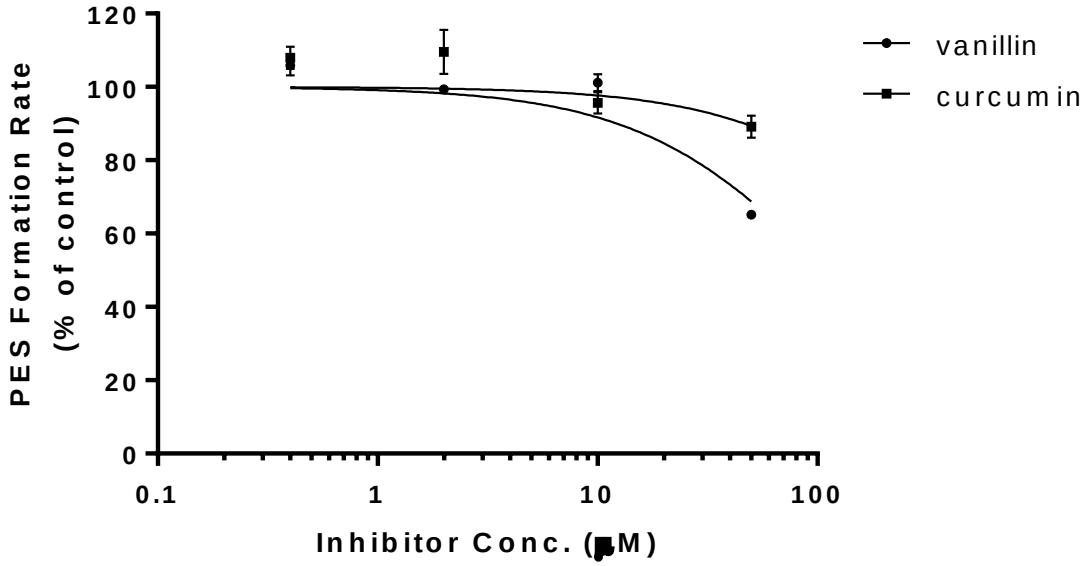
Y_{max} (control) of three determinations for Propyl Paraben: 720 pmol/hr, Pterostilbene: 484 pmol/hr, Quercetin: 484 pmol/hr, Isoeugenol: 486 pmol/hr

Figure 3.9 Inhibition of PES formation using resveratrol and zingerone



Y_{max} (control) of three determinations for Resveratrol and Zingerone: 444 pmol/hr

Figure 3.10 Inhibition of PES formation using vanillin and curcumin



Y_{max} (control) of three determinations for vanillin: 506 pmol/hr and curcumin: 578 pmol/hr.

Table 3.3 IC_{50} values for the inhibitors

Compound	IC_{50} (µM)	95% C.I
eugenol	3.5	3.0-4.0
resveratrol	6.0	4.2-8.5
zingerone	8.6	6.7-11
isoeugenol	10	7.7-13
ethyl vanillin	19	12-30
quercetin	33	21-53
pterostilbene	37	28-51
propyl paraben	46	31-68
raspberry ketone	>50	-
vanillin	>50	-
magnolol	>50	-
curcumin	>50	-

From the IC_{50} values obtained it was observed that resveratrol and eugenol were good inhibitors of PES formation using LS180 cell model as compared to other GRAS compounds.

In the previous studies curcumin showed a greater effect on preventing total PE disappearance. But curcumin showed low inhibition of sulfation of PE. Hence this tells us that curcumin may prevent the formation oxidation of PE and to a very less extent the formation of the sulfate metabolite. The calculated IC_{50} values obtained for raspberry ketone, vanillin, magnolol and curcumin were beyond the experimental range of the inhibitor concentration used. Hence the IC_{50} values are reported as greater than 50 µM.

3.3.4 Investigation of the unknown metabolite

From the PES saturation study, an unknown peak at about 1.9 minutes was observed with the HILIC assay method. The intensity of this unknown peak was found to increase proportionally with respect to the concentration of the substrate used. Also from the time course study with LS180 cell model, we know that there other pathways involved other than sulfation. Furthermore the unknown metabolite is less retained as compared to the sulfate metabolite on the HILIC column which is in line with our hypothesis that it could be an MAO metabolite as they are less hydrophilic as compared to the sulfate metabolite.

The major metabolites of PE when given orally are sulfate and the MAO metabolite. Hence further experiments were done in order to confirm whether the unknown peak is an MAO metabolite. In an attempt to determine the structure of this metabolite, the HPLC eluent containing the unknown peak was collected and pooled from several injections of LS180 cell samples incubated with PE. The eluent was concentration by evaporation, and injected using the HILIC method described above, but passing the column eluent through a Waters QDa mass detector (Waters Corp., Milford, MA) instead of the fluorescence detector. Broad-range scanning from 150 to 300 Da in both positive ion and negative ion modes was performed. However, at the retention time of the unknown peak (1.9 minutes), no discernible peak masses were detected. Additionally, small peaks at other retention times were seen but did

not match with any expected molecular masses other than PE. Hence the identification of the unknown metabolite was not completed.

3.3.4.1 Experimental setup

The HILIC assay method developed was used in order to study the PES formation. LS180 cells were seeded at a concentration of 1.9 x 10^5 cells/mL in the 24 well plate (14). The experiment was carried out on the 4^{th} day after plating the cells (14). Hence the effect of the following MAO inhibitors on the intensity of the unknown peak was investigated:

Table 3.4 Type and concentration of MAO inhibitors used (22)

Inhibitor	Target	IC_{50} values(µM)		Concentration
		MAO-A	MAO-B	(µM)
pargyline	Non-selective	-	-	10.00
selegiline	MAO-B	1.7	0.01	0.08
clorgyline	MAO-A	0.016	3.5	0.16
resveratrol	MAO-A	0.3	15.8	3.00
pterostilbene	MAO-B	13.4	0.14	1.40

The concentrations of MAO inhibitors used were much above the IC_{50} value for that particular MAO-A or MAO-B activity in order to ensure nearly complete inhibition of the formation of the MAO metabolite. The concentrations were chosen in such a way that it is much above the IC_{50} value for the particular enzyme it is selective for and much below the IC_{50} value for the particular MAO enzyme it is non-selective. The concentrations of selegiline and clorgyline used were based on IC_{50} values obtained from the literature. The concentration of clorgyline used was 10 times more than the IC_{50} value for MAO-A but it was much less as compared to IC_{50} value for MAO-B. Similarly the concentration of selegiline used was 10 times higher than IC_{50} value of MAO-B and much less than IC_{50} value for MAO-A. The concentrations for resveratrol and pterostilbene were selected based on IC_{50} values obtained from previous work done in the lab (14). The concentration of resveratrol used was 10 times

more than the IC_{50} value for MAO-A but it was much less as compared to IC_{50} value for MAO-B. Similarly the concentration of pterostilbene used was 10 times higher than IC_{50} value of MAO-B and much less than IC_{50} value for MAO-A. This was done so that they selectively inhibit either MAO-A or MAO-B. In order to study the effect of these MAO inhibitors on the formation of the unknown metabolite, LS180 cells were treated with 250 µL of these start solutions with various inhibitor for 5 hours (from incubation time optimization study) at 37°C with 5% CO_2. The concentration of PE used was 50 µM. LS180 cells containing control samples with PE alone and no inhibitor were also incubated. At the end of 5 hours, the metabolic reactions were stopped using 1.0 mL ACN containing 24 µM E2-3G as the internal standard. The samples were processed in the same way as described earlier. The samples at each concentration were prepared in triplicate.

Figure 3.11 Inhibition of the unknown metabolite in presence of MAO inhibitors

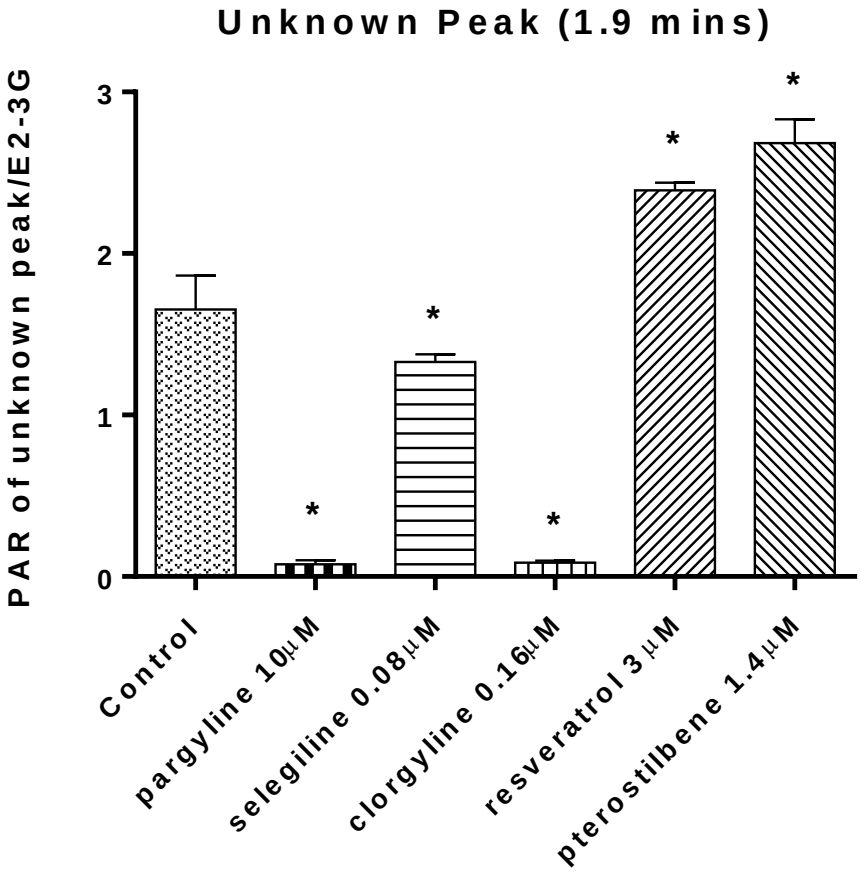


Figure 3.12 Inhibition of the PES formation in presence of MAO inhibitors

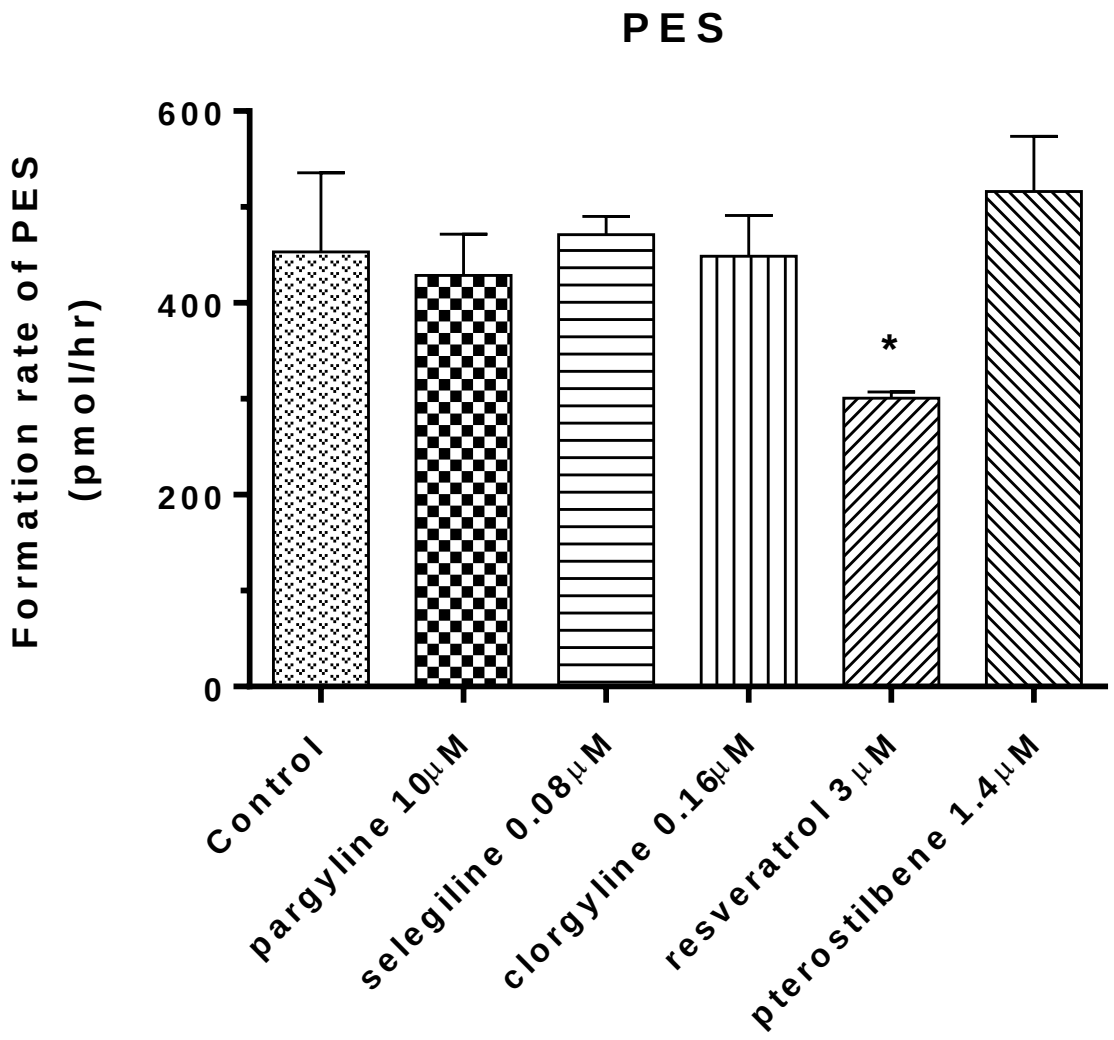
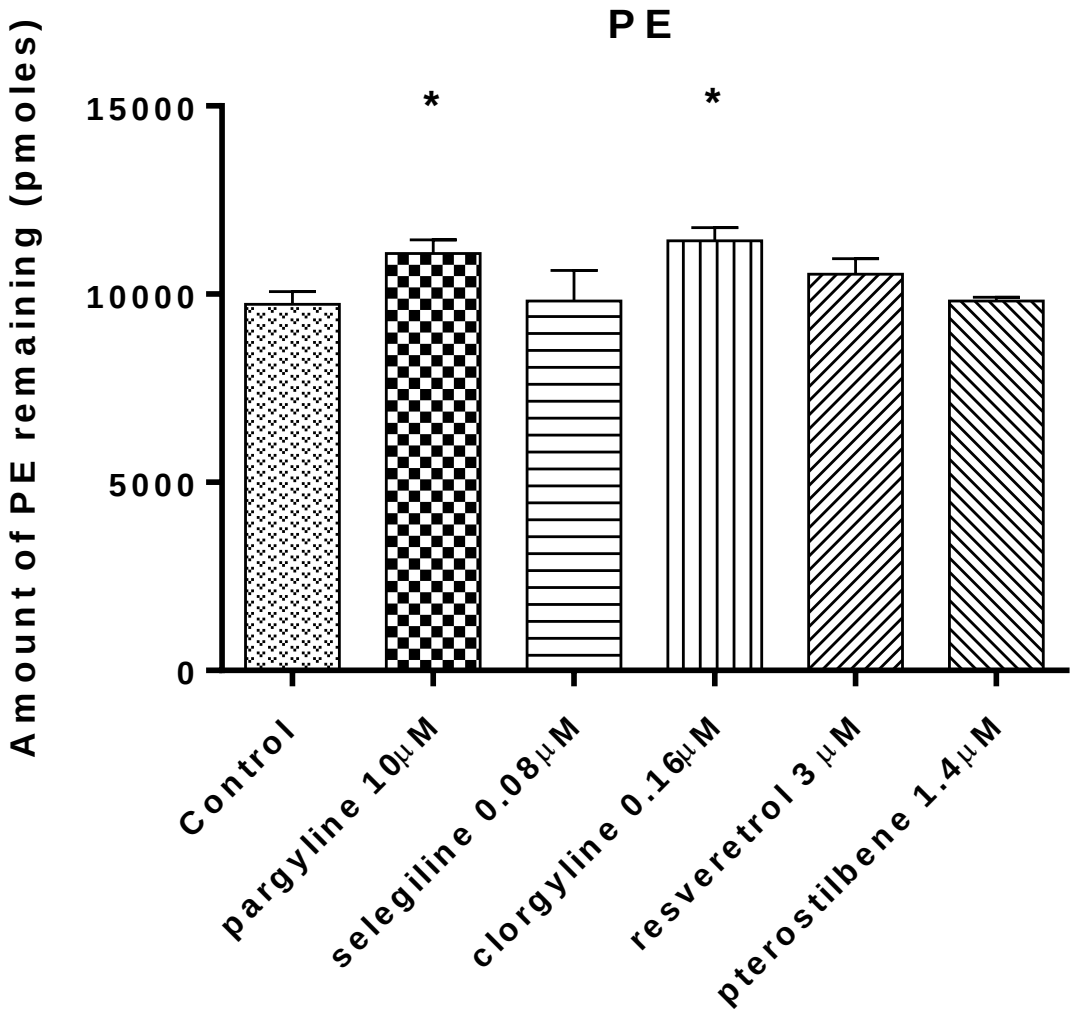


Figure 3.13 Amount of PE remaining in presence of MAO inhibitors



3.3.4.2 Results

From the data obtained from the figure 3.11 it was observed that pargyline (non-selective for MAO-A and MAO-B at a high concentration of 10 μM) and clorgyline (selective inhibitor for MAO-A at low concentration) almost completely inhibit the formation of the unknown metabolite whereas the formation of the unknown metabolite is statistically significantly inhibited in presence of selegiline which is a selective inhibitor for MAO-B. Hence we can infer that the unknown compound is mainly an MAO-A metabolite. Also the amount of PE

remaining with pargyline and clorgyline was not greater than 15% with respect to the control. This tells us that MAO pathway is a minor pathway for metabolism of PE in the LS180 cell model at 50 µM PE. Structural elucidation of this metabolite was not achieved. The possible reason could be, 3-hydroxy mandelaldehyde which is one of the metabolite of MAO pathway is highly reactive and can form combination products in presence of cellular amines. Carbonyl compounds like 3-hydroxy mandelaldehyde react with amines to form Schiff bases which are combination products from various possibilities, hence it becomes difficult to determine their structures. Also 3-hydroxy mandelaldehyde can react with aldehyde dehydrogenase to form an oxidation product that is 3-hydroxy mandelic acid. Also the contribution of the sulfation and MAO pathway could not be determined due to lack of identification of the unknown MAO metabolite(s).

3.4 Discussion

LS180 cell model served as a good source to investigate the sulfation of PE. The optimization experiments with the cell model helped in determining the incubation time and the substrate concentration to be used in order to test the effect of GRAS compounds on inhibition of formation of PES. From the IC_{50} values obtained with GRAS compounds using the LS180 cell model, resveratrol and eugenol showed highest inhibitory activity and were chosen for further studies with recombinant SULTs and HIC. The metabolism of PE in LS180 cells could be inhibited either at the entry step in to the cell, or after getting in to the cell, it inhibits the SULT enzyme. Further studies with recombinant SULTs and HIC were done in order to confirm the same. At this point it was of interest to determine which SULT enzyme is majorly responsible for sulfation of PE and also to determine the mechanism of inhibition with GRAS compounds using HIC. The study with MAO inhibitors (selective for MAO-A and MAO-B) suggested that the unknown metabolite is mainly an MAO-A metabolite.

4 Determination of PES formation in *in vitro* systems like Recombinant Enzymes and Human Intestinal Cytosol

4.1 Introduction

Experiments done using LS180 human intestinal cell model demonstrated inhibition of PE sulfation. Recombinant enzymes are over expressed systems and have greater total activity (per mg of total protein) than those produced by native sources. The advantages of using this recombinant enzyme system are the lack of interfering enzymes activity present in the cytosol and microsomes of cells, the flexibility to optimize reaction components, and the high expression level of the enzyme (thus facilitating product detection). It also helps in identifying the isoform responsible for metabolism, kinetic analysis, inhibitor screening and metabolite production. For isoform identification, recombinant enzymes are used to correlate the data obtained from microsomes or cytosol. Previous studies have demonstrated that SULT1A3 is expressed in many organs including the brain, gastrointestinal tract, kidney, liver and lung (23, 24). In order to further investigate the metabolism of PE, studies were done using recombinant SULT1A3 and human intestinal cytosol (HIC). The work done by Yamamoto et.al. suggested that SULT1A3 was the major human SULT responsible for the sulfation of phenylephrine (9). Taskinen et al. determined the sulfation rates for 53 catechol

compounds by six expressed human SULT isoforms and found the highest activity and broadest reactivity with SULT1A3 (25).

The studies also revealed that the kinetics of SULT1A3-mediated sulfation of phenylephrine appeared to be in the same order of magnitude as that of sulfation of dopamine (9). The Km value of phenylephrine was 17.54 µM, and the Vmax /Km (mL/min/mg) was 1.92 (9). The reported Km values of SULT1A3 toward dopamine ranged 2.2 to 8.56 µM and the Vmax/Km (mL/min/mg) ranged 9.73 to 228 (9). This can be due to the structural similarity between dopamine and phenylephrine. Mutagenesis studies and analysis of the crystal structure of SULT1A3 indicate that Glu146 is primarily responsible for the differences in the substrate specificity of SULT1A3 compared to SULT1A1 (26). The presence of the Glu146 is also proposed to interact with the Tyr240 residue to enhance hydrogen bonding with catechols (26). Overall rates of sulfation with SULT1A3 in the intestine tend to be higher than the liver (up to 10-fold) (10, 12).

Thus intestine represents a considerable barrier to the oral bioavailability of sulfotransferase substrate drugs (10, 12, 27). Human intestinal cytosol would provide an enzyme source with a physiologic expression level of SULTs.

4.2 Objective

The objective of the presented work to check the feasibility of the GRAS or dietary compounds on inhibition of PES formation using recombinant enzymes and HIC which would help in depicting a picture closer to the physiological level. The specific aims included (1) Optimization experiments using SULT1A3 and HIC (2) Generation an IC_{50} curve for resveratrol using HIC. (3) Determination of the mechanism of inhibition of PES formation using resveratrol in HIC system.

4.3 Methods

Human SULT1A3, 1 µg/mL protein (Cypex) batch no: S1A3002A and Catalogue no: CYP101 was used. PAPS (Sigma) was used and stock was prepared in pH 8.0 phosphate buffer. The concentration of PAPS in the body is approximately 23 nmoles/g body tissue.(28) Assuming a tissue density of ~1g/ml, it approximates 23 µM. The intestinal concentration of PAPS is a bit lower (14 nmoles/g), hence 20 µM is used typically.(28) All the solutions were prepared in water and pH of potassium phosphate solution was adjusted to 7.4 with potassium hydroxide solution. The following experimental design was setup based on the supplier's specifications.

4.3.1 Incomplete reaction solution

Table 4.1 Incomplete reaction solution

Component	Stock Concentration	Potency (X)	Desired final conc.	Stock Volume to add (µL)
Potassium Phosphate, pH 7.4	100 mM	2	50mM	375
Dithiothreitol (DTT)	1M	100	10mM	7.5
Magnesium Chloride	1M	200	5mM	3.75
Enzyme (SULT1A3)	10µg/uL	1000	1µg/mL	0.75
Substrate: PE	5mM	500	10µM	1.5
BSA	1%w/v	20	0.05%w/v	42.5
Water (q.s to volume)			-	244
Trigger solution: added to incomplete reaction solution				
PAPS	200 µM	10	20µM	75 µL

The enzyme was thawed on ice and incomplete reaction mixtures were prepared as listed in table 4.1 and kept on ice. Trigger solution (PAPS 5µL) was aliquoted in PCR tubes. To this

trigger solution 45 µL of incomplete reaction was added and mixed well. Care should be taken not to add the trigger solution to the bulk incomplete reaction solution, or the experimental design is ruined. The reaction times were 10 minutes and 60 minutes at 37°C. In order to stop the PES reaction, ACN (4 volumes) was added. The samples (n=3) were then centrifuged at 6000g for 13 minutes at 4°C. Supernatant (25 µL) was injected into the HPLC system. Blank samples were also prepared using only water instead of the trigger solution (PAPS) to check for any interferences. E2-3G was not used for SULT1A3 assay as there was no involvement of the extraction procedure as done for the cell-based assays. There was no blank interference observed. This study provided a good estimate for the incubation time (less than 60 minutes) to be used and also confirmed that PES formation could be detected using the same HILIC assay used for the cell-based assays.

4.3.2 Preparation of standard curves

Standard curve stock solution (A)

ACN (12 mL), 1.5 mL of potassium phosphate (100 mM pH 7.4), 30 µL of DTT (1M), 15 µL of $MgCl_2$ (1M) and 1.48 mL water were mixed. *Note: potassium phosphate (100mM) is expected to form precipitate in 80% ACN.*

Preparation of first standard containing 200 µM PE and 32 µM PES

Stock solution A (399.5 µL), 0.52 µL of PES (31mM PES), 100 µL of PE (1 mM) were added and mixed.

Serial dilutions were done using this first standard. To 250 µL of higher concentration of standard, 250 µL of stock solution A was added. Standard solutions containing PE (200, 100,

50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 µM) and PES (32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 µM) were prepared.

4.3.3 Chromatographic conditions

***Preparation of buffer for mobile phase*:** To 250 mL of HPLC grade water, 1250 µL of TEA and 780 µL of ammonium hydroxide was added. pH was adjusted to 4.5 with formic acid.

Mobile phase A: ACN: MeOH: Buffer (72: 8: 20)

Mobile phase B: 100% ACN

Separation of analytes was performed using a Phenomenex EC 100X4.6 mm Nucleodur HILIC, 3µm (Macherey – Nagel) column preceded by a HILIC Security Guard Cartridge. The HPLC system (Waters) consisted of the Alliance 2695 separations module and a 2475 fluorescence detector. Data were collected and analyzed using Empower 2 or Empower 3 software.

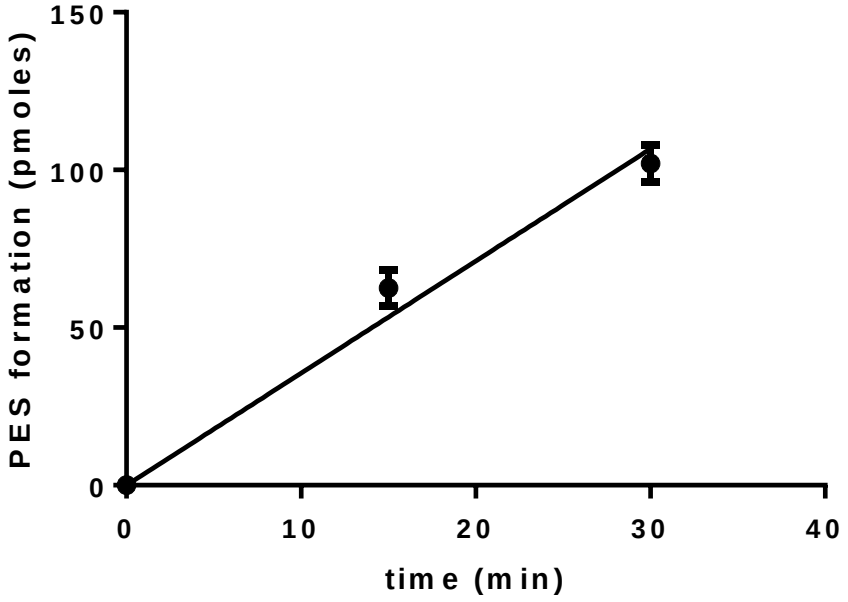
Determination of PE, PES and E2-3G was performed withµ isocratic elution (mobile phase A: mobile phase B, 90:10) at a flow rate of 0.8 mL/min. The injection volume was 25 µL. The runtime was 6.0 mins. The column temperature was maintained at 30°C and the autosampler compartment was set to 4°C**.** PE and PES were detected at the excitation wavelength of 268 nm and emission wavelength of 293 nm with retention times as follows: PES: 3.2 minutes, PE: 4.3 minutes.

4.3.4 Time course study with SULT1A3

4.3.4.1 Experimental setup

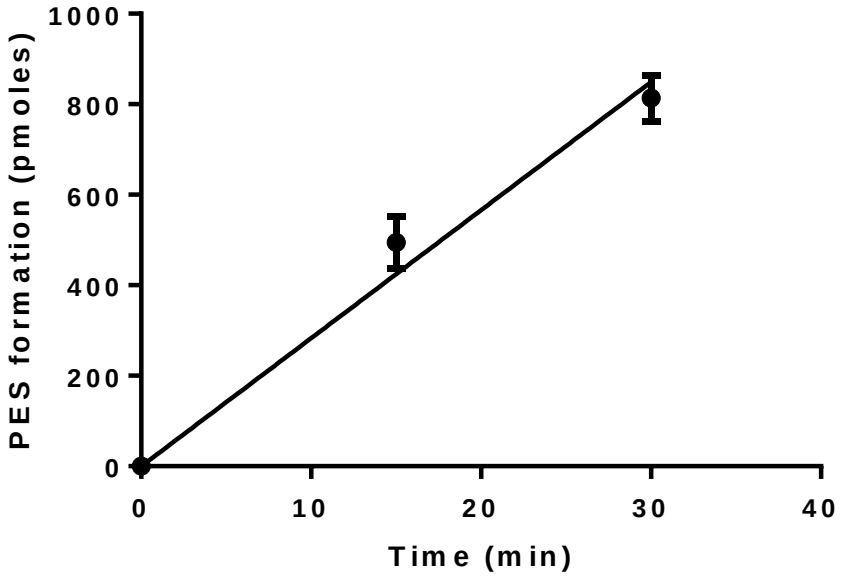
In order to optimize the incubation time with SULT1A3 the same experimental setup was used as discussed in Table 4.1. Standard curves were prepared in the same way as described in section 4.3.2. The incubation was terminated at 0, 15 and 30 minutes with 5 µM and 200 µM PE

Figure 4.1 Time course of PES formation with SULT1A3 at 5 µM PE



R^2 = 0.9754 , Slope = 3.6 pmoles/min (95% C.I: 3.3 - 3.9)

Figure 4.2 Time course of PES formation with SULT1A3 at 200 µM PE



R^2 = 0.9730, Slope = 28 pmoles/min (95% C.I: 26 - 31)

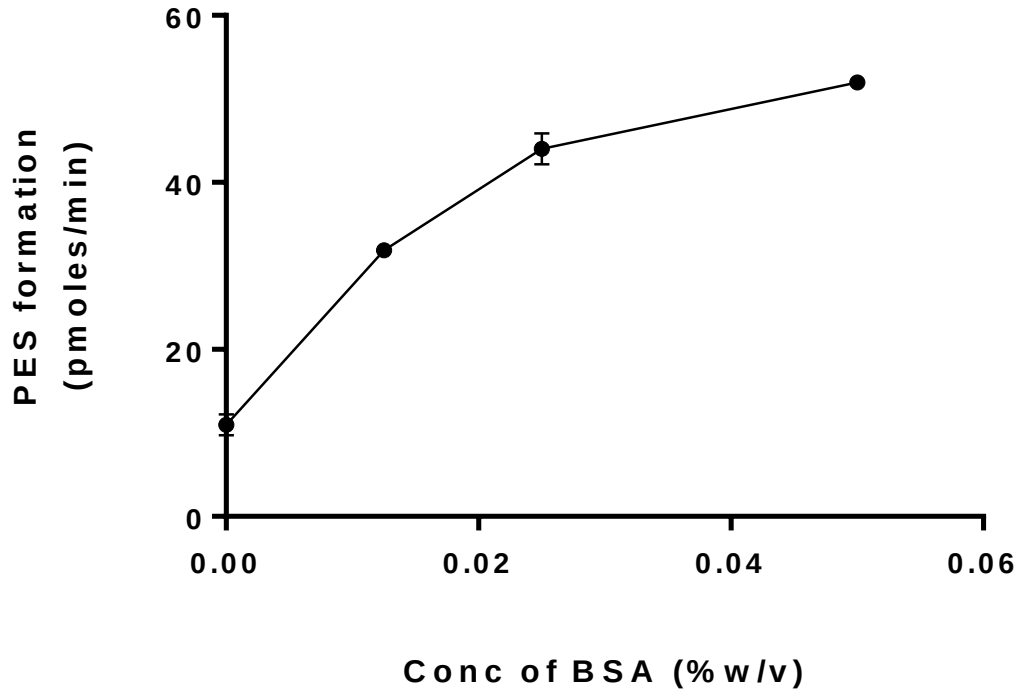
4.3.4.2 Results

The formation rate of PES was linear up to 30 minutes with 5 µM and 200 µM PE with good R^2 estimates. When slopes of this linear regression were compared at 5 µM and 200 µM PE, it was also observed that formation rate of PES was saturated with increasing substrate concentration from 5 µM to 200 µM. Clearance (CL) is the ratio of metabolism or excretion rate (dx/dt) and concentration (C). So although the actual formation rate is higher at the higher concentration, when formation rate is normalized to concentration, a decrease in clearance is observed as the system approaches saturation. This tells us that the enzyme is saturated at higher substrate concentrations and hence the sulfation pathway is a saturable process. At higher substrate concentrations the contribution of sulfation pathway is reduced as compared to lower substrate concentrations. From this study the chosen incubation time was 15 minutes in order to study the enzyme kinetic parameters using SULT1A3.

4.3.5 Optimization of concentration of BSA to be used

Bovine serum albumin is added to prevent non-specific binding of the protein and also it stabilizes the protein in the solution. During the time course study, non-specific binding of protein and/or inactivation were observed at lower concentration of SULT1A3 (0.25µg/mL). For this study 1% w/v of BSA solution was prepared using pH 7.4 phosphate buffer solution. Substrate concentration of PE used was 5µM and formation rate of PES was determined using 0%, 0.01%, 0.025% and 0.05% w/v BSA solutions. Reaction mixture was prepared as mentioned in Table 4.1. In order to stop the PES formation, ACN (4 volumes) was added. The samples (n=3) were then centrifuged at 6000g for 13 minutes at 4°C. Supernatant (25 µL) was injected into the HPLC system. The same HILIC assay method was used in order to analyze the samples. Standard curve was prepared as described under 4.2.2.

Figure 4.3 PES formation using various concentrations of BSA



SULT1A3 used = 1µg/mL

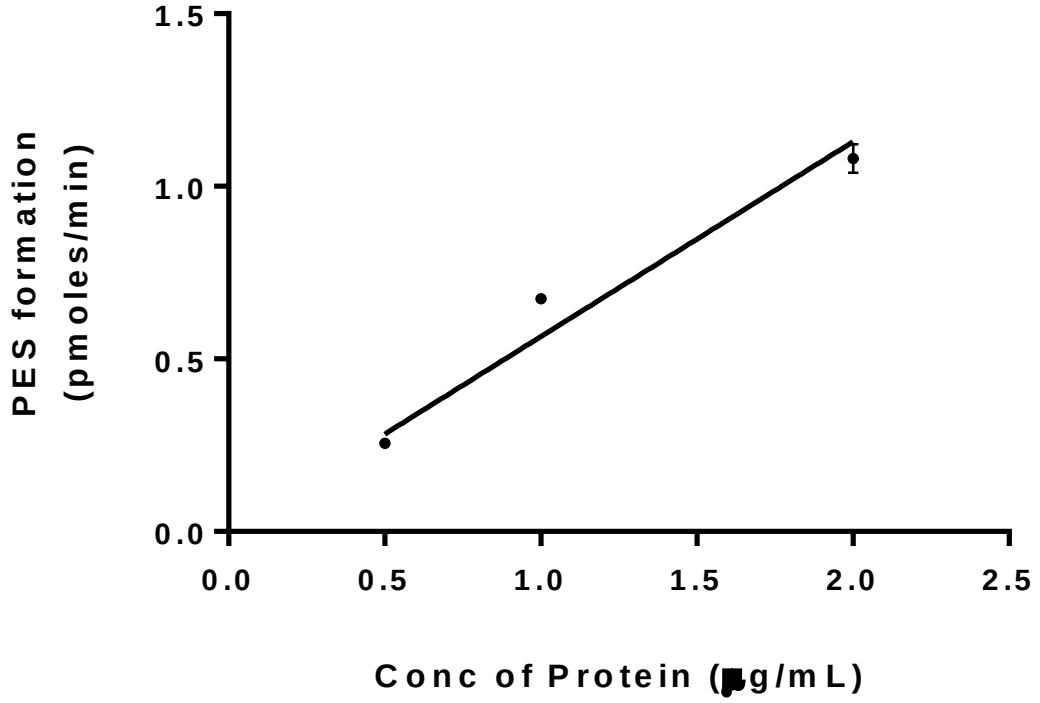
It was observed at 0.05% w/v BSA, the effect of BSA appeared optimal (a five-fold increase in PES formation) and hence 0.05% w/v BSA was selected as the BSA concentration to be used for further optimization experiments.

4.3.6 Optimization of protein concentration to be used for SULT1A3 assay

4.3.6.1 Experimental setup

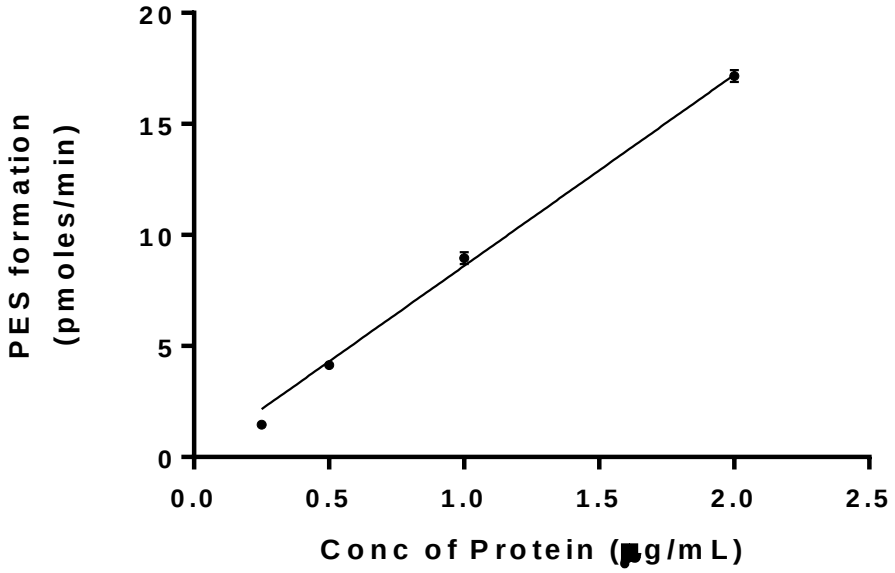
In order to optimize the protein (SULT1A3) concentration to be used for SULT1A3 assay the same experimental setup was used as discussed in Table 4.1. The study was done at 0.25 µg/mL, 0.5 µg/mL, 1.0 µg/mL and 2.0 µg/mL protein. Substrate concentrations used were 5 µM and 200 µM PE. BSA (0.05%w/v) was also added to the reaction mixture. Sample preparation was done in triplicate. Standard solutions was prepared as described under 4.2.2.

Figure 4.4 PES formation using various concentrations of SULT1A3 at 5 µM PE



Slope = 0.56 pmoles/min/µg/mL protein

Figure 4.5 PES formation using various concentrations of SULT1A3 at 200 µM PE



Slope = 8.6 pmoles/min/µg/mL protein (95% C.I: 8.4-8.9)

4.3.6.2 Discussion

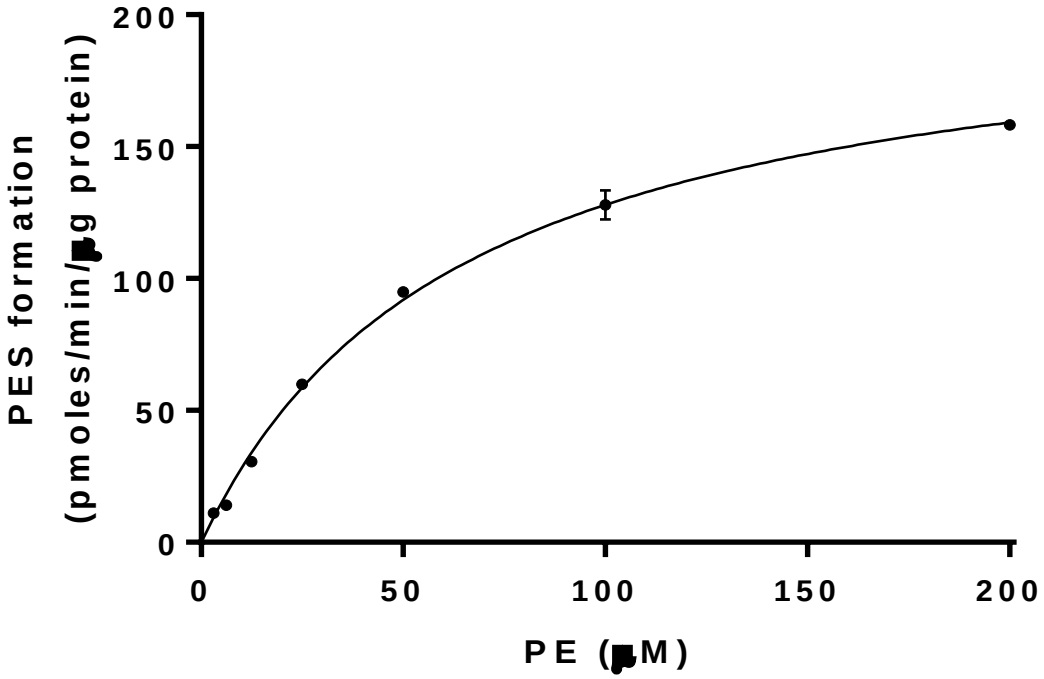
The SULT1A3 enzyme protein concentration was optimized to 1.0 µg/mL as it was found in the linear range and had good R^2 estimates at both 5 µM and 200 µM PE as shown in Figures 4.4 and 4.5.

4.3.7 Saturation of PES formation with SULT1A3

4.3.7.1 Experimental Setup

In order to optimize the substrate concentration to be used for further studies for SULT1A3 assay, the same experimental setup was used as discussed in Table 4.1. The study was done at 200, 100, 50, 25, 12.5, 6.25, 3.12 µM PE. The SULT1A3 protein concentration used was 1.0 µg/mL and the reaction time was 15 minutes with 0.05% w/v BSA as previously optimized. At the end of 15 minutes the metabolic reactions were stopped with 200 µL ACN. The samples (n=3) at each substrate concentration were then centrifuged at 6000g for 13 minutes at 4°C. Supernatant (25 µL) was injected into the HPLC system. The same HILIC assay was used as described under 4.2.3. The Michaelis-Menten non-linear regression model was used to fit the data using Graph Pad Prism v5 software.

Figure 4.6 Saturation of PES formation with SULT1A3



Protein Concentration used = 1 µg/mL

Table 4.2 Enzyme kinetic parameters using SULT1A3 enzyme

Parameter	Value
K_m	65 µM (95% CI: 58.1 to 71.2)
V_{max}	211 pmol/min/µg protein (95% CI: 202 to 220)

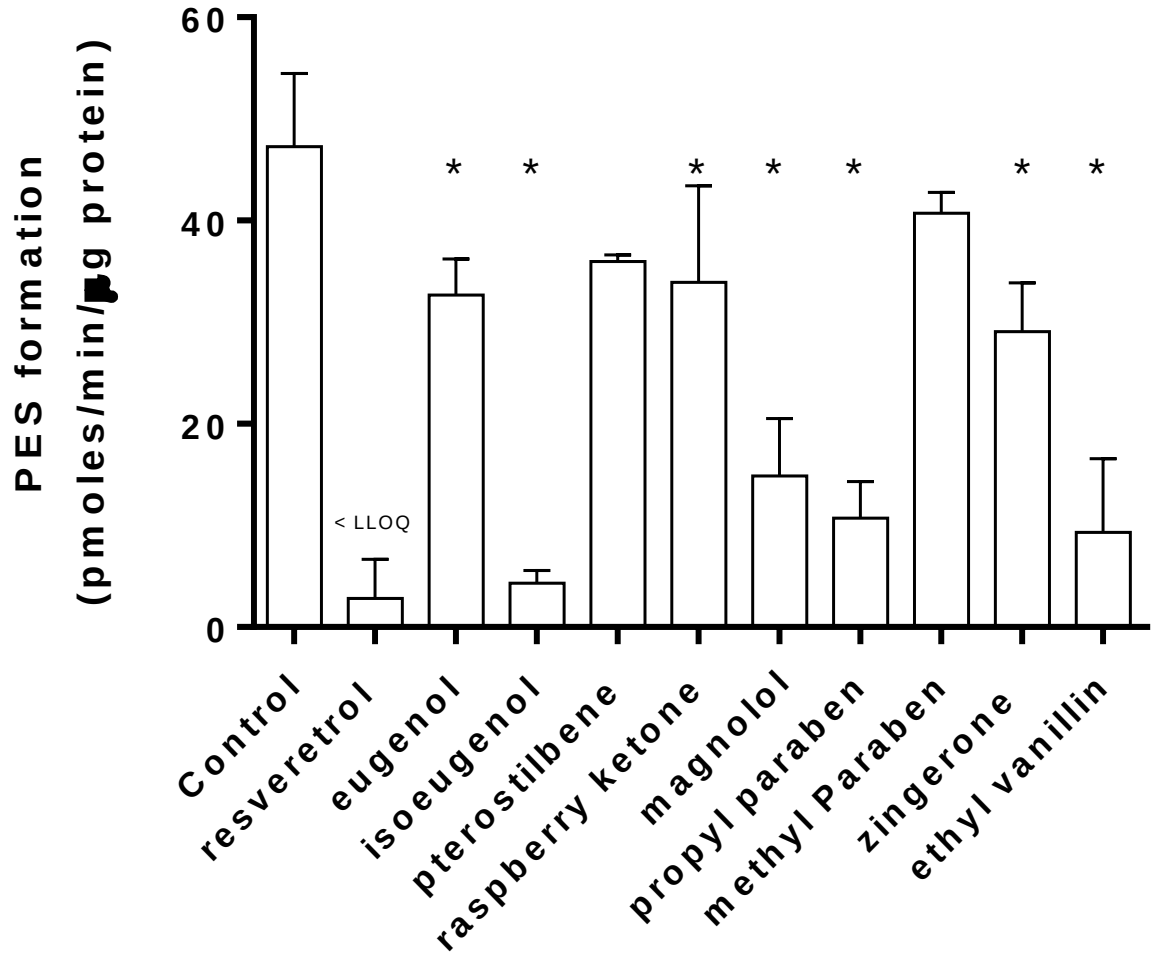
4.3.7.2 Results

It was observed that the formation of PES is a saturable process at higher concentrations of PE (Figure 4.6). The K_m value observed for PE with SULT1A3 enzyme was 65 µM (Table 4.2) which was almost two fold lower as compared to those observed with the LS180 cell model which was 149 µM. This difference in K_m can be attributed to the permeability of PE into the LS180 cells. V_{max} obtained with SULT1A3 and LS180 cell model could not be compared as the V_{max} for LS180 cell model was not normalized with amount of SULT1A3 protein present in the cells. This study gave a fair idea of the substrate concentration to be used for inhibitor screening below K_m value that is 65 µM.

4.3.8 Screening of inhibitors (GRAS compounds/dietary compounds) with SULT1A3

In order to study the mechanism of inhibition, various GRAS compounds were tested to select the best GRAS compound with maximum inhibitory activity on PES formation. The GRAS/dietary compounds that were tested are resveratrol, eugenol, isoeugenol, pterostilbene, raspberry ketone, magnolol, propyl paraben, methyl paraben, zingerone, ethyl vanillin. Almost complete inhibition of PES formation was seen at 50 µM with these GRAS compounds with LS180 cell model, hence 30 µM of inhibitor concentration was selected to test the inhibitory activity of these GRAS compounds using SULT1A3. At 30 µM inhibitor concentration the PES formation would be above LLOQ of the method and hence it would be quantifiable. The substrate concentration of 15 µM was chosen which was well below K_m value based on the PES formation saturation study with SULT1A3. Hence the formation of PES would be linear with respect to substrate concentration used.

Figure 4.7 Inhibitor Screening with SULT1A3



PE = 15 µM and Inhibitor = 30 µM. The error bar represents (±S.D).

4.3.8.1 Results

One way ANOVA analysis with a Dunnett's post-hoc test was used to compare the PES formation using various inhibitors with respect to control. Significant inhibition was seen was seen with almost all of the inhibitors except methyl paraben and pterostilbene. The highest inhibition of PES formation was seen with resveratrol. Hence resveratrol was selected to study the mechanism of inhibition study.

4.3.9 Time course study with Human intestinal cytosol (HIC)

Human intestinal cytosol (HIC) (mixed gender, 4mg/mL) was obtained from Xenotech, LLC (Lexena, KS) was prepared from mature enterocytes of the subcellular fractionization of intestinal villus tips.

4.3.9.1 Experimental setup

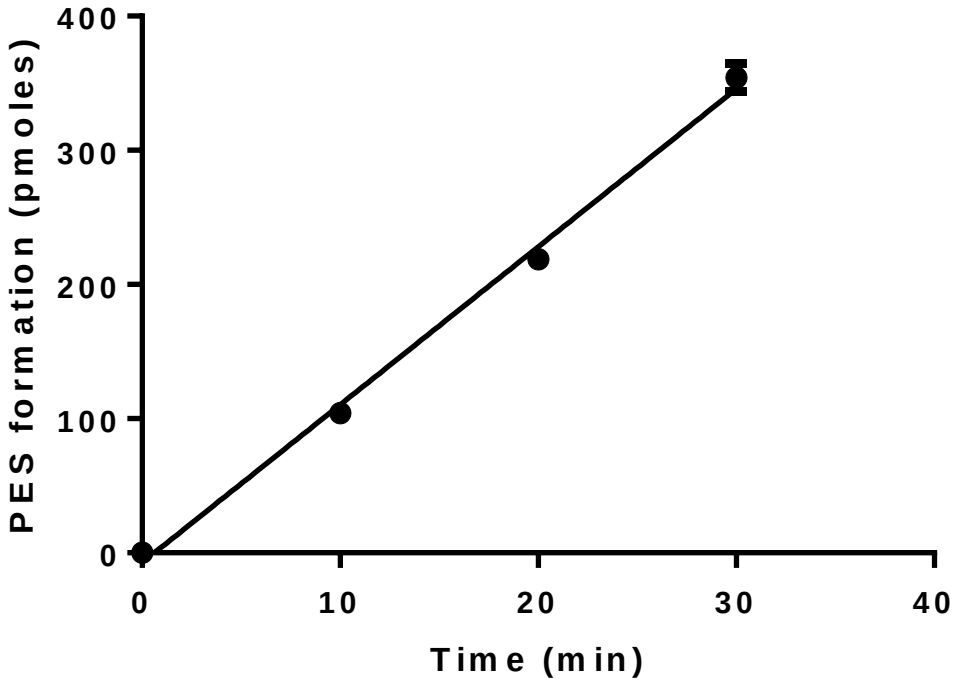
Table 4.3 Incomplete reaction solution

Component	Stock Concentration	Potency (X)	Desired final conc.	Stock Volume to add (µL)
Potassium Phosphate, pH 7.4	100 mM	2	50mM	375
Dithiothreitol (DTT)	1M	100	10mM	7.5
Magnesium Chloride	1M	200	5mM	3.75
HIC	4 mg/mL	16	250 µg/mL	42.20
Substrate: PE	5mM	500	10µM	1.5
BSA	1%w/v	20	0.5%w/v	33.75
Water (q.s to volume)			-	170.1
Trigger solution: added to incomplete reaction solution				
PAPS	200 µM	10	20µM	75 µL

The enzyme source (HIC) was thawed on ice and incomplete reaction mixtures were prepared as above and kept on ice. Trigger solution (PAPS 200µM, 5µL) was aliquoted in PCR tubes. To this trigger solution 45 µL of incomplete reaction was added and mixed well. Care should be taken not to add the trigger solution to the bulk incomplete reaction solution, or the experimental design is ruined. The time points used for the study were 0, 10, 20 and 30 minutes at 37°C at 5 µM and 200 µM PE. In order to stop the PES reaction, ACN (4 volumes) was added. The samples (n=3) were then, centrifuged at 6000g for 13 minutes at 4°C. Supernatant (25 µL) was injected into the HPLC system. And blank samples were also

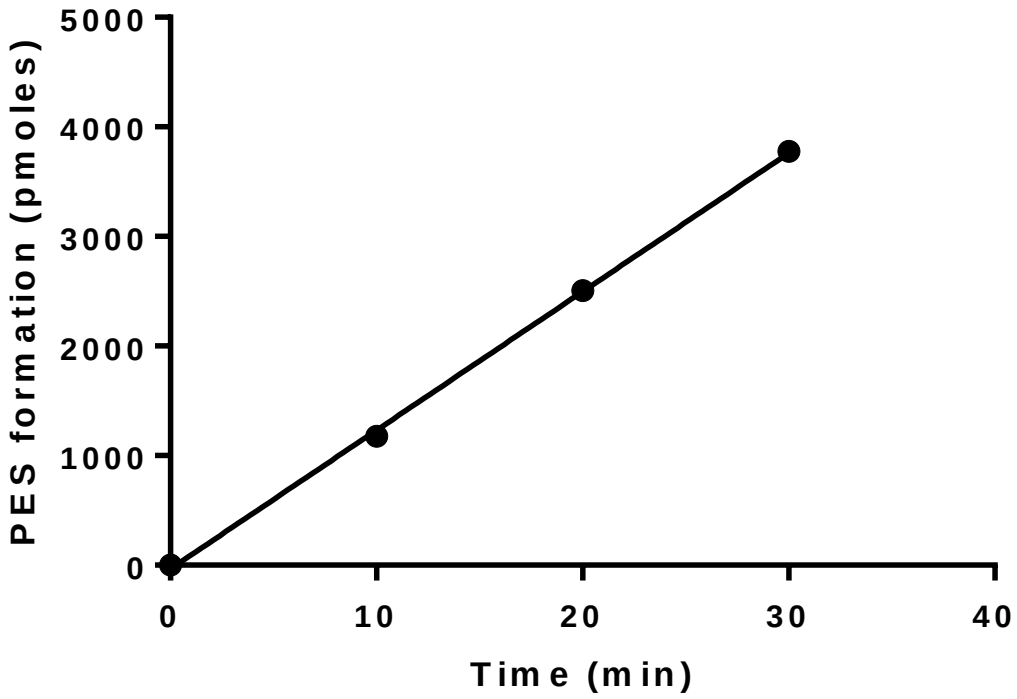
prepared using only water instead of the trigger solution (PAPS) to check for any interferences. E2-3G was not used for HIC assay as there was no involvement of the extraction procedure as done for the cell-based assays. There was no blank interference observed. This study provided a good estimate for the incubation time to be used and also confirmed that PES formation could be detected using the same HILIC assay used for the cell-based assays.

Figure 4.8 Time course study with HIC at 5 µM PE



Slope = 11.8 ± 0.3 pmoles/min (95% C.I: 11.2 – 12.3), R^2 = 0.9952

Figure 4.9 Time course study with HIC at 200 µM PE



Slope = 127 ± 1 pmoles/min (95% C.I: 124 – 129), R^2 = 0.9992

4.3.9.2 Results

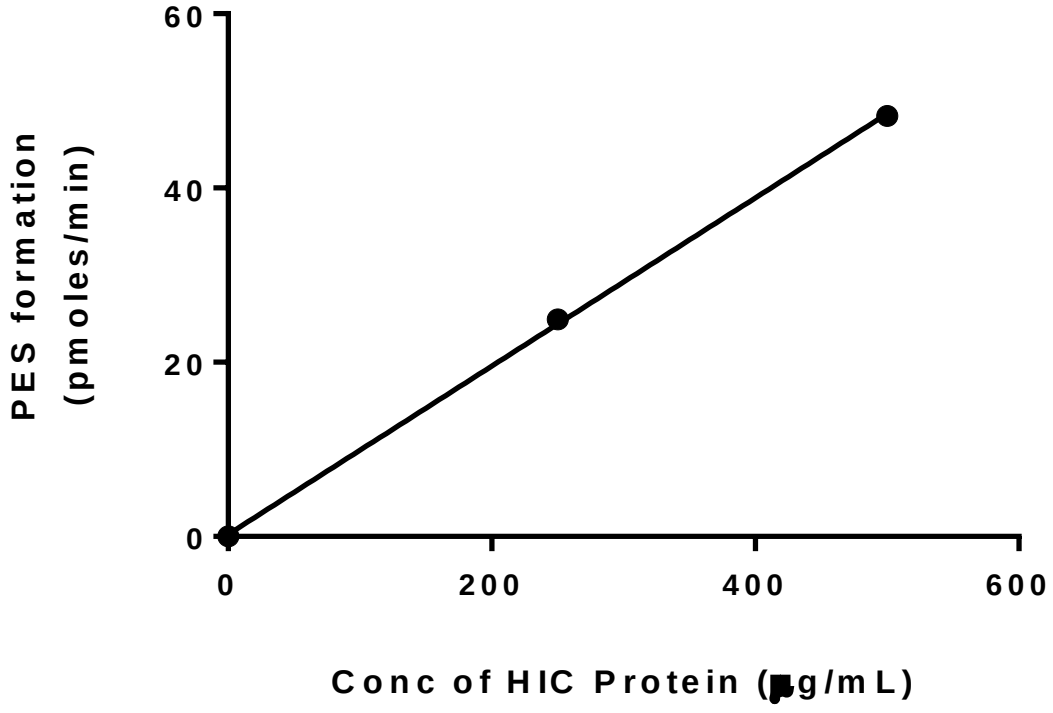
The formation rate of PES was linear up to 30 minutes with 5 µM and 200 µM PE with good R^2 estimates. When slopes of this linear regression were compared at 5 µM and 200 µM PE, it was also observed that formation rate of PES was saturated with increasing substrate concentration from 5 µM to 200 µM. This tells us that the enzyme may be saturated at higher substrate concentrations, consistent with the sulfation pathway being a saturable process. At higher substrate concentrations the contribution of sulfation pathway is reduced as compared to lower substrate concentrations. From this study the incubation time was optimized to 30 minutes in order to study the enzyme kinetic parameters using HIC.

4.3.10 Optimization of protein concentration to be used for HIC assay

4.3.10.1 Experimental Setup

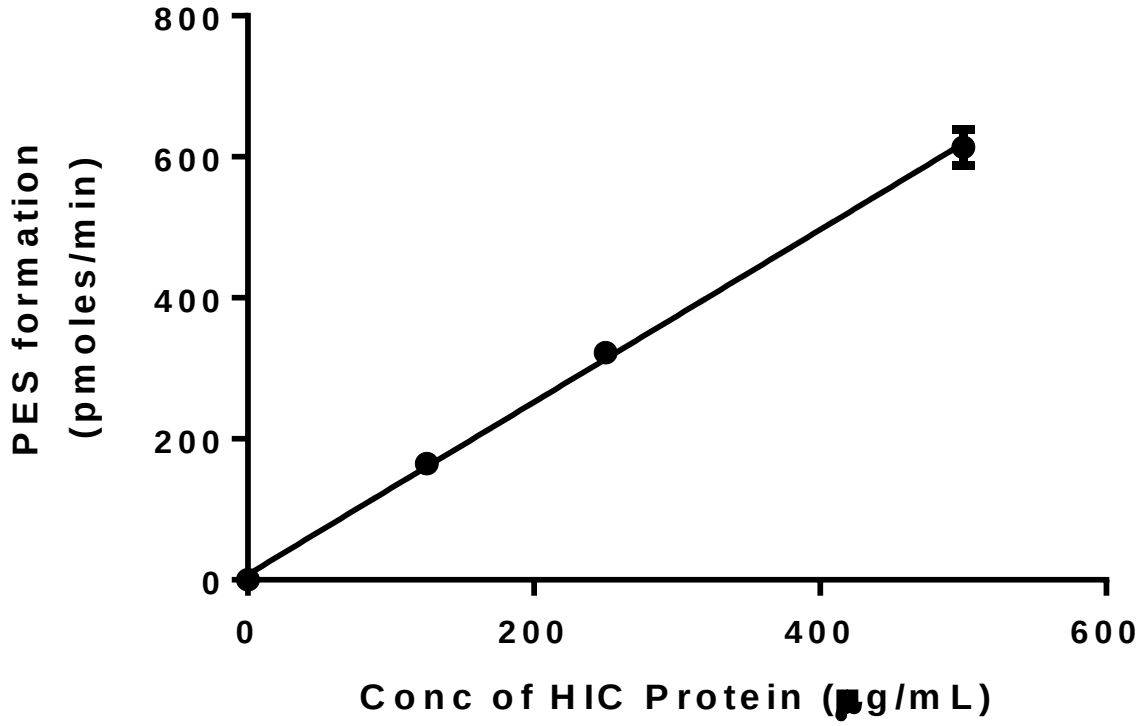
In order to optimize the HIC protein concentration to be used for HIC assay the same experimental setup was used as discussed in section 4.8.1. The study was done at 0 µg/mL, 250 µg/mL and 500 HIC µg/mL protein. Substrate concentrations used was 5 µM and 200 µM PE. BSA (0.05%w/v) was also added to the reaction mixture. Sample preparation was done in triplicate. Standard solutions was prepared as described under 4.2.2.

Figure 4.10 PES formation using various concentrations of HIC at 5 µM PE



R^2 = 0.9993; slope = 1.94 (pmol/min)/µg HIC protein

Figure 4.11 PES formation using various concentrations of HIC at 200 µM PE



R^2 = 0.9962. Slope = 24 (pmol/min)/µg HIC protein

4.3.10.2 Discussion

The protein concentration of HIC chosen for further studies was 250 µg/mL as it was in the linear range of PES formation rate. The slopes of the graphs at 5 µM and 200 µM PE, when normalized for PE concentrations, demonstrate evidence of a saturable process.

4.3.11 Saturation of PES formation with HIC

4.3.11.1 Experimental Setup

In order to optimize the substrate concentration to be used for further studies for HIC assay, the same experimental setup was used as discussed in section 4.8.1. The study was done at 200, 100, 50, 25, 12.5, 6.25, and 3.12 µM PE. The protein concentration used was 250 µg/mL and the reaction time was 30 minutes. At the end of 30 minutes the metabolic reactions were stopped with 4 volumes of ACN. The samples (n=3) at each substrate concentration were then centrifuged at 6000g for 13 minutes at 4°C. Supernatant (25 µL) was injected into the HPLC system. The same HILIC assay was used as described under 4.2.3. The Michaelis-Menten non-linear regression model was fitted the data obtained using Graph Pad Prism v5 software.

Figure 4.12 Saturation of PES formation with HIC

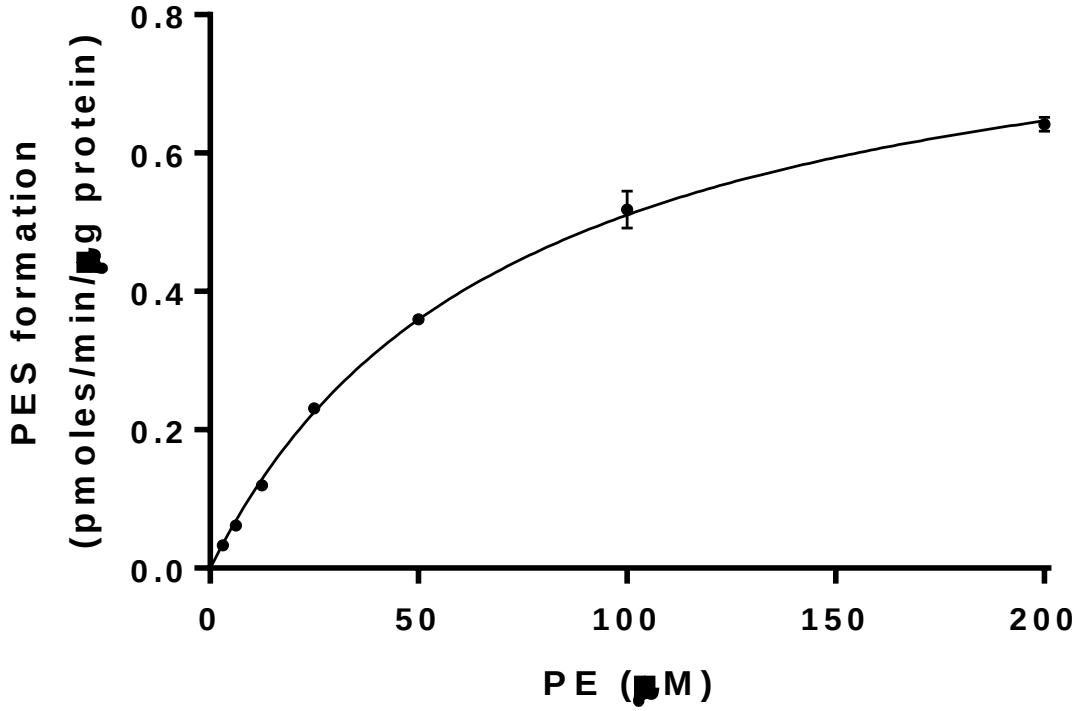


Table 4.4 Enzyme kinetic parameters using HIC

Parameter	Value
K_m	73 µM (95% CI: 66.0 to 79.1)
V_{max}	0.88 pmol/min/µg protein (95% CI: 0.85 to 0.92)

4.3.11.2 Results

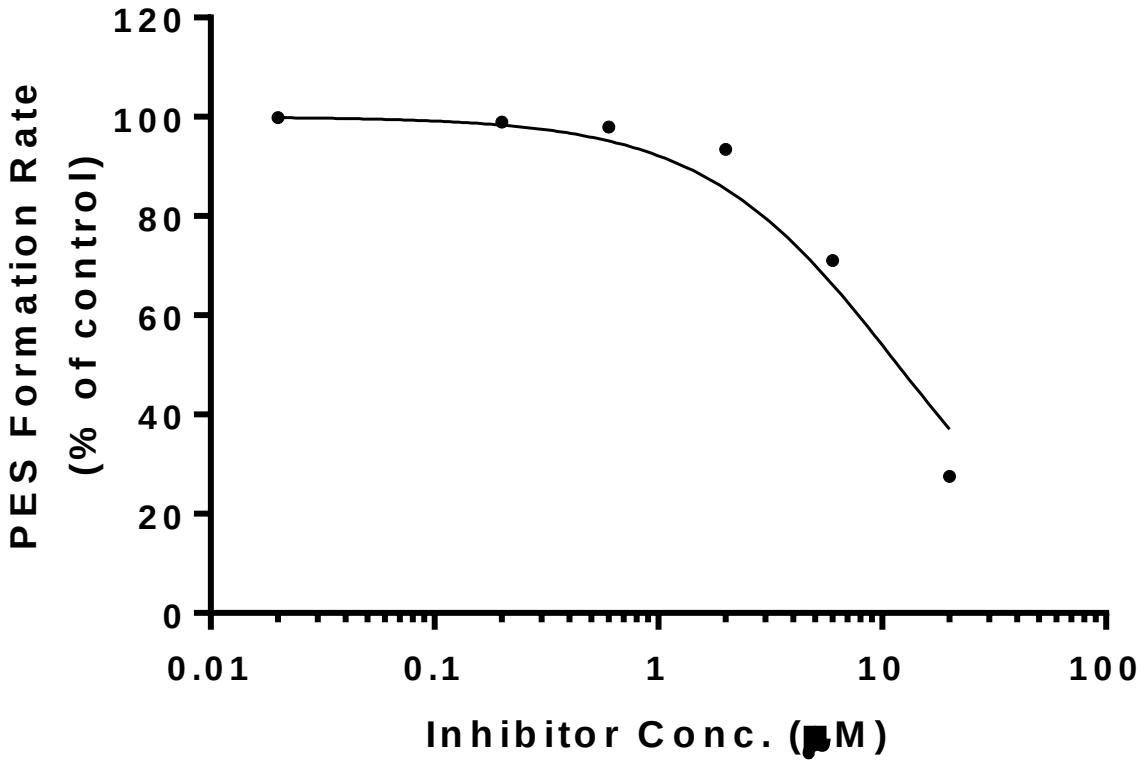
The Michaelis Menten model was fitted to the data (from Figure 4.12 and Table 4.4) and non-linear regression analysis was performed. It was observed that the formation of PES is a saturable process at higher concentrations of PE. The K_m value observed for PE with HIC was 73 µM (95% CI: 66.0 to 79.1) and with SULT1A3 was 65 µM (95% CI: 58.1 to 71.2). Thus there is no statistically significant difference in K_m values. The V_{max} value obtained with HIC is almost 240 fold lower per mg of total protein as compared to the V_{max} value obtained with SULT1A3. This is due to the enriched expression level of the recombinant SULT1A3 as compared to HIC which has presence of many other proteins too. This study gave a guidance for the choice of non-saturating substrate concentrations to be used for further experiments.

4.3.12 IC_{50} Curve for Resveratrol using HIC

4.3.12.1 Experimental Setup

Based on the inhibitor screening data obtained from the SULT1A3 experiments, resveratrol was selected as the inhibitor with the maximum inhibitory capacity among the tested GRAS compounds. In order to estimate the IC_{50} value for resveratrol using HIC- 0, 3.5, 7 and 21 µM of inhibitor concentrations of resveratrol were used. The substrate concentration of PE used was 50 µM and concentration of HIC used was 250 µg/mL. Control samples were also prepared with no inhibitor (0 µM). The same experimental setup was used as described under section 4.8.1 and Table 4.3. The reaction time was 30 minutes and the end of 30 minutes the metabolic reactions were stopped with 200 µL (4 volumes) of ACN. The samples (n=3) at each substrate concentration were then centrifuged at 6000g for 13 minutes at 4°C. Supernatant (25 µL) was injected into the HPLC system. The same HILIC assay was used as described under 4.2.3.

Figure 4.13 IC_{50} curve for Resveratrol



Control rate was 0.391 pmoles/min/µg protein

4.3.12.2 Results

The equation $Y=YMAX/1+10^{(X-IC50)}$ was used to fit the data (figure 4.13) of inhibition of PES formation (30 minutes incubation, PE=50µM using HIC, were fitted to in the presence of various inhibitors concentrations of resveratrol. YMAX was set to the average PES formation rate from 3 determinations in the absence of inhibitor (0.391 pmoles/min/µg protein). The model assumed that there was no PES formation at infinitely high concentrations of the inhibitor and at 0 µM concentration of the inhibitor, the PES formation was 100% (i.e. bottom = 0 and top = 100). Hill co-efficient was not included in the model. The IC50 non-linear regression model was fitted to the data (Figure 4.13) using GraphPad Prism v5 software. The IC_{50} value obtained was 12 µM (95% C.I: 9.7-14). This IC_{50} value gave a fair idea of the concentration range of the inhibitor to be used for the mechanism of

inhibition study using HIC. Assuming competitive inhibition, the Ki value (~7 µM) was calculated using this IC_{50} value using Cheng and Prusoff equation using GraphPad Prism 5. This Ki value was used to determine the inhibitor concentrations (0.5x, 1x, 3x Ki) to be used for the mechanism of inhibition experiment.

4.3.13 Determination of mechanism of inhibition

4.3.13.1 Experimental Setup

In order to determine the mechanism of inhibition of PES formation using resveratrol in the HIC system, the same experimental setup was used as discussed in Section 4.8.1. The study was done at 200, 100, 50, 25, 12.5, 6.25, 3.12 µM PE and 0, 3.5, 7 and 21 µM PE. The protein concentration used was 250 µg/mL and the reaction time was 30 minutes. At the end of 30 minutes the metabolic reactions were stopped with 200 µL ACN (4 volumes). The samples (n=3) at each substrate concentration were then centrifuged at 6000g for 13 minutes at 4°C. Supernatant (25 µL) was injected into the HPLC system. The same HILIC assay was used as described under 4.2.3. The non-linear regression model was used to fit the data using GraphPad Prism v5 software.

4.3.13.2 Results

Figure 4.14 Fit of non-competitive inhibition model on a linear scale

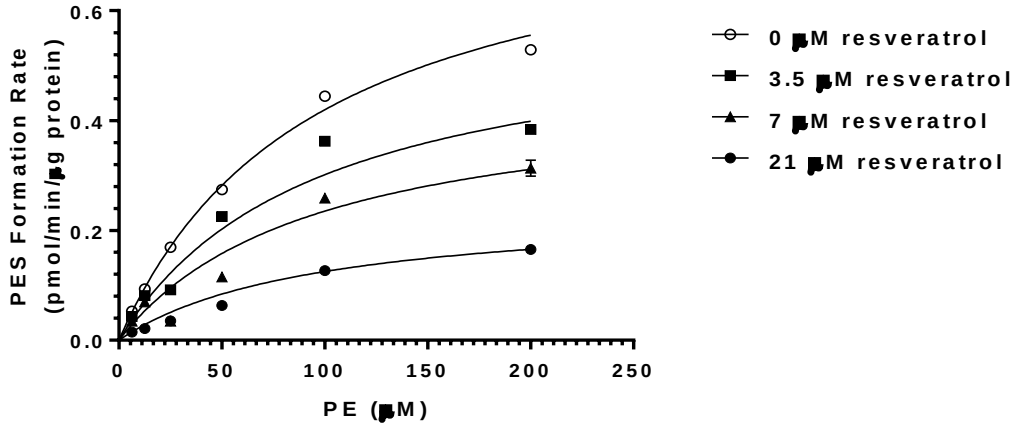
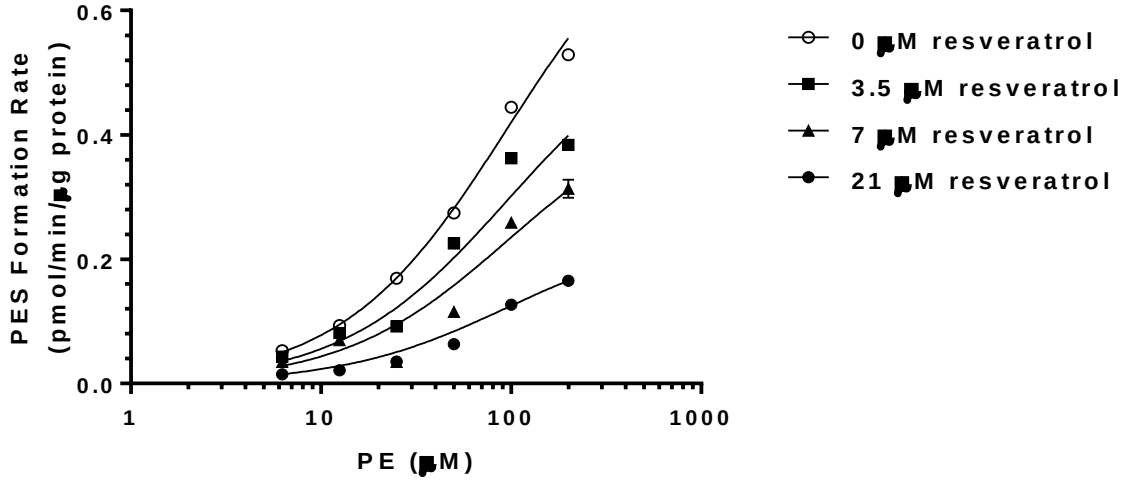


Figure 4.15 Fit of non-competitive inhibition model on a semilog scale



Equation used for non-competitive inhibition model

$$V = [V_{max}/(1+I/Ki)]^* [S]/[K_m+S]$$

In this case V_{max} is decreased by a factor of $(1+I/K_i)$

Figure 4.16 Fit of competitive inhibition model on a linear scale

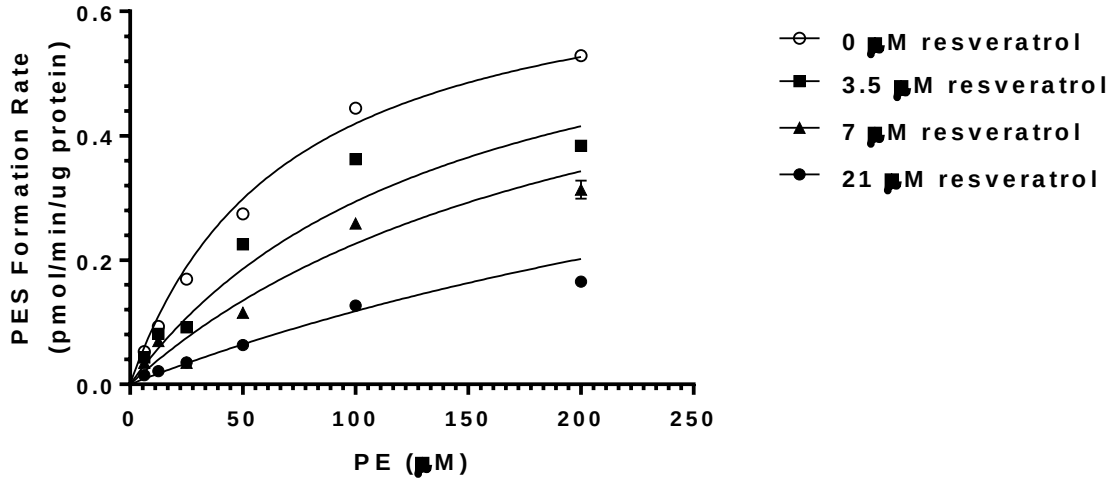
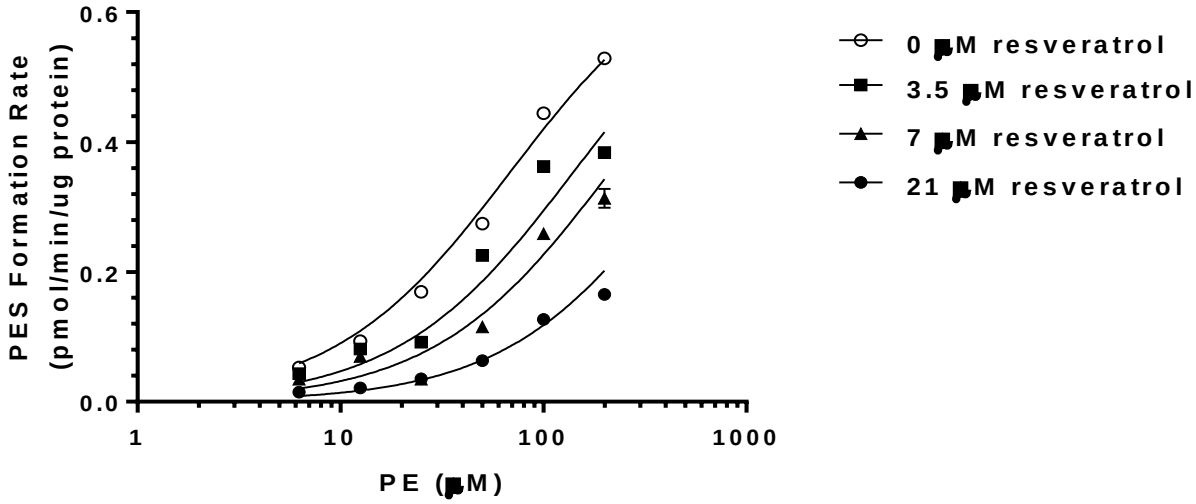


Figure 4.17 Fit of competitive inhibition on a semi-log scale



Equation used for competitive inhibition model

$$V = V_{max}[S]/[K_m(1+I/K_i)+S]$$

In this case $K_{m(app)}$ is increased by a factor of $(1+I/K_i)$

K_i = dissociation constant of an inhibitor when it binds to the enzyme only

Figure 4.18 Fit of uncompetitive inhibition model on a linear scale

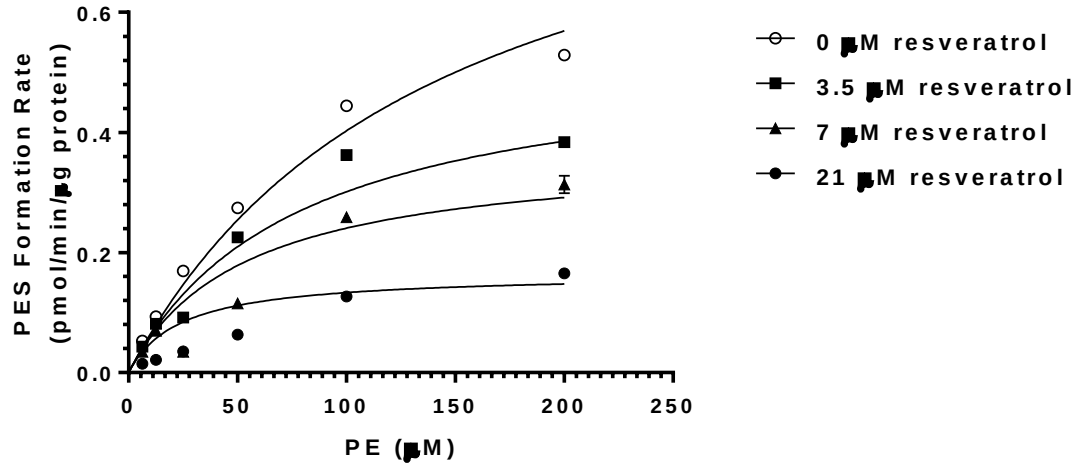
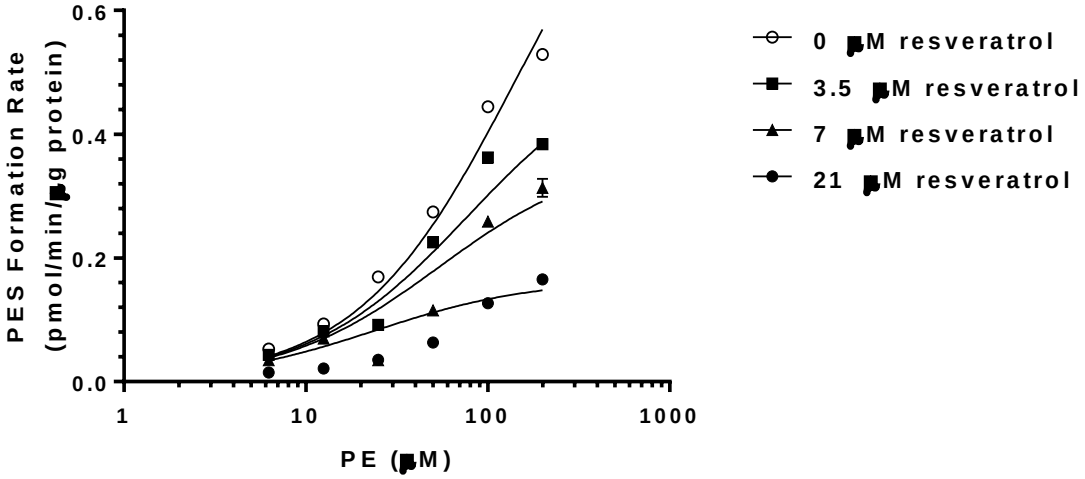


Figure 4.19 Fit of uncompetitive inhibition on a semi-log scale



Equation used for uncompetitive inhibition model

$$V= \{[V_{max}/(1+I/K_i')]*[S]\}/\{[K_m/(1+I/K_i')+S]\}$$

In this case both $K_{m(app)}$ and $V_{max(app)}$ are increased by a factor of $(1+I/K_i')$

K_i' = dissociation constant of an inhibitor when it binds to the enzyme-substrate complex

Figure 4.20 Fit of mixed inhibition model on a linear scale

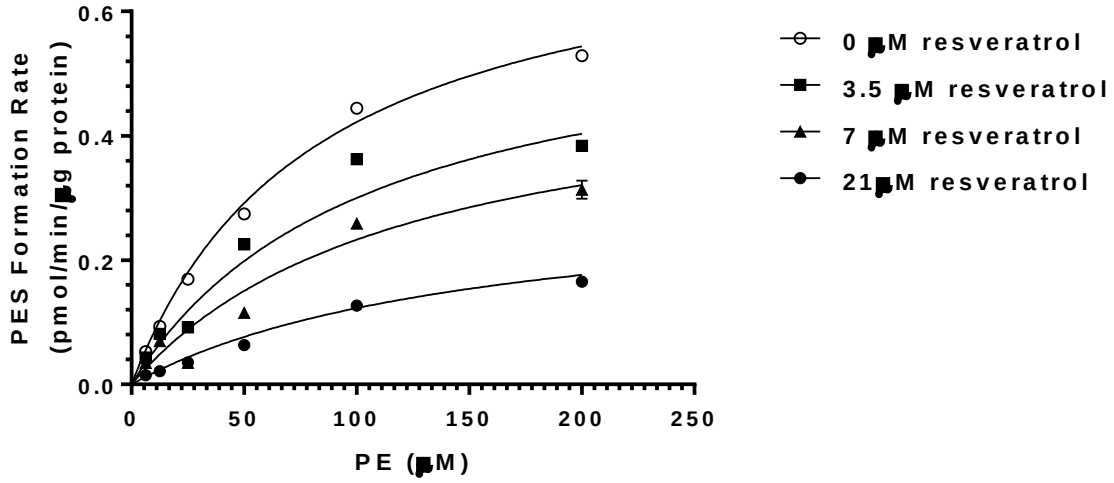
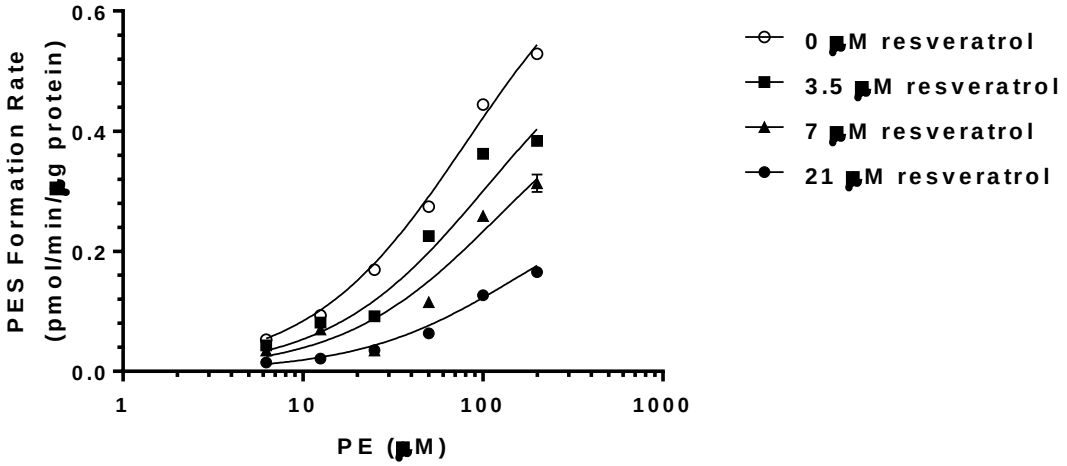


Figure 4.21 Fit of mixed inhibition model on a semi-log scale



Equation used for mixed inhibition model

$$V = \{[V_{max}/(1+I/K_i')]*[S]\}/\{[K_m(1+I/K_i)/(1+I/K_i')+S]\}$$

In this case both $K_{m(app)}$ can either be increased or decreased and $V_{max(app)}$ is decreased by a factor of $(1+I/K_i')$

K_i' = dissociation constant of an inhibitor when it binds to the enzyme substrate complex

Table 4.5 Comparison for models of inhibition

Parameter	Competitive inhibition	Non-competitive inhibition	Uncompetitive inhibition	Mixed inhibition
R^2	0.9667	0.9716	0.9431	0.9738
K_m (µM)	69 (95% CI: 49.7 to 87.6)	96 (95% CI: 75.0 to 117)	141 (95% CI: 89.2 to 193)	81 (95% CI: 59.1 to 104)
V_{max} (pmol/min/µg protein)	0.71 (95% CI: 0.626 to 0.789)	0.82 (95% CI: 0.734 to 0.910)	0.97 (95% CI: 0.767 to 1.17)	0.76 (95% CI: 0.672 to 0.858)
K_i (µM)	3.34 (95% CI: 2.53 to 4.15)	8.91 (95% CI: 7.48 to 10.4)	4.31 (αK_i) (95% CI: 2.97 to 5.65)	5.75 (95% CI: 3.04 to 8.46)
α	-	-	-	2.51 (95%C.I: 0.0-5.30)
Model selection criteria (MSC)	-1.9233	-1.8689	-1.9345	-3.9396

When mechanistic fit model of competitive vs non-competitive inhibition, non-competitive vs uncompetitive, mixed vs non-competitive inhibition were compared, the preferred model was non-competitive inhibition in all cases. There were three parameter estimates (V_{max}, K_m and Ki) for competitive, non-competitive and uncompetitive inhibition model whereas the mixed model inhibition included (V_{max}, K_m and Ki and a constant α). When goodness of fit was compared across the four models (competitive, non-competitive, uncompetitive and mixed inhibition), highest R^2 values were obtained for the mixed inhibition and non-competitive inhibition model. Also the values of the parameter estimates (K_m and V_{max}) obtained for the non-competitive model were closer to the values obtained from the PES formation saturation study with HIC with tight 95% confidence intervals. Model Selection Criteria was used to compare the models as the models had different number of parameter estimates. MSC calculations were done as follows:

$$R^2 = 1-(SSQ/TSQ)$$

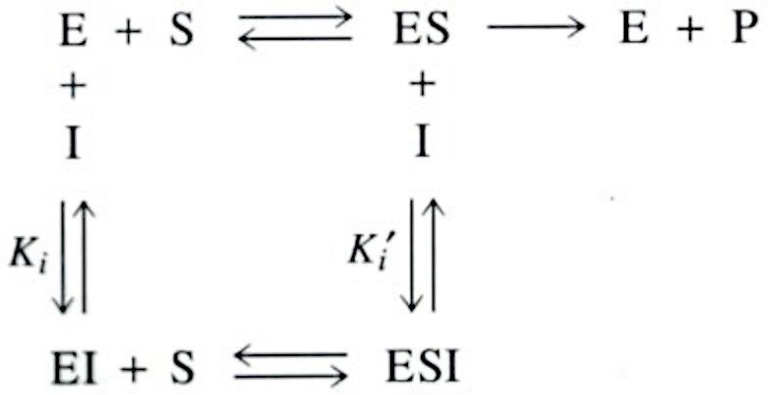
$$MSC = [\ln(SSQ/TSQ)]-2p/n \qquad (30)$$

Where SSQ = sum of squares, TSQ = total sum of squares, ln = natural log, p = number of parameter estimates, n = sample size

The highest MSC value was obtained for the non-competitive inhibition and hence non-competitive inhibition model was selected. The K_i values obtained from different models were comparable. It appears to be non-competitive inhibition since K_m values are comparable across the concentration range of the inhibitor used whereas the V_{max} decreases with the increase in inhibitor concentration because the enzyme is not catalytically efficient in presence of the inhibitor. During non-competitive inhibition, inhibitor either binds to the enzyme or the enzyme-substrate complex as shown in Figure 4.22.

The possible reason for this non-competitive inhibition could be due to the product inhibition caused due to PAP or depletion of PAPS. At higher concentrations of the inhibitor more amount of PAP is formed which inhibits the PES formation. Also it might bind to SULT1A3-PE complex and prevent the conversion of PE to PES, although further studies needs to be done in order to confirm this hypothesis. The amount of PAP into the reaction mixture needs to be analyzed at the end of 30 minutes. The major drawback of this study was that the range of the substrate concentration used was not wide enough to have a better confidence on the parameter estimate values. It was observed from linear and log linear plots for all the four models that the PES formation was not completely saturated even at the highest substrate concentrations.

Figure 4.22 Kinetic scheme illustrating noncompetitive binding (31)



E = enzyme, S = Substrate, P = Product, ES = Enzyme-substrate complex, EI = Enzyme-inhibitor complex, ESI = Enzyme-substrate-inhibitor complex

Table 4.6 Table indicating enzyme kinetic parameters for non-competitive inhibition

Conc. of inhibitor (μM)	0	3.5	7	21	Global (shared)
Noncompetitive inhibition					
Best-fit values					
Vmax	0.822	0.822	0.822	0.822	0.822
I	= 0.0	= 3.50	= 7.00	= 21.0	
Ki	8.91	8.91	8.91	8.91	8.91
KM	95.9	95.9	95.9	95.9	95.9
Std. Error					
Vmax	0.0438	0.0438	0.0438	0.0438	0.0438
Ki	0.711	0.711	0.711	0.711	0.711
KM	10.4	10.4	10.4	10.4	10.4
95% Confidence Intervals					
Vmax	0.734-0.910	0.734-0.910	0.734-0.910	0.734-0.910	0.734-0.910

Ki	**7.48 to 10.4**	**7.48 to 10.4**	**7.48 to 10.4**	**7.48 to 10.4**	**7.48 to 10.4**
KM	**75.0 to 117**	**75.0 to 117**	**75.0 to 117**	**75.0 to 117**	**75.0 to 117**
Goodness of Fit					
Degrees of Freedom					**45**
R square	**0.9922**	**0.9489**	**0.9095**	**0.9617**	**0.9716**
Absolute Sum of Squares	**0.00291**	**0.0114**	**0.0130**	**0.00146**	**0.0288**

4.4 Discussion

Kinetic studies done with the recombinant SULT1A3 and the data available in the literature (9) indicated that SULT1A3 is the major enzyme responsible for sulfation of PE. The K_m values obtained with HIC and SULT1A3 were comparable whereas the V_{max} values for SULT1A3 were more than 200-fold higher as compared to HIC. This can be attributed to higher expression of SULT1A3 in the recombinant system, although experiments with HIC would be physiologically more relevant. The optimization experiments done with SULT1A3 and HIC indicated the reaction conditions to be used for the PES formation saturation studies. Although the mechanism of inhibition experiment indicated that there might be non-competitive inhibition mechanism involved, further studies needs to be done in order to confirm the same. This non-competitive inhibition can be due to the product inhibition caused by PAP or might be resveratrol is binding to different binding site other than PE on the protein.

5 Overall Conclusions, Limitations and Future Directions

The efficacy of PE is controversial due to its extensive pre-systemic metabolism through sulfation to form PES. Hence quantitation of PES is essential in order to study the metabolism of PE. There are no published methods available for direction detection of PES. Methods are available for the determination of PE in pharmaceutical formulations by HPLC method using ion-pair and hydrophilic interaction liquid chromatography (HILIC) coupled with fluorescence detection as described by Dousa et.al. (15). However these methods do not enable the simultaneous quantitation of highly hydrophilic metabolite PES as well as PE in the in vitro system like LS180 human intestinal cell model, recombinant enzymes and human intestinal cytosol. Moreover the LLOQ of PE reported by Dousa et.al was 0.23 mg/L and a run time of 3.6 minutes and this method was employed on commercial samples of PE in pharmaceutical formulations (15). The method does not enable us to detect PES. The observed LLOQ for our method for PE and PES was 0.39 µg/L and 0.062 µg/L respectively with a run time of 6.0 minutes. Due to zwitterionic and hydrophilic character of PES, its retention on RP-HPLC column was very poor. Also LC-MS/MS technique could not be used due to matrix effects and unreliable chromatography. We have developed and validated a hydrophilic interaction liquid chromatography (HILIC) method for the direct detection of

phenylephrine 3-O-sulfate (PES) and simultaneous detection of phenylephrine (PE) to study the enzyme kinetics and metabolism of PE to enable approaches to reduce the presystemic metabolism of PE. This is the first method which facilitates direct detection of PES and also simultaneous detection of PE using a zwitterionic HILIC column with improved sensitivity in a single short run. The intensity of PE and PES in HILIC chromatography was 2-fold higher as compared to RP-HPLC and the observed LLOQ for PES was 62.5 nM. The method was fully validated for the determination of PE and PES in LS180 human intestinal cell model. Furthermore, the method was adapted for use with recombinant enzymes and human intestinal cytosol (HIC). HILIC chromatography involves hydrophilic partitioning which incorporated the use of a liquid-liquid extraction technique for sample preparation using 50%w/v dextrose. Development of the HILIC assay method was one of the most challenging part of the research project. Validation of the HILIC assay method as per the US-FDA guidelines confirmed that the method was specific, linear, precise, accurate, and stable over the concentration range of analytes tested.

PE has poor oral bioavailability due to its presystemic metabolism in the intestinal gut wall majorly through Phase II conjugation that is sulfation pathway. Hence in order to improve the bioavailability of PE and to improve its efficacy, a strategy of using phenolic compounds from FDA "GRAS" /dietary compounds list which are generally regarded as safe to inhibit metabolism of PE were tested. The validated HILIC assay method was employed to determine IC_{50} values using these GRAS compounds using LS180 cell model. Optimization studies with the LS180 cell model suggested that the formation of PES is linear with respect to substrate concentration up to 8 hours and the substrate concentration of 50 µM which was much below the K_m value (149 µM). Saturation of formation of PES at higher substrate concentrations indicated sulfation is a minor pathway at those concentrations. Resveratrol, eugenol, isoeugenol and zingerone showed good inhibitory activity towards PES formation in

this model. It also helped in screening of inhibitors and also to estimate the concentration of inhibitors to be used for further studies. Studies with LS180 cell model showed SULT1A3-like activity, where 1-naphthol and PE were sulfated which are two known SULT1A3 substrates (9, 26) DPBS buffer used for the study did not include sulfate and hence the system was falling short of PAPS which gave an apparent Hill co-efficient (0.69 ± 0.06) showing apparent negative cooperativity. Also the extraction process was time consuming and tedious. The LS180 cell culture model is a human colon adenocarcinoma cell line, which does not exactly represent small intestine, where most of the drug absorption occurs. The enzymatic activities are usually lower than the small intestine (29). These were the major limitations of the LS180 cell studies.

These cell-based assays showed presence of an unknown metabolite apart from PES. The intensity of this unknown metabolite peak was proportional with respect to the substrate concentration and it eluted much earlier as compared to PES on the HILIC column. This provided a basis that it could be an MAO metabolite as they more lipophilic as compared to sulfate metabolite (PES). In order to confirm this hypothesis, the formation of this unknown metabolite was observed in presence of various MAO inhibitors. The formation of this unknown metabolite was significantly inhibited in presence of clorgyline (MAO-A inhibitor) and pargyline (MAO inhibitor used non-selectively at 10μM), which indicated that the unknown metabolite is majorly an MAO-A metabolite. Also in presence of resveratrol and pterostilbene the intensity of the unknown metabolite was significantly greater than the control, which suggests that there might be a shift to MAO pathway due to inhibition of sulfation pathway through resveratrol and pterostilbene. This still needs to be confirmed. Although it could be inferred that the unknown metabolite was an MAO-A metabolite, identification of this metabolite was not possible. This can be due to the formation of various combination products between 3-hydroxy mandelaldehyde and amines to form Schiff bases.

Future studies may involve to elucidating the structure of this metabolite, study the enzyme kinetic parameters of this pathway and its contribution towards PE metabolism in this LS180 cell model. Also the relative contribution of the sulfation and MAO pathways could not be quantitatively determined due to lack of information of the unknown MAO metabolite.

Kinetic studies on sulfation inhibition with phenolic GRAS or dietary compounds using recombinant SULT1A3 and human intestinal cytosol (HIC) were done in order to estimate the K_i values of the GRAS compound with the maximum inhibitory capacity towards sulfation of PE. The optimization experiments done with SULT1A3 suggested that the formation of PES was linear up to 30 minutes at 5 µM and 200 µM PE and the protein concentration was optimized to 1 µg/mL. The PES formation saturation study with SULT1A3 using a wide range of substrate concentrations provided a valuable information of K_m and V_{max} values. These optimization experiments provided a basis for the incubation time, protein and substrate concentrations to be used in order to screen the inhibitors using SULT1A3. When the inhibitors were screened using SULT1A3, maximum inhibition was obtained using resveratrol. The K_m value obtained with the LS180 cell model was almost 2.5 times higher as compared to K_m value obtained with SULT1A3.

Further studies involved to determine the mechanism of inhibition for the selected inhibitor using HIC. HIC depicted a picture which was closer to what would happen at the physiological level. The optimization experiments with HIC suggested that the formation of PES was linear up to 30 minutes at 5 µM and 200 µM PE and the protein concentration was optimized to 250 µg/mL. The K_m value obtained using PES formation saturation study with HIC 73 µM (95% CI: 66 to 79) was not statistically different as compared to the K_m value of 65 µM (95% CI: 58 to 71) obtained using SULT1A3. The V_{max} value obtained using SULT1A3 was above 200-fold higher as compared to that obtained using HIC. This was due

to the increased expression of SULT1A3 in the recombinant overexpression system as compared to HIC, as HIC has presence of many other proteins too. In order to determine the mechanism of inhibition, preliminary study was done to estimate the IC_{50} value of resveratrol using HIC. This study provided a fair idea of the concentration range of the inhibitor to be used for the mechanism of inhibition study. The limitation to this study was that the concentration range used for inhibitor was not broad enough at the higher end and hence complete inhibition of PES formation was not observed. The broad concentration range of the inhibitor would have provided a better estimate of the IC_{50} value and hence the Ki for resveratrol.

For the mechanism of inhibition study, substrate concentration (3.12 µM – 200 µM) and inhibitor concentration range (0 µM – 21 µM) was used. When different models were compared the non-competitive inhibition model gave the best fit as compared to others. The K_m and V_{max} values obtained using the non-competitive inhibition model were comparable with those obtained from the PES formation saturation study using HIC without any inhibitor. This provided a stronger basis for the non-competitive inhibition model. The goodness of fit, the higher R^2 value and 95% CI intervals for the parameter estimates were obtained for non-competitive inhibition model. Also the published literature has shown that the sulfation of 17α-ethinyloestradiol was inhibited by vanillin. Vanillin was found to inhibit 50% of liver 17α-ethinyloestradiol sulfotransferase activity (IC_{50}) at a concentration of approximately 1.3 µM and the mode of inhibition was non-competitive (25). The major drawback of this study was that the substrate concentration range used was not broad enough and it is clearly seen in the semi-log plot were PES formation is not saturated at 200 µM PE. A broader substrate concentration range would have provided a better confidence over the parameter estimates.

Future studies needs to be done in order to determine the amount of PAP and resveratrol in the reaction mixture at the end of 30 minutes. This would confirm whether the non-competitive inhibition is due to the product inhibition caused due to PAP. The permeability of these GRAS/dietary compounds across the intestinal cell membrane also needs to be studied. The K_i value obtained for resveratrol using all the four models were between 3-10 µM. In order to clinically investigate this approach, maximum dose which can be given safely should be studied. Also we need to know the concentration of resveratrol in the cytosol of the intestinal cells. The concentration of inhibitor would be chosen upon its capacity to inhibit SULT1A3, maximum dose of the inhibitor that can be used *in vivo* and toxicity of the inhibitor itself.

All in all, HILIC assay method developed provided a quick and reliable way for direct detection of PE, PES and the unknown MAO metabolite. The developed method could be tried on the in vivo detection of PE and the metabolites. The strategy of using GRAS or dietary compounds gave promising results *in vitro* but the clinical relevance of this approach still needs to be reconfirmed. By using the appropriate scaling factors (amount of SULT1A3 in the gut, weight of the intestine and fraction unbound for PE) would help in determining the intrinsic clearance *in vivo* through the sulfation pathway. The studies done *in vitro* provided data to be used to predict *in vivo* intrinsic clearance through the sulfation pathway. The future goal is to develop a product containing PE in combination with one or more GRAS compounds in order to reduce its first pass metabolism in the gut through sulfation and in turn increase the oral bioavailability of PE.

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