PURIFICATION AND CHARACTERIZATION OF HUMAN UTERINE GLUTATHIONE S-TRANSFERASES: EXPRESSION IN CANCEROUS AND FETAL TISSUES

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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This passion of our kind
for the process of finding out
is a fact one can hardly doubt
but I would rejoice in it more,
if I knew more clearly what
We wanted the knowledge for.....

W.HAuden
20th century poet

DEDICATED

TO

MY

TEACHERS'

UNIVERSITY OF HYDERABAD

(A Central University Established in 1974 by an act of Parliament)

HYDERABAD-500 046,INDIA

DECLARATION

I hereby state that the work embodied in this thesis entitled " **Purification and Characterization of Human Uterine Cytosolic Glutathione S-transferases : Expression in Cancerous and Fetal tissues"** has been carried out by me under the supervision of Prof. P. Reddanna and that this has not been submitted for any degree or diploma of any other University earlier.

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CERTIFICATE

This is to certify that Mr.B.RamaKrishna has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis **"Purification and Characterization of Human Uterine Cytosolic Glutathione S-transferases : Expression in Cancerous and Fetal Tissues"** for submission for the degree of Doctor of Philosophy of this University.

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Abbreviations

BCIP		5-bromo-4-chloro-3-indoyl phosphate
BSP		bromosulfophthalein
cm	:	centi meter
CuOOH	:	cumene hydroperoxide
CDNB		1-chloro-2,4-dinitrobenzene
DCNB	:	1,2-dichloro-4-nitrobenzene
DNA	:	deoxy ribonucleic acid
EA	:	ethacrynic acid
EDTA	:	ethylene diamine tetraacetic acid
ELISA	:	enzyme linked immunosorbent assay
GSH	:	Glutathione reduced
GST	:	Glutathione S-transferases
g	:	gram
h	:	hour
HPLC	:	high pressure liquid chromatography
IgG	:	Immuno globulin
kDa	:	kilo dalton
LMW	:	low molecular weight
min	:	minute
mM	:	milli molar
Mol. Wt	:	molecular weight
MDR	:	multidrug resistance
M	:	molar
n	:	nano
nm	:	nano meter
NBT	:	nitroblue tertrazolium
PAHs	:	Polycyclic Aromatic Hydrocarbons
s	:	second

SDS PAGE	:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	:	trifluroacetic acid
Tris	:	tri (hydroxy methyl) aminomethane
UV-VIS	:	ultraviolate-visiable
μ	:	micro
Δ^5AD	:	Δ^5androstene-3,17-dione
4-HNE	:	4-hydroxynonenal
A e /mM/cm	:	molar extinction coefficient
X max.	:	lambda maximum
2D	:	two dimensional

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Finally to the almighty.....Lord Venkateshwara...

Badugu RamaKrishna.

INTRODUCTION

Existence of life is not sufficient enough to live, the judicious role of life supporting systems are required. Detoxification is one such life supporting system which exerts its function via a number of enzyme systems, which are broadly classified into two, phase I and phase II detoxification systems (fig 1). The phase I detoxification systems include the cytochrome P_{450} monoxygenases and other enzymes that catalyze the oxidation and reduction reaction. Glutathione S-transferases belong to the phase II category. Glutathione S-transferases **(GSTs E.C 2.5.1.18)** are a family of multigene, multifunctional enzymes (Booth *et.al*, 1961, Jakoby *et.al,* 1978, Mannervik *et.al,* 1985a, 1985b) which have evolved along with glutathione in aerobic organisms. GSTs were first observed by Booth *et.al* as glutathione binding proteins way back in 1961. Later on a systematic study was done and many isoforms have been defined both structurally and functionally and still many more are yet to be deciphered. These are abundantly expressed and are vertically and horizontally distributed in varied forms of life in biological kingdom. The complexity in comprehending a comprehensive subject note on GSTs lies in numerous factors responsible for their expression, of which species, tissue, sex, age and the genetic composition are of importance. The primary role of GSTs

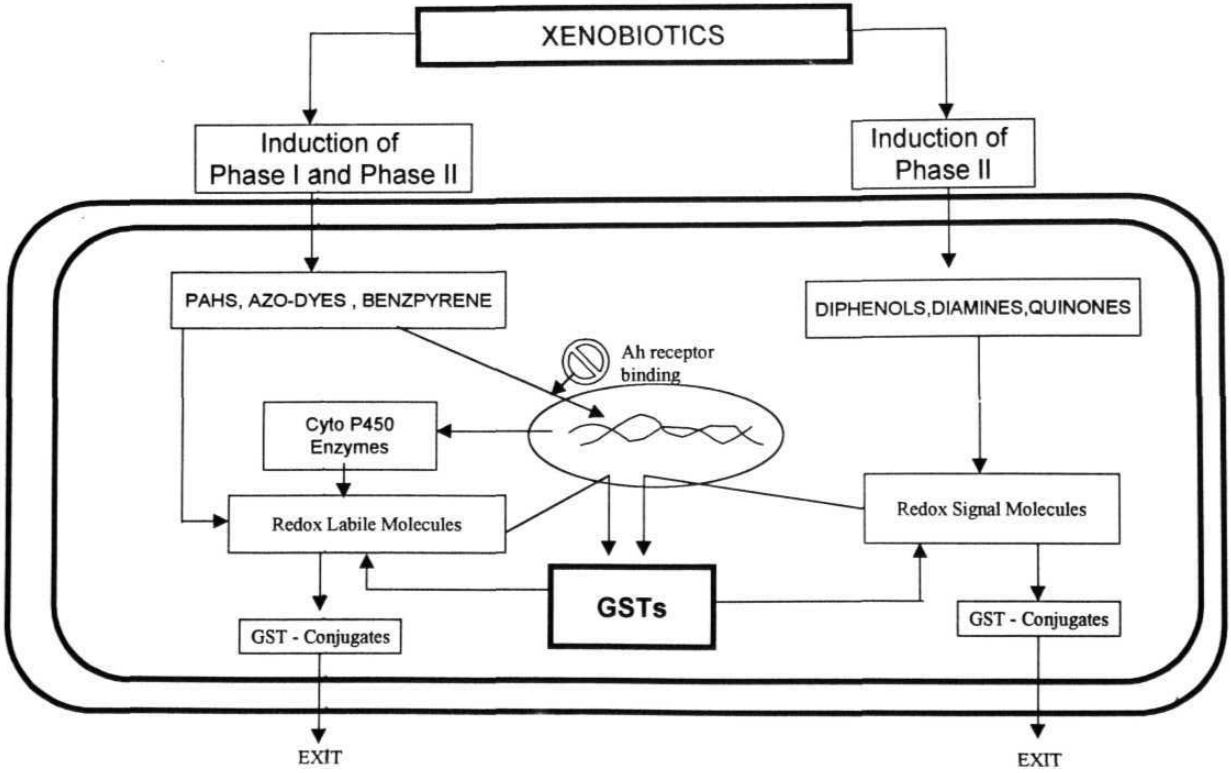


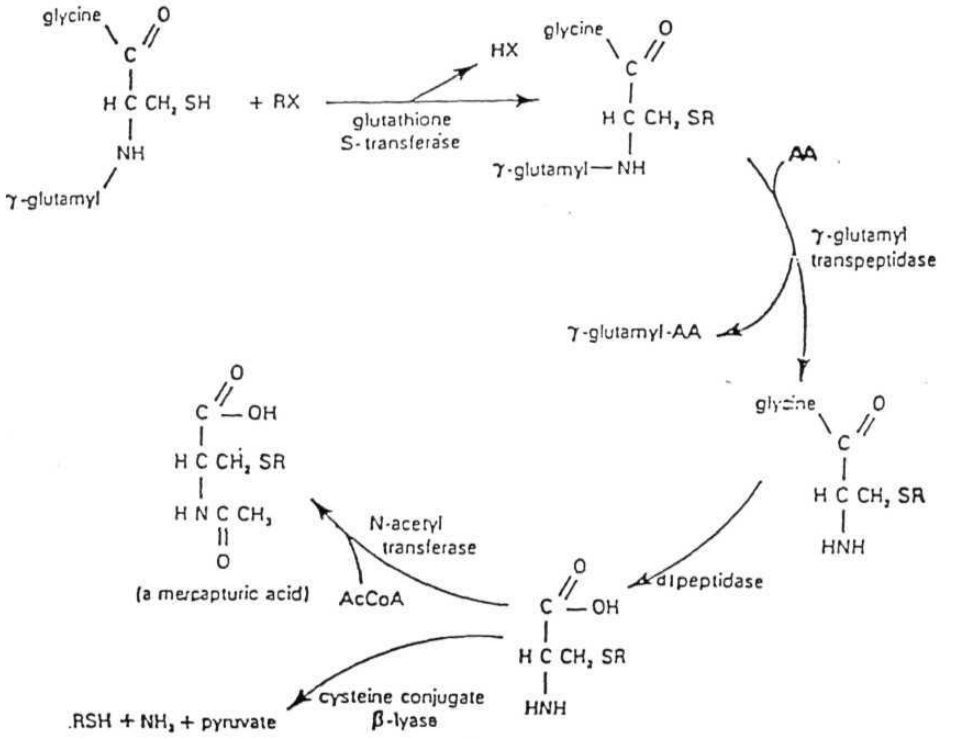
Fig. 1: Phase I and Phase II drug detoxification mechanisms

is to augment the detoxification of exogenous (xenobiotic and their metabolites) and endogenous (primarily products of oxidative stress) toxic compounds in the phase II reaction of drug detoxification pathway. They do so by conjugating the electrophilic center of toxic, hydrophobic compounds to the sulfur atom of glutathione (GSH) and the nature of the reaction is nucleophilic, and the resultant water soluble S-conjugate is processed and eliminated vie the classical mercapturate pathway (fig 2) (Chasseaud *et.al*; 1979, Boy land *et. al*; 1969)

1.1 Classification of GSTs

Classification of GSTs is a very difficult process, the major obstacle being the overlapping substrate specificities and also the highly conserved sequence homology. To date GSTs are classified in to seven classes, five classes represent the cytosolic GST a, μ, π,θ & σ and two represent the membrane bound GSTs. Initially the classification of GSTs was done by the "Y" configuration instituted by Bass (Bass *et.al*; 1977). The GST enriched cytosols were named as the "Y" fraction or ligandin containing fractions and on SDS PAGE resolved into three bands, which were named as Ya, Yb and Yc according to their decreasing anodal mobility. Later on when these were further analyzed for their substrate specificities it was reveled that Ya and Yc had overlapping substrate specificities, further the N-terminal sequencing had revealed that Ya and Yc represent the class a and Yb represent the class |i. Jackoby (Jackoby

Fig.2: Mercapturic acid pathway



et.al, 1984) had devised an Arabic numerical nomenclature for GST. It is of value because it projects the subunit combination but at the same time lacks information on to which gene family they belong. Similarly the Y nomenclature also suffers from a drawback where in interspecies comparison is not possible.

In 1992, a consultant group (Mannervik *et.al,* 1992) had conclusively classified GSTs, which was designed to classify the human GSTs but is generally applicable to other species. In this classification system single capital letter abbreviation is used to represent the class, A for Alpha, M for Mu, P for Pi, S for sigma and T for Theta. Arabic numerals are employed for numbering each of the separate gene products. A new GST will be given an asterisk and a preliminary class letter unless and until the primary structure of the new class of GST is determined and differentiated with the existing structures with in the class. A small letter is usually suffixed to indicate the class alphabet to identify the source of the enzyme h, m and r referring to human, mouse and rat.

Example: r GST A 1-3

Heterodimeric alpha class GST between subunit 1 and 3, from rat.

1.2 GENE FAMILIES ENCODING CYTOSOLIC GSTs

A large number of cytosolic GST isoenzymes have been purified from rat and human organs based on their primary structure. In comparison if

the primary structure matches more than 40 % of identity they are included in the same class. If the homology is less than 30 % then they are assigned to a separate class. This is only an arbitrary system they have followed, it will get further complicated if the homology is between 30 and 40%. Because of this, the emphasis is more on the "N" terminal sequence of the protein as with in the class this is the most conserved region.

The hypothesis that each class epresents a separate gene family is supported by the distinct structure of their genes and their chromosome localization

TABLE 1: Gene families encoding cytosolic GSTs

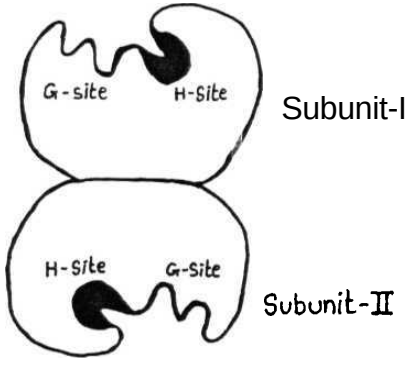
CLA	SOUR	Kb LEN	EXO	% HOMOLO	References
Alpha	Rats Mice Humans	**11 to 12**	**7**	55	Telakowski et al 1986 Daniel,V et,al 1987 Suzuki,T et.al 1993
Mu	Rats Mice Humans Hamster	5 to 6 **5**	8 8	65	Lia,H,-c.J et.al 1988 Reinhurt,J et.al 1993 Pearson,W R et al 199 Fan,W et.al 1992.
Pi	Rats Mice Humans	**3**	**7**	?	Okuda.A et al 1987 Bammler,T.K et.al 199 Cowell,I G et al 1988
Theta	Rat	**4**	**5**	50	Ogura,K et al 1994

1.3 MICROSTRUCTURE AND REACTIONS CATALYZED BY GSTs

GSTs are generally found to occur in the molecular weight range of 17 to 29 kDa. Each isoenzyme is a dimer, and has two domains, the smaller domain contains the GSH binding site or the "G-site" and the larger domain features the binding site for electrophilic substrate or the "H-site" (Mannervik *et.al*; 1985b)(Fig.3). The common property expressed by all GSTs is that they have high binding affinity toward GSH except the θ class. This ability of high affinity towards GSH has been thoroughly exploited for the preparation of affinity columns in an endeavor to purify GSTs from different tissues.

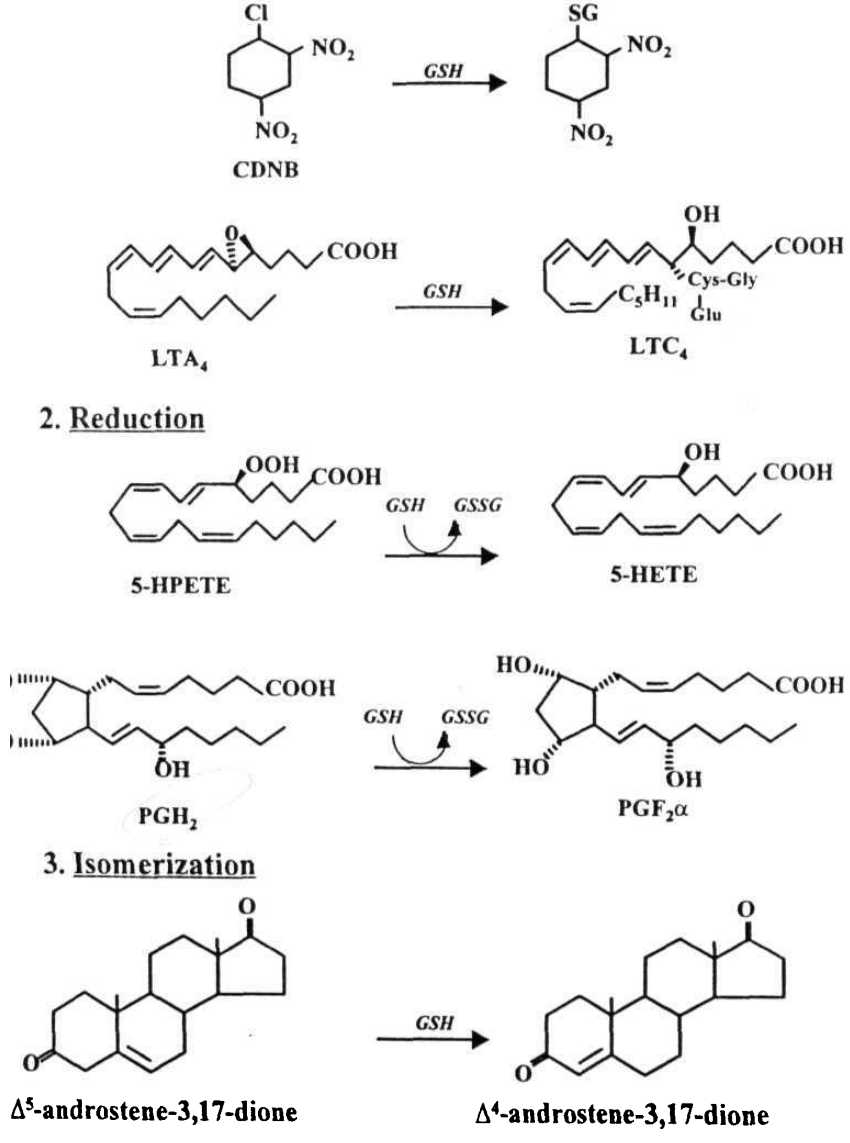
The reactions catalyzed by GSTs can be classified broadly as the conjugation reaction, oxidation-reduction reaction and isomerization reaction, in each of these reactions GSH is the nucleophilic reactant towards an electrophilic substrate (fig 4) (Mannervik, 1986). GSTs can conjugate GSH with compounds that possess an electrophilic center. The electrophilic functional group to a compound can be provided by a carbon, nitrogen, or a sulphur atom, aliphatic and aromatic halides, unsaturated carbonyls, organic nitrate esters and organic thiocyanates. The formation of a thioether bond between electrophiles and GSH almost always yields a conjugate that is more stable thermodynamically than the parental compound. Thus from the functional point of view, GSH conjugation is

Fig.3: Model of the topology for the two active sites of a dimeric glutathione S-transferase molecule



Shows a complete active site in each individual subunit, including a subsite for glutathione (G-site) as well as a subsite for the hydrophobic electrophilic substrate (H- site) (Mannervik *et.al.,* 1985a)

Fig.4: The catalytic and non-catalytic functions of GSTs



thought to be of value not only because it removes harmful electrophilic moieties from the cell but also it increases the solubility of hydrophobic xenobiotics and there by preventing their partitioning into membrane lipid. The exact mechanism of GST-catalyzed conjugation reaction is not yet clarified unequivocally and is still at the level of debate. Jakoby and Habig in 1980 proposed the proximity effect in which GSH and the electrophilic substrate bind, which is a chance occurrence and the second substrate, then determines the rate of the reaction. Offlate in 1994 Armstrong (Armstrong *et.al*; 1994) proposed that the most likely method involved is lowering of pKa value of GSH from 9 in aqueous solution to 6.5 in the bound state, which results in the formation of the potent nucleophilic species, the thiolate anion (GS^-). The stability to this anion is provided by the hydrogen bonding with tyrosine in the α,μ,π and σ classes and serine in θ class at the N-terminus of the polypeptide. The oxidation-reduction reaction spans around the selenium-independent glutathione peroxidase activity of GST (Mosialou *et.al*; 1993). This type of reaction is thought to occur in two steps, and proceeds via the formation of sulfenic acid and represents nucleophilic attack by GSH on electrophilic oxygen.

1. ROOH + GSH —> ROH + [GSOH]

2. [GSOH] + GSH—> GSSG + H_20

The reduction of organic hydroperoxides, and endoperoxides by GST forms an important antioxidant defense mechanism of cellular

systems as these compounds directly or indirectly, through their decomposition to reactive free radicals, damage vital biomacromolecules. In man the α and the 0D class excel in this activity. In rat specific isoform studies have shown that GSTs1-2, 2-2, 5-5 & 7-7 show activity toward lipid peroxides. GST 5-5 also shows activity towards DNA hydroperoxides and GST 8-8, shows high conjugative activity toward 4-hydroxynonenal, a potent aldehyde product of lipid peroxidation (Hoff *et.al;* 1992, 1993). The reduction of endoperoxides prostaglandin H_2 to $PGF_{2\alpha}$, is catalyzed by GST 1-1 (Chang et.al; 1987). GST 6-6 is involved in the biosynthesis of leukotrienes. GSTs also catalyze the reaction of positional isomerization. In this reaction GSTs act catalytically and hence do not undergo metabolism. For example, 5-ketosteroids to 4 ketosteroids and PGH_2 to biologically active PGE_2 and PGD_2, (Keen *et.al]* 1978). The other possible enzymatic functions are in the metabolism of ethanol (Bora *et.al;* 1989) and the catalysis of disulfide exchange with non-polar disulfides. Taniguchi (Taniguchi *et.al,* 1989) reported that GSTs undergo protein kinase-C dependent phosphorylation. The non-enzymatic binding, which was discovered in 1971, helps in the transport of large molecules like steroids and hormones. This actually helps in the buffering of hormonal levels to mitigate the transient flux and also small molecular weight proteins (Listowsky *et.al;* 1993 and Litwack *et.al;* 1971)

TABLE 2: Preferential Catalytic Activities of Human GST Isoenzymes

Substrat	CD	Δ^5A	BSP	DC	EA	4-HN	CuO
Isoform	Mu/	Alp	Alph	M	Alpha/	Alph	Alph

CDNB:1-chloro-2,4-dinitrobenzene;Δ^5AD:Δ^5androstene-3,17-dione;BSP: bromosulfophthalein; DCNB: 1,2-dichloro-4-nitrobenzene; EA: Ethacrynic acid; 4-HNE: 4-hydroxynonenal; CuOOH: cumene hydroperoxide

1.4 IMPLICATION OF GSTs IN CARCINOGENESIS

Initiation in carcinogenesis is the process of irreversible committed conversion of individual cell into a preneoplastic cell wherein the metabolic pathways are distorted for the prevalence of conversion, the subsequent step is the progression stage where the cellular reproductive potential is immensely increased at the cost of the neighboring cells. Molecular phenomena of initiation are the covalent modification of the DNA structure by the exogenous and/or endogenous electrophilic molecules. GSTs being the sequestering enzymes of electrophilic molecules, they are involved in the prevention of initiation in carcinogenesis. In preneoplastic and neoplastic cells it is observed that higher levels of specific isoforms of GSTs are expressed and this is dependent on the tissue in which it is taking place. The most paradoxical

aspect of these enzymes is, their role in multidrug resistance (MDR)(Tew *et.al,* 1994). GSTs have been implicated in carcinogenesis associated with multidrug resistance phenotype or pleotropic resistance. This is mainly because there is no quantitative correlation between the degree of resistance and alterations in net drug accumulation suggesting that other mechanisms besides alteration in drug accumulation may also be involved in the development of resistance.

The phenomena of multidrug resistance or the pleotropic resistance is the culmination of different processes including the 170 kDa membrane bound protein called the P- glycoprotein (Juliano *et.al,* 1976), GSTs in the cytoplasm and the topoisomerase II in the nucleoplasm (Pommier *et.al,* 1993). Apart from these, liphophilicity of the molecules and the membrane structures are also involved. Most of the substrates for GSTs are also inducers of these enzymes, which suggest that adaptive response is an inherent mechanism. But there is no uniformity in the over expression of a particular isoform of GSTs in relation to tissue, under the administration of chemotherapeutic agents. In some of the experiments even the enantiomers were recognized for GSTs induction suggesting "stereo-structural selectivity". Nevertheless the induction profiles of particular isoform is influenced by the normal level of expression and type of isoforms predominant in a particular tissue. The above mentioned two factors play an important role in the development of resistance towards a

drug. In this process GSTs reduce the efficacy of the drug resulting in the nullification or reduction in the potency of the drug. Studies on the inhibition of the GSTs and that too at the isoform specific level will make a positive intervention at the patient treatment methods.

1.5 CARCINOGENESIS AND OVER EXPRESSION OF *n* FORM OF GSTs

With the present knowledge at hand it is well understood that *n* class of GSTs produce only one type of isoform which is termed as the hGSTP1-1 with molecular weight of 24,500 kDa. The isoelectric point of this isoform is in the acidic range. One of the unique aspects which it does not share with the other isoforms is that it gets posttranslationally modified and this is identified as the glycosilation at the N-terminal end of the protein. In the course of studies by various workers on isoenzymic alteration during chemical carcinogenesis, it was shown conclusively that *n* isoform expresses strongly in preneoplastic and neoplastic cells (Sato, 1989). It is established that though *n* is expressed in many carcinomas but not in all. Koo (Koo *et. at,* 1994) had clearly showed that this isoform has a multilevel regulation, as there is no quantitative compatibility with the transcript, protein and enzyme activity. This finding led to the understanding of the actual mechanism of the induction of π form of GST. Initially rat model was studied but it was shown that in rat and human the regulatory pathways are of different nature. In rat the p21ras protein plays

an important role in the regulation where as this is not observed in the human (Sakai *et.al,* 1988 and Okuda era/; 1990).

In humans it was shown that methylation of the cytidine residues in the promoter sequence of GSTP1 is involved in the regulation, hypomethylation resulted in increased promotion and the hypermethylation resulted in the decreased promotion (Lee *et.al,* 1994) Apart from these insulin increases the expression through a cis-acting element in the intron 1 of the gene (Xia ***et.al;*** 1993). On the contrary the retinoic acid down regulates the expression with the products of the c-fos and c-jun oncogenes, through the interaction at the TRE (transcriptional regulatory element) (Moffat *et.al;* 1994). It was shown that the substrate specificity was very broad and its enzymatic activity was found to be highly insensitive to the inhibitory actions of various compounds (Fehring *et.al;* 1989). The detection of this enzyme (marker enzyme) facilitates the analysis of carcinogenic progression and provides the basis for new methods of screening for carcinogens and carcinogenic modifiers.

1.6 EXPRESSION OF μ ISOFORM OF GST AND CARCINOGENESIS

The polymorphic nature of the μ gene inherently depicts the broad spectrum of substrate specificities and also makes the individual more sensitive towards these compounds in a condition where it is not expressed (Hayes, 1989). But this problem to a certain extent is

compensated by the high expression of other proteins with similar if not same substrate specificities. To understand the polymorphic nature of μ gene in a population various studies were undertaken around 1980(Board *et.al;* 1981 and Widersten *et.al;* 1991).

Relation between the presence of a disease and the absence of an isoenzyme was studied at large by many workers and incidentally the influence of this relation does not depict the scientific approach towards the diseased state. But this work had encouraged the molecular epidemiological studies in different geographical areas. This approach on GSTs expression in Caucasian population revealed that about 50% of the population is devoid of μ gene. This lack of μ gene as a result of gene deletion was attributed to the increased risk of lung cancer observed in the population (Board *et.al;* 1981). In recent findings 50% of Japanese population also lacks μ gene and this was not only implicated in lung cancer but in cancer of urothelium (a transitional cell lining of the renal, pelvis, ureter and bladder) (Takahiko *et.al;* 1995). The μ isoform is very specific to polycyclic aromatic hydrocarbons (PAHs) like benzo[a]pyrene-4-5-oxide, the pyrolytic products of tobacco, since smokers constitute a broad reservoir of high risk individuals in the population, the risk therefore depends on the genetic composition of the individual. Hence a thorough screening of the interindividual variation from the genetic constitution point of view is required, which increases the efficacy of the diagnostic as well as prognostic systems.

Thus there is ever increasing information on the expression of new isoform of GSTs to act on varied forms of synthetic and natural exogenous and endogenous toxic substrates to which the animals are exposed, as part of cellular defense mechanism. Failure of expression of the appropriate isoform of GSTs results in the susceptibility of the organism to the toxic metabolites leading to diseased condition, especially the onset of carcinogenesis.

The action of GSTs on chemotherapeutic drugs, aimed for the treatment of a diseased condition, on the other hand, results in the development of drug resistance leading to the aggravation of the diseased state. Hence studies on the expression of specific isoform(s) of GSTs in the development of isoform - specific inhibitors in different types of cancer would be of immense value in the treatment of cancer patients (fig. 5). Also studies on GSTs in fetal tissues might throw light on the role of GSTs in actively proliferating normal cells.

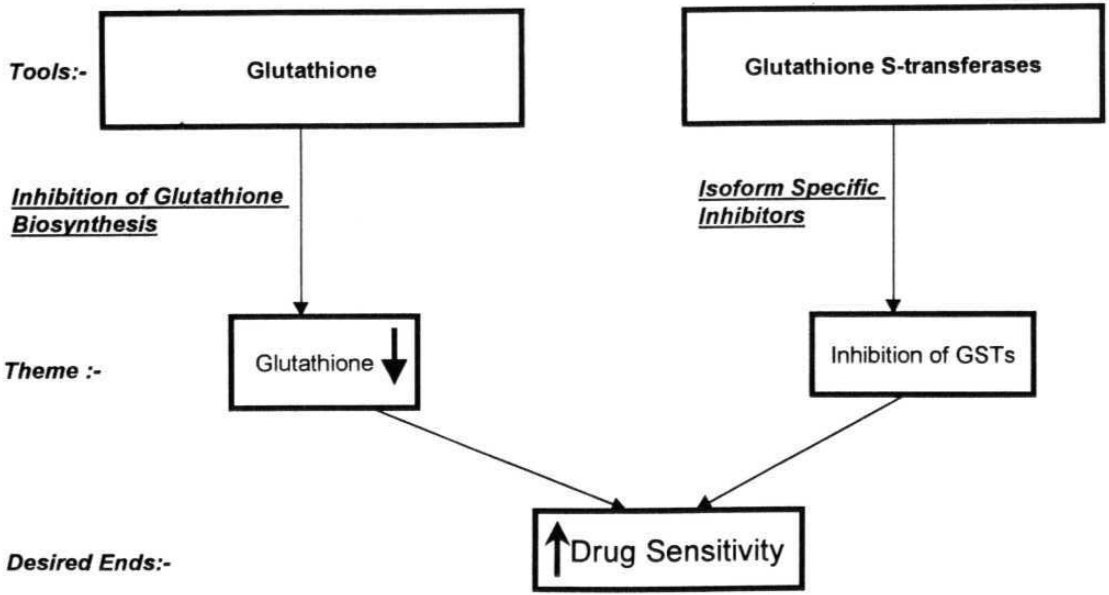


Fig. 5:- Hypothetical model to overcome multidrug resistance

METHODOLOGY

2.0 MATERIALS:

Glutathione reduced (GSH), S-hexyl glutathione, 1-chloro-2,4,-dinitrobenzene (CDNB), dithiothreitol (DTT), epoxy-activated Sepharose 6B, phenylmethylsulfonyl fluoried (PMSF), Tris, glycine, L-serine, Tween-20, Triton X-100, sodium chloried, Δ^5androstene-3,17-dione(Δ^5AD), bromosulfophthalein (BSP), 1,2-dichloro-4-nitrobenzene (DCNB), Ethacrynic acid (EA),4-hydroxynonenal (4-HNE).Freund's complete and incomplete adjuvant were purchased from Sigma Chemicals Company (St.Louis,USA)

Acrylamide (99.9%), N,N'-methylene-bis-acrylamide,N,N,N',N'-tetramethyl-ethylenediamine (TEMED), 2-mercaptoethanol, natriumlauryl-sulfat (SDS), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), comassie brilliant blue G-250, coomassie brilliant blue R-250, bio-lyte ampholines and bromophenol blue were purchased from Bio-Rad laboratories (Richmond, USA).

Low molecular weight markers for SDS-PAGE were purchased from Pharmacia Biotech (Uppsala, Sweden). Ultrafiltration units were procured from Amicon (MA,USA). Nitrocellulose membranes for immunoblotting were

from Amersham (England, UK). C18 and silica cartridges were purchased from Waters, India.

Class specific antibodies of GSTs were a kind gift from Yogesh.C.Awasthi (University of Texas, Galveston). HPLC solvents like acetonitrile, methanol were purchased from Spectrochem India Ltd. All other chemicals procured were from the local companies and were of high quality.

2.1 TISSUE HANDLING:

Human uteri were procured from local hospitals, immediately after hysterectomy. The normal portions of the collected uterus were confirmed by a pathologist at the source facility and were stored in -80° C till further use.

Cancerous and surrounding normal tissues were obtained from Mehidi Nawaz Jung Cancer Hospital (Regional Cancer Center), Hyderabad. All the tissues were collected in liquid nitrogen and stored at - 80 ° C till further use. A portion of each tissue was examined by a pathologist at the source facility and confirmed as being cancerous or normal. All the tissues were classified into different stages of cancer (stage I = Tumor cells found only in original site, stage II = Tumor cells found in original site and regional lymph nodes, stage III = Tumor cells found in original site, regional lymph nodes and in distant lymph nodes and stage IV = Metastatic tumor cells found in many body areas) by the pathologist.

2.2 PROCESSING OF TISSUE FOR GST ACTIVITY:

Normal and cancerous tissues were slightly thawed, minced with scissors and homogenized in 50 mM Tris - HCl buffer, pH 8.0 containing 0.25 M sucrose and 1mM PMSF using a glass homogenizer. Homogenization was done by keeping the glass homogenizer in an ice jacket and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheesecloth and the resulting supernatant was centrifuged at 10,000 X g on high speed refrigerated centrifuge (KUBOTA 6700, rotor RA-4) for 30 min. The resulting supernatant was used as the enzyme source.

2.3 GST ACTIVITY ASSAY:

GST activity was assayed by the conventional method of Habig *et al* (1981). The typical enzyme mixture in a volume of 1 mL consists of 1 mM CDNB (1, chloro, 2,4- dinitrobenzene), 1 mM glutathione (GSH) and 100mM potassium phosphate buffer (pH 6.5). Thioether formation was determined by reading the absorbance at 340 nm and quantification was done using 9.6 $-mM^{-1}.cm^{-1}$ as the extinction coefficient.

2.4 AFFINITY CHROMATOGRAPHY OF GSTs:

2.4.1 Preparation of affinity matrix

Affinity matrix was prepared by coupling glutathione (GSH) to epoxy activated Sepharose -6B as per the method of Simmons and Vander Jagt (1977). About 4 g of epoxy - activated Sepharose 6B was washed with 500

mL of distilled water followed by 40 mL of 44 mM phosphate buffer pH 7.0. Gel was transferred to another flask and the volume was adjusted to 20 mL with the same buffer and nitrogen gas was passed through for 5 min. To this 4 mL of GSH (400 mg of GSH in 4 mL of distilled water, pH was adjusted to 7.0 with KOH) was added and allowed to stay for 24 hr at 37° C with constant stirring

The coupled gel was washed with 100 mL of distilled water followed by 100 mL of 0.5 M KCl in 0.1 M sodium acetate, pH 4.0 and 0.5 M KCl in 0.1 M sodium borate buffer, pH 8.0. Finally the gel was transferred to 10 mM potassium phosphate buffer, pH 7.0 containing 150 mM KCl.

2.4.2 Cytosol preparation

Normal and cancerous tissues were homogenized in four volumes of 10 mM potassium phosphate buffer, pH 7.0 containing 0.25 M sucrose, 1mM EDTA, 2 mM dithiotheritol. Homogenization was done in potter Elvejahm homogenizer by keeping the glass homogenizer in an ice jacket and care was taken to minimize the froth formation and centrifuged at 10,000 X g for 30 min. The resultant supernatant was passed through two layers of cheesecloth to remove the cell debris. The supernatant was again centrifuged at 1,05,000 X g on Hitachi ultracentrifuge with P50AT2 rotor for one hour. The supernatant obtained was referred to as the cytosolic fraction.

2.4.3 Purification of GSTs

Cytosolic fractions after dialysis were loaded on to GSH-Sepharose 6B affinity column previously equilibrated with 10 mM potassium phosphate buffer pH 7.0 containing 0.15 M KCl and then washed with the same buffer till the protein content dropped to zero (by spectrophotometric detection). The affinity bound GSTs were eluted with 50 mM potassium phosphate buffer pH 7.5 containing 10 mM GSH and 1 mL fractions were collected Active fractions were pooled and concentrated by using centricon concentrators (AMICON).

2.4.4 Protein determination:

Protein content in the chromatographic fractions was determined spectrophotometrically by the procedure of Warburg and Christian (1941) by measuring the absorbance at 280 nm and 260 nm.

Protein content in the samples like crude homogenate and cytosol were assayed by the method of Bradford (Bradford *et.al*, 1976).

2.5 SUBSTRATE SPECIFICITIES:

In order to screen different GST isozymes for substrate specificities, they were assayed for activity with ethacrynic acid, 1,2 epoxy - 3(-p-nitrophenoxy)- propane, Sulfobromopthalein, 4-nitropyridine-N-oxide and 3,4 dichloronitrobenzene in addition to 1-chloro-2,4-dinitrobenzene, the classical substrate for GSTs.

Table 3: Reaction conditions for the assay of GSTs with different substrates.

Substrates	Mol. Wt	Con. mM	pH	X max.	GSH mM	A c /mM/cm
1- Chloro- 2,4 - dinitrobenzene	192.0	1	6.5	340	1	9.6
Ethacrynic acid	303.1	0.2	6 5	270	1	5 0
1,2- Epoxy - 3(-p-nitrophenoxy) propane	195.2	5	6.5	360	1	0 5
Sulfobromopthalein	8380	0.03	7.5	330	1	4.5
4 - Nitropyridine - N - oxide	140.1	0.2	7.0	295	1	7 0
3,4,-Dichloronitrobenzene	192.0	1	7.5	345	5	8.5

2.6 SDS-PAGE ANALYSIS

Vertical slab gel electrophoresis was performed according to the method of Laemmli (1970). Electrophoresis was carried out in 12% acrylamide gel with 5% stacking gel. Samples were treated with sample buffer containing 2% SDS, 5% p- mercaptoethanol, 0.01% bromophenol blue for five min. in boiling water bath. Samples were subjected to electrophoresis at constant voltage (100 Volts) till the dye reached the bottom.

2.7 TWO-DIMENSIONAL GEL ELECTROPHORESIS:

Two-dimensional electrophoresis was performed as described by O'Farrell (1975). Isoelectric focusing gels were cast in glass tubes (130X2.5 mm). To setup the pH gradient the ampholyte polyacrylamide gels were prefocused at 200 V for 15 min, at 300V for 30 min and at 400V for 30 min. The samples were loaded and the gels were run at 400 V for 12 h and 800 V for 1 h with 0.01 M H_3PO_4 as the anolyte and 0.02 M NaOH as the catholyte. The gels were extruded into 5 mL of 0.0625 M Tris- HCl (pH 6.8) buffer containing 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and equilibrated for 2 h at room temperature with shaking. The gels were stored frozen at -20° C till use. The second dimension separation was carried out using 3.3 % stacking gel and 12% resolving gel. Pharmacia LMW markers were run at the acidic end of the gel. The gels were run at 25 mA until the dye (bromophenol blue) reached the bottom of the gel The gels were fixed and sliver stained. To determine the pH gradient of the IEF gels, parallel

gels were cut into pieces of 0.5 cm length and incubated for 2 h in 0.5 mL degassed distilled water. The pH of the eluant was determined electrometrically and was taken as isoelectric point (pI) of the protein present in the gel.

2.8 SILVER STAINING

Gels were stained with silver nitrate by the method of Blum (Blum et.al 1981). Gels were treated with a solution of 50% methanol and 7.5% acetic acid for five minutes followed by washing thrice (5 min. each) with 10% ethanol and 5% acetic acid. Gels were treated with 0.1% potassium dichromate in nitric acid (24 mL /100 mL) for five min and washed thoroughly with distilled water. The gels were treated with 0.2 % silver nitrate solution for 15 min, rinsed with distilled water and color was developed with 3% sodium carbonate solution containing formaldehyde (500 mL / 1000 mL). Color development was stopped by 1% acetic acid and gels were preserved in 5% acetic acid.

2.9 MOLECULAR WEIGHT ANALYSIS

Molecular weight of the proteins was determined from the calibration curve generated using Pharmacia low molecular weight (LMW) markers using gel documentation system (UVP, San Gabriel Inc. UK). The LMW markers included phophorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and a lactalbumin (14.4 kDa).

2.10 PRODUCTION OF ANTISERA

Purified protein from affinity as well as HPLC eluants were used for immunization Rabbits (New Zealand white male, 2 months old), prior to the injection of sample, were bled a week before the serum was collected and stored as pre-immune sera. The samples were injected subcutaneously which were emulsified in Complete Freund's adjuvant in 1:1 ratio. The booster injections were given with the sample in incomplete Freund's adjuvant after 15 days and continued for 3 months giving booster for every 15 days. Rabbits were bled a week after the final booster injection, serum was collected, Ig G was purified using the protein -A agarose and stored in aliquots at -20° C as immune sera (primary antibody).

2.11 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

Affinity purified GSTs were further subjected to RP-HPLC (Shimadzu 6A) using Waters bondapack C-18 (3.9 X 300 mm) column by employing gradient elution. The mobile phase was 20 % acetonitrile with 0.1 % (v/v) trifluoroaceticacid (solvent A) and 65 % acetonitrile with 0.1 % of trifluoroaceticacid (solvent B). Affinity purified sample (100 µg) was injected in 100 % of solvent A and the elution was done with a linear complex gradient from 0 to 100 % of solvent B over a period of 50 min.

The gradient was constructed in the linear increasing concentration of solvent B in four successive steps, (0-10 % in 1st 10 min, 10-40 % from

15th to 20th min, 40-50 % from 25th to 35th min and 50 - 100 % from 40th to 50th min, using SCL- 6A system controller. The eluted polypeptides were monitored at 214 nm using SPD-6AV UV-VIS spectrophotometric detector.

2.12 ENZYME LINKED IMMUNOSORBENT ASSAY

Enzyme linked immunosorbent assay (ELISA) was performed with the normal and cancerous samples. The samples were loaded into the wells of microtitre plate (200 μL/well) and the plate was incubated at 37° C for 90 min in a humid container. The plate was washed thrice with Phosphate Buffer Saline with 0.05% Tween-20 (PBS-T) at three min interval. Primary antibody diluted (a 1:1000, μ_1 1.5:1000, $\mu_2$1.5:1000 & π1:1000) in PBST with 2% Polyvinylpyrroliden - 40,000 and 0.2% Ovalbumin (PBS - TPO) was added (200 μL / well) and incubated at 37 ° C for 90 min and washed with PBS - T. Secondary antibody conjugated with alkaline phosphatase (Genie, Bangalore) diluted (1:5000) in PBS - TPO was added (200 μL / well) in to the wells and incubated at 37 ° C for 90 min. After incubation, the wells were washed with PBS - T and the substrate, paranitrophenyl phosphate (5mg / 10 mL Diethanolamine buffer pH 9.8) was added (200 μL / well). The plate was incubated at room temperature till the desired color was developed (10 min.) and the color was read at 405 nm.

2.13 WESTERN BLOTTING

Immunoblot analysis was carried out on nitrocellulose membranes according to the published procedures of Towbin *et al* (1975). The cytosolic

GSTs separated on SDS-PAGE were transferred on to the nitrocellulose membrane. The gels were initially soaked in 25 mM Tris, 192 mM glycine & 20 % methanol. The separated peptides were transferred with a current of 0.8 mA/cm^2 for four hours.

After the transfer process, the membrane was air dried for few seconds. Immediately the membrane was made wet in Tris buffer saline (TBS) & thorough rinsing was done. Then the membrane was transferred in to TBS, which contains 5% nonfat milk for 30 min or more to block the nonspecific binding sites. The membrane was immersed in TBST (Tris buffer saline with 0.05% Tween 20) with nonfat milk containing the primary antibody and incubated for 30 min. The unbound primary antibody was removed by washing with TBST, 3 times (5-10 min each). Membrane was incubated for 30 min in TBST with 5% non-fat milk containing secondary antibody linked to alkaline phosphatase. The membrane was washed with TBST 5 times (5 min each) and was subjected to color development.

CHAPTER I

3.1 - PURIFICATION AND CHARACTERIZATION OF HUMAN UTERINE CYTOSOLIC GSTs

3.1.0 INTRODUCTION

Knowledge of the type of glutathione S-transferases in normal tissues is a prerequisite for a sound understanding of their variations in abnormal conditions, as seen in many diseased states. Hence in the present study GSTs are purified from human uteri by affinity chromatography. Individual GST subunits were separated on RP-HPLC. Polyclonal antibodies raised against individual subunits in rabbits, were employed for probing the variations in GST subunits in cancer and fetal tissues

3.1.1RESULTS:

3.1.1.1 AFFINITY PURIFICATION

Glutathione S-transferases have been purified to electrophoretic homogeneity from human uterus by a combination of affinity chromatography and RP-HPLC.

A 10% homogenate was prepared and centrifuged at 10,000 X g for 30 min and later the 10,000 X g supernatant was subjected to 1,05,000 X

g for 1 h, and the following supernatant was termed as cytosol. The cytosol was further subjected to overnight dialysis against 10mM phosphate buffer (pH 7.0), to remove the endogenous GSH.

The dialyzed cytosol was re-centrifuged at 10,000 X g for 30 min at 4°C to remove precipitated proteins. The supernatant thus obtained was loaded on to the GSH-Sepharose affinity column, previously equilibrated with 10mM potassium phosphate buffer pH 7.0. Subsequently the column was washed thoroughly with 50 mM potassium phosphate buffer pH 7.0 supplemented with 150 mM KCl to remove proteins bound non-specifically. The proteins with GST activity were eluted with 50 mM Tris-HCl buffer pH 9.6 containing 5 mM GSH. The typical elution profile of GSTs from GSH affinity column was represented in Fig 6. As shown in the figure, GSTs eluted in a single sharp peak. All the active fractions were pooled and concentrated to 5mL by ultra filtration using amicon concentrators with 30 kDa cut off. The purification achieved was 329 folds with an overall yield of 67% in single step of affinity chromatography (Table 4).

The affinity purified GSTs were subjected to SDS-PAGE (12%) to check the purity of the preparation and to analyze the molecular weights of the GSTs. Calibration of the relative molecular weight was done by loading the molecular weight standards along with the affinity purified GSTs side by side in a single gel (Fig. 7). As shown in the figure the

Table 4: Typical Purification Profile of Human Uterine GSTs

Fraction	Total Protein (mg)	Total Activity (Units)	Specific Activity* (Uints/mg protein)	Fold Purification	Yield (%)
Homogenate (10,000X g supt)	**6500**	**960**	**0.147**	**1**	**100**
Cytosol **(1,0,5000X g supt)**	**5620**	**900**	**0.160**	**1.08**	**93.7**
GSH Affinity Purified	**13.23**	**640**	**48.37**	**329**	**66.6**

** One unit is defined as one μ mole of thioether formed per min.*

Fig.6:Elution Profile of Human Uterine GSTs on GSH-Affinity Column

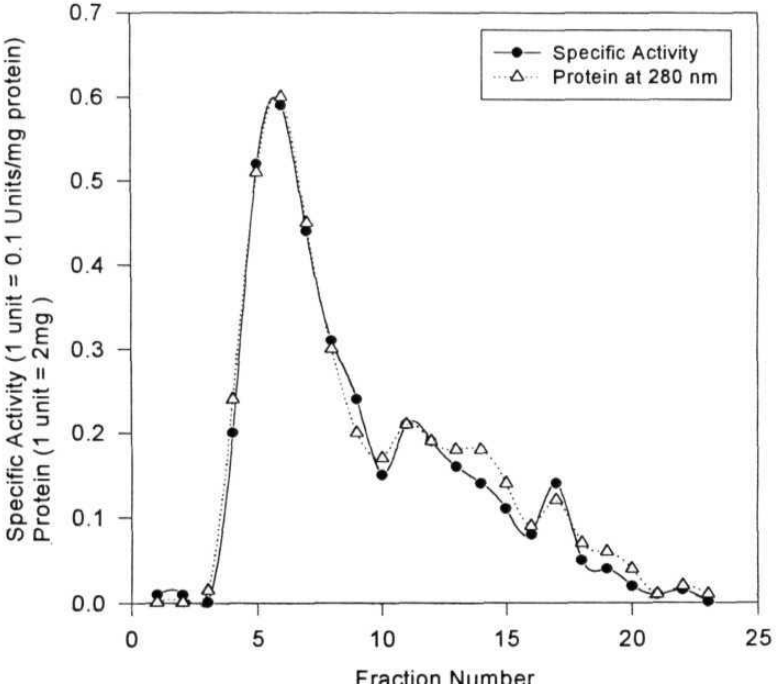
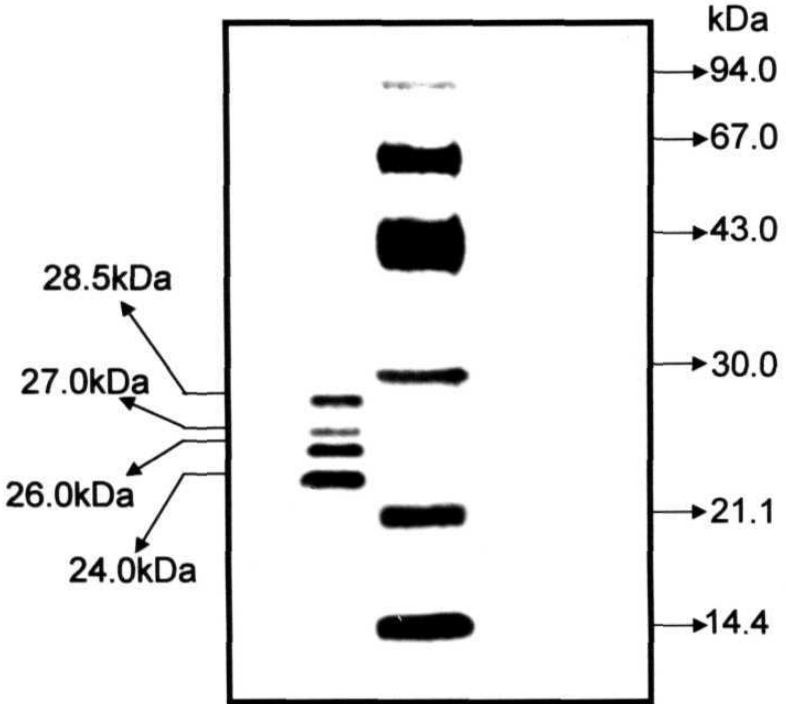


Fig.7: SDS PAGE analysis of affinity purified human uterine GSTs



Lane 1: Affinity Purified Human Uterine GSTs
Lane 2: Low Molecular Weight Markers

affinity purified GSTs (lane 1) resolved in to four bands with relative molecular weights of 24 kDa, 26 kDa, 27 kDa and 28.5 kDa.

3.1.1.2 RP-HPLC ANALYSIS

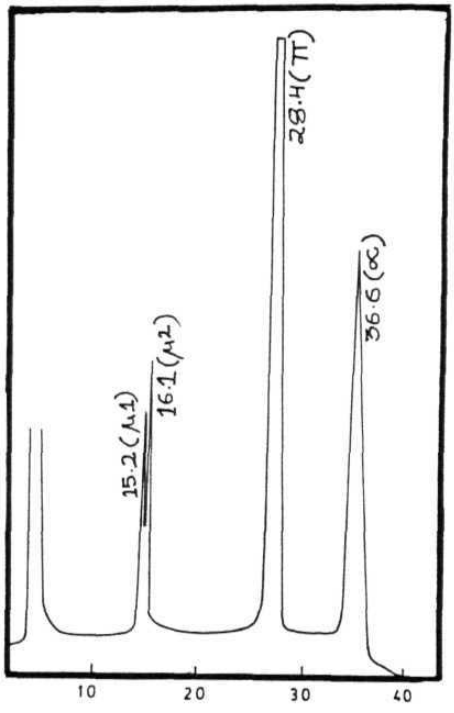
The affinity purified GSTs were separated on RP-HPLC using a complex gradient on a C_{18} column. A typical RP-HPLC profile of the affinity purified GSTs is shown in Fig 8. As shown in the figure the affinity purified GSTs resolved into four peaks with the retention times of 15.2 min (peak 1), 16.1 min (peak 2), 28.4 min (peak 3) and 36.6 min (peak 4). SDS PAGE analysis of the individual peaks separated on RP-HPLC is shown in Fig 9,10 & 11. As shown in the figures, the peaks with RT 15.2min showed a molecular weight of 27 kDa, 16.1 min peak showed 26 kDa MW, 28.4 min peak showed molecular weight of 24 kDa MW, and 36.3 min peak showed 28.5 kDa MW.

The relative composition was calculated using the data from CR4A integrator. Table 5 shows the relative subunit composition of GSTs from human uterus. Of all the subunits, peak 3 is the major subunit (62 %) followed by peak 4 (22%), peak 2 (10%) and lowest being the peak 1 (6%) in the decreasing order.

3.1.1.3 WESTERN BLOT ANALYSIS

Western blot analysis was performed with the polyclonal antibodies raised to each individual peaks separated on RP-HPLC and also cross checked with the antibodies provided by

Fig.8: RP-HPLC analysis of affinity purified human uterine GSTs



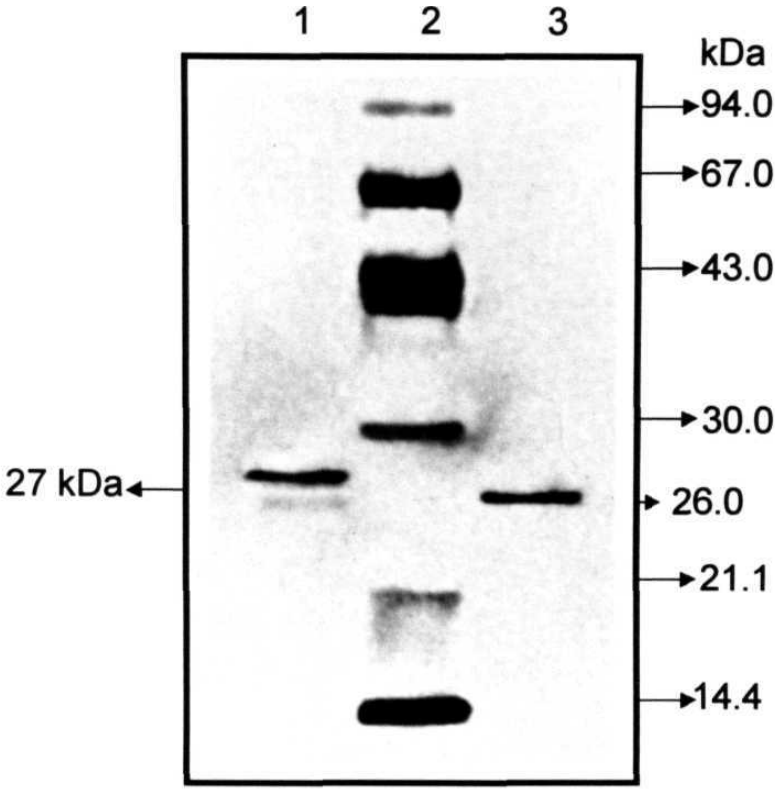
Column: Waters μ Bondapak C_{18} (0.39 X 30 cm)
Solvent: 0.1% TFA in 20% Acetonitrile (Solvent A)
0.1% TFA in 65% Acetonitrile (Solvent B)
Gradient: Step
Flow rate: 1mL/min
Detection: 214 nm
Sample: 75 μg of affinity GSTs

Table 5: Relative Abundance of GST Subunits in Human Uterus

Subunit	RT(min)	Abundance*
μ1	15.2	6%
μ2	16.1	10%
π	28.4	62%
a	36.6	22%

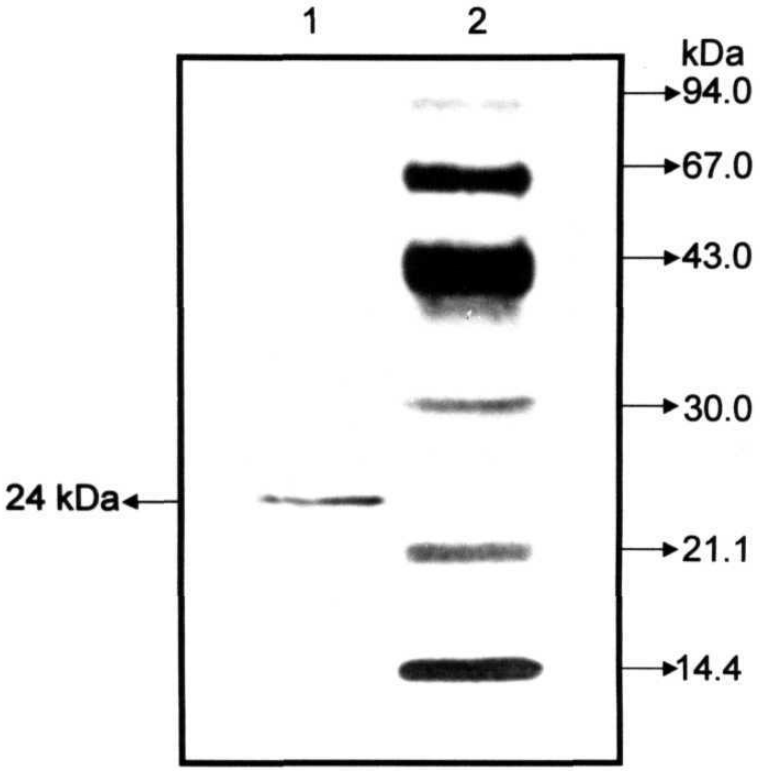
Relative abundance calculated based on RP-HPLC data as shown in Fig. 8, RT= Retention Time

Fig.9: SDS PAGE analysis of 15.2min & 16.1min HPLC peak



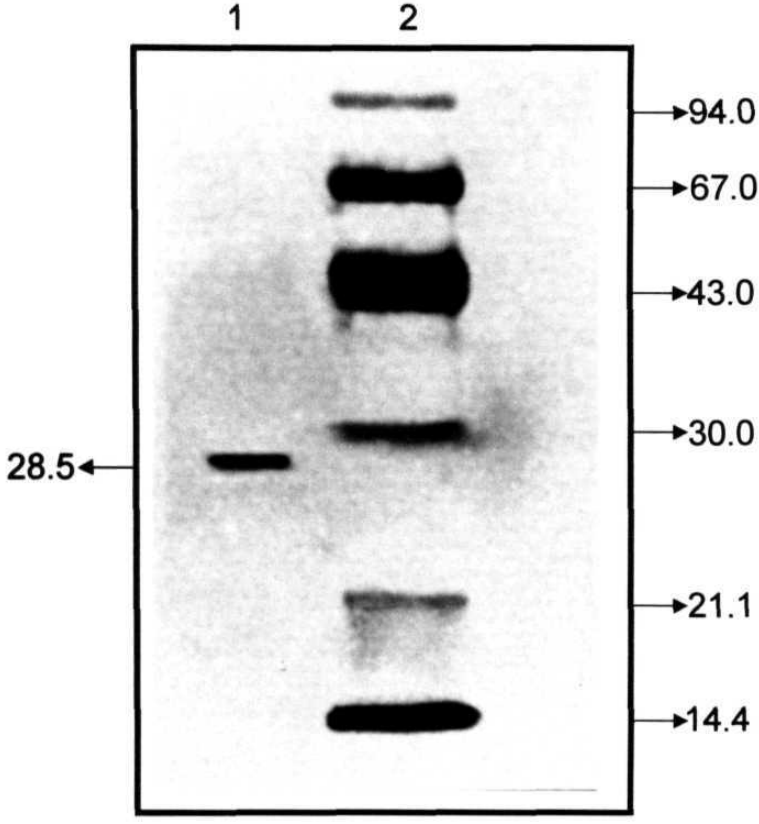
Lane 1: RP - HPLC Peak with RT 15.2
Lane 2: Low Molecular Weight Markers
Lane 3: RP - HPLC Peak with RT 16.1

Fig.10: **SDS PAGE analysis of 28.4min HPLC peak**



Lane 1: RP - HPLC Peak with RT 28.4
Lane 2: Low Molecular Weight Markers

Fig.11: **SDS PAGE analysis of 36.6min HPLC peak**



Lane 1: RP - HPLC Peak with RT 36.6
Lane 2: Low Molecular Weight Markers

Dr.Yogesh C.Awasthi, University of Texas at Galveston. As shown in the Figure 12, peak 1 and peak 2 showed cross reactivity with anti-Mu antibodies (Fig 12-P1), peak 3 with anti-Pi antibodies (Fig 12-P2) and peak 4 with anti-Alpha antibodies (Fig 12-P3). Apparently no cross reactivity was observed between the classes.

Based on the retention times, relative molecular weight and immunoblot analysis. The peak 1 is identified as μ1, peak 2 as μ2, peak 3 as π and peak 4 as a subunits of GSTs.

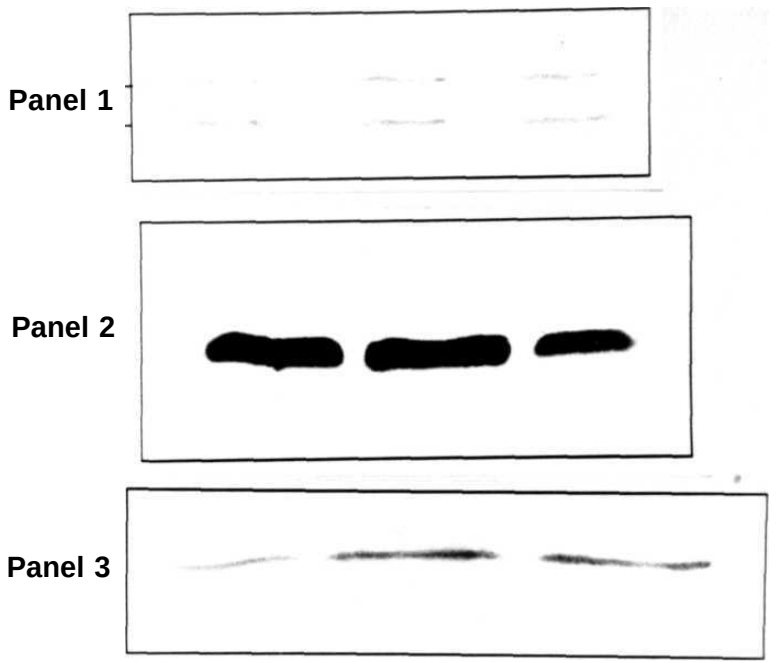
3.1.1.4 SUBSTRATES SPECIFICITIES

Apart from the structural characterization functional studies of human uterine GSTs were conducted by studying the activity levels of affinity purified GSTs with six different substrates (Table 6). As shown in the table, highest activity of affinity purified GSTs was observed with CDNB as the substrate, followed by sulfobromophthalein & DCNB. Lowest activity was observed with 4-nitropyridien-N-oxide.

3.1.1.5 Discussion

In the present study human uterine cytosolic GSTs were purified, individual subunits were isolated and then characterized. The study revealed structural multiplicity of GST subunits in human uterus. In view of the detoxification role of the GST against electrophilic compounds their presence in the uterine tissue might contribute to the protection of fetus during pregnancy. Of the four GST isoenzymes in the human uterus, the

Fig 12: Immunoblot analysis of affinity purified human uterine GSTs



In the three lanes of each panel 30 μg of affinity purified GSTs were loaded
Panel 1: probed with Anti-Mu IgG
Panel 2: probed with Anti-Pi IgG
Panel 3: probed with Anti-Alpha IgG

Table 6: Substrate Specificities of Human Uterine GSTs

S.No	Substrates Studied	Specific Activity
1	1-Chloro-2,4-dinitrobenzene	210
2	Ethacrynic acid	130
3	1,2 Epoxy-3(-p-nitrophenoxy)-propane	90
4	Sulfobromopthalein	180
5	4-Nitropyridine-N-oxide	70
6	3,4 Dichloronitrobenzene	160

Specific Activity expressed as n moles/minXmg protein
Each Value is the mean of atleast six different observation

major isoenzyme, which accounted for approximately 60% of the cytosolic GSTs, was the *n* class. Similar results were obtained by Dililo (Dillio et.al 1988) in fibridoma operated human uteri. However, Dililo had observed five forms of GSTs in the fibridoma uteri. The extra isoform observed was reported to be of the a form. Awasthi and his group (Singhal *et.al,* 1996) and Murakoshi *et.al,* (1990) also observed that π form of GST as the major cytosolic GST in rat uterus. Human as well as rat placenta also were shown to have majorily 7t form of GSTs in the cytosol (Sato *et.al,* 1984) It is reported that uterus is more resistant to lipid peroxidation than the liver (Devasagayam, 1986). This may be due to low availability of substrate for lipid peroxidation and/or higher concentration of factor(s) that protect uterus from lipid peroxidation. The relatively lower level of lipid peroxidation observed in the uterus is probably because of the higher expression of GST *n* in the uterus (Singhal *et.al;* 1996) which may be playing a protective role against lipid peroxides. This probably works as a good defense system especially at the time of pregnancy where in there will be a deluge of immune cells like mast cells, macrophages and granular cells, which contribute to the oxidative stress (Hunt. Et.al, 1994).

Until recently characterizing of GSTs was done mainly by SDS PAGE and immunoblotting (Tu et.al 1983 & Hayes et.al 1986b). However, there were still problems with cross reactivity of GST antibodies, quantification by immunoblotting and co-migration of GST subunits, which

differ by less than 500 MW on SDS PAGE (Hayes et.al 1986a). A number of analytical techniques have been developed to study the GST isoform separation like electrospray mass spectrometry (Yeh *et.al;* 1995), isoelectric focusing (Hales *et.al;* 1978) and the electron ionization spray chromatography (Rouimi *et.al;* 1995). But off late after the development of HPLC techniques, many labs (Ostlund Farrants et.al 1987, Ketterer et.al 1988, Kispert et.al 1989, Meyer et.al 1989, Hirutsuka et.al 1990, Hayes et.al 1990, Johnson et.al 1990, Veera Reddy et.al 1994) have significantly improved the ability to identify and quantify GST subunits. In the present study the HPLC technique was slightly modified so as to separate all subunits of human uterine cytosolic GSTs with near baseline resolution.

The present investigation establishes quantitative values of GST subunit expression in normal human uterus, data that was previously not available. These values can be used to determine the changes in subunit-specific effects under the influence of varied chemical compounds or under different patho-physiological conditions. Benson et.al, (1989) have used this technique to show the sex-specific differences in GST expression and subunit-specific induction of GSTs by two anticarcinogenic compounds, 2(3)-t-butyl-4-hydroxyanisole and bisethylxanthogen in mice. From this lab also (Veera Reddy et.al 1995) RP-HPLC technique was employed to demonstrate the induction of Ya1 subunit of GST in rat liver under oxidative stress. Further more HPLC can be used to identify and

characterize subunits expressed under varied pathophysiological conditions, including cancer

CHAPETR 2

3.2: Glutathione S-transferases in Normal Colon and Cancerous Colon & in Squamous Cell Carcinoma of Uterine **Endometrium,** Cervix and Penis.

3.2.0 INTRODUCTION

In order to study the pattern of changes in the expression of GSTs, GSTs were analyzed in cancer tissues of adenomatous colon and squamous cell carcinoma of uterine endometrium, cervix and penis. The data obtained was compared with those from corresponding normal tissues.

Tissue samples from twenty-three adenocarcinoma colon and matched normal tissues were collected from Mehdinawaj jung cancer hospital in liquid nitrogen immediately after resection. All the tissues were stored at -80°C in deep freezer. The collected tissues were confirmed to be either normal or cancerous by a pathologist at the source facility. Clinical characteristics of the patients with adenocarcinoma of colon are presented in table 7. As shown in the table the samples collected were from different age groups (31-82 years) and also from both the sexes (M: 15;F: 8). Except for four, which are sigmoid colon, all the other tissues were of adenocarcinoma of colon. Most of the samples (~ 44%) were widely differentiated and the other were either moderately differentiated

Table 7: Clinical Characters of Patients with Adenocarcinoma of Colon

Case	Age	Sex	Differentiation	Location	Histology	Stage
1	43	F	WD	Sigmoid Colon	AC	II
2	S3	M	PD	Ceacum	AC	II
3	61	F	PD	Ceacum	AC	I
4	32	F	WD	Colon	Dysplacia	II
5	57	M	WD	Colon	AC	I
6	68	M	WD	Colon	AC	II
7	72	M	PD	Ceacum	AC	III
8	49	M	PD	Ceacum	AC	I
9	56	F	WD	Ceacum	Dysplacia	III
10	73	M	MD	Ceacum	Dysplacia	I
11	31	F	MD	Colon	AC	III
12	82	M	MD	Sigmoid Colon	AC	II
13	64	M	PD	Sigmoid Colon	AC	I
14	67	F	WD	Colon	AD	I
15	78	F	WD	Colon	AD	II
16	58	F	PD	Colon	AC	I
17	53	M	WD	Colon	AC	III
18	48	M	WD	Colon	AC	II
19	67	M	WD	Sigmoid Colon	AC	III
20	49	M	PD	Colon	AC	III
21	56	M	MD	Colon	AC	III
22	80	M	MD	Ceacum	AC	II
23	38	M	MD	Colon	AC	III

WD= Widely Differentiated; MD= Moderately Differentiated
PD= Poorly Differentiated; AC= Adenocarcinoma; AD= Adenodysplacia

Table 8: Purification Profile of normal and adenocarcinomatous colon GSTs

Tissue Type	Total Activity (Units)	Total Protein (mg)	Yield(%)
Normal Colon	820 + 114	13.4 + 2.0	44.9 + 2.6
Cancerous Colon	994 + 73	18.9 + 3.4	43.4 + 2.3

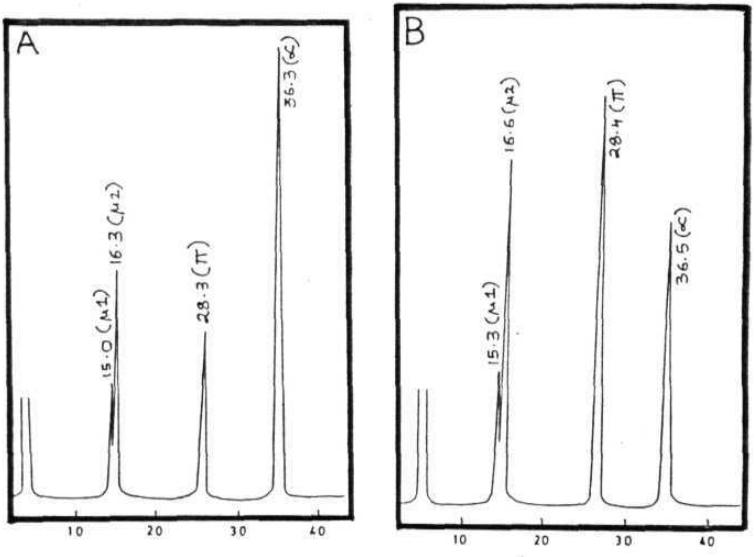
One Unit is defined as one μ mole of Thioether formed per min.
GSTs from normal colon and cancerous colon tissues (5-7g) were purified by GSH-affinity chromatography. Data presented is the mean ± SD of atleast six different observations.

Table 9: Substrate Specificities of affinity purified GSTs from normal and cancerous colon

S.No	Substrates Studied	Normal	Cancerous
1	1-Chloro-2,4-dinitrobenzene	280	750
2	Ethacrynic acid	130	270
3	1,2 Epoxy-3(-p-nitrophenoxy)-propane	50	110
4	Sulfobromopthalein	220	410
5	4-Nitropyridine-N-oxide	60	130
6	3,4 Dichloronitrobenzene	230	340

Units of Activity: n mole/minXmg protein
Activity levels of Affinity Purified GSTs with different substrates were measured as per the assay methods described in the methodology. Each value is the mean of atleast six different observations.

Fig.13: RP-HPLC analysis of cytosolic GSTs from normal and adenocarcinomatous colon



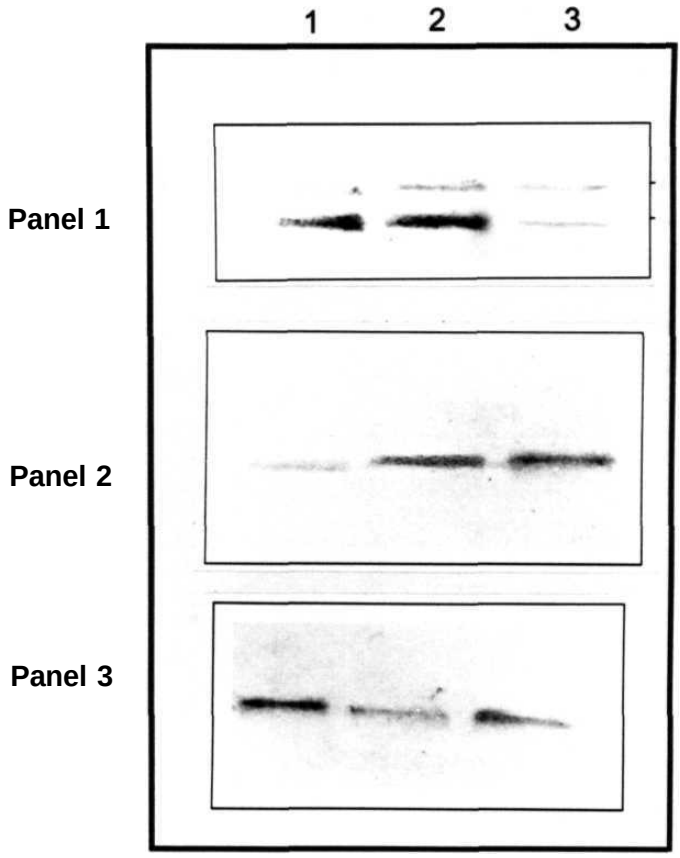
Column: Waters μ Bondapak C_{18} (0.39 X 30 cm)
Solvent: 0.1% TFA in 20% Acetonitrile (Solvent A)
0.1% TFA in 65% Acetonitrile (Solvent B)
Gradient: Step
Flow rate: 1mL/min
Detection: 214 nm
Sample: 75 μg of affinity GSTs

Table 10: Relative abundance of GST subunits in normal and cancerous colon

	Normal Colon		Cancerous Colon	
Subunit	RT(min)	Abundance*	RT(min)	Abundance*
μ1	15	2%	15.3	2%
	16.3	20%	16.6	28%
π	28.3	12%	28.4	46%
a	36.3	66%	36.5	24%

** Relative abundance calculated based on RP-HPLC data. The data obtained is from a typical RP-HPLC analysis data presented in Fig. 13*

Fig. 14: Immunoblot analysis of normal and cancerous colon



In all the Panels Lane 1 Normal Colon, Lane 2: Cancerous Colon & Lane 3: Human uterine GSTs
Panel 1: Probed with anti-Mu IgG
Panel 2: Probed with anti-Pi IgG
Panel 3: Probed with anti-Alpha IgG

(26%) or poorly differentiated (30%) and almost all the patients were suffering from adenocarcinoma of colon. The normal tissues were removed from the same patients by taking care to eliminate the local metastases of cancer. Final confirmation was done based on the pathologist's report.

3.2.1 Glutathione S-transf erases from Colon Cancer Tissues:

3.2.1.1 AFFINITY PURIFICATION OF GSTs

GST purification was carried out separately in normal and cancerous colon by GSH-affinity chromatography. The over all yield in cancerous colon is 43.4% + 2.3 % and 44.9% + 2.6% in normal colon. (Table 8)

3.2.1.2 SUBSTRATE SPECIFICITIES

Substrate specificities studies were carried out using the affinity mixture as the enzyme source. Six classical substrates were used for the study (Table 3). CDNB and DCNB showed high activity both in normal and cancerous colon (Table 9) followed by sulphobromopthalein, ethacrynic acid, 4-nitropyridine-N-oxide and 1,2 epoxy-3(-p-nitrophenoxy)-propane. In cancerous colon GSTs showed high activity compared to normal towards all the six substrates studied.

3.2.1.3 RP-HPLC ANALYSIS

The mixture of GSTs obtained after affinity chromatography was subjected to RP-HPLC analysis to separate different isoforms of GSTs.

The separation was done by employing a complex step gradient on Waters μ bondapak C18 column. The mobile phase applied was 20% acetonitrile as solvent A and 65% acetonitrile as solvent B, with 0.1% trifloroacetic acid as the organic modifier in both the solvents. Detection of the eluted peaks was done at 214 nm at a constant flow rate of 1mL/min.

The affinity purified cytosolic GSTs from both normal and adenocarcinomatous colon showed four peaks (Fig 13) with retention times of, 15.0 min (Peak1), 16.6 min (Peak 2) and 28.4 min (Peak 3) and 36.5 min (Peak 4). These peaks were collected, lyophilized and later subjected to Western blotting with the polyclonal antibodies raised against the human uterine GST isoforms and also cross checked with antibodies provided by Dr.Yogesh.C.Awasthi. Based on the western blot analysis the peak with RT 15.0 min, 16.6 min, 28.4 min and 36.5 min were identified as μ1, μ2, π and α form of GSTs (Fig.14) respectively. The relative abundance of each of the separated GST subunits were calculated from the area normalization method obtained from the CR4A integrator. The comparative analysis of the data showed (Table 10), that there is a clear increase of 34% in *n* form and 8% in μ2. On the contrary 42% decrease was observed in the a from of GST.

3.2.1.4 ELISA ANALYSIS

To further confirm the RP-HPLC results, ELISA analysis was done using the class specific polydonal antibodies for μ, *n* and a form of GSTs

(Table 11). The numeric values given in the table were calculated as the ratios of the intensity of each sample compared to the intensity of the authentic standard. The pictrol representation is shown in fig 16,17,18 and 19

3.2.1.5 STATISTICAL ANALYSIS

To assess the significance of the ELISA results, students t-test, (two tail -paired) was conducted. The significance was set at both $p< 0.01$ and $p < 0.05$ levels From the statistical analysis, except for the $\mu 1$ date $\mu 2$, π and α are significantly different when compared to the normal condition (Table 11).

3.2.1.6 WESTERN BLOT ANALYSIS

To further confirm the elevated levels of $\mu 2$ and *n* and decreased levels of a form of cytosolic GSTs, the tissue homogenate of the colon cancer patients and the corresponding normal tissue homogenate were subjected to Western blotting by using the polydonal antibodies raised from human uterine GSTs, which are separated on RP-HPLC (Fig. 20). As shown in the figure the data here confirms the results obtained by RP-HPLC and ELISA analysis.

3.2.1.7 Glutathione S-transferases from Squamous Cell Carcinoma Tissues

Similar to colon cancer tissue GST isoform analysis was carried in squamous cell carcinoma tissues (cervix, uterine endometrium and penis).

Table 11: ELISA Analysis of Affinity Purified GSTs in Normal and Cancerous Colon

Case Number	Alpha		Mu				Pi	
			M1		M2			
	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor
1	0 19	005	0 04	0 02	008	0 142	0 07	0 142
2	028	003	0.08	0 07	003	0232	0089	0341
3	0.64	0 16	0.08	0 04	0.05	0319	0069	0 129
4	0 89	0 12	005	0.09	0.07	0.431	0 041	0.179
5	**0.21**	0 06	0 001	0 003	0.08	0 469	0 034	0249
6	0.36	0.04	008	0 07	007	0.342	0031	0.142
7	**0.19**	0005	009	0 010	0089	0 564	0 069	0 147
8	0 24	003	0 02	0 018	0 091	0342	0.08	0.08
9	0.36	002	0 03	0 014	0 042	0 042	0 012	0 314
10	093	001	0 02	001	003	0 561	0 067	0 431
11	0.19	0.08	003	0 03	004	0 641	0 049	0561
12	0.23	0.03	0 08	007	0.08	0 142	**0 052**	0 698
13	046	003	005	0.03	0043	0419	0 043	0.731
14	0.59	0.08	004	003	0051	0.673	0032	0891
15	0 87	0.07	0 09	0 08	008	0412	0 063	0.721
16	0.45	0 09	0 07	0.08	0.07	0.842	0.012	0819
17	0 37	0.08	0.06	005	0.03	0947	0.016	0731
18	0 45	0 04	0.001	0 001	0 081	0 563	0.049	0432
19	052	006	0 02	0 04	0042	0 289	0.081	0.193
20	1.42	0.09	0 03	0.01	0051	0 378	0 001	0.148
21	0 14	007	004	004	0040	0.641	0 021	0343
22	082	006	0.03	0.01	0 039	0 764	0031	0 492
23	0 39	0.04	004	005	0 041	0 849	0 081	0 729

Values were determined from the optical density of ELISA Calculated as the ratio of intensity of each sample compared to the intensity of the authentic standard All the values are mean of atleast six different observations

All the data was significant at both the levels P≤ 0 01 and P<005 when compared between normal and cancerous colon by Student t test (Two Tail) except the M1 data

Fig.16:ELISA Analysis of μ1 GST in Normal and Cancerous Colon

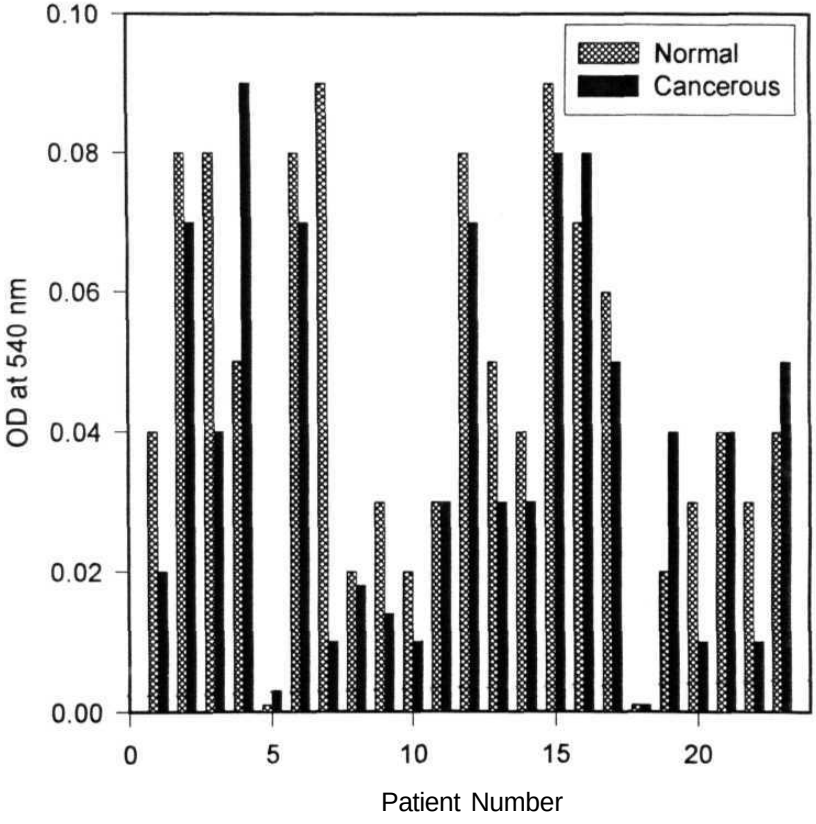


Fig.17: ELISA Analysis of μ2 GST in Normal and Cancerous Colon

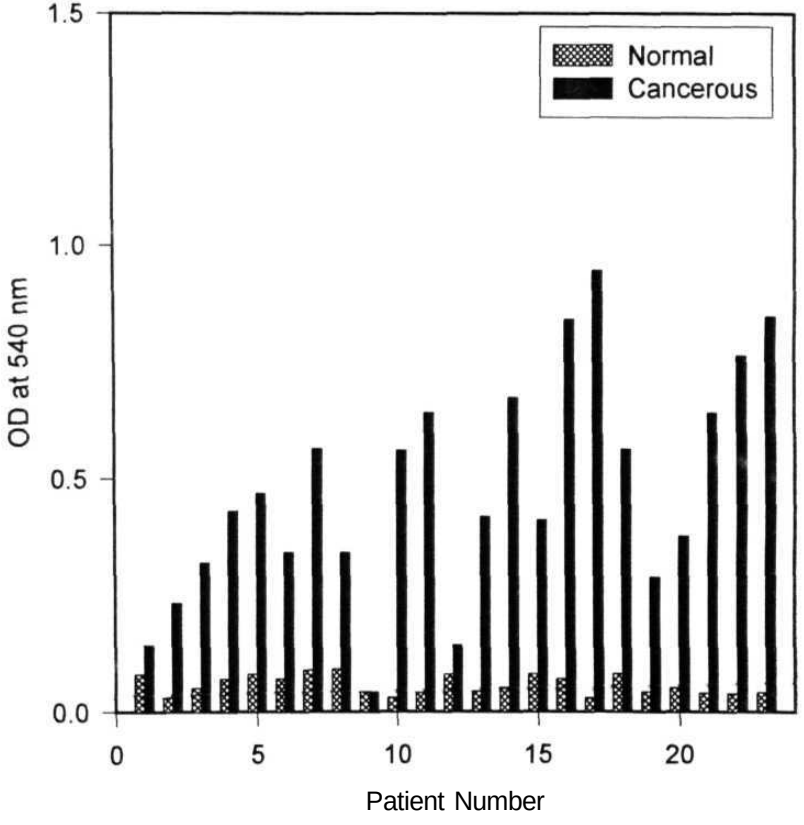


Fig.18: ELISA Analysis of *n* class GST in Normal and Cancerous Colon

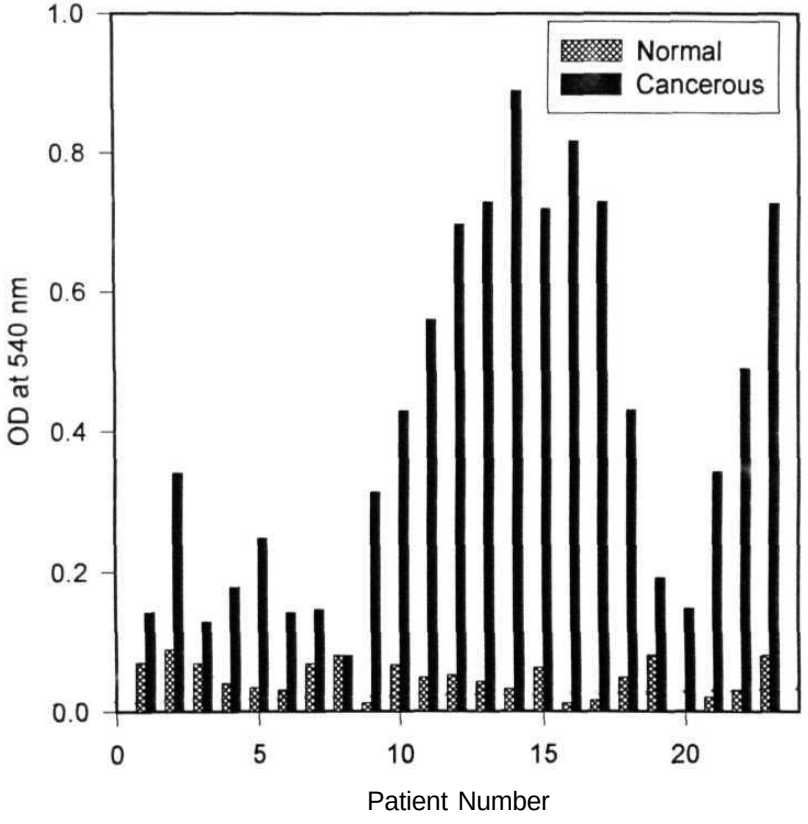
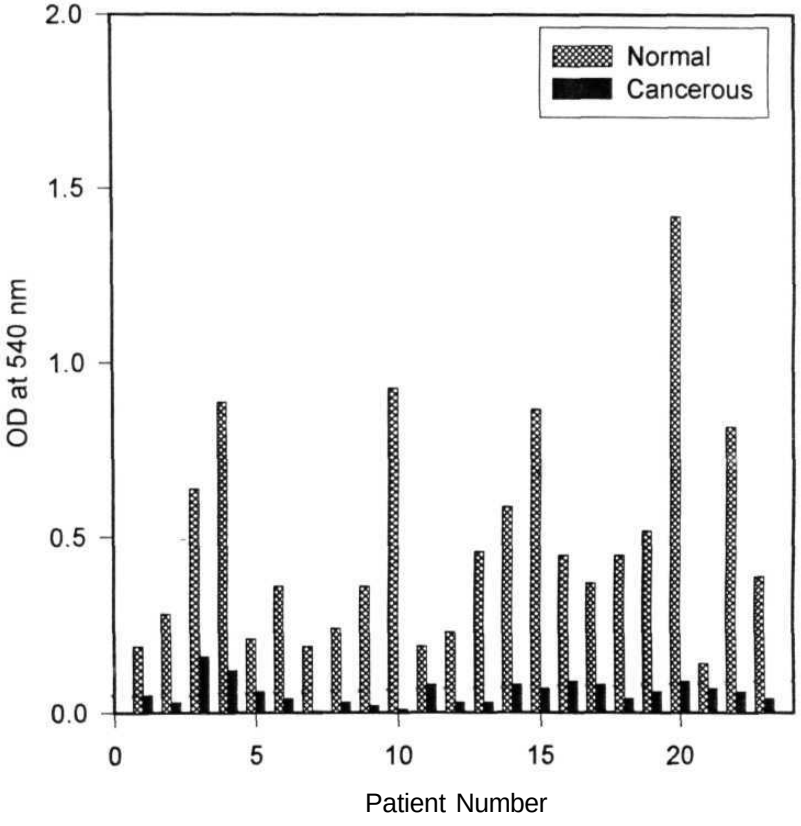


Fig. 19: ELISA analysis of a class GST in Normal and Cancerous Colon



In this aspect the idea was to analyze expression of GST isoforms in different tissues with the same kind of cancer i.e. squamous cell carcinoma. The clinical characters of the patients with squamous cell carcinoma are represented in Table 12. The tissues were separately pooled and processed for GSTs purification by affinity chromatography. In all the three tissues the total activity remained similar and the overall yield was between 43% and 48%.(Table 13)

The purified GSTs were separated on RP-HPLC for subunits, under similar conditions as was done to adenocarcinoma of colon. The RP-HPLC profile of GSTs from cervix, uterine endometrium and penis showed almost similar pattern (Fig. 20, A,B,C). In all the tissues *n* from of GST was the highest followed by a, μ1 and μ2 (Table 14). Further the Westernblot analysis also reveled the similar results (Fig 21).

3.2.1.8 DISCUSSION

Cancers of colon and rectum are the fourth most commonly diagnosed cancers and rank second among cancer deaths in the United States (Miller *et al*; 1996) and is on rise in Indian population. Many studies have been conducted on GSTs from colon cancer patients in American and Japanese populations (Shiratori *et al*; 1987 and Peter *et al*; 1992) and on the Indian scenario no studies were conducted so far. Generally carcinogenesis is classified into initiation, promotion and progression (Farber 1980). Initiation is the covalent modification of DNA with

Table 12: Clinical Characters of Patients with Squamous Cell Carcinoma

Cervix

CASE	AGE	DIFFERENTIATION	HISTOLOGY	STAGE
1	40	PD	SCC	II
2	44	WD	SCC	I
3	53	PD	SCC	I
4	62	MD	SCC	II
5	32	MD	SCC	II
6	37	MD	SCC	I
7	45	MD	SCC	III
8	46	WD	SCC	III
9	43	WD	SCC	I
10	39	PD	SCC	III
11	36	WD	SCC	II
12	40	WD	SCC	II
13	32	WD	SCC	I

Uterine Endometrium

CASE	AGE	DIFFERENTIATION	HISTOLOGY	STAGE
1	29	WD	SSC	III
2	33	WD	SSC	III
3	41	WD	SSC	I
4	32	PD	SSC	II
5	39	MD	SSC	III
6	35	MD	SSC	II
7	28	MD	SSC	I
8	27	MD	SSC	II
9	38	MD	SSC	II
10	36	PD	SSC	II

Penis

CASE	AGE	DIFFERENTIATION	HISTOLOGY	STAGE
1	64	MD	SCC	I
2	28	PD	SCC	II
3	32	WD	SCC	I
4	48	WD	SCC	D
5	51	MD	SCC	III
6	42	MD	SCC	III

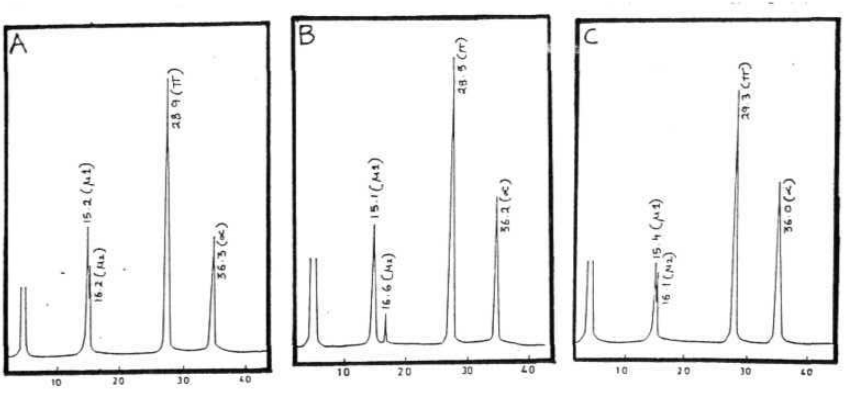
WD= Widely Differentiated; MD= Moderately Differentiated
PD= Poorly Differentiated; SSC= Squamous Cell Carcinoma

Table 13: Purification Profile of GSTs in Squamous Cell Carcinoma Tissues

Tissue Type	**Total** Activity (Units)	Total Protein **(mg)**	Specific Activity **(Units/mg)**	**Yield (%)**
Cervix	634	13	**48.7**	**48**
Uterine **Endometrium**	589	17	**34.7**	**45**
Penis	**732**	15	**48.8**	**43**

One *Unit is defined as one μmole of Thioether formed per min*

Fig.20: RP-HPLC analysis of cytosolic GSTs from squamous cell carcinoma in A) cervix, B) uterine endometrium and C) penis



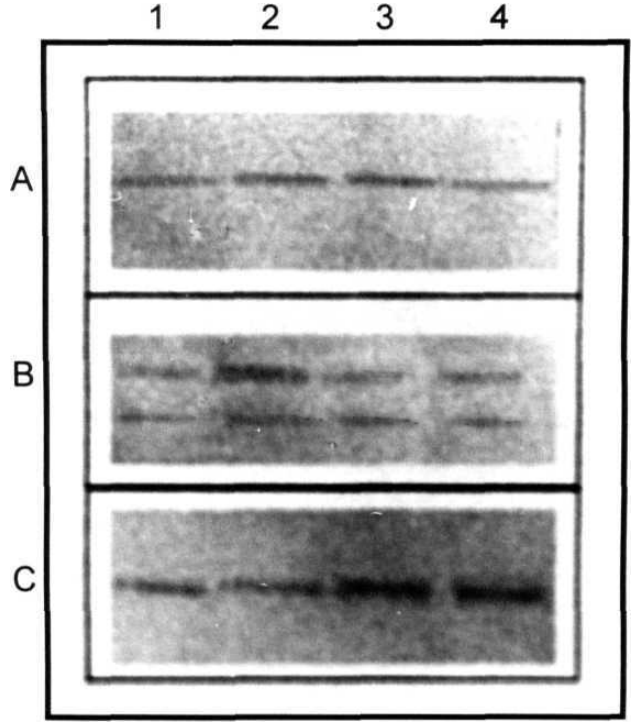
Column: Waters μ Bondapak C_{18} (0.39 X 30 cm), Solvent: 0.1% TFA in 20% Acetonitrile (Solvent A), 0.1% TFA in 65% Acetonitrile (Solvent B), Gradient: Step, Flow rate: 1mL/min Detection: 214 nm, Sample: 75 μg of affinity GSTs

Table 14: Relative abundance of GST subunits in squamous cell carcinoma of cervix, uterine endometrium and penis

	Cervix		UE		Penis	
Subunit	**RT(min)**	**Abundance***	**RT(min)**	Abundance*	**RT(min)**	**Abundance***
μ1	15.2	**13%**	15.1	19%	**15.4**	6%
	16.2	9%	16.6	2%	**16.1**	4%
π	28.9	50%	28.5	48%	29.3	**55%**
a	36.3	28%	**36.2**	31%	36.0	35%

**Relative abundance calculated based on RP-HPLC data as shown in Fig.20, UE= Uterine endometrium; RT= Retention Time in min*

Fig.2V. Immunoblot analysis of GSTs from squamous cell carcinoma in cervix, uterine endometrium and penis



Panel A: Probed with Anti-Alpha, Panel B: Probed with Anti-Mu, Panel C:Probed with Anti-Pi antibodies
In all panels Lanes 1 - 4: Affinity Purified GSTs from Cervix(1) Uterine Endometrium(2), Penis(3) and Normal Uterus(4)
10 μg of protein loaded in each lane.

electrophilic metabolites derived from carcinogens. The role of GSTs is particularly important at this stage as they remove the harmful electrophilic molecules from the body. Numerous reports have been published about the induction of GST isoforms during cancer (Batist et.al 1986, Shea et.al 1988, Tsuchida et.al 1989). The elevated expression of GSTs is also observed in spontaneous cancers (Mitaka *et.al,* 1987, Oyamado *et.al,* 1988 and Masuda *et.al;* 1989). The induction is attributed to more than one factors like increase in the half life of the protein and m-RNA • tability (Hongxie *et.al;* 1995) and because of gene amplification (Chao *et.al;* 1987). GST π induction is not only observed in cancers but also in preneoplastic tissues like the colon adenomas (Kodat *et.al*; 1986) and also in dysplasia of uterine cervix (Shiratori *et.al;* 1987). These studies suggest that GST *n* subunit can be considered as a potent biomarker for the detection of colon cancer in the early stage. In the present study GST *n* was found to be the major subunit in normal colon compared to other isoforms of GSTs. In colon cancer patients compared to the normal, GST π is further increased by ~ 34%. However a form of GST was decreased by 42% in colon cancer compared to normal colon. Similar increase in GST π with concomitant decrease in a form was observed at m-RNA level in renal cell carcinoma of rats, after treating the rats with ferric nitriotriacetate an iron chelator (Tomoyuki Tanaka, *et.al;* 1998) and human renal cell carcinoma (Di Ilio *et.al;* 1991).

As of now there are numerous reports which conclusively established the fact that *n* is the major form of GSTs in almost all cancer types including lung cancer (Howie *et.al;* 1990), oral cavity cancer and bladder cancers (Hirata *et.al;* 1992). Hence GSTs are being used as markers for the detection of cancers. Very recently Harris *et.al;* (1998) have reported the existence of polymorphic nature of the gene. Further the population studies conducted on different racial groups (Indian population included) reveled that there is no significant relation between the GSTP1 polymorphism and colorectal cancers. At present two allelic variants of *n* form of GSTs have been identified GST P 1 * A (coding for GST P 1-1/ He -105) and GST P 1 * B (coding for GST P 1-1/ Val-105). Population studies conducted by Katharin Sundberg (Katharin Sundberg *et.al;* 1998) showed that population with high frequency of variant B is at high risk for tumor formation. This report was also supported by the analysis of catalytic efficiency (k_{cat}/k_m) of both the variants by using polycyclic aromatic hydrocarbons as a model substrates *invitro,* which also differ with respect to there heat stability (Zimniak et.al 1994).

Similar to the present studies, Tsuchida (Tuschida et.al 1997) have clearly revealed that many cancers, histologically classified as adenocarcinoma or squamous cell carcinoma express high GST P 1-1. A significant increase in GST *n* m-RNA ($p<0.01$) by 1.28 and 1.57 -fold was reported in P388/ADR and L1210/DPP resistance cell lines compared to

the normal, suggesting that the elevated expression is contributing to the drug resistance.

Other than the multidrug resistance mechanisms, lack of a GST isoform and susceptibility to cancer was also studied extensively. Many population studies have been conducted in elucidating the underlying fact of GST isoforms expression and their susceptibility to one or the other types of cancers. Very recently it was identified that 50% of the Caucasian population and Japanese population is null phenotype for GST μ (Board *et.al;* 1981 and Takahiko *et.al;* 1995). In the present study 2 null phenotypes for μ1 (n=23) were recorded. Apart from this observation there exists interindividual variation more in μ1 subunit compared to μ2 and other classes GST isoforms. In adenomatous colon, however, significant increase in the m2 subunit (8%) was observed with no significant changes in m1, when compared to that from normal colon.

Another significant observation of the present study is a 38% decrease in the levels of a GST subunit in colon cancer tissue compared to normal. Twe and his group (Ranganathan *et.al,* 1991) have observed that there is an induction of the π form of GSTs in cancerous colon compared to normal colon, similar to the present study results. However they have not observed any clear pattern in the a form of GSTs

The GSTs levels in the squamous cell carcinoma tissues showed similar results, though they were studied in different tissues. Of all the

isoforms of GSTs, *n* form is the major GST subunit observed as evidenced by western blotting and RP-HPLC analysis. Since the corresponding normal tissues could not be procured no meaningful conclusions were drawn, except that rcfrom is the predominant subunit in all squamous cell carcinoma studies. Similar observation was reported by Moscow et.al (1989), where in a 7 fold increase in the GST *n* m-RNA levels in five squamous cell carcinoma of head and neck cancers as compared to the adjacent normal tissue. Riou et.al (1991) also reported increase GST π m-RNA levels in squamous cell carcinoma of the cervix.

CHAPTER 3

3.3: EXPRESSION OF GLUTATHIONE S-TRANSFERASES IN HUMAN FETAL LIVER.

3.3.0 INTRODUCTION

The major aim of the study was to analyze the expression of GSTs in he normal proliferating cells so a comparison can be drawn with those of cancerous tissues. In this connection human fetal livers from different gestation periods viz; 21, 29, 31 and 33 weeks were studied for the GSTs isoform profile. Further the affinity purified human fetal GSTs were used for substrate specificities and RP-HPLC analysis followed by immunoblotting.

3.3.1 RESULTS

3.3.1.1 AFFINITY PURIFICATION

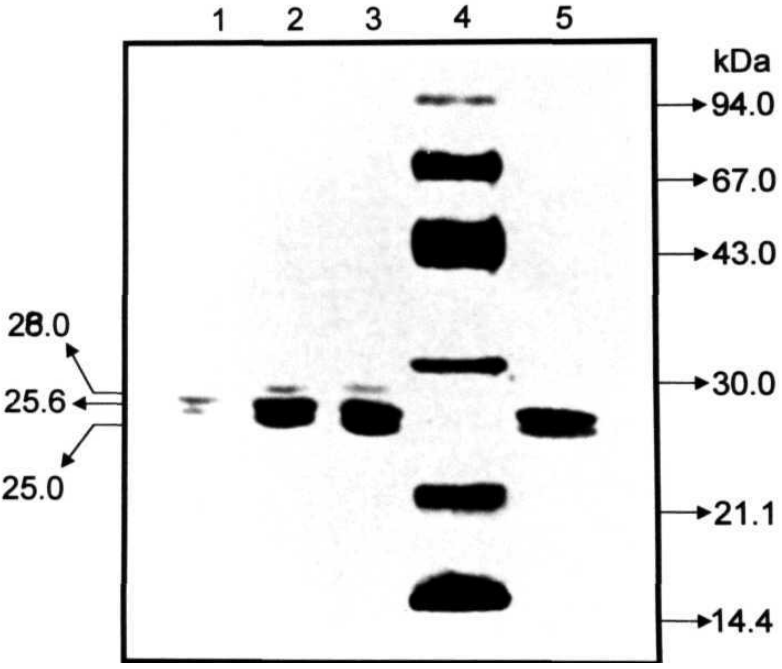
At different stages of gestation (21, 29, 31, and 33 weeks) human fetal liver tissues were collected from Deccan Medical college, and GSTs were purified by GSH affinity chromatography and the data on purification profile is presented in Table 15. The GSTs activity levels (Fig. 24) showed a change in the 31 week fetal liver which is not in consistence with the protein value as seen in the other gestation week. The purified proteins from fetal livers, on SDS PAGE majority resolved into two bands with 25.0 kDa, 25.6 kDa molecular weights (Fig.23). However in 29 and 31 week

Table 15: Purification Profile of human fetal Hepatic GSTs

Gestation week	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg.min protein)	Yield
21	280	8.1	34.5	45.8
29	430	8.6	50.0	45.2
31	620	8.3	74.6	45.8
33	640	13.4	47.7	46.2

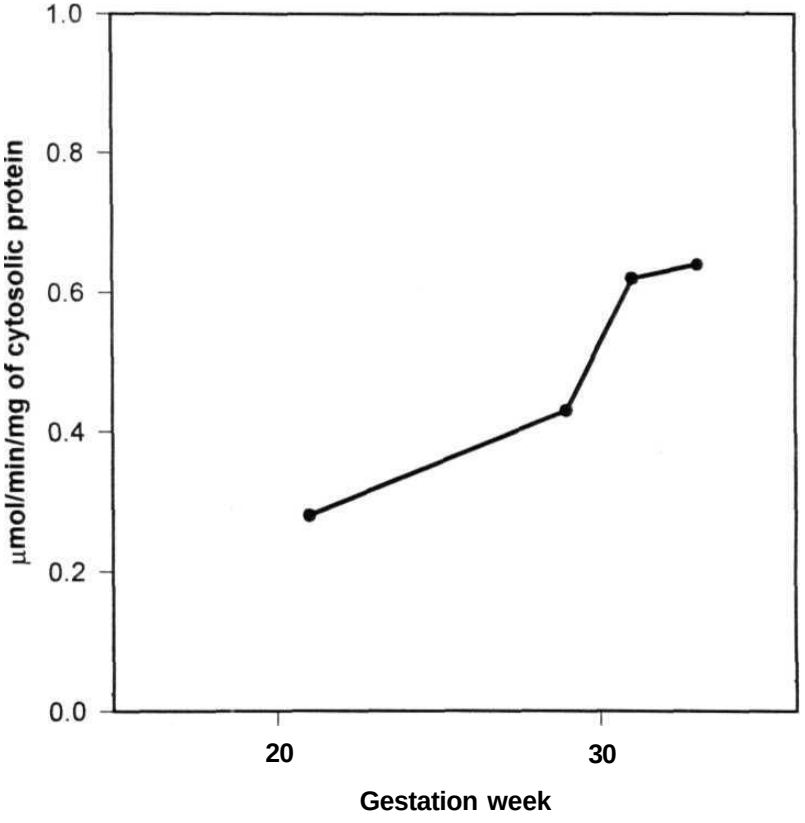
One *Unit is defined as one n mole of Thioether formed per min.*

Fig.23: SDS PAGE analysis of affinity purified human fetal liver GSTs



Lane 1: 21 Week Fetal Liver (10 ug)
Lane 2: 29 Week Fetal Liver (10 µg)
Lane 3: 31 Week Fetal Liver (10 µg)
Lane 4: LMW Markers
Lane 5: 33 Week Fetal Liver (10 ug)

Fig. 24: Activity levels of liver GSTs from human fetuses at different weeks of gestation



fetal livers another additional band with 28 0 kDa MW band was also present

3.3.1.2 RP-HPLC ANALYSIS

RP - HPLC analysis of the affinity purified GSTs from 21 and 33 week fetal livers showed a single peak with retention times 38 7 min and 38.5 min respectively (Fig. 28 a & d). In case of GSTs from 29 and 31 week fetal livers however, two peaks with retention times around 38 min and 45 min were observed (Fig.28 b & c). The extra peak observed in the 29 and 31 week fetal liver GSTs (RT~ 45 min) belonged to the additional 28 kDa protein band as observed on SDS-PAGE (Fig 25). To understand the subunit composition of the peak with RT 45.3 min from 29 week fetal liver, two-dimensional gel electrophoresis was performed (Fig 26) from the 2D-PAGE data, the isoelectric point of the protein was found to be around 7.5 to 8.0 pH.

3.3.1.4 SUBSTRATE SPECIFICITIES

Apart from the subunit composition, the substrate specificities of the affinity purified GSTs were also studied. As shown in the table (Table 16), the affinity of the GSTs towards CDNB, ethacrynic acid and 1,2-epoxy- 3-propane increased with the age of the fetus, the increase ranging from 2 fold in case of CDNB to 10 fold in case of 1,2-epoxy-3-propane. In case of sulphobromopthalein, the activity was not detected by the GSTs from 21 and 33 week old fetuses. This activity was maximum in the GSTs from 29

Fig.28: RP-HPLC analysis of GSTs from human fetal livers at different gestational age

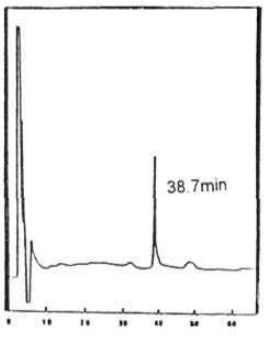


Fig. A: 21 week fetal liver

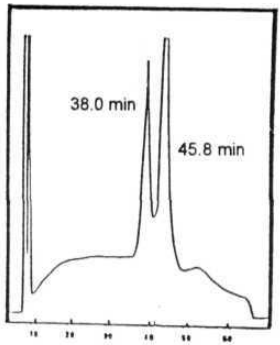


Fig. B: 29 week fetal liver

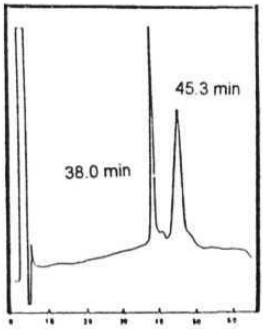


Fig. C: 31 week fetal liver

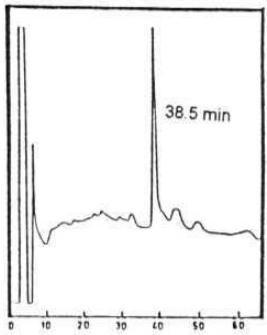
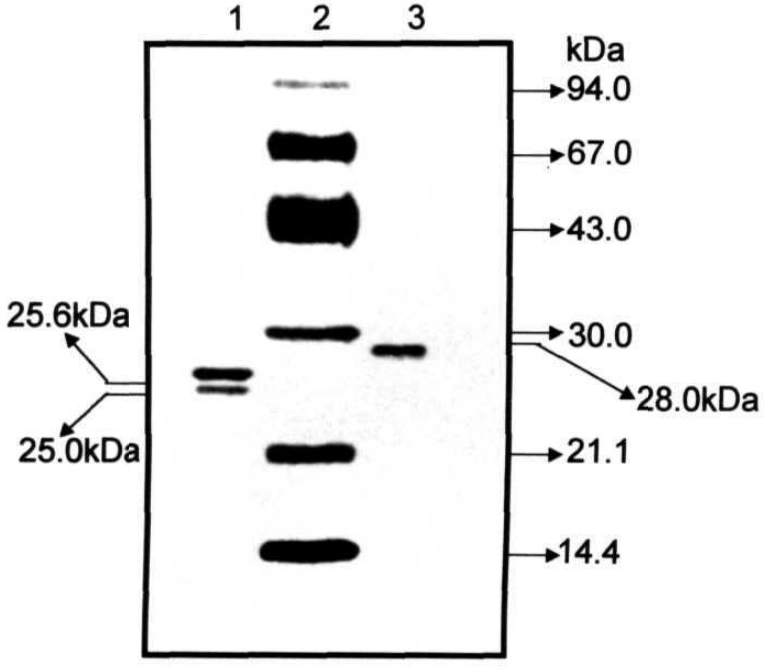


Fig. D: 33 week fetal liver

Column: Waters μ Bondapak C_{18} (0.39 X 30 cm)
Solvent: 0.1% TFA in 20% Acetonitrile (Solvent A)
0.1% TFA in 65% Acetonitrile (Solvent B)
Gradient: Step
Flow rate: 1mL/min
Detection: 214 nm
Sample: 75 μg of affinity GSTs

Fig.25: SDS PAGE analysis of 38.0 min & 45.8 min RP-HPLC peaks from human fetal liver



Lane 1: RP - HPLC Peak with RT 38.0
Lane 2: Low Molecular Weight Markers
Lane 3: RP - HPLC Peak with RT 45.8

Fig.26: 2D electrophoresis of 35.4 min RT peak from 29 week old fetal liver (20μ g)

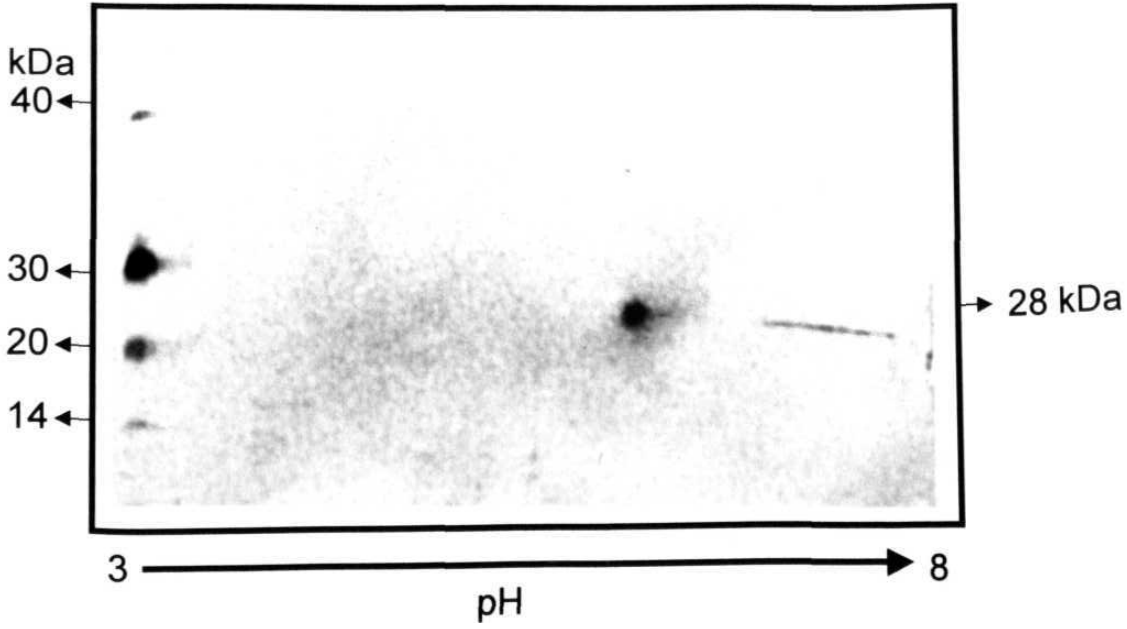


Table 16: Substrate Specificities of Human Fetal Hepatic GSTs at different weeks of gestation

Substrates	Age of the fetus in weeks			
	21 Week	**29 Week**	**31 Week**	**33 Week**
1,-chloro 2,4,- dinitrobenzene	**280**	**430**	**620**	**640**
Ethacrynic acid	**150**	280	**300**	**260**
1,2,-Epoxy-3-(-p nitrophenoxy)-propane	**20**	**140**	**230**	**290**
Sulfobromopthalein	**Nd**	**120**	**90**	**20**
4-Nitropyridine-N-oxide	**90**	**160**	**120**	**110**
3,4,- Dichloronitrobenzene	**220**	**280**	**250**	**310**

Units of activity: n mole/min.mg protein, nd = not detected

week fetus followed by that of 31 week old fetus. This transient appearance of activity with sulphobromopthalein was increased in the 29 and 31 week fetal livers, may be due to the extra isoform of GST that was observed only in these two stages.

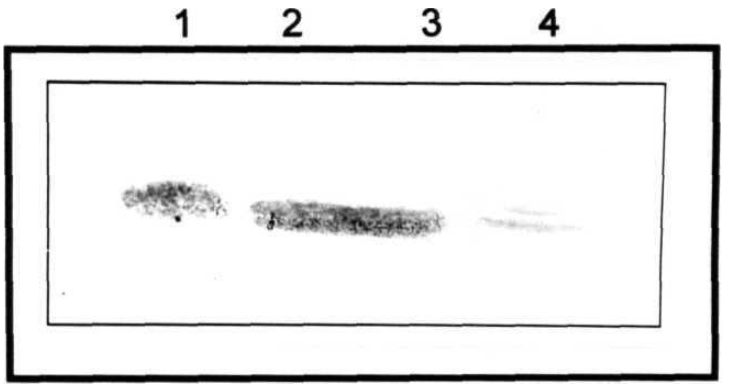
3.3.1.5 WESTERN BLOT ANALYSIS

In order to characterize the GST subunits expressed during the course of development, GSTs separated on SDS PAGE were transferred to nitrocellulose paper and probed with human a, μ and π class specific GST antibodies (Fig 27). As shown in the Fig.27, α form of GSTs were present in the livers from all the stages of gestation periods studied. But in the 29 and 31 week fetal liver apart from the two bands another band was also recognized by the a class specific antibodies, similar to that seen on SDS PAGE. How ever no protein was detected when the GSTs separated on SDS PAGE were probed with μ and *n* class specific antibodies.

3.3.1.6 DISCUSSION

GSTs as the detoxifying enzymes play an important protective role in fetal demise. Though many reports have been published regarding the GSTs profiles in developing tissues none of the studies were complete as the availability of fetal tissue is a scarcity. Greengard (Greengard *et.al*; 1970) proposed that changes in the expression of liver-specific proteins generally occurred at three specific developmental stages, (I) Late gestation, (II) at or directly after birth and (III) just before weanling. During

Fig.27: Immunoblot analysis of fetal liver GSTs , probed with a class specific antibodies



Affinity Purified GSTs from livers of 33 week (lane 1), 31 week (lane 2),29 week (lane 3) and 21 week (lane 4) human fetuses. 10 μg of protein was loaded in each lane

these periods the liver undergoes a significant anatomical, morphological and physiological changes in response to (a) commitment of embryonal cells to become hepatocytes, (b) preparation for extra-uterine function during late gestation and (c) maturation of newborn liver.

In the present study we observed that there is a variation in the specific activity during the course of gestation, suggesting that there could be an ontogenic change in the titer of different isoforms of GSTs, which was also observed by K.Datta (Kaushik Datta et.al 1994). The substrate specificity studies have further reveled that there will be a variation in the GSTs profiles Guthenberg (Guthenberg *et.al;* 1986) reported two distinct forms of GSTs in human fetal livers an alpha and a pi form. But in the present study only one form of GST was found is alpha and no pi or mu forms were detected. This observation is in consonance with with the report published by Sato (Sato.K *et.al;* 1989). This could be because of the difference in gestation weeks, Guthenberg studied in the early term fetal livers where as the present study was on late term fetal livers. Strange (Strange R C. *et.al;* 1985) while studying GSTs from fetal tissues observed three subunits of GSTs and termed them as GST1,GST2 and GST3. They also have reported that, after 30 weeks of gestation the GST3 decreases, which is in accordance with the present findings. Edward (Edward *et.al;* 1977) had proposed that human development is often accompanied by a generalized increase in the expression of basic

isoenzymes and decreased expression of acidic forms of GSTs in fetal livers. A continuous expression of GST alpha in fetal livers with minor changes was reported during the course of gestation (Strange *et.al,* 1984). On the contrary the pi form of GST was reported only in the early stages of development (Van lieshout *et.al,* 1998). In all these studies no effort is made to study the changes in individual subunits and the stages at which variations take place.

Increase in the Ya form of GST during the course of development especially in the late term of pregnancy was reported in rats (Listowsky *et.al,,)* which is in accordance with the present study. Apart from this they have observed the presence of Yp (p form) which decreased as age progressed. In the present study, however no p form of GST was observed, may be because the studies were concentrated on the late term fetuses.

In case of rat development, a differential expression of GSTs was observed with reference to the two subunits of GST alpha (Lisa.B.G.Tee et.al 1992), Subunit 1 was found to be dominant in adult liver where as subunit 2 fetal liver. This clearly shows that there is a developmental regulation of GST in mammalian systems.

The physiological significance for the presence of alpha form of GSTs in the liver is not quite clear. However, it can be proposed that the a from of GSTs with high peroxiciases activity might be playing a role in the

protection of the fetus during pregnancy from the oxidative stress. The extra uterine environment is markedly hyperoxic compared to that of fetus (Bruke et.al 1978). This could be the reason for the increase in the alpha forms of GSTs in the late term of human fetal liver, which is also seen in rat liver development (Lisa et.al 1992). Though different GST isoforms share common substrates such as CDNB, the varied developmental expression emphasizes that their *invivo* substrates are likely to be very different from those of *invitro*

From the foregoing studies it is quite clear that in actively proliferating normal fetal tissues a from of GSTs are predominant. This observation, however, is quite different from those of actively proliferating abnormal tissues such as cancer, where in p is the predominant form of GST. In these tissues, the a form is on the other hand is decreased. This differential expression of GST isoforms in actively proliferating normal and pathological tissues, may be due to the varied environments to which the tissues are exposed. Further studies, however, are required to understand the role of a form of GSTs in normal dividing tissues and *n* form of GSTs in cancer tissues.

SUMMARY AND CONCLUSIONS

Myriad number of intercalated factors play a pivotal role in maintaining normal physiological conditions. Malfunctioning of any one of these might result in pathophysiological conditions, which is deleterious to life. Cancer- as a diseased state is one such a culmination of more than a factor, where in the normal cells are irreversibly converted to a state where the neoplastic cells loose the potential to control the proliferation Some of these factors are either toxic electrophiles generated endogenously or xenobiotics to which one is exposed Cellular system, however, are equipped with detoxification systems, among which glutathione S-transferases constitutes a primary pathway. Preclinical studies have correlated enhanced metabolism of electrophiles with increased levels of GST isoenzymes within various tissues. Expression of GSTs in an individual can therefore provide an indicator about the metabolic potential of their tissues and possible deficiencies in the susceptibility to dietary or environmental carcinogens. GSTs are over expressed in certain tumor types, therefore measurement of GST and their subunits in serum or in pathological specimens can be used as diagnostic markers for certain types of cancer. Also overexpression of GSTs have been implicated for the development of drug resistance during the course of treatment of cancers. Therefore, measurement of GSTs can be used to follow the course of disease and to monitor the success of intervention. The

present study in an attempt to isolate and characterize GSTs expressed in rapidly proliferating normal and cancerous tissues. In order to develop rapid assay methods to identify individual GST subunits such as ELISA and Western blot studies, GSTs were purified from human uterus, individual subunits separated on RP-HPLC and polyclonal antibodies were raised. The salient findings of the present study are summarized below.

1) Human Uterine GSTs were purified to electrophoretic homogeneity by GSH-Affinity chromatography.

2) SDS-PAGE of affinity purified cytosolic uterine GSTs resolved into four bands with relative molecular weights of 23,26,27 and 28.5 kDa respectively.

3) Further separation of affinity purified cytosolic uterine GSTs on RP-HPLC showed four peaks with retention times of 15.2, 16.1, 28.4 & 36.6 min respectively.

4) Based on the order of elution on RP-HPLC and immunoblot analysis, the four peaks obtained on RP-HPLC were identified as μ1(15.2 min), μ2 (16.1 min), *n* (28.4 min) and a (36.6 min).

5) Polyclonal antibodies were raised in rabbits to different subunits of human uterine GSTs separated on RP-HPLC.

6) In order to understand the role of GSTs in colon cancer, GSTs were purified and characterized from colon cancer

tissues. Using the class specific polyclonal antibodies and RP-HPLC data it is identified that there is an increase in μ2 (7%) and π (38%) and decrease in a (28%) in adenocarcinoma of colon. These results suggest that a and *n* subunits of GSTs can be used as potential biomarkers for detection of colon cancer.

7) Similar studies on squamous cell carcinoma of cervix, penis and uterine endometrium reveled that *n* form of GST is the major subunit. Also no significant difference was observed in the levels of different subunits of squamous cell carcinoma tissues.

8) In order to study the involvement of GSTs in actively proliferating normal tissues, GSTs were purified from fetal livers at different stages of development. These studies reveled that a class GSTs are the only type of GSTs expressed in fetal livers. Fetal liver is conspicuous in the absence of *n* and μ class of GSTs. These studies on fetal livers thus reveled that normal proliferative cells do not mimic the expression of GSTs in cancerous tissues i.e. abnormal proliferative cells. Further it can be suggested that different regulatory mechanisms are controlling the expression of

GSTs in cancer (abnormal proliferating) fetal (normal proliferating) tissues.

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