ANALYSIS OF LOW MOLECULAR WEIGHT THIOLS PRESENT IN A HUMAN PROSTATE CANCER CELL LINE (LNCAP) USING 4-DIMETHYLAMINOAZOBENZENE-4'-MALEIMIDE (DABMA) AS A LABELLING AGENT: APPARENT GLUTATHIONE CONTENT.

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ABSTRACT

Low molecular weight thiols play important roles in cell homeostasis, drug metabolism, cancer formation and progression. The major component in this fraction is commonly assumed to be the tripeptide glutathione but there have been reports of the presence of other unknown thiols in tumour cells. To investigate this further the composition of the non protein sulphydryl components of the acid soluble fraction (ASF) of LNCap cells (a lymph node human prostate cancer) have been investigated after labelling with 4-dimethylaminoazobenzene-4'-maleimide (DABMA) at pH 5.5.
A preliminary isolation of the adducts formed was achieved using reverse phase chromatography on C-18 silica gel (ODS-AQ) giving three crude fractions. The first fraction contained the glutathione adduct and this could be separated from the other thiol adducts by flash chromatography on silica gel followed by ion exchange chromatography on a cellulose anion exchanger.
The molar extinction coefficient of the glutathione derivative at the λ max of 455nm (pH5.5) was estimated by amino acid analysis to be 26,600 M^{-1} cm^{-1} (DABMA in methanol 32,000 M^{-1} cm^{-1}). Using this value it was calculated that, in the total ASF, not more than 40% of the total measured thiol could be attributed to glutathione.
Other thiol adducts were shown to be present in variable amounts; separation and analysis proved difficult but using HPLC and TLC techniques a number of unknown components were shown to be present. Amino acid analysis revealed that they did not contain cysteine or peptide material and UV/visible spectra ruled out the presence of nucleic acid derivatives. MS and NMR analyses indicated that unknown water soluble thiols are present which are probably lipid in nature. Further analysis of one isolated adduct indicated the presence of 5-mercapto-pentanol.

Key words: Thiols; acid soluble fraction; glutathione; prostate cancer cells; analysis; maleimide adducts; silica gel chromatography; HPLC; TLC

1. Introduction

As one of the most reactive chemical grouping present in the cell, thiol (sulfhydryl, mercapto or –SH) compounds play a central role in cellular metabolism; the vital role of thiols in the maintaining the redox balance and homeostasis within eukaryote cells is now widely recognised (e.g. see [1]). Thiols play major roles in drug metabolism [2] and detoxification, radiation sensitization and protection, and the prevention of oxidative stress [3]. They also play vital roles in nucleic acid synthesis and cell signalling mechanisms [4]; in addition, cell thiols have been implicated in the regulation of both necrotic or apoptotic death [5]. There is also a growing body of evidence that an abnormal thiol redox state is involved with the pathogenesis of a number of diseases ranging from various neurological diseases to cancer (e.g. see [6])

The most mobile form of cellular thiols, the low molecular weight thiols, therefore play a vital role in cell metabolism. Over the years, it has become accepted that in eukaryote cells the ubiquitous tripeptide glutathione is the dominant low molecular weight thiol present. The presence of other species of low molecular weight thiols has also been reported but only small amounts and in specialized cells. However, recent studies on the human and mouse cancer cell lines [7], have shown the presence of low molecular weight thiols other than glutathione in these cells; in fact glutathione constituted less than 40% of the total thiols isolated from the acid soluble fraction (deproteinized extract) of these cells. Hence further investigations have been carried out to characterize and identify the other thiols present in the acid soluble fraction of the human prostate cancer cell line, LNCap.

Because of the highly reactive nature of thiol compounds it was deemed necessary in these studies to block the -SH group prior to analysis. Furthermore, as these thiols were found to rapidly oxidize or decompose at pH values above 5, the use of any thiol blocking reagents which require a higher pH for reaction was ruled out. For this purpose, compounds containing the **maleimide** group were considered to be the best option for labeling. It was shown in 1949 [8] that compounds containing this grouping react rapidly, stoichiometrically and specifically with thiols at pH 5 to 8. Since this discovery various dye chromophore derivatives of maleimide have been prepared to visualize and analyze cellular thiols [9]. Chang et al. [10] reported the use of derivatives of the dye dimethylaminoazobenzene (DAB) for thiol peptide analysis by introducing N-ethylmaleimide and iodoacetamide into the dye structure. This paper describes attempts to use the former derivative for the analysis of the non-protein thiols of LNCap cells. The reaction of thiols with this maleimide derivative is shown in figure1 below.

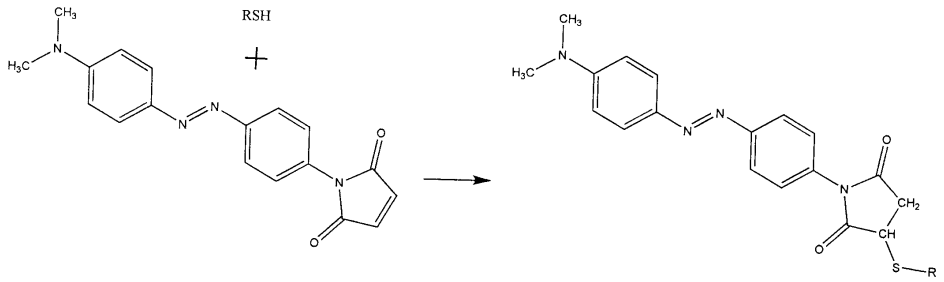

Figure 1

2. MATERIALS AND METHODS

2.1 Materials

All reagents and chemicals were of analytical or higher grade. Ellman reagent, (5,5'-dithio-bis-(2-nitrobenzoic acid) and other chemicals used were obtained from Sigma Aldrich. Solvents for HPLC were Merck LiChroSolv grade.
ODS AQ silica gel (product 12S50) was purchased from YMC Europe GMBH.
Silica gel for flash chromatography was obtained from R&L Slaughter/VWR International, Essex

4-dimethylaminoazobenzene-4'-maleimide (DABMA) was prepared by the method of Chang et al (1983) and recrystallized several times to purity, Chemical analysis of this DABMA gave C 67.44%, H 5.11%, N 17.15%; calculated C 67.50%, H 5.00%, N 17.50%. It gave one peak on several HPLC analytical systems.

LNCap cells (clone FGC-ECACC no. 89110211) were supplied by ECACC - HPA at Porton Down. They were grown in monolayer culture in medium consisting of RPMI 1640 + 2mM glutamine + 1mM sodium pyruvate +10mM HEPES containing 10% Zone 2 FBS. For large scale preparations antibiotics were added and the cells grown in cell factories to yield batches of 10^9 cells. Cells were removed by trypsinization, collected by centrifugation and the pellet frozen until required. They were initially harvested when confluent but later it was found that better yields of thiols were obtained after 4 hours re-incubation in fresh medium before harvesting.

2.2 Preparation of the acid soluble fraction containing low molecular weight thiols

All extractions were carried out at 4ºC.

The cell pellets were resuspended in an equal volume of distilled water at 4ºC and 100% w/v trichloracetic acid (TCA) solution was added to a final concentration of 10% w/v TCA. After mixing the suspension was sonicated for up to 2 minutes (in ice) in a Sanyo MSE Soniprep 150 ultrasonic disintegrator at full power until no whole cells could be seen. . The mixture was sonicated again briefly before centrifuging at 2000 rpm/1700 x g for 2 minutes to give a clear supernatant. The pellet was then re-extracted in a similar fashion with an equal volume of 10% w/v TCA and centrifuged down as before.

The thiol content of the extract was measured using the Ellman reagent [11] in 0.5 M sodium phosphate pH 7.6 (0.5 mg/ml) using a molar extinction coefficient of 14,100 (at 412 nm) for the yellow anion formed on reaction with thiols. Usually 100 µl of ASF was added to 1900 µl of the Ellman reagent solution to give a suitable A_{412} reading. An average of 3-4 readings was used to calculate the thiol content. Two extractions of the cell pellet were found to be sufficient to remove 95%+ of the available low molecular weight cellular thiol.

2.3 Preparation of 4-dimethylaminoazobenzene-4'-maleimide (DABMA) derivatives

The combined ASF 10% w/v TCA supernatants were extracted 3 times with an equal volume of ethyl acetate to remove the bulk of the TCA. Then the pH was adjusted to 5.5 by the addition of 2M sodium acetate pH 5.5 and the thiol content was re-checked. . The resulting solution was immediately added drop wise with stirring to 8 volumes of acetone:methanol (1:1 v/v) containing an **equimolar** amount of DABMA to the thiol present. The mixture was incubated for 2 hours at 40°C and a buff coloured precipitate formed was filtered off.

After removal of the acetone and methanol (in a rotary evaporator), water was added back to give the original concentration of 0.2M sodium acetate; 6 volumes of acetone:methanol (1:1 v/v) were added to 5 volumes of the aqueous reaction mix to give a clear dark brown solution.

This mixture was crudely separately into three main fractions by absorption onto a 10 x 5 cms diameter column of "hydrophilic" C18 reversed phased silica gel, YMC ODS-AQ 12nm, S-50µm(supplied by YMC Europe GmbH) in 20%methanol. After applying the sample the column was given an initial wash with 0.2M sodium acetate in 20% methanol followed by 20% methanol until the conductivity dropped to about 5µS. This removed all other contaminants usually found in the cellular ASF (e.g. nucleotides) leaving the DABMA derivatives firmly bound on the column. These were eluted batchwise with increasing concentrations of methanol in water until no yellow colour remained on the column.
The crude fractions were filtered and evaporated to near dryness in a rotary evaporator and made up to 5-10 absorbancy units (455nm) per ml in 50%methanol.

Standards were prepared from known low molecular weight thiols, glutathione, cysteine, dl homocysteine and a- lipoic acid using the above protocol.

2.4 HPLC analysis

HPLC analysis was carried out in Waters Alliance 2690 HPLC automated equipment fitted with a 2487 detector using a 150 x 4.6 mm Ascentis™ RP-Amide C18 60Å 4 µm column (Supelco) for the separations
Solvent A was10% methanol (in water) and solvent B was methanol.
Flow rate 1ml/min, column temp.25°C, detector set at 455nm;
Elution: start 100%A, 3min 25% B, 16min 40%B, 22 min 40%B, 26min 100%B, 30min 100%B, 33min 100%A, 37min 100%A.

2.5 Amino acid analysis

For amino acid analysis samples were made up at an estimated concentration of approximately 40-60nmoles/ml, generally based on the molar extinction coefficient (32,000) of the original dye labelling reagent, DABMA. 100µl of these solutions were acid hydrolysed and the amino acid composition determined in an ion exchange auto analyser (4-5nmole of norleucine being used as an internal standard) by the Protein & Nucleic Acid chemistry facility of the Department of Biochemistry at the University of Cambridge.

2.6 MS and NMR analysis

This work was contracted out to Intertek ASG, Hexagon Tower, Blackley, Manchester, UK and M-Scan Ltd., 3 Millars Business Centre Wokingham, UK

3. RESULTS

3.1 Preparation and HPLC analysis of standard thiol adducts

Adducts were prepared from standard thiol compounds in the reaction mixture described and, after processing though ODS AQ, were run in the HPLC system described.
The results are illustrated in **table 1**, revealing that the DABMA:glutathione adduct prepared in this way can give up to six peaks in this HPLC; probably as different ionic forms and/or aggregates with unreacted DABMA

TABLE 1
Properties of DABMA adducts prepared from standard thiols

Thiol addduct	Elution from ODS AQ % methanol	Emergence in HPLC (min)
Glutathione	55	Main 5.87/6.40 Others at 11.5/12.5; 14.8/15.7
Cysteine	55	19.55 (± 0.15)

dl homocysteine	60	27.23 (± 0.21)
α- lipoic acid	70	27.4 (± 0.11)
DABMA (labelling compound)	85	28.32 (± 0.12)

3.2 HPLC analysis and separation of the LNCap acid soluble thiol DABMA adducts

Attempts to analyse the original reaction mixture directly by HPLC analysis gave non reproducible, artifactual patterns, presumably due to strong aggregations of the components present. This problem could be partially resolved by the initial separation step on ODS AQ gel. The results are illustrated in **table 2** showing the percentage distribution of the yellow coloured adducts, λ max 455-458nm on this gel. Nearly 100% recovery of 455-458nm material was obtained. In this preliminary separation of LNCap adducts on ODS AQ, although considerable variation was obtained with different batches of cells, it can be seen that a major proportion of the material elutes in 55% methanol. The labelling reagent (DABMA) elutes in methanol concentrations above 80%.

TABLE 2

Initial separation of DABMA adducts on a 10x5cms column of ODS AQ silica gel
Percentages of A_{455} eluted in different methanol concentrations

Elution; percent methanol	55	65	70	80+
LNCap cells reaction mix Average 4 runs (range)	64(57-70)%	4-5%	32(25-36)%	0
Glutathione:DABMA mix (stoichiometric 1:1)	100	0	0	0

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All of glutathione adduct peaks can appear in the HPLC pattern of the 55% methanol ODS AQ fraction isolated from the LNCap cells which is shown in **figure 2A**. However, here two extra major peaks are present in this fraction these constituting 36-41% of the AU but not corresponding with any of the peaks found in the glutathione standard preparations; in line with previous findings that glutathione constitutes only a maximum of 40% of the total thiol in the ASF of these cells [7].

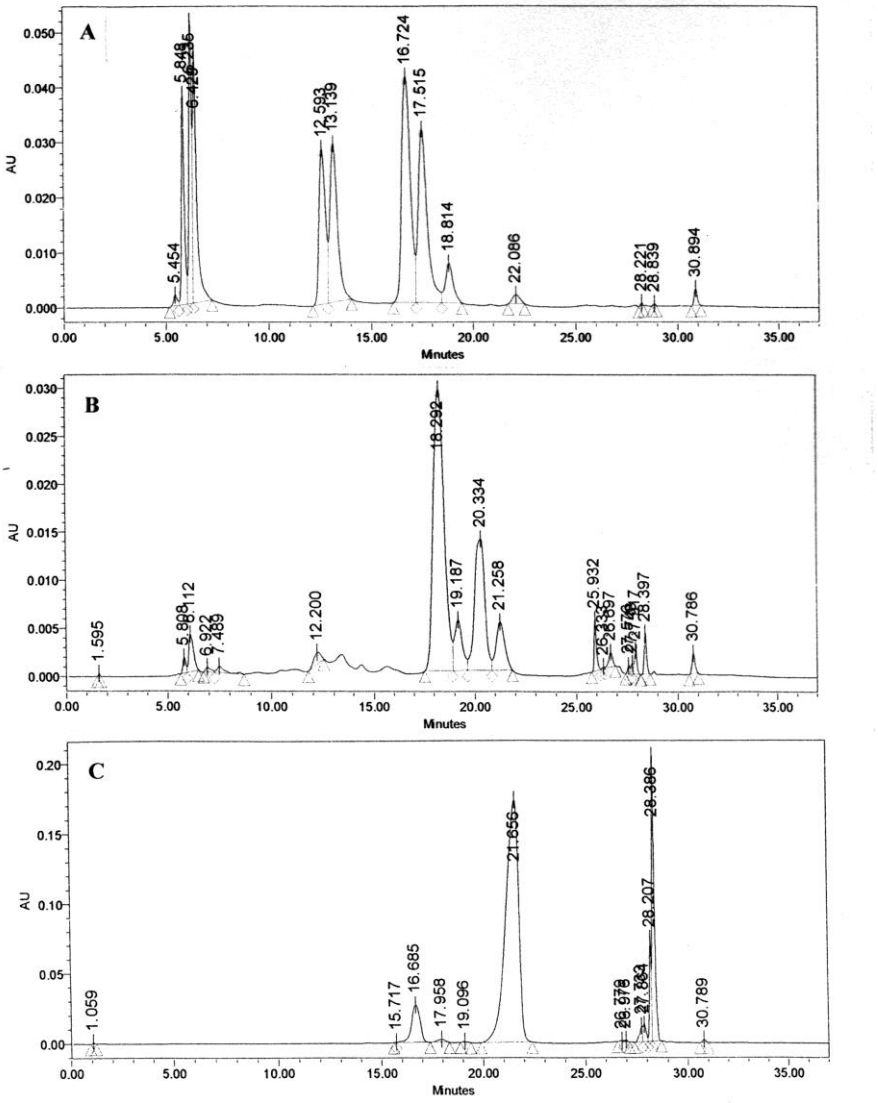

Figure 2

Auto scaled HPLC chromatograms obtained from the 1:1 DABMA reaction with the thiols of the acid soluble fraction of LNCap cells after initial fractionation on ODS AQ silica gel; eluted with aqueous methanol

A) 55% methanol; B) 65% methanol; C) 70% methanol v/v

Column: 150 x 4.6 mm Ascentis™ RP-Amide C18 60Å 4 µm (Supelco)
Solvent A :10% methanol (in water)
Solvent B: methanol.
Gradient: start 100%A, 3min 25% B, 16min 40%B, 22 min 40%B, 26min 100%B, 30min 100%B, 33min100%A, 37min 100%A.
Flow rate: 1.0mL/min
Temp.: 25^{0}C
Detection: 455nm sample 30µl

HPLC analysis on components eluted in methanol concentrations above 55% showed that

these fractions did not contain any of the commonly expected low molecular weight thiols. **Figure 2B** shows the HPLC pattern of the components eluted in 65% methanol but, as only small amounts were present, further analytical separations were not attempted, except for aminoacid analysis, which was negative.

Components found in the 70% methanol ODS AQ elute are shown in **Figure 2C**. Two major peaks are evident at 21.65 and 28.38 min but the former contains nearly 70% of the AU Evaporation of the methanol from this fraction to an aqueous phase leads to the precipitation of some material which was shown to be the labelling compound DABMA on HPLC analysis (eluting at 28.38 min)

Examination of the UV/Visible spectra of the isolated adducts revealed very little absorbance in the 260nm region that can be seen in thiol derivatives containing nucleotide bases, such as CoA. . The LNCap 55% methanol eluate in 50% methanol containing 0.2M sodium acetate buffer pH 5.5 has a λ max of around 460nm. . In 0.1M HCl the λ max of the adducts shifts to 548nm with a higher extinction coefficient in agreement with the findings of Chang et al. [9]. The glutathione adduct and other isolated adducts gave a very similar spectra. [The labelling compound DABMA is insoluble in aqueous solutions containing less than 80% methanol and has a λ max at 412nm].

3.3 Further Fractionation of the 55% methanol/ glutathione containing fraction

The 55% methanol eluate from LNCap cells (table 2) could be further fractionated by flash chromatography on silica gel. After evaporation of this fraction to dryness samples were dissolved in methyl ethyl ketone (MEK) : ethanol : water (10:10:1,v/v/v) and applied to a column of silica gel made up in this solvent. Typically 600 dye absorbancy units (AU) were applied to a 10 x 5 cms column. Broadly three fractions could be eluted as shown in **table 3** below.

TABLE 3
Flash chromatography of isolated 55% ODS AQ fraction LNCaP DABMA adducts on Silica Gel

Elution solvent	Fraction eluted	Percentage of AU recovered Average (5 runs)	Range
MEK : EtOH : H_2O 10:10:1 (v/v)	**I**	**38.8**	**33.2 - 44.7**
80% methanol	**II**	**54.7**	**46.1 - 63.5**
50% methanol	**III**	**6.5**	**2.8 - 10.2**

Some resolution of the components present in **Fraction I** can be achieved by TLC on a silica gel plate in the elution solvent as illustrated in **Figure 3**.

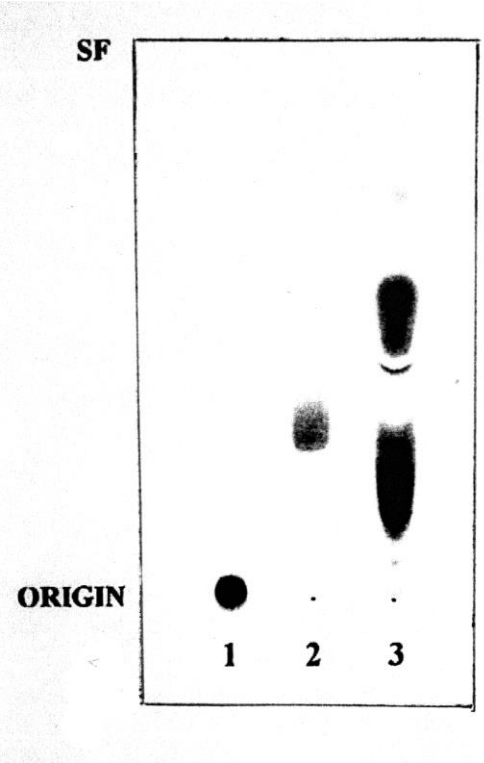

FIGURE 3

8 x 5cms Silica gel TLC plate run in methylethyl ketone: ethanol: water(10:10:1v/v)

LNCaP DABMA adducts from initial ODS AQ separation (eluted in 55% methanol) isolated by Silica gel flash chromatography
1. Adduct A from the 80% methanol fraction; mainly GSSE.
2. Standard Cysteine DABMA adduct (eluted in 55% methanol from ODS AQ)
3. Fraction I eluted in methylethyl ketone: ethanol: water

At least 3-4 components are present none of which was the cysteineDABMA adduct. Re-chromatography on a finer grade silica TLC plate revealed the presence of further minor components. However enough of a major component, R_f 0.26 was isolated to carry out an MS and NMR investigation. The glutathione adduct (Adduct A - see below) did not migrate in this solvent and remained at the origin of the TLC plate. DABMA has an R_f of about 0.8 in this system

Fraction II was eluted with 80% v/v methanol and contained the **glutathione** adduct. This fraction accounted for 46-60% of the applied AU. It could be further fractionated a DE52 cellulose anion exchange column as follows:-
About 800 AU of adduct dissolved in 20mM tris-HCl pH 7 containing 30% v/v methanol were applied to a 5 x 5cms column equilibrated in this buffer.
Batchwise elution produced two fractions :-
i) The bulk of the AU eluted in 0.6M NaCl 20mM tris-HCl pH 7 containing 30% v/v methanol, designated **Adduct A**
ii) The residual AU, usually 15-20% of the total applied, could only be removed from the column in 1M NaCl 20mM tris-HCl pH 7 containing 65% v/v methanol; designated

Adduct B

Fraction III; the remaining AU eluted in 50% v/v methanol; it contained approximately 4-10% of the applied AU. HPLC analysis showed several components were present. This fraction could be further separated on a fresh ODS AQ column into two components which were present in equal amounts. The first fraction eluted in 30% methanol was designated as **Adduct C**, and a further fraction eluted in 50% methanol as **Adduct D**,

3.4 Amino acid analysis of fractions

All the fractions isolated by flash chromatography and 70% methanol eluate (from the initial separation) were analysed for amino acid content after acid hydrolysis by standard ion exchange-ninhydrin analysis, which was carried out by the Protein & Nucleic Acid chemistry facility of the Department of Biochemistry at the University of Cambridge. The results are given in **table 4.**

TABLE 4
AMINO ACID ANALYSIS OF THE ISOLATED DABMA ADDUCTS FROM LNCaP CELLS

Adduct isolated		**7.4 min peak***	**Glu**	**Gly**	**Asp**	**Thr**	**Ser**
Fraction I (First ODS AQ 55% methanol eluate) Silica Gel: MEK: ethanol:water (10:10:1v/v/v) eluate		✘	0	0	0	0	0
Fraction II ODS AQ: 55% ethanol Silica Gel **80% v/v** methanol eluate DE52 fractions	ADDUCT A	✓	4.47	4.72	0	0	0
	ADDUCT B	✘	0.10	0.26	0	0	0
Fraction III ODS AQ 55% methanol Silica Gel **50% v/v** methanol eluate, RP C18 fractions	ADDUCT C	✓	4.05	5.06	0.25	0.10	0.18
	ADDUCT D	✘	0	trace	0	0	0
First ODS AQ 70% v/v methanol eluate		✘	0.17	0.29	0	0	0.19
Glutathione standard		✓	5.75	6.06	0	0	0
Cysteine standard		✓	0	0	0	0	0

*** ✓Peak obtained on ion-exchange/ninhydrin chromatographic analysis for acid hydrolysed cysteineDABMA or cysteineNEM adducts**
Other AA present at zero or insignificant levels. No homocysteine could be detected

Initially amounts required for hydrolysis and analysis were calculated from the A_{455} using the molar extinction value of the labelling dye (32,000). From the amounts of glycine and glutamic present in the glutathione adduct the molar extinction coefficient was found to be 26,600.

On acid hydrolysis the cysteine adduct gives an undesignated ninhydrin positive peak at around 7.4 minutes (just before aspartic acid emerges on the ion exchange column). Acid hydrolysis of an adduct prepared by reacting cysteine with N-ethylmaleimide gave the same peak; this is almost certainly the cysteine malic acid adduct formed from the hydrolysis of the maleimide ring. As can be seen in the table, two of the isolated fractions were found to give the 7.4 minute peak but only one is clearly glutathione – adduct A. Adduct C had an odd glutamic acid and glycine ratio (not 1:1 as in glutathione) and the presence of other amino acids indicated that this was a mixture. HPLC using the method previously described revealed at least three components to be present in nearly equal amounts, none of them corresponding to the glutathione adduct confirmed in adduct A.

From the absence of the cysteine malic acid adduct in the other fractions of the adduct mixture it is apparent that other thiols present in the ASF are not derived from this amino acid. The hydrolysed adduct of homocysteine emerges at 11.02 mins from the ion exchange column. This peak was not detected in any of the in any of the ninhydrin traces of the analysed samples.

The result obtained from a similar analysis of the 70% v/v methanol extract from the first ODS AQ separation shows that, apart from insignificant traces, virtually no amino acids, **or cysteine,** was present. A similar result was obtained from the 65% v/v methanol extract.

3.5 Attempted analysis of isolated adducts by Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (NMR) techniques

Electrospray Ionization Mass Spectrographic (ESMS) analysis was carried out on the isolated DABMA adducts by in the positive mode. The labelling compound itself, DABMA, gave a clear major molecular weight ion of 321.4m/z $[M+H]^+$ and the ammonium salt of the glutathione adduct gave a strong positive ion at 646m/z $[M+NH4]^+$

However, attempts to obtain meaningful MS data/information on the isolated adducts not containing amino acids were less successful (despite the fact that the samples were sent to two different laboratories for analysis). The 70% methanol extract of the original ODS AQ was purified by further chromatography on CM52 cation exchanger (indicating the presence of a positively charged moiety) followed by a further step on silica gel. The isolated adduct seemed to be homogeneous in that it gave a single peak/component on HPLC and TLC analysis. However the MS pattern obtained on this material as shown in **figure 4** indicated a degree of heterogeneity, positive ions being produced ranging from 882m/z to 239m/z. On limited MSMS analysis of the m/z603 to m/z 441 fragments, clusters were produced 14amu apart, indicative of glyceride structures being present; the 239 fragment was interpreted as a palmitoyl residue.

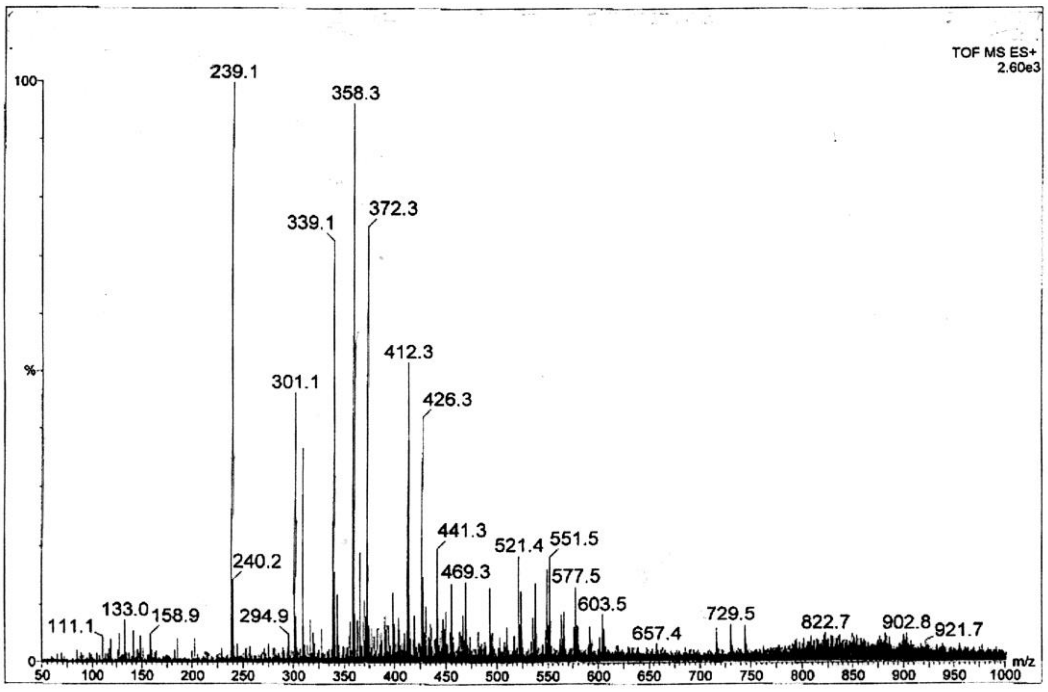

FIGURE 4

Electrospray Ionisation Mass Spectrometry analysis (positive ion mode) of the purified 70% methanol extract from initial ODS AQ separation

The presence of the m/z 339 peak, which on further analysis seemed to be a hydrolysis fragment produced by the opening of the maleimide ring of DABMA indicates that some decomposition of the adduct (s) may be occurring.

NMR analysis was attempted but meaningful data were not obtained due to the addition of acid to the sample prior to analysis. Later HPLC studies demonstrated the rapid breakdown of this adduct in acid solutions.

The first elute from silica gel flash chromatography i.e. in MEK:ethanol:water (10:10:1,v/v/v) (see figure 3.3) was re-run on large thin layer silica gel plate and a main component was isolated with an R_f value of 0.26.

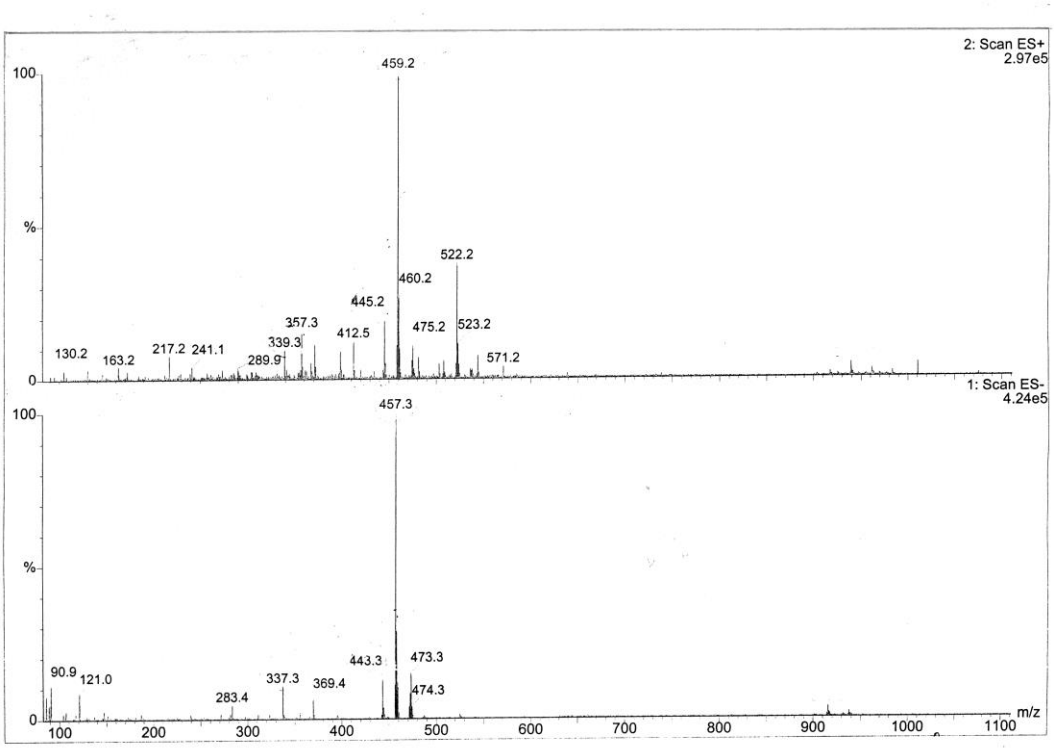

FIGURE 5

Electrospray Ionisation Mass Spectrometry analysis (positive and negative ion modes) of Fraction I component R_f 0.26 on silica gel TLC.

MS analysis of this material as shown in **figure 5** gave weak molecular ions at m/z of 445 $[M+H]^+$ and 443 $[M-H]^-$ from a species of molecular weight of 444.
Further ions were present at 459$[M+H]^+$ and 457$[M-H]^-$ indicative a species of MW of 458 which is probably a product of the 444 species formed from the hydrolysis of the amide ring.

NMR analysis carried out by an external commercial laboratory on this adduct produced complicated ^1H and ^{13}C NMR spectra showing numerous components but an expert concluded that the use of TFA in this analysis placed the sample integrity at risk. However, these spectra, together with the MS findings, indicated that the 5- thiopentanol adduct of DABMA, FW 444, could be present in the R_f 0.26 component.

4. DISCUSSION

N-ethylmaleimide and various derivatives of this thiol reactive compound have been widely used to for blocking protein and non-protein thiols [12]. The reaction proceeds stoichiometrically to give stable adducts at lower pH values (below 7). Chromophoric derivatives are stated to enable the spectral detection of adducts at sensitivity in the 2-5 pmole range. According to Chang et al. [9] DABMA reacts specifically and quantitatively with thiol groups at pH 5.

When this reagent is used at pH 5.5 to label the thiols of the ASF of LNCap cells a complex mixture of adducts is produced which has proved to be difficult to separate and analyse. Furthermore, as could be expected, considering high metabolic activity of thiol compounds, the composition of the mixture was found to be highly variable. In four bulk batches of LNCap cells the division of adduct $A_{455\text{-}460}$ in the various fractions was highly variable probably reflecting the dynamic nature of this class of cellular constituents and the redox "milieu".

There has been much published data on one of the most dominant species of low molecular weight thiol, the ubiquitous tripeptide, glutathione, which have clearly established the importance of this thiol in detoxification, removal of drugs, carcinogens and other xenophobic substances (for review see [13]). Since its discovery by De Rey-Pailhide in 1888 (and later rediscovery by Hopkins in 1921) numerous cellular functions and roles have been attributed to this compound [14,15]. In thousands of publications glutathione has been widely mooted as the major low molecular weight thiol in the cell but almost all investigators have had problems in estimating this thiol in cell and tissue preparations. It is generally thought that glutathione accounts for greater than 90% of the cellular non-protein, low molecular weight thiols of eukaryote cells.

In these studies the glutathione adduct, although initially present in several chiral and ionic forms, was isolated in three chromatographic steps and identified by standard amino acid analysis. Of the isolated fractions only adduct C contained significant amounts of amino acids. Other adducts isolated did not seem to be derived from cysteine or homocysteine. Using an molar extinction coefficient of 26,600(455nm) it was estimated that the glutathione adduct isolated from the ASF of LNCap cells only accounts for about 40% (range 35-45%) of the total low molecular weight thiol found in these cells, in agreement with previous findings using a different thiol label [7]. In 1962 Calcutt and Doxey [16], using a somewhat crude analytical technique to analyse the thiols of the ASF of normal and tumour tissues concluded that glutathione accounted for a maximum of 33% of the total thiol present. Since this early report many workers have reported difficulties in determining the glutathione content of cells. For example, Eady et al. [17] have attempted to analyse the glutathione contents of a panel of six human cell lines by several methods and found considerable variations and discrepancies between the results obtained; their work highlighted the difficulties in obtaining consistent data on cellular glutathione content.

Other than glutathione the thiol adducts isolated in these studies proved to be quite soluble in aqueous media though they do not seem to be any of the usual thiols found in eukaryote cells. Amino acid analysis showed clearly the no cysteine was present although traces of other amino acids were found in some fractions, probably as contaminants. One of these adducts isolated by TLC in sufficient quantities for MS and NMR studies contained a fragment of 5-thiopentanol; not previously reported to be present in eukaryote cells.

MS studies on another adduct isolated indicated that it contained palmitoyl fragments. Palmitoylation is known to play a major role in membrane protein micromodifications

which are known to have diverse effects on cellular regulatory networks and signalling molecules [18], a process in which thiol related compounds are known to be involved. For example, molecules such as the soluble N-ethylmaleimide-sensitive factor-attachment protein receptors (SNARE's) have been shown to be intimately involved with membrane trafficking of proteins and lipids [19].

5. CONCLUSION

As expected from metabolically active, water soluble, low molecular weight fraction of a cell, the thiol composition of the ASF of this tumour cell line was found to be variable and heterogeneous. From the dye maleimide adducts prepared it was clear that the glutathione adduct isolated from the mixture constituted no more than 35 - 45% of the total thiol adducts formed from the ASF preparations. The bulk of the remaining adducts, ca.60%, are not peptide, cysteine or amino acid containing compounds and these have proved difficult to analyse even using MS and NMR analytical techniques indicating that this maleimide based labelling compound turned out not to be the most suitable reagent for use in identifying these unknown thiols. However the results do indicate that some water soluble lipid-type thiol compounds are present in this (and other) tumour cell lines which could play important roles in membrane interactions involved in cellular drug metabolism.

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