UNIVERSITÄT
DES
SAARLANDES

ADVANCE PRACTICAL COURSE

IN VITRO EXPERIMENTS FOR VIABILITY

SUBMITTED BY

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1 INTRODUCTION

1.1 HepG2 cell line

Liver impairment caused by drug is a major cause for acute liver failure in many countries. The mortality rate is 80% in patients with acute liver failure [1]. This has led to the urgent requirement of human models to predict hepatoxicity [2]. The animal based toxicity assays predict about 70 percent of the toxicity in human beings. Only half of the new compounds that caused clinical hepatotoxicity were in agreement with animal hepatoxicity [3]. Primary hepatocytes represent the gold-standard for the analysis of hepatocytic potential of a new drug. The cellular models can be used for the same prediction task [4]. There are several cell lines which are available for the analysis of toxicity mechanisms. From the entire cell lines, the human cell line HepG2, established in 1979, is the best characterized and the most frequently used cell line and has been used to examine various mechanisms of hepatotoxicity [5]. The evaluation of multiple endpoints HepG2 cells allows the prediction of hepatotoxicity with 80 to 90 percent specificity [6]. These have led to the belief that Hep G2 serves well in the prediction of overall toxicity using hepatospecific endpoints [2].

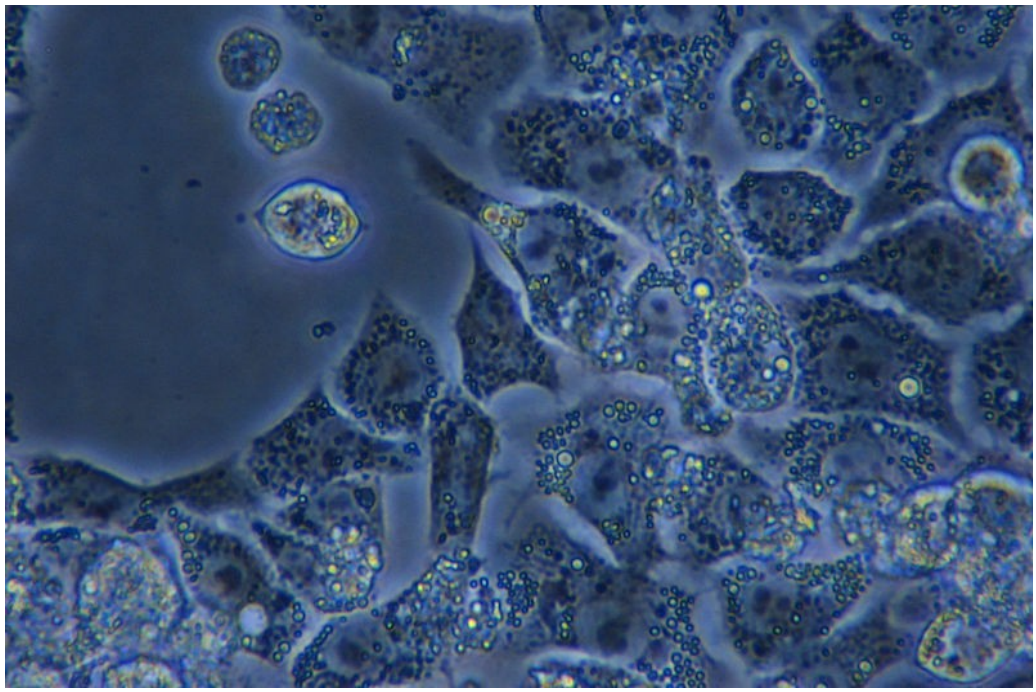


Fig 1. Human hepatocellular carcinoma cell line – HepG2

1.2 Cell viability assays

- **Sulforhodamine B (SRB) assay:**

The Sulforhodamine B (SRB) assay was originally developed by Skehan et.al to measure drug-induced cytotoxicity and cell proliferation for large-scale drug screening applications. The principle of SRB assay is based on the ability of the protein dye i.e., Sulforhodamine B, to bind electrostatically on protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to and under mild basic conditions it can be extracted from cells and solubilized for measurement [9].

- **Alamar blue assay:**

Assay is designed to measure quantitatively the proliferation of various human and animal cell lines, bacteria and fungi. The bioassay may also be used to establish relative cytotoxicity of agents within various chemical classes [10]. The toxicologist can establish baseline data for predicting the toxicity of related novel agents by comparing such baseline data with known in-vivo toxicity. The assay is simple to perform since the indicator is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays. The alamar blue Assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth [11]. The specific (fluorometric/colorimetric) REDOX indicator incorporated into alamarBlue has been carefully selected because of several properties. First, the REDOX indicator exhibits both fluorescence and colorimetric change in the appropriate oxidation-reduction range relating to cellular metabolic reduction. Second, the REDOX indicator is demonstrated to be minimally toxic to living cells. Third, the REDOX indicator produces a clear, stable distinct change which is easy to interpret. The REDOX indicator has no current or past indication of carcinogenic capacity. As cells being tested grow, innate metabolic activity results in a chemical reduction of alamar blue. Continued growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the REDOX indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form [12].

1.3 GC-TOF mass spectrometry

Gas chromatography mass spectrometry incorporates both analytical as well as combined features of gas-liquid chromatography for separating the chemical mixture into pulses of pure chemicals and TOF mass spectrometry in order to identify and quantify the chemicals. The Time of flight method in GC helps in separating these different ionic substances based on the time required by each to transverse a fixed distance [7].

- **Gas chromatography (GC)**

  - ***Injection port***

    One microliter (1 µl, or 0.000001 L) of solvent containing the mixture of molecules is injected into the GC and the sample is carried by inert (non-reactive) gas through the instrument, usually helium. The inject port is heated to 300° C to cause the chemicals to become gases.

  - ***Oven***

    The outer part of the GC is a very specialized oven. The column is heated to move the molecules through the column. Typical oven temperatures range from 40° C to 320° C.

  - ***Column***

    Inside the oven is the column which is a 30 meter thin tube with a special polymer coating on the inside. Chemical mixtures are separated based on their votality and are carried through the column by helium. Chemicals with high volatility travel through the column more quickly than chemicals with low votality.

- **Mass Spectrometer (MS)**

  - ***Ion Source***

    After passing through the GC, the chemical pulses continue to the MS. The molecules are blasted with electrons, which cause them to break into pieces and turn into positively charged particles called ions. This is important because the particles must be charged to pass through the filter.

- ***Filter***

  As the ions continue through the MS, they travel through an electromagnetic field that filters the ions based on mass. The scientist using the instrument chooses what range of masses should be allowed through the filter. The filter continuously scans through the range of masses as the stream of ions come from the ion source.

- ***Detector***

  A detector counts the number of ions with a specific mass. This information is sent to a computer and a mass spectrum is created. The mass spectrum is a graph of the number of ions with different masses that travelled through the filter.

- **Computer**

  The data from the mass spectrometer is sent to a computer and plotted on a graph called a mass spectrum [8].

Fig 2. Gas Chromatography – Mass spectrometry

- **Applications of GC-MS**

  There are various application for GC-MS of which the major ones include drug detection, fire investigation, environmental investigation, explosives investigation, identification of unknown samples. GC-MS is also widely popular because of its ability to detect trace elements in materials which were thought to

have disintegrated beyond identification. Also GC-MS has been used widely as a gold-standard for forensic substance identification [7].

1.4 Drug induced metabolism

It was shown that HepG2 cell line has the ability to retain many cellular functions which are lost by the cells in the cell culture. The acetaminophen bioactivation can enhance the activity of CYP2E1 activity, and CYPIA1/CYPIA2 activity which was demonstrated in non-induced HepG2 microsomes. This showed that HepG2 cells are good candidates to be an invitro model for assessing human xenobiotic metabolism of acetaminophen and other drugs [13]. The metabolism of Acetaminophen (N-acetyl-p-aminophenol, APAP) also referred to as paracetamol, by the oxidase system, cytochrome P450 (CYP) leads to the formation of N-acetyl-p-benzoquinoneimine (NAPQI), which is reactive intermediate and detoxified by conjugation with reduced glutathione (GSH) [14]. APAP is biotransformed into glucouronic acid and sulphate conjugates which are nontoxic and eliminated. When a high dose of APAP is given, its conjugation with glucouronide and sulphate exceeds during the process of its removal and more and more NAPQ1 is formed. These formed NAPQ1 will bind covalently to the proteins leading to cell death [15].

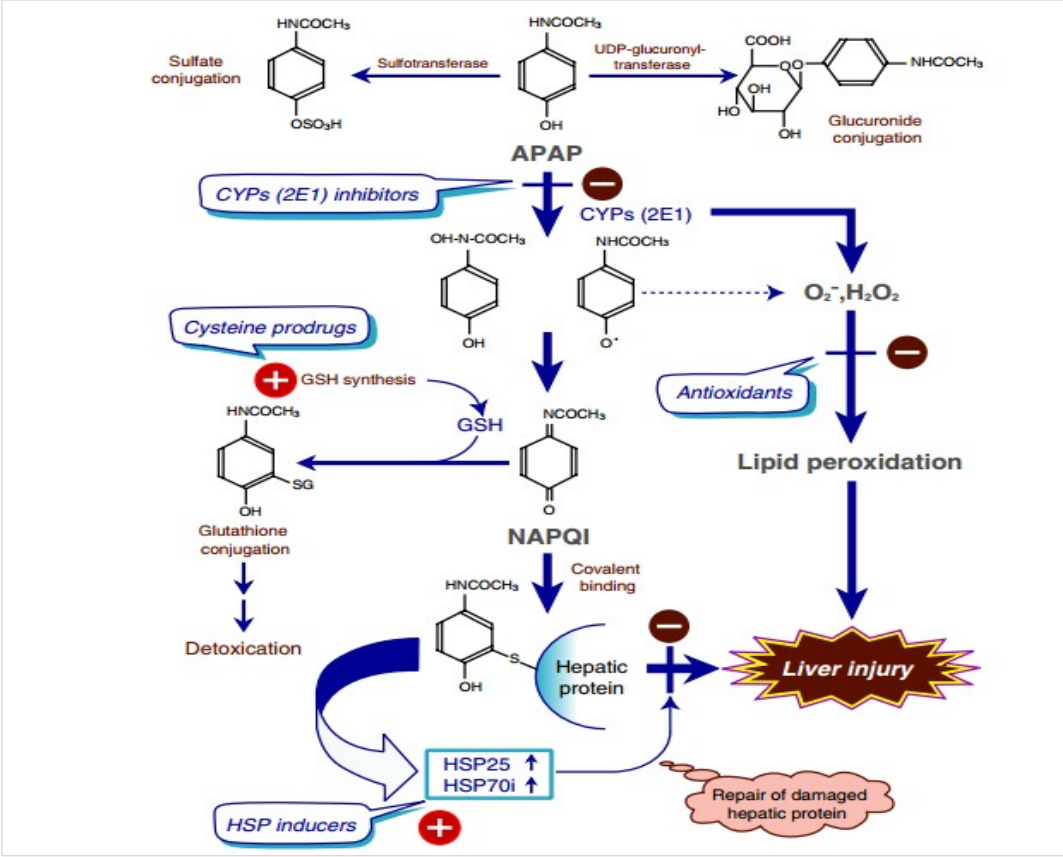


Fig 3. Acetaminophen induced Hepatotoxicity

2 OBJECTIVE

Measure the drug induced cytotoxicity of acetaminophen using HepG2 cells and analysing the metabolites using GC-TOF mass spectroscopy.

3 MATERIALS AND METHODS

3.1 Cell Passaging and Counting

HepG2 cells are grown in 25 cm^2 flasks in William's Medium E (WME) (PAN Biotech, Aidenbach, Germany) containing 1 % Penicillin/Streptomycin and 10 % Fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria) at 37^0C in a humidified incubator in a 5 % CO_2 atmosphere . The cells were passaged when they reached a confluency of 80 % by removing the medium; washing with 5 mL pre-warmed WME with 10 % FCS was then added to inactivate the trypsin. The 10 mL cell suspension was then transferred into a 15mL Falcon tube and centrifuged (Labofuge 400R, Thermo Scientific, Schwerte, Germany) at 800 rpm and 23^0C for 5 min. After aspirating the supernatant, the cell pellet was resuspended in 2mL WME.

Quater of the suspension was then added to a labelled new culture flask containing 6mL of medium, and placed in the incubator. The cell number determined the cell confluency using Neubauer chamber. In an Eppendorf tube, 30µL of the cell suspension and 30µL of the Trypan-Blue dye were mixed and 10µL were introduced into the chamber. Under the microscope cells in the four big squares (each square having a volume of 0.1 µL) on the periphery were counted.

The concentration of the cells per mL of culture volume was calculated using the formula:

Cell Concentration (Number of cells/mL) = (Number of cells / 4) * 2 * 1000

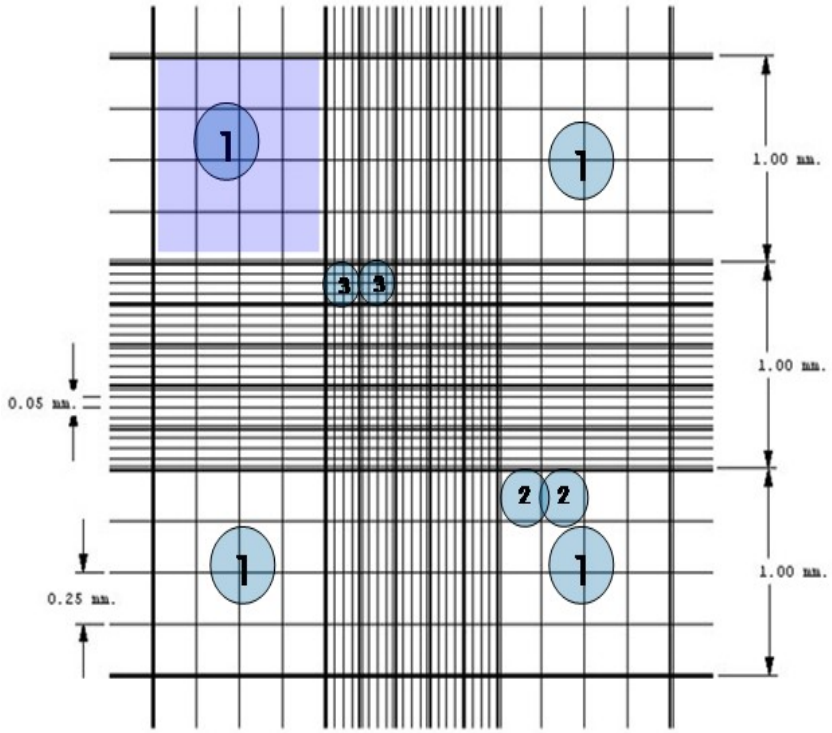


Fig 4 . Cell counting in the four big squares of Neubauer chamber

3.2 Viability assays

The viability assays namely SRB and Alamar blue were carried out in which the cells were seeded in different cell numbers per well (5000, 10000, 25000, 40000, 50000, 75000, 90000, 100000, 125000, 150000) in 200µL WME with 10 % FCS in a 96-well plate. Each cell number was seeded in triplicate. Finally the plates were then placed in an incubator for an equilibrium period of 1 to 12 hours. This allows the cells to attach before carrying out the viability assays.

3.2.1 SRB assay

First 200µL of the culture medium on 96 well plates were aspirated. Then the microtiter plate from the incubator was taken into the laminar flow hood. After that ¼ volume of cold TCA (trichloroacetic acid, 50 %) solution was gently used as a layer on top of the growth medium. The calculation is that, if the wells contain 100µL then we use 25µL of TCA. It should be noted that if the medium contains added proteins or fetal bovine serum (FBS), then the culture has to be washed twice with medium without added supplements of proteins or FBS before fixation. Next the original amount of medium which contains no medium or proteins over the cells before adding ¼ volume of TCA on top of it was layered. Now the plate is ready for incubation. The plate is then incubated for 1 hour at 4^0C on ice and then rinse with distilled water times to remove TCA. The plate was left to air dry in laminar flow. Lastly the plate was put with water into the fridge.

Next, Sulforhodamine B solution (50 % of initial culture medium volume) was added on cells in each well and stained the cells for 30 minutes. Next the dye was removed by aspirating the wells and washed swiftly with 1 % acetic acid, volume same as that of SRB solution, about 5 times to remove unincorporated dye. Air drying the culture till no remaining fluid is visible was the next step done. Then Tri base was added in volume equal to that of the original culture volume, and incubated for 10 minutes at room temperature on a plate shaker to solubilize the dye. Lastly the absorbance was measured at a wavelength of 540 nm against a background absorbance of 660 nm.

3.2.2 Alamar blue assay

On Removing the 96 well plates from the incubator, 40 µL of the thawed alamar blue Reagent (Promega, Mannheim, Germany) was added to each of the wells containing cells and 200µL culture medium. Then we placed the plate on a shaker for 10 seconds and incubated at 37^0C. In later repeats of the experiment, we measured the absorbance after approximately 1 hour of incubation and again after approximately 2 hours of incubation.

3.2.3 Acetaminophen exposure

First 6 Falcon tubes were taken for preparing different concentrations of Acetaminophen drug (APAP). The concentrations of APAP added were 40Mm, 20mM, 10mM, 5mM, 2.5mM and 0mM. APAP, which was initially kept in a waterbath at 37^0C, was taken out and 1mL APAP (80mM) was taken out of it and along with 1mL of WME (40Mm) was added immediately to the first falcon tube. From the 40mM tube 1mL was transferred to the 2^{nd} Falcon tube and 1mL WME (20mM) was added again. This serial dilution was carried out till 5^{th} concentration. We didn't add the APAP in the last tube as that is the control. We then took the incubated plate containing 500,000 cells. The wells of the plate were then aspirated. After that, the drug of each of the concentration taken, were transferred to the aspirated wells and was kept for incubation. The supernatant is then taken from 20mM, 5mM, 2.5Mm and 0Mm to 4 * 3 (i.e., 12) Eppendorf tubes. All the wells are then aspirated, including the 40mM and 10mM wells. After that 100µLof WME solution was added to the aspirated wells along with 2.5 µL of alamar blue. Then the plate was kept for incubation.

3.3 GC-TOFMS

3.3.1 Sample preparation for GC-TOF mass spectrometry

The supernatant samples were centrifuged at 13.000 g for 5 minutes in order to remove the dead cells. 200µL methanol was added to 50µL supernatant or blank medium samples for protein precipitation. The mixture was vortexed for 3 minutes and incubated for 1 hour on ice. Afterwards, samples were again centrifuged for 10 min, 13.000 g and at 4^0C. Supernatant was transferred into the glass vials. These samples were then freeze died at -80^0C (the process is known as lufilization) prior to a two-step derivatization or the volatilization step (approximately at $2 * 10^{-1}$ mbar pressure). Firstly, 50µL of methoxyaminin pyridine (20g/l) was added and the mixture stirred for 30 min at 80^0C for methoximation. This was followed by derivatization with 50µL of the reagent MSTFA (N-Methyl-N-(trimethylsilyl) trifluro-acetamide) for 30 min at 80^0C. Derivatization was automatically carried out using MPS 2XL autosampler equipped with an agitator (both from Gerstel, Karlsruhe, and Germany). Each sample was measured in triplicate, resulting in both three biological and three technical replicates, i.e. 9 samples per test group.

3.3.2 GC-TOF mass spectroscopy measurement

The GC-TOF mass spectroscopy consisted of an Agilent 7890 gas chromatograph (Hewlett-Packard, Atlanta, USA) coupled to a Pegasus HT ToF mass spectrometer (Leco, MÖnchengladbach, Germany). A HP5-ms capillary column of 60 m length, 0.25 mm inner diameter and 0.25 µm film thickness was used for separation. Splitless injection (Volume 1µL) was formed. The initial GC-oven temperature was set at 70^0C with a ramp of 50 C/min and a final temperature of 320^0C. Helium was used as carrier gas and constant flow rate of 1mL/min was adjusted. The transfer line temperature was set at 250^0C. Mass spectra were acquired within a range of 70 to 700 m/z and scan rate was 20 spectra per second. Ion source voltage was set at 70 eV and temperature at 200^0C.

3.3.3 Precision and accuracy of GC-TOF mass spectroscopy based metabolite profiling

For assessment of the precision and reproducibility of the method, blank medium samples were prepared for GC-TOF mass spectroscopy analysis including the internal standard as described in section **I**. and measured the triplicates .15 medium components were analyzed and the relative standard deviations (% RSD) were determined to assess the precision and accuracy of sample preparation and of the analytical method. Peak area normalization was done peak areas of the internal standard α-aminobutyric acid.

3.3.4 Data processing, normalization and multivariate statistical analysis

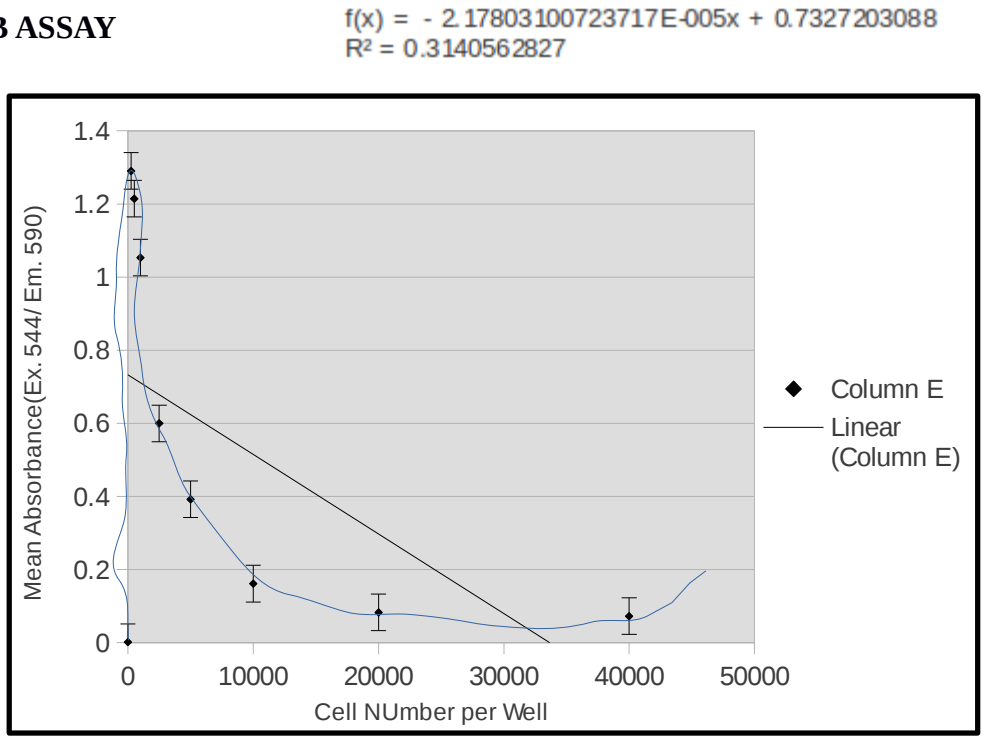
Chromatogram acquisition, automated peak deconvolution, identification of suitable fragment mass to charge ratio for peak area determination and reference library search were carried out using ChromaTof 4.22 software (LECO Corporation, Michigan, USA). Similarly threshold, which determines the minimum similarity of the obtained spectrum with the reference library spectrum, was set at 600. Annotation of metabolites were performed using the reference library or by the assessment of retension time and mass spectra from standard substance measurements.

Furthermore, the Golm Metabolome Database was used for mass spectra analysis of non-identified metabolites (NIMs). The database supports a decision-tree based prediction of functional groups allowing classification of the compounds. Known artifact peaks such as solvent contamination, column bleeding, plasticizers or reagent peaks were manually excluded. Peak areas of biologically relevant metabolites which were present in both drug-exposed and control samples were normalized to the respective peak area of the internal standard (m/z = 130) for each sample. Peak area of metabolites with more than one derivatization product (eg. Aspartate) were summed as well as peak areas of metabolites with several isomers upon methoximation such as glucose.

A range of 25-30 metabolites was analyzed by this method. To assess if a metabolite was released or taken up by the cells, blank medium controls were prepared, measured and analysed exactly as described and the resulting peak areas were compared to peak areas of the cell samples. Normalized peak were auto-scaled using standard deviation and then used for PCA to reduce the dimensions of the dataset. The *t*-standard was used to identify significant quantitative changes in exometabolome of untreated control and drug exposed cells. Fold changes were calculated compared to the control. A threshold of significance was defined as p-value < 0.05 and fold changes $>= 1.15$.

4 RESULTS

4.1 SRB ASSAY



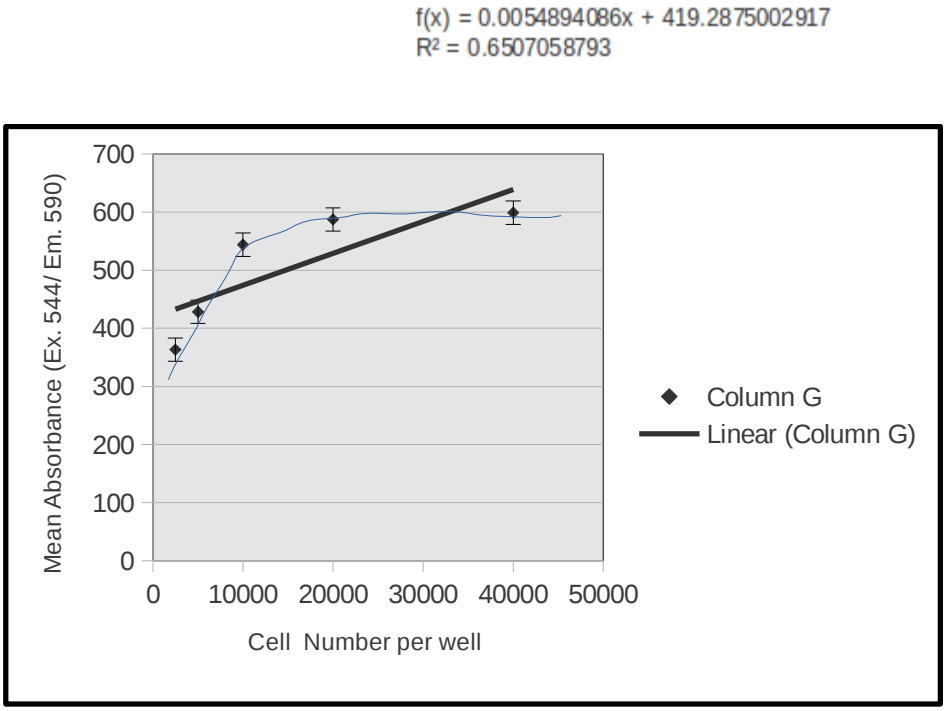
Cell Number	Area	Mean	SD
0	0	0.004375	0.00159799
250	0.25	1.264666667	1.29021332
500	0.5	0.889666667	1.214555761
1000	1	0.86966667	1.053246741
2500	2.5	0.43533333	0.599546257
5000	5	0.277	0.392174888
10000	10	0.114	0.161498728
20000	20	0.05766667	0.082998709
40000	40	0.04633333	0.072560812

Results of the SRB assay must be linear with cell number and cellular protein measured at cellular densities ranging from 1 to 200 % of confluence. From the above results its clear that there was an error in the measurements as the absorbance increases and then is decreases with increasing cell number number. This cannot be possible. This shows the experiment had an error.

SRB sensitivity is comparable with that of several fluorescence assays and superior to that of Lowry or Bradford.

The signal-to-noise ratio is favourable and the resolution is 1000-2000 cells/well. It performed similarly compared to other cytotoxicity assays such as MTT or clonogenic assay. The SRB assay possesses a colorimetric end point and is non-destructive and indefinitely stable. These practical advances make the SRB assay an appropriate and sensitive assay to measure drug-induced cytotoxicity even at large-scale application [9].

4.2 ALAMAR BLUE ASSAY



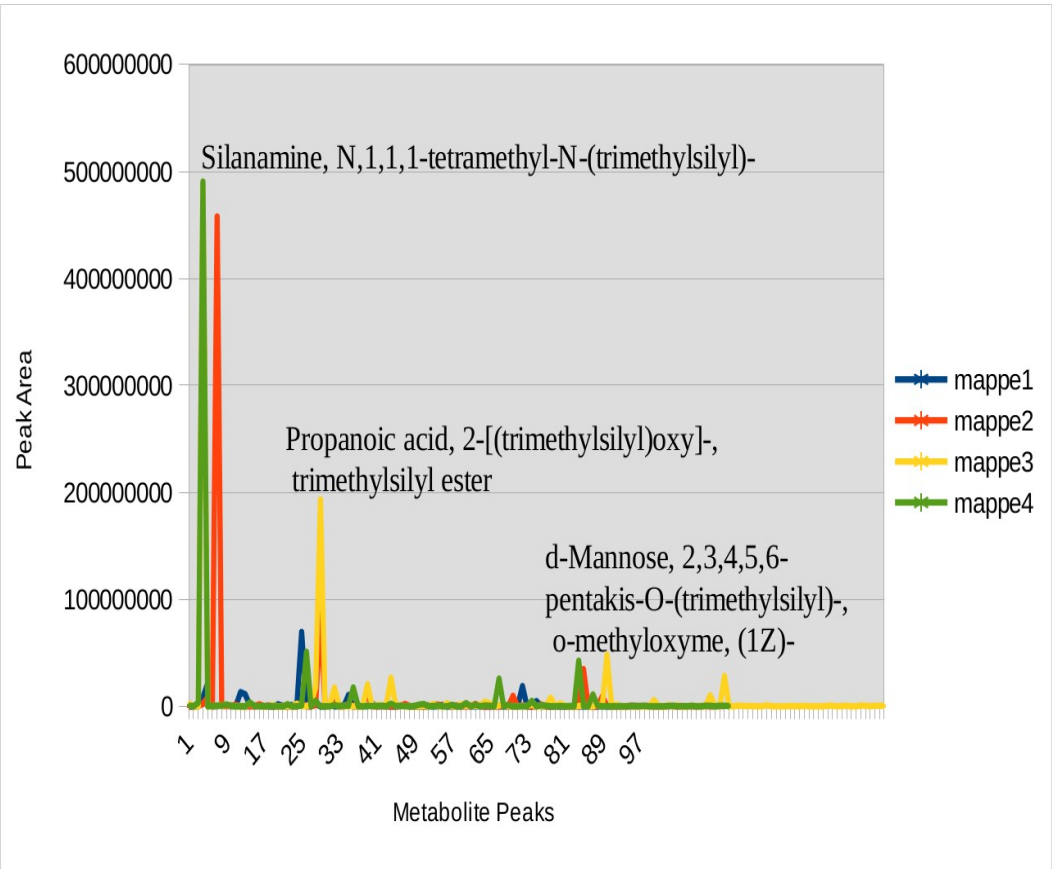
Cell Number	AREA	Mean	SD
0	0	345	36.20621494
2500	2.5	363.366667	55.22330426
5000	5	428.266667	226.1506651
10000	10	543.966667	62.93618461
20000	20	587.3	5.284884105
40000	40	598.966667	4.406056438

Pre-warmed alamar blue reagent was added according to 1/5th of the volume of the cell culture volume. Absorbance was measured after 3-4 hours at expulsion wavelength of 544 nm and emission wavelength at 590 nm.

4.3 GCTOF MASS SPECTROMETRY RESULTS

A total of 4 runs were carried out in GC-TOF mass spectroscopy and the results were obtained. Each of the the runs indicated many missing protein metabolites with different reaction time and peak area.Sampling, extraction and derivatization takes less than 50% of the time, but more effort and time,

approximately between 2 and 5 days, is needed for the comprehensive data analysis. Further, annotation of a given peak to a compound (with known or unknown chemical structure), needs to be done in order to identify a particular ion of interest and depends on two known factors, namely the Retension time index (RI) and the mass spectrum.



Mappe	Peak#	Weight	Name	Similarity	R. T (s)	Unique Mass	Quant Mass	Area
4	4	175	Silanamine, N,1,1,1-tetramethyl-N-(trimethylsilyl)-	967	625.45	86	86	490432752
2	7			974	622.9			457766762

1	25	234	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	969	981.2	147	147	69340252
4	26			974	980.35	117	117	50994686
3	29			966	979.6	117		193186582
4	84	569	d-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-	929	2297	73	73	42662539
2	85			928	2297.1			34711595

5 DISCUSSIONS

I have used Human HepG2 cells for my study. This is one of the best characterised and very frequently used cell lines to examine various mechanisms of hepatotoxicity. The initial stages of my praktikum includes culturing the HepG2 cells and allowing the cells to split to keep the cells alive under cultured conditions for longer periods of time. Passaging is done when the cells are 90 % to 100 % confluent. The cells are subjected to cell counting by using hemocytometer in which the cells are counted manually under microscope using Neubauer chamber. Using such technologies for counting cells yields results which show the consistency in the future to check the optimal cell number for experiments such as Sulforhodomine B assay and Alamar blue assay for the culturing of different cell numbers and checking which cell number suitable for 96 well plate . Due to the time constraints, the experiments yeild mere acceptable correlation values of 0.58 and 0.67 respectively. The results of alamar blue assay shown above says that the cell numbers are not highly diverged from standard deviation in the concentration of cell viability. The cells got saturated at 70,000 cell number. But the places where the deviation occurs may cause due to the presence of cell lumps. After the cell viability tests GCTOF mass spectroscopy was carried out for the profiling of different metabolites. Due to lack of time GC-MS data was not analysed.

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