Modulation of the Activity of Human Plasma Esterases by Binary and Ternary Mixtures of Water Miscible Organic Solvents during *In Vitro* Drug Metabolism Studies

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Organic solvents are commonly used in *in vitro* drug metabolism studies as co-solvents for lipophilic substrate/ new chemical entities. The effects of 1 % v/v final concentration of individual, binary and ternary mixtures of methanol, acetonitrile and dimethyl sulfoxide on human plasma esterases were investigated. The hydrolysis of p-nitrophenyl acetate to p-nitrophenol was monitored by ultraviolet spectrophotometry to follow esterase activity. Results indicated that the mixtures always showed less inhibition of esterase as compared to theoretically calculated additive inhibition based on individual solvent data. Results also indicated that dimethyl sulfoxide was the most-inhibitory solvent (59-64 % inhibition at 1 % v/v final concentration in incubation) and mixtures containing the least concentration of dimethyl sulfoxide caused the least inhibition of human plasma esterases.

Key words: Binary solvents mixtures, ternary solvent mixtures, human plasma esterases

Drug metabolism is an important determinant of the pharmacokinetic properties of drugs and in many cases, is the reason for bioavailability problems, inter-individual variations, and drug-drug interactions[1-4]. Recent advances of *in vitro* enzyme systems, the advent of commercial liquid chromatography-mass spectrometry instrumentation, the development of high-field nuclear magnetic resonance, liquid chromatography-nuclear magnetic resonance techniques, *in silico* modelling techniques, together with the explosion of our knowledge on various Drug Metabolizing Enzymes (DMEs) have strengthened the capability to study the metabolism of new drugs in an *in vitro* setting, well before the initial clinical studies[5,6]. In this regard, the *in vitro* metabolism assays developed should be appropriate in their design; an error in this may affect extrapolation of the results to in vivo parameters. In general, the incubation conditions of *in vitro* assays are optimized in such a way that the enzyme activity is linear with protein content and incubation period. *In vitro* metabolism assays generally involve incubation of model substrate/New Chemical Entities (NCEs) with the enzyme system containing necessary cofactors for the appropriate time[5-7]. *In vitro* studies are usually carried out in aqueous physiological buffers. Most NCEs are lipophilic in nature. Thus, to perform the enzyme activity determination, the NCEs are dissolved in the organic water miscible solvent. There are numerous reports available on the effect of organic solvents on DMEs like Cytochromes P450s (CYP450s), sulfotransferases (SULTs) and Uridine diphosphate-Glucuronosyltransferases (UGTs), but only a few studies have been reported for other DMEs[8-11]. In this regard, organic water miscible solvents are reported to show an inhibitory effect on carboxylesterase activity in incubation studies using up to 2 % v/v concentration of solvent[12]. Preliminary studies in our laboratory have also shown that organic solvents modulate esterase activity[13]. In these studies, Dimethyl Sulfoxide (DMSO) was observed to show more inhibitory effect of plasma carboxylesterases as compared to Methanol (MeOH) and Acetonitrile (ACN). The aim of the present study was to evaluate the effect of combinations of organic solvents on the activity of carboxylesterases. Binary

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and ternary combinations of DMSO, MeOH, and ACN were prepared at 1 % v/v total strength. The rate of conversion of p-Nitrophenyl acetate (PNPA) to p-Nitrophenol (PNP) in the presence of a single organic solvent at 1 % v/v concentration and in the presence of a binary/ternary combination of organic solvents at 1 % v/v total strength was determined, calculated and compared. The objective was to investigate whether binary and ternary mixtures show less inhibition of esterases and would be more suitable for use as co-solvents for NCEs while performing *in vitro* studies to investigate carboxyesterase mediated metabolism. PNPA was obtained from Hi Media. PNP, ethylenediaminetetraacetic acid and hydrochloric acid were purchased from S. D. Fine-Chem Ltd, Mumbai. Tris (hydroxyl) aminomethane (analytical research grade) was obtained from Sigma Chemical Co. Calcium chloride dihydrate was purchased from Research Lab Fine Chem Industries. ACN and MeOH (High-Performance Liquid Chromatography (HPLC) grade), was obtained from Merck India Ltd. DMSO was obtained from Thermo Fisher. All other chemicals were of analytical grade. Human plasma was used for esterase activity. There is a marked difference in the plasma hydrolyzing ability between different donors, and thus pooled human plasma was used in the assays[14,15]. Plasma was obtained from King Edward Memorial Hospital, Parel, Mumbai. For the enzymatic assay, an appropriate volume of plasma in diluted form (1:3 dilution in buffer) was used to ensure that the turnover was less than 20 % and the product formation was linear; for adherence to Michaelis Menten assumptions of steady state enzyme kinetics. Plasma fraction (30 µl) was incubated with 300 µl of 1200 µM PNPA in 0.1 M Tris-HCl buffer containing 0.01 M $CaCl_2$ and 100 µl organic solvent containing mix was added from the appropriate stock solutions to achieve desired final solvent concentration in the incubation mixture, at ambient temperature. The total incubation volume was 3 ml. Control incubations were performed in absence of solvent. The rate of hydrolysis to PNPA was measured by an increase in absorbance at 400 nm over a time course of 10 min using continuous spectrophotometry assay method. The instrument used was Jasco (Model No V-530) and spectra were analyzed using spectra manager. The concentration of PNP formed was obtained by dividing the absorbance values with its molar extinction coefficient (10.9 $mM^{-1}cm^{-1}$). This data was further mathematically treated to calculate the velocity of the reaction. During the ultraviolet assay the reference cuvette was always set up without the addition of enzyme. Each experiment was conducted in quadruplicate. Addition of pure solvents to desired extent in final incubation mixture individually is cumbersome. Therefore, binary and ternary mixtures of solvents were prepared at 30X total strength in 5 ml of volumetric flask with solvent (MeOH, ACN and DMSO) and 0.1 M Tris-HCL (pH 6.85) buffer, respectively. HPLC grade organic solvents were used in present study. Table 1 and Table 2 show the protocol for preparation of 30X total strength binary and ternary organic solvent stock solutions to be used in incubation mixture for plasma carboxylesterases. The substrate used for determination of esterase activity was para-nitrophenyl acetate, which is a hydrophilic probe substrate for esterases and shows sufficient solubility in aqueous media. Substrate stock solution was prepared in water, to achieve a true solvent free control. Substrate concentration selected for enzyme assays is dependent on various factors like Km of the enzyme substrate pair, solubility of the substrate, enzyme inhibiting ability of the substrate at high concentrations. Reported Km for PNPA ester and paraoxonases (PON) is 1.5±0.2 mM. But due to water solubility constraints of PNPA (0.53 g/l), 1.2 mM stock solution of PNPA was prepared. Thus, final concentration of PNPA in incubation mixture was 0.12 mM. Table 3 depicts percent inhibition exhibited by 1 % v/v binary mixture of ACN and MeOH. Increase in level of relatively higher inhibitory solvent (ACN) did not show drastic increase in total percent inhibition by the mixture. This may be because the difference between percent inhibition showed by ACN and MeOH is low at concentrations up to 1 % v/v. In contrast, data from Table 4 indicates that an appreciable decrease is seen in percent inhibition by binary mixture, when level of DMSO in 1 % v/v binary mixture of DMSO and ACN is lowered. Binary mixture of 0.25 % v/v DMSO and 0.75 % ACN showed 40 % inhibition, which was 19 % lower than that shown by 1 % v/v DMSO (59 %). Table 5 shows a similar trend, as the level of DMSO in 1 % v/v binary mixture of DMSO and MeOH is reduced from 0.75 % v/v to 0.25 % v/v, the percent inhibition reduces from 51 to 40 %. Likewise, the effect of ternary mixture at 1 % v/v on the plasma carboxylesterases activity was studied in a different set of experiments. The comparison of

inhibition by different combination of ternary mixture with that of single organic solvent at 1 % v/v is shown in Table 6. It was observed that when concentration of DMSO decreases in the ternary mixture, the inhibition observed decreased from (48 % to 38 %). Further, this inhibition was less as compared to 1 % DMSO alone (64 % inhibition) It was also observed that, when the concentration of each organic solvent in the ternary mixture is kept equal e.g. in case of 1 % v/v ternary mixture MeOH 0.333 % v/v+ACN 0.333 % v/v+DMSO 0.333 % v/v, the inhibition was found to be 42 % (22 % less than 1 % DMSO alone). In all the experiments, with respect to binary and ternary mixtures, the inhibition caused by each individual solvent alone at the concentration present in the mixture was also determined. This allowed one to calculate the theoretical inhibition that would be expected if the inhibition caused by each component in the mixture was independent of each other and additive. These theoretical inhibition values are also presented in Table 3-Table 6. In almost all cases it was observed that the actual inhibition observed was always less that the theoretical value indicating that the inhibition in binary and ternary mixtures are not simply an additive phenomenon. The reason for these observations is not clear at present. It is possible that inter-solvent interactions result in less individual solvents being available to interact with the enzyme and modulate its activity. The catalytic activity of enzymes is highly related to enzymic hydration[16-20]. Non-covalent interactions like hydrogen bonding, ionic interactions, van der Waal's and hydrophobic forces help maintain the enzyme in its catalytically active conformation[16-19]. Water is involved in these noncovalent interactions. Consequently, enzyme dehydration may cause conformational changes and lead to catalytic inactivity. Evidence suggests that, tightly bound structural water (and not bulk water) is crucial for enzyme activity[18]. A study reported by Zaks *et al*.[16] proposed that organic solvents interact with the enzyme bound water causing loss of enzyme activity. A broader study was performed by Gorman *et al*.[17] on chymotrypsin, subtilisin and peroxidase to study ability of various polar and nonpolar organic solvents to desorb enzyme bound water. The bound water from the freeze-dried enzymes was replaced by tritiated water (T_2O), which is radioactive. These enzymes suspended in organic solvents were then analyzed for amount of T2O desorbed by liquid scintillation counting method. It was observed that solvents with higher polarity showed more desorption of T_2O from enzymes. But, unlike chymotrypsin and subtilisin, peroxidase enzyme retained lesser T2O in moderate polarity. Thus, desorption was both solvent and enzyme dependent[17]. This may be allied to the fact that; same solvent shows varying effects on different enzymes. For example, CYP1A2, CYP2C8, and CYP2C9 appear to be the resistant to inhibition by DMSO, ACN, methanol and ethanol while CYP1A1, CYP2B6, CYP2C19, and CYP2D6 seem very sensitive to inhibition by these solvents[20]. Furthermore, from the Gorman *et al*.[17], solvent dielectric constant proved to be a good indicator of water desorbing ability of solvents. Water is bound by electrostatic forces to the charged residues of proteins. Higher solvent dielectric constant greatly weakens these electrostatic forces, enabling water molecules to desorb from the enzyme. Therefore, solvents with high dielectric constant may have greater effect on enzymes than those with a lower dielectric constant. On similar lines, reported dielectric constant of DMSO is 48.9, ACN is 38.8 and methanol is 33.6[21]. Percent inhibition at 1 % v/v concentration, shown by DMSO was 59-64 %, ACN was 24-28 % and methanol was 20-26 %. Consequently, higher dielectric constant seems to enable DMSO to cause efficient desorption of bound water molecules from the esterase enzyme and may be one of the factors leading to higher inhibition of esterase activity by DMSO. The choice of solvent mixture to be used for solubilizing an NCE in *in vitro* drug metabolism studies involving esterases, primarily depends on the solubility and polarity of the NCE. If the NCE is soluble in ACN and MeOH, binary mixture of 0.25 % v/v ACN and 0.75 % v/v MeOH can be used. But, DMSO is the solvent of choice for solubilization of chemical libraries for high throughput screening applications for pharmacological activity. In cases where use of DMSO is inevitable, binary mixture of DMSO and MeOH should be preferred. The mixture showing least inhibition at 1 % v/v level was 0.25 % v/v DMSO+0.75 % v/v MeOH. If use of binary mixture of DMSO and ACN is desired, the mixture showing least inhibition at 1 % v/v level was 0.25 % v/v+0.75 % v/v ACN. It should be noted that PNPA is a nonspecific substrate for esterase enzymes and the results from this study designate the combined effect of solvents and their mixtures on a mixed group of esterases and not a specific type of esterase. Reports indicate that, inhibitory effects of solvents on a given

cytochrome P-450 are substrate-dependent[20]. Such may also be the case regarding the esterase enzymes i.e. inhibitory effects of organic solvents on specific esterases may differ, depending on different selective probe substrates. To the best of our knowledge, this is the first report on the effects of binary and ternary water miscible organic solvent mixtures on enzyme activity that may prove valuable in the design on i*n vitro* drug metabolism studies.

TABLE 1: PROTOCOL FOR PREPARATION OF BINARY ORGANIC SOLVENT STOCKS (30 % V/V) FOR SPIKING INTO INCUBATIONS

Solvent		Buffer	Total
MeOH % v/v (Vol)	ACN % v/v (Vol)		
7.5 % (375 ml)	22.5 % (1125 ml)	3500 ml	5000 ml
15 % (750 ml)	15 % (750 ml)	3500 ml	5000 ml
22.5 % (1125 ml)	7.5 % (375 ml)	3500 ml	5000 ml
30 % (1500 ml)	0	3500 ml	5000 ml
0	30 % (1500 ml)	3500 ml	5000 ml

TABLE 2: PROTOCOL FOR PREPARATION OF TERNARY ORGANIC SOLVENT STOCKS (30 % V/V) FOR SPIKING INTO INCUBATIONS

Solvent			Buffer	Total
MeOH % v/v (Vol)	ACN % v/v (Vol)	DMSO % v/v (Vol)		
7.5 % (375 ml)	7.5 % (375 ml)	15 % (750 ml)	3500 ml	5000 ml
7.5 % (375 ml)	15 % (750 ml)	7.5 % (375 ml)	3500 ml	5000 ml
15 % (750 ml)	7.5 % (375 ml)	7.5 % (375 ml)	3500 ml	5000 ml
10 % (500 ml)	10 % (500 ml)	10 % (500 ml)	3500 ml	5000 ml
30 % (1500 ml)	0	0	3500 ml	5000 ml
0	30 % (1500 ml)	0	3500 ml	5000 ml
0	0	30 % (1500 ml)	3500 ml	5000 ml

TABLE 3: PERCENT INHIBITION OF ESTERASE ACTIVITY BY METHANOL (M), ACETONITRILE (A), AND THEIR BINARY MIXTURES AT A FINAL CONCENTRATION OF 1 % V/V IN THE INCUBATION

Solvent	% v/v	% I±SD	% v/v	% I + SD	% v/v	% I + SD
M	0.25	9.2±4.1	0.5	7.5±5.3	0.75	10.9±6.7
A	0.75	29.3±6.2	0.5	21.5±9.6	0.25	10.5±5
Binary mix (M:A)	0.25:0.75	26.6±6.5	0.5:0.5	31.4±4	0.75:0.25	36.2±12.8
Theoretical Inhibition	38.5	29	21.4	0	0	0
M	1	20.3±3.9				
A	1	24.5±1.4				

Note: M: Methanol, A: Acetonitrile, D: Dimethyl sulfoxide, I: Inhibition. All values for percent inhibition are mean±standard deviation (SD) (n=4)

TABLE 4: PERCENT INHIBITION OF ESTERASE ACTIVITY BY DIMETHYL SULPHOXIDE (D), ACETONITRILE (A), AND THEIR BINARY MIXTURES AT A FINAL CONCENTRATION OF 1 % V/V IN THE INCUBATION

Solvent	%v/v	% I±SD	%v/v	% I±SD	%v/v	% I±SD
M	0.25	6.9±5.3	0.5	4.7±2	0.75	24.3±7.8
D	0.75	53.5±2.7	0.5	48.2±5.4	0.25	42.2±18.1
Binary Mix (M:D)	0.25:0.75	51.2±1	0.5:0.5	46.5±4.3	0.75:0.25	39.6±1.3
Theoretical Inhibition	60.4	52.9	66.5	0	0	0
D	1	59±5.3				
M	1	20.3±3.9				

Note: M: Methanol, A: Acetonitrile, D: Dimethyl sulfoxide, I: Inhibition. All values for percent inhibition are mean±standard deviation (SD) (n=4)

TABLE 5: PERCENT INHIBITION OF ESTERASE ACTIVITY BY DIMETHYL SULPHOXIDE (D), METHANOL (M), AND THEIR BINARY MIXTURES AT A FINAL CONCENTRATION OF 1% V/V IN THE INCUBATION

Solvent	% v/v	% I±SD	% v/v	% I±SD	% v/v	% I±SD
M	0.25	6.9±5.3	0.5	4.7±2	0.75	24.3±7.8
D	0.75	53.5±2.7	0.5	48.2±5.4	0.25	42.2±18.1
Binary Mix (M:D)	0.25:0.75	51.2±1	0.5:0.5	46.5±4.3	0.75:0.25	39.6±1.3
Theoretical Inhibition	60.4	52.9	66.5	0	0	0
D	1	59±5.3				
M	1	20.3±3.9				

Note: M: Methanol, A: Acetonitrile, D: Dimethyl sulfoxide, I: Inhibition. All values for percent inhibition are mean±standard deviation (SD) (n=4)

TABLE 6: PERCENT INHIBITION OF ESTERASE ACTIVITY BY DIMETHYL SULPHOXIDE (D), METHANOL (M), ACETONITRLE (A), AND THEIR TERNARY MIXTURES AT A FINAL CONCENTRATION OF 1 % V/V IN THE INCUBATION

Solvent	% v/v	% I±SD	% v/v	% I±SD	% v/v	% I±SD	% v/v	% I±SD
M	0.25	11±1.96	0.25	6±0.19	0.5	15±1.22	0.33	10±3.18
A	0.25	12±7.22	0.5	15±0.31	0.25	13±4.75	0.33	14±3.26
D	0.5	47±5.37	0.25	30±0.29	0.25	28±6.42	0.33	35±1.37
Ternary Mix (M:A:D)	0.25:0.25:0.5	48±1.84	0.25:0.5:0.25	38±3.11	0.5:0.25:0.25	40±0.96	0.33:0.33:0.33	42±1.05
Theor. Inhib.	70		51		56		59	
M	1	26±2.06						
A	1	28±0.32						
D	1	64±9.16						

Note: M: Methanol, A: Acetonitrile, D: Dimethyl sulfoxide, I: Inhibition. All values for percent inhibition are mean±standard deviation (SD) (n=4)

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