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Comparative Study of *in vivo* Gastrointestinal Absorption of Mustard Oil Emulsions Prepared with Different Types of Medium Chain Fatty Acids

Susmita Roy[1], Sohini Mukherjee[1], Avery Sengupta[1*], Tanmoy Kumar Dey[2], Pubali Dhar[2] & Mahua Ghosh[1]

[1] *Department of Chemical Technology, University of Calcutta, India*
[2] *Department of Home Science, University of Calcutta, India*

ABSTRACT

Introduction: Absorption of dietary fats is generally in the form of emulsions. The present study assessed the preparation and gastrointestinal absorption efficiency of three emulsions of mustard oil containing three types of medium chain fatty acids (MCFAs) in a rat model. **Methods:** Caprylic acid ($C_{8:0}$), capric acid ($C_{10:0}$) and lauric acid ($C_{12:0}$) were chosen as the MCFAs. Mustard oil emulsions were formulated using each of the MCFAs and lecithin as an emulsifier. The characteristics of the formulations including optical microscopy, particle size, zeta potential analysis and viscosity studies were assessed. Thereafter the intestinal digestion patterns of the three MCFA rich mustard oil emulsions were compared using a single pass perfusion test. **Results:** The particle size of the emulsions varied between 212.70 nm and 312.70 nm. Physical characterisation such as a zeta potential study confirmed that all emulsions were thermodynamically stable. The absorption study was monitored at 30 min intervals of up to 2 h The absorption of $C_{8:0}$ emulsion was found to be maximum (27.78%) followed by $C_{10:0}$ emulsion (24.81%) and $C_{12:0}$ emulsion (22.50%). The differences in absorption efficiency of the emulsions could be attributed to the smaller chain length of $C_{8:0}$ which was more rapidly absorbed by the intestine. **Conclusion:** *In vivo* gastrointestinal absorption of MCFA rich mustard oil emulsions was compared; caprylic acid-rich mustard oil showed the highest absorption rate in comparison to the other two emulsions. Further *in vivo* studies are required to establish the mechanism of absorption of structured lipids containing MCFAs.

Key words: Intestinal absorption, medium chain fatty acids, oil-in-water emulsion

INTRODUCTION

Medium chain TGs (MCT, with fatty acids C_6-C_{12}) are well known for their rapid metabolism, higher energy expenditure and low deposition in adipose tissues. Thus they can provide instant energy and reduce obesity (Seaton *et al*., 1986). Essential fatty acids (EFA) on the other hand are also of great importance in the human system as they cannot be produced by the body but play several distinguishing roles in preventing the body from succumbing to various diseases. Thus a combination of EFA and medium chain fatty acids (MCFA) may be useful to deliver optimum nutrition and combat against specific diseases and metabolic conditions.

Mustard oil has been chosen as the vehicle/backbone for the preparation

Correspondence: Avery Sengupta; Email: *avery.sgupta@gmail.com*

of structured triacylglycerol because it contains a unique monounsaturated fatty acid, erucic acid, along with other monounsaturated fatty acids and also has an appreciable amount of polyunsaturated fatty acids like linoleic and linolenic acids that are low in saturated fatty acids. It is a popular edible oil in India. The presence of appreciable amounts of unsaturated fatty acids and lower amounts of saturated fatty acids render mustard oil good for the heart and is said to lower the risk of heart disease among Indians (Ghosh & Bhattacharjee, 2013). The oil is also rich in tocopherol which not only acts as an anti-oxidant but also extends the shelf-life of the oil. In our previous studies, we prepared MCFA rich mustard oil using a packed bed bioreactor (Sengupta *et al*., 2015) which is a structured oil containing both EFA and MCFA but is low in saturated fatty acids.

In the present study, emulsions of different MCFA rich mustard oils were prepared and their comparative effect on gastrointestinal absorption was observed in rats. Emulsions were used as a vehicle to transport the lipid of interest (in this case MCFA rich mustard oils) inside the rat model and observe the pattern of absorption of the MCFA rich mustard oils. It is well-known that emulsification of lipids is the elementary step in fat digestion and thus administration of structured mustard oil in the form of emulsions can increase the bio-availability of the oil. This is due to the fact that the generation of an oil/water interface by preparation of an emulsion instead of directly administering the oil can facilitate the interaction between water soluble lipases. This is important for lipid digestion and water insoluble lipids that temper bioavailability of lipid nutrients (Dey *et al*., 2012). In the prepared oil-in-water emulsion oil was dispersed in water. Lecithin was added to act as an emulsifier and casein protein as an emulsion stabiliser to reduce the interfacial tension between the two phases and to contribute to the stabilisation of dispersed droplets with electrostatic or steric effects.

The present study had the following aims: (i) to prepare 10% oil-in-water (O/W) emulsions using three different types of MCFA rich mustard oil, namely, caprylic acid ($C_{8:0}$) rich mustard oil, capric acid ($C_{10:0}$) rich mustard oil and lauric acid ($C_{12:0}$) rich mustard oil; (ii) characterisation of prepared emulsions including optical microscopy, particle size and zeta potential analysis and determination of viscosity studies; and finally (iii) an *in vivo* absorption study in rats by a single pass perfusion test to study the comparative intestinal digestion of three MCFA rich mustard oil formulations in the rat model. Perfusion was given instead of oral ingestion as the rat was in anaesthesised condition during the course of the experiment. This *in vivo* perfusion absorption study can provide us a better insight into the absorption pattern with minimal error, in comparison to an oral ingestion study in a non-anaesthesised condition.

METHODS

Preparation of oil-in-water emulsion

From our previous study, it was observed that medium chain fatty acids (MCFA) such as caprylic acid ($C_{8:0}$), capric acid ($C_{10:0}$) and lauric acid ($C_{12:0}$) could be successfully incorporated into mustard oil resulting in the formation of caprylic acid, capric acid and lauric acid rich mustard oils respectively by an enzymatic transesterification process in a packed bed bioreactor (PBBR) (Sengupta *et al*., 2015). These three types of MCFA rich mustard oils were used as the dispersed phase in oil-in-water (O/W) emulsions and double glass distilled water as the continuous phase. Fifty grams of the prepared oil was added to 25 g of de-oiled lecithin (emulsifier) and 25 g of casein protein (emulsion stabiliser) and mixed with 500 ml of double distilled water under constant

stirring at 800 rpm by a mechanical stirrer for 1 h at normal room temperature (25°C) as described by Huang, Yu & Ru (2010) to prepare 10% o/w emulsions. Thus, three different types of emulsions were prepared with three different MCFA rich mustard oils. After mixing the contents, each emulsion was homogenised at 10,000 rpm by Ultra Turrax T25 (IKA Werke, GmbH & Co.). This procedure was followed by High Pressure Homogenisation using Electric Bench-top Ultra High Pressure Homogenizer (Nano DeBEE, BEE INTERNATIONAL, INC., South Easton, MA 02375) for 6 passes at 35,000 psi at 4°C. The resultant formulations were stored at 30°C.

Characterisation of emulsion

Optical microscopy

A thin film of emulsion (5 µl) was prepared in a grease-free glass slide which was perceived under optical microscope (Leica DM 750 optical microscope, Leica Microsystems, Germany) to observe the droplet topography. Images of the droplet topography were taken with a camera module (ICC 50, Leica Microsystems GmbH, Wetzlar, Germany) attached to the microscope.

Particle size and zeta potential

Average diameter and zeta potential of the emulsion droplet was measured by Malvern Zetasizer Version 6.0 (Malvern Instruments Ltd, Serial Number: MAL1032012) using water as dispersant (Refractive index 1.330 and viscosity 0.8872 cP).

Viscosity

Refractive index of three different O/W emulsions was determined by Brookfield viscometer at 32.9°C with a spindle torque of 90 rpm.

***In vivo* absorption study - animals and experimental set-up**

To comprehend the absorption of emulsion through the intestine, a single pass perfusion test was performed for each prepared emulsion. This animal experiment was monitored by the Bioethics Committee for Animal and Human Research Studies of the University of Calcutta, West Bengal, India. Nine adult male Sprague Dawley rats (170-250 g), with 3 rats in each group, were accommodated and accustomed for a week and given food and water *ad libitum*. The animals were divided into three groups and each group was fed with a different type of emulsion. Each animal was fasted overnight prior to the experiment with access to water *ad libitum* and then anesthetised with intra-muscular injection of Ketajet® 50 @ 35 mg/kg body weight and an intra-peritoneal injection of Xylocaine 2% @ 5 mg/kg body weight. The experimental procedure was set up following the method of Dey *et al.* (2012). The peritoneal cavity of the rat was then opened by a midline slit and the small intestine was cannulated proximally (just downstream to the pyloric sphincter) and distally (about 5 mm upstream to the illeo-caecal junction) so that the perfusate (emulsion) entering the small intestine, spanned through it and lastly left through the distal cannula which was provided with a stoppered cap. The cannula was tied primed with a loop of silk suture placed firmly around the intestine at both ends. A similar association was given to the gastric end of the oesophagus to avert any fortuitous backflow of the emulsion through the nasopharyngeal channel and consequent clogging of the respiratory system. The cannulated intestine was kept covered during the experiment with cotton pad wet in phosphate buffer solution (PBS) to avoid drying. At the beginning of the experiment, the entire GI tract was

flushed with PBS until the fluid from the outlet became clear. Then, a fixed volume (10 ml) of the emulsions were injected in the cannulated intestine and observed for different time intervals. In this study the emulsion absorption was observed at 30 min intervals for 120 min after injecting the emulsions. At the beginning of each time interval, the cannulated small intestine was injected with 10 ml of the experimental emulsion (10% $C_{8:0}$ emulsion or 10% $C_{10:0}$ emulsion or 10% $C_{12:0}$ emulsion) and at the end, the outflow fluid was collected; the difference in volume between the perfusate-emulsion and the outflow-fluid was taken as the *absorbed volume*. Lipid content was determined by the process of the Folch method (Folch, Lees & Stanley, 1957) which was modified before application. The outflow fluid and initial emulsions were measured and the difference of these two was assumed to be the absorbed lipid.

Gas-liquid chromatographic analysis

The fatty acid compositions of the total lipid separated from the initial emulsions and the total lipid separated from the passed through out-flow emulsions were analysed by gas-liquid chromatography (GLC). TGs in lipid fraction were converted into their methyl esters according to the method of Litchfield (Litchfield, 1972). About 1 ml of 0.5 (N) methanolic KOH was added to the small quantity of the isolated lipid dissolved in chloroform, shaken vigorously for 10 min and then neutralised with 1 ml of 1 (N) HCl. Fatty acid methyl esters (FAME) were then extracted with petroleum ether (boiling point 40°C - 60°C) and dried under nitrogen gas-flow. FAMEs were analysed by an Agilent 6890 N computerised gas chromatograph (network GC system- G 1530 N). A glass capillary column, DB-WAX (30 meter × 0.32 mm; film 0.25 µm) was used as the stationary phase. The temperature for the analysis was programmed as follows: oven temperature starting at 150 °C for 2 min and rising up to 200 °C immediately after injection, followed by linear heating (15°C/ min) up to 250 °C and maintained at this temperature for 10.33 min; finally oven temperature was maintained at 270°C for 16.67 min rising with 4°C/ min. Carrier gas was N_2 (flow rate 1ml/ min). The gas chromatograph was calibrated prior to sample injection and the corresponding concentration of fatty acids was expressed as %w/w.

Statistical analysis

The absorption study comparing three different o/w emulsions in the rat model was plotted using OriginLab software, version 8.0.

RESULTS AND DISCUSSION

Preparation of oil-in-water emulsion

Mustard oils rich in three types of medium chain fatty acids (MCFA) were emulsified into an aqueous phase containing double distilled water and de-oiled lecithin as emulsifier and casein as emulsion stabiliser. It is well known that emulsifying parameters such as the type and emulsifier concentration, homogenisation parameters and concentration of lipid phase can affect the physico-chemical properties of the emulsion (Cheong *et al*., 2008; Wulff-Pérez *et al*., 2009; Oh *et al*., 2011; Yuan *et al*., 2008). Consequently, all parameters including the concentrations of different MCFA containing oils in different emulsions were kept constant during the emulsion preparation and therefore three emulsions were prepared with three different types of mustard oils keeping constant all other conditions like amount of oil and amount of emulsifier and stabiliser.

Characterisation of emulsion

Physico-chemical properties of the three types of emulsions were characterised by optical microscopy, particle size and zeta potential and viscosity studies.

Optical microscopy

Although optical microscopy was developed a century ago, it still remains a valuable tool for the determination of micro-structure of emulsions. Optical microscopy provides valuable information about the size distribution of droplets in emulsions and can be used to differentiate between flocculation and coalescence. Figure 1 shows the microscopic structures of three different types of emulsions. Each figure shows a distinctive morphology of the droplets indicating the size distribution of the droplets is 350-200 nm. The figures also show that no agglomeration took place between the emulsion droplets and the particles were well distributed in the emulsions.

Particle size and zeta potential

Table 1 showed the emulsion particle size of the three MCFA rich emulsions based on measurements of particle size analyser. It is very clear from the particle size results

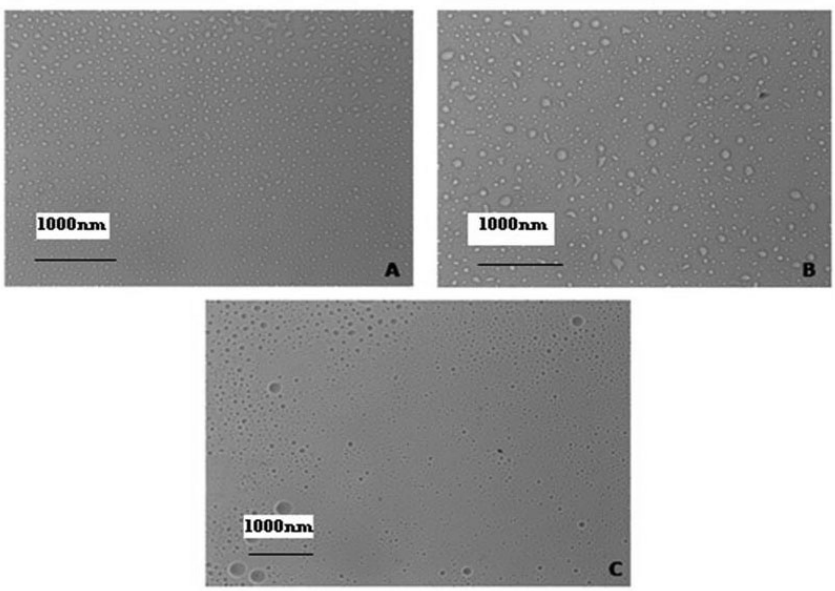

Figure 1. Optical microscopy of 10% O/W emulsion using (A) caprylic acid ($C_{8:0}$), (B) capric acid($C_{10:0}$) and (C) lauric acid ($C_{12:0}$) rich mustard oil

Table 1. Particle size (nm), polydispersity index (PdI), zeta potential (mV) and viscosity (cP) of three types of emulsions made by three different MCFA rich mustard oils

Emulsion type	*z-average diameter (nm)*	*Polydispersity Index (PdI)*	*Zeta potential (mV)*	*Viscosity (cP)*
Caprylic acid emulsion (C8:0)	312.70	0.267	-36.90	18.4
Capric acid emulsion (C10:0)	265.60	0.516	-36.30	20.3
Lauric acid emulsion (C12:0)	212.70	0.270	-36.00	25.3

● For each group, n= 3.

that the emulsions are homogeneously distributed in the system. From the table it can be seen that particle size is in the range of 212.7nm to 312.7nm. The zeta potential results confirm that all the emulsions were highly stable given their negative charge. The negative charge may be due to the emulsifier lecithin used in the preparation of the emulsions. It imparts a high negative charge to the emulsion droplet surface as the phosphate moieties align towards the aqueous phase from the o/w interface. The lecithin clearly causes major changes to the charge potential even at small concentrations preventing flocculation within the emulsion.

Viscosity

Viscosity of the emulsion samples, measured at 32.9°C with a spindle torque of 90 showed an increasing trend from caprylic (18.4 cP) to lauric acid (25.3 cP) rich MCFA-emulsion (Table 1). These changes may be due to the increasing chain length of the MCFA used in the emulsions.

Influence of droplet size of prepared emulsions on *in vivo* absorption in rat intestine

Three different types of emulsions of similar dilution levels, an *in vivo* study based on intestinal perfusion model was designed. The emulsions were formulated with oils with less z-average diameter of droplets and low polydispersity index as shown in Table 1. In this study the effect of droplet diameter and chain length of fatty acids on the absorption efficiency by the body was monitored by comparing the intestinal absorption of three types of emulsions prepared using three types of MCFAs of different chain lengths. Both lipid absorption and volume absorption were found to be significantly different in the three emulsions at different time intervals as shown in Figure 2. Based on the volume of absorption of three different types of emulsion, caprylic acid rich mustard oil emulsion showed the highest value whereas lauric acid rich mustard oil emulsion showed the lowest (Table 2). Based on the lipid content of the

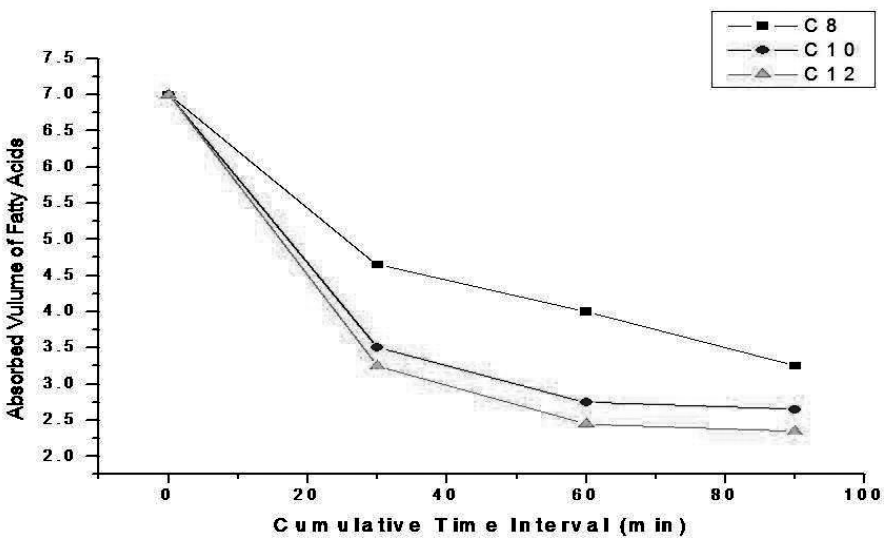

Figure 2. Comparison of absorption of three different 10% O/W emulsion made by three different MCFA rich mustard in rats, with respect to absorbed volume percentage of emulsion atdifferent time intervals within the intestine [Mean±SD, n=6]

out-flow fluid from the intestine, caprylic acid rich mustard oil emulsion had the highest absorption as the out-flow fluid contained the least lipid among the three, whereas lauric acid rich O/W emulsion showed the maximum lipid content in the out-flow liquid. So, it is observed that there was a positive correlation between volume absorbed and lipid absorbed within the intestine which is shown in Figure 2 ($p \leq 0.05$). So, it could be stated that caprylic acid rich MCFA emulsion showed the best absorption efficacy among the three similar characterised emulsions.

Gas liquid chromatographic analysis

Fatty acid composition of the isolated lipid extracted from the out-flow fluid is shown in Table 3. In all three cases, a steady trend of fatty acid composition was seen. Apart from caprylic ($C_{8:0}$), capric ($C_{10:0}$) and lauric acid ($C_{12:0}$), the fatty acids palmitic acid ($C_{16:0}$), staeric acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic acid ($C_{18:3}$) were also seen in the out-flow lipid due to their presence in mustard oil. Table 3 shows that the outflow lipid was rich in palmitic acid, especially in the initial run, in comparison to the amount present in the original lipid administered. Triacylglycerols are generally broken down in the human system to produce monoacylglycerol and fatty acids with the help of gastric and pancreatic lipases which are sn-1,3 specific enzymes, that is, they hydrolyse the ester bond present in sn-1 and in palmitic acid in the outflow lipid indicates that the structured lipids contain a significant amount of palmitic acid in sn-2 position and thus form 2-monopalmitin after lipase hydrolysis. Moreover, the shorter chain fatty acids are absorbed at a faster rate leaving behind the longer chains and thus the absorption of palmitic acid does not occur at a rapid rate but remains in the outflow lipid. But later it gets absorbed, as can be seen in the data presented in Table 3.

From Figure 3 it can be concluded that at different time frames, the absorption of caprylic acid is much better than that of capric and lauric acids as the outflow lipid caprylic acid percentage was least in comparison to capric acid and lauric acid.

CONCLUSION

The above study describes the formation of three types of emulsions using three different types of MCFA rich mustard oils. All the three emulsions were shown to

Table 2. Time intervals of collection of outflow fluid (min), volume of absorbed emulsion (mL) and total lipid content of absorbed emulsion (g/dL) made by three different MCFA rich mustard oils

Groups	*Cumulative time intervals of collection of out-flow fluid (min)*	*Volume of emulsion absorbed (mL)*	*Total lipid content of out-flow fluid (g/dL)*
Caprylic acid	30	4.65	1.086 ± 0.427
	60	4.00	0.525 ± 0.013
	90	3.25	1.23 ± 0.389
Capric acid	30	3.50	0.611 ± 0.008
	60	2.75	1.204 ± 0.286
	90	2.65	1.55 ± 0.304
Lauric acid	30	3.25	1.039 ± 0.098
	60	2.45	0.823 ± 0.006
	90	2.35	2.012 ± 0.892

For each group, n= 3; Values are represented as Arithmetic Mean ± Standard Error of Mean.

Table 3. Fatty acid compositions of three different 10% O/W emulsion made by three different MCFA rich mustard oils in three time intervals

Parameters / *Groups*	*Time Intervals (minutes)*	*Percentage of fatty acids of out-flow fluid (% w/w)*								
		C8:0	*C10:0*	*C12:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*C18:3*	*C22:1*
Caprylic acid group (C8:0)	Initial	15.51	-	-	13.15	11.12	9.24	10.67	8.72	28.40
	30	0.60	-	-	42.86	13.98	8.31	5.25	3.38	25.62
	60	2.68	-	-	36.06	15.05	8.08	3.05	5.80	29.28
	90	2.74	-	-	45.75	20.59	5.59	8.40	6.15	10.78
Capric acid group (C10:0)	Initial	-	15.66	-	18.86	13.12	15.20	10.90	8.98	17.28
	30	-	0.70	-	41.62	16.78	3.83	14.29	5.80	16.98
	60	-	4.84	-	35.79	3.96	13.85	18.94	3.79	18.83
	90	-	6.01	-	25.34	7.57	16.13	10.29	8.77	25.89
Lauric acid group (C12:0)	Initial	-	-	12.43	23.86	13.12	9.92	7.03	8.55	25.09
	30	-	-	1.85	43.97	15.51	12.15	3.43	12.23	10.86
	60	-	-	3.01	43.82	17.40	10.99	4.89	7.76	12.13
	90	-	-	9.09	28.03	12.15	7.92	5.83	5.31	31.67

For each group, n= 3.

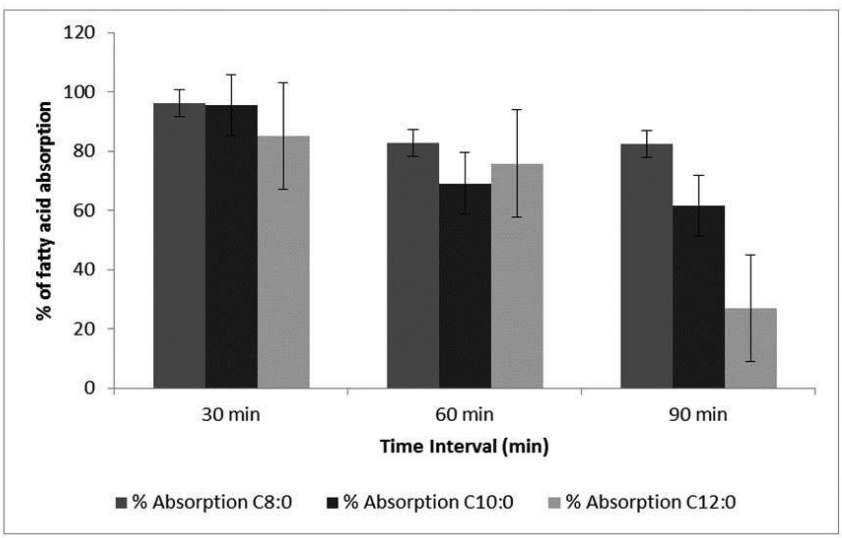

Figure 3. Absorption of fatty acids (%) of three different emulsions in rat intestine in accordance-with time intervals (min)

be stable and no flocculation was seen in the emulsions as indicated by the optical microscopic study. From this study, it could be concluded that absorption of caprylic acid rich O/W emulsion within the rat intestine was comparatively higher in comparison to capric acid and lauric acid rich O/W emulsions. The highest absorption of caprylic acid rich emulsion could be attributed due to the smallest size of the $C_{8:0}$ fatty acid compared to the other two ($C_{10:0}$ and $C_{12:0}$), which helps in more rapid intestinal absorption. Further *in vivo* studies are required to establish the mechanism of absorption of structured lipids containing MCFAs.

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