Synthesis, spectral studies, hydrolysis kinetics and pharmacodynamic profile of mefenamic acid prodrugs

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ABSTRACT

Prodrugs of mefenamic acid with natural compounds eugenol and vanillin have been synthesized by dicyclohexylcarbodimide coupling method. Purified synthesized ester prodrugs were characterized by Melting Point, Thin Layer Chromatography, Fourier Transform Infra Red Spectroscopy, Proton Nuclear Magnetic Resonance Spectroscopy and Mass Sspectroscopy. Prodrugs were also characterized by solubility studies, partition coefficient and hydrolytic studies. The synthesized derivatives are screened for their anti-inflammatory, analgesic and ulcerogenic study which show retention of anti-inflammatory activity with reduced ulcerogenicity.

Keywords: Prodrug, Eugenol, vanillin, dicyclohexylcarbodimide.
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INTRODUCTION

Non Steroidal Anti-inflammatory Drug's (NSAIDs) are the most commonly prescribed drugs worldwide today. NSAIDs are widely used for the treatment of pain and inflammation. Currently it is well established that cyclooxygenases (COXs) exist in two isoforms COX-I and COX-II [1]. The potentially deleterious effects of NSAIDs on gastrointestinal tract are caused by inhibition of cytoprotective COX-I and by physical contact mechanism in stomach. [2,3] Long term use of available acidic NSAIDs is most of the time restricted due to gastric irritation, ulcers & bleeding [4, 5]. The free carboxylic acid group is crucial in maintaining the effectiveness and is also responsible for gastric side effects [6]. As a result several selective COX-II inhibitors like celecoxib, Rofecoxib and valdecoxib have been introduced in clinical use. But serious cardiovascular side effects on long term use resulted in withdrawal of this drug [7]. It is now well known that local generation of Reactive Oxygen Species (ROS), plays a key role in gastric ulceration associated with NSAIDs therapy [8, 9]. These indicate that antioxidants can be used to prevent NSAIDs induced gastric ulcers & bleeding. Recently several natural antioxidant compounds are considered promising in treatment of free radical mediated diseases [10]. These phytoconstituents have been traditionally in use for their medicinal & flavoring properties and are safe. Naturally occurring antioxidants like thymol, menthol, eugenol and sesamol are the suitable promoieties for mutual prodrug as they provide additional antioxidant & analgesic activity [11, 12, 13].

Mutual prodrug is an area of increasing interest in recent years, which involves combining two different pharmacophore with similar pharmacological spectrum to give synergistic activity. Several mutual prodrugs of NSAID's with natural phenolic & alcoholic antioxidant compounds are reported such as diclofenac with thymol, guiacol, eugenol, sesamol, menthol, vanillin and umbelliferone [14], biphenyl acetic acid with thymol, guiacol, menthol & eugenol [15], Ibuprofen eugenol ester[16] and ibuprofen thymol, menthol and eugenol ester [17]. Mefenamic acid is the potent NSAID used for headache, rheumatic, muscle pain, & tooth ache. But the side effects like nausea, dyspepsia, ulceration of stomach & intestinal tract are reported [18]. Literature reveals that many efforts

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have been made to synthesize mutual prodrug of mefenamic acid. Acyloxyethyl and glycolic acid esters of mefenamic acid with lower gastrointestinal toxically have been reported [19]. Several mefenamic acid mutual prodrug are reported, mefenamic thymol & menthol esters [20], mefenamic acid guiacol ester [21], mefenamic acid with paracetamol & Salicylamide [22] and mefenamic acid glucosamine [23]. Mefenamic acid derivatives with sesamol, eugenol, cinnamyl alcohol, 7-hydroxy-4-methylcoumarin and mesitol were synthesized and evaluated for antioxidant and anticoagulant activities [24].

In this present work, an effective NSAID mefenamic acid was conjugated with vanillin and eugenol to design prodrugs. Also the synthesized prodrugs were confirmed by spectral characterization and evaluated for physicochemical properties, hydrolytic stability, anti-inflammatory activity and gastro protective effect.

MATERIALS AND METHODS

Mefenamic acid was obtained from BlueCross Laboratories Ltd (Nasik, India). Eugenol, vanillin, Dimethylaminopyridine and N, N-Dicyclohexyl carbodimide were commercially obtained from Loba Chemicals Pvt. Limited, Mumbai, India. Other reagents and solvents used were of analytical/HPLC grade as the case desired. The purity of the synthesized compound was confirmed by thin layer chromatography using precoated TLC plates (Merck, 20×20, 60F 254). Visualization of spot was done using iodine vapors and UV cabinet. Melting points were recorded in open capillary tubes and are uncorrected. IR spectra were recorded in Bruker, Germany 3000 Hyperion Microscope with Vertex 80 FTIR System using KBr disks. ^{1}H NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) multi nuclei solution NMR Spectrometer using DMSO-d_6 as the solvent and TOPSPIN -2 software. Mass spectra were recorded on JEOL GCMATE II GC-MS mass spectrometer/Data System by electron ionization (EI) technique. The high-performance liquid chromatography (HPLC) system consisted of a pump (Jasco PU-2089 plus Quaternary Gradient Pump), a UV/Vis detector (Jasco UV-2075 Plus intelligent UV/Vis detector) with a C-18 column (Finepak SIL, 250 x 4.6 mm, 5 μm) using acetonitrile: phosphate buffer pH 3 (70:30 v/v) as mobile phase and flow rate 1.0 ml/min with UV detection at 285 nm. The HPLC software used was Jasco-ChromNAV (1.19.1 Version).

Synthesis of mefenamic acid esters [25]

Mefenamic acid (1.20 g, 5 mmol) was dissolved in dichloromethane in round bottom flask with condenser and drying tube at its upper end in order to maintain anhydrous conditions. The acid solution was cooled to 0°C. The alcohol/phenol (eugenol and vanillin, 5 mmol) was added to acid solution at 0°C followed by addition of N, N-Dicyclohexyl carbodimide (1.03 g, 5 mmol) and dimethylaminopyridine (0.12 g, 1 mmol). The reaction mixture was allowed to stir at 0°C for one hour. The ice bath was removed and reaction mixture was stirred at room temperature for next 12 hours attached with drying tube. Then reaction mixture was filtered to separate N, N' dicyclohexyl urea (DCU) precipitate. The filtrate was washed twice with 25 ml of 5% NaHCO$_3$ solution to remove excess/unreacted acid. The aqueous layer was separated and organic layer was then washed twice with 5 ml, 5% NaOH solution. The organic layer was then made completely anhydrous by adding Na$_2$SO$_4$. The dichloromethane was then distilled out under reduced pressure in order to avoid thermal degradation of ester. The products mefenamic acid-eugenol ester (MAEU) and mefenamic acid-vanillin ester (MAVAN) obtained were then recrystallized by dissolving it in little quantity of n-hexane to give products. The products were characterized for purity and subjected to spectral analysis. The schematic representation of synthesize of mefenamic acid prodrugs is shown in figure 1.

### 2-methoxy-4-(2-propenyl) phenyl-2-[(2,3-dimethylphenyl)amino]benzoate.

The percentage yield of MAEU was found to be 65%. UV (λ_{max}): (MeOH) 289 nm, (PBS, pH 7.4) 291 nm. IR (KBr) cm^{-1} : 3327.55 N-H stretching of amide, 3125.86 aromatic C-H stretching, 1693.60 C=O stretching of esters, 1237.62 C-O stretching ester, 1448.12 C-N stretching. ^{1}H NMR (500 MHz, DMSO): δ 2.08 (s, 3H, ArCH3), δ 2.28 (s, 3H, ArCH3), δ 3.77 (m, 3H, OCH$_3$), δ 3.41 (d, 2H, -CH$_2$-), δ 5.06-5.16 (m, 2H, =CH$_2$), δ 5.98-6.03 (m, 1H, -CH=), δ 6.69-6.81 (m, 3H, Ar-H), δ 6.83-67.07 (m, 4H, Ar-H), δ 7.16-8.11 (m, 3H, Ar-H), δ 9.04 (s, 1H, -NH-). Mass: (70 eV) m/z 387

4-formyl-2-methoxyphenyl-2-[(2,3-dimethylphenyl)amino]benzoate.

The percentage yield of MAVAN was found to 71%. UV (λ_{max}): (MeOH) 286 nm, (PBS, pH 7.4) 294 nm. IR (KBr) cm^{-1} : 3348.97 N-H stretching of amide, 3124.32 aromatic C-H stretching, 1697.74 C=O stretching of esters, 1237.49 C-O stretching ester, 1456.06 C-N stretching. ^{1}H NMR (500 MHz, DMSO): δ 2.08 (s, 3H, ArCH3), δ 2.28 (s, 3H, ArCH3), δ 3.98 (m, 3H, OCH$_3$), δ 6.70-7.08 (m, 3H, Ar-H), δ 7.14-7.56 (m, 4H, Ar-H), δ 7.64-8.11 (m, 3H, Ar-H), δ 8.98 (s, 1H, -NH-), δ 10.02 (s, 1H, -CHO). Mass: (70 eV) m/z 375

Figure 1: Scheme of Synthesis of mefenamic acid ester prodrugs. DCM- dichloromethane, DCC- N, N'- dicyclohexylcarbodimide, DMAP- dimethylaminopyridine. MAEU- mefenamic acid eugenol ester, MAVAN-

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mefenamic acid vanillin ester.

Mefenamic acid   Mefenamic acid Esters

MAEU   MAVAN
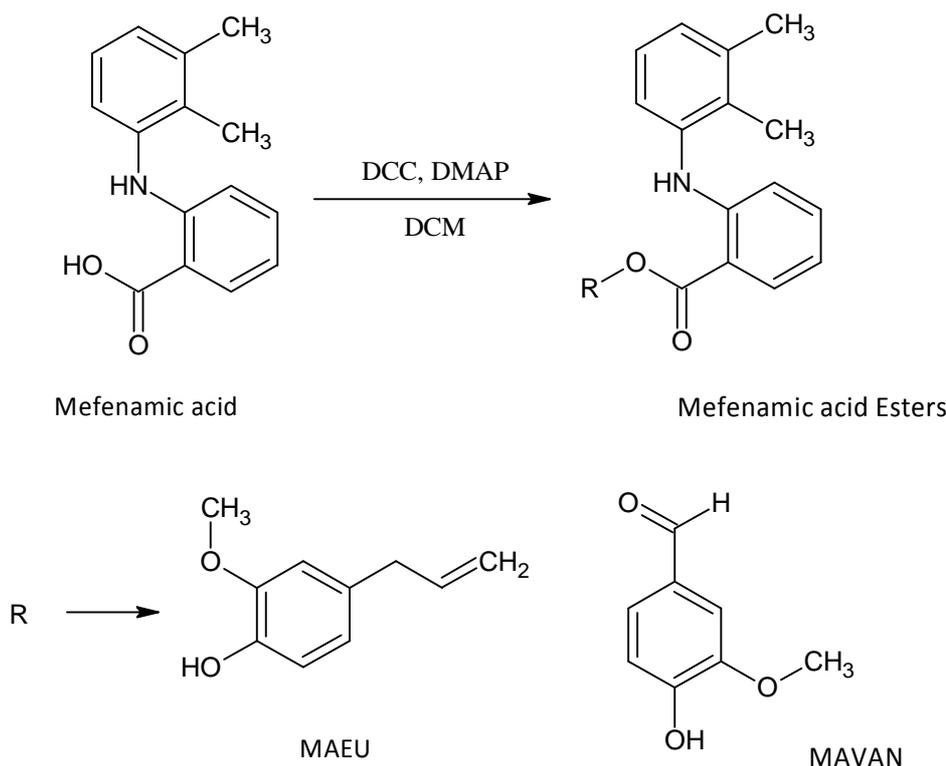
Characterization of synthesized prodrug
Solubility
10 mg of synthesized prodrug (MAEU and MAVAN) were dissolved in 0.5 ml of each solvent in test tubes. After gentle shaking solubility was observed. Further 0.5 ml solvent was added in case compound was insoluble, till the compound was completely dissolved.

Partition coefficient [26]
The partition coefficients of synthesized prodrugs (MAEU and MAVAN) were determined in octanol-phosphate buffer (pH 7.4). Prodrugs, 100 mg, were added to 10 ml of aqueous phase followed by addition of 10 ml of n-octanol. The contents were thoroughly shaken for 2 hrs at room temperature and left for 1hr. Layers were separated out using separating funnel. The concentration in aqueous and organic phase was determined by using HPLC and partition coefficient was calculated as the ratio of concentration of drug in organic phase to the concentration of drug in aqueous phase.

***In vitro* hydrolysis [27]**
The hydrolytic stability of synthesized prodrugs was studied in simulated gastric fluid (SGF) at pH 1.2 and simulated intestinal fluid (SIF) at pH 7.4. Solutions of 10 mg of prodrug prepared in 90 mL of SGF (pH 1.2) or SIF (pH 7.4) were kept in screw capped tubes maintained at 37 ± 0.5 °C. At definite time interval (15, 30, 60, 120, 240 min), aliquots were withdrawn from tubes and analyzed by HPLC method for the amount of drug released after the hydrolysis of prodrugs. Pseudo first order rate constants (K_{obs}) for the individual reactions were calculated with equation, $K_{obs}=2.303/t \times \log (a/a-x)$, where 'a' is initial concentration of prodrug, 'x' is the amount of prodrug hydrolyzed and 't' is time in minutes. The corresponding half life ($t_{1/2}$) was then obtained from the equation: $t_{1/2} = 0.693/ K_{obs}$

Pharmacological evaluations
Mefenamic acid and synthesized prodrugs (MAEU and MAVAN) were evaluated for analgesic, anti-inflammatory and ulcerogenic activity. The prodrugs were compared with mefenamic acid for these activities. Animals were procured from animal house of the institute and the study protocol was approved by Institutional Animal Ethics Committee (SND/IAEC/2013-14/14).

Anti-inflammatory activity [28]

The anti-inflammatory activity of mefenamic acid and prodrugs determined by the hind paw edema method utilizing carrageenean (0.1 ml 1%) as phlogistic agent. Wistar rats (albino rats) of either sex weighing 100 to 200 g were divided into four groups, each comprising of six rats, including a control and standard group. The initial volume of right hind paw was measured using a plethysmometer without administration of drug/prodrug. The Mefenamic acid (MA standard, 80 mg/kg), prodrugs MAEU (135 mg/kg) and MAVAN (130 mg/kg) was administered orally in a 1 % suspension of sodium carboxymethylcellulose. Control animals were given the corresponding amount of vehicle. After 30 min of drug/prodrug administration, the carrageenean (0.1 mL, 1 %) solution in normal saline was injected into the sub planter region of the left hind paw and edema volume was measured before injection and at the interval of every hour upto 6 h.

Analgesic activity [29]

Analgesic activity was carried out using the acetic acid induced writhing method in albino mice of swiss strain. A 1% v/v solution of acetic acid was used to induce writhings. Mice were divided into 5 groups of 6 animals each. Group I served as a control group, group II received standard drug, groups III and group IV received test drugs. The drug/prodrug (dose of prodrug molecularly equivalent to mefenamic acid) was administered orally in a 1 % suspension of sodium carboxymethylcellulose. Drugs were administered as a homogenous suspension in an aqueous solution of sodium CMC (0.5%w/v) orally. Acetic acid was administered intraperitoneally at 1 mL/100g body weight of the animal. Test compounds were administered orally 3 h prior to acetic acid injection. The number of writhings in 10 min with the control and test compounds were counted and compared. Analgesic activity was measured as percentage decrease in writhing in comparison to control.

Ulcerogenic study [30]

Gastrointestinal toxicity of the synthesized prodrugs was measured and compared with the parent drug by measuring the ulcer index. For this purpose rats were divided in four groups of six animals each and fasted for 24 h prior to administration of drug/prodrug. The Mefenamic acid (standard, 800 mg/kg), prodrugs MAEU (1350 mg/kg) and MAVAN (1300 mg/kg) was administered orally as suspension in 0.5 % acacia. The control group was administered only a 0.5 % acacia suspension. Animals were sacrificed 12 h after the treatment. The stomach was removed, opened along the curvature, rinsed with 5 mL saline and was examined by means of magnifier. The ulcer index was calculated as mean for all animals in group.

Statistical Analysis

Statistical analysis was carried out for pharmacological evaluation data using analysis of variance (ANOVA) test, followed by Dunnet's Test for determining level of significance. P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The prodrugs mefenamic acid-eugenol ester (MAEU) and mefenamic acid-vanillin ester (MAVAN) were synthesized by using N, N'- dicyclohexyl carbodimide (DCC). Purity of synthesized prodrug was ascertained by melting point and thin layer chromatography (TLC). The synthesized compounds were confirmed by FTIR, ^{1}HNMR and Mass spectroscopy. Infrared spectra of products shows the characteristics band at 1693.60 (C=O, ester) for MAEU and at 1697.74 (C=O, ester) for MAVAN. The C-O (ester) stretching is also observed at 1237.62 and 1237.49 for MAEU and MAVAN respectively. The ^{1}H NMR spectrum of synthesized prodrugs shows the chemical shift value for the anticipated compounds. The mass spectroscopic analysis gives the parent peak confirming molecular weight of the targeted compounds.

The synthesized ester prodrugs of mefenamic acid (MAEU and MAVAN) were subjected to solubility studies. It was observed that mefenamic acid was highly soluble in 0.1 N sodium hydroxide solution. Prodrugs were found very slightly soluble in 0.1 N NaOH. All the prodrugs showed increased solubility then drug in organic solvents such as methanol, ethanol, chloroform and dichloromethane which indicate lipophilic behavior of the prodrugs. The Partition coefficient of mefenamic acid and prodrugs were found in octanol-aqueous buffer (pH 7.4) system. The result indicates that synthesized esters were found to be more lipophilic than parent drug, mefenamic acid. (Table 1)

Table 1: Physical constants and physicochemical characteristics of mutual prodrugs

| Prodrug	Molecular Formula	Colour	Yield (%)	Melting point (ºC)[a]	Rf Value[*]	Partition coefficient
MA-EU	$C_{25}H_{25}NO_3$	Creamy white	59	73-74	0.48	6.12
MA-VAN	$C_{23}H_{21}NO_4$	Yellow	66	119-120	0.63	5.17

[a]Uncorrected; [*]TLC (n-hexane: methanol, 3:1)

Kinetics of prodrug hydrolysis was studied in aqueous buffer solution at pH 1.2 and pH 7.4. The decrease in concentration of ester was monitored by HPLC. The result shows longer half life of prodrugs in acidic pH 1.2 compared to pH 7.4, which implies it may pass unhydrolyzed through stomach and posses enough stability to be absorbed from intestine. The values of the rate parameters K_{obs} for hydrolysis of prodrugs at different pH and 37° C are listed in Table 2 along with the half-lives ($t_{1/2}$). The plot of log concentration of residual prodrug v/s time (Figure 2 and 3) obeys first order kinetics and a straight plot was obtained.

Table 2 Kinetic data for the hydrolysis at different pH at 37°C

pH	MAEU		MAVAN	
	K_{obs}	$t_{1/2}$(h)	K_{obs}	$t_{1/2}$(h)
1.2	6.261×10^{-4}	18.44	7.058×10^{-4}	16.36
7.4	1.524×10^{-3}	7.57	1.865×10^{-3}	6.19

Figure 2: First order hydrolysis plot of mefenamic acid prodrugs in phosphate buffer pH 7.4
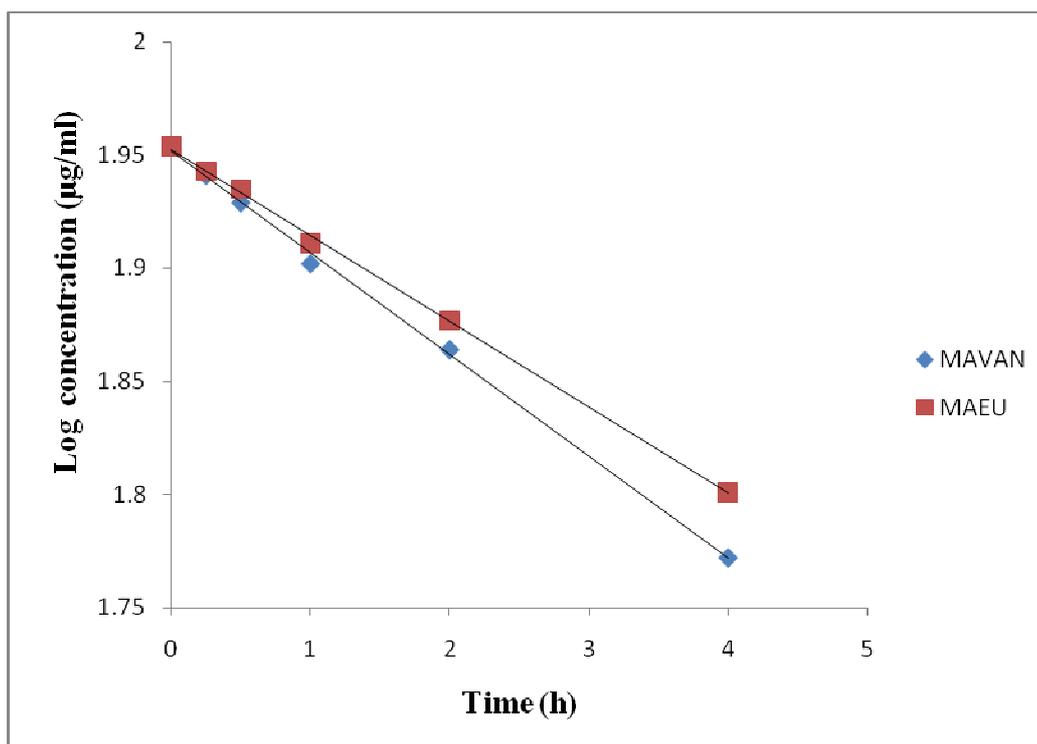
Synthesized prodrugs were evaluated for anti-inflammatory, analgesic and ulcerogenic activity. Antiinflammatory activity was determined by using Carrageenan induced rat paw edema model. The prodrugs (in molecularly equivalent dose) showed comparable inhibition of carrageenan induced inflammation (Table 3). Statistical significance testing using one way analysis followed by Dunnet's Test indicated that prodrugs have comparable activity to parent drugs. For analgesic activity, the abdominal writhing method was used. The decrease in number of writhings was expressed as percentage protection by test compounds with reference to control for analgesic activity. Prodrugs showed considerable retention of analgesic activity. (Table 4) MAEU showed higher activity compared to MAVAN. The synthesized prodrugs showed lower ulcer index value of 5.83 and 6.34 for MAEU and MAVAN as compared to 9.67 for Mefenamic Acid thus indicating decrease in gastrointestinal sideeffects through successful masking of free carboxylic group of drug (Table 4).

Table 3: Anti-inflammatory activity of mutual prodrugs of mefenamic acid

Group	Difference in paw volume (mean ± SD)[a]				% Inhibition			
	3 h	4 h	6 h	24 h	3 h	4 h	6 h	24 h
Vehicle	1.61 ± 0.042	1.33 ± 0.120	1.21 ± 0.029	1.01 ± 0.068	-	-	-	-
MA	0.80 ± 0.016	0.68 ± 0.018	0.45 ± 0.030	0.31 ± 0.014	50.31	48.61	62.48	69.45
MA-EU	0.86 ± 0.031	0.73 ± 0.022	0.53 ± 0.028	0.37 ± 0.026	46.65	45.10	55.81	63.37
MA-VAN	0.90 ± 0.256	0.84 ± 0.025	0.60 ± 0.021	0.41 ± 0.016	44..16	36.98	50.04	58.94

[a] Statistical analysis was performed with ANOVA followed by dunnett test P < 0.05 with respect to control

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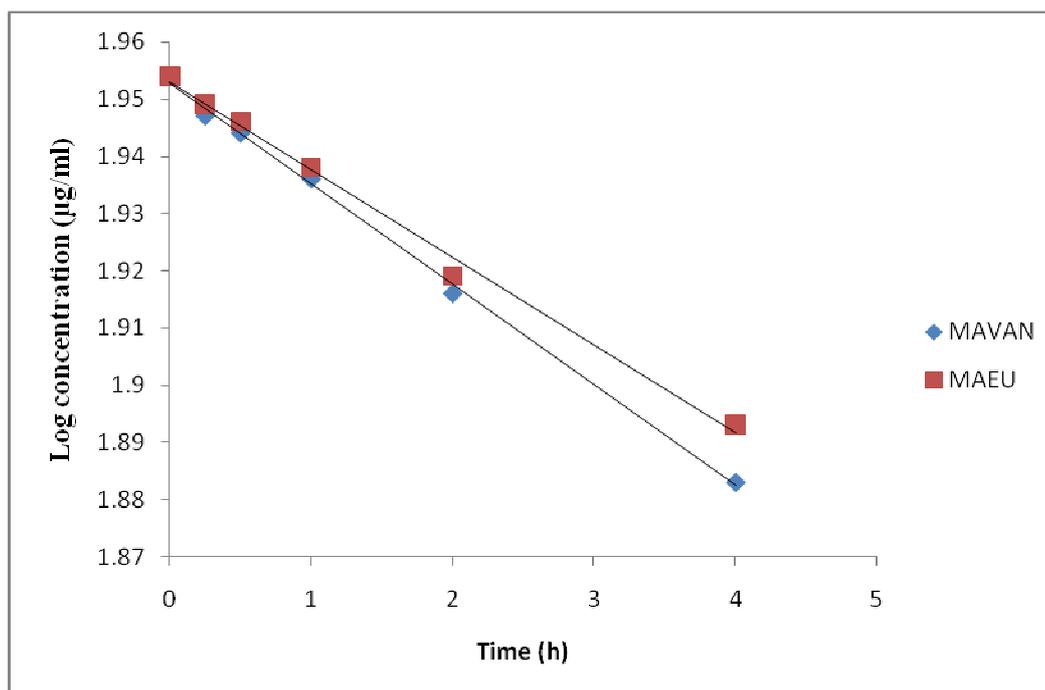**Figure 3: First order hydrolysis plot of mefenamic acid prodrugs in Hydrochloric acid buffer pH 1.2**

Table 4: Analgesic and ulcerogenic activity of mutual prodrugs of mefenamic acid

Group	Number of writhing	% Inhibition in writhing	Ulcer Index[a] (± SEM)
Vehicle (Control)	12.50 ± 1.378	-	2.59 ± 0.42
MA	5.167 ± 1.169	58.72	9.67 ± 0.55
MA-EU	6.617 ± 0.758	50.72	5.83 ± 0.60
MA-VAN	6.667 ± 1.033	46.72	6.34 ± 0.49

[a] *Statistical analysis was performed with ANOVA followed by dunnett test $P < 0.05$ with respect to control*

CONCLUSION

In present study mefenamic acid ester prodrugs with eugenol and vanillin were synthesized by dicyclohexylcarbodimide coupling and characterized by Melting Point, Thin Layer Chromatography, Fourier Transform Infra Red Spectroscopy, Proton Nuclear Magnetic Resonance Spectroscopy and Mass Spectroscopy to confirm its structure. Prodrug showed improved solubility in organic solvents which implies lipophilic character of ester prodrugs. The prodrugs were found chemically stable and biolabile. The mefenamic acid ester prodrugs showed comparable analgesic and anti-inflammatory activities with reduced ulcerogenicty. Retention of activity along with reduction in ulcerogenicty may be due to analgesic properties of eugenol [31] and prevention of direct contact of carboxylic group with gastric mucosa.

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