European Journal of Pharmaceutical Sciences 43 (2011) 99–108

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Synthesis, characterization and in vitro hydrolysis of a gemfibrozil-nicotinic acid codrug for improvement of lipid profile

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ARTICLE INFO

Article history:
Received 3 November 2010
Received in revised form 8 March 2011
Accepted 28 March 2011
Available online 3 April 2011

Keywords:
Codrug
Hyperlipidemia
Gemfibrozil
Nicotinic acid
Hydrolysis
Arrhenius equation

ABSTRACT

Combination therapy of fibrates and nicotinic acid has been reported to be synergistic. Herein, we describe a covalent codrug of gemfibrozil (GEM) and nicotinic acid (NA) that was synthesized and characterized by ^{1}H NMR, ^{13}C NMR, FT-IR, MS analysis and elemental analysis. A validated HPLC method was developed that allows for the accurate quantitative determination of the codrug and its hydrolytic products that are formed during the in vitro chemical and enzymatic hydrolysis. The physico-chemical properties of codrug were improved compared to its parent drugs in term of water solubility and partition coefficient. The kinetics of hydrolysis of the codrug was studied using accelerated hydrolysis experiments at high temperatures in aqueous phosphate buffer solution in pH 1.2, 6.8 and 7.4. Using the Arrhenius equation, the extrapolated half-life at 37 °C were 289 days at pH 1.2 for the codrug and 130 and 20,315 days at pH 6.8 for the codrug and gemfibrozil 2-hydroxyethyl ester (GHEE), respectively. The shortest half-lives were at pH 7.4; 42 days for the codrug and 5837 days for GHEE, respectively. The hydrolysis of the latter was studied, alone, at 80 °C and pH 1.2 and compared to its hydrolysis when it is produced from the codrug using similar conditions. The k_{obs} was found in both cases to be 1.60×10^{-3} h^{-1}. The half-lives in plasma were 35.24 min and 26.75 h for the codrug and GHEE, respectively. With regard to liver homogenate, the hydrolysis half-lives were 1.96 min and 48.13 min for the codrug and GHEE, respectively. It can be expected that in vivo, the codrug will liberate NA immediately in plasma then GEM will be liberated from its 2-hydroxyethyl ester in the liver.

1. Introduction

Combined hyperlipidemia is typically characterized by elevations in total cholesterol and triglycerides with decreased high-density lipoprotein cholesterol (HDL-C). Large-scale population studies have demonstrated that HDL-Cholesterol (HDL-C) is a strong and independent inverse predictor of coronary heart disease (CHD), even in subjects with normal low density lipoprotein LDL-Cholesterol (LDL-C) levels (Birjmohun et al., 2005). Achieving recommended cholesterol and triglyceride targets is difficult and frequently requires the use of more than one lipid-lowering medication (Taher et al., 2002). Unfortunately, no single agent or class of agents is able to correct all of the lipoprotein disorders (Steiner, 2005). Statins, fibrates, ezetimibe and nicotinic acid exert their effects via different mechanisms and impact multiple pathways in patients. When used alone, or in combination, these drugs decrease the risk for the development and progression of atherosclerotic disease (Davidson and Toth, 2004). Gemfibrozil, a fibrate derivative, can lower plasma triglyceride-rich lipoproteins mainly very low density lipoproteins VLDL (Todd and Ward, 1988; Zovko et al., 2005) and can increase HDL in a variety of primary and secondary (e.g., diabetes mellitus, renal disease) dyslipoproteinemias including endogenous hypertriglyceridemia, dysbetalipoproteinemia and combined hyperlipidemia (Kashyap, 1984; Manninen et al., 1982). It has been shown that an increase of 6% in HDL-C levels in the group receiving gemfibrozil was associated with a 22% decrease in the incidence of fatal and non-fatal myocardial infarction and coronary heart disease (CHD) mortality rates, although LDL-C levels in the two groups (treated with gemfibrozil and placebo) were similar (Dean et al., 2004; Manninen et al., 1998). On the other range of the spectrum there is nicotinic acid (Niacin, Vitamin B_3), which, as a vitamin, serves as a precursor for two essential coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). While niacin as a vitamin is potent in milligram doses, at gram doses it can act as a broad-spectrum lipid-regulating agent because of its diverse effects on the lipid profile (Carlson, 2005; Ganji et al., 2003). The HDL-raising properties of nicotinic acid occur with doses as low as 1–1.5 g/day whereas, the VLDL- and LDL-lowering effects are typically seen with higher doses (3 g/day) (Samson, 2002). Nicotinic acid is rapidly absorbed with an oral bioavailability of

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doi:10.1016/j.ejps.2011.03.012

60–70% (Carlson, 2004). These high doses of niacin are associated with various side effects the most common of which is cutaneous flushing (Davidson, 2008; Meyers et al., 2007; Raza et al., 2004; Taylor and Stanek, 2008). It has been reported that the combination of gemfibrozil and nicotinic acid was significantly superior to monotherapy in increasing the levels of HDL-C (Zema, 2010). In addition, it has been shown that a combination of a fibric acid derivative and nicotinic acid offered greater improvement in lipoprotein subclass distribution and apolipoprotein ratios than nicotinic acid monotherapy (Superko et al., 2009). It is worth mentioning that the combination of gemfibrozil and nicotinic acid lead to reduction in coronary artery disease (CAD) events (Gurakar et al., 1985; Link et al., 2007; Ramires et al., 1995; Superko et al., 2009, 2006; Zema, 2010).

The implementation of the prodrug approach in the early stages of drug discovery is a growing trend as about 5–7% of drugs approved worldwide can be classified as prodrugs (Rautio et al., 2008). A codrug or a mutual prodrug consists of two synergistic drugs chemically linked together (Cynkowska et al., 2005; Dhaneshwar et al., 2007; Hamad et al., 2006; Kiptoo et al., 2006, 2008). In this scenario, the therapeutic agents act as promoieties for each other. The active moiety selected may have the same biological action as that of the parent drug and thus might give synergistic action, or this moiety may have some additional biological action that is lacking in the parent drug, thus ensuring some additional benefit. The active moiety may also be a drug that might help to target the parent drug to a specific site or organ or cells or may improve site specificity of a drug and may be used to overcome some side effects of the parent drugs as well (Abdel-Azeem et al., 2009). The codrug approach is of a great interest, because combination therapy is used for the management of many diseases where therapeutic agents can be co-administered in separate dosage forms, however, there are potential advantages in delivering co-administered agents as a single chemical entity using this approach such as improved delivery, improved pharmacokinetic properties, improved targeting to specific sites of action, in addition to improved patient compliance (Hamad et al., 2006). For example, Etofibrate, Fig. 1, shows a codrug of clofibric acid with niacin (Sposito et al., 2001). Pharmacologically, etofibrate can decrease the circulating levels of triacylglycerols and cholesterol (Herrera et al., 1988). Unfortunately, etofibrate has seen limited clinical use because of reports that, in rodents, clofibric acid can cause an increase in malignancies and produce peroxisome proliferation and hepatomegaly. In addition, clofibrate has been shown to increase the risk of gallbladder disease and has been associated with an increased incidence of non-coronary mortality in humans.

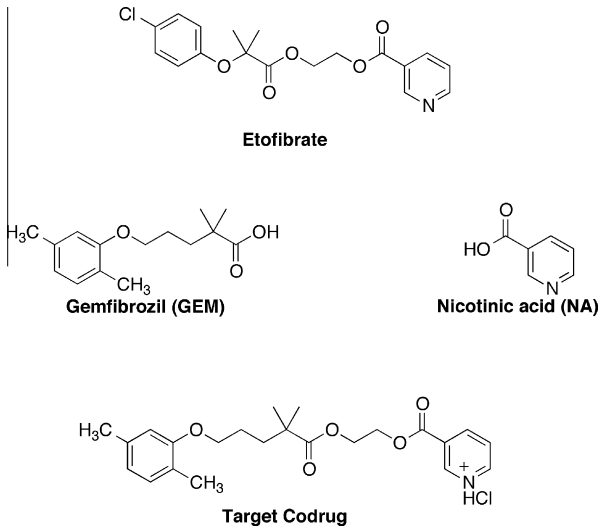


Fig. 1. Chemical structure of the traget codrug.

This work reports a codrug or a mutual prodrug of gemfibrozil (GEM) and nicotinic acid (NA) as shown in Fig. 1. These drugs possess complementary pharmacological activities and the combination is expected to offers greater improvement in detailed lipoprotein subclass distribution and apolipoprotein ratios than monotherapy. Moreover, among clinically used fibrates, gemfibrozil (GEM) is the most widely used and appears to have a lower tendency for causing many of the observed side effects of fibrates (Steiner, 2005; Thomas et al., 1999). Although it is difficult to predict a dose for the target codrug which contains equalimlolar amounts of GEM and NA, the case of etofibrate (which is a similar equimolar codrug of clofibric acid and NA) providse a promising premise. The typical dose of etofibrate is 500 mg/day (Martindale: The Complete Drug Reference, 2010b) despite the fact that the dose of clofibrate, when administered alone, is 0.75–1.50 g/day (Martindale: The Complete Drug Reference, 2010a) which is not far from the dose of GEM which is 1.20 g/day (Martindale: The Complete Drug Reference, 2010c). This reduction in dose might be due to the synergistic action of fibrates and NA or to the fact that the codrug will have a different pharmacokinetic profile than the individual agents. In addition, both nicotinic acid (NA) and gemfibrozil (GEM) suffer from problems in elimination, bioavailability and half-life, that the codrug might be able to address. Herein, the synthesis and characterization of the codrug will be detailed. In addition, its physicochemical properties; i.e. aqueous solubility and partition coefficient will be determined. Finally, the effect of pH and temperature on the stability of the codrug as well as its enzymatic stability will be reported.

2. Materials and methods

2.1. Chemicals and reagents

Gemfibrozil (GEM) was a generous gift from the United Pharmaceuticals, Amman, Jordan. Nicotinoyl chloride hydrochloride (97%) and 2-bromoethanol (95%) were obtained from Aldrich Chemical Company (USA). Other chemicals and HPLC solvents used were obtained form either Fluka chemical (UK), Biosolve (Netherlands, Holland), TEDIA (USA), Frutarom LTD (UK), Riedel-de Haen (Germany), SupraSolv, Merch (Germany), Scharlau Chemie (Barcelona, Spain), Carlo Erba reagents (France), GCC Biotech (UK) and Sure Chem products (UK).

2.2. Chemistry

2.2.1. 2-Hydroxyethyl 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoate (Gemfibrozil 2-hydroxyethyl ester, GHEE)

Potassium carbonate (8.28 g, 59.90 mmol) was added to a stirring solution of GEM (10.00 g, 39.90 mmol) in acetonitrile (750 mL) at reflux. To the mixture, 2-bromoethanol (7.49 g, 59.90 mmol) was added drop wise. TLC (50% ethyl acetate in hexane) showed a complete disappearance of starting material after 24 h. The mixture was filtered and the filtrate was evaporated to obtain an oily residue. The residue was re-dissolved in ethyl acetate (400 mL) and washed with cold 1 N sodium hydroxide solution (300 mL × 3) and the organic layer was then dried over $MgSO_4$ and the solvent was evaporated to afford a colorless oil (10.63 g, yield 90.54%) which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.00 (1H, *d*, *J* = 7.5 Hz, Ar-*H*), 6.66 (1H, *d*, *J* = 7.5 Hz, Ar-*H*), 6.62 (1H, *s*, Ar-*H*), 4.19 (2H, *m*, O–CH_2–CH_2–O–), 3.91 (2H, m, O–CH_2–CH_2–O–), 3.78 (2H, m, Ar–O–CH_2–), 2.48 (1H, s, OH), 2.30 (3H, s, Ar–CH_3), 2.18 (3H, s, Ar–CH_3), 1.76 (4H, s, –CH_2–CH_2–CH_2–), 1.24 (6H, s, $(CH_3)_2$). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 160.99, 159.59, 139.20, 133.06, 126.30, 123.59, 114.94, 70.72, 68.77, 63.91, 44.93, 39.80, 24.10 and 18.50. IR

(KBr): 3442.94 (OH), 1729.29 (C=O), 1614.42 and 1585.49 (Ar C=C), 1265.30 and 1193.94 (C–O), 804.32 (Ar=C–H). LC–MS (ESI) *m*/*z*: MH + (294.9, 100.0%), MH + 1 (296.1, 21.24%), MH + 2 (297.0, 1.43%).

2.2.2. 2-(5-(2,5-Dimethylphenoxy)-2,2-dimethylpentanoyloxy)ethyl nicotinate (codrug)

GHEE (10.00 g, 34.00 mmol) was dissolved in dichloromethane (500 mL) and to it, nicotinoyl chloride hydrochloride (6.06 g, 34.00 mmol) was added at room temperature. To the mixture, triethylamine (6.88 g, 68.00 mmol) in dichloromethane (100 mL) was added drop wise. After, the addition was complete; it was left to stir for 3 days at room temperature. The reaction progress was followed up by TLC (20% ethyl acetate in hexane in an ammonia atmosphere). Upon reaction completion, the solution was washed with distilled water once (500 mL) then by cold 1 N NaOH (300 mL × 3). The organic layer was then dried over anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure to yield an oily residue. The oily residue was converted to hydrochloride salt by dissolving it in methanolic HCl. The resulting solution containing the salt was evaporated and the residue was by crystallization from ethyl acetate and ether to afford 8.10 g (54.40%) of white crystals. m.p.: 102–105 °C, ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 9.21 (1H, *d*, *J* = 1.7 Hz, Py-H), 8.99 (1H, *dd*, *J* = 5.7, 1.0 Hz, Py-H), 8.79 (1H, *dt*, *J* = 8.1, 1.7, 1.0 Hz, Py-H), 7.87 (dd, *J* = 8.1, 5.7 Hz, Py-H), 6.85 (1H, *d*, *J* = 7.5 Hz, Ar-H), 6.54 (1H, *d*, *J* = 7.5 Hz, Ar-H), 6.44 (1H, *s*, Ar-H), 4.58 (2H, m, O–CH_2–CH_2–O–), 4.37 (2H, m, O–CH_2–CH_2–O–), 3.75 (2H, m, Ar–O–CH_2), 2.20 (3H, s, Ar–CH_3), 1.99 (3H, s, Ar–CH_3), 1.62 (4H, s, CH_2–CH_2–CH_2-), 1.13 (6H, s, –CH_{32}). ^{13}C NMR (CDCl3, 100 MHz): δ = 177.60, 161.29, 156.77, 145.56, 144.50, 142.11, 136.75, 130.45, 129.47, 127.44, 123.40, 120.98, 112.14, 67.85, 65.05, 61.69, 42.27, 36.89, 25.32, 25.24, 21.52, 15.82. Elemental analysis: calculated % (C: 63.37, H: 6.94, N: 3.21); found % (C: 64.225, H: 7.089, N: 3.669). IR (KBr): 1729.29 (C=O), 1290.38 and 1265.30 (C–O), 804.46 (Ar=C–H), 742.59–800.46 (Ar=C–H). LC–MS (ESI) *m*/*z*: MH+ (437.1, 100.0%), MH + 1 (438.1, 8.81%), MH + 2 (439.0, 2.40%).

2.2.3. Nicotinic acid 2-hydroxyethyl ester (NAHEE)

A solution of ethylene glycol (11.13 g, 17.90 mmol) in dichloromethane (500 mL) and triethylamine (7.25 g, 71.70 mmol) was stirred at room temperature and to it nicotinoyl chloride hydrochloride (6.39 g, 35.80 mmol) was added in several portions. TLC (20% ethyl acetate in hexane in ammonia atmosphere) showed completion of the reaction after 2 h. Upon completion of the reaction, the solution was washed with distilled water once (400 mL) and then with cold 1 N NaOH (300 mL × 3) then dried over anhydrous $MgSO_4$ and evaporated. The product was treated with methanolic HCl to convert it to a hydrochloride salt, then, the solution was evaporated and the residue was crystallized from ethanol to afford 0.35 g (4.82%) of white crystals. m.p.: 217–219 °C. ^{1}H NMR (H_2O + D_2O, 400 MHz): δ = 9.24 (1H, *s*, Py-H), 8.98 (1H, *d*, *J* = 8.2 Hz, Py-H), 8.87 (1H, *d*, *J* = 5.6 Hz, Py-H), 8.08 (1H, *dd*, *J* = 8.2, 5.6 Hz, Py-H), 4.65 (4H, s, O–CH_2–CH_2–O). Elemental analysis: calculated % (C: 47.19, H: 4.95, N: 6.88); found % (C: 47.963, H: 4.331, N: 8.777). IR (KBr): 1724.36 (C=O), 1313.52 and 1286.52 (C–O) *m*/*z*: MH + 1 (205.2, 100.0%).

2.3. Analysis of the codrug

2.3.1. Method of analysis of codrug and degradation products

The quantitative analysis and separation of the codrug and its possible degradation products were performed using high performance liquid chromatography (HPLC). The analytical (HPLC) system consisted of a SCL-10A *VP* system controller, FRC-10A fraction collector, LC-10 AD *VP* liquid chromatography pump, DGV-12A *VP* degasser, SIL-10AD *VP* auto injector, SPD-10AV *VP* UV–visible detector and was connected to computer furnished with the appropriate software Class *VP* (Shimadzu, USA). The chromatographic separation was carried out under isocratic reversed-phase conditions using a Thermo RP-18C column (250 mm × 4.6 mm, 5 μm) (UK). The injection volume was 20 μL and the detection wavelength was 270 nm. The mobile phase was a mixture of 0.02 M phosphate buffer pH 5.75 and acetonitrile (70:30 v/v) to obtain a final pH 6.8. The mobile phase was filtered through 0.45 μm membrane filters. In the same chromatographic run, GEM, GHEE, the codrug, and NAHEE were detected with different retention times, Fig. 1. NA itself is much more polar than the other compounds thus it was masked (elute together) in the solvent front/void volume.

2.3.2. Preparation of standard solutions

Stock solutions for the GEM, GHEE and codrug were prepared by dissolving 100.00 mg of each compound in 100 ml of acetonitrile in a volumetric flask to have a final concentration equal to 1000 μg/mL. Standard solutions were prepared by serial dilution of these stock solutions with acetonitrile to obtain standard concentrations ranging from 0.1 to 200 μg/mL. Each standard concentration was injected in triplicate into the HPLC system. The calibration curves were constructed by plotting the area under the curve for each standard solution versus concentration.

2.3.3. Method validation

The HPLC method was validated for the quantitation of GEM, GHEE and codrug in terms of linearity, accuracy, precision, selectivity, recovery, limit of detection (LOD) and limit of quantitation (LOQ). Linearity was performed by triplicate injection of nine freshly prepared standard concentrations corresponding to each point in the calibration curve. Accuracy and precision were carried out by injecting freshly prepared control solutions of concentrations 7, 80, 160 μg/mL six times per day for three consecutive days. Signal to noise ratio was used to detect LOD and LOQ. Recovery of GEM, GHEE and codrug was calculated by comparing the theoretical concentration to the concentration that was recovered after spiking samples of each of the compounds in plasma and liver homogenate at concentration levels of 10 and 40 μg/mL.

2.3.4. Physicochemical properties of the codrug

2.3.4.1. Aqueous and organic solubility. The aqueous solubility of the codrug was determined at 25 °C in aqueous 0.05 M phosphate buffer solution at pH 1.2, pH 6.8 and pH 7.4 as well as in 0.1 N HCl. Excess amounts of the codrug were added to 1.00 mL of each of the previously mentioned aqueous or organic media. The vessels were kept in a temperature controlled shaking water bath at 25 °C for 24 h then the saturated solutions were filtered using Millipore filters 0.45 μm and the concentration of each compound was determined by HPLC system. Additionally GEM aqueous solubility was determined in the same manner as the codrug at pH 6.8, pH 7.4 and distilled water at 25 °C for 24 h.

2.3.4.2. Apparent partition coefficient and pK_a. The apparent partition coefficient expressed as log P_{app} of the codrug was determined at 25 °C between *n*-octanol and 0.05 M aqueous phosphate buffer solution at pH 1.2, 6.8 and pH 7.4, as well as 0.1 M HCl. *n*-Octanol was firstly saturated with each of the aqueous solutions by vigorous stirring using magnetic stirrer for 24 h.

Log P_{app} was measured by adding 5 mg of the codrug in 10 mL of pre-saturated *n*-octanol/aqueous media in a glass screw-capped test tube. The test tube was shaken at 25 °C in a water bath for 12 h. The layers were separated by centrifugation (5000 rpm at 25 °C) for 5 min. The aqueous layer was sampled using a syringe with a removable needle, which was partially filled with air that was gently expelled during the passage through *n*-octanol layer,

and an adequate volume of aqueous layer was withdrawn into the syringe, which was quickly removed, from the solution and the needle detached. The concentration of the codrug in the aqueous and organic layers was analyzed by HPLC. The experiments were performed in triplicate. The apparent partition coefficient was calculated by dividing the concentration of codrug in the *n*-octanol layer by its concentration in the aqueous phase.

2.3.5. Chemical hydrolysis

The rates of the chemical hydrolysis of the codrug were studied in 22% acetonitrile in aqueous phosphate buffer solutions of pH values 1.2, 6.8, 7.4 and 9. All aqueous phosphate buffers were prepared at a concentration of 0.05 M and their ionic strength was adjusted to 0.15.

The reactions were initiated by adding 22 ml of 400 μg/mL (0.913 mM) codrug stock solution in acetonitrile to 100 mL volumetric flask and preheated phosphate buffer was added to complete the volume to 100 mL resulting in a final codrug concentration of 88 μg/mL (0.2 mM). The solutions were placed in screw capped test tubes and kept in a temperature-controlled shaker water baths at 60, 70, 80 °C at pH 1.2, 6.8 and 7.4 and at 90 °C at pH 6.8 and 7.4. Hydrolysis of the codrug at pH 9 was only studied at 80 °C. At appropriate time intervals, depending on the stability of the codrug at each temperature and pH, 1 mL samples were withdrawn cooled with iced water and immediately analyzed for remaining codrug, the levels of GHEE and appearance of GEM by HPLC. All experiments were carried out in triplicate. Additionally, the stability of GHEE was studied separately in 0.05 M phosphate buffer of pH 1.2 at 80 °C in the same manner as the codrug by using 22% acetonitrile as cosolvent.

The rate constants of the degradation of the hydrolysis of the codrug at selected temperatures for pH 1.2, 6.8 and 7.4 were obtained and then used to construct an Arrhenius plot where the activation energy was calculated from the slope of the linear plot of the natural logarithm (ln) of the rate constant versus the reciprocal of temperature to extrapolate the rate constants and half-lives at the human body temperature 37 °C and room temperature 25 °C.

2.3.6. In vitro enzymatic hydrolysis

The rates of enzymatic hydrolysis of the codrug and GHEE were assessed in human plasma and liver homogenate.

2.3.6.1. In vitro enzymatic hydrolysis in human plasma. The rates of the enzymatic hydrolysis of the codrug and GHEE were studied in 10% buffered human plasma at 37 °C. Pooled plasma solutions (4 mL) were prepared by centrifugation of citrated human venous blood and dilution of the resultant plasma supernatant with 0.05 M phosphate buffer of pH 7.4 followed by incubation at 37 °C in a shaking water bath.

Stock solutions of the codrug and GHEE were prepared by dissolving 5.50 mg of each compound in 1 mL methanol. The reactions were initiated by adding 80 μL of these stock solutions to 3.92 mL of plasma in a test tube kept in a shaking water bath at 37 ± 1 °C for 15 min. At appropriate time intervals, 150 μL aliquots were withdrawn and transferred to 1.5 mL Eppendorf tubes containing 450 μL methanol to quench the reaction and precipitate proteins, then the mixture was vortexed immediately and centrifuged at 13,000 rpm for 7 min at 4 °C, and the clear supernatant was then directly injected into the HPLC system and analyzed for remaining codrug and/or GHEE.

2.3.6.2. In vitro enzymatic hydrolysis in 20% liver homogenate. A volume equal to 3.92 mL of the supernatant from liver homogenate was placed in a test tube and incubated for 5 min in a shaking water bath equilibrated at 37 ± 1 °C. Then, 80 μL from codrug or GHEE solution (5.50 mg/mL in methanol) was mixed well with supernatant using vortex mixer to give initial concentration equal to 25 nM. At predetermined time intervals, 150 μL samples were withdrawn by micropipette and added to 450 μL methanol to quench the reaction and precipitate the proteins. The mixture was then centrifuged at 13,000 rpm for 7 min at 4 °C and the clear supernatants obtained was analyzed by HPLC.

3. Result and discussion

3.1. Chemistry

The synthetic scheme of the target codrug is depicted in Scheme 1. Synthesis of gemfibrozil 2-hydroxyethyl ester (GHHE) was accomplished by a simple nucleophilic substitution reaction in which gemfibrozil carboxylate anion, as the nucleophile, was reacted with the 2-bromoethanol as the electrophile. The reactions was conducted at reflux in acetonitrile. After appropriate workup, the GHHE was obtained in an excellent yield as an oil and its chemical structure was determined by ^{1}H NMR, ^{13}C NMR, FT-IR and MS analysis. To obtain the target codrug, a nucleophilic acyl substitution was effected in which the resultant 2-hydroxyethyl ester, the nucleophile, was coupled with nicotinoyl chloride, the electrophile, in the presence of a base. After the appropriate workup, the obtained residue was converted to the hydrochloride salt, which was purified by crystallization to afford white crystals of the codrug in moderate yields. The chemical structure of the salt was confirmed by ^{1}H NMR, ^{13}C NMR, FT-IR and MS analysis and its purity was determined by elemental analysis.

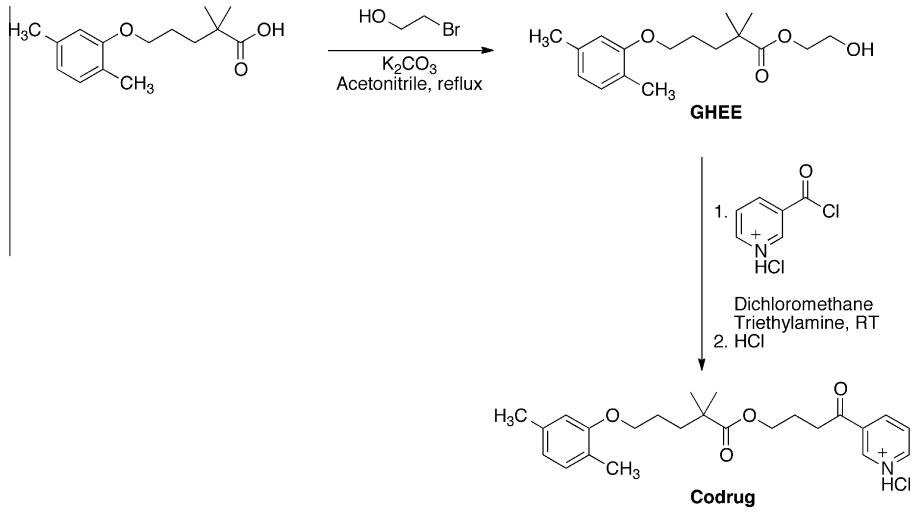
Nicotinic acid 2-hydroxyethyl ester (NAHEE) is, theoretically, a possible intermediate of the hydrolysis of the codrug. As seen Scheme 2, this ester was synthesized by adding nicotinoyl chloride hydrochloride to an excess of ethylene glycol in the presence of triethylamine as a base, Scheme 3. After the appropriate work-up, the product was converted to the hydrochloride salt and crystallized from ethanol. The chemical structure of the salt was confirmed by ^{1}H NMR, FT-IR and MS analysis and its purity was determined by elemental analysis.

3.2. HPLC method validation

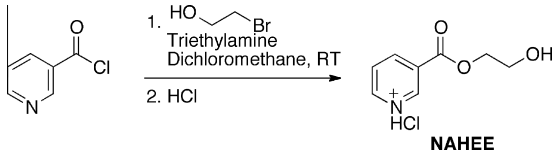
For the establishment of linearity, a minimum of 5 concentrations is recommended (International Conference on Harmonisation, 2005). Calibration curves were constructed for GHEE, codrug, and GEM by plotting concentration versus peak area of the standard solutions prepared in the range of 0.1–200 μg/mL. The coefficient of correlation of the linear regression equation R^2 for the calibration curves was calculated by the method of least square and all were in the order of 0.9992–0.9999 (Shabir, 2003).

Nicotinic acid 2-hydroxyethyl ester (NAHEE) was prepared as a control since it, theoretically, a possible hydrolytic product of the degradation of the synthesized codrug. Since it was not observed in any of the chemical hydrolyses experiments that were conducted, it is only included as a part of validation in terms of selectivity. There was no overlap between the codrug as indicated by retention time (rt), for the observed hydrolytic products, rt = 3.7 min for GEM, rt = 4.65 min for GHEE and rt = 7.65 min for the Codrug or the theoretically possible hydrolytic product, rt = 2.2 min for NAHEE. The selectivity of the HPLC method in plasma and in liver homogenate was also confirmed for samples containing the GHEE, codrug and GEM.

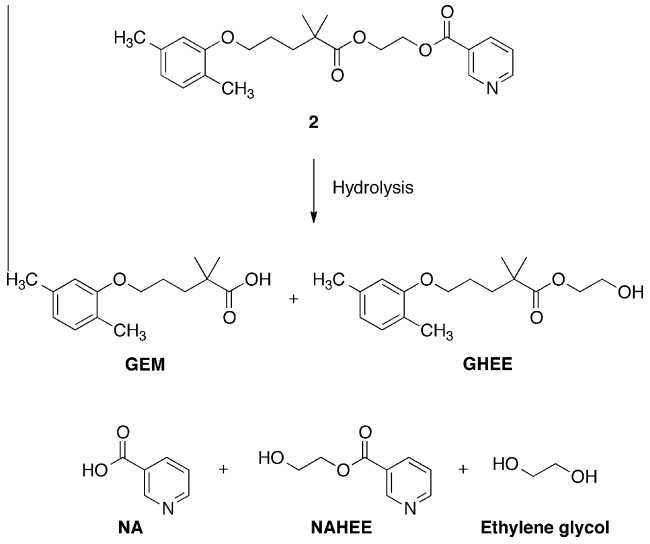
The accuracy was assessed by triplicate analysis of three control samples at concentration levels of 7, 80 and 160 μg/mL for each of GHEE, codrug and GEM. The samples were injected daily for 3 days. The error in the measured value for each compound was found to



Scheme 1. Synthetic scheme of the codrug.



Scheme 2. Synthetic scheme of nicotinic acid 2-hydroxyethyl ester (NAHEE).



Scheme 3. A schematic presentation for the possible hydrolytic products of the hydrolysis of the codrug.

be between 0.09 and 11% of the true value during the 3 days of study which indicates accurate method of analysis (FDA, 2001).

The intra- and inter-day precision was determined by replicate injection ($n = 6$) for three different concentration (7, 80 and 160 μg/mL) of GHEE, codrug and GEM for 3 days. The %RSD can be calculated from the equation: %RSD = (SD/mean) × 100, where SD is the standard deviation (Karnes and March, 1993; Lindholm, 2004). The %RSD obtained for intra-precision was 0.093–1.474 and %RSD for inter-day precision was 0.538–1.727 which is below 1.5% for repeatability and 2% for intermediate precision and comply with the acceptance criteria (Ermer and Miller, 2005).

The percentage of recovery of the GHEE, codrug, and GEM from human plasma and liver homogenate were measured by spiking human plasma or liver homogenate samples with a known concentration, 10 and 40 μg/mL, for each compound. The percentage of recovery for the three analytes was between 89.45 and 101.67% in plasma and between 96.17–100.45% in liver homogenate. The lower recovery of GEM in plasma may be due to protein binding.

LOD were calculated according to signal to noise ratio. Signal-to-noise ratios of 3:1 and 10:1 are generally considered acceptable for estimating the detection limit and quantification limit, respectively (International Conference on Harmonisation, 1997). LOD and LOQ were, respectively, for GHEE, 14 and 47 μg/mL, for codrug, 14 and 48 μg/mL and 11 and 38 μg/mL for GEM.

3.3. Physicochemical properties of codrug

3.3.1. Solubility, apparent partition coefficient and log P_{app}

Table 1 shows the solubility of the codrug and GEM and log P_{app} (Apparent Partition Coefficient) of the codrug in different media.

Table 1
Solubility of codrug and GEM.

Media	Solubility (mg/mL) ± SD*		Log P_{app} ± SD*
	Codrug	GEM	Codrug
pH 1.2**	$1.71 \times 10^{-1} \pm 9.88 \times 10^{-3}$	–	3.11 ± 0.12
pH 6.8**	$6.95 \times 10^{-4} \pm 9.59 \times 10^{-5}$	$4.37 \times 10^{-1} \pm 1.98 \times 10^{-2}$	3.32 ± 0.04
pH 7.4**	$4.81 \times 10^{-4} \pm 1.58 \times 10^{-5}$	$1.39 \pm 0.14 \times 10^{-1}$	3.37 ± 0.04
0.1 M HCl	$2.33 \times 10^{-1} \pm 9.62 \times 10^{-3}$	$<3.00 \times 10^{-2}$***	2.84 ± 0.02

* Standard deviation ($n = 3$).
** 0.05 M phosphate buffer.
*** Literature report (Luner et al., 1994).

Table 2
Observed first-order rate constant for hydrolysis of the codrug using different cosolvents at pH 6.8 and 7.4 at 70 °C.

Cosolvent	pH 6.8			pH 7.4		
	k_{obs} (h^{-1})	R^2	$t_{0.5}$ (h)	k_{obs} (h^{-1})	R^2	$t_{0.5}$ (h)
22% Acetonitrile	1.69×10^{-2}	0.9996	41.01	5.87×10^{-2}	0.9997	11.81
22% Isopropanol	1.56×10^{-2}	0.9995	44.42	5.51×10^{-2}	0.9995	12.58
35% Methanol	3.04×10^{-1}	0.9993	2.28	–	–	–

It is clear that the solubility increases by decreasing the pH of the media since the pyridine ring in the codrug is expected to be weakly basic. On the other hand, GEM, which is a weak acid, pK_a 4.7, is more soluble at higher pH values, 6.8 and 7.4 than acidic at pH (Luner et al., 1994).

With regard to nicotinic acid, its water solubility is reported to be 15 mg/mL in water at 20 °C (IPCS Inchem, 1995).

Apparent partition coefficient (P_{app}) is expressed as the ratio of the equilibrium concentrations of a dissolved substance in the two immiscible solvent system, *n*-octanol–water system (Hassan et al., 2004). The calculated log P_{app} of the synthesized codrug is shown in Table 1. It is clear, and expected, that the log P_{app} of the codrug increases as the pH of the aqueous media increases due to the decrease in the extent of ionization of the nitrogen atom of the pyridine moiety. Also, all the values are below that of the log P_{app} of GEM, 6.72 (Hassan et al., 2004). Due to the presence of both acidic and basic groups in nicotinic acid, it exhibits low log P_{app} in acidic and basic media; −0.59 and −2.34 at pH 4 and 7, respectively (IPCS Inchem, 1995). The value of log P_{app} of codrug complies with the criteria for good oral absorption (Yalkowsky and Morozowich, 1980).

3.4. Chemical hydrolysis

3.4.1. Effect of the cosolvent

Table 2 shows the rate constant (k_{obs}), half-life ($t_{0.5}$) of the codrug in three different solvents at pH 6.8 and 7.4 at 70 °C.

Acetonitrile and isopropanol, which were used in the same proportion, resulted in first order rate constants that were in the same order. On the other hand, the use of methanol at 35%, resulted in a rate constant that is 19 times those in either acetonitrile or isopropanol. From the known nucleophilic abilities of methanol, it can be expected that it can participate as a reactant in such hydrolytic reactions. Therefore, acetonitrile, which is expected to be completely inert, was chosen as a cosolvent (Simões et al., 2009).

3.4.2. Effect of pH on hydrolysis of the codrug

The hydrolysis of the codrug in theory should afford all or some of the following compounds, GEM, GHEE, NA, NAHEE and ethylene glycol, Scheme 3.

While, the synthesized codrug is chemically a diester, its two ester linkages have different steric and electronic properties which are expected to affect the rate of their respective hydrolysis and hence it will determine which linkage will be hydrolyzed first. Fig. 2 shows the hydrolysis of the codrug at pH 7.4 at 70 °C.

The complete hydrolysis profile is seen in Fig. 2a which clearly shows that GEM is librated from the formed GHEE. While in Fig. 2b, which represents the hydrolysis of the codrug in the first 72 h it can be observed that at pH 7.4, the codrug was hydrolyzing quantitatively to GHEE without any formation of GEM. In addition, NAHEE ester was not detected as a hydrolysis product that is also evident by the equimolar formation of GHEE from the codrug. This differential hydrolysis rate can be explained largely by the relative steric bulk around the two ester linkages. The ester linkage beside

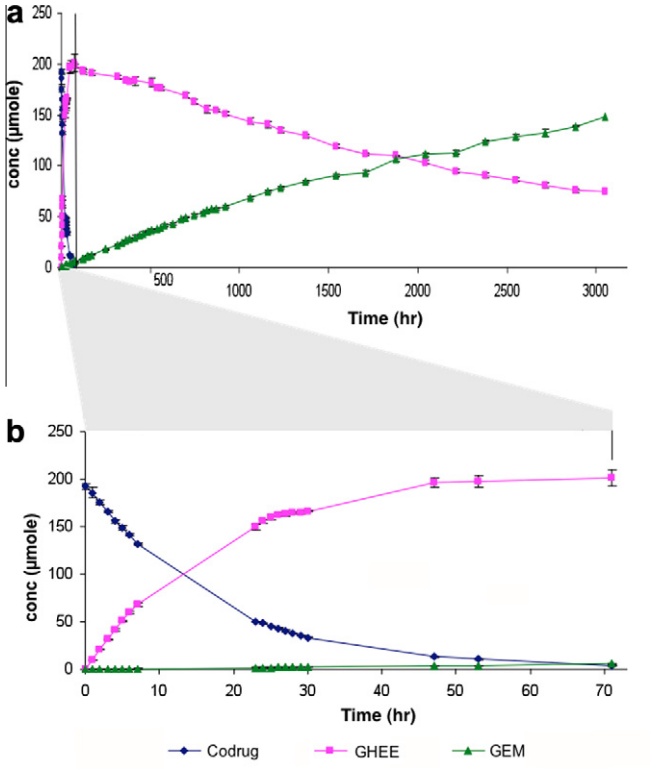


Fig. 2. Degradation of codrug in 0.05 M phosphate buffer pH 7.4 at 70 °C (a) is the complete degradation profile, (b) is the first 71 h of the profile.

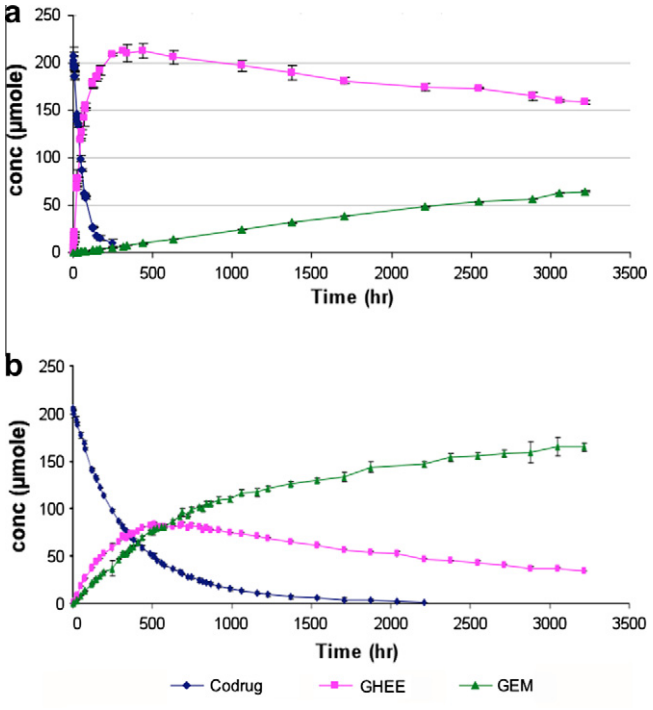


Fig. 3. Degradation of codrug in 0.05 M phosphate buffer at (a) pH 6.8 at 70 °C and (b) pH 1.2 at 70 °C.

Table 3
Observed first-order-rate constant for hydrolysis of codrug and for the liberated GHEE at different pH (60, 70, 80 and 90 °C).

Temp (°C)	pH	Codrug			GHEE		
		k_{obs} (h^{-1}) ± SD	R^2	$t_{0.5}$ (h) ± SD	k_{obs} (h^{-1}) ± SD	R^2	$t_{0.5}$ (h) ± SD
60	1.2	$1.10 \times 10^{-2} \pm 0.00$	0.998	630.00 ± 000	–	–	–
	6.8	$4.40 \times 10^{-3} \pm 5.77 \times 10^{-5}$	0.997	159.33 ± 2.12	–	–	–
	7.4	$1.58 \times 10^{-2} \pm 1.00 \times 10^{-4}$	0.999	43.96 ± 0.32	$1.00 \times 10^{-4} \pm 0.00$	0.998	6930.00 ± 000
70	1.2	$2.70 \times 10^{-3} \pm 1.00 \times 10^{-4}$	0.997	253.61 ± 5.29	$3.67 \times 10^{-4} \pm 5.77 \times 10^{-5}$	0.997	1925.00 ± 334.42
	6.8	$1.69 \times 10^{-2} \pm 2.00 \times 10^{-4}$	0.999	41.09 ± 0.28	$9.67 \times 10^{-5} \pm 5.77 \times 10^{-6}$	0.993	7187.67 ± 444.60
	7.4	$5.87 \times 10^{-2} \pm 2.10 \times 10^{-3}$	0.999	11.81 ± 0.04	$3.33 \times 10^{-4} \pm 5.77 \times 10^{-5}$	0.996	2117.50 ± 333.42
80	1.2	$6.80 \times 10^{-3} \pm 2.00 \times 10^{-4}$	0.999	101.43 ± 1.73	$1.63 \times 10^{-3} \pm 1.53 \times 10^{-4}$	0.974	426.71 ± 38.90
	6.8	$4.52 \times 10^{-2} \pm 3.00 \times 10^{-4}$	0.999	15.34 ± 0.10	$3.33 \times 10^{-4} \pm 5.77 \times 10^{-5}$	0.981	2117.50 ± 333.42
	7.4	$1.76 \times 10^{-1} \pm 3.70 \times 10^{-3}$	0.998	3.94 ± 0.08	$1.20 \times 10^{-3} \pm 1.00 \times 10^{-4}$	0.985	580.19 ± 48.52
	9	$2.40 \pm 1.37 \times 10^{-2}$	0.996	0.29 ± 0.00	$5.07 \times 10^{-2} \pm 1.16 \times 10^{-3}$	0.954	13.68 ± 0.308
90	6.8	$1.36 \times 10^{-1} \pm 6.00 \times 10^{-4}$	0.999	5.12 ± 0.02	$8.67 \times 10^{-4} \pm 5.77 \times 10^{-5}$	0.992	802.08 ± 55.57
	7.4	$5.40 \times 10^{-1} \pm 1.40 \times 10^{-3}$	0.998	$02.60 \times 10^{-3} \pm 0.00$	$2.60 \times 10^{-3} \pm 2.00 \times 10^{-4}$	0.990	267.60 ± 20.65

GEM is very stable due to the steric hindrance imparted by the bulky dimethyl group which hinders the attack of the hydroxide anion, the rate limiting step in base-catalyzed hydrolysis. In addition, the pyridine in NA is an electron-withdrawing group which renders the adjacent carbonyl more electrophilic, hence facilitating the hydrolysis at the NA side. A similar trend is observed at pH 6.8, Fig. 3a, but the hydrolysis rate is slower compared to that at pH 7.4. The hydrolysis of codrug in 0.05 M phosphate buffer pH 1.2 at 70 °C is shown in Fig. 3b. It can be seen that the codrug is more stable at pH 1.2 than at pH 7.4 or 6.8, which is attributed to the mechanism of hydrolysis that is expected to be an acid-catalyzed hydrolysis. In acid-catalyzed hydrolysis the carbonyl of the ester group must be protonated first and this protonation is the rate-limiting step. Protonation of either of the carbonyl groups in the codrug is possible and steric bulk plays a less pronounced role, which explains the relatively faster disappearance of the GHEE (and appearance of GEM) at pH 1.2 than at either 7.4 or 6.8. Here, the formation of GHEE peaked at 500 h producing less than 50% of the expected amount. It is also important to mention that no NAHEE was seen at this pH. This means that in acidic medium either GHEE is hydrolyzed while it is formed, yet its formation seems to be faster than its hydrolysis or that NAHEE, if formed, is being hydrolyzed rapidly so it cannot be detected, still, a combination of both explanations might be also possible.

For a more complete profile, the effect of pH on the hydrolysis of codrug was studied in 0.05 M phosphate buffer at pH 1.2, 6.8 and 7.4 at 60, 70, 80 and 90 °C. In addition, the hydrolysis at pH 9 at 80 °C was studied to get a better feel for the effect of pH in hydrolysis using a relatively quick experiment. The observed first-order-rates constants and the corresponding half-lives at each pH are presented in Table 3.

At 80 °C, Fig. 4, it is clear that the codrug and GHEE exhibited hydrolysis in all the studied pH values with highest rate of hydrolysis being at pH 9. For the codrug, the hydrolysis is pH-dependant and it increases, as the pH of the medium becomes more alkaline, Fig. 4a. As seen in Fig. 4c, the value of log k_{obs} of codrug increases sharply and linearly with increasing pH (6.8–9) at which the slope equals 0.7687 (R^2 = 0.994). This slope may indicate a combination of general-base and specific-base (OH^-) catalysis. For GHEE, the hydrolysis is also pH-dependant, but its lowest rate is at pH 6.8

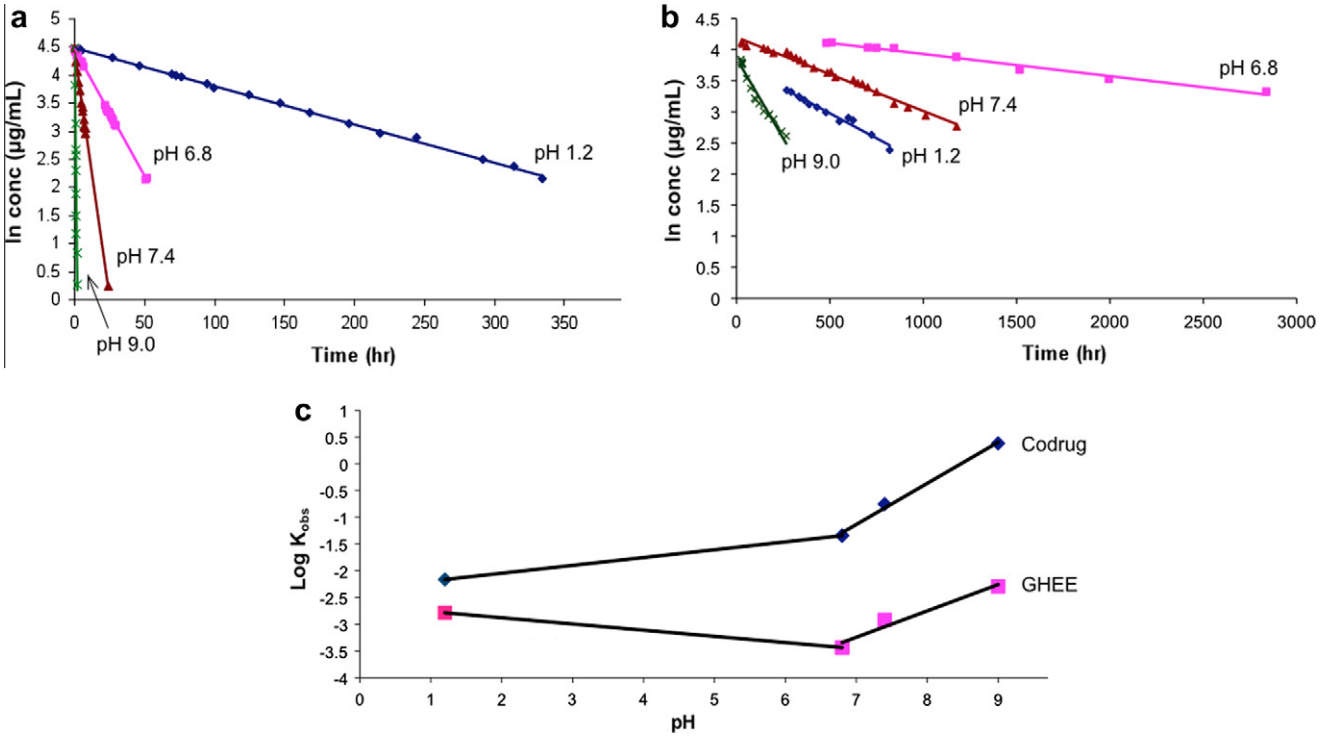


Fig. 4. (a) Influence of pH on the hydrolysis of codrug at 80 °C (b) Influence of pH on the hydrolysis of GHEE at 80 °C (c) pH rate profile for both the codrug and GHEE.

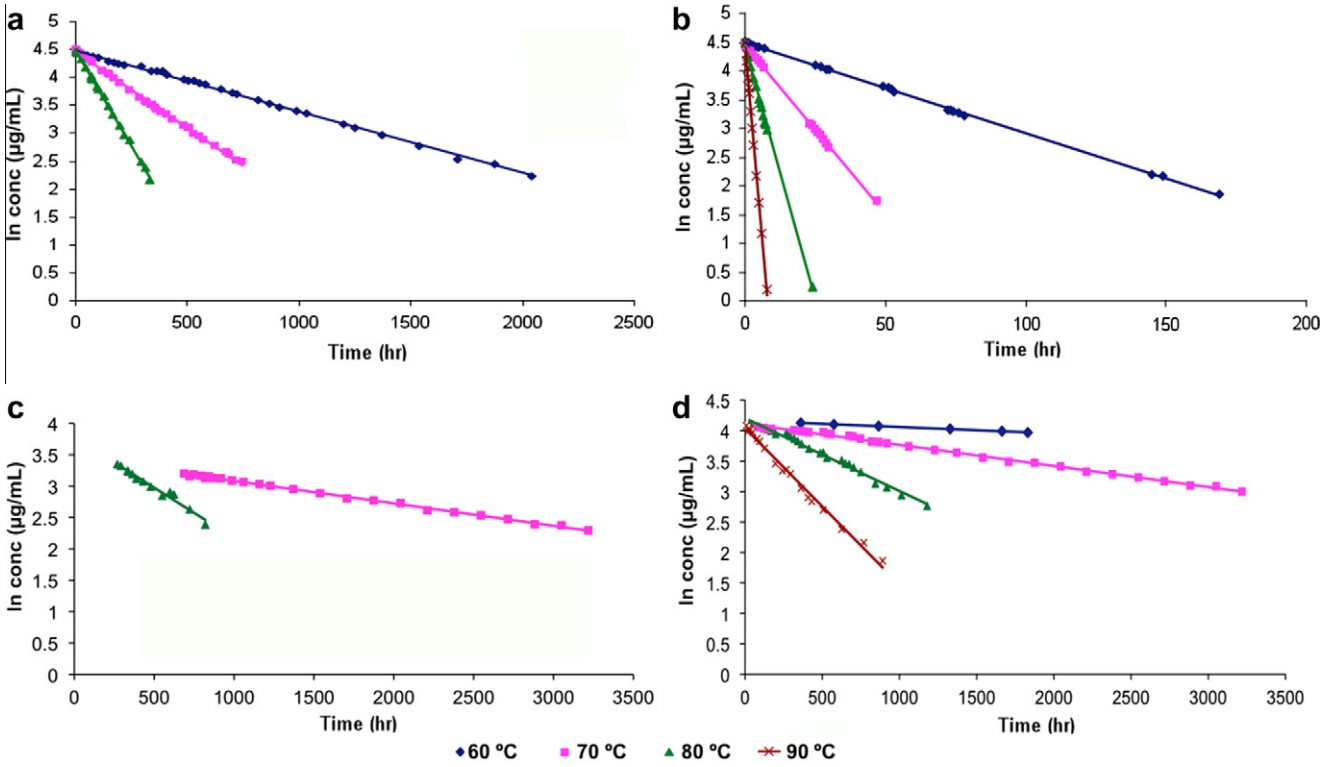


Fig. 5. Influence of temperature on the hydrolysis of (a) codrug at pH 1.2, (b) codrug at pH 7.4, (c) GHEE at pH 1.2 and (d) GHEE at 7.4.

rather than 1.2, Fig. 4b. This is another clear evidence that the bulk imparted by the dimethyl group of GEM retards the hydrolysis in alkaline media and its effect is less pronounced at acidic pH. The acceding line in the hydrolysis of the GHEE, Fig. 4c, has a slope of 0.4927, which not as steep as that for the codrug indicating that is mainly hydrolyzed by general-base catalysis.

3.4.3. Effect of temperature on hydrolysis of the codrug

It can be seen in Fig. 5 which shows the effect of temperature on the hydrolysis of the codrug and GHEE at pH 1.2 and 7.4 that the hydrolysis is affected by temperature and its rate increases as the temperature increases.

Form the data presented in Table 3, in a linear plot was obtained indicating that the degradation of the codrug and the liberated GHEE follows the Arrhenius relationship over the temperature region studied at the selected pH values, Fig. 6. By Extrapolating the obtained relationship between k_{obs} and temperature to room temperature, 25 °C (298 K) and 37 °C (310 K), Table 4, it's obvious that codrug exhibits high stability at 37 °C (310 K), at the studied pH range which indicates that it will most likely pass unhydrolyzed through the GIT after oral administration. At 310 K (37 °C), the codrug showed high stability at pH 1.2 which implies that it will pass unhydrolyzed through stomach after oral administration, and it showed a sufficient stability at pH 6.8 to be absorbed intact from intestine. At 298 K (25 °C), the codrug exhibits very high stability, which is beneficial since a prodrug should be chemically stable in order to be easily formulated into an appropriate pharmaceutical dosage form.

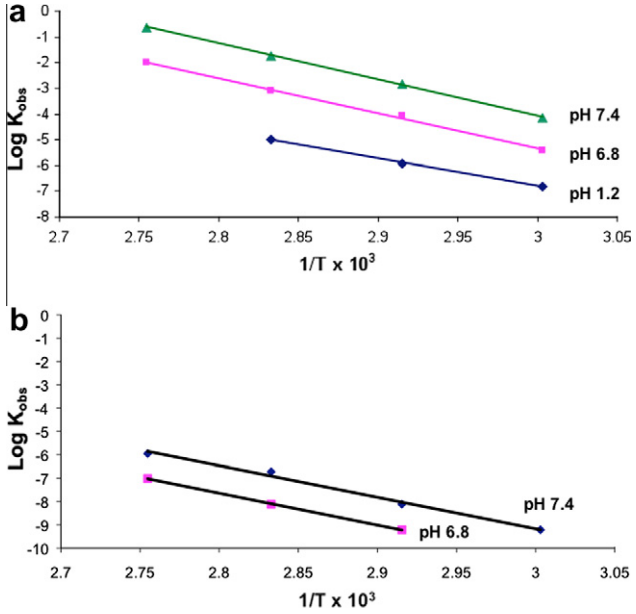


Fig. 6. Arrhenius plot for (a) codrug at phosphate buffer of pH 1.2, 6.8 and 7.4 and (b) the liberated GHEE at phosphate buffer of pH 6.8 and 7.4

3.4.4. Chemical hydrolysis of GHEE

The chemical hydrolysis of GHEE, which is the stable hydrolytic product of the codrug was studied separately using 0.05 M phosphate buffer pH 1.2 at 80 °C. This experiment was conducted as a control and these conditions resulted in a relatively fast hydrolysis of ester (1). Fig. 7 shows the hydrolysis of GHEE when subjected to hydrolysis separately, (a), in comparison with its hydrolysis when it is formed as product from codrug hydrolysis, (b). It can be seen the two curves follow similar pattern as the k_{obs} was $1.60 \times 10^{-3}\ h^{-1}$ in both cases. It is worth mentioning that R^2 for curve (a) was 0.9714 and for curve (b) was 0.9744.

3.5. In vitro enzymatic hydrolysis

3.5.1. In vitro enzymatic hydrolysis in 10% buffered human plasma

The rates of enzymatic hydrolysis were studied in 10% buffered human plasma at 37 °C as seen in Fig. 8. The observed pseudo-

Table 4
The observed rate constants and half-lives for degradation of codrug at 310 K and 298 K as calculated from Arrhenius plot.

Codrug	pH				
	Codrug			GHHE	
	1.2	6.8	7.4	7.4	6.8
R^2	0.9994	0.9977	0.9995	0.9919	0.9997
E_a (Kcal mol^{-1})	21.25	27.07	28.08	26.82	27.16
k_{obs} (h^{-1}) at 310 K	1.00×10^{-4}	2.22×10^{-4}	6.95×10^{-4}	4.95×10^{-6}	1.42×10^{-6}
$t_{0.5}$ at 310 K (days)	288.53	129.89	41.56	5836.96	20315.19
k_{obs} (h^{-1}) at 298 K	2.49×10^{-5}	3.79×10^{-5}	1.11×10^{-4}	8.56×10^{-7}	2.40×10^{-7}
$t_{0.5}$ at 298 K (days)	1158.61	762.89	260.83	33737.61	120028.28

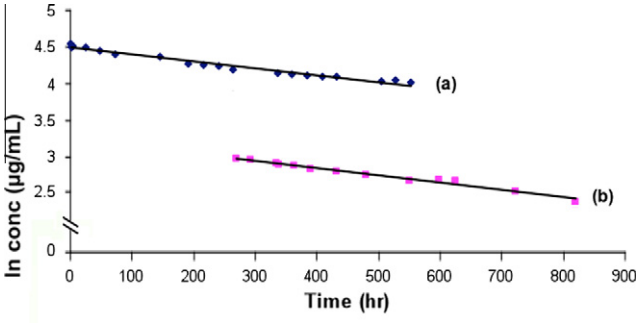


Fig. 7. Pseudo-first-order plots for hydrolysis in 0.05 M phosphate buffer pH 1.2 at 80 °C of (a) GHEE studied separately with (b) a plot of its degradation phase as a result of codrug hydrolysis.

first-order rate constant for enzymatic hydrolysis of the codrug in human plasma was 1.18 h^{-1} and the half-life was 35.24 min (R^2 = 0.979). It is clear that GHEE is quite stable in plasma where a small amount of GEM is formed. The stability of GHEE in plasma prompted us to study it separately, and even then it did not show appreciable hydrolysis even after 24 h. This further confirms the protection effect exerted by the dimethyl group adjacent to GEM carbonyl group against attack by nucleophiles.

3.5.2. In vitro enzymatic hydrolysis in 20% liver homogenate

The rates of enzymatic hydrolysis were studied in 20% liver homogenate at 37 °C as seen in Fig. 8. Also, here the hydrolysis of the codrug was studied and compared to the hydrolysis of GHEE which was studied separately. The pseudo first-order rate constants were 0.35 min^{-1} for the codrug (R^2 = 0.999) and 0.014 min^{-1} for GHEE (R^2 = 0.999) and the half-lives were 1.96 min and 48.13 min for the codrug and GHEE, respectively. It is clear that the hydrolysis of the codrug and GHEE is much a faster in liver homogenate than it is in plasma. In addition, this enzymatic hydrolysis leads to the expectation that, in vivo, the codrug yields its two parent drug, first it will release NA and following that there will be delayed release of GEM.

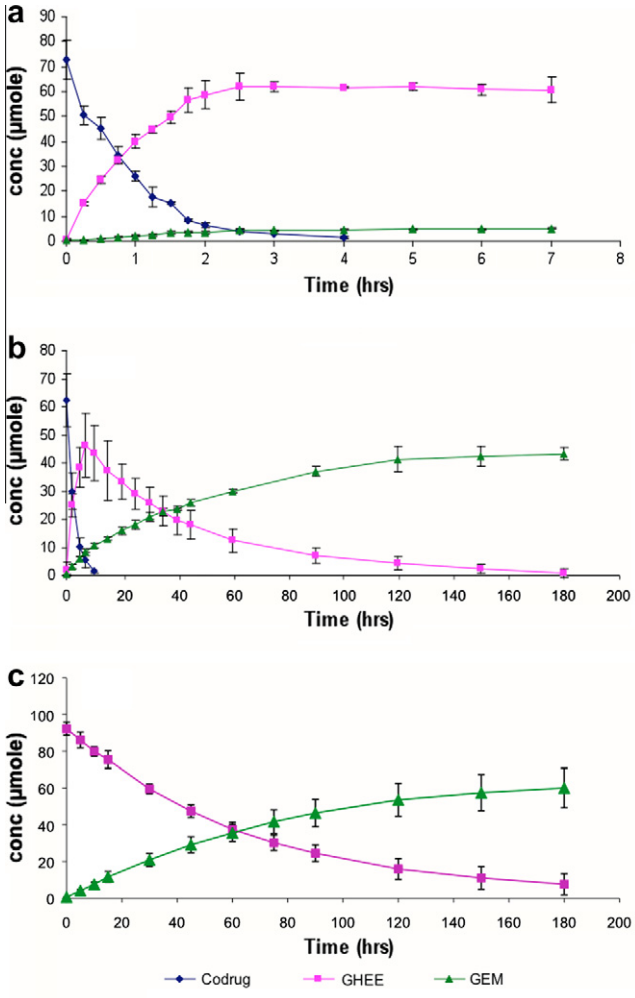


Fig. 8. Enzymatic hydrolysis of (a) codrug in human plasma at 37 °C to GHEE and GEM, (b) codrug in liver homogenate at 37 °C to GHEE and GEM and (c) GHEE in liver homogenate at 37 °C to GEM.

4. Conclusion

The synthesis of a codrug of gemfibrozil and nicotinic acid was successfully effected in a rather simple and scalable scheme that consists of two steps only. The chemical structures of the codrug and the intermediate were confirmed by ^{1}H NMR, ^{13}C NMR, FT-IR, MS analysis and elemental analysis. A validated HPLC method was also developed that allowed for the determination of three analytes in aqueous and biological samples simultaneously. The physico-chemical properties of the codrug were improved, in term of water solubility and partition coefficient compared to the two parent drugs; the lipophilic gemfibrozil and the hydrophilic nicotinic acid. The codrug was found to be stable in aqueous buffer solution and its hydrolysis product, GHHE was found to be much more stable. The hydrolysis of the codrug and its hydrolysis product intermediate GHEE in human plasma and liver homogenate was found to be faster than the chemical hydrolysis. It can be concluded that, in vivo, the codrug is expected to be converted to its parent drugs releasing nicotinic acid rapidly in plasma and liver followed by a relatively delayed release of gemfibrozil in liver. Future work should include in vivo investigations of the codrug to determine its pharmacokinetic parameters compared to its parent

drugs in addition to evaluation of its therapeutic efficiency in improving the lipid profile in a suitable animal models.

Acknowledgments

This work was funded by a grant from the Deanship of Research at Jordan University of Science and Technology. The authors would like to convey their sincere thanks to Mr. Eyad Hamzeh and Mr. Farouk Al-Zogoul for their help.

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