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Chemical and in vitro enzymatic stability of newly synthesized celecoxib lipophilic and hydrophilic amides

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ABSTRACT

Five celecoxib (**CXB**) acylamide sodium salts, **MP-CXB**, **Cy-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB** were synthesized and characterized. Two simple, fast and validated RP-HPLC methods were developed for simultaneous quantitative determination of the amides and celecoxib in aqueous and biological samples and LOD and LOQ were ≤13.6 and ≤40 ng/mL, respectively. The solubility and log P_{app} of the amides, in relevant media, were determined. The chemical hydrolysis, at 60, 70 and 80 °C, of **MP-CXB** was studied at GIT-relevant pH (1.2, 6.8 and 7.4) and of **CY-CXB** was studied at skin relative pH (5.4 and 7.4). Significant hydrolysis was observed for **MP-CXB** at pH 1.2 only with half-lives 28.28, 11.64 and 3.53 h at 60, 70 and 80 °C, respectively, with extrapolated half-lives of 2060 and 443 h at 25 and 37 °C, respectively. The hydrolysis of all amides was studied in rat live homogenate and only **Cy-CXB** was hydrolyzed with half-life of 3.79 h. The hydrolysis of **MP-CXB** and **Cy-CXB** was studied in human plasma and neither was hydrolyzed. It is finally suggested that hydrophobic interactions plays a role in the binding of susceptible acylamides to the hepatic hydrolyzing enzyme since only amides with saturated hydrocarbon chains underwent hydrolysis.

1. Introduction

Non steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of the cyclooxygenase (COX) enzyme in the biosynthesis of prostaglandins and they are generally used for the management of acute and chronic pain, inflammation and fever (Duttaa et al., 2009; Kısmet et al., 2004). To date, cyclooxygenases exit in three isoforms, COX-1, COX-2 and COX-3, among which, only COX-1 and COX-2 are utilized clinically (Vane and Botting, 1998). COX-1 is a constitutive enzyme that, mainly, plays a cytoprotective role (El-Medanya et al., 2005). On the other hand, COX-2 is primarily inducible by a variety of inflammatory stimuli including exposure to growth factors, cytokines, tumor promoters, carcinogens, and endotoxins and so it induces inflammation, pain, and fever (Radi and Khan, 2006). Traditional NSAIDs, which are nonselective COX inhibitors, exhibit an anti-inflammatory action and can, sometimes, cause severe gastrointestinal side effects. On the other hand, selective COX-2 inhibitors have been demonstrated to produces anti-inflammatory action while at the same time exert less untoward effects on the gastrointestinal tract tissues (Bombardier et al., 2000; Silverstein et al., 2000; Vonkeman and van de Laar, 2010). Celecoxib (**CXB**), Fig. 1, the prototype of the Coxib family of selective COX-2 inhibiting NSAIDs is a weak acid with a p*K*a value of about 11, very lipophilic and is practically insoluble in acidic, neural and moderately alkaline environment including the physiological pH (Subramanian et al., 2004; Ventura et al., 2005). **CXB** capsules have a low absolute bioavailability of only 22–40% (Paulson et al., 2001) due to celecoxib's very low aqueous solubility, low wettability and low dissolution rate. **CXB**, also, has low intrinsic permeation capacity through skin which is mainly due to its poor aqueous solubility (Baboota et al., 2007).

Prodrug design can be employed to optimize a drug's permeation properties and consequently affecting its oral bioavailability and transdermal penetration. A product of such design is parecoxib, a sodium salt of the propionylamide of valdecoxib which has been shown to be suitable for of IV administration (Talley et al., 2000). Like valdecoxib, **CXB** is an arylsulfonamide that can be transformed into a hydrolysable amide by acylation (Larsen and Bundgaard, 1987; Larsen et al., 1988). For example, **CXB** butyrylamide was shown to exhibit 5-fold enhancement in oral bioavailability compared to its parent drug (Mamidi et al., 2002).

Herein, we report the design, synthesis and in vitro hydrolysis of a relatively diverse group of **CXB** acylamides with chemical structures shown in Fig. 1. Due to the differences in the chemical structure of **MP-CXB** compared to the rest of the acylamides, two different validated RF-HPLC methods have been developed and employed for the quantitative determination of **CXB** and its acylamides. There are limited reports of HPLC methods in which **CXB**

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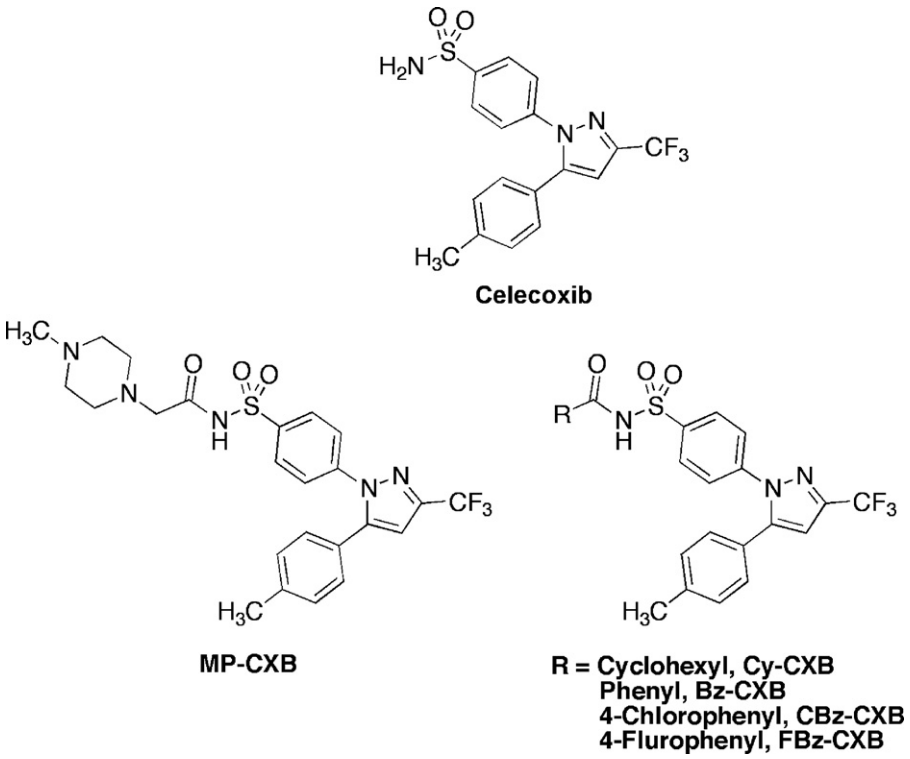


Fig. 1. The chemical structures of the target **CXB** acylamides.

is being analyzed with its derivatives and none if that derivative is not lipophilic. One method for the analysis of **CXB** and three acylamides was long with retention times up to 22.3 min and uses a 25 cm column (Mamidi et al., 2002) and another for the analysis for **CXB** alone uses a similar length column with no mention of retention time (Baboota et al., 2007).

MP-CXB is designed with the main goal of enhancement the solubility of **CXB** beyond what a sodium salt of its alkanoylamides can achieve which is reported to be approximately 15 mg/mL (Mamidi et al., 2002). In the GIT **MP-CXB** which contains a piprazine ring is expected to be ionizable both, in basic and acidic media and hence, maintaining high solubility over a large pH range. **Cy-CXB** is an alicyclic amide that is designed as a prodrug for transdermal delivery. The promoiety was chosen because it has a similar size to that in **MP-CXB**, hence allows for direct comparison specially in enzymatic hydrolysis. Accordingly, in addition to enzymatic hydrolysis, the hydrolysis of **MP-CXB** will be studied in GIT-relevant pH values and the hydrolysis of **Cy-CXB** will be studied in skin-relevant pH values. In addition, **Cy-CXB** is an alicylic amide rather than a straight chain amide and it will be interesting to compare its enzymatic hydrolysis to that of the other **CXB** aliphatic acylamides reported in the literature. The benzamides are synthesized to give a more comprehensive idea about the in vitro hydrolysis of this class of prodrugs in liver homogenate. Finally it is worth mentioning that the hydrolysis in liver homogenate of the labile bond in these prodrugs, sulfonyl acylamide, has not been fully studied before and this work provide an insight into this accept of the functional group.

2. Experimental

CXB was kindly donated by the Jordanian Pharmaceutical Manufacturing Company (JPM). Bulk solvents were purchased through local vendors. The acyl and benzoyl chlorides were obtained from Aldrich Chemical Company (USA). Other reagent grade, fine chemicals and HPLC solvents were obtained from Scharlau chemicals (Spain) and BDH organics (England). The deuterated solvents were obtained from Acros Chemicals (Belgium). Deionized water was used in the HPLC separation. Melting points were determined using Stuart Scientific-melting point apparatus (UK) and were uncorrected. ^{1}H and ^{13}C NMR spectra were obtained using 400 MHz Bruker Avance UltraShield Spectrometer (Switzerland). NMR data are reported in ppm (δ) using automatic calibration to the residual proton peak of the solvent, CD_3OD and DMSO-d6; the splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet; bs, broad singlet. Atomic pressure chemical ionization (APCI) mass spectra were obtained using Agilent 1100 series LC–MS (USA) at the Pharmaceutical Research Center (PRC), Jordan University of Science and Technology. The FT-IR spectra were recorded on Nicolet avatar 360 FT-IR (USA) using KBr disks. TLC analysis was performed on Albet aluminium TLC plate (Silica 60 and UV 254 (Spain). Log p*K*a values for the compounds were calculated using Pipline Pilot by Accelrys, USA (http://accelrys.com).

2.1. Synthesis and characterization

2.1.1. Sodium 2-chloro-N-(4-(5-p-tolyl-3-(trifluoromethyl)-1H-pyrazol-1-yl)-phenylsulfonyl) acetamide (*CAc-CXB*)

To a stirring solution of triethylamine (1.09 mL, 0.79 g, 7.86 mmol) and **CXB** (3.00 g, 7.86 mmol) in dichloromethane (200 mL), chloroacetyl chloride (0.62 mL, 0.88 g, 7.86 mmol) was added drop wise and the reaction mixture was allowed to stir at room temperature for 24 h. The reaction progress was followed by TLC (50% ethyl acetate in hexane). Upon completion of the reaction, the solution was washed with distilled water (200 mL× 3), then with 0.1 N HCl solution (300 mL× 3). The organic layer was then dried over sodium sulfate and the solvent was evaporated. The resultant compound was dissolved in absolute ethanol (200 mL) and was then titrated with 2 M sodium hydroxide. The mixture

was then concentrated and the residue was crystallized from ethyl acetate to afford 1.23 g of **CAc-CXB** as off-white crystals (30.20%). ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 8 (2H, d, J = 8.6 Hz, Ar-H), 7.42 (2H, d, J = 8.6 Hz, Ar-H), 7.18 (4H, q, J = 8 Hz, Ar-H), 6.9 (1H, s, pyr-H), 3.98 (2H, s, CH_2), 2.36 (3H, s, Ar-CH_3). IR (KBr, cm^{-1}): 1612.49, 1408.04, 1342.49, 1236.3, 1114.8. LC–MS (APCI) m/z: M (479.9, 100%), MH (480.9, 52.3%), MH + 1 (481.9, 48%). Elemental analysis (calculated) C: 47.56; H: 2.94; N: 8.76; (found) C: 46.72; H: 3.15; N: 9.34.

2.1.2. Sodium 2-(4-methylpiperazin-1-yl)-N-(4-(5-p-tolyl-3-(trifluoromethyl)-1H-pyrazol-1-yl)-phenyl sulfonyl) acetamide (*MP-CXB*)

CAc-CXB (1.00 g, 2.08 mmol) was dissolved in acetonitrile and to the solution, 1-methylpiperazine (0.46 mL, 0.41 g, 4.16 mmol) and sodium iodide (0.44 g, 2.08 mmol) were added. The reaction mixture was allowed to stir at room temperature for 3 days. The reaction progress was followed by TLC (10% methanol in dichloromethane). Upon completion of the reaction it was filtered and the solvent was evaporated under vacuum and the obtained solid was crystallized from acetonitrile to yield 0.90 g of **MP-CXB** as white crystals (90.00%). ^{1}H NMR (CD_3OD, 400 MHz), δ (ppm): 7.99 (2H, d, J = 8.6 Hz, Ar-H), 7.42 (2H, d, J = 8.6 Hz, Ar-H), 7.2 (4H, q, J = 8.3 Hz, Ar-H), 6.91 (1H, s, pyr-H), 3.04 (2H, s, CH_2), 2.50 (8H, bs, pip-H), 2.38 (3H, s, N-CH_3), 2.28 (3H, s, Ar-CH_3). ^{13}C NMR (CD_3OD, 100 MHz), δ (ppm): 179.23, 147.67, 146.33, 145.38 (q, J = 38 Hz, CF_3), 143.39, 141.61, 131.40, 130.81, 129.97, 128.06, 127.04, 124.89, 122.22, 107.54, 65.33, 56.20, 54.52, 46.80, 22.17. FT-IR (KBr, cm^{-1}): 1600.92, 1408.04, 1303.88, 1263.37, 1151.50. LC–MS (APCI) m/z: MH (544.1, 100%), MH + 1 (545.1, 42.5%), MH + 2 (546.1, 46.4%). Elemental analysis (calculated) C: 53.03; H: 4.64; N: 12.88; (found) C: 53.8; H: 4.66; N: 13.84.

2.1.3. General procedure for the synthesis of *Cy-CXB*, *Bz-CXB*, *CBz-CXB* and *FBz-CXB* as the sodium salts

To a stirring solution of triethylamine (1.09 mL, 0.79 g, 7.86 mmol) and **CXB** (3.00 g, 7.86 mmol) in dichloromethane (200 mL), the appropriate acylchloride or benzoylchloride (7.86 mmol) was added drop wise and the reaction mixture was allowed to stir at room temperature for 24 h. The reaction progress was followed by TLC (5% methanol in dichloromethane). Upon completion of the reaction the solvent was evaporated and 300 mL of ether was added and the solution washed with 1N HCl aqueous solution (200 mL× 3), then with 1 M sodium bicarbonate (300 mL× 3). The organic layer was then dried over sodium sulfate and the solvent was evaporated. The resultant residue was dissolved in absolute ethanol (200 mL) and then was titrated with 2 M sodium hydroxide. The resultant mixture was then concentrated and the residue was washed extensively with boiling diethylether to afford the desired compound as white solids with 99–96% purity according to elemental analysis.

*2.1.3.1. Sodium N-(4-(5-p-tolyl-3-(trifluoromethyl)-1-H-pyrazol-1-yl)-phenylsulfonyl) cyclohexane carboxamide (**Cy-CXB**).* Cyclohexane carbonyl chloride (1.05 mL, 1.15 g, 7.86 mmol). Yield = 66.00%. ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 7.76 (2H, d, J = 8.6 Hz, Ar-H), 7.32 (2H, d, J = 8.6 Hz, Ar-H), 7.23–7.13 (5H, m, Ar-H), 7.15 (1H, s, pyr-H), 2.31 (3H, s, Ar-CH_3), 1.89 (1H, m, Cyc-H), (5H, m, Cyc-H), 1.16 (5H, m, Cyc-H). ^{13}C NMR (DMSO-d_6, 100 MHz), δ (ppm): 183.51, 149.54, 147.70, 144.38 (q, J = 37.8 Hz, CF_3), 142.03, 141.52, 131.97, 131.28, 130.25, 128.12, 127.46, 125.37, 122.70, 108.3, 49.70, 32.90, 28.56, 28.22, 23.42. FT-IR (KBr, cm^{-1}): 2926.01, 2852.72, 1593.20, 1409.96, 1321.24, 1238.3, 1134.1. LC–MS (APCI) m/z: MH (514.1, 100%). MH + 1 (515.1, 15.4%), MH + 2 (516.1, 16%). Elemental analysis (calculated) C: 56.13; H: 4.51; N: 8.18; (found) C: 54.76; H: 4.59; N: 7.99.

*2.1.3.2. Sodium N-(4-(5-p-tolyl-3-(trifluoromethyl)-1-H-pyrazol-1-yl)phenylsulfonyl) benzamide (**Bz-CXB**).* Benzoyl chloride (0.91 mL, 1.10 g, 7.86 mmol). Yield = 83.00%. ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 7.82–7.92 (4H, m, J = Hz, Ar-H), 7.24–7.39 (5H, m, Ar-H), 7.17 (4H, m, Ar-H), 7.13 (1H, s, pyr-H), 2.28 (3H, s, CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz), δ (ppm): 172.67, 147.72, 144.44 (q, J = 37.5 Hz, CF_3), 142.31, 141.68, 140.11, 132.63, 132.03, 131.64, 131.31, 130.99, 130.59, 130.06, 129.70, 128.13, 127.42, 108.32, 23.43. FT-IR (KBr, cm^{-1}): 1595.1, 1409.96, 1330.88, 1238.30, 1170.79. LC–MS (APCI) m/z: MH (508.2, 100%), MH + 1 (509.2, 35.8%), MH + 2 (510.2, 34.48%). Elemental analysis (calculated) C: 56.80; H: 3.38; N: 8.28; (found) C: 52.49; H: 3.28; N: 7.81.

*2.1.3.3. Sodium 4-chloro-N-(4-(5-p-tolyl-3-(trifluoromethyl)-1-H-pyrazol-1-yl)phenylsulfonyl) benzamide (**CBz-CXB**).* p-Chlorobenzoyl chloride (0.99 mL, 1.37 g, 7.86 mmol). Yield = 76.6%. ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 7.78–7.98 (4H, m, Ar-H), 7.26–7.42 (4H, m, Ar-H), 7.06–7.24 (5H, m, Ar-H and pyr-CH), 2.28 (3H, s, Ar-CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz), δ (ppm): 171.64, 148.8, 147.7, 144.47 (q, J = 37.7 Hz, CF_3), 142.44, 141.6, 140.49, 137.5, 133.54, 132.87, 132.03, 131.3, 130.58, 130.16, 129.76, 128.12, 127.55, 108.37, 23.43. FT-IR (KBr, cm^{-1}): 1597.06, 1409.96, 1332.8, 1238.3, 1136. LC–MS (APCI) m/z: MH (543.2, 100%), MH + 1 (544.2, 93.3%). Elemental analysis (calculated) C: 53.19; H: 2.98; N: 7.75; (found) C: 49.93; H: 2.92; N:7.09.

*2.1.3.4. Sodium 4-fluoro-N-(4-(5-p-tolyl-3-(trifluoromethyl)-1-H-pyrazol-1-yl)phenylsulfonyl) benzamide (**FBz-CXB**).* p-Fluorobenzoyl chloride (1.67, 1.24 g, 7.86 mmol). Yield = 92.00%. ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 7.88–7.97 (2H, m, Ar-H), 7.85 (2H, d, J = 8.4 Hz, Ar-H), 7.34 (2H, d, J = 8.4 Hz, Ar-H), 6.99–7.23 (7H, m, Ar-H and pyr-H), 2.28 (3H, s, Ar-CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz), δ (ppm): 166.17 (d, J = 246.3 Hz), 148.88, 147.72, 144.47 (q, J = 37.5 Hz), 142.43, 141.6, 138.08, 133.46 (d, J = 8.7 Hz), 132.35, 131.31, 130.54, 128.1, 127.55, 125.35, 122.69, 116.82 (d, J = 29.3 Hz), 108.35, 23.42. FT-IR (KBr, cm^{-1}) 1608.63, 1409.96, 1325.10, 1238.86, 1168.86. LC–MS (APCI) m/z: MH + (526, 100%). Elemental analysis (calculated) C: 54.86; H: 3.07; N: 8.00; and (found) C: 53.05; H: 3.05; N: 8.40.

2.2. High performance liquid chromatography (HPLC)

The HPLC system consisted of a SCL-10 AVP system controller, LC-10 ADVP isocratic pump, DGV-14AVP degasser, SPD-M 10AVP diode array detector, SIL-10ADVP auto injector, and CTO-10ASVP column oven connected to a computer prepared with appropriate software. Chromatographic separation and quantitative analysis were performed using a LiChroCART C18 (125 mm × 4.6 column mm ID), 5 μm (RP-select B) with a flow rate of 2 mL/min. The temperature of the column oven was set to 25 °C ± 1. The wavelength was set at 254 nm and the injection volume was 30 μL. In method A, for **MP-CXB**, the mobile phase for isocratic elution was a mixture of 0.02 M phosphate buffer and acetonitrile (60:40, v/v) and 1% triethylamine, pH 3.5. In method B, for **Cy-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB**, the mobile phase for isocratic elution consisted of 0.01 M phosphate buffer and acetonitrile (45:55, v/v), pH 3.2. The mobile phases were filtered through 0.45 μm regenerated cellulose membrane filter (VIVID, USA). **CXB** was analyzed simultaneously with its acylamides and benzamides in each of the two methods.

2.2.1. Calibration curves

A stock solution (400 μg/mL) was prepared by dissolving 40 mg of each of the analytes in 100 mL HPLC grade methanol. Standard

solutions for the calibration curves of each of the analyzed compounds were obtained by serial dilution of the stock solution of the corresponding compound with HPLC grade methanol. Calibration curves for **CXB** and five amides were constructed by injecting samples from the standard solutions of each of the compounds in the concentration range from 1.5 to 200 μg/mL. The area under the curve (AUC) for each compound was determined and plotted versus the concentration (μg/mL).

2.2.2. Method validation

HPLC method A was validated for the quantitation of **CXB** and **MP-CXB** while method B was validated for the quantitation of **CXB**, **Cy-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB** in terms of linearity, accuracy, precision, selectivity, and recovery, limit of detection (LOD) and limit of quantitation (LOQ).

Linearity was performed by constructing six calibration of curve for all the analytes in each method. Accuracy and precision were carried out by injecting freshly prepared control solutions at 10, 80, and 160 μg/mL six times a day for three consecutive days. Signal to noise ratio was used to determine the LOD and LOQ. Relative recovery of the analytes from biological samples was calculated by comparing the theoretical concentration to that recovered from spiking liver homogenate and plasma with known concentrations of the analytes.

2.3. Physico-chemical properties

2.3.1. Aqueous solubility

All solubility determinations was done at room temperature. The aqueous solubility of the five amides was determined both in water and in aqueous phosphate buffer solution (0.16 M) at pH 7.4. In addition, the solubilities of **Cy-CXB** and **MP-CXB** werewas also determined in aqueous phosphate buffer solution (0.16 M) at pH 5.4 or 6.8, respectively. In each case, excess amounts of compound were added to 1 mL of the dissolution media and were incubated at 25 ± 1 °C for 48 h. The saturated solutions were centrifuged at 4000 rpm for 5 min, and filtered using Millipore filters 0.45 μm and the concentration of each compound was determined by HPLC.

2.3.2. Partition coefficient and log P_{app}

The apparent partition coefficients expressed as log P_{app} of the five amides was determined, at room temperature, in *n*-octanol and water or aqueous phosphate buffer solution (0.16 M) pH 7.4 systems. The log P_{app} of **Cy-CXB** and **MP-CXB** were also determined using *n*-octanol and aqueous phosphate buffer solution (0.16 M) pH 5.4 or 6.8 systems, respectively. *n*-Octanol was firstly saturated with aqueous phosphate buffer solution of the appropriate pH by vigorous stirring for 24 h. Log P_{app} was measured by initially suspending 2 mg of each compound in 10 mL of the mixture of *n*-octanol water or aqueous phosphate buffer solution in a glass screw-capped test tube. The test tube was shaken at 25 °C in water bath for 2 h and then centrifuged at 5000 rpm for 8 min to assure complete separation of the two phases. The partition coefficient was calculated by dividing the concentration of each **CXB** acylamide or benzamide in the *n*-octanol layer by its concentration in aqueous phase. The experiments were performed in triplicate.

2.4. Chemical hydrolysis

*2.4.1. Hydrolysis of **MP-CXB** in aqueous phosphate buffer*

Hydrolysis of **MP-CXB** was investigated in aqueous 0.16 M phosphate buffer solution at GIT relevant pH values of 1.2, 6.8 and 7.4 at 80 °C ± 0.2. The hydrolysis was furthered studied at pH 1.2 at 60 and 70 °C ± 0.2. The ionic strength was always adjusted to 0.44 by adding a calculated amount of sodium chloride. The reactions were initiated by preparing 100 mg/mL solution of **MP-CXB** in 0.16 M phosphate buffer at pH 1.2 at pH 6.8 and pH 7.4 (except for pH 1.2 at 80 °C where a 50 mg/mL initial concentration was used) and the solutions were placed in a thermostatically controlled water bath at the respective temperatures. At appropriate time intervals, samples were withdrawn, cooled with iced water and immediately analyzed for **CXB** and remaining **MP-CXB** by HPLC. All experiments were carried out in triplicate.

*2.4.2. Hydrolysis of **Cy-CXB** in aqueous phosphate buffer*

Hydrolysis of **Cy-CXB** was investigated in aqueous 0.16 M phosphate buffer solution in the presence of 30% acetonitrile as a cosolvent at pH 5.4 and 7.4 at 80 ± 0.2 °C. The ionic strength was maintained at 0.44 by adding a calculated amount of sodium chloride. The reactions were initiated by preparing 50 μg/mL solutions of **Cy-CXB** in 0.16 M phosphate buffer at pH 5.4 and pH 7.4 in the presence of 30% acetonitrile and the solutions were placed in a thermostatically controlled water bath 80 °C ± 0.2. At appropriate time interval, samples were withdrawn, cooled with iced water and immediately analyzed for **CXB** and remaining **Cy-CXB** by HPLC. All experiments were carried out in triplicate.

2.5. In vitro enzymatic hydrolysis

2.5.1. Hydrolysis in liver homogenate

The liver homogenate was prepared according to published procedure (Mamidi et al., 2002). The hydrolysis of **Cy-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB** was assessed in liver homogenate by placing 980 μL of the supernatant from a liver homogenate in an eppondorf test tube and incubating it for 15 min in a shaking water bath equilibrated at 37 ± 1 °C. Then, 20 μL from a 2 mg/mL solution of each of the amides in methanol was mixed well with supernatant using vortex mixer leading to an initial concentration of 40 μg/mL. For the more water soluble **MP-CXB**, 900 μL of the supernatant from liver homogenate was placed in an eppondorf test tube and incubating it for 15 min in a shaking water bath equilibrated at 37 ± 1 °C. Then 100 μL (**MP-CXB**) from a 2 mg/mL solution in methanol was mixed well with supernatant using vortex mixer leading to an initial concentration of 200 μg/mL. At appropriate time intervals, 100 μL samples were withdrawn by micropipette and added to 300 μL methanol to precipitate the proteins. The mixture was then centrifuged for 5 min at 10,000 rpm and the clear supernatants obtained were analyzed for remaining amide and released **CXB** by HPLC. The rate of hydrolysis of each amide was determined from the slope of the linear plot of Ln (residual amide concentration) versus time. The half-life of the hydrolysis was also calculated.

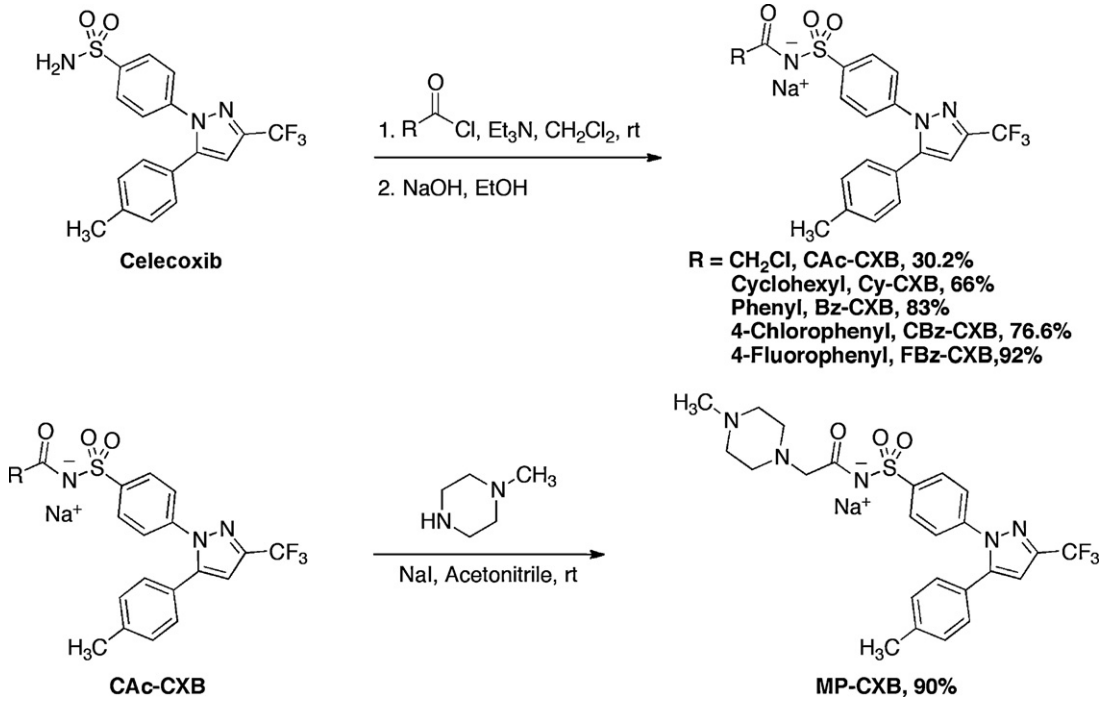
2.5.2. Hydrolysis in 10% human plasma

The rate of hydrolysis of **MP-CXB** and **Cy-CXB** was studied in human plasma at 37 °C. For **Cy-CXB**, from a solution of 2 mg/mL solution in methanol, 20 μL were added to 980 μL of 10% human plasma. The mixture was kept in a water bath for 48 hat 37 °C. While for **MP-CXB**, from a 2 mg/mL solution in methanol, 100 μL were added to 900 μL of 10% human plasma. The mixture was kept in a water bath for 48 hat 37 °C. At appropriate time intervals, 100 μL of the mixture was withdrawn and added to 300 μL of absolute methanol to precipitate proteins and then the mixture was centrifuged for 5 min at 10,000 rpm. The supernatant was analyzed for the remaining **MP-CXB** and **Cy-CXB** and released **CXB** by the HPLC.

3. Results and discussion

3.1. Chemistry

As seen in Scheme 1, the first step in the synthesis of **MP-CXB** was to acetylate **CXB** with α-chloroacetyl chloride followed by conversion of the resultant amide to the sodium salt to afford



Scheme 1. Synthesis of the target **CXB** acyl and benzamide.

CAc-CXB in modest yield. **CAc-CXB** was then reacted with 1-methylpiperazine in the presence of sodium iodide as the catalyst to afford MP-CXB, after crystallization, in excellent yield. For the synthesis of the rest of the amides, **CXB** was acylated with either cyclohexane carbonyl chloride, benzoyl chloride, 4-chlorobenzoyl chloride or 4-fluorobenzoyl chloride in the presence of triethylamine to afford **Cy-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB** in good to excellent yields, Scheme 1. The crude amides were converted to the sodium salts but failed to crystallize after many attempts. The best way to obtain them with acceptable purity was to extensively wash the sodium salts with boiling diethylether. HPLC and elemental analyses clearly showed that the purity of all the synthesized amides was 96–99%.

3.2. HPLC method validation

3.2.1. Linearity

Acceptability of linearity data is often judged by examining the correlation coefficient of the linear regression line for the area under the curve versus concentrations. For establishment of linearity, a minimum of five concentrations is recommended (International Conference on Harmonisation (ICH), 1997) and a correlation coefficient of 0.999 is generally considered as evidence of acceptable fit of the data to the regression line (Shabir, 2003). For method A, six calibration curves were constructed for **CXB** and **MP-CXB** over the concentration, ranges of 1.56–400 μg/mL by triplicate injection of nine freshly prepared standard solutions. In the same manner, for method B, six calibration curves were constructed for **CXB** and **Cy-CXB**, **Bz-CXB**, **CBz-CXB**, **FBz-CXB** of the same concentration range. The linear regression equation and its coefficient of correlation, R^2, for each of the calibration curves is summarized in Table 1. It can be seen that R^2 was always greater than 0.999 indicating the methods linearity.

3.2.2. Selectivity

The selectivity of the method was confirmed by analysis of samples containing **CXB** and each of its amides spiked in either methanol, in supernatant obtained from the liver homogenate or in plasma. The chromatograms form methanol is shown in Figs. 2 and 3. The chromatograms for the same compounds in liver homogenate and plasma are essentially identical expect for the appearance of one sharp peak at around 0.6 min, which corresponds for the solvent front.

3.2.3. Accuracy

The accuracy of an analytical method expresses the closeness of agreement between true value of analyte in samples and test result obtained by the method (Ermer and Miller, 2005). The accuracy as confirmed by triplicate analysis of three control samples at concentration level of 10, 80 and 160 μg/mL for **CXB** and all the amides. The samples were injected daily for three days. The measured value for each compound was found to be within 15% of the true value during the three days of study which indicates accurate method of analysis (Center for Drug Evaluation and Research (CDER), 2001). Tables 2 and 3 summarize the results of accuracy of methods A and B for **CXB** and its amides.

3.2.4. Precision

The precision of an analytical method describes the closeness of individual measures of the same analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous sample (Center for Drug Evaluation and Research (CDER),

Table 1
The average linear regression equations from the six calibration curves for the analytes.

Compound	Calibration curve equation (average of six curves)	R^2
Celecoxib (method A)	$y = 31219x - 3201.4$	1
Celecoxib (method B)	$y = 45739x + 4080.6$	0.99998
MP-CXB	$y = 30300x - 416.72$	0.99999
Cy-CXB	$y = 32445x + 13159$	0.99999
Bz-CXB	$y = 31960x - 11883$	0.99999
CBz-CXB	$y = 45277x - 5651.2$	0.99999
FBz-CXB	$y = 31671x - 14274$	0.99994

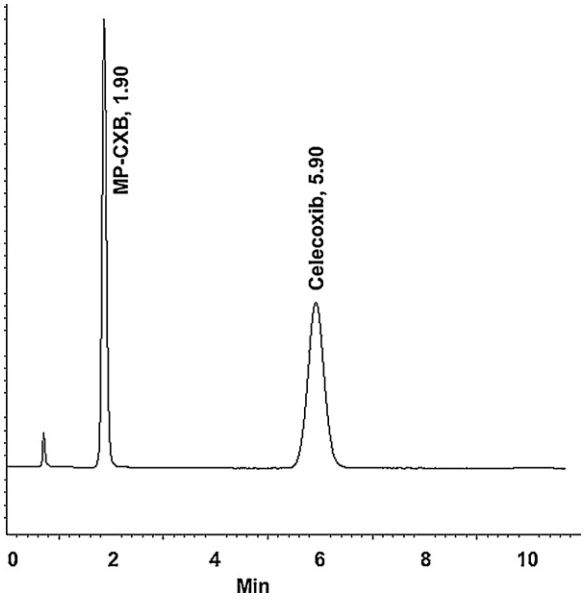


Fig. 2. HPLC chromatogram from methods A for **CXB** and **MP-CXB** from methanol.

Table 2
Accuracy of method A for the determination of **CXB** and **MP-CXB**.

Compound	Measured concentration (μg/mL) ± SD[a]		
	10	80	160
Celecoxib			
Day 1	9.85 ± 0.03	78.69 ± 0.38	162.40 ± 0.46
Day 2	10.06 ± 0.02	82.643 ± 0.28	162.48 ± 0.3
Day 3	10.11 ± 0.06	81.88 ± 0.1	160.60 ± 0.11
MP-CXB			
Day 1	10.01 ± 0.01	81.62 ± 0.06	160.32 ± 0.29
Day 2	10.05 ± 0.02	81.81 ± 0.13	161.16 ± 0.31
Day 3	10.11 ± 0.02	82.18 ± 0.09	161.72 ± 0.11

[a] Standard deviation ($n = 3$).

Table 3
Accuracy of method B for the determination of **CXB**, **Cy-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB**.

Compound	Measured concentration (μg/mL) ± SD[a]		
	10	80	160
Celecoxib			
Day 1	9.957 ± 0.03	78.09 ± 0.07	162.78 ± 0.1
Day 2	10.08 ± 0.01	77.99 ± 0.06	163.11 ± 0.4
Day 3	10.16 ± 0.02	78.17 ± 0.03	162.84 ± 0.09
Cy-CXB			
Day 1	10.26 ± 0.02	78.47 ± 0.15	160.16 ± 0.22
Day 2	10.09 ± 0.02	77.76 ± 0.45	159.84 ± 0.45
Day 3	10.12 ± 0.02	78.02 ± 0.46	161.14 ± 0.65
Bz-CXB			
Day 1	10.18 ± 0.05	80.92 ± 0.29	161.88 ± 0.22
Day 2	9.78 ± 0.06	80.67 ± 0.37	160.34 ± 0.35
Day 3	10.16 ± 0.03	78.76 ± 0.47	160.16 ± 0.66
CBz-CXB			
Day 1	10.04 ± 0.14	82.26 ± 0.17	160.90 ± 0.67
Day 2	10.22 ± 0.02	80.44 ± 0.42	161.59 ± 0.50
Day 3	9.85 ± 0.27	79.61 ± 0.07	159.43 ± 0.21
FBz-CXB			
Day 1	10.04 ± 0.14	82.26 ± 0.17	160.90 ± 0.67
Day 2	10.22 ± 0.02	80.44 ± 0.42	161.59 ± 0.50
Day 3	9.85 ± 0.27	79.61 ± 0.07	159.43 ± 0.21

[a] Standard deviation ($n = 3$).

2001). The precision is expressed as relative standard deviation (RSD) of the replicate measurement (Lindholm, 2004), where %RSD = (SD/mean) × 100 (Karnes and March, 1993). The precision is divided into three categories: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (between laboratories precision) which is not normally performed if intermediate precision is accomplished (USP, 1994). Repeatability expresses the precision under the same operating conditions over a short interval of time (International Conference on Harmonisation (ICH), 1997). Repeatability can be obtained by analysis of a minimum of three concentrations (low, medium and high) representing the entire range of the standard curve for at least five injections ($n \geq 5$) (Center for Drug Evaluation and Research (CDER), 2001). Intermediate precision shows variation affected in day to day analysis, at least two days is recommended for inter-day precision testing (International Conference on Harmonisation (ICH), 1995). Precision acceptance criteria for a method are that the intra-day precision (%RSD) will be ≤2.0% and the inter-day precision will be ≤3% (Ermer and Miller, 2005). The intra- and inter-day precision was determined by replicate injection ($n = 6$) for three different concentration (10, 80 and 160 μg/mL) of **CXB** and its amides for three consecutive days. The results obtained for intra- and inter-day precision studies are presented in Tables 4–10, respectively. As it can be seen method precision has a relative standard deviation (%RSD) below 1% for repeatability and 2% for intermediate precision.

3.2.5. Recovery

Recovery is expressed as the amount of the compound of interest analyzed as a percentage to the theoretical amount present

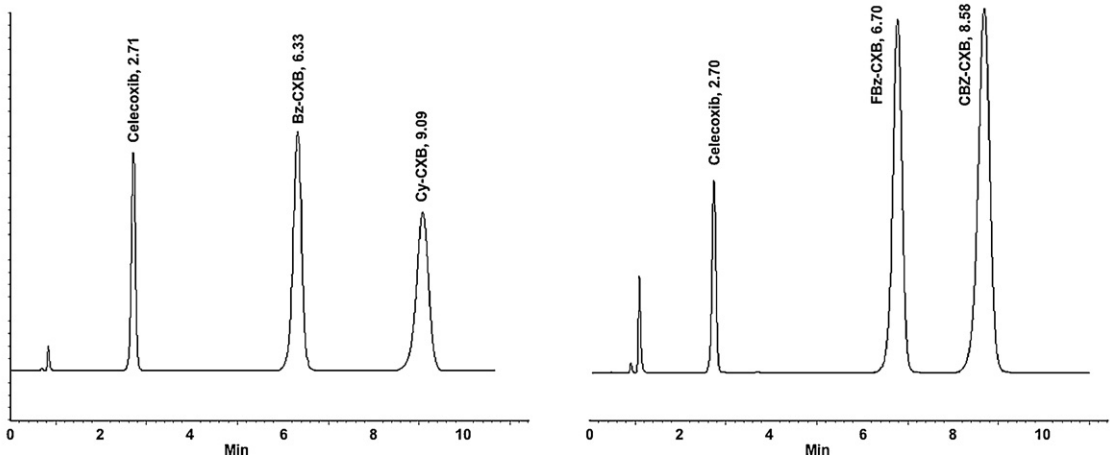


Fig. 3. HPLC chromatograms from methods B for **CXB**, **Cy-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB** from methanol.

Table 4
Inter- and intra-day precision of method A for the determination of **CXB**.

Concentration	Inter-day precision			
	Day 1	Day 2	Day 3	
10 μg/mL intra-day precision				
Average ($n=6$)	9.96 ± 0.03	10.08 ± 0.01	10.16 ± 0.02	10.06 ± 0.1
%RSD	0.28%	0.13%	0.25%	1.00%
80 μg/mL intra-day precision				
Average ($n=6$)	78.09 ± 0.07	77.99 ± 0.06	78.17 ± 0.03	78.08 ± 0.09
%RSD	0.08%	0.08%	0.04%	0.12%
160 μg/mL intra-day precision				
Average ($n=6$)	162.78 ± 0.10	163.11 ± 0.4	162.84 ± 0.09	162.91 ± 0.17
%RSD	0.06%	0.24%	0.05%	0.10%

Table 5
Inter- and intra-day precision of method A for the determination of **MP-CXB**.

Concentration	Inter-day precision			
	Day 1	Day 2	Day 3	
10 μg/mL intra-day precision				
Average ($n=6$)	10.02 ± 0.01	10.05 ± 0.02	10.12 ± 0.02	10.06 ± 0.05
%RSD	0.13%	0.23%	0.19%	0.51%
80 μg/mL intra-day precision				
Average ($n=6$)	81.62 ± 0.06	81.82 ± 0.13	82.19 ± 0.09	81.87 ± 0.29
%RSD	0.07%	0.15%	0.11%	0.35%
160 μg/mL intra-day precision				
Average ($n=6$)	160.32 ± 0.29	161.16 ± 0.3	161.72 ± 0.11	161.07 ± 0.70
%RSD	0.18%	0.07%	0.19%	0.43%

Table 6
Inter- and intra-day precision of method B for the determination of **CXB**.

Concentration	Inter-day precision			
	Day 1	Day 2	Day 3	
10 μg/mL intra-day precision				
Average ($n=6$)	9.85 ± 0.03	10.53 ± 0.03	10.11 ± 0.06	10.01 ± 0.14
%RSD	0.35%	0.49%	0.58%	1.38%
80 μg/mL intra-day precision				
Average ($n=6$)	78.70 ± 0.38	82.64 ± 0.28	81.88 ± 0.1	81.07 ± 2.09
%RSD	0.49%	0.34%	0.12%	2.58%
160 μg/mL intra-day precision				
Average ($n=6$)	162.40 ± 0.46	162.48 ± 0.29	160.62 ± 0.11	161.82 ± 1.06
%RSD	0.28%	0.18%	0.07%	0.657%

in the medium (Center for Drug Evaluation and Research (CDER), 1994). The percentage of recovery of **CXB** and its amides from liver homogenate was performed using the concentrations in Table 11. For amides that showed no hydrolysis in liver homogenate only one concentration was used. While in plasma, Table 12, only recovery for **Cy-CXB** and **MP-CXB** but not **CXB** itself because neither amide showed hydrolysis in plasma. In all cases recoveries were calculated by comparing recovered concentration to the theoretical one from spiked samples in a solution of equal volume of homogenates and plasma. The lower recovery of **Cy-CXB** in plasma compared to **MP-CXB** is most likely due to the predominant acidic nature of former, which results in higher percentage of protein binding.

3.2.6. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected or quantitatively determined, respectively, with suitable precision and accuracy (International Conference on Harmonisation (ICH), 1995). LOD and LOQ are 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

Table 7
Inter- and intra-day precision of method B for the determination of **Cy-CXB**.

Concentration	Inter-day precision			
	Day 1	Day 2	Day 3	
10 μg/mL intra-day precision				
Average ($n=6$)	10.27 ± 0.02	10.09 ± 0.02	10.12 ± 0.02	10.16 ± 0.09
%RSD	0.20%	0.22%	0.17%	0.90%
80 μg/mL intra-day precision				
Average ($n=6$)	78.48 ± 0.15	77.77 ± 0.45	78.03 ± 0.46	78.09 ± 0.36
%RSD	0.18%	0.57%	0.59%	0.46%
160 μg/mL intra-day precision				
Average ($n=6$)	160.17 ± 0.22	159.84 ± 0.45	161.15 ± 0.66	160.39 ± 0.68
%RSD	0.14%	0.27%	0.402%	0.42%

Table 8
Inter- and intra-day precision of method B for the determination of **Bz-CXB**.

Concentration	Inter-day precision			
	Day 1	Day 2	Day 3	
10 μg/mL intra-day precision				
Average (n = 6)	10.19 ± 0.05	9.78 ± 0.06	10.16 ± 0.03	10.17 ± 0.01
%RSD	0.54%	0.58%	0.34%	0.12%
80 μg/mL intra-day precision				
Average (n = 6)	80.92 ± 0.29	80.67 ± 0.37	78.76 ± 0.47	80.12 ± 1.18
%RSD	0.36%	0.46%	0.60%	1.47%
160 μg/mL intra-day precision				
Average (n = 6)	161.89 ± 0.22	160.35 ± 0.35	160.17 ± 0.65	160.78 ± 0.91
%RSD	0.14%	0.22%	0.41%	0.56%

Table 9
Inter- and intra-day precision of method B for the determination of **CBz-CXB**.

Concentration	Inter-day precision			
	Day 1	Day 2	Day 3	
10 μg/mL intra-day precision				
Average (n = 6)	10.04 ± 0.14	10.22 ± 0.23	9.86 ± 0.27	10.04 ± 0.18
%RSD	0.14%	0.23%	0.27%	1.79%
80 μg/mL intra-day precision				
Average (n = 6)	82.27 ± 0.17	80.44 ± 0.42	79.61 ± 0.07	80.77 ± 1.36
%RSD	0.2%	0.5%	0.08%	1.68%
160 μg/mL intra-day precision				
Average (n = 6)	160.91 ± 0.67	161.60 ± 0.50	159.43 ± 0.21	160.64 ± 1.10
%RSD	0.42%	0.31%	0.13%	0.68%

Table 10
Inter- and intra-day precision of method B for the determination of **FBz-CXB**.

Concentration	Inter-day precision			
	Day 1	Day 2	Day3	
10 μg/mL intra-day precision				
Average (n = 6)	9.92 ± 0.02	10.03 ± 0.02	10.10 ± 0.67	10.02 ± 0.09
%RSD	0.26%	0.23%	0.66%	0.93%
80 μg/mL intra-day precision				
Average (n = 6)	77.65 ± 0.41	80.51 ± 0.12	80.43 ± 0.20	79.53 ± 1.63
%RSD	0.53%	0.15%	0.25%	2.04%
160 μg/mL intra-day precision				
Average (n = 6)	159.71 ± 0.19	160.50 ± 1.24	161.61 ± 0.20	160.60 ± 0.95
%RSD	0.12%	0.77%	0.12%	0.59%

Table 11
Recovery data for **CXB** and its amides in liver homogenate.

	Compound						
	CXB	**MP-CXB**	**Cy-CXB**		**Bz-CXB**	**CBz-CXB**	**FBz-CXB**
Actual (μg/mL)	10	200	10	40	10	10	10
Mean recovered (μg/mL)	9.79	197.91	9.492	39.70	9.492	9.776	8.40
%Recovery	97.9	98.95	94.92	99.26	94.92	97.76	84
SD[a]	0.21	0.44	0.5	0.05	0.5	0.204	0.322

[a] Standard deviation (n = 3).

Table 12
Recovery data for **MP-CXB** and **Cy-CXB** from human plasma.

	Compound		
	MP-CXB	**Cy-CXB**	
Actual (μg/mL)	200	10	40
Mean recovered (μg/mL)	196.93	6.621	26.77
%Recovery	98.46	66.21	66.92
SD[a]	0.36	0.08	0.51

[a] Standard deviation (n = 3).

Table 13
LOD and LOQ for **CXB** and its amides.

	CXB[a]	CXB[b]	MP-CXB[a]	Cy-CXB[b]	Bz-CXB[b]	CBz-CXB[b]	FBz-CXB[b]
LOD (ng/mL)	9.74	1.06	13.6	0.40	0.59	0.36	1.39
LOQ (ng/mL)	29.50	3.2	40.00	1.20	1.80	1.10	4.20

[a] Method A.
[b] Method B.

Table 14
Physico-chemical properties of **CXB**, **MP-CXB**, **Cy-CXB** and the benzamides.

Cmpd	MP (°C)	log P_{app}			Solubility (μg/mL) at rt		
		Water	pH 5.4 (6.8)	pH 7.4	Water	pH 5.4 (6.8)	pH 7.4
CXB[a]	157–159	3.50	–	–	3.00–7.00	–	–
MP-CXB	296–298	0.99	1.5 (pH 6.8)	1.40 ± 0.04	>45,000	>45,000 (pH 6.8)	>45,000
Cy-CXB	289–290	2.52 ± 0.21	4.50 ± 0.68	3.59 ± 0.57	1587.60 ± 38.9	0.53 ± 0.05	53.3 ± 9.84
Bz-CXB	332–334	1.78 ± 0.03	ND	4.74 ± 0.34	1125.20 ± 69.36	ND	0.17 ± 0.01
ClBz-CXB	342–344	2.80	ND	4.62	234.37 ± 9.55	ND	5.83 ± 0.61
FBz-CXB	337–339	2.13 ± 0.03	ND	5.35 ± 0.371	624.71 ± 18.92	ND	0.27 ± 0.05

[a] Literature (Subramanian et al., 2004). ND: not determined.

limit, respectively (International Conference on Harmonisation (ICH), 1997). Table 13 shows the calculated LOD and LOQ for **CXB** and its amides.

3.3. Physico-chemical properties of CXB acylamides

3.3.1. Aqueous solubility

The **MP-CXB** was synthesized with the intention of enhancing solubility in the GIT over all the pH range. The solubility of this amide, CXB, Cy-CXB and the other benzamide in some dissolution media is summarized in Table 14.

It is important to mention that the solubility of **MP-CXB** is higher than 45 mg/mL regardless of the pH of the solution. This is due to the fact that it contains functional groups that are ionizable at both acidic and basic pH (calculated p*Ka*: N_{pip}-CH_3 = 7.44, N_{pip}-CH_2-CO = 4.53 and NH-SO_2 = 5.40). The halogenated benzamides (**CBz-CXB** and **FBz-CXB**) have the lowest water solubility, which is expected due the presence of the chlorine and fluorine atoms. The solubility of **Cy-CXB** in basic media, were the drug is expected to be ionized, is 100 more than that in a more acidic media, pH 5.4. It must be mentioned here that in general, the solubility of all amides, except **MP-CXB**, is lower at pH 7.4 compared to that in water (pH 6.9), which is may be due to the fact the buffer species have much higher solubility in water than the tested amides and hence can salt them out.

3.3.2. Partition coefficient

Again, as seen in Table 14, **MP-CXB** has the lowest log P_{app} due to the fact that it has a significant ionized fraction in the studied pH range. The rest of the amides showed high log P_{app} values at pH 7.4 (3.59–5.35) and the most lipophilic were the benzamides, which correlates with the solubility data in Table 14. This data is consistent with reported hydrophobicity constants of cyclohexane, benzene, chlorobenzene and fluorobenzene (Hung et al., 2010; Ruelle, 2000).

3.4. Chemical hydrolysis of MP-CXB in aqueous phosphate buffer

The chemical hydrolysis of **MP-CXB** was studied at GIT-relevant pH values, i.e. 1.2, and 6.8 and 7.4. In all experiments, 0.16 M aqueous phosphate buffer was used and the ionic strength was adjusted to 0.44. First, the hydrolysis was studied at 80 °C and as seen in Fig. 4, the chemical hydrolysis follows pseudo first-order kinetics.

The first order degradation rate constants (k_{obs}) and half-lives of **MP-CXB** in pH 1.2, 6.8 and 7.4 at 80 °C are summarized in Table 15. It can be seen that **MP-CXB** was hydrolyzed the fastest in pH 1.2 with half-life of 3.53 h, compared to 72 and 96 days in pH 6.8 and 7.4, respectively.

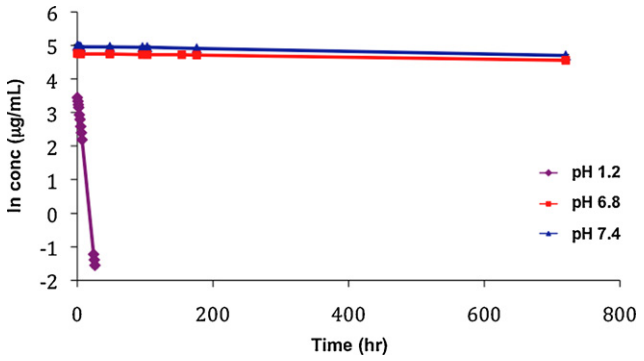


Fig. 4. The hydrolysis of **MP-CXB** in pH 1.2, 6.8, and 7.4, 0.16 M phosphate buffer at 80 °C, μ = 0.44.

Table 15
The first order degradation rate constants (k_{obs}) and half-lives of **MP-CXB** in pH 1.2, 6.8 and 7.4 at 80 °C.

pH	k_{obs} (h^{-1})	$t_{1/2}$ (h)
1.2	$1.96 \times 10^{-1} \pm 3.00 \times 10^{-3}$	3.53 ± 0.05
6.8	$4.00 \times 10^{-4} \pm 0.00$	1732.40 ± 0.00
7.4	$3.00 \times 10^{-4} \pm 0.00$	2310.00 ± 0.00

The stability of **MP-CXB** in relatively basic media was surprising, at first, since an extensive report by Larsen et al. (1988) have previously demonstrated that the introduction of an α-amino group (even a tertiary one) will facilitate the hydrolytic reaction both at acidic and basic media by intramolecular catalysis (Larsen et al., 1988). It is important to consider that while the amides reported by Larsen et al. are tertiary, **MP-CXB** is secondary and hence its amide group is prone to ionization, which renders it resistant to hydrolysis, Fig. 5. Another additional explanation can be attributed to the presence of another nitrogen atom in piperazine ring. The most basic nitrogen atom in **MP-CXB**, due to steric and electronic factors, will most likely be the *N*-methylated one and

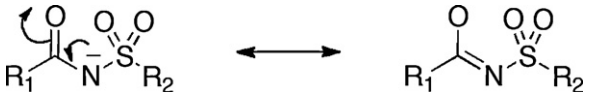


Fig. 5. The resonance stabilization of the deprotonated sulfonyl acylamide groups.

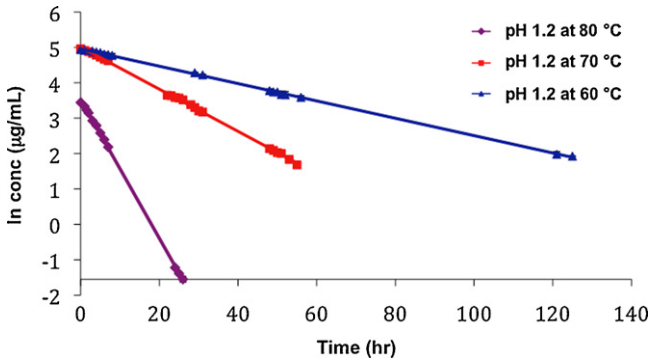


Fig. 6. The hydrolysis of **MP-CXB** in pH 1.2 in 0.16 M phosphate buffer at 60, 70, 80 °C, $\mu = 0.44$.

Table 16
The observed rate constants and half-lives for degradation of **MP-CXB** in aqueous buffer solutions pH 1.2 at different temperatures.

Temperature, T (K)	k_{obs} (h^{-1})	R^2	$t_{1/2}$ (h)
333	$2.45 \times 10^{-2} \pm 3.00 \times 10^{-4}$	0.999	28.28 ± 0.34
343	$5.95 \times 10^{-2} \pm 6.00 \times 10^{-5}$	0.999	11.64 ± 0.13
353	$1.96 \times 10^{-1} \pm 2.80 \times 10^{-3}$	0.9995	3.53 ± 0.05

hence, undermines the α-nitogen's participation in hydrolysis. On the other hand, in acidic media, **MP-CXB** is hydrolyzed in a faster rate and intramolecular catalysis described by Larsen et al. (1988) will play a role since both nitrogens of the piperazine can be ionized at this pH. In addition, at this pH, the amide of **MP-CXB** will not be ionized and there will be much less resistance to hydrolysis.

Since the hydrolysis of **MP-CXB** at pH 6.8 and 7.4 was slow, only the rate of its hydrolysis at pH 1.2 was investigated further by determining its hydrolysis rate at 60 and 70 °C in 0.16 M phosphate buffer of ionic strength of 0.44, Fig. 6 and Table 16.

It is obvious that the hydrolysis is influenced by temperature and the relation between $\ln k_{obs}$ and $1/T$ followed the Arrhenius relationship, $R^2 = 0.9911$, over the temperature region studied according to the following equation: $\ln k_{obs} = \ln A - (E_a/RT)$. Where ($E_a$) is the energy of activation and (A) is the pre-exponential coefficient for the degradation reaction. The activation energy for the hydrolysis of **MP-CXB** in aqueous solution was found to be 23.83 kcal mol^{-1} while the pre-exponential coefficient was found to be 32.27. The observed first-order constant and half-life at room temperature 298 K and at 310 K are also calculated from the linear plot of Arrhenius equation and found to be 3.36×10^{-4} h^{-1} and 2059.82 h (86 days), 1.60×10^{-3} h^{-1} and 433.42 h (18 days), respectively (Fig. 7).

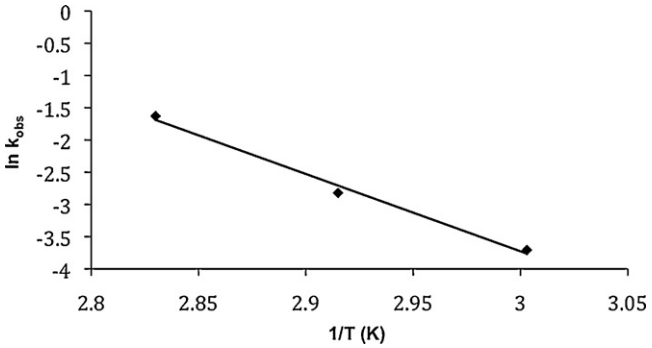


Fig. 7. Arrhenius plot of **MP-CXB** in 0.16 M phosphate buffer pH 1.2.

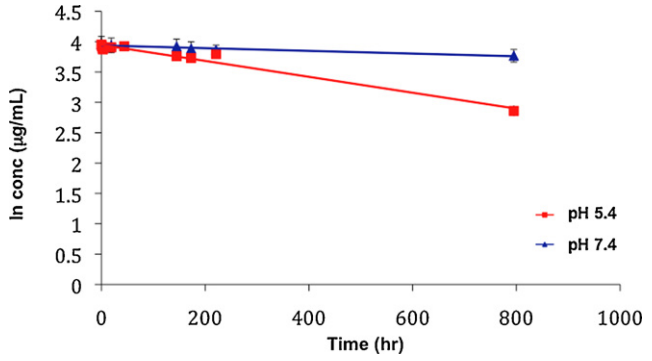


Fig. 8. Degradation of **Cy-CXB** in 0.16 M phosphate buffer pH 5.4 and 7.4 at 80 °C, $\mu = 0.44$.

3.5. Chemical hydrolysis of *Cy-CXB* in aqueous phosphate buffer

The effect of pH on the chemical hydrolysis of the synthesized **Cy-CXB** was studied at skin-relevant pH values, i.e. 5.4 and 7.4 in 0.16 M aqueous phosphate buffer solutions of ionic strength of 0.44 at 80 °C (Fig. 8).

The observed rate constants of **Cy-CXB** in pH 5.4 and 7.4 were 1.30×10^{-3} ($R^2 = 0.9675$) and 2.00×10^{-4} ($R^2 = 0.9386$), respectively. It will be difficult to compare these data to those of **MP-CXB** because of the difference in the dissolution media, i.e. the use of acetonitrile as a cosolvent in the study of the hydrolysis of **Cy-CXB**. The hydrolysis of **Cy-CXB** was slow at the selected pH values; hence, no further studies were performed.

3.6. In vitro enzymatic hydrolysis study

3.6.1. Hydrolysis in liver homogenate

The enzymatic hydrolysis of all acylamide was studied in 20% liver homogenate at 37 °C. Of all the studied amides only the ali-

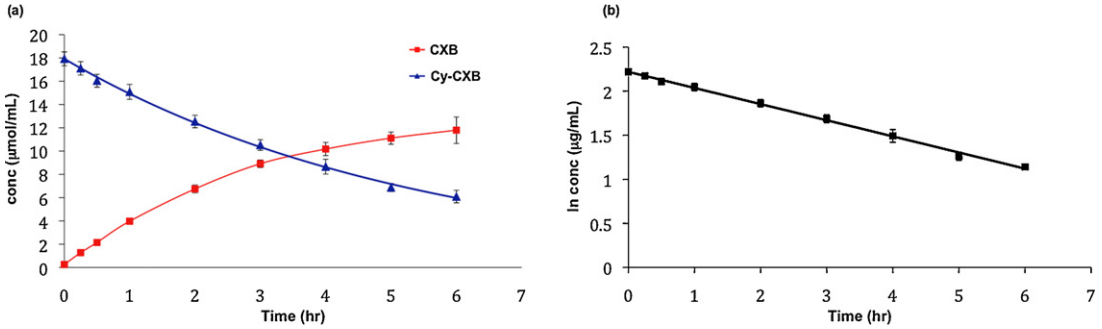


Fig. 9. Enzymatic hydrolysis of **Cy-CXB** in 20% liver homogenate at 37 °C.

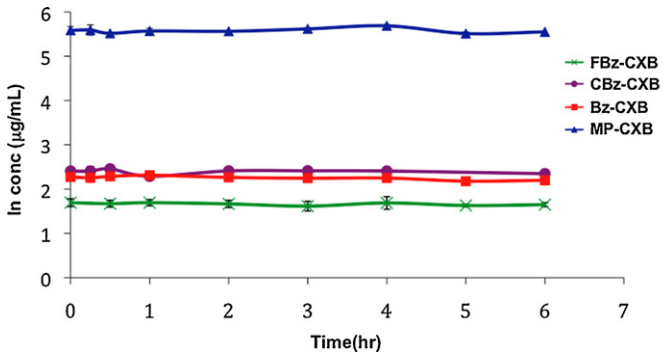


Fig. 10. Enzymatic hydrolysis of **MP-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB** in 20% liver homogenate at 37 °C.

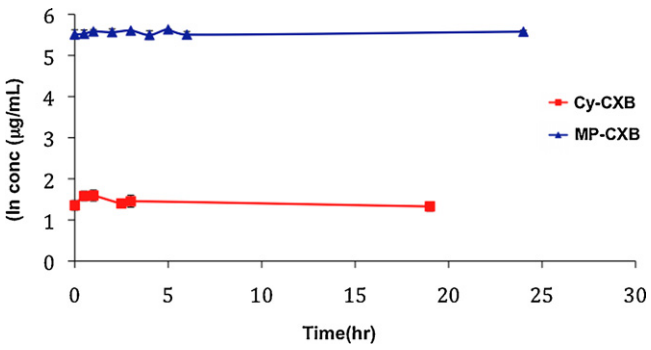


Fig. 11. Enzymatic hydrolysis of **MP-CXB** and **Cy-CBX** in 80% human plasma at 37 °C.

cyclic amide **Cy-CXB** was hydrolyzed under these conditions. The disappearance of **Cy-CXB** and the appearance of the parent drug, **CXB**, are shown in Fig. 9(a). As seen in Fig. 9(b), the hydrolysis of **Cy-CXB** follows pseudo-first order kinetic with a k_{obs} equal to 1.83×10^{-1} and half-life of 3.79 h ($R^2 = 0.9977$).

As mentioned earlier, **MP-CXB**, **Bz-CXB**, **CBz-CXB** and **FBz-CXB** did not show visible hydrolysis in 20% liver homogenate, Fig. 10. Further discussion of the point can be seen in the next section.

3.6.2. Hydrolysis in human plasma

The hydrolysis of Cy-CXB, which was the only amide that showed hydrolysis in liver homogenate, and MP-CXB were studied in 10% human plasma. As seen in Fig. 11, there is no detectable hydrolysis even after 18–24 h.

Examining the literature concerning prodrugs reveals that they are most commonly esters. The ester linkage can easily be hydrolyzed by various esterases, amidases, and proteases and sometimes can be rapidly hydrolyzed at pH 7.4 without the aid of enzymes (Qandil et al., 2008). The secondary sulfonyl acylamide moiety, on the other hand, is much more resistant to hydrolysis chemically and in plasma as demonstrated by this work and earlier reports, most likely due to ionization of the sulfonamide amide group (Larsen and Bundgaard, 1987; Mamidi et al., 2002) and only tertiary sulfonyl acylamide, which is unionizable due to the lack of the acidic proton, were shown to undergo chemical hydrolysis and in human plasma with relative ease (Larsen et al., 1988). On the other hand, Mamidi et al. (2002) shown that simple acylamides of **CXB**, Fig. 12, were hydrolyzed to variable degrees in live homogenates with the following approximate half-lives, **Ac-CXB** < 2 h and **Pr-CXB** = **Bu-CXB** < 30 min. It has also been reported that parecoxib, Fig. 12, is hydrolyzed to its parent drug valdecoxib by a non cytochrome P-450 hepatic enzyme (Karim et al., 2001).

The fact that only Cy-CXB underwent hydrolysis in liver homogenate and taking in consideration the report by Mamidi et al. (2002) leads to important observations. Firstly, it is obvious that the enzymes in liver homogenate can hydrolyze aliphatic amides of celecoxib whether they are straight chain or cycles. Secondly, the hydrolysis rate increases as the length of the side chain increases, but in the case of the cyclohexyl moiety it appears that its steric bulk lead to slower hydrolysis. Thirdly, from the data presented in this work, it is clear that lipophilicity is not the decisive factor in the rate of hydrolysis since lipophilic aromatic amide (**Bz-CXB**, **CBz-CBX** and **FBz-CZB**) did not show any hydrolysis. These observations can lead to the suggestion that the binding of the amide to its hydrolyzing enzyme might involve hydrophobic interactions rather than van der Waal interactions since amides bearing saturated hydrocarbon side chains were selectively hydrolyzed. Hydrophobic interactions increases in strength in a proportional manner to the side chain size (Davey et al., 1976; Ho et al., 1973; Lew et al., 2000) and it becomes weaker when the side chain is branched or cyclic (Imai et al., 2006). In addition, these interactions are entropy driven and the aromatic rings are generally much better hydrated than aliphatic or alicyclic side chains (Raschke and Levitt, 2004), hence aromatic rings do not form hydrophobic interaction as readily as saturated hydrocarbons. The lack of hydrolysis of the arylamides also suggests that van der Waal forces may not play a significant role in the amide-enzyme interaction. Finally, the above conclusion can easily predict that **MP-CXB** will not be hydrolyzed in liver homogenate. If this information were available to us before hand, our choice in a candidate for a prodrug with enhanced water solubility in the GIT conditions would have taken a different direction, which further emphasizes the importance of this work. It is worth mentioning that a report for the liver homogenate hydrolysis of amide prodrugs of ketorolac showed that the hydrolysis reaction can be selective with regard to the chemical structure of the promoiety distinguishing between

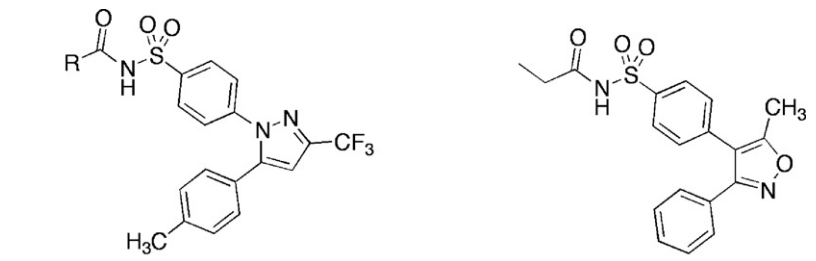


Fig. 12. The chemical structures of Mamidi et al. **CXB** acylamides and parecoxib.

the bulkiness of the group as well as its electronic properties (Kim et al., 2005).

4. Conclusion

Various sodium celecoxib acylamides were successfully synthesized. Two simple and relatively fast HPLC methods were developed and validated for the determination of theses amides and **CXB** in aqueous solution and from biological samples. The two methods were linear, precise, accurate with very good limits of detection of all analytes. These methods allowed for the simultaneous determination of celecoxib and its acylamides. The physico-chemical properties of the synthesized amides were determined. This work has shown that the sulfonyl acylamides are very stable at room temperature and resistant to hydrolysis in basic conditions even at high temperatures while they can be hydrolyzed in acidic pH 1.2, at high temperatures. Only aliphatic and alicylic acylamides of celecoxib can be hydrolyzed in liver homogenate which lead to the conclusion that hydrophobic interactions in the enzyme's catalytic site are a determining factor in this hydrolysis. The transdermal permeation **Cy-CXB** as potential prodrug of **CXB** and the oral bioavailability **CXB** from this amide will be fully investigated in the near future. **MP-CXB** will not be investigated further because of its resistance to enzymatic and chemical hydrolysis at physiological conditions although it possesses the desired physico-chemical properties.

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