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Determination of Duloxetine and Its Major Metabolites in Rabbit Plasma by High-Performance Liquid Chromatography

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ABSTRACT
A rapid and sensitive high performance liquid chromatographic method is described for simultaneous determination of duloxetine and its major metabolites, such as 4- hydroxy duloxetine (M7), Glucuronide conjugate of 5-hydroxy-6-methoxy duloxetine (M6) and Glucuronide conjugate of dihydrodiol duloxetine (M12) in rabbit plasma. HPLC analysis was carried out on a µ-Bondapak C_{18} column (250 mm × 4.6 mm, 5µm particle size) using methanol: phosphate buffer (pH 7.9, 50 mM) (7:3 v/v) as the mobile phase at a flow rate of 1.5 ml/min. Detection was carried out at 224 nm with an UV detector. The above metabolites present in the rabbit plasma were characterized by retro-synthesis followed by NMR and MS study for structure confirmation and finally injected separately into the HPLC system. All the three retention time matches with the metabolites present in the plasma sample.

Keywords: Duloxetine, Metabolites, HPLC, Retro-synthesis, Characterization.

INTRODUCTION

Duloxetine (N-Methyl-3-naphthlen-yloxy-3-thiophen-2-yl-propan-1-amine) a selective serotonin and norepinephrine reuptake inhibitor (SSNRI) is used for the treatment of major depressive disorder and anxiety. [1-3] It is used for the treatment of neuropathic pain associated with peripheral neuropathy especially diabetic polyneuropathy for which it is first-line, and as an add-on treatment in stress urinary incontinence instead of surgery [4-5] also indicated for the management of fibromyalgia. [6-7] It restores the balance of neurotransmitters in the brain like serotonin and norepinephrine. [8] Moreover it is also being used in the treatment of peripheral neuropathy caused by certain anticancer drugs. [9]

A literature survey indicated few methods for the determination of duloxetine and its key intermediate, desmethyl-duloxetine, in human serum by HPLC. [10-11] Reports were found regarding the characterization of phenolic impurities in duloxetine samples by MS, NMR spectrometry and X-ray analysis [12] and impurities formed by interaction of duloxetine with various enteric polymers. [13] A simple UV spectrophotometric method for the estimation of duloxetine in a formulation was reported. [14] An HPLC method to separate duloxetine and structurally related impurities using a combination of computer-based solvent strength optimization and solvent selectivity mixture design. [15] An HPTLC method for estimation of duloxetine in bulk and in tablet dosage form. [16] A capillary electrophoresis with laser-induced

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fluorescence detection method also reported for estimation of duloxetine in human plasma. [17] During our literature survey, very few articles related to the stability-indicating HPLC determination of duloxetine and its metabolites in rat were found [18-24] but no article related to the measurement of duloxetine and its metabolites in rabbit plasma was reported. Therefore the aim of the present work is to develop a novel, isocratic, RP- HPLC method for the determination of duloxetine and its metabolites in rabbit plasma and the structure of the metabolites were characterized through retro-synthesis followed by MS and NMR study. The characterization of the metabolites will open a new scope of research on toxicity study. The findings of toxicity study will help in scrupulous determination of expiry, adverse effects etc. This method has also been successfully applied in the pharmacokinetics study of duloxetine after orally administrating to New Zealand rabbits.

MATERIALS AND METHODS

Apparatus

Experiments were performed using a Waters (India) 510 HPLC system with Waters 486 tunable absorbance detector. The samples were injected manually using a 200µL sample loop. The Millennium-32 software was used for quantification and data processing.

Chemicals and reagents

Pure duloxetine (Fig. 1) was provided by Wockhardt Limited, Mumbai, India. Methanol and water of HPLC grade and were purchased from Merck (India) Ltd., Mumbai, India. The probable *in-vivo* metabolites were divined through retro-synthesis (Fig. 1) and were synthesized in our own laboratory (Fig. 2, 3, 4). All these and other chemicals used in the experiments were of analytical grade and were purchased from commercial sources.

Synthesis of *in vivo* metabolites present in rabbit plasma

The initial object was to prepare thiophene side chain i.e N-methyl-3-hydroxy-3-(2-thienyl) propylamine (A) for that 2-Acetylthiophene (A3) was prepared by the acetylation of thiophene (A1) with acetic anhydride (A2) in the presence of orthophosphoric acid. Placed (A1) and (A2) in a three necked flask, fitted with a thermometer, mechanical stirrer and reflux condenser. Heated the stirred solution to 70-75°C, removed the source of heat, added 87 percent orthophosphoric acid. An exothermic reaction occurred after 2-3 minutes and temperature raised up to 90°C; Immersed the flask in a bath of cold water to control the reaction. When the boiling subsides (ca. 5 minutes) refluxed the mixture for 2 h at 175-190°C, added water, stirred for 5 minutes, transferred the cold reaction mixture to a separatory funnel, removed the water layer, washed with 5 percent sodium carbonate solution and dried over anhydrous magnesium sulphate. Distilled the orange-red liquid through a short fractionating column at atmospheric pressure and recovered some portion of unchanged thiophene at 83-84°C. Distilled the residue under reduced pressure to collect (A3) at 89-90°C/10mm; this solidifies on cooling in ice, with this methylamine hydrochloride, paraformaldahyde, concentrated hydrochloric acid and isopropanol were added and this mixture was heated to reflux and stirred for 6 h. The mixture was then cooled to 0°C and stirred for one hour more. The slurry was then filtered, and the solid was washed with cold ethanol. The washed solid was dried for 16 hrs at 50°C to obtain 2-thienyl 2-methylaminoethyl ketone hydrochloride (A4), as a white solid. This intermediate product (A4) was stirred in presence of ethanol at ambient temperature, and the pH of the solution was raised to 11-12 by slow addition of sodium hydroxide. A required portion of sodium borohydride was added, and the mixture was stirred at ambient temperature for 4 h. Then acetone was added, and the mixture was stirred for 20 minutes more. The mixtures were then concentrated by evaporation to white slurry and add methyl t-butyl ether. The mixture was acidified to pH 1-1.5 by addition of concentrated hydrochloric acid, and the solution was stirred for ten minutes. The pH was then made basic to pH 12 by slow addition of sodium hydroxide. The layers were then separated, the aqueous phase was extracted with methyl t-butyl ether and the organic phases were combined and washed once with water. The organic phase was concentrated by evaporation to get a solid product (A).

Second objective was to prepare corresponding fluoronaphthols. The first compound, 1-fluoronaphth-6-ol (B) was synthesized from commercially available 1-aminonaphth-6-ol (B1) using a modified Schiemann reaction. [25-26] The diazonium tetrafluoroborate intermediate (B2) was isolated and dried. The dried powder was then heated in decahydronaphthalene at 160°C to give the desired fluoronaphthol, B. The 1-fluoronaphth-4-ol (E) was synthesized from the commercially available 1-fluoro-naphthalene (E1). Compound (E1) was formylated by stirring with 1, 1-dichloromethyl methyl ether and $SnCl_4$ at 0°C in methylene chloride to give 4-fluoro-1-naphthaldehyde (E2). [27] Baeyer Villiger rearrangement followed by saponification of the corresponding formate gave the desired product (E) in good overall yield. The hydroxy group on 1-fluoronaphth-6-ol (B) was protected as methyl ether. The resulting 1-fluoro-6-methoxynaphthalene (G1) was then formylated as described above. The formylation occurred exclusively at the C-5 position. [28-29] This aldehyde was then converted to the corresponding 1-fluoro-6-methoxynaphth-5-ol (G), by following the reactions shown in the reaction scheme.

To synthesize 4-hydroxy duloxetine (M7) and 5-ydroxy-6-methoxy duloxetine (M_{HMD}), thiophene side chain (A) was condensing with the corresponding fluoronaphthols, E and G. The hydroxyl groups were protected as ketals or as acetals, under the conditions

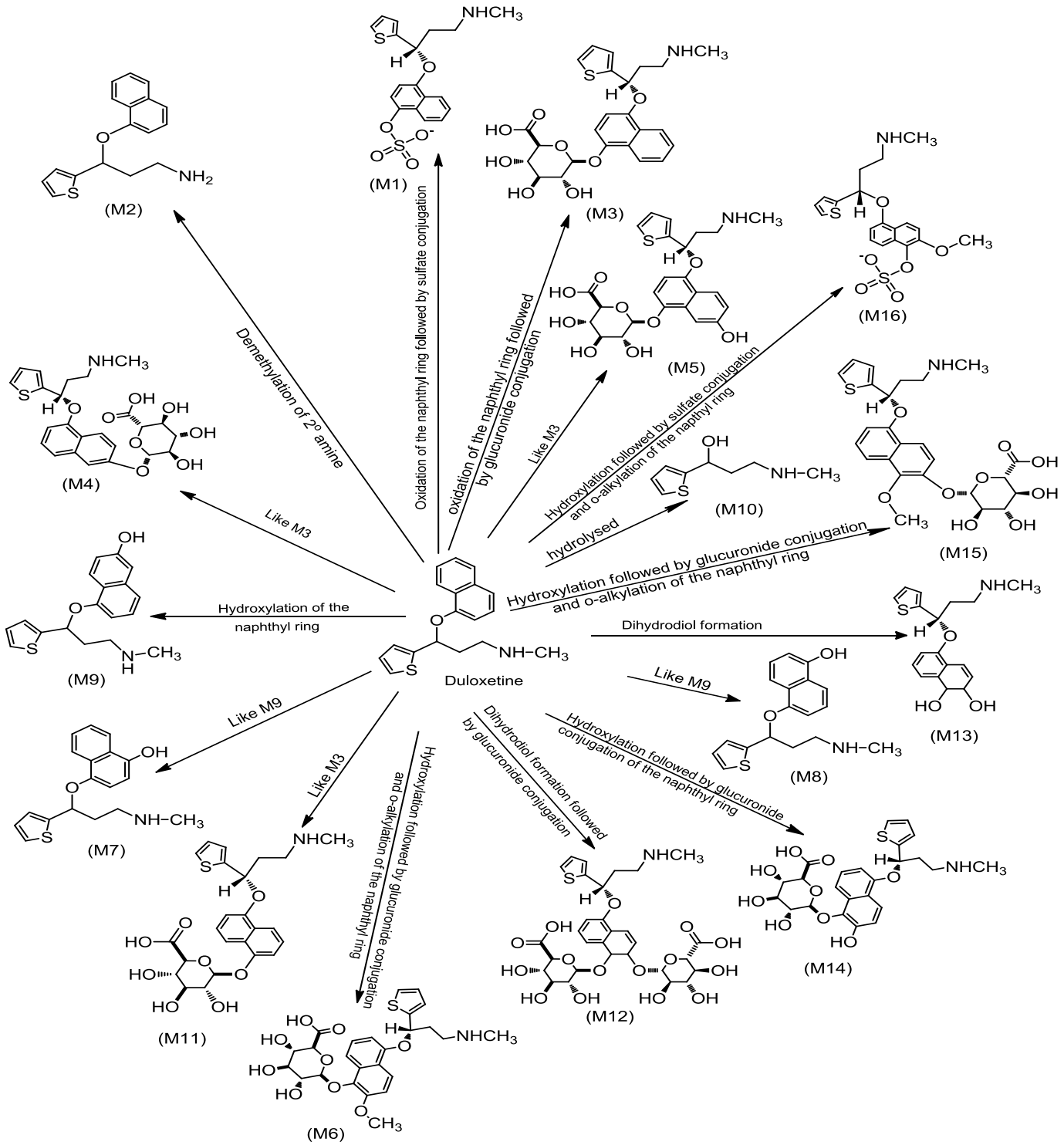

Fig. 1: Proposed *in vivo* metabolic scheme of duloxetine in rabbit after oral administration.

for the synthesis of duloxetine. [30] The protecting groups were then removed by acetic acid.

The glucuronide conjugate of 5-hydroxy-6-methoxy duloxetine (M6) was synthesized by o-alkylation of M_{HMD} with acetobromo-α-D- glucuronic acid methyl ester followed by saponification of the ester group.

Demethylation of G, with BCl_3 at room temperature yielded J1. Treatment of J1 with trimethyl orthoformate gave a cyclic ortho-formate J2. After condensation with A, the desired product was isolated as a cyclic ortho-formate and hydrolyzed to yield J3. Compound J3 was then stirred under oxygen atmosphere in the presence of large excess of sodium bromohydride in ethanol to compound J4. Thereafter, prepared J4 was treated with L and LiOH in methanol at room temperature gave the conjugate J5. Saponification with excess of LiOH in aqueous methanol gave M12.

M7: ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.381 (s, 3H, -NH-C**H$_3$**), 1.650 (m, 1H, -N**H**), 2.691 (t, 2H, J=5.848 Hz, **-**CH_2-C**H$_2$-**NH), 2.905 (m, 2H, **-**C**H$_2$-**CH_2-NH-), 4.965 (t, 1H, *J*= 5.769 Hz, **-CH-**CH_2-CH_2-NH-), 6.821(m,1H, Ar-H), 6.902 (1H, dd, J=8.633 Hz, J=4.870 Hz, Ar-H), 7.362 (dd, 1H, J=8.633 Hz, J=3.556 Hz, Ar-H), 6.495 (q, 1H, *J*= 7.489 Hz, Ar-H), 6.564 (q, 1H, *J*= 7.289 Hz, Ar-H), 5.36

(s, 1H, -OH), 7.629 (ddd, 1H, J=8.327 Hz, J=7.579 Hz, J=1.396 Hz, Ar-H), 7.650 (ddd, 1H, J=8.340 Hz, J=7.579 Hz, J=1.393 Hz, Ar-H), 8.216 (m,1H, Ar-H), 8.292 (m,1H, Ar-H); Mass (m/z) ESI TOF: 314.12 (M+H).

M6: ^{1}H NMR (400 MHz, $CDCl_3$): 2.431 (s, 3H,-NH-C**H$_3$**), 3.871 (s, 3H, O-CH_3), 1.652 (m, 1H, -NH), 2.693 (t, 2H, J=5.851, -C**H$_2$**-NH-), 2.027 (td, 2H, J=5.851, J=5.772, -C**H$_2$**-CH_2-NH-), 5.428 (t, 1H, J=5.772, **-CH-**CH_2-CH_2-NH-), 7.387 (dd, 1H, J=8.081, J=5.490, Ar-H), 7.320 (dd,1H, J=8.900, J=8.095, Ar-H), 7.483 (dd, 1H, J=5.490, J=1.385, Ar-H), 7.057 (ddd, 1H, J=8.095, J=3.900, J=1.748, Ar-H), 7.354 (dd, 1H, J=8.081, J=1.385, Ar-H), 6.504 (d, 1H, J=8.729,Ar-H), 7.407 (ddd, 1H, J=8.900, J=4.376, J=1.748, Ar-H), 7.567 (ddd, 1H, J=8.729, J=4.376, J=3.900, Ar-H), 3.774 (dd, 1H, J=10.160, J=3.450,CY-H), 3.276 (dd, 1H, J=10.260, J=10.160,CY-H), 3.925 (dd, 1H, J=3.450, J=2.680,CY-H), 5.042 (d, 1H, J=10.260, CY-H), 4.038 (d, 1H, J=2.680,CY-H), 3.631 (m, 1H, o, CY-O**H**), 10.921 (m, 1H, CY-COO**H**); ESI TOF: 520.16 (M+H).

M12: 2.431 (s, 3H,-NH-C**H$_3$**), 1.652 (m, 1H, -NH), 2.694 (t, 2H, J=5.857,-C**H$_2$**-NH-), 2.029 (td, 2H, J=5.857, J=5.777, -C**H$_2$**-CH_2-NH-), 5.429 (t, 1H, J=5.777, **-CH-**CH_2-CH_2-NH-),7.519 (dd, 1H, J=8.079, J=5.464, Ar-H), 7.320 (dd, 1H, J=8.900, J=8.117, Ar-H), 7.423 (dd, 1H, J=5.464, J=1.387, Ar-H), 7.038 (ddd, 1H, J=8.117, J=4.675, J=1.679, Ar-H), 7.354 (dd, 1H, J=8.079, J=1.387, Ar-H), 6.844 (d, 1H, J=8.722, Ar-H), 7.457 (ddd, 1H, J=8.900, J=4.759, J=1.679, Ar-H), 7.579 (ddd, 1H, J=8.722, J=4.759, J=4.675, Ar-H), 3.775 (m, 1H, o, CY-H), 3.257 (m, 1H, o, CY-H), 3.924 (m, 1H, o, CY-H), 5.238 (m, 1H, o, CY-H), 4.038 (m, 1H, o, CY-H), 3.695 (m, 1H, o, CY-O**H**), 10.924 (m, 1H, o, CY-COO**H**); Mass (m/z) ESI TOF: 684.19 (M+H).

Animals

Three healthy rabbits (New Zealander strain) of either sex weighing 1.4-2.5 kg were selected. They were housed under standard conditions for a week. For conducting this experiment permission has taken from the University Animal Ethics Committee, Berhampur University, Odisha, India. Animals were deprived of food for 12 h before administration of the duloxetine, while water was allowed ad libitum. Each rabbit was administered a dose of 45 mg/kg of duloxetine by oral route via oral gavage in the form of an aqueous suspension.

Biological sample preparation

For identification of circulating metabolites, blood samples (2 mL) were collected from the marginal ear vein at the intervals of 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 12 h after dosing in heparinized tubes containing sodium heparin. After sampling, whole blood was centrifuged (ELTEK RC 4815 F, India) at 5000 rpm for 25 min, and plasma (3 mL) was transferred to Eppendorf snap-capped tubes and stored at -20°C for future analyses of duloxetine and metabolites. Physiological saline (2 mL) was administered to compensate for the blood loss after each blood was withdrawn. In order to ensure the effective separation of duloxetine and its circulating metabolites from plasma components, the deproteinization method was used. Three (3) mL of acetronitrile was added to1 mL of plasma. The mixture was vortex-mixed for 1 min and centrifuged for 15 min at 1600 rpm. The supernatant was transferred to a tube and evaporated to dryness (approximately 15 min), under a stream of nitrogen. The resulting residue was reconstituted with mobile phase (200µL) and immediately subjected to analysis using the proposed HPLC method for estimation of duloxetine and its major metabolites.

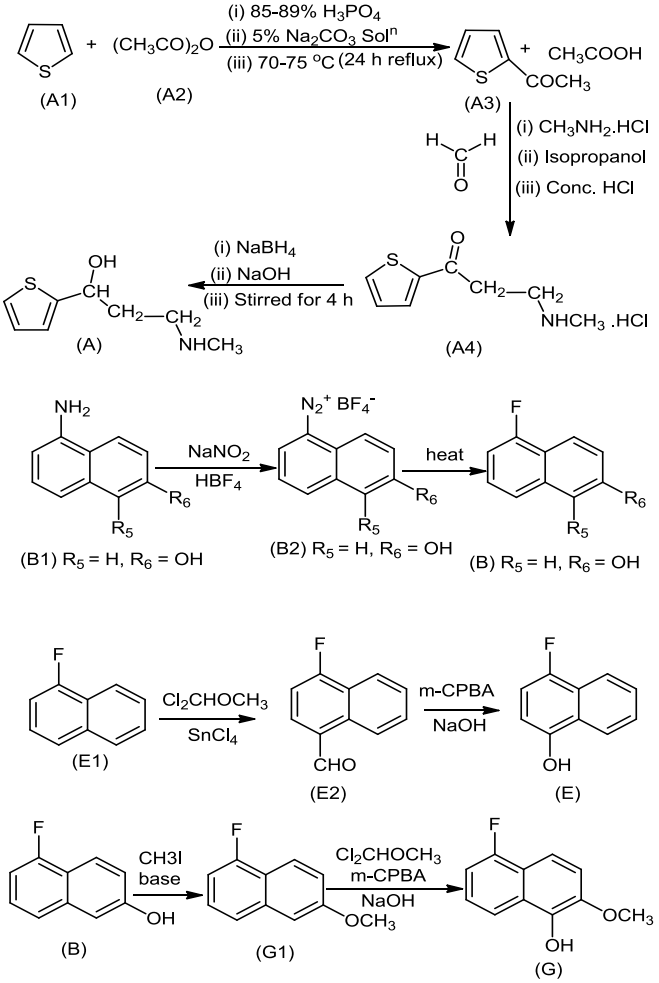

Fig. 2: Synthetic scheme for the metabolites of duloxetine (part-I).

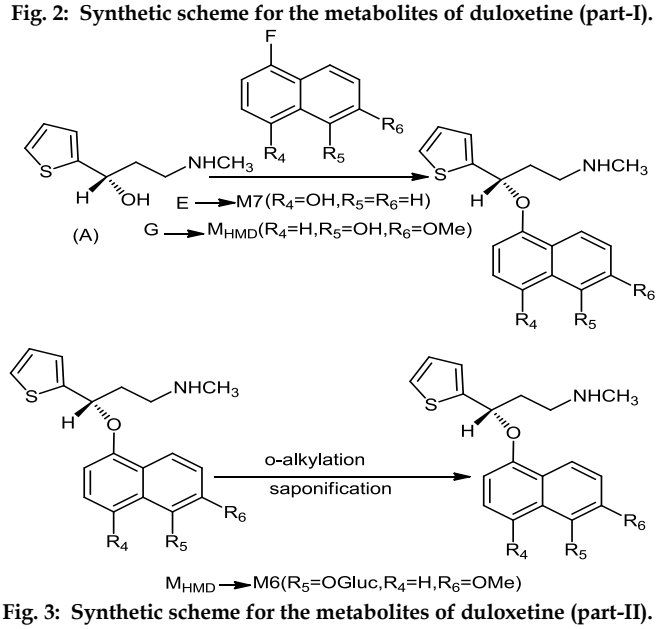

Fig. 3: Synthetic scheme for the metabolites of duloxetine (part-II).

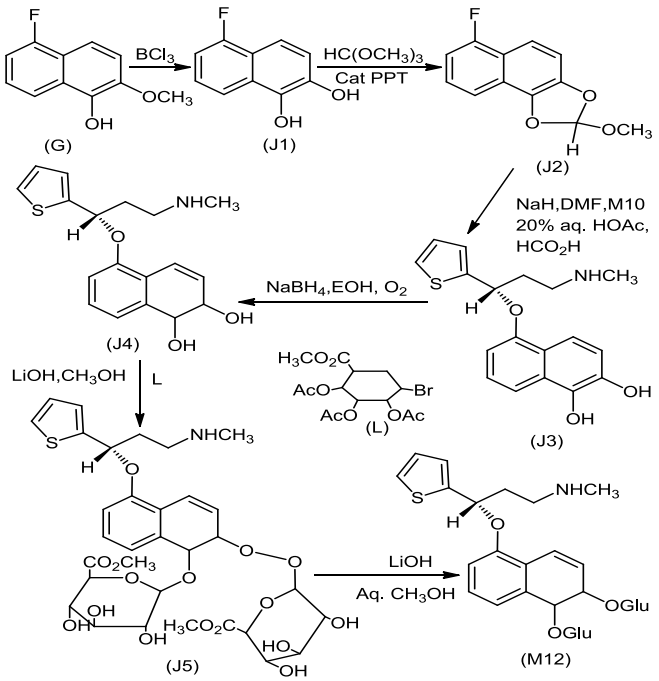

Fig. 4: Synthetic scheme for the metabolites of duloxetine (part-III).

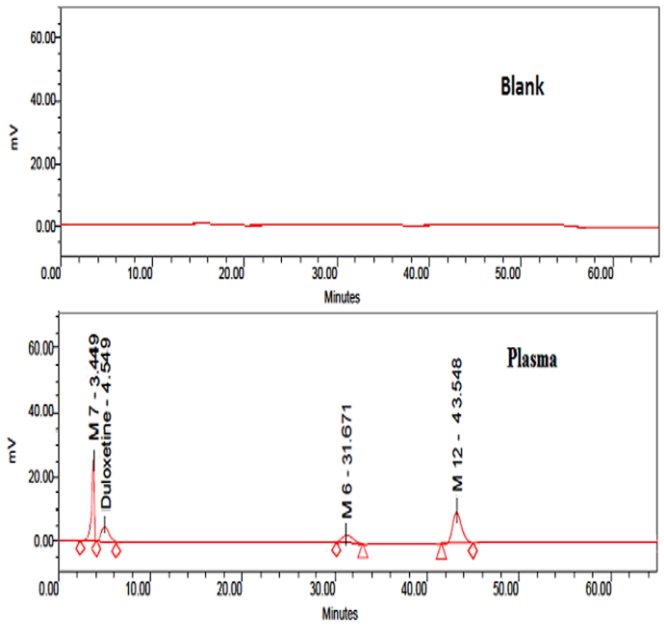

Fig. 5: Representative HPLC chromatograms of duloxetine and its metabolites present in rabbit plasma.

Table 1: HPLC peak area percentages of unchanged duloxetine and its major metabolites in rabbit plasma.

Compound	% Peak Area in plasma
Duloxetine	16
4-Hydroxy duloxetine (M7)	34
Glucuronide conjugate of 5-hydroxy-6-methoxy duloxetine (M6)	11
Glucuronide conjugate of dihydrodiol duloxetine (M12)	19

Chromatographic conditions

The experiment was performed on a Bondapak C_{18} (250 mm × 4.6 mm, 5µm particle size) column using methanol-phosphate buffer (pH 7.9; 50 mM) (7:3, v/v) as the mobile phase at a flow rate of 1.5 ml/min. The mobile phase was filtered through a nylon membrane filter paper (pore size 0.45 µm) and degassed with a sonicator for 10 min. The column temperature was maintained at 25°C and eluents were monitored at a wavelength of 224 nm. The volume of each injection was 200 µL. In all cases, metabolites were confirmed by co-injection.

RESULTS AND DISCUSSION

For identification of circulating metabolites, blood samples were collected at a particular time intervals followed by centrifugation, deproteinization and evaporation prior to HPLC analysis. Duloxetine, 4-hydroxy duloxetine (M7) and the glucuronide conjugate of 5-hydroxy-6-methoxy duloxetine (M6) and dihydrodiol duloxetine (M12) were identified from the extracted plasma sample by comparison with the synthetic standards. The retention time of the duloxetine peak matched that of the authentic duloxetine standard and the retention time of M7, M6 and M12 also matched that of the synthetic standards. These results confirmed the identification of the duloxetine, M7, M6 and M12 in plasma. The major metabolite in plasma was 4- hydroxy duloxetine (M7) and second most abundant metabolite in plasma was the Glucuronide conjugate of dihydrodiol duloxetine (M12). In addition to unchanged parent drug, a total of 3 metabolites were identified in plasma sample from male and female rabbits. The representative HPLC chromatograms of duloxetine and its metabolites in plasma from rabbit are shown in Fig. 5. A list of all the metabolites of duloxetine detected in rabbits, together with the associated information used for quantitative work, is summarized in Table 1. The free form of duloxetine and its metabolites after oral administration of a single dose of duloxetine were characterized in this study. Duloxetine was rapidly and extensively metabolized to form multiple conjugated and oxidative metabolites. Only a small portion of duloxetine (approximately 16% for AUC) was present in plasma. The most abundant metabolite, M7, appears in plasma may be due to oxidation of duloxetine at 4- position of the naphthyl ring. The presence of trace amount of glucuronide conjugate of 5-hydroxy-6-methoxy duloxetine (M6) in the plasma sample is due to initial oxidation at 5- and 6- position of the naphthyl ring followed by glucuronidation at 5- position and methylation at 6- position. A minor pathway for 5- or 6-hydroxy duloxetine is the formation of a dihydrodiol, which is then glucuronidated to form M12. An intermediate in the formation of the dihydrodiol is an epoxide. The epoxide intermediate was chemically unstable and was short-lived since it was not detected in the sample. These data indicated the very rapid formation of the dihydrodiol from the postulated epoxide intermediate. Other metabolites may be present but were not detected may be due to very low concentration in plasma.

A new HPLC method was developed for separation of *in vivo* metabolites present in rabbit plasma. These

metabolites were identified by HPLC analysis. Characterization of the metabolites was carried by synthesis followed by spectroscopic analysis. The newly developed HPLC method has also been successfully applied in the pharmacokinetics study of all metabolites. The method was found to be specific, accurate and precise, and can be used for the routine analysis as well as to monitor the stability studies.

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