ABSTRACT

Objective: A simple and sensitive reverse phase high performance liquid chromatographic (RP-HPLC) method for determination of duloxetine and four of its in vitro metabolites, 4-hydroxy duloxetine (M1), 5-hydroxy duloxetine (M2), 6-hydroxy duloxetine (M3) and N-desmethyl duloxetine (M4) in Wistar rat liver microsomes was developed.

Method: Analysis was carried out on a µ-Bondapak C18 column (250mm × 4.6mm, 5µm particle size) using methanol: phosphate buffer (pH 7.8, 50 mM) (6:4 v/v) as the mobile phase at a flow rate of 1ml/min. Detection was carried out at 221 nm with an UV detector.

Results: The above metabolites were characterized by comparison of their retention time with synthetic standards. All the four retention time matches with the metabolites present in the microsomal sample.

Conclusion: A new HPLC method was developed for separation of in vitro metabolites present in rat liver microsomes. This method has also been successfully applied in routine analysis, stability study as well as pharmacokinetics study of duloxetine after orally administrating the duloxetine to Wistar rat.

Keywords: Duloxetine, In vitro metabolites, Liver microsomes, HPLC, Retro-synthesis, Characterization.

INTRODUCTION

Duloxetine (N-Methyl-3-naphthlen-yl-ox-3-thiophen-2-yl-propan-1-amine) [1] a selective serotonin and norepinephrine reuptake inhibitor (SSNRI) is used for the treatment of major depressive disorder [2] and anxiety [3-5]. 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 [6-7] also indicated for the management of fibromyalgia [8-9]. It restores the balance of neurotransmitters in the brain like serotonin and norepinephrine [10]. Moreover it is also being used in the treatment of peripheral neuropathy caused by certain anti cancer drugs [11].

A literature survey indicated few methods for the determination of duloxetine and its key intermediate, desmethyl-duloxetine, in human serum by HPLC [12-13]. Reports were found regarding the characterization of phenolic impurities in duloxetine samples by MS, NMR spectrometry and X-ray analysis [14] and of impurities formed by interaction of duloxetine with various enteric polymers [15]. A simple UV spectrophotometric method for the estimation of duloxetine in a formulation was reported [16].

Fig. 1: Proposed metabolic pathways of duloxetine in rat liver microsomes.
An HPLC method to separate duloxetine and structurally related impurities using a combination of computer-based solvent strength optimization and solvent selectivity mixture design [17]. An HPTLC method for estimation of duloxetine in bulk and in tablet dosage form [18]. A capillary electrophoresis with laser-induced fluorescence detection method also reported for estimation of duloxetine in human plasma [19]. During our literature survey, very few articles related to the stability-indicating HPLC determination of duloxetine were found [20-26] but no article related to the measurement of duloxetine and its metabolites in rat liver microsomes by HPLC method 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 in vitro metabolites in rat liver microsomes and the structure of the metabolites were characterized through retro-synthesis followed by MS and NMR study.

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 200 µL 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 vitro metabolites were divined through retro-synthesis (Fig. 1) and were synthesized in our own laboratory (Fig. 2). All these and other chemicals used in the experiments were of analytical grade and were purchased from commercial sources.

Fig. 2: Different synthetic scheme for the microsomal metabolites of duloxetine.
Synthesis of in vitro metabolites present in rat liver microsomes

The initial object was to prepare thiophene side chain i.e. N-methyl-5-(4-fluorophenyl)-2-acetylamidine (A3) derived from 2-Acetylamine (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 between 7-8 minutes and temperature raised up to 90 °C. Immersed the flask in a bath of cold water to control the reaction. When the boiling subsided (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 chloroform to give a crude product (A). Compound (A) was formylated by stirring with an excess of formic acid in dry methanol at room temperature yielding M4. To synthesize some of the metabolites present in rat liver microsomes.

The first two compounds, 1-naphthalene (E1). Compound (E1) was formylated by stirring with an excess of formic acid in dry methanol at room temperature yielding M4. To synthesize some of the metabolites present in rat liver microsomes.

The second objective was to prepare corresponding fluoronaphthols. The first two compounds, 1-naphthalene (E1). Compound (E1) was formylated by stirring with an excess of formic acid in dry methanol at room temperature yielding M4. To synthesize some of the metabolites present in rat liver microsomes.

Animals

Male Wistar albino rats (body weight 230-250 gm) were supplied by M/s. Maharv Enterprises, Hyderabad, Andhra Pradesh, India. Before starting the experiment Wistar rats were kept in an environmentally controlled room for one week and fed with standard laboratory food and water ad libitum. Rats were fasted overnight before used for preparation of liver microsomes. For conducting this experiment permission has taken from the University Animal Ethics Committee, Berhampur University, Berhampur, India.

Preparation of microsomes

In order to minimize degradation of enzymes, all apparatus and solutions were frozen and stored at 4 °C prior to the start of the experiments. Rats were weighed and then killed by cervical dislocation. The livers were rapidly removed and immediately placed in ice-cold saline to wash off excess blood and to cool the tissue. The livers were then blotted dry, minced, weighed and transferred to a homogenizer with 4 times of their weight of KCl-survive buffer (pH 7.4) consisting of 0.15M KCl and 0.25M sucrose in 0.05M phosphate buffer (pH 7.5). The livers were then homogenized at 23,000 rpm for 1 min using F1-200 homogenizer (Shanghai, China). The homogenate was centrifuged (Sorvall Super T21, Du Pont, Wilmington, USA) at 20,000 rpm at 4 °C for 20 min. The supernatant was further ultracentrifuged (Sorvall Ultracen- trifuge OTD 55B, Du Pont, Wilmington, USA) at 100,000 rpm at 4 °C for 60 min. The supernatant was discarded and the washed microsomal pellets were resuspended in Glycerol/Phosphate buffer (20% glycerol (v/v), 80% 0.1M phosphate buffer pH 7.5) and stored at -70 °C until used.

Incubation study

Incubation study was performed using 500 μL microsome extract, 500 μL co-factor solution (2mM NADPH), dextrose (250mg/mL), 500μL and 100 nM sodium phosphate buffer (pH 7.4) added to bring a total volume of 2.0 mL. Incubation was done at 37 °C for 30 min. After 30 min reactions were terminated using an equal volume (2mL) of ice cold acetonitrile.

HPLC analysis and characterization of metabolites

Above sample was centrifuged at 14,000 rpm for 10 min and the supernatant was evaporated to dryness under a stream of nitrogen at 40 °C. Residue was redissolved in 1000 μL of LC mobile phase,
and an aliquot of 250 µL was injected into the HPLC system. Experiment was performed on a Bondapak C18 (250 mm x 4.6 mm, 5 µm particle size) column using methanol-phosphate buffer (pH 7.8; 50 mM) (6:4 v/v) as the mobile phase at a flow rate of 1 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. Ambient column temperature was maintained and eluents were monitored at a wavelength of 221 nm. The volume of each injection was 200 µL. In all cases, metabolites were confirmed by co-injection.

RESULTS AND DISCUSSION

Table 1 shows data for the in vitro metabolism of duloxetine using rat liver microsomal preparation. The experimental findings indicate that rat liver microsomal preparation in the presence of NADPH metabolizes an essential portion of the parent compound in thirty minutes. The detectable metabolites were 4-hydroxy duloxetine (M1), 5-Hydroxy duloxetine (M2), 6-hydroxy duloxetine (M3) and N-desmethyl duloxetine (M4). The metabolites were formed by the hydroxylation of the naphthyl ring and N-dealkylation of the parent duloxetine. Duloxetine and above metabolites were identified 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 M1, M2, M3 and M4 also matched that of the synthetic standards. These results confirmed the identification of the duloxetine, M1, M2, M3 and M4 in microsomal sample. The major metabolite in microsomal sample was the N-desmethyl duloxetine (M4) 17.34% and second most abundant metabolite was the 6-hydroxy duloxetine (M3) 15.02%. In addition to unchanged parent drug, a total of 4 metabolites were identified in microsomal sample. The representative HPLC chromatograms of duloxetine and its metabolites in microsomal sample are shown in figure 3.

Table 1: Retention time and HPLC peak area percentages of unchanged duloxetine and its metabolites in rat liver microsomes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duloxetine</td>
<td>5.671</td>
<td>57.14</td>
</tr>
<tr>
<td>4-Hydroxy duloxetine (M1)</td>
<td>9.035</td>
<td>9.11</td>
</tr>
<tr>
<td>5-Hydroxy duloxetine (M2)</td>
<td>10.552</td>
<td>1.24</td>
</tr>
<tr>
<td>6-Hydroxy duloxetine (M3)</td>
<td>13.978</td>
<td>15.02</td>
</tr>
<tr>
<td>N-desmethyl duloxetine (M4)</td>
<td>3.352</td>
<td>17.34</td>
</tr>
</tbody>
</table>

The ratio of metabolites to parent drug in incubates were very low, which were due to the limited formation of metabolites in vitro. A total of four metabolites of duloxetine were found in rat liver microsomal incubates by HPLC method. The identities of four major metabolites were confirmed by chromatographic comparison with synthetic standards. Based on the results, hydroxylation and N-dealkylation were the main metabolic pathways of duloxetine in rat liver microsomes. Due to very low concentration of M2, initially it was not detected. To detect M2, we investigated various elution systems and chromatographic procedures. In addition, two different HPLC columns, NovaPak C18 and Bondapak C18 from Waters India, USA, were utilized for this study. A spectrum of different wavelengths was tested for this purpose (data not shown). The satisfactory detection was obtained by using Bondapak C18 column at a wavelength of 221 nm.

CONCLUSION

A new HPLC method was developed for separation of in vitro metabolites present in rat liver microsomes. These metabolites were identified by HPLC analysis. Characterizations of the metabolites were confirmed by chromatographic comparison with synthetic standards. The newly developed HPLC method confirms the suitability of HPLC with UV detection for analysis of in vitro microsomal metabolites of duloxetine and does not re-quire use of hyphenated HPLC-MS. The method was found to be specific, accurate and precise, and can also be used for the routine analysis as well as to monitor the stability studies.

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