BIOLOGICAL SCREENING OF METABOLITE AND DEGRADATION PRODUCT (ADDITIONAL WORK)

7.1 Evaluation of nootropic effect of piracetam & its metabolite and degradation product

7.1.1 Introduction

This study was planned to study the nootropic effect of piracetam compare to its metabolite and degradation product, which would have some nootropic effect in scopolamine induced amnesia in Wistar rats.

Piracetam is a pyrrolodine derivative (2-oxo-1-pyrrolidine acetamide) that has been shown to facilitate learning and prevent the development of amnesia under different experimental conditions [1-5]. In clinical practice, the drug has been shown to enhance recovery from aphasia after stroke, and to improve cognitive function in the elderly and after coronary artery bypass. It also improved degenerative cerebellar ataxia and prevented alcohol withdrawal delirium [3, 4-6].

7.1.2 Review of the literature

During the last several years a number of studies have indicated that age-related dysfunctions in central cholinergic mechanisms play an important role in the memory loss observed in elderly humans and patients suffering from senile dementia [1-2]. One way to attempt to compensate for these possible age-related deficits would be to administer abundant amounts of choline while simultaneously giving a drug which might correct other critical age-related neuronal deficiencies. Although no drug yet
exists that is recognized as effective in correcting neuronal function in aged brain, one drug which is beginning to attract interest for its biochemical and pharmacological properties is piracetam (Table 7.1) [1, 3-4].

It has been reported to improve learning and memory in both animals and humans [2, 5-6] although its efficacy in aged subjects is not robust and is still controversial. Several lines of pharmacological evidence indicate that piracetam enables the central nervous system to function more effectively under hypoxic conditions [1, 5-6].

Table 7.1 Reported article on the nootropic effect

<table>
<thead>
<tr>
<th>Drug</th>
<th>Animal model</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mentat and donepezil</td>
<td>Scopolamine induced memory impairment in rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[7]</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Scopolamine induced memory impairment in rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[8]</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Scopolamine induced memory impairment in rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[9]</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Scopolamine induced memory impairment in rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[10]</td>
</tr>
<tr>
<td>Psychotropic agents</td>
<td>Rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[11]</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>Scopolamine induced memory impairment in rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[12]</td>
</tr>
<tr>
<td>Topiramate</td>
<td>Scopolamine induced memory impairment in rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[13]</td>
</tr>
<tr>
<td>Canscora decussate</td>
<td>Scopolamine induced memory impairment in rats</td>
<td>Conditioned avoidance response by using Cook’s pole climbing apparatus</td>
<td>[14]</td>
</tr>
<tr>
<td>Piracetam and pramiracetam</td>
<td>Rats</td>
<td>One trial recognition test</td>
<td>[15]</td>
</tr>
<tr>
<td>Choline and Piracetam</td>
<td>Rats</td>
<td>Passive avoidance task</td>
<td>[16]</td>
</tr>
<tr>
<td>Piracetam</td>
<td>haloperidol-induced catalepsy</td>
<td>Bar test</td>
<td>[17]</td>
</tr>
</tbody>
</table>

The aim of this study was to investigate the nootropic effect of the piracetam and comparison with its metabolite and its degradation product in an animal model of scopolamine-induced memory impairment.
7.1.3 Materials and Methods

7.1.3.1 Animals

Experimentally Wistar rats weighing between 180 and 200 g of either sex were used. The rats were maintained under standard conditions of temperature (25°C±5°C), relative humidity (55±10%) and a 12/12 hr light/dark cycle. The rats were fed with commercially available ‘Amrut rat pellet feed’ manufactured by Pranav Agro Food, Pune. Drinking tap-water supplied by Municipal Corporation was provided to the rats through the feeding bottles with stainless steel nozzle in each cage. Replenishment of food and water was done once daily. The study was approved by the Institutional Animal Ethics Committee.

7.1.3.2 Instruments, drugs and chemicals

Cook’s pole climbing apparatus were purchased from Inco Instruments Private Ltd (Pune, India). Piracetam was obtained as gratis sample from Micro Labs Ltd (Bangalore, India). Scopolamine was obtained from Sigma Aldrich, St Louis, USA.

7.1.3.3 Conditioned avoidance response

This model was used to study the nootropic effects of piracetam & its metabolite and degradation product. The rats were trained for conditioned avoidance response by using Cook’s pole climbing apparatus. The method of Cook’s was used with some modifications. Each rat was allowed to acclimatize for two minutes and was then exposed to a buzzer noise. After 5 seconds of putting on the buzzer, mild electric shocks were given through the stainless steel grid floor. The magnitude of the voltage was adequate (5-10V) to stimulate the rat to escape from the floor and climb the pole. As soon as the rat climbed the pole, both the buzzer and the foot shocking were switched off. At least 10 such trials were given to each rat at an interval of 1 min per day for 7 days. After about 7 days training schedule, most of the rats learned to climb the pole within 5 seconds of starting the buzzer, thus avoiding the electric foot shocks (Figure 7.1). Rats avoiding the foot shocks in all 10 out of 10 trials were considered to have developed conditioned avoidance response for further experiments.
7.1.3.4 Scopolamine induced disruption of memory

Rats trained for conditioned avoidance response (CAR) received scopolamine hydrobromide in a dose of 0.5mg/kg by intraperitoneal route before administration of study drugs. This is known to produce amnesia which will be used to evaluate the effect on learning and memory of study drugs.

7.1.3.5 Study drug administration

- Piracetam, M1 and Degradation product: All of these were given in a dose of 50 mg/kg by oral drug administration route for eight days in the animals after training for conditioned avoidance response.
- Scopolamine: Scopolamine was dissolved in 0.9% saline solution and given in a dose of 0.5mg/kg to all the group except control group by intraperitoneal route, 20 minutes before test run on the apparatus.
7.1.3.6 Grouping

The animals were divided into 5 different groups of 5 rats each after training for CAR. The animals received drugs by oral route depending on the group. The rats were divided into following groups (Figure 7.2):

- **Control**: Distilled water orally for 8 days (Not treated with scopolamine)
- **Piracetam**: Piracetam 50 mg/kg per orally for 8 days (treated with scopolamine (0.5mg/kg, i.p.), 20 minutes before administration of piracetam on 8\textsuperscript{th} day).
- **Vehicle**: Distilled water per orally for 8 days (treated with scopolamine (0.5mg/kg, i.p.), 20 minutes before administration of water on 8\textsuperscript{th} day).
- **M1**: Metabolite (M1) 50 mg/kg per orally for 8 days (treated with scopolamine (0.5mg/kg, i.p.), 20 minutes before administration of metabolite on 8\textsuperscript{th} day).
- **Deg. Pdt**: Degradation product of Piracetam 50 mg/kg per orally for 8 days (treated with scopolamine (0.5mg/kg, i.p.), 20 minutes before administration of deg. pdt on 8\textsuperscript{th} day).

On day 8, all rats were tested to see if they had retained the conditioned avoidance response. After 2 min of acclimatization period, each rat was exposed to the buzzer for 5 s. Ten such trials were given at an interval of 1 min, without giving any foot shock. Rats, responding by climbing the pole when exposed to the buzzer noise, were considered to have retained the conditioned avoidance response.
7.1.4 Results and Discussion

The percentage of rats showing retention of conditioned avoidance response (CAR) was calculated in each group. The result is shown in Table 7.2 and Figure 7.3.

Table 7.2 Percentage of rats showing retention of conditioned avoidance Response (n=5)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Conditioned stimulus*</th>
<th>Unconditioned stimulus**</th>
<th>Climbing (%)</th>
<th>Avoidance (%) (CAR Blockage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control@</td>
<td>2</td>
<td>3</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Piracetam# (50 mg/kg)</td>
<td>5</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle# (water)</td>
<td>1</td>
<td>4</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>M1# (50 mg/kg)</td>
<td>3</td>
<td>2</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Deg. Pdt # (50 mg/kg)</td>
<td>1</td>
<td>4</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

*Climbed on buzzer, **Did not climbed on buzzer
@Not treated with Scopolamine (0.5mg/kg, i.p), # Treated with Scopolamine (0.5mg/kg, i.p)

Figure 7.3 Graphical representation of comparison of the all the groups
7.1.5 Conclusion

The results showed that the piracetam is more effective in preventing memory loss in comparison with rest group in scopolamine induced memory impairment model. The effect of piracetam and its metabolite/degradation product are as: Piracetam>Metabolite (M1) > Degradation product ~ Vehicle.

7.1.6 References


7.2 Evaluation of uric acid plasma level and urine level in Wistar rats treated with febuxostat & its metabolite/ degradation product using Siemens Kit

7.2.1 Introduction

Gout is the most common inflammatory arthritis in men over 40 years and has an increasing prevalence among postmenopausal women [1]. It results from the deposition of monosodium uric acid crystals in and around the joints and soft tissues. It has been recognized that the formation of such crystals requires the presence of hyperuricaemia, defined as a serum uric acid concentration (serum uric acid levels) above its solubility limit [2-3] supersaturating the body fluids [2, 4].

The present study established the febuxostat, its metabolite (M3) and degradation product influence on the purine metabolism; the uric acid level was measured.

7.2.2 Review of the literature

7.2.2.1 Gout intervention

Lowering serum uric acid levels below saturating levels, at a target < 6.0 mg/dL, remains one of the major goals in the treatment of chronic gout to reduce or reverse clinical events [2, 5]. The pharmacological methods currently employed for that purpose are as following

- Reduction of uric acid production by use of the xanthine oxidase inhibitors;
- Enhancement of urinary uric acid excretion with uricosuric agents; and
- Promotion of the catabolism of uric acid with the pegylated recombinant uricase (pegloticase) [5].

Febuxostat, a novel non-purine analogue xanthine oxidase inhibitor, at daily dosages of 40 mg to 240 mg has been shown in studies to be at least as good as allopurinol (dose 300 mg/day) in lowering serumuric acid levels to < 6.0 mg/dL, and may require fewer dose adjustments in patients with mild to moderate renal dysfunction [3-5].

Tayar J.H. et al reported the various analysis and clinical trials on febuxostat including safety [6].
Daniel I. Feig et al. reported that increased serum uric acid is associated with increased risk for future hypertension in several large longitudinal clinical trials as well as an independent risk factor for prognosis [7].

Theodora Szasz et al. concluded that, at least *in vitro*, UA does not affect the ACh-induced relaxation of normotensive and DOCA-salt hypertensive rats [8].

Shiza Batool et al. concluded that the uric acid concentration increases when we take fructose up to 60% in our diet. It also increases superoxide dismutase concentration [9].

It was reported that ambroxol parenteral administration led to urinary bladder stone formation in rats. This study was undertaken to examine the serum uric acid levels and urine pH in rats after ambroxol parenteral treatment. Uric acid level was determined by the kit [10].

Fossati et al. reported that a new direct colorimetric procedure for uric acid assay in serum or urine is described, utilizing a 3, 5-dichloro-2-hydroxybenzene sulfonic acid/4-aminophenazone chromogenic system in the presence of horseradish peroxidase and uricase from Aspergillus flavus [11].

Marilida Mazzali et al. concluded that mild hyperuricemia causes hypertension and renal injury in the rat via a crystal-independent mechanism, with stimulation of the renin-angiotensin system and inhibition of neuronal NO synthase [12].

Kang et al. reported the various *in vitro* studies; cultured vascular smooth muscle cells incubated with uric acid also generated COX-2 with time-dependent proliferation, which was prevented by either a COX-2 or TXA-2 receptor inhibitor [13].

The goal of this study was to systematically review the formed metabolite/degradation product for febuxostat benefit and harms in treating chronic gout.

7.2.3 Materials and Methods

7.2.3.1 Animals

Experimentally Wistar rats weighing between 180 and 200 g of either sex were used. The rats were maintained under standard conditions of temperature (25°C±5°C), relative humidity (55±10%) and a 12/12 hr light/dark cycle. The rats were fed with
commercially available ‘Amrut rat pellet feed’ manufactured by Pranav Agro Food, Pune. Drinking tap-water supplied by Municipal Corporation was provided to the rats through the feeding bottles with stainless steel nozzle in each cage. Replenishment of food and water was done once daily. The study was approved by the Institutional Animal Ethics Committee. The apparatus used for the collection of the urine is given in Figure 7.4

![Figure 7.4 Apparatus used to collect urine](image)

7.2.3.2 Instruments, drugs and chemicals

UV-Visible Spectrophotometer was used for the analysis of plasma/urine samples (manufactured by Shimadzu, Japan). Uric acid estimation kit was purchased from Siemens Ltd (Vadodara, India). Febuxostat was obtained as gratis sample from Micro Labs Ltd (Bangalore, India). Oxonic acid was obtained from Sigma Aldrich, St Louis, USA.

7.2.3.3 Methodology

The animals were divided into 5 different groups of 3 rats. The animals received drugs by intra-peritoneal route for oxonic acid on the 1st day and 5th day and oral route for febuxostat, vehicle, and M3 and degradation product on each of the group on the 6th day. The plasma and urine samples were collected after 24 h of orally drug administration. Oxonic acid was used to induce hyperuricemia. The rats were divided into following groups:
Control: Distilled water on the 6th day (Not treated with oxonic acid)
Febuxostat: Febuxostat 5 mg/kg per orally on the 6th day.
Vehicle: A solution of 5% tween 80 + 95% of CMC (0.25%)
M3: Metabolite (M3) 5 mg/kg per orally on the 6th day.
Deg. Pdt: Degradation product of febuxostat 5 mg/kg per orally on the 6th day.

Oxonic acid: A dose of 250 mg/kg pretreated in all the groups on day 1st and 5th day except control group.

On day 7, all the rat plasma and rat urine samples were collected. The analysis of uric acid in samples was performed using Siemen kit followed by the UV-Vis spectrophotometer (Figure 7.5).

Figure 7.5 Working protocol for the estimation of uric acid in rats

7.2.3.4 Statistical analysis

Experimental values are expressed as mean±SD. Statistical analysis was performed by one-way ANOVA followed by Tukey’s test using Graph Pad Prism software. A value of P<0.05 was considered significant. Calculations of concentrations of uric acid were
performed using single point estimation method of UV-Vis spectroscopy using identity as follows:

\[
C_{\text{unknown}} = \frac{A_{\text{unknown}} \times C_{\text{std}}}{A_{\text{std}}}
\]

7.2.4 Result and Discussion

The comparison of mean plasma uric acid level and mean urine uric acid levels of febuxostat group with rest of the groups shown in Figure 7.6 and Figure 7.7, respectively.

Mean plasma level of uric acid was shown in Table 7.3. The mean plasma level of uric acid of control was decreased to 57.5% compared to vehicle group. The rest group plasma level of uric acid of febuxostat, M3 and Deg. Product is decreased to be 64.2%, 89.4% and 82.5%, respectively. On comparing control group with febuxostat group does not shows significant statistical correlation (P>0.05). The comparison of febuxostat group with rest groups i.e., vehicle group, M3 group and Deg. Product group was observed, which shows most significant statistical correlation (P<0.0001).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Absorbance</th>
<th>Mean Concentration (mg/dL)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1   R2   R3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.188 0.179 0.183</td>
<td>2.41</td>
<td>0.112</td>
</tr>
<tr>
<td>Febuxostat (5mg/kg)</td>
<td>0.205 0.207 0.202</td>
<td>2.69</td>
<td>0.083</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.317 0.319 0.321</td>
<td>4.19</td>
<td>0.132</td>
</tr>
<tr>
<td>M3 (5mg/kg)</td>
<td>0.283 0.288 0.285</td>
<td>3.75</td>
<td>0.118</td>
</tr>
<tr>
<td>Deg. Pdt (5mg/kg)</td>
<td>0.261 0.267 0.263</td>
<td>3.46</td>
<td>0.099</td>
</tr>
</tbody>
</table>

S.D.= Standard deviation, "A solution of 5% tween 80 + 95 % of CMC (0.25%)
Absorbance of standard uric acid solution (6mg/dL) = 0.457 A
" Not pretreated with oxonic acid on day 1" and 5" day.
" Pretreated with oxonic acid on day 1" and 5" day.
Mean urine level of uric acid was shown in Table 7.4. The mean urine level of uric acid of control was decreased to 46% compared to vehicle group. The rest group urine level of uric acid of febuxostat, M3 and Deg. Product is decreased to be 53%, 88% and 74%, respectively. On comparing control group with febuxostat group does not shows significant statistical correlation (P>0.05). The comparison of febuxostat group with rest group i.e., vehicle group, M3 group and Deg. Product group was observed, which shows most significant statistical correlation (P<0.0001).

**Table 7.4 Comparison of urine uric acid levels of all study groups (n=3)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Absorbance</th>
<th>Mean Concentration (mg/dL)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
</tr>
<tr>
<td>Control@</td>
<td>0.135</td>
<td>0.111</td>
<td>0.128</td>
</tr>
<tr>
<td>Febuxostat#</td>
<td>0.164</td>
<td>0.130</td>
<td>0.136</td>
</tr>
<tr>
<td>(5mg/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle $^*$</td>
<td>0.250</td>
<td>0.270</td>
<td>0.290</td>
</tr>
<tr>
<td>M3$^*$</td>
<td>0.224</td>
<td>0.267</td>
<td>0.229</td>
</tr>
<tr>
<td>(5mg/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deg. Pdt$^*$</td>
<td>0.209</td>
<td>0.210</td>
<td>0.190</td>
</tr>
</tbody>
</table>

S.D. = Standard deviation, $^*$ A solution of 5% tween 80 + 95 % of CMC (0.25%) Absorbance of standard uric acid solution (6mg/dL) = 0.457 A
*Dilution factor of 100
@Not pretreated with oxonic acid on day 1st and 5th day.
# Pretreated with oxonic acid on day 1st and 5th day.
7.2.5 Conclusion
The present study contains the comparison of febuxostat and its metabolite/degradation product in hyperuricemic induced model in rats. The uric acid concentration in febuxostat group was decreased to 64% in rat plasma and 53% in rat urine. All the groups (Vehicle, M3 & deg. Product group) except control group found to be most significant statistical correlation in comparison with febuxostat group.

7.2.6 References


