Section A
Simultaneous High-Performance Liquid Chromatographic Determination of Telmisartan and Hydrochlorothiazide in Pharmaceutical Preparation

Fig.1. Chemical structure of Telmisartan

Drug Profile
1) Telmisartan

Chemical Name : 4’-[(1,4-dimethyl-2’-propyl [2,6’-1H-benzimidazol]-1’-yl)methyl]-[1,1’-biphenyl]-2-carboxylic acid

Molecular Formula : C₃₃H₃₀N₄O₂

Molecular Weight : 514.62

Description : Off white color solid

Melting Point : 261-263°C

Solubility : Freely soluble in methanol and chloroform

Therapeutic Category : Antihypertensive

2) Hydrochlorothiazide

Fig.2. Chemical structure of Hydrochlorothiazide
Chemical Name : 6-Chloro-3,4-dihydro-2H-1,2,4-
benzothiadiazine-7-sulfonamide 1,1-dioxide
Molecular Formula : C$_7$H$_8$ClN$_3$O$_4$S$_2$
Molecular Weight : 297.74
Solubility : Methanol, ethanol, acetone, Practically insoluble in water
Properties : Off white powder
Melting Point : 273-275°C
Therapeutic Category : Diuretic

Introduction
Telmisartan (TLM), (Fig.1.) a nonpeptide molecule, is chemically 4’-[(1,4-
dimethyl-2’-propyl [2,6’-1H-benzimidazol]-1’-yl) methyl]-[1,1’-biphenyl]-2-
carboxylic acid. Hydrochlorothiazide (HCTZ) (Fig.2.) is chemically 6-Chloro-
3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide (1). TLM
is an angiotensin II receptor antagonist that is highly selective for type 1
angiotensin II receptor for the treatment of essential hypertension usually
given in combination with HCTZ. Angiotensin II is the principle agent of the
rennin-angiotensin system, with effects that include vasoconstriction,
stimulation of synthesis and release of aldosterone, cardiac stimulation, and
renal reabsorption of sodium. HCTZ is a thiazide diuretic. The combination is
useful in the treatment of mild to moderate hypertension, well tolerated with a
lower incidence of cough than ACE inhibitors (2). A combination of 40 mg of
TLM and 12.5mg of HCTZ is available commercially as tablets.

The literature survey reveals that several methods were reported for the
individual estimation of TLM and HCTZ. The methods (3-9) for TLM in
combination with other drugs in plasma, serum and are in tablets by (HPLC)
and (10-16) is for the estimation of HCTZ in combination with other drugs in
plasma, serum and is in tablets by (HPLC). However, recently there is two
methods were reported for the simultaneous determination of the TLM and
HCTZ in combined pharmaceutical - dosage form by HPTLC and HPLC (17,
18).

In the present research work, attempts were made to develop a rapid,
economical, precise and accurate method for the simultaneous estimation of
the ingredients of this combination. An internal standards method was used for the quantitation of TLM and HCTZ. Methyl paraben (MPB) was used as an internal standard. The proposed method is successfully applied for the simultaneous determination of TLM and HCTZ in combined -dosage form (tablets) available in the commercial market. It can be used for the quality control of formulation products.

Experimental

Materials and reagents
TLM and HCTZ standards were obtained from Lupin pharmaceutical Ltd. (Mumbai, India), ammonium acetate; acetic acid and acetonitrile (HPLC grade) were obtained from Qualigens Fine Chemicals (Mumbai, India). MPB was obtained from Merck Laboratories Ltd., (Mumbai, India). The 0.45 -Pump nylon filter was obtained from Advanced Micro devices Pvt. Ltd., (Ambala Cantt, India). The (Telista) tablets of the combination of TLM and HCTZ were purchased from market. Double -distilled water was used throughout the experiment. Other chemicals used were HPLC grade.

Chromatographic conditions
A chromatographic system (Shimadzu, Japan) consisting of quaternary solvent delivery pump, a degasser, an auto- injector, column oven and photodiode array detector, 10A-VP series with LC-10 software. Ace5 C_{18}, (4.6 x 250 mm, 5μm, Advance separation technology, US) column was used. The instrumental settings were a flow of 1 mL/min. The injection volume was 10 μL. The detection wavelength was 260nm for all three analytes. The UV spectra of HCTZ, TLM and MPB (IS) in methanol are shown in (Fig.3). The peak purity was checked with the photodiode array detector from 10A-VP series model with LC-10 software. The mobile phase consisted of buffer and acetonitrile in the ratio 60:40 (v/v). The pH of the mobile phase was adjusted to 5.5 with acetic acid. The buffer used in the mobile phase contained 50mM of ammonium acetate in double -distilled water. The mobile phase was premixed and filtered through a 0.45 μm nylon filter and degassed.

Preparation of standard stock solutions
Diluent used for solution preparation is diluent A- methanol and B-water: Acetonitrile (60:40, v/v).
50 mg sample of TLM (99.78%) was accurately weighed, transferred into a 50 mL volumetric flask, and dissolved with diluent A. 25 mg sample of HCTZ (99.56%) was accurately weighed, transferred into a 50 mL volumetric flask, 5 mg sample of MPB was accurately weighed, transferred in a 25-mL volumetric flask, and dissolved with diluent A. A mixed standard solution was prepared from these stock solutions by transferring 5 mL of each stock solution and in a 50 mL volumetric flask and diluted with diluent B. This solution contained 100 µg/mL of TLM, 50 µg/mL of HCTZ and 20 µg/mL of MPB. The calibration curve solutions containing 10-150 µg/mL of TLM, 5-75 µg/mL of HCTZ, and 20 µg/mL of MPB in each calibration level were prepared.

**Preparation of sample**

Ten tablets were weighed and finely powdered. A quantity of powder equivalent to one tablet containing 40 mg of TLM and 12.5 mg of HCTZ was transferred in a 100-mL volumetric flask. To this flask, 50 mL of diluent A was added, and the solution was sonicated for 10 min with intermittent shaking. An accurately measured volume of 10 mL acetonitrile was added to the flask and mixed well. Further sonication was performed for another 25 min with intermittent shaking. The solution was cooled to ambient temperature. An accurately measured volume of 20 mL methanol was added to the flask, and centrifuged at 10,000 rpm for 10 min. From the centrifuged solution, 5 mL of clear solution was transferred into a 50- mL volumetric flask, and 5 mL of internal standard solution was added into it and diluted to volume with diluent.

**Results and discussion**

**Optimization of the chromatographic conditions**

The primary target in developing this LC method is to achieve simultaneous determination of TLM and HCTZ in combined pharmaceutical dosage form, under common conditions that are applicable for the routine quality control of this product in ordinary laboratories.

The chromatographic separation was achieved using end capped C₁₈ (Ace5 C₁₈, 25-cm) column. The chromatographic method was optimized by changing the composition of mobile phase and pH of the mobile phase. From the development studies, finally a mobile phase consisting of mixture of 50mM
ammonium acetate in water and acetonitrile in the ratio of 60:40 (v/v) at pH 5.5 was adopted, which produces good resolution and reasonable retention and acceptable for both drugs and internal standard MPB and the chromatographic analysis was less than 10 min. A typical chromatogram for a tablet sample is shown in (Fig.4). The retention time is 3.75 for HCTZ, 6.17 for MPB and 8.23 for TLM, respectively. The run time is less than 10 min.

Validation of the method
 Specificity
 The specificity of the method was checked by a peak purity test of the sample preparation performed by a photodiode array detector. The peak purity for the peaks of TLM, HCTZ and internal standard (MPB) was observed to be 999, and 998 at wavelength 260 nm, which shows that the peaks of analyte were pure and also that formulation excipients were not interfering with the analyte peaks.

Calibration and linearity
 An internal standard method was used for quantitative determinations. Linearity of the method was tested from 10% to 150% of the targeted level of the assay concentration (TLM, 100 µg/mL and HCTZ 50 µg/mL) for both the analytes. Mixed standard solutions containing 10-150 µg/mL of TLM, 5-75 µg/mL of HCTZ, and 20 µg/mL of MPB in each linearity level were prepared. Linearity solutions were injected in triplicate. The calibration graphs were obtained by plotting peak area ratio against the concentration of the drugs. The equations of the calibration curves for TLM and HCTZ obtained were \( y = 18967x - 9710.7 \) and \( y = 8240.1x - 386.09 \), respectively. In the simultaneous determination, the calibration graphs were found to be linear in the aforementioned concentrations.

Precision (Repeatability)
 The precision of the method was studied by determining the concentrations of each drug in the tablets six times. The results of the precision study indicate that the method is reliable (RSD %< 2).

Accuracy (recovery test)
 The recovery experiments were performed by adding known amounts of the drugs in the placebo. The recovery was performed at three levels, 80%, 100%
and 120% of the label claim of the tablet (40mg of TLM and 12.5 mg of HCTZ). Placebo equivalent to one tablet was transferred into a 200-mL volumetric flask, and the amounts of TLM and of HCTZ at 80%, 100% and 120% of the label claim of the tablet were added to it. The recovery samples were prepared as per the procedure mentioned, and then 5 mL of each of the solutions were transferred into a 50-mL volumetric flask; 5 mL of internal standard solution was added to it and diluted to volume with diluent B. Three samples were prepared for each recovery level. The solutions were then analyzed, and the percentage recoveries were calculated by comparing the amount of drug added and recovered. The recovery value for TLM and HCTZ ranged from 100.79% - 101.55% & 100.24 - 100.87%, respectively (Table- I).

Intermediate precision (Reproducibility)
Intermediate precision of the method was determined by analyzing the samples six times on different days, by different chemists, by using different analytical columns of the same make and different HPLC systems. The percentage assay was calculated using amount added and with amount found.

Robustness
To determine robustness of the method, experimental conditions were purposely altered and chromatographic resolution between TLM and HCTZ were evaluated.

The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on resolution of TLM and HCTZ, it was changed 0.2 units from 1.0 to 1.2 mL/min. The effect of change in percent acetonitrile on resolution was studied by varying from -1 to +1% while the other mobile phase components were held constant. The effect of column temperature on resolution was studied at 20 °C and 25 °C instead of 30 °C while other mobile phase components were held constant. The resolution in robustness study was not less than 5 at in all conditions.

Determination of limit of quantification and Limit of detection (LOD & LOQ)
For determining the limit of detection (LOD) and limit of quantification (LOQ), the method based on signal to noise ratio (19). To determine the LOD and LOQ serially diluted the linearity solution. The LOD and LOQ for TLM
and HCTZ were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. The LODs for TLM and HCTZ were 0.018 and 0.022 µg/mL, and the LOQs were 0.052 and 0.068 µg/mL, respectively.

**Solution Stability**

The stability of solutions was determined by comparing results of the assay of the freshly prepared standard solutions. The RSD for the assay results determined up to 72 h for TLM, HCTZ and the MPB internal standard were 0.55 %, 0.74 %, and 0.59 %, respectively. The assay values were within 2 % after 72 h. Standard solutions were used, and the RSD of peak area ratio, column efficiency, resolution, and tailing factor of the peaks were calculated. The results are shown in (Table- III).

**Determination of active ingredients in tablets**

The contents of two drugs in tablets were determined by the proposed method using the calibration curve. The determinations were done in two sets, one for precision and the second for ruggedness, and six samples were prepared for each set. The results are shown in (Table- II). The chromatogram of the tablet sample is shown in (Fig.3).

**Conclusion**

The proposed LC method is rapid and accurate for the simultaneous determination of TLM and HCTZ in the dosage form. It can be used in the quality control departments for the assay and dissolution study.
References

**Figure 2.** UV spectra of hydrochlorothiazide (A), methylparaben (IS) (B), and telmisartan (C) all in methanol.

**Figure 3.** UV-spectra of HCTZ (A), MPB (B), and TLM (C) in methanol.

**Figure 4.** Chromatogram of the tablet: HCTZ (3.75), MPB (6.17), and TLM (8.23)
### Table I: Results of the recovery Tests for the drugs

<table>
<thead>
<tr>
<th>Level of addition</th>
<th>Ingredient</th>
<th>Amount added</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>TLM</td>
<td>32</td>
<td>101.55</td>
</tr>
<tr>
<td></td>
<td>HCTZ</td>
<td>10</td>
<td>100.24</td>
</tr>
<tr>
<td>100%</td>
<td>TLM</td>
<td>40</td>
<td>100.79</td>
</tr>
<tr>
<td></td>
<td>HCTZ</td>
<td>12.5</td>
<td>100.87</td>
</tr>
<tr>
<td>120%</td>
<td>TLM</td>
<td>48</td>
<td>101.32</td>
</tr>
<tr>
<td></td>
<td>HCTZ</td>
<td>15</td>
<td>100.53</td>
</tr>
</tbody>
</table>

### Table II: Assay results of active ingredients in tablets

<table>
<thead>
<tr>
<th>Set</th>
<th>Ingredient</th>
<th>Label</th>
<th>Found</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability</td>
<td>TLM</td>
<td>40</td>
<td>40.15</td>
<td>100.37</td>
</tr>
<tr>
<td></td>
<td>HCTZ</td>
<td>12.5</td>
<td>12.35</td>
<td>98.8</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>TLM</td>
<td>40</td>
<td>40.42</td>
<td>101.2</td>
</tr>
<tr>
<td></td>
<td>HCTZ</td>
<td>12.5</td>
<td>12.47</td>
<td>99.76</td>
</tr>
</tbody>
</table>

### Table III: System suitability parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HCTZ</th>
<th>MPB</th>
<th>TLM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates</td>
<td>18074</td>
<td>12879</td>
<td>11366</td>
</tr>
<tr>
<td>Resolutions</td>
<td>-</td>
<td>12.54</td>
<td>7.83</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.29</td>
<td>1.27</td>
<td>1.21</td>
</tr>
<tr>
<td>% RSD</td>
<td>-</td>
<td>0.35</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Section B
Simultaneous High-Performance Liquid Chromatographic Determination of Nitazoxanide and Ofloxacin in New Tablet Formulation

Fig. 1. Chemical Structure of Ofloxacin

Drug Profile
1) Ofloxacin
Chemical Name: 9-Fluoro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)- 7-oxo-7H- Pyrido [1, 2, 3-de]-1, 4-benzoaxazine-6-carboxylic acid
Molecular Formula: C₁₈H₂₀FN₃O₄
Molecular Weight: 361.37
Solubility: Freely soluble in methanol, acetonitrile and chloroform
Properties: Off white color solid
Melting Point: 250-257°C
Therapeutic Category: Antibacterial

2) Nitazoxanide

Fig. 2. Chemical Structure of Nitazoxanide
**Chemical Name**: 2-(Acetyloxy)-N-(5-nitro-2-thiazolyl) benzamide.

**Molecular Formula**: C₁₂H₉N₃O₅S

**Molecular Weight**: 307.29

**Properties**: Pale yellow powder

**Melting Point**: 202°C

**Solubility**: Freely solubles in alcohol, acetonitrile, slightly soluble in water

**Therapeutic Category**: Anthelmintic (cestodes), antiprotozoal

(Cryptosporidium)

**Introduction**

Ofloxacin (OFLX) (Fig.1.) is chemically 9-Fluoro-2, 3-dihydro-3 methyl-10 (4-methyl-1-piprazinyl)-7-oxo-7H-pyrido [1, 2, 3-de]-1,4-benzoxazine-6-carboxylic acid and NTZ (NTZ) (Fig.2) is chemically 2-(Acetyloxy)-N- (5-nitro-2-thiazolyl) benzamide. A combination of 200 mg of OFLX and 500mg of NTZ is available commercially as tablets (Netazox-OF). An OFLX and NTZ combination is indicated to antibacterial and antiprotozoal activity. The combination of NTZ and OFLX is antiparasitic and antibacterial which is effective against a wide variety of protozoa, helminthes and gram-negative organisms. Oral bioavailability is good and well tolerated, with mild gastrointestinal side effects. Used in Giardia intestinalis-induced diarrhea in patients (1). This new combination was recently developed by pharmaceutical companies. In the process of development, fast and reliable analytical method is required for the simultaneous determination of both drugs in this compound formulation.

The literature survey reveals that several methods were reported for the individual estimation of NTZ and OFLX. The method (2) is spectrophotometric method for the estimation of NTZ in tablets, the method (3) is LC in determination of NTZ in human plasma and the method (4, 5) is stability indicating (HPLC) and HPTLC methods were reported for the estimation of NTZ in dosage form respectively. The methods available for the determination OFLX in human plasma and serum with combination with other drugs by HPLC (6-8), and method (9-12) are for tablets by HPLC. The method (6) described HPLC assay of OFLX in human plasma and lung tissue, method
(7) described determination of mixture of fluoroquinolones, enoxacin, norfloxacin, OFLX and ciprofloxacin in pharmaceuticals and blood serum by HPLC, method (8) described meropenem- OFLX mixture analysis by HPLC, method (9) described simultaneous determination of OFLX, tetrahydrozoline hydrochloride and prednisolone acetate by HPLC, method (10) described separation of OFLX and its degradation products, method (11) described stability indicating HPLC method for simultaneous estimation of OFLX and ornidazole, method (12) described simultaneous estimation of five quinolones antibiotics and stability studies of OFLX in formulation.

However, no reference has been found for simultaneous determination of OFLX and NTZ in pharmaceutical preparations. LC with UV detection is often preferred in ordinary laboratories because of its wide suitability and availability. The reported LC methods for the individual determination of the drugs can not be easily applied for the simultaneous determination of both drugs in formulation owing to their large differences in physical and chemical properties such as polarity and solubility. If the reported individual methods are applied for the analysis of the tablets containing NTZ and OFLX, it would require double time for analysis, as compared with the method would not be rapid, less expensive or economical, whereas the simultaneous determination of the ingredients of the tablets would save analysis time and also economy.

The present research work describes a rapid, precise and accurate LC method for the simultaneous determination of OFLX and NTZ in the new tablet formulation. The developed method was validated in terms of selectivity, linearity, precision, accuracy, LOD, LOQ and robustness.

Experimental

Materials and reagents

NTZ and OFLX were obtained from Lupin pharmaceutical Ltd. (Mumbai, India), dipotassium hydrogen phosphate; orthophosphoric acid and acetonitrile (HPLC grade) were obtained from Qualigens Fine Chemicals (Mumbai, India). Caffeine was obtained from Merck Laboratories Ltd., (Mumbai, India). The 0.45 μm -Pump nylon filter was obtained from Advanced Micro devices Pvt. Ltd., (Ambala Cantt, India). The (Netazox-OF) tablets of the combination of NTZ and OFLX were purchased from Lupin pharmaceutical Ltd. (Mumbai,
India). Double distilled water was used throughout the experiment. Other chemicals used were have analytical or HPLC grade

**Chromatographic conditions**

A chromatographic system (Shimadzu, Japan) consisting of quaternary solvent delivery pump, a degasser, an auto-injector, column oven and photodiode array detector, 10A-VP series with LC-10 software. A Ymc pack-AM C\textsubscript{18}, (4.6 x 250 mm, 5 um, Ymc technology, Japan) column was used for separation. The column is end capped and carbon content of 17%. The instrumental settings were a flow of 1 mL/min. The analysis was performed at 30°C as column oven temperature and detection was carried out at 254 nm wavelength. The injection volume was 10 μL. The peak purity was checked with the photodiode array detector.

The Mobile Phase consisted of buffer and acetonitrile in the ratio 65:35 (v/v). The pH of the mobile phase was adjusted to 7.0 with orthophosphoric acid. The buffer used in the mobile phase contained 10mM of dipotassium hydrogen phosphate in double-distilled water. The mobile phase was premixed and filtered through a 0.45 μm nylon filter and degassed.

**Preparation of standard solutions**

Diluents used for the standards and sample preparation were prepared as follows: diluent A was composed of methanol and acetonitrile in the ratio of 50:50(v/v) and diluent B was composed of water and acetonitrile in the ratios of 65:35 (v/v). 50 mg sample of NTZ was accurately weighed, transferred in to a 50-mL volumetric flask, and dissolved with the diluent A. 20 mg sample of OFLX was accurately weighed, transferred in to a 50-mL volumetric flask, and dissolved with the diluent A. 20 mg sample of caffeine was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with the diluent A. Mixed standard solution was prepared from these stock solutions by transferring 5 mL of NTZ standard solution, 5 mL of OFLX by transferring standard solution, and 2.5 mL of caffeine standard solution in to a 50 mL volumetric flask and diluted to volume with the diluent B. This solution contained 100 μg/mL of NTZ, 40 μg /mL of OFLX and, 20 μg /mL of caffeine. The calibration curve solutions containing 20-200 μg/mL of NTZ,
8-80 μg/mL of OFLX and 20 μg/mL of caffeine in each calibration level were prepared.

**Preparation of sample**

Ten tablets were weighed and finely powdered. A quantity of powder equivalent to one tablet containing 500 mg of NTZ and 200 mg of OFLX was transferred in to a 200-mL volumetric flask. To this flask, 100 mL of diluent A was added, and the solution was sonicated for 10 min with intermittent shaking. An accurately measured volume of 10 mL diluent was added to the flask and mixed well. Further sonication was performed for another 10 min with intermittent shaking. An accurately measured volume of 10 mL acetonitrile was added to the flask, and centrifuged at 10,000 rpm for 5 min. From the centrifuged solution, 5 mL of clear solution was transferred into a 50- mL volumetric flask, and 2.5 mL of internal standard solution was added into it and volume made up with the diluent B.

**Method development**

The primary target in developing this LC method is to achieve simultaneous determination of NTZ and OFLX in combined pharmaceutical dosage form under the common set of conditions that are applicable for the routine quality control of this product in ordinary laboratories. Taken in to account the instability of NTZ in strong acidic and basic media, a mobile phase with weakly acidic or neutral pH value is preferred. The optimal pH value was found to be 7.0. At the time of method development number of stationary phase like C₈, C₁₈, CN and NH₂ and different mobile phases were employed. In C₁₈ stationary phase using ammonium acetate and phosphate buffer at different pH the resolution between NTZ, OFLX and caffeine was achieved but broad peak shape of OFLX was obtained having tailing factor is about 2.5. To avoid tailing for OFLX we used NH2 and CN column, in case of NH2 stationary phase peak shape of OFLX was not improved (tailing factor 2.4) and resolution between caffeine and OFLX decreased. In case of CN stationary phase peak shape of OFLX was improved but peak of caffeine, OFLX and NTZ was eluted at 2.9, 4.1 and 18.5, respectively.

Finally used high carbon loading, double end capped C₁₈ (Ymc pack-AM C₁₈, 25-cm) column. Mobile phase was selected in terms of its components and
proportions. This work began with a binary mixture of acetonitrile and 10mM dipotassium hydrogen phosphate in the ratio of 50:50 (v/v) at different pH range of pH 8.0 to pH 5.5. It was observed that at 50% aqueous dipotassium hydrogen phosphate pH-8.0 peak of caffeine, OFLX and NTZ was eluted at 1.8, 3.1 and 14.5 respectively, while at pH 5.5 of mobile phase resolution between caffeine and OFLX was reduced. Finally a mobile phase consisting of mixture of 10mM dipotassium hydrogen phosphate and acetonitrile in the ratio of 65:35 (v/v) at pH 7 was adopted, which produces good resolution and reasonable retention and acceptable for both drug and internal standard caffeine. The chromatographic analysis time was less than 10 min. A typical chromatogram of a tablet sample solution is shown in (Fig. 3.). The retention time is 3.27 for Caffeine, 3.9 for OFLX and 5.36 for NTZ, respectively. The run time is less than 10 min.

**Specificity**

The specificity of the method was checked by a peak purity test of the sample preparation performed by a photodiode array detector. The peak purity for the peaks of NTZ, and OFLX was observed to be 995, and 998 at wavelength 254 nm, which shows that the peaks of analyte were pure and also that formulation excipients were not interfering with the analyte peaks.

**Validation of the method**

**Calibration and linearity**

An internal standard method was used for quantitative determinations. Linearity of the method was tested from 20% to 200% of the targeted level of the assay concentration (NTZ 100 μg/mL and OFLX 40 μg/mL) for both the analytes. Mixed standard solutions containing 20-200 μg/mL of NTZ, 8-80 μg/mL of OFLX, and 20 μg/mL of caffeine in each linearity level were prepared. Linearity solutions were injected in triplicate. The calibration graphs were obtained by plotting peak area ratio against the concentration of the drugs. The equations of the calibration curves for NTZ and OFLX obtained were y = 5267.5x – 4505.5 and y = 5580.6x - 14477, respectively. The correlation coefficient for NTZ and OFLX are 0.9994 and 0.9992, respectively. The calibration graphs were found to be linear in the aforementioned concentrations.
**Precision**

The system precision is a measure of the method variability that can be expected for given analyst performing the analysis and was determined by performing six replicate analysis of the same working solution. The obtained relative standard deviation (R.S.D.) for OFLX and NTZ was 0.62% and 0.48%, respectively.

The intraday precision of developed LC method was determined by preparing the tablet of samples of same batches in nine determinations with three concentrations and three replicate each. The R.S.D. of the assay results, expressed as percentage of the label claim, was used to evaluate the method precision. The R.S.D. values were 1.5% for OFLX and 1.4% for NTZ. The inter-day precision R.S.D was also determined by assaying the tablets in triplicate for consecutive three days, which was found to be 1.6% for OFLX and 1.5% for NTZ, respectively. The results indicated good precision of the developed method.

**Accuracy (Recovery Test)**

Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of the drugs in the placebo. The recovery was performed at three levels 80%, 100% and 120% of the label claim of the tablet (500mg of NTZ and 200 mg of OFLX). Placebo equivalent to one tablet was transferred into a 200-mL volumetric flask, and the amounts of NTZ and OFLX at 80%, 100% and 120% of the label claim of the tablet were added to it. The recovery samples were prepared as per the procedure mentioned, and then 5 mL of each of the solutions were transferred into a 50-mL volumetric flask 2.5 mL of internal standard solution was added to it and diluted to volume with diluent B. Three samples were prepared for each recovery level. These solutions were then analyzed, and the percentage recoveries were calculated from the amount added and amount found. The recovery values for NTZ and OFLX are shown in (Table- I). The obtained results suggested the accuracy of developed method for the simultaneous determination of the two drugs in the formulation.

**Limit of quantification and limit of detection (LOQ & LOD)**

The LOD and LOQ were determined at signal-to-noise ratio of 3:1 and 10:1,
respectively, by injecting series of dilute solutions of known concentration (13). The LODs for NTZ and OFLX were 0.022 and 0.008 μg/mL, and the LOQs were 0.070 and 0.028 μg/mL, respectively.

**Assay of tablets**

The validated LC method was applied to the determination of OFLX and NTZ in tablets. Two batches of the tablets were assayed and the results shown in (Table- II), indicating that the amount of each drug in tablet samples met the requirement.

**Robustness**

To determine robustness of the method, experimental conditions were purposely altered. The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on resolution of OFLX and NTZ, it was changed 0.2 units from 0.8 to 1.2 mL/min. The effect of change in percent acetonitrile on resolution was studied by varying from -1 to +1% while the other mobile phase components were held constant. The effect of column temperature on resolution was studied at 25 °C and 35 °C instead of 30 °C while other mobile phase components were held constant. The resolution in robustness study was not less than 5 at in all conditions.

**Solution stability**

The stability of solutions was determined by comparing results of the assay of the freshly prepared standard solutions. The RSD for the assay results determined up to 72 h for NTZ, OFLX and the caffeine internal standard were 0.65 %, 0.34 %, and 0.39 %, respectively. The system suitability results are shown in (Table- III).

**Conclusion**

The proposed LC method is rapid and accurate for the simultaneous determination of OFLX and NTZ in new formulation. It can be used in the quality control departments for the assay and dissolution of tablets of the combined pharmaceutical-dosage forms containing NTZ and OFLX.
References

Fig.3. Chromatogram of the tablet: Caffeine (3.27), OFLX (3.9), and NTZ (5.36)
Table- I- Results of the Recovery Tests for the Drugs

<table>
<thead>
<tr>
<th>Level of addition</th>
<th>Ingredient</th>
<th>Amount added</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>NTZ</td>
<td>400</td>
<td>101.25</td>
</tr>
<tr>
<td></td>
<td>OFLX</td>
<td>160</td>
<td>100.54</td>
</tr>
<tr>
<td>100%</td>
<td>NTZ</td>
<td>500</td>
<td>100.97</td>
</tr>
<tr>
<td></td>
<td>OFLX</td>
<td>200</td>
<td>100.65</td>
</tr>
<tr>
<td>120%</td>
<td>NTZ</td>
<td>600</td>
<td>101.23</td>
</tr>
<tr>
<td></td>
<td>OFLX</td>
<td>240</td>
<td>100.87</td>
</tr>
</tbody>
</table>

Table- II- Assay Results of Active Ingredients in Tablets

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Ingredient</th>
<th>Label value (mg)</th>
<th>Found (mg)</th>
<th>Recovery %</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NTZ</td>
<td>500</td>
<td>501.99</td>
<td>100.39</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>OFLX</td>
<td>200</td>
<td>199.58</td>
<td>99.79</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>NTZ</td>
<td>500</td>
<td>503.45</td>
<td>100.69</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>OFLX</td>
<td>200</td>
<td>198.12</td>
<td>99.06</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Table –III- System suitability parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Caffeine</th>
<th>OFLX</th>
<th>NTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates</td>
<td>6112</td>
<td>5509</td>
<td>12839</td>
</tr>
<tr>
<td>Resolutions</td>
<td>3.29</td>
<td>-</td>
<td>6.74</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.21</td>
<td>1.55</td>
<td>1.1</td>
</tr>
<tr>
<td>% RSD</td>
<td>-</td>
<td>0.21</td>
<td>0.18</td>
</tr>
</tbody>
</table>
Section C


\[
\text{H}_2\text{N}\begin{array}{c}
\text{COOH}
\end{array}
\]

Fig. 1. Chemical Structure of Gabapentin

Drug Profile

Gabapentin
- **Chemical Name**: 1-(Aminomethyl) cyclohexane acetic acid
- **Molecular Formula**: C9H17NO2
- **Molecular Weight**: 171.24
- **Solubility**: In water at pH 7.4 exceeds 10%
- **Properties**: White powder
- **Melting Point**: 162-166°C
- **Therapeutic-Category**: Anticonvulsant

Introduction

Gabapentin (GBP) (1(amo10 methyl) cyclohexane acetic acid) is a new generation of anti-epileptic drug in treatment of pain of reflux sympathetic dystrophy. It is structurally (Fig.1) related to \(\gamma\)-aminobutyric acid (GBP) an inhibitory neurotransmitter (1). It has very low toxicity in humans and it is well associated and extracted completely (2). The new anti-convulsant drug GBP its action is attributed to the irreversible inhibition of the enzyme GABA transaminase which their by prevent the physiological degradation of GABA in brain, thus it increases GABA level in the brain clinically (3).

A variety of separation techniques are available for assay determination of GBP in biological fluids. This includes LC-MS (4), HPLC (5-8). GBP has no significant ultraviolet, or visible absorption, or fluorescence. Derivatization by chromophoric reagents increases the sensitivity of GBP detection. Several HPLC methods for determination of GBP in human plasma have been...
published using different derivatizing reagents such as 2,4,6-trinitrobenzenesulphonic acid (9-10). O-phthalaldehyde (OPA) was used in most published methods (11-17). Using OPA as a derivatizing reagent, the fluorescent OPA-drug derivative should be injected immediately after preparation because of the instability of the adduct. Therefore, the method is difficult to apply for routine studies especially when automated instrumentation is not available. Derivatization with phenylisothiocyanate (PITC) is simple, but this reagent degrades in contact with water (18).

Evaporative light scattering detection (ELSD) an alternative means of detection of non-chromophoric compounds, in which the chromatographic elution is nebulized by gas stream and the vapour, enters a heated funnel, where the solvent evaporates. The resulting analyte particles pass through a narrow light beam and the scattered light is collected by a photo multiplier. The ELSD response depends on the numbers and size of analyte particles. It provide flat baseline even with gradient elution. ELSD has been successfully applied to the analysis of non-volatile compounds such as sugars, lipids and surfactant. We developed first time ELSD method for GBP. The aim of present study was to develop a rapid, accurate and precise HPLC with ELSD for determination of GBP in bulk and pharmaceutical formulation. This method permits to quantitation of GBP in bulk drug and pharmaceutical capsules.

**Experimental**

**Chemicals**

GBP pure drug was obtained from Alkem laboratories limited Mumbai, India. GBP capsules obtained from pantacare (Gabata 300), ammonium acetate and acetic acid obtained from Qualigens (Mumbai, India). Acetonitrile obtained from Rankem (Mumbai, India). The 0.45 μ nylon filter was obtained from Advanced Micro devices Pvt. Ltd., (Ambala Cantt, India). Double distilled water passed through pure lab classic (US Filters) and UHP grade nitrogen gas was used during studies. Other chemicals used were analytical or HPLC grade.

**Equipment**

HPLC system used was Agilent – 1100 series comprised of degasser, quaternary pump, auto injector, column compartment, variable wavelength detector and integrated with Alltech ELSD 2000ES detector from Alltech
associates. The system was controlled through Chemstation software. HPLC column Ace5, C18 (250 x 4.6 mm, 5μ) Advanced separation technique Inc., Lab India model PICO pH meter was used for measure the pH of mobile phase.

**Chromatographic conditions**

The suitable chromatographic conditions of peak was achieved on Ace5,C18 (250 x 4.6 mm, 5μ) column using the mobile phase composed of aqueous solution of 50mM ammonium acetate  (pH 7.0 by acetic acid and ammonia) and acetonitrile (60:40 v/v). The instrumental settings were flow rate of 0.7 ml/min, column oven temperature at 30°C, and a detector drift tube temperature set at 97°C, a detector nitrogen gas flow set at 2.6 standard liters per minute (SLPM), gain 1 and 10 μl injection volume.

**Preparation of standard and sample solution**

A stock solution of GBP (1 mg/mL) was prepared by dissolving 25 mg of drug substance in 25 ml diluent (60:40 v/v, Water: ACN). Working solution 200 μg/mL was prepared from stock solution for assay determination. A typical chromatograph of diluent (Fig.2.) and standard GBP shown in (Fig. 3)

**Capsules**

Twenty capsules (300 mg) of each one separately emptied out and homogenized. A portion of a powder equivalent to 10.0 mg of GBP was taken into 10 mL flask, added 5 mL of diluent, shaked for 3 min and made up to the volume with the diluent. An appropriative concentration of sample (0.2 mg/mL) was prepared in the diluent. A typical chromatogram of GBP capsule solution shown in (Fig. 4).

**Result and discussion**

The aim of this work is to estimation of GBP from capsule but the GBP is highly polar compound and lacks of sufficient UV chromophore. However, in routine HPLC-UV method the peak response and peak shape was not proper that’s why we have selected evaporative light scattering detector.Various mobile phase compositions were studied in order to improve the peak response, peak shape and sensitivity, however only volatile buffers and mobile phase additives was used. Started with 50mM ammonium acetate was
examined, owing to the poor peak shape, response and sensitivity. Therefore the pH of ammonium acetate was adjusted with acetic acid and ammonia. In ELSD, the nebulizer gas flow rate affords the signal response significantly when the gas flow rate is too low. Large droplets are formed resulting in spikey and noisy signals on the other hand when the gas flow rate is too high, the droplets decrease in size, which results in a decreased signal response. The drift tube temperature is also an importance parameter affecting the signal response. At low temperature mobile phase evaporation is not complete and at higher temperature the detector response is decreased owing to the decrease in particle size by the partial vaporization of the analyzer. Method development started with impactor on and off positions. Impactor temperature is 50°C and in impactor off position tempreature is 101°C.

**ELSD optimization conditions**

The ELSD is relatively independent of molecular functional group within a chemical entity. The GBP does not have a suitable chromophore, in this situation the ELSD proves to be a more suitable detector than UV because the ELSD response is independent of molecular functional groups. ELSD were optimized to obtain the GBP signal to noise ratio by controlling both the nitrogen gas flow into to the nebulizer and the temperature of the drift tube. The nitrogen gas flow into the nebulizer controls droplets size which is critical for obtaining efficient vaporization of mobile phase and maximum sensitivity of the detector. A nitrogen gas flow of 2.6 L/min and drift tube temperature was 97°C for validation in impactor off position. It is necessary to evaporate the mobile phase completely prior to detector.

**Optimization of chromatographic conditions.**

Because of the complexity of the sample analyzed, To develop method different stationary phases like C18, CN, different mobile phases containing buffers like ammonium acetate, ammonium formate acetonitrile, methanol additives (acetic acid, formic acid, trifluoroacetic acid and ammonia) and flow rates (from 0.6 to 1.2 mL/min) were examined to achieve appropriate chromatographic separation. GBP is small highly polar molecule which can exist as a cation, anion or zwitterions due to its acid pKa of 3.7 and base pKa of 10.7. Thus, it is poorly retained on most reversed-phase HPLC columns.
Therefore it is difficult to elute GBP efficiently using a simple isocratic system. Hence, the mobile phase modified to include a pH 7.0 by ammonia and acetic acid of ammonium acetate buffer (50 mM). Finally a mobile phase consisting of a mixture of ammonium acetate and acetonitrile in the ratio of 60:40 (v/v) at pH 7 was adopted, which produces good peak shape and reasonable retention and analysis time is less than 10 min. A flow rate of 0.7 mL/min which resulted in a satisfactory S/N ratio was selected.

The specificity test of the proposed method demonstrated that the excipients from capsules did not interfere in the drug. Thus the proposed HPLC method was useful to quantify GBP in formulation.

**Method validation**

**Precision:**

The system precision is measure of the method variability was determined by performing six replicate analysis of the same working solution. The relative standard deviation (R.S.D.) for GBP was 0.3%.

The intraday precision of developed LC method was determined by preparing the capsules of samples of same batches in six determinations with three concentrations and three replicate each. The R.S.D.of the assay results, expressed as percentage of the label claim, was used to evaluate the method precision. The obtained R.S.D. values were 0.26% and results indicated good precision of the developed method shown in (Table- I).

**Limit of detection (LOD) and limit of quantification (LOQ)**

The LOD and LOQ were determined at signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting series of dilute solutions of known concentration. The limit of detection and limit of quantification was achieved at 0.4 μg/mL and 1.2 μg/mL respectively. The precision for six individual preparations of GBP at LOQ level showed the % RSD of peak area 1.5.

**Linearity**

Test solutions were prepared from stock solution at six concentration levels from 20 to 200% of assay concentration (40, 60, 100, 150, 200, 220, 240, 300 and 400 μg/mL). Linearity solutions were injected in triplicate. The calibration graphs were obtained by plotting peak area ratio against the concentration of the drugs. The equations of the calibration curves for GBP obtained was y =
18967x – 9710.7. The calibration graph was found to be linear in the aforementioned concentrations with correlation coefficients 0.998.

**Accuracy**
The recovery experiments were performed by adding known amounts of the drugs in the placebo. The recovery was performed at three levels, 80%, 100% and 120% of the label claim of the tablet (300 mg of GBP). Placebo equivalent to one capsule was transferred into a 100 mL volumetric flask, and the amount of GBP at 80%, 100% and 120% of the label claim of the capsule was added to it. The recovery samples were prepared as stated in 2.4. The recovery value ranged from 99.58% to 100.84%.

**Robustness**
The flow rate of the mobile phase was 0.7 mL/min. To study the effect of flow rate it was changed by 0.1 units from 0.6 to 0.8 mL/min. while the other mobile phase components were held constant as stated in section 2.3. The effect of change in the composition of organic modifier was checked by changing the mobile phase composition to 58:42 (v/v) (buffer: acetonitrile) and also 62:38 v/v. The effect of column temperature was studied at 28°C and 32°C while the other mobile phase components were held constant as stated in section 2.3.

In all deliberate varied chromatographic conditions assay value of GBP within 98 to 101%, illustrating the robustness of the method (Table-II).

**Solution stability and mobile phase stability**
The % RSD of assay of GBP during solution stability and mobile phase stability experiments (up to 48 h) were within 2% RSD.

**Determination of active ingredients in capsules**
The developed method was applied to quantitative determination of GBP in capsules. The results were shown to be in good agreement with the labeled amount. The recovery in the range of 100.3 to 100.6% shown in (Table-III).

**Conclusion**
The isocratic HPLC-ELSD method developed for the quantitative determination of GBP in pharmaceutical dosage form capsule is precise, accurate and with short run time. This method does not require derivatization of GBP.
References

1) D. Chadwick, Lneet, 343:89, **1994**.
2) J. P. Leach, *et al*. Scizure, 4:59, **1994**.
3) D. Oullet, *et al*. Epilepsy Res. 47 (3): 229, **2001**.
Fig. 2. Chromatogram of diluent

Fig. 3. Chromatogram of GBP standard solution

Fig. 4. Chromatogram of GBP capsule solution
Table- I- Result of precision of test method

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Analyst-I (Intra-day)</th>
<th>Analyst-II (Inter-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98.51</td>
<td>99.33</td>
</tr>
<tr>
<td>2</td>
<td>99.72</td>
<td>98.99</td>
</tr>
<tr>
<td>3</td>
<td>99.25</td>
<td>99.54</td>
</tr>
<tr>
<td>4</td>
<td>97.98</td>
<td>99.13</td>
</tr>
<tr>
<td>5</td>
<td>99.23</td>
<td>98.77</td>
</tr>
<tr>
<td>6</td>
<td>98.44</td>
<td>99.22</td>
</tr>
<tr>
<td>Mean</td>
<td>99.02</td>
<td>99.16</td>
</tr>
<tr>
<td>RSD</td>
<td>0.67</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Table- II- Results of robustness study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>99.25</td>
</tr>
<tr>
<td>0.8</td>
<td>99.98</td>
</tr>
<tr>
<td>Column Temp.</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>100.15</td>
</tr>
<tr>
<td>30</td>
<td>98.95</td>
</tr>
<tr>
<td>Mobile phase</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>99.78</td>
</tr>
<tr>
<td>38</td>
<td>100.21</td>
</tr>
</tbody>
</table>

Table- III- Assay Results of GBP capsule

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Ingredient</th>
<th>Lable (mg)</th>
<th>Found</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GBP</td>
<td>300</td>
<td>301.99</td>
<td>100.39</td>
</tr>
<tr>
<td>2</td>
<td>GBP</td>
<td>300</td>
<td>303.45</td>
<td>100.69</td>
</tr>
</tbody>
</table>