Development and Validation of RP-HPLC Method for the Estimation of Raloxifene in Marketed Formulations

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Development and Validation of RP-HPLC Method for the Estimation of Raloxifene in Marketed Formulations

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Abstract: A Simple, selective, accurate, economical reverse phase high performance liquid chromatography (RP-HPLC) was developed for estimation of Raloxifene in pharmaceutical formulations. Chromatographic separation achieved on a C 18 column (Use INERTSIL, C 18 , 5 μ, 250×4.6 mm i.d.) with mobile phase containing acetonitrile and ammonium acetate buffer in the ratio 75:25 v/v. The flow rate was 1.0 mL/min and effluent was monitored at 254 nm. The retention time was 4.413 min. The method was validated in terms of linearity, accuracy and precision. The linearity curve was found to be linear over 2.5 – 12.5 μg/mL. The limit of detection and limit of quantification were found to be 0.0202 and 0.202 μg/ml respectively. The proposed method was successfully used to determine the drug content of marketed formulations.

Key words: Raloxifene, HPLC, linearity, validation.

Introduction
Raloxifene 1 chemically, [6-hydroxy-2- (4-hydroxyphenyl) benzo [b]thien-3-yl]-[4-[2-(1-piperidinyl) ethoxy]phenyl] is a selective estrogen receptor modulator used for the treatment and prevention of osteoporosis in postmenopausal women. Clinically it is effective in the treatment of breast cancer 2,3 and in reduction of fracture risk. Literature survey reveals that a few LC/MS/MS technique 4, Reverse phase HPLC with UV detection 5,7 and Gradient RP-HPLC methods 8 have been reported for the estimation of Raloxifene in bulk and pharmaceutical formulations. In the present investigation a new RP-HPLC method has been reported for the estimation of Raloxifene from marketed formulations.

Experimental
Chemicals and materials
The pharmaceutical grade pure sample of Raloxifene was procured from Hetro Laboratories limited, Andrapradesh. Ammonium acetate and acetonitrile solvent of analytical grade were obtained from E Merck Ltd, Mumbai, India. The HPLC grade water was obtained from a Milli-QRO water purification system, sonicated and used.

Equipment and apparatus
Waters Alliance series (HPLC 2487) HPLC system equipped with two reciprocating pumps and waters series 996 photo diode array detector was used for the analysis. The data was recorded...
using window based single channel software. The purity determination performed on a stainless steel column INERTSIL, C\textsubscript{18}, 5μ, 250×4.6 mm i.d. A downer electronic balance was used for weighing the materials.

**Preparation of mobile phase**

Accurately weighed 0.5 gm of ammonium acetate in 250 ml water. 750 ml of acetonitrile was added and final volume was made up to 1000 ml with distilled water.

**Preparation and assay of standard stock solution**

An accurately weighted sample of 25 mg of Raloxifene was dissolved in 100 ml of mobile phase to give standard stock solution of 100 μg/ml. A series of working standard solutions (1.0 μg/mL-6.0 μg/mL) were obtained by diluting the stock solutions with mobile phase (acetonitrile and ammonium acetate buffer in the ratio 75:25 v/v). All the volumetric flasks containing Raloxifene were wrapped with aluminium foil and stored in the dark.

**Preparation and assay of pharmaceutical formulations**

An average of ten tablets of Raloxifene were weighed and ground to fine powder. Accurately weighed powder sample equivalent to 25 mg of Raloxifene was dissolved in mobile phase in a 100 mL volumetric flask. The flask was placed in a mechanical shaker at room temperature for 10 min. After through mixing, the solution was allowed to stand for 5.0 min. 1.0 mL was transferred into a 100 mL volumetric flask and diluted to the mark with mobile phase. A sample of 0.5 μL of this solution was directly injected. The average content of the tablets was determined either from the calibration graph or using the corresponding regression equation.

**Results and discussion**

**Chromatographic conditions**

Waters Alliance series (HPLC 2487) HPLC system equipped with two reciprocating pumps and waters series 996 photo diode array detector was used for the analysis. The data was recorded using window based single channel software. The purity determination performed on a stainless steel column INERTSIL, C\textsubscript{18}, 5μ, 250×4.6 mm with the mobile phase containing acetonitrile and ammonium acetate buffer in the ratio 75:25 v/v at a flow rate 1.0 mL/min at ambient temperature. The elution was monitored at 254 nm and the chromatographic conditions employed for the analysis of Raloxifene are shown in Table 1. The typical chromatogram of Raloxifene was shown in Fig. 2.

**Range and linearity:**

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. The linearity of the method was observed within the expected concentration range demonstrating its suitability for analysis. The regression equation of absorbance on concentration gave equation:

\[ y = 1657883.6C + 284589.2 \quad (r^2 = 0.9998, \quad n = 5) \]

The results showed that an excellent correlation exists between response factor and concentration of drugs within the concentration range indicated above (Table 2).

**Limits of detection and quantitation:**

The detection limit (LOD) is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated
experimental conditions. It may be expressed as a concentration that gives a signal-to-noise ratio of 2:1 or 3:1. The limit of detection for Raloxifene is 0.0202 μg/mL in reference material and formulation. Limit of Quantitation (LOQ) is the lowest amount analyte in a sample that can be detected with a specified degree of confidence.

Table 1. Optimized chromatographic conditions

<table>
<thead>
<tr>
<th>Chromatographic parameters</th>
<th>Peak HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution</td>
<td>Gradient</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>(Acetonirile and Ammonium acetate buffer in the ratio 75:25v/)</td>
</tr>
<tr>
<td>Column</td>
<td>INERTSIL,ODS C18 RP (4.6 mm i.d x 250 mm)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 min/ml</td>
</tr>
<tr>
<td>Detection</td>
<td>UV at 254 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10 μl</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Retention time</td>
<td>4.4 minutes</td>
</tr>
<tr>
<td>Run time</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Area</td>
<td>132023.2 mAU</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>3978</td>
</tr>
<tr>
<td>Pressure</td>
<td>30-35 Mpa</td>
</tr>
</tbody>
</table>

Table 2. Calibration of the RP-HPLC for the estimation of Raloxifene

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation (Y = a + bx) Slope (b)</td>
<td>1657883.6</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>284589.2</td>
</tr>
<tr>
<td>Standard deviation on intercept (Sa)</td>
<td>11190.97</td>
</tr>
<tr>
<td>Standard deviation on slope (S_b)</td>
<td>19398.86</td>
</tr>
<tr>
<td>Standard Error of Estimation (S_e)</td>
<td>153361.4</td>
</tr>
<tr>
<td>Correlation co-efficient (r^2)</td>
<td>0.9998</td>
</tr>
</tbody>
</table>
determined with acceptable precision and accuracy under the stated experimental conditions. A signal-to-noise ratio of 10:1 can be taken as LOQ of the method (USP 2004). The LOQ values were found to be 0.202 μg/mL for raw material, formulations.

**Precision**

Precision is the degree of reproducibility or repeatability of the analytical method under normal operating conditions. The method passed the test for repeatability as determined by % RSD of the area of the peaks of five replicate injections at 100 % test concentration. The results of intra- and inter-day variation are shown in Table. 3.

**Accuracy (Recovery studies)**

The accuracy of an analytical method is the closeness of test results obtained by that method to true value. In case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which known amount of analyte has been added within the range of method. If it is not possible to obtain samples of all drug product components, it may be acceptable to add known quantities of the analyte to the drug product (i.e. “to spike”). In our studies, the later technique was adopted and Raloxifene was spiked in drug product. The result of accuracy given in (Table.4) revealed that the method was found accurate.

**Ruggedness and robustness**

The ruggedness of the method was determined by carrying out the experiment on different instruments like Shimadzu HPLC (LC2010AHT), Agilent HPLC and Water’s Breeze HPLC by different operators using different columns of similar type like Hypersil C18, Phenomenex and Gemini C18. Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed, are rugged and robust.

**Conclusion**

The results of our study indicate that the proposed RP-HPLC method is simple, rapid, precise and accurate. The developed HPLC method was found suitable for determination of Raloxifene in bulk drug and in marketed formulations without any interference from the excipients. Statistical analysis proves that, the method is repeatable and selective for the analysis of Raloxifene. It can therefore be concluded that use of the method can save much time and money.

**Table 3. Precision data of HPLC method**

<table>
<thead>
<tr>
<th>Day</th>
<th>Precession area mean</th>
<th>R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-1</td>
<td>4388803</td>
<td>1.095</td>
</tr>
<tr>
<td>Day-2</td>
<td>4357584</td>
<td>0.977</td>
</tr>
</tbody>
</table>

a = values are the averages of five determinations

**Table 4. Results of recovery studies of tablet containing Raloxifene**

<table>
<thead>
<tr>
<th>Pharmaceutical formulation</th>
<th>Amount of Raloxifene</th>
<th>% of Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labelled</td>
<td>Found</td>
</tr>
<tr>
<td>Ralista (Cipla, Mumbai)</td>
<td>60</td>
<td>59.89 ± 0.25</td>
</tr>
<tr>
<td>Fiona (Dr.Reddy’s Ltd, India)</td>
<td>60</td>
<td>60.05 ± 0.16</td>
</tr>
</tbody>
</table>

a = values are the averages of three determinations
and it can be used in small laboratories with very high accuracy and a wide linear range.

Acknowledgements
The authors are grateful to Hetero Laboratories limited, Hyderabad for providing gift sample of Raloxifene. The authors are also grateful to Head, Dept of Chemistry, Krishna University-P.G. Centre, Nuzvid for providing necessary facilities to carry out the research work

References
Extractive spectrophotometric determination of trimethoprim in pharmaceutical formulations

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ABSTRACT
Two new, simple, sensitive, rapid and economical extractive spectrophotometric methods (A, B) have been developed for the determination of Trimethoprim in pharmaceutical bulk and tablet dosage forms. These methods are based on the formation of yellow colored chromogen by ion association reaction of Trimethoprim with Bromophenol Blue (BPB) and Bromocresol purple (BCP) exhibiting maximum absorbance at 422 and 418nm respectively against the corresponding reagent blank. Beer’s law is obeyed in the concentration range of 4.0-24 µg/ml for method A and and 5.0-25 µg/ml for method B. The methods have been statistically evaluated and were found to be precise and accurate. The proposed methods have been successfully applied for the analysis of the bulk drug and its tablet dosage forms. © 2014 Trade Science Inc. - INDIA

INTRODUCTION
Trimethoprim[1,2] is 5-(3,4,5-trimethoxybenzyl) pyrimidin-2,4-diylidiamine. The empirical formula is C_{14}H_{18}N_{4}O_{3}, representing a molecular weight of 290.3 g/mole. The molecular structure was presented in Figure 1. Its closed formula C_{14}H_{18}N_{4}O_{3} and molecular weight 290.3 g/mol. Trimethoprim has bacteriostatic effect with broad-range of Gram positive and Gram negative bacteria as it structurally resembles in pytheridine of dihydrofolic acid and is strong inhibitor of dihydrofolat reductase which converted dihydrofolate into tetrahydrufolate that in turn blocks purines and finally DNA, RNA and protein synthesis.

Various analytical procedures have been reported in combination forms[3-7] and only two visible spectrophotometric methods in single dosage forms are available for the assay of Trimethoprim[8,9]. Visible spectrophotometric methods involving ion-pair complexes of acidic dyes viz, Bromophenol Blue (BPB), and Bromocresol purple (BCP) have not been reported with this drug and this prompted the authors to develop extractive spectrophotometric methods for the determination of trimethoprim using the above mentioned dyes. The reported methods are simple and sensitive and are based on ion–pair complexation of the drug with acidic dyes (Bromophenol Blue (BPB) and Bromocresol purple (BCP)).

KEYWORDS
Trimethoprim; Beers law; Validation; Accuracy; Spectrophotometry; Linearity.

Figure 1: Chemical structure of trimethoprim
purple (BCP) and subsequent extraction of colored complexes into chloroform and measurement of absorbance of color complexes at their absorption maximum.

EXPERIMENTAL

Instrument

Elico UV Visible spectrophotometer SL 159 with 1.0 cm matched quartz cells was used for all spectral measurements. A digital pH meter Model Elico L1 120 was used for pH measurements.

Reagents

All the reagents and solvents used were of analytical reagent grade. Double distilled water was used throughout the investigation. Trimethoprim was received as a gift sample from cipra Lab Limited, sanath nagar, Hyderabad, India.

Solution of Bromophenol Blue (BPB)(0.1% v/v) of Method-A, and Bromocresol Purple solution (0.1% v/v) of Method-B were prepared by weighing and dissolving 100 mg of appropriate dyes (BPB and BCP) separately in 100 ml of double distilled water.

Buffer solution, (pH 3.0) was prepared by mixing 50 mL of 0.2 M Glycine acetate solution with 22.4 mL of 0.2 M HCl solution and diluted to 200 mL with doubly distilled water. The pH of the solution was adjusted to an appropriate value with the aid of a pH meter.

Standard drug solution of trimethoprim was prepared by dissolving 100 mg pure trimethoprim into 100 mL volumetric flask with to double distilled water obtain 1000 µg/ml of stock solution from which desired concentrations 80 µg/ml for BPB and 100 µg/ml for BCP were prepared.

Procedure for Method A, B

Different aliquots of drug solution were transferred into a series of 100 ml separating funnels. To this add 5.0 ml of glycine -acetate buffer, 5.0 ml of various dye solutions (BPB and BCP), were added and total volume was made upto 15 ml with distilled water. To this 10 ml of chloroform was added, and the contents were shaken for 5 minutes. The organic layer was separated and the absorbance of yellow colored solution is measured spectrophotometrically 422 nm for BPB (Method-A), and 418 nm for BCP(Method-B) against blank similarly prepared) which is stable for 24 hrs. For the two proposed methods, standard calibration plots were prepared by plotting the absorbance versus drug concentration, and the concentration of the unknown was read from the plotted calibration graphs or computed from the respective regression equations derived using the absorbance concentration data.

Preparation of sample solution

Tablets containing Trimethoprim were successfully analyzed by the proposed methods. Twenty tablets of commercial samples of Trimethoprim were accurately weighed and powdered. Tablet powder equivalent to 100 mg of Trimethoprim was dissolved in 50 ml double distal water. The solution was suitably diluted and analyzed as given under the assay procedure for bulk samples. The results were represented in Table I. None of the excipients usually employed in the formulation of tablets interfered in the analysis of Trimethoprim by the proposed methods.

RESULTS AND DISCUSSION

In the proposed methods(A, B) the drug Trimethoprim in its protonated form reacts with the anionic dyes viz, BPB, and BCP in aqueous solution at pH 3.0 ± 0.01 to form yellow colored ion pair extractable complex. The optimum conditions for the proposed methods were established by varying on parameter at a time and keeping the others fixed and observing the effect on absorbance. The effect of temperature of the reaction, quantity, concentration and addition of various reagents and buffer were studied, optimized after several experiments and incorporated in the procedure. The yellow color developed in methods A, B was stable for more than 24 hours. Wavelength of maximum absorbance for colored ion-pair complexes of Trimethoprim were selected at 422 nm for BPB and ND imental association complex418 nm for BCP and were used for the quantitative determination. Linearity for Trimethoprim was observed in the concentration ranges and the regression analysis of the Beer’s law data indicated a linear relationship between absorbance and concentration (TABLE 1) which is corroborated by high values (close to unity) of the correlations coefficients for all three methods. The calculated molar absorptivity
and Sandell sensitivity values are summarized in Table I. The high values of ε and low values of Sandell sensitivity indicate the high sensitivity of the proposed methods. Precision studies for the proposed methods were carried out by one fixed concentration six times on the same day and the results of this study were summarized in Table I. The percentage relative standard deviation (%RSD) values indicating high precision of the proposed methods respectively. The accuracy of the proposed methods was determined by the percent mean deviation from known concentration, at one fixed concentration and these results are also presented in Table I. The percent relative error (%RE) values demonstrated the high accuracy of the proposed methods. The proposed methods were applied for the quantification of Trimethoprim in marketed formulations. The results of statistical analysis of did not detect any significant difference between the proposed method and reference method with respect to accuracy and precision as revealed by the Students t-value and variance ratio F-value. The results of assay are given in Table 2.

**Full Paper**

TABLE 1: Results of optical characteristics and precision of the proposed methods for trimethoprim assay

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BPB</th>
<th>BCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>λmax (nm)</td>
<td>422</td>
<td>418</td>
</tr>
<tr>
<td>Beer’s law limits (µg/ml)</td>
<td>4.0 - 24</td>
<td>5.0 - 25</td>
</tr>
<tr>
<td>Molar absorptivity (1 mol⁻¹.cm⁻³)</td>
<td>3.416 x 10³</td>
<td>4.013 x 10³</td>
</tr>
<tr>
<td>Sandell’s sensitivity (µg.cm⁻²/0.001 absorbance unit)</td>
<td>0.03100</td>
<td>0.0178</td>
</tr>
<tr>
<td>Optimum photometric range (µg/ml)</td>
<td>5.5 - 22.5</td>
<td>7.5 - 22.5</td>
</tr>
<tr>
<td>Regression equation (Y=a+bc); slope (b)</td>
<td>0.0121</td>
<td>0.0190</td>
</tr>
<tr>
<td>Standard deviation on slope (Sb)</td>
<td>0.000391</td>
<td>0.000572</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>0.0062</td>
<td>0.0153</td>
</tr>
<tr>
<td>Standard deviation on intercept (Sa)</td>
<td>0.000225</td>
<td>0.0003303</td>
</tr>
<tr>
<td>Standard error on estimation (Se)</td>
<td>0.00495</td>
<td>0.00455</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9969</td>
<td>0.9973</td>
</tr>
<tr>
<td>Relative standard deviation (%)*</td>
<td>1.600</td>
<td>0.8593</td>
</tr>
<tr>
<td>% Range of error (confidence limits)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 level</td>
<td>1.338</td>
<td>0.7186</td>
</tr>
<tr>
<td>0.01 level</td>
<td>1.982</td>
<td>1.0630</td>
</tr>
</tbody>
</table>

Average of six determinations

TABLE 2: Assay of Trimethoprim in dosage forms

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BPB</td>
<td>100</td>
<td>99.95 ± 0.20</td>
<td>0.314</td>
<td>199.99 ± 0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t (value)</td>
<td>F (Value)</td>
<td>99.95 ± 0.603</td>
</tr>
<tr>
<td>BCP</td>
<td>100</td>
<td>99.93 ± 0.22</td>
<td>0.527</td>
<td>99.93 ± 0.346</td>
</tr>
</tbody>
</table>

*Average of six determinations

**Linearity graph of Trimethoprim with BPB**

**Linearity for Trimethoprim with BCP**

**CONCLUSIONS**

The present research work demonstrated the fea-
sibility of the use of visible spectroscopy and ion complexation reaction for the determination of Trimethoprim in pure and its dosage formulations using two acid dyes. The proposed methods make use of simple reagents and are found to be simple, precise, economical and rapid, which an ordinary analytical laboratory can afford. The proposed methods were statistically evaluated and results obtained are accurate, precise, sensitive and free from the interferences of other additives present in the formulation. The proposed extractive visible spectrophotometric methods can be applied for determination of Trimethoprim in pure and dosage forms with high precession and good accuracy in quality control laboratories.

ACKNOWLEDGEMENTS

The authors are thankful to Cipra Laboratories limited, Hyderabad for providing gift sample of trimethoprim.

REFERENCES


Simultaneous Determination of Glucosamine and Diacerein in Pharmaceutical Dosage Form by RP-HPLC

Keywords: Glucosamine, Diacerein, RP-HPLC, Simultaneous analysis, Tablets

ABSTRACT

The chromatographic analysis was performed on Agilent, Zebra C18 reversed phase column with mobile phase consisting Ammonium acetate: acetonitrile in the ratio 60:40% v/v, at a flow rate of 1.0 mL/min and eluents monitored at 267nm. The method was validated for linearity, accuracy, precision, robustness and application for assay as per International Conference on Harmonization (ICH) guidelines. The retention times of glucosamine and diacerein were 2.710 and 3.203min, respectively. The calibration curves of peak area versus concentration, which was linear from 3000-9000 µg/mL for glucosamine and 100-300µg/mL for diacerein, having regression coefficients (r²) greater than 0.999. The method had the requisite accuracy, precision, and robustness for simultaneous determination of glucosamine and diacerein in tablets. The proposed method is simple, economical, accurate and precise, and could be successfully employed in routine quality control for the simultaneous estimation of glucosamine and diacerein in tablets.
INTRODUCTION
Glucosamine [1-9] [FIG.1.01.A] is an amino sugar and a prominent precursor in the biochemical synthesis of glycosylated proteins and lipids. It is absorbed rapidly from the intestine and transported to the connective tissues and helps in the restoration of damaged joint tissue in osteoarthritis. It has been used for osteoarthritis, back pain, joint pain and glaucoma, by itself or in combination with chondroitin sulfate, diacerein. It is one of the most commonly used supplementary medicines as non-vitamin, non-mineral and natural product. It may decrease catabolic activity by inhibiting the synthesis of proteolysis enzymes and other substances that contribute to damage of the cartilage matrix. Glucosamine is required for the synthesis of glycoprotein, glycolipids and glycosaminoglycans (mucopolysaccharides).

Diacerein [10] [FIG.1.01.B], also known as diacetylrhein, is a (4, 5-diacetoxy-9, 10-dihydro9, 10 di-oxo-2 anthracene carboxylic acid) is a new anti-inflammatory, analgesic and antipyretic drug used in the treatment of osteoarthritis. It has a novel mode of action that differentiates it from NSAIDs and other conventional form of drug therapy. It also significantly reduces severity of pathological changes of osteoarthritis compared to placebo and increases the expression of transforming growth factor (TGF) - beta land TGF-beta 2, with, potential cartilage repairing properties. Diacerein does not alter renal or platelet cyclo-oxygenase. In addition to effect on macrophage migration and phagocytosis, it also inhibits superoxide production, chemotaxis and phagocytic activity of neutrophils. However, Diacerein lacks cyclooxygenase inhibitory activity and hence shows no effect on prostaglandin synthesis.

Glucosamine [11-15] and Diacerein [16-23] have reported methods by chemical and instrumental methods in pure drug, pharmaceutical dosage forms and in biological samples either in single or in combined forms.

The objectives of this study were, therefore, to develop a simpler, economic, rapid, precise, isocratic, and accurate RP-HPLC method with good sensitivity for quantitative analysis of glucosamine and diacerein in pharmaceutical dosage forms and to validate the method in accordance with International Conference on Harmonization (ICH) guidelines [30]. The direct use of the mobile phase for dilution of the formulations for quantitative analysis would minimize errors that might occur during tedious extraction procedures.
EXPERIMENTAL

i) Chemicals and Reagents:
HPLC grade acetonitrile, analytical reagent grade Ammonium acetate and phosphoric acid were purchased from Merck, Darmstadt, Germany. High pure water was prepared by using Millipore Milli-Q plus water purification system.

ii) Mobile Phase:
The mobile phase selected was Ammonium acetate buffer (pH 3.5): acetonitrile in the ratio of 60:40(v/v), and before analysis mobile phase was degassed.

iii) Standard preparation:
100 mg of Glucosamine sulphate and Diacerein and were accurately weighed and transferred into a 100 ml clean dry volumetric flask, about 70 ml of diluents (mobile phase) was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give a concentration of 1.0mg/ml glucosamine and diacerin and filtered through 0.45 μm nylon filter. (stock solution). From the above stock solutions different aliquots was transferred into 10ml volumetric flask and diluted up to the mark by the diluent to achieve a concentration of 3000-9000μg/ml and of 100-300μg/ml of μg/ml glucosamine and diacerin respectively.

iv) Sample Preparation:
20 Tablets were weighed and triturated in glass mortar. The quantity of powder equivalent to 100mg of active ingredient present in 20 tablets was transferred into a 10 ml clean dry volumetric flask, 7 ml of diluent was added to it and shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 μg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution). 0.8 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark to give the respective concentrations as par with standard solution. The solution was filtered through 0.45 mm nylon filter before injecting into HPLC system.
HPLC Instrumentation and Chromatographic Conditions:
Chromatography was performed with Shimadzu HPLC equipment comprising an LC-10A VP quaternary pump, a variable-wavelength programmable UV–visible detector, an SPD-10AVP column oven, and an SCL10AVP system controller. A Rheodyne injector fitted with a 20-μL loop was also used and data were recorded and evaluated by use of Class-VP 5.032 software. Glucosamine and diacerein was separated, at ambient temperature (30°C) on a 250 mm × 4.6 mm i.d., 5-μm particle, Agilent, Zorbax C18 reversed phase column with Ammonium acetate buffer (3.5): acetonitrile in the ratio of 60:40(v/v) as mobile phase at a flow rate of 1.0 mL.min\(^{-1}\). Before use the mobile phase was filtered through a 0.45μm Nylon filter. UV detection was performed at 267nm.

RESULTS AND DISCUSSION

HPLC METHOD DEVELOPMENT
To optimize the chromatographic conditions, different combinations of Ammonium acetate buffer (pH 3.5): acetonitrile (40:60, 50:50 and 60:40). Ammonium acetate: acetonitrile in the ratio (60:40) was preferred because it resulted in a greater response to glucosamine and diacerein. The composition and flow rate of the mobile phase were changed to optimize the separation conditions. Decreasing the organic modifier content resulted in a decrease in the retention time (RT) of the drug. The effect of the flow rate was studied in the range 0.8 to 1.2 mL.min\(^{-1}\). High acetonitrile content and flow rate resulted in prolonged analysis time. A low acetonitrile concentration was therefore used at a flow rate of 1.0 mL.min\(^{-1}\), keeping in mind the possibility that potential minor degradation products could appear after stress studies and might co elute with the drug because of the reduced RT if the flow rate was increased. High flow rates also reduce the life time of both column and pump. Under these conditions, the analyte peak was well-defined and free from tailing (Fig.1.02.C). The retention time (RT) of glucosamine and diacerein were 2.710 and 3.203min respectively. Other advantages of this mobile phase included its low cost and simplicity. The short retention time achieved implied that many samples can be run using a small quantity of mobile phase, thus minimizing analysis time and cost per analysis.

Citation: C Rambabu et al. Ijppr.Human, 2015; Vol. 2 (2): 139-151.
METHOD VALIDATION

The developed RP-HPLC method is validated in accordance with ICH guidelines for assay of glucosamine and diacerein using the following Parameters.

A. SPECIFICITY

1. BLANK AND PLACEBO INTERFERENCE

A study to establish the interference of blank and placebo were conducted. Diluent and placebo was injected into the chromatograph in the defined above chromatographic conditions and the blank and placebo chromatograms were recorded. Chromatogram of blank solution (Fig.1.02.A) showed no peaks at the retention time of glucosamine and diacerein peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of glucosamine and diacerein in tablets. Similarly chromatogram of placebo solution (Fig.1.02.B) showed no peaks at the retention time of glucosamine and diacerein peak. This indicates that the placebo used in sample preparation do not interfere in estimation of glucosamine and diacerein in their formulations.

B. LINEARITY OF DETECTOR RESPONSE

The standard curve was obtained in the concentration range of 3000-9000μg/ml for glucosamine and 100-300μg/mL for diacerein. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient \([r^2]\) of standard curve were plotted and calculated and are given in Fig.1.03.A & Table:1.02.A for glucosamine Fig.1.03.B & Table:1.02.B and for diacerein demonstrating the linearity of the proposed method. The LOD value for glucosamine and diacerein were found to be 2.939μg/mL and 2.985μg/mL, respectively and the LOQ value 9.79μg/mL and 9.95μg/mL and are reported in Table:1.02.A&B respectively.

C. PRECISION

The method precision study for six sample preparations in marketed samples showed a % RSD of 0.5% and glucosamine and diacerein respectively revealing high precision of the proposed RP-HPLC method (Table.2.04)
D. ACCURACY
The accuracy of the method was determined on three concentration levels by recovery experiments. The recovery studies were carried out in triplicate preparations on composite blend collected from 20 tablets of glucosamine and diacerin, analyzed as per the proposed method. The percentage recoveries was found to be %100 with an overall %RSD of 0.5 for glucosamine and the percentage recoveries with found in the range of 100 with an overall %RSD of 0.2 for diacerin. From the data reported in Table: 1.04.A&B reported that the developed RP-HPLC method was found to be accurate for glucosamine and diacerin assay.

E. ROBUSTNESS STUDIES
The robustness study of the developed assay method for glucosamine and diacerein were established in all variance conditions. Assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence, the analytical method would be concluded as robust.

F. ANALYSIS OF MARKETED FORMULATION
Analysis of marketed tablets ((T-MINIC Tab, Novartis) was carried out using optimized mobile phase and HPLC conditions. The % drug content of tablets obtained by the proposed method for glucosamine and diacerein was found to be 99.98 and 99.96, respectively. This showed that the estimation of dosage forms was accurate within the acceptance level of 95% to 105%. The results are given in Table.1.05.

CONCLUSION
A simple, rapid, sensitive and economical RP-HPLC method has been developed for the estimation of Glucosamine and Diacerin single and also in combined dosage forms. The credibility of the proposed method has been established by validation as per the ICH guidelines. The results of validation were in good agreement with acceptable limits. Therefore the method has proven to be accurate, precise, linear, specific and robust. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine quality control analysis of Glucosamine and Diacerin in pure and also in combined dosage forms.
ACKNOWLEDGEMENT

The authors are thankful to Bio Lee. Labs- Hyderabad, Andhra Pradesh and Dept. of Chemistry, Acharya Nagarjuna University, Guntur, AP, India for providing necessary for providing laboratory facilities to carry out this research and the authors greatly acknowledge INVENTIS drug Pvt.ltd, for providing the gifted samples of the above studied drugs.

REFERENCES


Fig. 1.01.A&B. Structure of Glucosamine and Diacerein

Fig: 1.02.A - Typical HPLC Chromatogram Showing the No Interference of Blank for Glucosamine And Diacerein
Fig: 1.02.B - A Typical HPLC Chromatogram Showing the No Interference of Placebo for Glucosamine and Diacerein

Fig: 1.02.C – Validated HPLC Chromatogram of Glucosamine and Diacerein
Fig: 1.03. A&B. Linearity Curve Of Glucosamine (A) and Diacerein (B)

Table.1.01: System Suitability Parameters of Glucosamine and Diacerein

<table>
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<tr>
<th>NAME OF THE COMPOUND</th>
<th>RETENTION TIME</th>
<th>THEORETICAL PLATES</th>
<th>TAILING FACTOR</th>
<th>USP RESOLUTION</th>
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<td>8719</td>
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<td>DIACEREIN(B)</td>
<td>5.916</td>
<td>18971</td>
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1.02. A. Linearity Data Results of Glucosamine

<table>
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<th>LINEARITY STUDY FOR GLUCOSAMINE</th>
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</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>75</td>
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<td>100</td>
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<tr>
<td>125</td>
</tr>
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<td>150</td>
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<td>Slope</td>
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<td>RSQ(r2)</td>
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<td>LOD</td>
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<td>LOQ</td>
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</table>

Citation: C Rambabu et al. Ijppr.Human, 2015; Vol. 2 (2): 139-151.
1.02. B. Linearity Data Results of Diacerein

<table>
<thead>
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<th>% LEVEL (APPROX.)</th>
<th>CONC. µg/mL</th>
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</thead>
<tbody>
<tr>
<td>50</td>
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<td>75</td>
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</tr>
<tr>
<td>150</td>
<td>300</td>
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Slope 19288  
RSQ(r2) 0.9999  
LLD (µg/ml) 2.985  
LLQ (µg/ml) 9.95

Table 1.03: Method Precision (Inter and Intraday) Studies for Glucosamine and Diacerein by the Proposed Method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Injection</th>
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</tr>
<tr>
<td>2</td>
<td>Injection 2</td>
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</tr>
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<td>3</td>
<td>Injection 3</td>
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<td>Injection 4</td>
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<td>Injection 5</td>
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<td>Injection 6</td>
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<tr>
<td>% RSD</td>
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</table>

*Average of six determinations

Citation: C Rambabu et al. Ijppr.Human, 2015; Vol. 2 (2): 139-151.
### Table: 1.04.A: Recovery Studies For Glucosamine and Diacerein by the Proposed Method

<table>
<thead>
<tr>
<th>Spiked Level</th>
<th>Sample Weight</th>
<th>Sample Area</th>
<th>µg/ml added</th>
<th>µg/ml found</th>
<th>% Recovery</th>
<th>% Mean</th>
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</thead>
<tbody>
<tr>
<td>50%</td>
<td>936.23</td>
<td>2163710</td>
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### Table: 1.04.B: Recovery Studies For Glucosamine and Diacerein by the Proposed Method

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<tr>
<th>Spiked Level</th>
<th>Sample Weight</th>
<th>Sample Area</th>
<th>µg/ml added</th>
<th>µg/ml found</th>
<th>% Recovery</th>
<th>% Mean</th>
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<tr>
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Citation: C Rambabu et al. Ijprr.Human, 2015; Vol. 2 (2): 139-151.
Table 1.05: Analysis of Marketed Tablets

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<tr>
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<th>LABEL CLAIM</th>
<th>QUANTITY FOUND*</th>
<th>% RSD</th>
<th>% ASSAY</th>
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<tbody>
<tr>
<td>GLUCOSAMINE</td>
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<tr>
<td>DIACEREIN</td>
<td>50mg</td>
<td>49.98</td>
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<td>99.96</td>
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</table>

*Average of six determinations