3.0.1. DRUG PROFILE

Raloxifene hydrochloride (RLX) is a selective estrogen receptor modulator that belongs to the benzothiophene class of compounds. The chemical designation is methanone,[6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl]-[4-[2-(1-piperidinyl)ethoxy]phenyl]-, hydrochloride.

It was first prepared by C.D. Jones et al. [1]. It is having a molecular formula of C\textsubscript{28}H\textsubscript{27}NO\textsubscript{4}S.HCl with molecular weight of 510.05. Its structural formula is:

\[
\begin{align*}
\text{HO} & \quad \text{S} \quad \text{OH} \\
\text{O} & \quad \text{N} + \text{H} \\
\text{O} & \quad \text{Cl}^- \\
\text{O} & \quad \text{N}^+ \text{H}
\end{align*}
\]

RLX is an off-white to pale-yellow solid that is very slightly soluble in water. RLX is used for the treatment and prevention of osteoporosis in postmenopausal women [1a].
3.0.2. LITERATURE SURVEY OF SPECTROPHOTOMETRIC, HPLC AND HPLC-MS-MS METHODS FOR RALOXIFENE HYDROCHLORIDE

Various analytical methods are found in the literature for the determination of RLX in pharmaceuticals.

**Visible spectrophotometric and HPLC methods for RLX in pharmaceuticals**

Only two reports available in literature. Dharuman *et al.* [2] have reported two spectrophotometric methods (A and B) for the determination of RLX. Method A is based on the oxidation of the drug with ferric chloride and coupling with potassium ferric cyanide and measured the absorbance at 735 nm; method B is based on the reduction of the drug with Fehling’s reagent and measured the absorbance at 430 nm. Annapurna *et al.* [3] reported three spectrophotometric methods for the determination of RLX. Method A is based on the formation of yellow coloured chromogen with 0.1 N sodium hydroxide and measured the absorbance at 425 nm. Method B is based on the reaction of RLX with ferric chloride and 1,10 phenanthroline to form blood red coloured chromogen, measured at 510 nm and method C is based on reaction of RLX with ferric chloride and 2,2’ bipyridyl to form blood red coloured chromogen, measured at 521 nm.

The HPLC methods available for determination of RLX in bulk drugs and in dosage forms are scanty.

Jin Yicui [4] has analysed RLX by using the mobile phase of acetonitrile-0.05 M ammonium acetate (3:7, v/v) and the separation was achieved on octadecyl-bonded silica column (5µm, 6x150 mm) at 286 nm. A method reported by Trontelj *et al.* [5] consists of coulometric and UV detection of RLX with limit of quantification of 0.336 and 0.610 mg L⁻¹, respectively. Wang *et al.* [6] have used reversed phase HPLC method for the determination of RLX. The mobile phase consists of acetonitrile-0.01 M sodium dodecyl sulphate (55:45; v/v, adjusted to pH 4.0 with glacial acetic acid) and monitored at 286 nm. Nandini Pai *et al.* [7] have described a reversed phase HPLC method in the linearity range of 250-750 µg mL⁻¹. Venkata Reddy *et al.* [8] have reported RP-HPLC method using Inertsil C₁₈ column. The mobile phase consists of acetonitrile-phosphate buffer (pH 2.0) at 1 mL min⁻¹ in the concentration range of 0.5-200 µg mL⁻¹. One more method reported by Pavithra and Lakshmi [9] employ water : methanol (50:50, v/v) as a mobile phase at 1 mL min⁻¹, performed on Waters Symmetry C₁₈ (150x4.6 mm) column in the range of 10-60 mg mL⁻¹.
Other techniques

For the determination of this drug in dosage forms, several other techniques including UV-spectrophotometry [10], electrophoresis [11] and resonance rayleigh scattering [12] have been reported. But most of these techniques are tedious, time-consuming and often difficult to perform besides involving expensive instrumental set up, which many laboratories in underdeveloped/developing countries can ill afford.

Methods for RLX in urine

The determinations of RLX have mainly been focused on High performance liquid chromatography (HPLC) techniques in body fluids such as plasma [13] and rat tissue [14].

There is only one method reported for the determination of drug in human plasma. Trontelj et al. [15] have reported determination of RLX and its metabolites in human plasma using HPLC with tandem mass spectrometry in the range of 0.088-60 µg L⁻¹.

The literature survey revealed that no method has been reported for the determination of RLX in human urine.

With a view to overcome the above shortcomings of the reported methods, the author has developed some spectrophotometric methods and one HPLC method for RLX. Spectrophotometric methods make use of bromate-bromide and potassium permanganate as reagents. HPLC method relies on the use of an Agilent system with UV detection for the assay.

In addition, one LC-MS-MS method has also been developed for the determination of RLX in spiked urine. The details of above methods are presented in this chapter.
SECTION 3.1
SPECTROPHOTOMETRIC METHODS FOR
THE DETERMINATION OF RALOXIFENE HYDROCHLORIDE IN
PHARMACEUTICALS USING BROMATE-BROMIDE MIXTURE AND TWO
DYES, METHYLENE BLUE AND RHODAMINE-B AS REAGENTS*

3.1.1.0. INTRODUCTION

The utility of bromate-bromide reagent in spectrophotometric assay of organic
compounds of pharmaceutical importance has been reviewed in Chapter 2.0, Section
2.1.1.0.

In this section, the investigation that led to the use of bromate-bromide for the
spectrophotometric determination of RLX is presented. Two new spectrophotometric
methods are proposed for the determination of raloxifene hydrochloride (RLX) using
bromate-bromide mixture and two dyes, methylene blue and rhodamine B, as
reagents. The methods entail the addition of a known excess of bromate-bromide
mixture to RLX in hydrochloric acid medium followed by determination of residual
bromine by reacting with a fixed amount of either methylene blue and measuring the
absorbance at 665 nm (Method A) or rhodamine B and measuring the absorbance at
555 nm (Method B). Details concerning the development and validation of these
methods are compiled in this Section (3.1.).

3.1.2. EXPERIMENTAL

3.1.2.1. Apparatus

The instrument is the same that was described in section 2.1.2.1.

3.1.2.2. Reagents

Bromate-Bromide mixture: Preparation of 1000 µg mL⁻¹ KBrO₃ in the presence of
large excess of bromide was described in Section 2.1.2.2. For the present
investigation, the above solution was diluted appropriately with water to get 25 and 10
µg mL⁻¹ concentrations for method A and method B, respectively. Preparation of HCl
(5M) was also described in the same section.

*This work has been published in J. Chil. Chem. Soc, 2008, 53,1126-1130
**Methylene blue (40 µg mL⁻¹):** First, a 400 µg mL⁻¹ dye solution was prepared by dissolving 57.14 mg of dye (Qualigen fine-chem., Mumbai, assay 70%) in water and diluting to 100 mL in a calibrated flask, and filtered using glass wool. This was appropriately diluted to get required concentration for use in method A.

**Rhodamine B (50 µg mL⁻¹):** First, a 500 µg mL⁻¹ rhodamine B solution was prepared by dissolving 62.5 mg of dye (s.d.fine-chem Ltd., Mumbai, 80% assay) in water and diluting to 100 mL, and filtered. This was appropriately diluted with water to get 50 µg mL⁻¹ for use in method B.

**Standard drug solutions:** Pharmaceutical grade RLX, certified to be 99.8% pure was procured from Cipla India Ltd, Mumbai, India, and was used as received. A 200 µg mL⁻¹ solution of RLX was prepared by dissolving accurately weighed 20 mg of pure drug in minimum quantity of glacial acetic acid and warmed to get clear solution, followed by diluting to 100 mL with water in a calibrated flask. This stock solution was diluted with water to get working concentrations of 10 and 5 µg mL⁻¹ RLX for method A and method B, respectively.

### 3.1.3. PROCEDURES

#### 3.1.3.1. Spectrophotometric method using methylene blue (Method A)

Aliquots of pure RLX solution (0.5 to 5.0 mL; 10 µg mL⁻¹) were transferred into a series of 10 mL calibrated flasks and the total volume was adjusted to 5.0 mL with water. To each flask were added 1 mL of 5M hydrochloric acid followed by 1 mL of bromate-bromide mixture (25 µg mL⁻¹). The content was mixed well and the flasks were set aside for 10 min with occasional shaking. Finally, 1 mL of 40 µg mL⁻¹ methylene blue solution was added to each flask, diluted to the mark with water and the absorbance of solution was measured at 665 nm against reagent blank after 10 min.

#### 3.1.3.2. Spectrophotometry with rhodamine B (Method B)

Varying aliquots (0.5-4.0 mL) of standard 5 µg mL⁻¹RLX solution were measured accurately and delivered into a series of 10 mL calibrated flasks and the total volume was brought to 4.0 mL with water. To each flask were added 1 mL each of 5 M hydrochloric acid and 10 µg mL⁻¹ bromate-bromide mixture successively; the flasks were let stand for 10 min with occasional shaking. Then, 1 mL of 50 µg mL⁻¹ rhodamine B solution was added to each flask, the volume was adjusted to the mark
with water and mixed well. The absorbance of each solution was measured at 555 nm against a reagent blank after 10 min.

In either spectrophotometric method, the concentration of the unknown was read from the calibration graph or computed from the regression equation derived from the Beer’s law data.

**Assay procedure for formulations**

Pharmaceutical preparations such as Fiona-60 (Dr. Reddy’s laboratories Ltd., Hyderabad, India), Ralista-60 (Cipla Ltd., Mumbai, India), Gynista-60 (Reddy’s Ltd, Hyderabad, India), Ronal-60 (Blue cross Ltd., India) and Ralofen (Lupin laboratories Ltd., Mumbai, India) were purchased from local market.

An amount of finely ground tablet powder equivalent to 20 mg of RLX was accurately weighed into a beaker, 10 mL of glacial acetic acid was added and stirred for 20 min, and warmed. Then, the content was transferred to a 100 mL calibrated flask, the beaker was washed with water and the washings were also transferred to the flask, and the volume was diluted with water to the mark, mixed well, and filtered using a Whatman No 42 filter paper. First 10 mL portion of the filtrate was discarded and a suitable aliquot of the subsequent portion (200 µg mL⁻¹ RLX) was diluted appropriately to get 10 and 5 µg mL⁻¹ concentrations for analysis by method A and method B, respectively.

### 3.1.4. RESULTS AND DISCUSSION

#### 3.1.4.1. Method Development

The proposed spectrophotometric methods are indirect and are based on the determination of the residual bromine (*insitu* generated) after allowing the reaction between RLX and a measured excess of bromine to be complete. The surplus bromine was determined by reacting it with a fixed concentration of either methylene blue or rhodamine -B dye. The possible reaction scheme is given below:

```
RLX + Known excess of *insitu* Bromine → Reaction product + Unreacted bromine
```

Unreacted bromine + Methylene blue → Absorbance measured at 665 nm (method A)

Unreacted bromine + Rhodamine B → Absorbance measured at 555 nm (method B)
RLX when added in increasing concentrations to a fixed concentration of *insitu* generated bromine, RLX consumes the latter proportionately and there occurs a concomitant fall in the concentration of bromine. When a fixed concentration of dye is added to decreasing amounts of bromine, a concomitant increase in the concentration of dye results. Consequently, a proportional increase in the absorbance at the respective $\lambda_{\text{max}}$ is observed with increasing concentration of RLX (Fig. 3.1.1 and 3.1.2).

![Fig. 3.1.1. Beer’s law curve for method A](image1)

![Fig. 3.1.2. Beer’s law curve for method B](image2)

Preliminary experiments were performed to fix the upper limits of the dyes that could be determined spectrophotometrically, and these were found to be 4 and 5 $\mu$g mL$^{-1}$ for methylene blue and rhodamine B, respectively. A bromate concentration of 2.5 $\mu$g mL$^{-1}$ was found to irreversibly destroy the blue colour of 4 $\mu$g mL$^{-1}$ methylene blue whereas 1.0 $\mu$g mL$^{-1}$ bromate was required to bleach red colour due to 5 $\mu$g mL$^{-1}$ rhodamine B. Hence, different concentrations of RLX were reacted with 1 mL of 25 $\mu$g mL$^{-1}$ bromate in method A and 1 mL of 10 $\mu$g mL$^{-1}$ bromate in
method B followed by determination of the residual bromine as described under the respective procedures.

For both the steps, i.e., reaction of RLX with bromine, and bleaching of dye by bromine, hydrochloric acid medium was found well suited. One mL of 5 M hydrochloric acid in a total volume of ~4-5 mL was adequate for the bromination/oxidation step which was complete in 10 min in both methods and the same quantity of acid was employed for the estimation of the dye. Contact time of 10 min is not critical and any delay upto 20 min for method A, and 30 min for method B, had no effect on the absorbance. The absorbance of either dyes solution even in the presence of reaction product was found to be stable for several days.

3.1.4.2. Method Validation

Linearity range and sensitivity

A linear correlation was found between absorbance at $\lambda_{\text{max}}$ and concentration of RLX.

The graphs showed negligible intercept and are described by the equation:

$$Y = a + bX$$

(Where $Y$ = absorbance of 1-cm layer of solution; $a$ = intercept; $b$ = slope and $X$ = concentration in $\mu$g mL$^{-1}$). Regression analysis of the Beer’s law data using the method of least squares was made to evaluate the slope ($b$), intercept ($a$) and correlation coefficient($r$) for each system and the values are presented in Table 3.1.1. The optical characteristics such as Beer’s law limits, molar absorptivity, Sandell sensitivity values, LOD and LOQ of both methods are also given in Table 3.1.1.
Table 3.1.1 - Analytical and regression parameters of methods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$, nm</td>
<td>665</td>
<td>555</td>
</tr>
<tr>
<td>Beer’s law limits, $\mu$g mL$^{-1}$</td>
<td>0.5 – 5.0</td>
<td>0.25 – 2.0</td>
</tr>
<tr>
<td>Molar absorptivity, L mol$^{-1}$ cm$^{-1}$</td>
<td>$7.0 \times 10^4$</td>
<td>$1.1 \times 10^5$</td>
</tr>
<tr>
<td>Sandell sensitivity, $\mu$g cm$^{-2}$</td>
<td>0.0073</td>
<td>0.0048</td>
</tr>
<tr>
<td>Limit of detection, $\mu$g mL$^{-1}$</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Limit of quantification, $\mu$g mL$^{-1}$</td>
<td>0.18</td>
<td>0.13</td>
</tr>
<tr>
<td>Regression equation, $Y^*$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>-0.0090</td>
<td>-0.0050</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.1410</td>
<td>0.2140</td>
</tr>
<tr>
<td>Correlation coefficient, (r)</td>
<td>0.9997</td>
<td>0.9997</td>
</tr>
<tr>
<td>$S_a$</td>
<td>0.0061</td>
<td>0.0039</td>
</tr>
<tr>
<td>$S_b$</td>
<td>0.0019</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

*Y = a+bX, where Y is the absorbance and X concentration in $\mu$g mL$^{-1}$

$S_a$= Standard deviation of intercept.
$S_b$= Standard deviation of slope.

Intra-day and inter-day accuracy and precision

The precision of the methods was evaluated in terms of intermediate precision (intra-day and inter-day). Three different concentrations of RLX (within the Beer’s law range) were analysed in seven replicates during the same day (intra-day precision) and five consecutive days (inter-day precision) by preparing all solutions afresh each day. The RSD values of intra-day and inter-day precision studies for both methods showed that the precision was satisfactory (Table 3.1.2).

The accuracy was evaluated as percentage relative error between the measured mean concentrations and added concentrations of RLX (Bias, %). The results obtained for three different concentrations are shown in Table 3.1.2, from which it is clear that the accuracy is fairly good in both the methods.
Table 3.1.2. Intra-day and inter-day accuracy and precision studies

<table>
<thead>
<tr>
<th>Method*</th>
<th>RLX taken</th>
<th>Intra-day accuracy and precisiona</th>
<th>Inter-day accuracy and precisionb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RLX found</td>
<td>RE, %</td>
<td>RSD, %</td>
</tr>
<tr>
<td>Spectrophotometric</td>
<td>1.5</td>
<td>1.47</td>
<td>2.00</td>
</tr>
<tr>
<td>method A</td>
<td>2.5</td>
<td>2.49</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>3.45</td>
<td>1.43</td>
</tr>
<tr>
<td>Spectrophotometric</td>
<td>0.5</td>
<td>0.49</td>
<td>2.00</td>
</tr>
<tr>
<td>method B</td>
<td>1.0</td>
<td>0.95</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.45</td>
<td>3.33</td>
</tr>
</tbody>
</table>

RE, relative error; RSD, relative standard deviation.
*The quantities are in µg mL⁻¹
  a. n=7; b. n=5.

Robustness and ruggedness

To evaluate the robustness of the method, the reaction time was slightly altered (10±1 min), and the same was found to have no significant effect on the precision of the methods when studies were made on a single concentration of RLX. The intermediate precision was quite satisfactory with RSD values less than 3%. The ruggedness of the methods was assessed by calculating the RSD for results obtained by performing the analysis using three different instruments and by three different persons. The inter-instrumental RSD values ranged from 2.9-5.5% whereas the inter-personal RSD values varied from 1.5-3.5% for three concentrations of RLX employed for accuracy and precision. The study suggested that the methods were robust as well as rugged.

Selectivity

To determine the selectivity of the methods, a synthetic mixture with the composition: RLX, talc, starch, lactose, calcium gluconate, calcium dihydrogen orthophosphate, sodium alginate and magnesium stearate, in the mass ratio of 1:2.5:3.0:0.3:0.5:0.2:0.7:1 was prepared and subjected to analysis by the proposed methods after preparing the solution using the procedure described for tablets. The percent recoveries of RLX were 99.58 ± 0.72 (n=5) and 100.40 ± 1.78 (n=5) by method A and method B, respectively, suggesting non-interference by the excipients in the assay of RLX under the described optimum conditions. In addition, the slope of
the calibration plot for standards in each method was compared with that prepared from the synthetic mixture extract. It was found that there was no significant difference between the slopes which indicated that excipients did not interfere in the determination of active ingredient.

**Application to analysis of commercial samples**

In order to check the validity of the proposed methods, RLX was determined in some commercial formulations. Table 3.1.3 gives the results of the determination from which it is clear that there is close agreement between the results obtained by the proposed methods and the label claim. The results were also compared statistically by a Student’s t- test for accuracy and variance ratio F- test for precision with those of the reference method [2] (The method is based on the oxidation of the drug with ferric chloride and coupling with potassium ferric cyanide and the absorbance measured at 735 nm), at 95% confidence level. The calculated t- and F-values (Table 3.1.3) did not exceed the tabulated values (t=2.77, F=6.39) for four degrees of freedom indicate that there was no significant difference between the proposed methods and the reference method in respect to accuracy and precision.

### Table 3.1.3-Results of determination of raloxifene hydrochloride in formulations and statistical comparison with the reference method

<table>
<thead>
<tr>
<th>Formulation Brand name*</th>
<th>Nominal amount, mg</th>
<th>Reference method</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIONA a</td>
<td>60</td>
<td>100.3±0.46</td>
<td>99.8±1.05</td>
<td>101.1±1.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t=1.05</td>
<td>t=1.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=5.21</td>
<td>F=5.01</td>
<td></td>
</tr>
<tr>
<td>GYNISTA b</td>
<td>60</td>
<td>102.3±0.51</td>
<td>101.95±1.02</td>
<td>101.5±1.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t=0.72</td>
<td>t=1.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=4.00</td>
<td>F=5.17</td>
<td></td>
</tr>
<tr>
<td>RONAL c</td>
<td>60</td>
<td>101.2±0.65</td>
<td>99.95±1.02</td>
<td>101.9±1.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t=2.37</td>
<td>t=1.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=2.46</td>
<td>F=4.19</td>
<td></td>
</tr>
</tbody>
</table>

*Mean value of five determinations


Tabulated t-value at 95% confidence level is 2.77

Tabulated F-value at 95% confidence level is 6.39.
**Recovery Study**

The accuracy and validity of the proposed methods were further ascertained by performing recovery studies. Pre-analysed tablet powder was spiked with pure RLX at three different levels and the total was found by the proposed methods. Each determination was repeated three times. The recovery of the pure drug added was quantitative and revealed that co-formulated substances did not interfere in the determination. The results of recovery study are compiled in Table-3.1.4.

**Table 3.1.4- Results of recovery experiments by standard addition method**

<table>
<thead>
<tr>
<th>Formulation studied</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RLX in tablet, µg mL(^{-1})</td>
<td>Pure RLX added, µg mL(^{-1})</td>
</tr>
<tr>
<td>FIONA 60</td>
<td>0.50</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>3.0</td>
</tr>
<tr>
<td>RONAL 60</td>
<td>0.50</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*Mean value of three determinations
SECTION 3.2

SPECTROPHOTOMETRIC METHODS FOR THE ASSAY OF RALOXIFENE HYDROCHLORIDE USING POTASSIUM PERMANGANATE AS REAGENT*

3.2.1. INTRODUCTION

Potassium permanganate is the chemical compound, KMnO₄. In this salt, manganese is in the +7 oxidation state. The salt is also known as "permanganate of potash." The permanganate ion is a strong oxidizing agent [16]. As a strong oxidant it does not generate toxic byproducts.

Potassium permanganate can also be used to quantitatively determine the total oxidisable organic material in an aqueous sample. The value determined is known as the permanganate value. In analytical chemistry, a standardized aqueous solution of KMnO₄ is sometimes used as an oxidizing titrant for redox titrations. In a related way, it is used as a reagent to determine the Kappa number of wood pulp. For the standardization of KMnO₄ solutions, reduction by oxalic acid [17] is often used.

The Mn-containing products from redox reactions depend on the pH. Acidic solutions of permanganate are reduced to the faintly pink manganese(II) sulfate ([Mn(H₂O)₆]²⁺). In neutral solution, permanganate is only reduced by 3e⁻ to give MnO₂, wherein Mn is in a +4 oxidation state. This is the material that stains one's skin when handling KMnO₄. KMnO₄ spontaneously reduced in an alkaline solution to green K₂MnO₄, wherein manganese is in the +6 oxidation state. Potassium permanganate and sulfuric acid react to produce some ozone, which has a high oxidising power and rapidly oxidises the alcohol, causing it to combust. As a similar reaction produces explosive Mn₃O₇, this should only be attempted with great care. An approximate equation for the ozone formation is shown below.

$$6 \text{KMnO}_4(aq) + 9 \text{H}_2\text{SO}_4(aq) \rightarrow 6 \text{MnSO}_4(aq) + 3 \text{K}_2\text{SO}_4(aq) + 9 \text{H}_2\text{O}(l) + 5 \text{O}_3(g)$$

KMnO₄ has been a useful oxidimetric reagent in pharmaceutical analysis, for example thioxanthines [18], isoniazid [19], methyl thiouracil [20], chloramphenicol [21], amidopyrine [22], valdecoxib [23], nicotine [24], tramadol HCl [25], cefuroxime [26], diloxamide [27] and pentacozone [28], to name a few.

*This work has been communicated to Arch. Pharm. Res (Revised version submitted)
The literature survey presented in Section 3.0.1 reveals that no spectrophotometric method has ever been reported using permanganate. The author studied the reaction of RLX with permanganate in acid medium based on which two sensitive spectrophotometric methods were developed. The details of method development and method validation are contained in this Section, 3.2.

3.2.2.0. EXPERIMENTAL

3.2.2.1. Apparatus

The instrument used is the same as the one described in section 2.1.2.1.

3.2.2.2. Reagents and Standards

All chemicals used were of analytical reagent grade purity and solutions were made in distilled water.

**KMnO₄ (0.01 M):** Prepared by dissolving the calculated amount of the chemical (S. D. Fine Chem., Mumbai, India) in water and standardized with pure arsenous oxide [29]. It was diluted appropriately with water to get a working concentration of 600 µg mL⁻¹ KMnO₄.

**Acetic acid (0.1 and 10 M) and sulphuric acid (5 M):** Prepared by diluting the concentrated acids (both procured from Merck Ltd., Mumbai, India) with water.

**Preparation of standard RLX solution**

A stock solution was prepared as described in Section 3.1.2.2. The stock solution was diluted with water appropriately to get working concentrations of 12 and 30 µg mL⁻¹ for method A and method B, respectively.

3.2.3. PROCEDURES

3.2.3.1. Method A

Different aliquots (0.0, 0.5, 1.0, 2.0...5.0 mL) of standard RLX solution (12 µg mL⁻¹) were accurately measured by means of a micro burette and transferred into a series of 10 mL calibrated flasks and the total volume was brought to 5.0 mL with 0.1 M acetic acid. To each flask was added 1 mL of 600 µg mL⁻¹ KMnO₄, content mixed and the flasks were set aside for 20 min with occasional shaking. Finally, the volume was diluted to the mark with 0.1 M acetic acid, mixed and absorbance of each solution was measured at 430 nm against the reagent blank.

3.2.3.2. Method B

Varying aliquots (0.0, 0.5, 1.0, 2.0...5.0 mL) of standard RLX solution (30 µg mL⁻¹) were measured accurately and delivered into a series of 10 mL calibrated flasks
and the total volume was adjusted to 5 mL by adding water. To each flask were added 1 mL each of 5 M H₂SO₄ and 600 µg mL⁻¹ KMnO₄, the last being measured with a microburette, and mixed. After 10 min, the volume was brought to the mark with 10 M acetic acid and absorbance measured at 550 nm against water blank.

The calibration graphs were prepared by plotting the increasing absorbance values in method A and decreasing absorbance values in method B at the respective λ_max versus concentration of RLX in µg mL⁻¹. The concentration of the unknown was computed from the calibration graph or deduced from the regression equation derived using the absorbance-concentration data.

**Procedure for tablets**

Tablet extract was prepared as described in Section 3.1.3 and a subsequent portion was diluted according to the need, and then analysed as per the recommended procedures.

### 3.2.4. RESULTS AND DISCUSSION

In the present work, the author examined the utility of KMnO₄ for the assay of RLX. The first method (method A) is based on the formation of a yellowish-brown coloured product when the drug is reacted with KMnO₄ in acetic acid medium. RLX was also found to react with KMnO₄ in H₂SO₄ medium consuming the oxidant in proportion to its concentration. These two observations served as the basis for the spectrophotometric assay of RLX.

#### 3.2.4.1. Method development

**Method A**

KMnO₄ was found to react with RLX in dilute acetic acid medium to form a yellowish-brown product with an absorption maximum at 430 nm at which the reagent blank had negligible absorbance (Fig. 3.2.1).

![Absorption spectra for method A](image-url)

**Fig. 3.2.1.** Absorption spectra for method A
Below and above 430 nm, the blank absorbance showed increasing trend, and hence all absorbance measurement were made at 430 nm. Optimum conditions of the reaction with respect to acid concentration, reaction time and the permanganate concentration were investigated by univariate method (changing one parameter at a time). The formation of yellowish-brown colour was found to be specific in acidic acid medium since no such colour formation resulted in $\text{H}_2\text{SO}_4$, HCl and $\text{H}_3\text{PO}_4$ media. The effect of acetic acid concentration on the reaction was examined by changing the volume of 1.0 M acetic acid from 0.1 to 0.5 mL in a total volume of ~ 5 mL. The absorbance was nearly constant over this range (Fig. 3.2.2).

![Effect of acetic acid concentration](image)

**Fig. 3.2.2.** Effect of acetic acid concentration. (RLX 6.0 µg mL$^{-1}$, Method A)

Higher acetic acid concentrations were undesirable since they failed to produce a constant absorbance even after 30 min contact time. Reaction sensitivity and stability of the coloured species depended on the diluent. Best results were obtained when 0.1 M acetic acid was used as the diluent.

The optimum time for the completion of the reaction between RLX and KMnO$_4$ was 20 min and the colour was stable for the next 15 min (Fig. 3.2.3).

![Effect of reaction time](image)

**Fig. 3.2.3.** Effect of reaction time. (RLX 6.0 µg mL$^{-1}$, Method A)
Optimum permanganate concentration was determined by varying the volume of 600 µg mL\(^{-1}\) KMnO\(_4\) from 0.5 to 3.0 mL in a total volume of 10 mL. Maximum absorbance was reached for 1 mL, and any further increase in permanganate volume did not significantly alter the measured absorbance (Fig.3.2.4), but the blank absorbance showed a gradual rising trend with the permanganate volume. Hence, 1 mL of 600 µg mL\(^{-1}\) KMnO\(_4\) was used in subsequent experiments.

![Figure 3.2.4](image)

**Fig.3.2.4.** Effect of KMnO\(_4\) volume (RLX 6.0 µg mL\(^{-1}\), Method A)

**Method B**

In this method, RLX was reacted with measured excess of KMnO\(_4\) in H\(_2\)SO\(_4\) medium, and after the oxidation was determined to be complete, the absorbance of the residual permanganate was measured at 550 nm. The decrease in absorbance compared with the reagent blank was taken as a measure of the drug concentration. When a fixed concentration of permanganate was reacted with increasing concentrations of RLX, there occurred a concomitant fall in the concentration of permanganate which resulted in the decrease in absorbance at 550 nm with increasing concentration of RLX. This is corroborated by the regression coefficient value of -0.9960.

Preliminary experiment was performed to assess the linear range over which KMnO\(_4\) could be determined spectrophotometrically at 550 nm in H\(_2\)SO\(_4\) medium, and this was found to be 6.0-60.0 µg mL\(^{-1}\). Hence, different concentrations of RLX were reacted with 1 mL of 600 µg mL\(^{-1}\) KMnO\(_4\) in H\(_2\)SO\(_4\) medium before measuring the absorbance of the residual permanganate. This enabled to fix the linear range of applicability of the method for RLX.

The experimental conditions of assay were optimized in ways similar to method A. H\(_2\)SO\(_4\) concentration was varied over a wide range (0.1-1.0 M, overall) and its effect was examined on the rate and sensitivity of the reaction. The reaction
was found to be quantitative when 0.3 M $\text{H}_2\text{SO}_4$ was maintained and any increase up to 1.0 M did not alter the sensitivity of the reaction (Fig. 3.2.5).

**Fig. 3.2.5.** Effect of $\text{H}_2\text{SO}_4$ concentration (Method B)

At $\text{H}_2\text{SO}_4$ concentration <0.3 M, turbid and unclear solution resulted, and acid concentrations >1.0 M were also undesirable since the reaction between RLX and permanganate did not stop even after a 30 min standing time. Hence, 1 mL of 5 M $\text{H}_2\text{SO}_4$ in a total volume of 10 mL (0.50 M, overall) was used in all subsequent work.

At 0.5 M $\text{H}_2\text{SO}_4$, the reaction was faster than in method A, reaching completion in 10 min and the absorbance was constant for the next 15 min. Very small changes in absorbance were observed beyond this stability time.

Two blanks were prepared in this method. The reagent blank which consisted of all the reactants except RLX showed maximum absorbance (equal to the intercept of the regression equation). The second blank was prepared in the absence of permanganate and RLX to assess the contribution of others to the absorbance of the system. Since the absorbance of the second blank was negligible, all measurements were made against water blank.

**3.2.4.2. Method Validation**

**Analytical data**

Under the described experimental conditions of the proposed methods, the plots of absorbance versus concentration within the range studied (Table 3.2.1) showed linear relationships. In method B, Beer’s law was obeyed in the inverse manner. The regression analyses of these plots using the method of least squares were made to evaluate intercept ($a$), slope ($b$), correlation coefficient ($r$), confidence limits of intercept ($\pm t S_a$) and slope ($\pm t S_b$) at 95% confidence level and variance ($S_D^2$). The
results are summarized in Table 3.2.1. The linearity of the calibration plots was validated by high values of correlation coefficients of regression equations and intercept value (method A) which was close to zero. In method B, intercept was equal to the absorbance of the reagent blank because of the inverse relationship between absorbance and concentration. The high sensitivity of the proposed methods was indicated by the high values of molar absorptivity and low values Sandell sensitivity, and detection limit (LOD). The small values of variance also confirmed the negligible scattering of experimental data points from the line of best-fit for both the methods.

**Table 3.2.1. Sensitivity and regression parameter**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{max}} ), nm</td>
<td>430</td>
<td>550</td>
</tr>
<tr>
<td>Beer’s law limits, ( \mu g \text{ mL}^{-1} )</td>
<td>0.6-6.0</td>
<td>1.5-15.0</td>
</tr>
<tr>
<td>Molar absorptivity, ( L \text{ mol}^{-1} \text{ cm}^{-1} )</td>
<td>( 7.01 \times 10^{4} )</td>
<td>( 2.8 \times 10^{4} )</td>
</tr>
<tr>
<td>Sandell sensitivity, ( \mu g \text{ cm}^{2} )</td>
<td>0.0073</td>
<td>0.0146</td>
</tr>
<tr>
<td>Limit of detection, ( \mu g \text{ mL}^{-1} )</td>
<td>0.08</td>
<td>0.45</td>
</tr>
<tr>
<td>Limit of quantification, ( \mu g \text{ mL}^{-1} )</td>
<td>0.25</td>
<td>1.35</td>
</tr>
<tr>
<td>Regression equation, ( Y^{*} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>-0.0416</td>
<td>0.7760</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.1591</td>
<td>-0.0500</td>
</tr>
<tr>
<td>Correlation coefficient, ( r )</td>
<td>0.9992</td>
<td>-0.9962</td>
</tr>
<tr>
<td>( S_a )</td>
<td>0.0150</td>
<td>0.1400</td>
</tr>
<tr>
<td>( S_a^{2} )</td>
<td>0.0002</td>
<td>0.019</td>
</tr>
<tr>
<td>Confidence limit, intercept</td>
<td>-0.0416 ( \pm 0.041 )</td>
<td>0.7760 ( \pm 0.388 )</td>
</tr>
<tr>
<td>( S_b )</td>
<td>0.0032</td>
<td>0.0111</td>
</tr>
<tr>
<td>( S_b^{2} )</td>
<td>0.00001</td>
<td>0.00012</td>
</tr>
<tr>
<td>Confidence limit, slope</td>
<td>0.1591 ( \pm 0.009 )</td>
<td>-0.050 ( \pm 0.031 )</td>
</tr>
</tbody>
</table>

\( *Y = a+bX \), where \( Y \) is the absorbance and \( X \) concentration in \( \mu g \text{ mL}^{-1} \).

\( S_a \) = Standard deviation of intercept.

\( S_b \) = Standard deviation of slope.

**Precision and accuracy**

The precision of the methods was evaluated in terms of intermediate precision (intra-day and inter-day). Three different concentrations of RLX within the Beer’s
limits were analysed in seven replicates during the same-day [intra-day precision]. The results are presented in Table 3.2.2. The intra-day RSD (%) values were <0.74% and < 0.83%, for method A and method B, respectively. The inter-day precision was somewhat poor with the RSD values in the range 0.56-0.95%.

The accuracy of an analytical method expresses the closeness between the reference value and found value. Accuracy was evaluated as percent relative error (RE,%) between the measured mean concentrations and taken concentrations. The results obtained for three concentrations (within the Beer’s law range) are shown in Table 3.2.2. With % RE values in the range 0.42-1.11, the accuracy of the methods can be considered to be satisfactory.

**Table 3.2.2. Evaluation Intra-day and Inter-day precision and accuracy**

<table>
<thead>
<tr>
<th>Method</th>
<th>Nominal concentration taken, µg mL (^{-1})</th>
<th>Intra-day accuracy and precision (n=7)</th>
<th>Inter-day accuracy and precision (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RLX found, µg mL (^{-1})</td>
<td>RE, %</td>
<td>RSD, %</td>
</tr>
<tr>
<td>A</td>
<td>1.2</td>
<td>1.21</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>2.41</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>3.62</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>6.05</td>
<td>0.83</td>
</tr>
<tr>
<td>B</td>
<td>9.0</td>
<td>9.07</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>12.07</td>
<td>0.58</td>
</tr>
</tbody>
</table>

RE. relative error; RSD. relative standard deviation.

*The quantities are in µg mL \(^{-1}\)

a. \(n=7\); b. \(n=5\).

**Selectivity**

To determine the selectivity of the methods, the analytical placebo was prepared and subjected to analysis by the proposed methods. It was confirmed that the change in absorbance with respect to the reagent blank was caused only by the analyte. To identify the interference by common tablet excipients, a synthetic mixture with the composition: RLX, talc, starch, lactose, calcium gluconate, calcium dihydrogenorthophosphate, sodium alginate and magnesium stearate, in the mass ratio of 1: 2.5: 3.0: 0.3: 0.5: 0.2: 0.7: 1 was prepared and subjected to analysis by the proposed methods after preparing the solution using the procedure described for
tablets. The percent recoveries of RLX were 98.58 ± 0.72 (n=5) and 101.40 ± 1.28 (n=5) by method A and method B, respectively, suggesting non-interference by the excipients in the assay of RLX under the described optimum conditions. In addition, the slope of the calibration plot for standards in each method was compared with that prepared from the synthetic mixture extract. It was found that there was no significant difference between the slopes which indicated that excipients did not interfere in the determination of active ingredient.

**Robustness and ruggedness**

Robustness of the proposed methods was checked by deliberately altering two important parameters; acid concentration and reaction time as shown in Table 3.2.3. The performance of the methods was found to be unaffected as revealed by small values of percent RSD values (intermediate precision). The intermediate precision as a measure of the ruggedness is obtained when the assay is performed by multiple analysts and using multiple instruments in the same laboratory. In order to study these effects, RLX solution at three concentration levels was analysed by four different analysts; and also using three different instruments by a single analyst. The low percent RSD values presented in Table 3.2.3 indicate a good precision of the analytical procedures.

### Table 3.2.3. Robustness and ruggedness expressed as intermediate precision (%RSD)

<table>
<thead>
<tr>
<th>Method</th>
<th>RLX taken, µg mL⁻¹</th>
<th>Method robustness</th>
<th>Method ruggedness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Parameter altered</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acid concentration (n = 3)</td>
<td>Reaction time (n = 3)</td>
</tr>
<tr>
<td>A</td>
<td>1.2</td>
<td>0.92</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>0.85</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>0.66</td>
<td>0.77</td>
</tr>
<tr>
<td>B</td>
<td>6.0</td>
<td>1.06</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>0.98</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>0.74</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*In method A, acetic acid concentrations used were 0.02, 0.03 and 0.04 M; and in method B, H₂SO₄ acid concentrations were 0.4, 0.5 and 0.6 M

**Reaction times were 18, 20 and 22 min in method A and they were 8, 10 and 12 min in method B.**
Application to analysis of tablets

The assay of RLX in commercial tablets was performed using the proposed methods and the reference method [2]. The results obtained were compared statistically by Student’s t-test and the variance ratio F-test, and are summed up in Table 3.2.4. It is clear from the Table that the calculated t-values are less than the theoretical (tabulated) values at the 95% confidence level indicating no significant difference between the proposed and reference methods with regard to accuracy and precision.

Table 3.2.4. Assay results in tablets and statistical comparison with the reference method.

<table>
<thead>
<tr>
<th>Tablet brand name</th>
<th>Nominal amount, mg</th>
<th>Percent of label claim ± SD</th>
<th>Reference method</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiona</td>
<td>60</td>
<td>97.58 ± 0.62</td>
<td>98.72 ± 1.24</td>
<td>t = 1.94</td>
<td>t = 1.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F = 4.0</td>
<td>F = 5.25</td>
</tr>
<tr>
<td>Ralista</td>
<td>60</td>
<td>102.5 ± 0.74</td>
<td>101.3 ± 1.32</td>
<td>t = 1.84</td>
<td>t = 1.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F = 3.18</td>
<td>F = 2.99</td>
</tr>
<tr>
<td>Ralofen</td>
<td>60</td>
<td>99.74 ± 0.42</td>
<td>100.3 ± 1.14</td>
<td>t = 1.07</td>
<td>t = 2.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F = 4.81</td>
<td>F = 3.41</td>
</tr>
</tbody>
</table>

*Marketed by: a. Dr. Reddy’s laboratories Ltd., Hyderabad, India, Hyderabad; b. Cipla Ltd., Mumbai, India; c. Lupin laboratories Ltd., Mumbai, India. Tabulated t-value at the 95% confidence level is 2.77. Tabulated F-value at the 95% confidence level is 6.39.

Recovery study

As an additional demonstration of accuracy and reliability of the proposed methods, recovery experiments were performed via standard-addition procedure. Pre-analysed tablet powder was spiked with pure RLX at three different levels and the total was found by the proposed methods. Each determination was repeated three times. The results are summarized in Table 3.2.5. The mean percent recoveries of pure RLX obtained by the proposed methods suggest that there was not interference from the common excipients present in tablets. The good reproducibility was manifested by low SD values as evident from Table 3.2.5.
Table 3.2.5. Results of recovery study via standard-addition method.

<table>
<thead>
<tr>
<th>Tablet Studied</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RLX in tablet, µg mL⁻¹</td>
<td>Pure RLX added, µg mL⁻¹</td>
</tr>
<tr>
<td>Fiona, 60 mg</td>
<td>1.97</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.97</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>1.97</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*Mean value of three determinations*
SECTION 3.3

GRADIENT HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF RALOXIFINE HYDROCHLORIDE IN PHARMACEUTICALS*

3.3.1. INTRODUCTION

The reported HPLC methods for RLX were surveyed in Section 3.0.2. By introducing certain modifications in respect of column and mobile phase composition, the author has been able to develop an HPLC method for RLX which does not require an internal standard. The method is applicable over a wide dynamic concentration range. The details of method development and method validation are presented in this Section.

3.3.2. EXPERIMENTAL

3.3.2.1. Apparatus

The chromatographic system consisted of an Agilent 1100 series chromatograph equipped with an in built solvent degasser, quaternary pump, photo diode array detector with variable injector and auto sampler, and a reversed phase 5 µm Hypersil ODS column (250x4.6 mm,i.d.). Data was processed using class VP software 5.032 software.

3.3.2.2. Reagents and Standards

All chemicals used were of analytical reagent grade. Ammonium acetate, acetic acid (sd. Fine Chem. Ltd, India) and HPLC grade acetonitrile (Merck. Ltd, India) were used. Distilled water was filtered through 0.45 µm filter paper (Millipore). A diluent consisting of acetonitrile: water (60:40, v/v) was used for the sample preparations.

Mobile phase

Mobile phase A: 0.154% ammonium acetate (w/v) was dissolved in water and pH was adjusted to 4.0 with acetic acid.

Mobile phase B: acetonitrile.

Preparation of standard solution

An accurately weighed 50 mg of RLX was dissolved in and diluted to volume with the diluent solution in a 50 mL calibrated flask to obtain a concentration of 1000 µg mL⁻¹ RLX.

*This work has been published in Acta. Pharm, 2008, 58, 347-356
3.3.3. PROCEDURES

Chromatographic conditions

The separation was achieved at ambient temperature on the column using the mobile phase at a flow rate of 1.2 mL min\(^{-1}\). The detector wavelength was set at 284 nm with a sensitivity of 0.2 a.u.f.s.

Gradient composition was as follows:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>35</th>
<th>40</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>%A</td>
<td>50</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>%B</td>
<td>50</td>
<td>90</td>
<td>90</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Calibration

Working standard solutions equivalent to 50 to 600 µg mL\(^{-1}\) RLX were prepared by appropriate dilution of stock standard solution (1000 µg mL\(^{-1}\)) with the diluent solution. Twenty µl aliquot of each solution was injected automatically onto the column in duplicate and the chromatograms were recorded. Calibration graph was prepared by plotting the mean peak area \textit{versus} concentration of RLX.

The concentration of the unknown was read from the calibration graph or computed from the regression equation derived from the mean peak area-concentration data.

Assay in dosage forms

A quantity of tablet powder equivalent 100 mg of RLX was accurately weighed into a 100 mL calibrated flask, 60 mL of diluent solution was added and content shaken for 20 min; then, the volume was diluted to the mark and mixed well. A small portion of the extract (say 10 mL) was withdrawn and filtered through 0.45-µm filter to ensure the absence of particulate matter. The filtered solution was appropriately diluted with the diluent solution for analysis as described already. The results are compared in Table. 3.3.2.

3.3.4. RESULTS AND DISCUSSION

3.3.4.1. Method development

A solution of RLX was injected in duplicate on to the column and was monitored by UV-detection at 284 nm. A gradient method was selected rather than isocratic to get the faster elution with lesser retention time. At a flow rate of 1.2 mL min\(^{-1}\), the retention time was 6.65 min (Fig. 3.3.1.a). Under the described experimental conditions, the peak was well defined and free from tailing. RLX was determined by measuring the peak area. A plot of mean peak area against
concentration gave a linear relationship (correlation coefficient, R=0.9991, n=6) over the concentration range 50-600 µg mL⁻¹. Using the regression analysis, the linear equation, \( Y = (-184.40) + (57.88) \times X \) was obtained, where, \( Y \) is the mean peak area and \( X \) concentration in µg mL⁻¹ (Fig. 3.3.1.b)

![Typical chromatogram of pure RLX (400 µg mL⁻¹)](image)

![Calibration curve](image)

**b.**

Fig. 3.3.1. **a.** Typical chromatogram of pure RLX (400 µg mL⁻¹); **b.** Calibration curve

### 3.3.4.2. Method validation

#### Specificity

In order to determine the adequate resolution and reproducibility of the method, suitability parameters including retention time, plate number and tailing factor were investigated, and they were found to be 6.65 min, 4047 and 1.72, respectively, which amply demonstrate the method suitability. The specificity of an analytical method may be defined as the ability to unequivocally determine the analyte in the presence of additional components such as impurities, degradation products and matrix. The specificity was evaluated by preparing the analytical placebo and it was confirmed that the signal measured was caused only by the

112
analyte. A solution of analytical placebo (containing all the tablet excipients except RLX) was prepared according to the sample preparation procedure, and injected. The resulting chromatogram is shown in Fig. 3.3.1.c. To identify the interference by these excipients, tablet extract was chromatographed. The resulting chromatogram did not show any other peaks, (Fig. 3.3.1.d) which confirmed the specificity of the method. In addition, the slope of the calibration curve for standards was compared with that prepared from tablet extract. It was found that there was no significant difference between the slopes which indicated that excipients did not interfere with RLX.

Fig. 3.3.1. Chromatograms: c. Placebo blank and d. tablet extract (300 µg mL⁻¹ RLX)

**Accuracy and Precision**

To determine the intra-day accuracy and precision, pure RLX solution at three different concentrations was analysed in seven replicates on the same day. The percent relative error which is an index of accuracy is <1.0% and is indicative of good accuracy. The peak area based RSD was less than 1% and retention time based RSD was less than 0.22% indicating high accuracy and precision of the method. These results are presented in Table. 3.3.1. The inter-day precision was established by performing analyses over a period of five days on solutions prepared afresh each day.
The peak-area based and retention-time-based RSD values were <2.0 % and <1 %, respectively.

**Table 3.3.1. Intra day accuracy and precision**

<table>
<thead>
<tr>
<th>RLX taken (µg mL⁻¹)</th>
<th>RLX found (µg mL⁻¹)ᵃ</th>
<th>RE (%)</th>
<th>RSD (%)ᵇ</th>
<th>RSD (%)ᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>249.5</td>
<td>0.20</td>
<td>0.41</td>
<td>0.15</td>
</tr>
<tr>
<td>400</td>
<td>398.2</td>
<td>0.45</td>
<td>0.47</td>
<td>0.21</td>
</tr>
<tr>
<td>550</td>
<td>548.6</td>
<td>0.25</td>
<td>0.24</td>
<td>0.18</td>
</tr>
</tbody>
</table>

ᵃMean value of seven determinations;ᵇBased on peak area;ᶜBased on retention time

**Linearity and Range**

Linearity was assessed in the range of 50 to 150% of the working level concentration including working level concentration. First and last level of linearity was carried out in six replicates and other levels in duplicates. The Linearity coefficient of mean response of replicate determination plotted against respective concentration was found to be 0.9991. The percent y-intercept as obtained from the linearity data was less than 2%. The % RSD for peak area response of six replicates of first and last level was less than 2.0% and 1.0% for retention time.

**Ruggedness (Intermediate Precision)**

Intermediate precision of six replicate determination of assay of a sample was analysed by different analyst with different instrument in different day after specifying the system suitability of the method. The %RSD of assay was less than 2.0% and the cumulative %RSD of assay of precision study and intermediate precision was also less than 2.0%.

**Robustness**

Robustness of the method was checked by deliberately altering two critical parameters by minor variation: the flow rate was changed from 1.2 mL min⁻¹ to 1.1 mL min⁻¹ and the pH of the mobile phase A was changed from 4.0 to 3.9. The differences in the retention time and peak area (for a given RLX concentration) caused by the above minor alterations were insignificant.

**Limit of Quantitation and Limit of Detection**

LOQ and LOD were established based on signal-to-noise ratio, performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples. The signal to noise ratio of 3:1 and 10:1 were taken as
LOD and LOQ, respectively and were found to be 0.04 µg mL\(^{-1}\) and 0.16 µg mL\(^{-1}\), respectively.

**Selectivity testing**

A separate selectivity test was performed by applying the proposed methods to the determination of RLX in a synthetic mixture consisting of RLX, talc, starch, lactose, calcium gluconate, calcium dihydrogenorthophosphate, sodium alginate and magnesium stearate, in the mass ratio of 1: 2.5: 3.0: 0.3: 0.5: 0.2: 0.7: 1 RLX was extracted with three 20 mL portions of diluent and filtered. The filter was washed with diluent; the filtrate and washings were collected in a 100-mL calibrated flask and diluted to volume with diluent and mixed well. A convenient aliquot of the extract was subjected to analysis as stated earlier.

**Application**

The developed and validated method was applied to the determination of RLX in three brands of tablets each containing 60 mg per tablet. Evaluation was performed using the calibration curve method since no significant difference between the slopes of the calibration curves for standards and tablet extracts was observed. The results obtained by the proposed method were statistically compared with those of the literature method \cite{2} by applying the student’s t-test for accuracy and F-test for precision. As shown by the results compiled in Table 3.3.2, the calculated t- and F-values did not exceed the tabulated values at the 95\% confidence level for four degrees of freedom suggesting that the proposed method and the literature method do not differ significantly with respect to accuracy and precision.

**Table 3.3.2.** Determination of raloxifene hydrochloride in tablets and statistical comparison with the reference method

<table>
<thead>
<tr>
<th>Formulation Studied(^a)</th>
<th>Nominal amount (mg)</th>
<th>Found (%)(^a)</th>
<th>t-value</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Literature method</td>
<td>Proposed method</td>
<td></td>
</tr>
<tr>
<td>Fiona(^a)</td>
<td>60</td>
<td>101.3±0.62</td>
<td>100.8±1.29</td>
<td>0.83</td>
</tr>
<tr>
<td>Gynista(^b)</td>
<td>60</td>
<td>98.2±1.06</td>
<td>96.9±1.48</td>
<td>1.62</td>
</tr>
<tr>
<td>Ronal(^c)</td>
<td>60</td>
<td>101.5±0.85</td>
<td>102.8±1.61</td>
<td>1.67</td>
</tr>
</tbody>
</table>

\(^a\)Mean± SD, n=5
Tabulated t-value at 95\% confidence level is 2.77
Tabulated F-value at 95\% confidence level is 6.39
#Marketed by: a. Reddys Ltd; b. Micro Nova Ltd; c. Blue Cross Ltd.
Recovery study

The accuracy and validity of the proposed methods were further ascertained by performing recovery experiments. Pre-analysed tablet powder was spiked with pure RLX at three different levels and the total was found by the proposed methods. Each determination was repeated three times. The recovery of pure drug added in the range 97.7-103.2% with the RSD values of 1.6-2.2%. The results of this study given in Table 3.3.3 reveal that the common tablet excipients did not interfere in the determination.

Table 3.3.3. Results of Recovery study

<table>
<thead>
<tr>
<th>Formulation studied</th>
<th>RLX in formulation, (µg mL⁻¹)</th>
<th>Pure RLX added, (µg mL⁻¹)</th>
<th>Total found (µg mL⁻¹)</th>
<th>Pure RLX recovered (%)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fional-60</td>
<td>201.6</td>
<td>50</td>
<td>251.75</td>
<td>100.3±1.7</td>
</tr>
<tr>
<td></td>
<td>201.6</td>
<td>200</td>
<td>408.00</td>
<td>103.2±1.9</td>
</tr>
<tr>
<td></td>
<td>201.6</td>
<td>350</td>
<td>550.90</td>
<td>99.80±2.1</td>
</tr>
<tr>
<td>Ronal-60</td>
<td>205.6</td>
<td>50</td>
<td>255.45</td>
<td>99.7±1.8</td>
</tr>
<tr>
<td></td>
<td>205.6</td>
<td>200</td>
<td>401.00</td>
<td>97.7±1.6</td>
</tr>
<tr>
<td></td>
<td>205.6</td>
<td>350</td>
<td>560.15</td>
<td>101.3±2.2</td>
</tr>
</tbody>
</table>

ᵃMean ± SD, 
n=3
SECTION 3.4

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC – MASS SPECTROMETRIC DETERMINATION OF RALOXIFENE HYDROCHLORIDE IN URINE*

3.4.1.0. INTRODUCTION

The reported LC-MS-MS methods for the determination of RLX were surveyed in Section 3.0.2. The details of method development and method validation are presented in this Section 3.4.

3.4.2. EXPERIMENTAL

3.4.2.1. Apparatus

The instrument used is the same as the one described in section 2.6.2.1.

Reagents and Standards

All chemicals used were of analytical reagent grade and HPLC grade acetonitrile (Merck. Ltd, Mumbai) was used. Distilled water filtered through 0.45 µm filter (Millipore, Bangalore, India) was used to prepare the solutions. For sample preparations Oasis HLB cartridge (Waters, Bangalore) was used.

Mobile phase

10 mM ammonium acetate (adjusted to pH 4.0 with formic acid) and acetonitrile in the ratio of 60:40, was used as the mobile phase. Acetonitrile was used as the diluent.

Preparation of standard solution

A stock standard containing 100 µg mL⁻¹ RLX solution was prepared by dissolving accurately weighed 10 mg of pure drug in diluent and diluting to 100 mL in a calibrated flask with the same solvent. It was subsequently diluted to obtain a working concentrations of RLX.

3.4.3. PROCEDURES

LC-MS conditions

The chromatographic separation was achieved at ambient temperature(25°C) on the column (Inertsil ODS, 3.5µm; 50x4.6 mm i.d) using the mobile phase of 10mM ammonium acetate (pH 4.0, adjusted with formic acid) and acetonitrile in the ratio of 60:40(v/v) at a flow rate of 0.6 mL min⁻¹. The mobile phase was degassed before use.

*This work has been communicated to Chem. Ind. Chem. Engg. Qtly.,(Revised paper)
Quantitation was performed using multiple reaction monitoring (MRM) of the transitions of m/z 474.6 → m/z 269.1 for raloxifene and m/z 284.2 → 257.5 m/z for letrozole (Fig. 3.4.1) (IS) with a scan time of 0.2 s per transition. Fig. 3.4.2 shows the product ion spectra of [M+H]^+ for raloxifene and letrozole.

![Structure of letrozole](image)

**Fig. 3.4.1. Structure of letrozole**

![Product ion spectra](image)

**Fig. 3.4.2. Full scan product ion spectra of raloxifene (A) and letrozole (B)**

In order to optimize all the MS parameters, a standard solution (1 µg mL$^{-1}$) of the analyte and IS (1 µg mL$^{-1}$) was infused into the mass spectrometer.

Analysis was performed in positive mode (ESI) with a turbo ion spray interface under the conditions: ion source potential, 5500 V; declustering potential, 70
V; focusing potential, 400 V, capillary temperature, 350°C; entrance potential, 10 V
with nitrogen as nebuliser gas at 25 Psi. The column eluent was introduced into the
electron spray ionization chamber of the mass spectrometer with a split ratio of 3:7.
Mass fragmentation studies were performed by maintaining the normalized collision
energy at 30 eV.

Sample preparation

The urine samples collected from healthy volunteers were spiked with known
corcentration of RLX and I.S. and the resulting sample was diluted 1:1 with water
prior to loading on solid phase extraction(SPE) cartridge (Oasis HLB-60 mg). The
SPE cartridge was conditioned with 1 mL of methanol and equilibrated with 1mL of
water, and then 1 mL of diluted urine was loaded on. The cartridge was then washed
with 1mL of 10% methanol and then eluted with 100% methanol. Sample was
evaporated with nitrogen at 40°C, and reconstituted the residue with water:
acetonitrile (1:1) to 500 µL. Then 20 µL of the above solution was injected into LC-
MS-MS for analysis. This procedure was carried out under yellow monochromatic
light.

Preparation of standard and quality control samples

Stock solutions of RLX and IS (both 10 µg mL⁻¹) were prepared in acetonitrile
and diluted to 1000 ng mL⁻¹ with acetonitrile:water(1:1). The stock solutions were
kept in amber colored bottle and stored in refrigerator when not in use.

Calibration curve was prepared by diluting the stock solution with
acetonitrile:water (1:1) to get appropriate concentration. From this dilution, 20 µL of
appropriate standard solution was added to 1 mL of urine blank to get an effective
concentration of 20, 80, 100, 500, 850 and 1000 ng mL⁻¹ for RLX.

The quality control (QC) samples were separately prepared in the blank urine
sample at the concentrations of 100, 500 and 850 ng mL⁻¹, respectively. The spiked
urine samples (standards and quality controls) were then subjected to Solid phase
extraction.

3.4.4. RESULTS AND DISCUSSION

Mass spectrometry

The signal intensity obtained for RLX in positive mode was much higher than
that in negative mode. Then, the possibility of using electrospray ionization (ESI) or
atmospheric pressure chemical ionization (APCI) sources under positive ion detection
mode was evaluated during the early stage of method development. ESI spectra revealed higher signals for the molecule compared to APCI source. Further assay development was therefore limited to ESI source. The Q1 full spectra of RLX and IS were dominated by protonated molecules \([M+H]^+\) and no significant solvent adduct ions and fragments ions were observed. The tuning of the ESI source such as capillary temperature, flow of sheath and auxiliary gas \((N_2)\) and spray voltage on the transition of RLX and IS further improved the sensitivity.

**Chromatography**

Although in the aspect of chromatographic separation the determination of the analyte was not interfered by endogenous substances in the urine sample, yet the ionization of the analyte, especially of low concentration, was easily suppressed, which resulted in the linearity of narrow concentration range. In order to avoid the ion suppression induced by endogenous substances, the influence of the mobile phase that composed of different percentage of organic phase to the ion suppression was evaluated during the experiment. It was found that when mobile phase consists of ammonium acetate\((pH 4.0)\)-acetonitrile (60:40, v/v), the spiked sample demonstrated good linearity between 20 to 1000 ng mL\(^{-1}\) for RLX. Under the present chromatographic conditions, the run time of each sample was 6.0 min. The retention times were 0.91 min and 0.90 min for RLX and IS, respectively (Fig. 3.4.3).

![Representative chromatogram of raloxifene (20 ng mL\(^{-1}\)) and IS (500 ng mL\(^{-1}\))](image-url)
3.4.4.1. Method Validation

Specificity

There was no significant interferences or ion suppression from endogenous substances observed at the retention times of the analytes in the blank chromatograms of RLX and IS (Fig. 3.4.4).

Fig. 3.4.4. Representative blank chromatogram for raloxifene(A) and IS(B)

Linearity of calibration curves and lower limit of quantification

Urine samples were quantified using ratio of the peak area of RLX to that of IS as the assay parameter. Peak area ratios were plotted against RLX concentrations and standard curve in the form of \( Y = a + bX \) was calculated using weighed (1/x^2) least squares linear regression.

The linear range of calibration curve was 20-1000 ng mL\(^{-1}\) for the analyte. The representative calibration curve was as follows: \( Y=0.00675 +0.0751x \) (\( r=0.9997 \))

The lower limit of quantification was defined as the lowest concentration on the calibration curve for which the acceptable accuracy of ± 15% and a precision below ± 15% were obtained. The present LC-MS-MS method offered an LLOQ of 20 ng mL\(^{-1}\).
Intra-day and Inter-day Accuracy and Precision

The intra-day accuracy and precision were assessed by determining QC samples in a set of six replicates within one day. The accuracy was expressed by (mean observed concentration)/(spiked concentration)x100% and the precision by relative standard deviation(RSD). The inter-day precision was established by performing analyses over a period of five days on solutions prepared afresh each day. Table-3.4.1 summarizes the precision and accuracy for the RLX evaluated by assaying the QC samples.

Table. 3.4.1 Accuracy and intra-day precision

<table>
<thead>
<tr>
<th>RLX taken ng mL⁻¹</th>
<th>RLX found* ng mL⁻¹</th>
<th>Intra-day RSD, %</th>
<th>Inter-day RSD, %</th>
<th>RE %</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>99.4</td>
<td>9.3</td>
<td>12.5</td>
<td>0.60</td>
</tr>
<tr>
<td>500</td>
<td>495.8</td>
<td>8.2</td>
<td>8.6</td>
<td>0.84</td>
</tr>
<tr>
<td>850</td>
<td>846.6</td>
<td>7.7</td>
<td>7.8</td>
<td>0.40</td>
</tr>
</tbody>
</table>

RE, relative error; RSD, Relative standard deviation;
*Mean value of seven determinations

Recovery study

Absolute recoveries of RLX at three QC levels were determined by assaying the samples as described above and comparing the peak areas of both RLX and IS with those obtained from direct injection of the compounds dissolved in the blank urine. The recovery of RLX, determined at three concentrations (100, 500, 850 ng mL⁻¹) were 75.4± 6.4%, 79.4± 6.9% and 76.4± 7.4% (n=6) respectively.
SECTION 3.5

CONCLUSION ON CHAPTER III-Assessment of Methods

Table 3.5.1. Comparison of performance characteristics of proposed methods with existing methods

### A. Spectrophotometry

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Reagent/s used</th>
<th>( \lambda_{\text{max}} )</th>
<th>Beer’s law Limit ( \mu \text{g mL}^{-1} )</th>
<th>Molar absorptivity ( \text{L mol}^{-1} \text{cm}^{-1} )</th>
<th>Remarks</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>a) Ferric chloride-Potassium ferricyanide b) Fehling’s reagent</td>
<td>735</td>
<td>----</td>
<td>----</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>430</td>
<td>----</td>
<td>----</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>a) 0.1 N Sodium hydroxide b) ferric chloride+1,10 phenanthroline c) ferric chloride+2,2’ bipyridyl</td>
<td>425</td>
<td>5-150</td>
<td>3.62 x10³</td>
<td>Less sensitive, involves heating step.</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>510</td>
<td>1-10</td>
<td>6.3 x10⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>521</td>
<td>2-25</td>
<td>2.1 x10⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>a) ( \text{BrO}^-_3 )-Br(^-) methylene blue b) ( \text{BrO}^-_3 )-Br(^-) rhodamine-B</td>
<td>665</td>
<td>0.5-5.0</td>
<td>7.0 x10⁴</td>
<td>Sensitive, uses stable reagent solution No heating /extraction step</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>555</td>
<td>0.25-2.0</td>
<td>1.1 x10⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>a). Acetic acid + KMnO(_4) b). H(_2)SO(_4) + KMnO(_4)</td>
<td>430</td>
<td>0.6-6.0</td>
<td>7.01x10⁴</td>
<td>Wide linear dynamic range, highly precise (intra-day and inter-day RSD &lt;0.95%) and accurate (( \epsilon_R &lt; 1.1% ))</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>550</td>
<td>1.5-15.0</td>
<td>2.8x10⁴</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### B. High Performance Liquid Chromatography.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Chromatographic conditions</th>
<th>$\lambda_{\text{max, nm}}$</th>
<th>Linear range</th>
<th>Remarks</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Octadecyl-bonded silica column (5µm, 6x150 mm) with mobile phase acetonitrile:0.05M ammoniumacetate (3:7)</td>
<td>286</td>
<td>-----</td>
<td>---</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>Not available</td>
<td>--</td>
<td>0.5-10 mg L$^{-1}$</td>
<td>--</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>Mobilephase: MeCN-0.01M Na dodecyl sulphate(55:45, pH 4.0 with glacial acetic acid)</td>
<td>286</td>
<td>---</td>
<td>--</td>
<td>6</td>
</tr>
<tr>
<td>4.</td>
<td>Octadecyl silane (250x4.6mm, 5µ) with mobile phase acetonitrile: 0.1% triethyl amine (pH 3)(40:60) at 1 mL min$^{-1}$</td>
<td>--</td>
<td>250-750 µg mL$^{-1}$</td>
<td>Low sensitivity</td>
<td>7</td>
</tr>
<tr>
<td>5.</td>
<td>Inertsil C18, mobile phase of acetonitrile-phosphate buffer (pH 2.0)</td>
<td>280</td>
<td>0.5-200 µg mL$^{-1}$</td>
<td>Highly acidic pH used</td>
<td>8</td>
</tr>
<tr>
<td>6.</td>
<td>Symmetry C18(150x4.6 mm); mobile phase- methanol:water (50:50, v/v)</td>
<td>230</td>
<td>10-60 mg mL$^{-1}$</td>
<td>Least sensitive, narrow linear range</td>
<td>9</td>
</tr>
<tr>
<td>7.</td>
<td>Hypersil ODS column (5 µm; 250 X4.6 mm i.d) using a mobile phase (1.2 mL min$^{-1}$)</td>
<td>284</td>
<td>50-600 µg mL$^{-1}$</td>
<td>Wide linear dynamic range; highly sensitive</td>
<td>This work</td>
</tr>
</tbody>
</table>

### C. Liquid chromatography-Mass spectrometry

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Chromatographic conditions</th>
<th>Linear range ng mL$^{-1}$</th>
<th>Remarks</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Inertsil ODS, (3.5µm; 50x4.6 mm i.d) with mobile phase of ammonium acetate (pH 4) and acetonitrile(60:40) at 0.6 mL min$^{-1}$</td>
<td>20-1000</td>
<td>Wide linear dynamic range of applicability</td>
<td>This work</td>
</tr>
</tbody>
</table>
Raloxifene hydrochloride has been assayed in pharmaceutical formulations by two techniques, viz., visible spectrophotometry and HPLC. The developed methods are characterized by simplicity, speed, sensitivity besides accuracy and precision. The spectrophotometric methods offer one of the simplest and the sensitive approaches for the assay of RLX in pharmaceuticals. The bromate-bromide/rhodamine-B (Sl. No. 3, Table 3.5.1) is the most sensitive method developed for RLX. All the methods are free from heating or extraction step. The methods, in addition, don’t entail any stringent experimental variable such as rigid pH control or longer contact time which would affect the reliability of the results. However, for reasons stated earlier (Section, 2.7), bromatometric methods lack specificity. As shown by the results of assay, commonly added tablet excipients did not interfere.

The present HPLC method is superior to many reported previously in terms of sensitivity, linear range of response and analysis time. Though the method is intended to determine RLX in single component formulations, it can conveniently be applied for combined dosage forms, since the method is specific for RLX under the described chromatographic conditions.

The proposed LC-MS method is most sensitive with LLOQ of 20 ng mL⁻¹. Thus, four spectrophotometric, one HPLC method for the assay of RLX in pharmaceuticals and one LC-MS-MS method have been developed and validated for the determination of RLX in urine sample. The methods have been demonstrated to be fairly accurate and precise in addition to being highly sensitive (except HPLC). The spectrophotometric methods can usefully be employed in routine use in areas/countries which lack modern instrumental facilities such as HPLC, LC-mass spectrometry, spectrofluorimetry, capillary electrophoresis, etc.,
REFERENCES


1a. Current Index of Medical Specialities (CIMS), CMP Medica India Pvt Ltd., Bangalore, India, July-October, 2007, p. 433.


