A Validated Isocratic RP-HPLC Method
determination for Rubiadin in the roots of *Rubia cordifolia* Linn

Khodke A.S.¹, Potale L.V.¹, Patole S.M.¹, and Damle M.C.²*

¹Department of Quality Assurance, A.I.S.S.M.S. College of Pharmacy, Kennedy Road, Near R.T.O., Pune - 411 001, India. (amolkhodke86@gmail.com)
²Department of Pharm. Chemistry, A.I.S.S.M.S. College of Pharmacy, Kennedy Road, Near R.T.O., Pune - 411 001.

*Corres. author: mcdamle@rediffmail.com
Ph. No. +91-20-26058204, + 91-9860230912

Abstract: Herbal medicines have good efficacy, safety, and lesser side effects. They have great demand in developed world for primary health care. India has rich traditional knowledge, heritage of herbal medicines and large biodiversity but despite it India has dismal share of world market. Many Pharma companies marketed herbal preparation as neutraceutical and took excuses from quality control parameter set by W.H.O. India has thousands of medicinal plants but in Indian Pharmacopoeia, Quality Control parameter of only 56 plants have been included. *Rubia cordifolia* Linn has good pharmacological activity. Both the stem and roots of *Rubia cordifolia* Linn have pharmacological activity like blood purifier, anti-cancer, anti-oxidant. But I.P. (2007) has given marker designation only for stem of *Rubia cordifolia* Linn. Hence we have developed and validated Isocratic HPLC method for determination of Rubiadin in roots of *Rubia cordifolia* Linn. Rubiadin was isolated from roots *Rubia cordifolia* Linn and purified. RP-HPLC method for Rubiadin was developed using mobile phase Methanol: water in the ratio of 80:20(v/v). Stationary phase use for this method was Hi-Qsil C18 (250×4.6mm). Using this mobile phase marker gets eluted at Rt 8.657 min. This RP-HPLC method was validated as per ICH guidelines.

Keywords: ICH, RP-HPLC, *Rubia cordifolia* Linn., W.H.O, Rubiadin

Introduction and Experimental

*Rubia cordifolia* Linn. is a flowering plant species. It is commonly known as Manjistha. Roots and stems are active part of plant. Plant has many pharmacological actions like blood purifier activity, anticancer, astringent, antidyseretic, antiseptic, deobstruent properties and antirheumatic, hepatoprotective¹-². Hepatoprotective action is mainly shown by Rubiadin³. Plant contains various chemical constituents like Anthraquinones⁴, Iridoïds⁵, Hexapeptides, Rubiprasins, Quinones, and Triterpenoids⁶. Literature survey reveals that Quality control protocol was developed for stems of *Rubia cordifolia* Linn⁷. Literature survey revealed that there are number of HPLC and HPTLC methods developed for chemical constituent of stem and root of *Rubia cordifolia* Linn like HPLC method developed for Hexapeptide from roots of *Rubia cordifolia* Linn⁸, a gradient HPLC method for Rubiadin from stem of *Rubia cordifolia* Linn⁹ and HPTLC methods were developed for Purpurin¹⁰ and Quinonoids¹¹ from roots of *Rubia cordifolia* Linn. The aim of the present study accordingly was to develop RP-HPLC method for Rubiadin and validate it as per ICH guideline¹².
**Materials and Method**

Powder of roots of *Rubia cordifolia* Linn. was purchased from M/S Total Herb Solution firm, Mumbai and authenticated by Botanical Survey of India, Pune. Chemical used for the experimentation were of HPLC grade and analytical reagent (AR) grade. Chemicals used for this experiment were Methanol, Acetone, Ethyl acetate, Toluene, and Water.

**Instrumentation**

Instruments used during research work were HPLC (Make JASCO) with UV detector, UV spectrophotometer (Make JASCO), Rotary Evaporator, Electronic Balance (Make SHIMDZU Model AY-120). Isolation of Marker was done by column chromatography using small bore column as per procedure reported in research paper by Singh R and Geetanjali. RP-HPLC Chromatographic method was developed using column Hi-Qsil C\text{18} (250×4.6mm) using UV detector (Model 2050).

**Isolation of Marker**

**Acetone: water extraction**

The powder of air dried roots of *Rubia cordifolia* was macerated three times in acetone: water (1:1) for 48Hrs at room temperature. The combined decanted solvent was distilled by simple distillation to remove acetone. A brown coloured solid separated after removal of acetone.

**Ethyl acetate extraction**

After removal of brown color solid, remaining aqueous portion was partitioned with ethyl acetate and separated ethyl acetate portion. The ethyl acetate was removed under reduced pressure and solid residue was obtained.

**Column Chromatography**

Ethyl acetate residue was chromatographed over silica gel (60-120 mesh) using Pet ether (60-80) and ethyl acetate in the ratio (4:1 v/v). Then eluant was collected in 2×15ml fractions and TLC of these fractions was performed using mobile phase Toluene : ethyl acetate (4:1 v/v). This TLC shows presence of two compounds at Rf 0.56 and 0.82 upon detection at 300nm.

**Preparative TLC**

Two compounds were separated using silica gel (60-120 mesh) as a stationary phase and Toluene: ethyl acetate (4:1% v/v) as a mobile phase. Compound present at Rf 0.56 was scratched from the plate and isolate it using chloroform. Identification of marker (Rubiadin) was confirmed by IR and NMR by comparing with reported spectra.

**Preparation of standard stock solution**

10mg of Rubiadin was accurately weighed and dissolved in 10 ml of methanol to obtain stock solution (1000µg/ml).

**b) Working standard solution**

1ml of standard standard stock solution was diluted to 10ml with methanol.

**Selection of Detection Wavelength**

The solution of Marker was scanned over range 400nm to 200nm. From the UV spectrum of Marker, it was found that marker shows good absorbance at 300nm; hence detection wavelength selected was found to be 300nm.

**Method development**

Selection of Mobile phase and chromatographic condition

Chromatographic studies were carried on working standard solution using C\text{18} column. Mobile phase consisting of different proportion of methanol and water was tried. After several trials optimum mobile phase was found to be Methanol and water in the ratio of 80:20. This mobile phase gave peak with acceptable retention time 8.568min and acceptable peak parameters.

**Summary of chromatographic parameters selected:**

- Column: HiQ-Sil C\text{18} (250×4.6mm) column
- Mobile phase: Methanol: water (80:20% v/v)
- Flow rate: 1.00 ml/min
- Detection Wavelength: 300 nm
- Sample injector: 50 µl loop
- Temperature: ambient

**Method Validation**

Validation of method was performed by using parameters as per ICH guideline.

**Linearity and Range**

Linearity of the method was checked using five different concentrations of Rubiadin in the range of 10µg/ml to 50µg/ml. The relation between concentration and area under curve was determined. The data of concentration and area under curve for marker peak was subjected to linear regression analysis.

**Precision**

Inter-day and Intra-day Precision were evaluated by analyzing standard solutions, six times and % RSD value was calculated to determine any intra-day and Inter-day variation.

**Accuracy**

To check accuracy of the method, recovery studies were carried out by addition of standard drug solution to sample solution at three different levels 80, 100 and 120 %. Mean percentage recovery was determined.

**Limit of detection and limit of quantitation**

The limit of detection (LOD) and limit of quantitation (LOQ) were obtained by using the standard formula as per the ICH guidelines.
LOD = $\frac{3.3 \sigma}{S}$

LOQ = $\frac{10 \sigma}{S}$

Where $\sigma$ is Standard deviation of the response and $S$ is slope of the calibration curve.

**Selectivity**

Selectivity is the ability to assess unequivocally the marker in presence of other chemical constituent of plant. It is indicated by Rubiadin peak getting well resolved from peaks of other chemical constituents.

### Quantitation of marker

**Preparation of Sample solution**

Extraction was carried out as per the procedure given in Ayurvedic Pharmacopoeia as follows:

2 gm root powder of *Rubia cordifolia* was accurately weighed and dispersed in 20 ml of ethyl acetate. This solution was kept aside overnight at ambient temperature. The solution was filtered through Whatman filter paper No. 41 and filtrate was evaporated on a water bath to obtain a solid mass of extract. The extract was dissolved in methanol and diluted appropriately.

Chromatograph of extract is shown in Fig. 2

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**Fig. 1. Chromatogram of Rubiadin having RT 8.675 min.**

**Fig. 2. Chromatogram showing presence of Rubiadin in mixture of compounds in root extract of *Rubia cordifolia* Linn.**
Table 1: Validation Summary of marker

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Validation Parameter</th>
<th>Rubiadin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linearity(r²)</td>
<td>0.999</td>
</tr>
<tr>
<td>2</td>
<td>Regression Equation</td>
<td>y=13610x + 10658</td>
</tr>
<tr>
<td>3</td>
<td>Range</td>
<td>10-50µg/ml</td>
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<tr>
<td>4</td>
<td>Intra-day Precision (%RSD)</td>
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<tr>
<td>5</td>
<td>Inter-day Precision (%RSD)</td>
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<tr>
<td>6</td>
<td>Accuracy</td>
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<tr>
<td>7</td>
<td>LOD</td>
<td>55.75 ng/ml</td>
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<tr>
<td>8</td>
<td>LOQ</td>
<td>184 ng/ml</td>
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<tr>
<td>9</td>
<td>Selectivity</td>
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</tr>
</tbody>
</table>

**Results and Discussion**

**Development of the optimum mobile phase**

The optimum mobile phase was found to be Methanol: Water (HPLC grade) in the ratio of 80: 20. Retention time of Rubiadin was found to be 8.675min as shown in Fig.1.

**Validation of the developed method**

A linear relationship between peak areas and concentrations was obtained in the range of 10-50µg/ml. This shows that method is linear. Repeatability studies show %RSD to be less than 2%.

**References:**


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