International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.2, No.4, pp 2256-2260, Oct-Dec 2010

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]*

[1]Department of Quality Assurance , A.I.S.S.M.S. College of Pharmacy, Kennedy Road, Near R.T.O., Pune - 411 001,India.

[1,2]Department of Pharm. Chemistry, A.I.S.S.M.S. College of Pharmacy, Kennedy Road, Near R.T.O., Pune - 411 001,India.

*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.

Introductioin 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, antidysentric, antiseptic, deobstruent properties and antirheumatic, hepatoprotective[1, 2]. Hepatoprotective action is mainly shown by Rubiadin[3]. Plant contains various chemical constituents like Anthraquinones[4], Iridoids[5], Hexapeptides, Rubiprasins, Quinones, and Triterpenoids[6]. Literature survey reveals that Quality control protocol was developed for stems of *Rubia cordifolia* Linn[7]. 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* Linn[8], a gradient HPLC method for Rubiadin from stem of *Rubia cordifolia* Linn[9] and HPTLC methods were developed for Purpurin[10] and Quinonoids[11] 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 guideline[12].

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.[13] RP-HPLC Chromatographic method was developed using column Hi-Qsil C_{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 (4:1v/v). Then eluant was collected in 2×15ml fractions and TLC of these fractions was performed using mobile phase Toluene : ethyl acetate (4:1v/v). This TLC shows presence of two compounds at R_f 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.[14]

Preparation of standard stock solution

a) 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_{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:

a)Column: HiQ-SiL C_{18} (250×4.6mm) column
b) Mobile phase : Methanol: water (80:20 %v/v)
c) Flow rate: 1.00 ml/min
d) Detection Wavelength : 300 nm
e) Sample injector : 50 µl loop
f) 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,

$$LOD = \frac{3.3\,\sigma}{S} \qquad LOQ = \frac{10\,\sigma}{S}$$

Where σ 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

2gm 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.

Table 1: Validation Summary of marker

Sr.No.	Validation Parameter	Rubiadin
1	Linearity(r^2)	0.999
2	Regression Equation	y=13610x + 10658
3	Range	10-50µg/ml
4	Intra-day Precision (%RSD)	1.70
5	Inter-day Precision (%RSD)	1.92
6	Accuracy	98-102%
7	LOD	55.75 ng/ml
8	LOQ	184 ng/ml
9	Selectivity	Selective

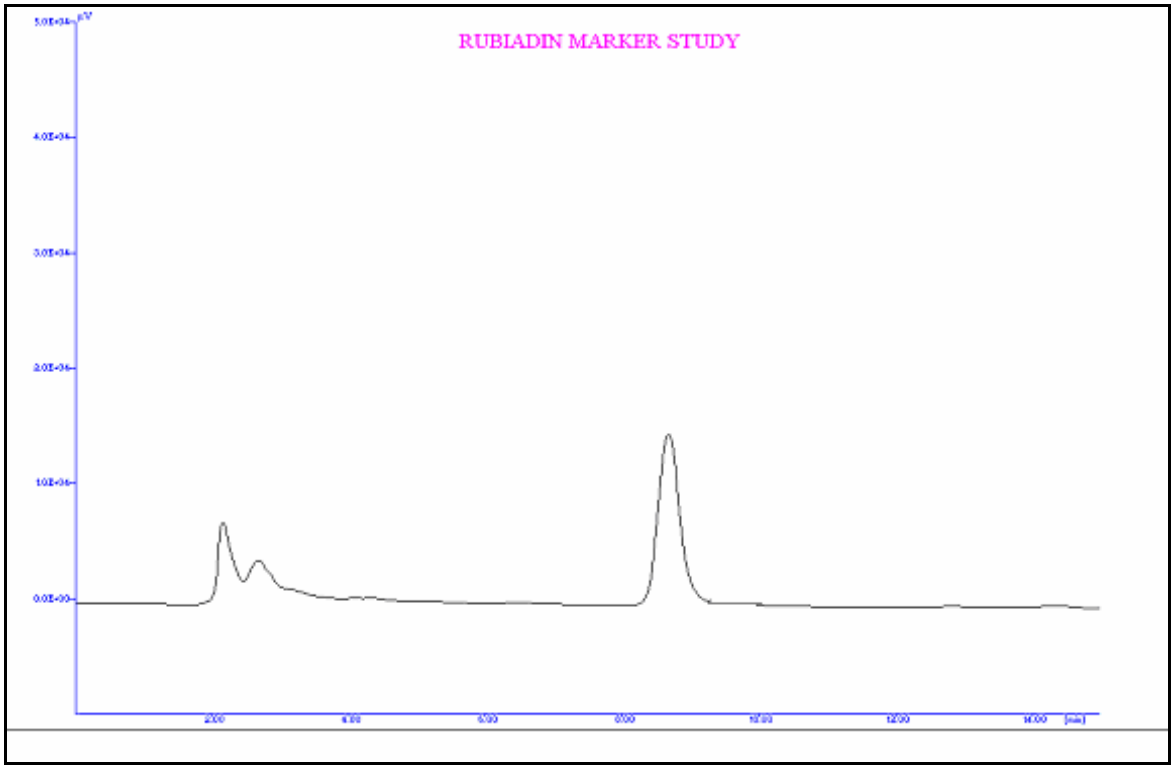

Fig.1. Chrmatogram of Rubiadin having RT 8.675min.

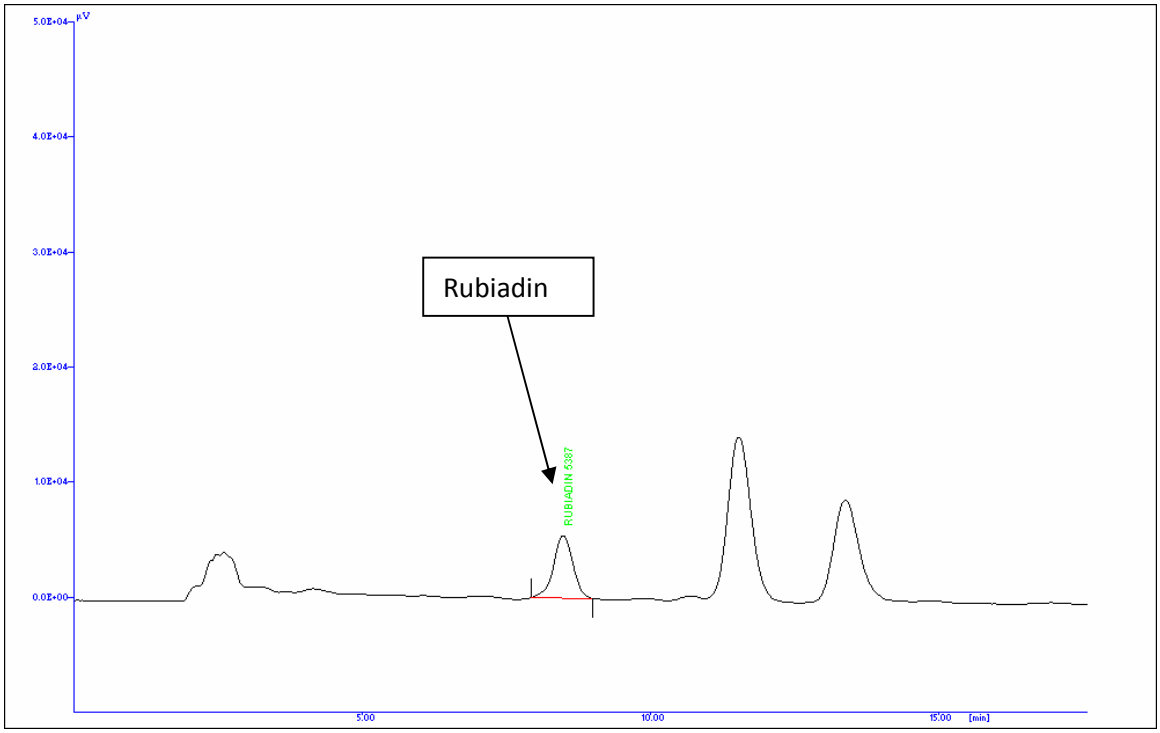

Fig.2. Chromatogram showing presence of Rubiadin in mixture of compounds in root extract of *Rubia cordifolia* Linn.

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%. This shows that method is precise. %RSD for inter-day precision was higher than that of intra-day precision. Excellent recoveries were obtained at each level of added concentration as the mean recovery found to be within 98% to 102% for Rubiadin. The limit of detection and limit of Quantitation of method was found to be 55.75ng/ml and 184ng/ml. As it was found that Rubiadin peak gets well resolved from peaks of other chemical constituents, hence we conclude that method is selective.

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