



ISSN 2278-4136  
 SSN 2349-8234  
 PJP 2014; 2 (5): 172-176  
 Received: 08-12-2013  
 Accepted: 28-12-2013

**Javed Ahamad\***  
 Phyto-pharmaceuticals Research  
 Laboratory, Department of  
 Pharmacognosy and Phytochemistry,  
 Hamdard University, Hamdard  
 Nagar, New Delhi, India.

**Saima Amin**  
 Department of Pharmaceutics, Faculty  
 of Pharmacy, Hamdard University,  
 PO Hamdard Nagar, New Delhi,  
 India.  
 E-mail: [samin@jamiyahamdard.ac.in](mailto:samin@jamiyahamdard.ac.in)

**Showkat R Mir**  
 Phyto-pharmaceuticals Research  
 Laboratory, Department of  
 Pharmacognosy and Phytochemistry,  
 Faculty of Pharmacy,  
 Hamdard University, Hamdard  
 Nagar, New Delhi 110 062, India.  
 E-mail: [showkatrmir@gmail.com](mailto:showkatrmir@gmail.com)

**Correspondence**  
**Showkat R Mir**  
 Phyto-pharmaceuticals Research  
 Laboratory, Department of  
 Pharmacognosy and  
 Phytochemistry, Faculty of  
 Pharmacy,  
 Hamdard University, Hamdard  
 Nagar,  
 New Delhi 110 062, India.  
 E-mail: [showkatrmir@gmail.com](mailto:showkatrmir@gmail.com)  
 Tel: +91-11-26059688/5616  
 Fax: +91-11-26059663

## Development and validation of HPTLC densitometric method for estimation of charantin in *Momordica charantia* fruits and herbal formulation

Javed Ahamad, Saima Amin, Showkat R Mir

### ABSTRACT

A simple, sensitive, precise, rapid, and reliable HPTLC method for the estimation of charantin in *Momordica charantia* fruits as well as in herbal formulation was developed. In this method, Aluminum backed silica gel 60 F<sub>254</sub> plates (20x10 cm: 200 µm thickness) were used as stationary phase and toluene-ethyl acetate-methanol-formic acid (68:20:10:02, v/v) as a optimized mobile phase. Developed chromatogram was scanned at 525 nm, the wavelength of maximum absorption for charantin after derivatization with anisaldehyde-sulphuric acid reagent. Regression analysis of the calibration data showed an excellent linear relationship between peak-area vs drug concentration. Linearity was found to be in the range of 100-500 ng/band. The suitability of developed HPTLC method for estimation of charantin was established by validating it as per the ICH guidelines. The limits of detection (LOD) and quantification (LOQ) for charantin were found to be ≈30 and ≈90 ng/band. The developed method was found to be linear (r<sup>2</sup>=0.9943), precise (RSD < 1.5% and < 2% for intra-day and inter-day precision), accurate (mean recovery of within the range of 98.68-100.20%), specific and robust. The developed method has been successfully applied for the determination of charantin in *M. charantia* fruits and herbal formulation without any interference.

**Keywords:** *Momordica charantia*, Cucurbitaceae, charantin, HPTLC, ICH guidelines.

### 1. Introduction

*Momordica charantia* Linn. belongs to family Cucurbitaceae and commonly known as Bitter gourd. Its fruits and seeds are widely reported to have antidiabetic, antihyperlipidaemic, anticancer, anti-HIV, anti-ulcer, anti-tumor, antiviral, analgesic, anti-inflammatory, hypotensive, anti-fertility, hepatoprotective and antioxidant activities [1]. Their antidiabetic effects are reported by several workers [2-4]. *M. charantia* fruits reduced blood glucose levels, improved body weight and glucose tolerance [5,6]. Its extract decreased insulin resistance in rats fed on high-fructose diet [7], increased the mass of β-cells and enhanced insulin production in pancreas [8]. Charantin has been found to be more potent than oral hypoglycemic agent tolbutamide in an animal study [9].

HPTLC is a planner chromatographic technique used for detection and quantification of analytes/drugs. The advantages of HPTLC over other analytical methods are accurate sample application and *in-situ* scanning which facilitate reliability, rapidity and accuracy of analysis. It also allows simultaneous estimation of several samples utilising only a small quantity of a mobile phase, hence minimising the analysis time and cost [10]. A thorough review of literature revealed that few analytical techniques have been reported for the analysis of charantin individually in plants [11], however no method is available for its estimation in herbal formulations. The main aim of this study was to develop and validate an accurate and reproducible HPTLC densitometric method for estimation of charantin in *M. charantia* fruits extract and in-house herbal formulation containing *M. charantia* as an ingredient. The validated was done as per ICH guidelines.

## 2. Materials and Methods

### 2.1. Plant Materials and Chemicals

Fresh unripe fruits of *M. charantia* were collected from Meerut, Uttar Pradesh, India (28.99 °C N, 77.70 °C E) in the month of September. A voucher specimen (PRL/JH/11/03) was deposited in Phyto-pharmaceuticals Research Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi. The in-house herbal formulation was prepared by mixing dried powders of *Gymnema sylvestre*, *Enicostemma littorale* and *M. charantia* in the ratio of 1:1 w/w in a mixer. Charantin was isolated in Phyto-pharmaceuticals Research Laboratory. Silica gel 60 F<sub>254</sub> HPTLC plates were purchased from Merck, Mumbai, India. Anisaldehyde was purchased from Thomas Baker, Mumbai, India. Methanol, sulphuric acid and potassium hydroxide were purchased from S.D. Fine Chemicals, Mumbai, India.

### 2.2. Preparation of Sample Solution

Fruits were cleaned and cut into small pieces and dried in an oven at 45 °C. Dried sample was pulverized to a powder using a grinder. About 10 g of powder of *M. charantia* and herbal formulation were placed separately in a stoppered conical flask and in an ultra-sonicator (Toshniwal, India) at 200 W ultrasonic power. Extraction was carried out with MeOH-H<sub>2</sub>O (80:20 % v/v) at 45 °C temperature for 120 min time using a solid to solvent ratio of 1:25. The extracts were filtered and concentrated *in-vacuo* in a rotary evaporator (Buchi, Switzerland). The residue was then extracted with ethyl acetate (3 × 100 ml). Ethyl acetate layer was pooled and concentrated. The residue was reconstituted in 10 ml methanol and filtered through 0.45 µm membrane filter.

### 2.3. Preparation of standard stock solution and quality control (QC) samples

Accurately weighed 10 mg of charantin reference standard and transferred to 10 ml volumetric flask. Methanol was added and sonicated in ultrasonic water bath and final volume made up to 10 ml. This gives concentration of 1 mg/ml, which was further diluted to give 0.1 mg/ml and used as standard stock solution. Quality control (QC) samples at three different concentration levels (100, 200 and 300 ng/band arbitrary units as low, medium and high) was prepared independent of the calibration standards.

### 2.4. HPTLC instrumentation and chromatographic conditions

Chromatography was performed on 20×10 cm aluminum backed plates coated with 200 µm layer of silica gel 60 F<sub>254</sub> (Merck, Mumbai, India). Samples were spotted (started from the point, X=10 mm and Y=10 mm) in the form of distinct bands (4 mm in width; 10 mm apart) with a CAMAG 100 ml syringe using a Linomat V (CAMAG, Muttenz, Switzerland) sample applicator. Constant application rate 160 nl s<sup>-1</sup>; slit dimension 3×0.45 mm and scanning speed 20 mm s<sup>-1</sup> were chosen as optimized equipment parameters. Linear ascending development was carried out in a 20×10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland), previously saturated with optimized mobile phase for 10 min at room temperature (25±2°C) and relative humidity (RH) of 60±5% (Fisher Scientific). The development of chromatogram included run height 8 cm, mobile phase, 20 ml and saturation time of development chamber, 10 minutes. Densitometric analysis was carried out at 525 nm in absorption mode with CAMAG TLC scanner III, using tungsten lamp as a radiation source and operated by winCATS software (Version 1.2.0).

### 2.5. Method Development

Various solvent mixtures were tried for the development of a suitable mobile phase such as toluene-ethyl acetate-methanol-formic acid (70:20:15:05, 68:20:10:02 and 60:30:15:05 % v/v) and ethyl acetate-methanol-water (60:30:10, 70:20:10 and 80:15:5 % v/v). The suitability of a solvent mixture was decided by band separation, shape, lack of tailing, sensitivity of the assay and the time required for development. Different TLC chamber saturation times were also tried. Due to lack of conjugation, charantin has poor UV absorption. Various derivatization methods like spraying with vanillin-sulphuric acid, modified vanillin-sulphuric acid and anisaldehyde-sulphuric acid reagent were tried.

### 2.6. Calibration curve

Different volumes of standard stock solution (1-5 µl/band) were applied to a TLC plate in triplicate and then the calibration curves were constructed by plotting the peak areas versus concentration ranging from 100-500 ng/band.

### 2.7. Method validation

Method validation was carried out to confirm that the developed analytical method employed for this specific analysis was suitable for its intended use. The method was validated by determining linearity, precision, accuracy, robustness, specificity, limit of detection (LOD) and limit of quantitation (LOQ) as per ICH guidelines [12].

#### 2.7.1. Precision

The precision and accuracy of the system was determined by measuring repeatability of sample application and measurement of concentration for six replicates of the bands (100, 200 and 300 ng/band). Intra and inter-day variation for the determination of drugs were carried out. The intraday precision was carried out on the same day while inter-day precision (intermediate precision) was studied by comparing assays performed on three different days. The precision of the system and method were expressed as percent relative standard deviation (% RSD) and standard error of mean (SEM).

#### 2.7.2. Accuracy

Accuracy was determined as percent recovery by the standard addition method. The pre-analysed samples (100 ng/band) were spiked with 50, 100 and 150 % of the standard and the mixtures were reanalysed in triplicate by the developed method. Percent recovery and % RSD were calculated at each concentration level.

#### 2.7.3. Robustness

Robustness is a measure of the capacity of a method to remain unaffected by little but intentional changes in the method conditions, and is an indicator of the stability of the method. Robustness was studied in triplicate at 200 ng/band by making small changes to the volume of mobile phase, composition of mobile phase, and saturation time of development chamber. The effects on the results were examined by calculation of RSD (%) and R<sub>f</sub> values.

#### 2.7.4. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD of an analytical procedure is the lowest amount of analyte in a sample which can be detected. LOQ of an analytical procedure is the lowest amount of analyte in a sample which can be

quantitatively determined with suitable precision and accuracy. In order to estimate LOD and LOQ limits, blank solution (methanol) was spotted six times following the same method as explained above. The standard deviation ( $\sigma$ ) of the magnitude of analytical response was determined for six replicate determinations. The LOD was expressed as  $3.3\sigma/\text{slope}$  of calibration curve, whereas LOQ was expressed as  $10\sigma/\text{slope}$  of calibration curve.

### 2.7.5. Specificity

The specificity of method was ascertained by analysing standard and test samples. The analyte in the test samples was confirmed by comparing  $R_f$  and UV spectra of the spots with that of the standard. Peak purity of the analyte was assessed by comparing the spectra at three different levels i.e. peak start, peak apex and peak end position of the spot.

### 2.8. Applications of method

The test samples (4  $\mu\text{l}$ ) were applied in triplicate and

chromatograms were obtained under same conditions as that of standard. Area under the peak corresponding to that of standard was recorded and content of the same was calculated from the regression equation obtained from calibration curves.

## 3. Results and Discussion

### 3.1. Chromatographic conditions

Ascending development to a distance of 8 cm was performed at laboratory conditions (temperature:  $28 \pm 2$  °C and % RH:  $65 \pm 5$ ), with toluene-ethyl acetate-methanol-formic acid (68:20:10:02 % v/v) as mobile phase and this gave a sharp, symmetrical and well-resolved band at the  $R_f$  value of  $0.71 \pm 0.02$ . The optimum saturation time was found to be 10 min. The band showed maximum absorbance at 525 nm after derivatization with anisaldehyde-sulphuric acid reagent and therefore it was chosen as the wavelength for densitometric determination. HPTLC chromatograms of *M. charantia* fruits extract shown in Figure 1.

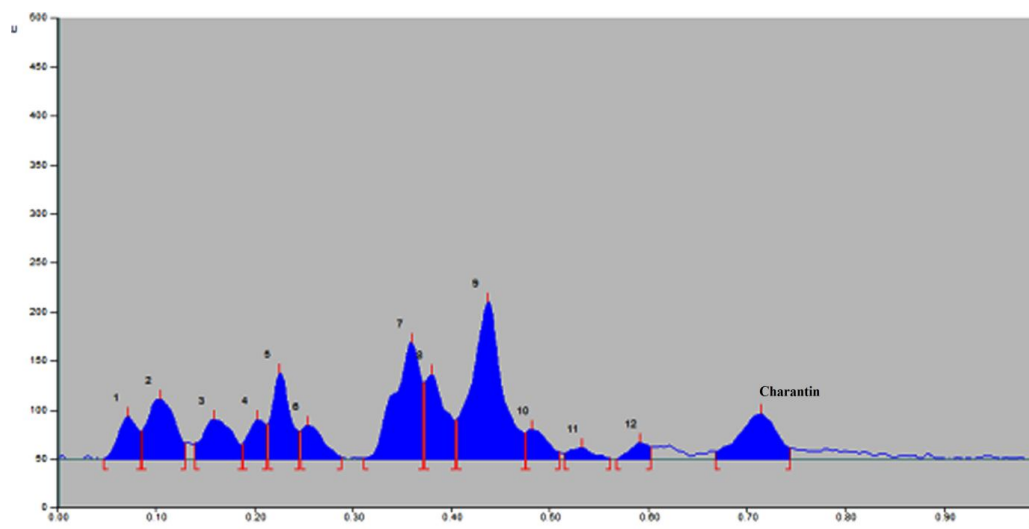


Fig 1: HPTLC chromatogram of *M. charantia* fruit extract

### 3.2. Method validation

#### 3.2.1. Linearity

The linearity range of charantin was found as 100-500 ng/band.

The regression equation was  $Y=8.734X-151.016$  with a correlation coefficient ( $r^2$ ) of 0.9943 as shown in Table 1.

Table 1: Linear regression data for the calibration curve

Linearity range (ng/band)	100 - 500
Regression equation	$Y = 8.734 X - 151.016$
Correlation coefficient ( $r^2$ )	0.994
Slope $\pm$ SD	$8.68 \pm 0.045$
Intercept $\pm$ SD	$144.45 \pm 7.61$
Slope without intercept $\pm$ SD	$8.28 \pm 0.034$
Standard error of slope	0.026
Standard error of intercept	4.397
95% Confidence interval of slope	8.572 - 8.793
95% Confidence interval of intercept	125.53 - 163.38

n = 3, SD = standard deviation

#### 3.2.2. Precision

The RSD (%) for repeatability of sample application were done at three concentration levels (100, 200, 300 ng/band). All the three concentrations were applied 6 times and included for both intra-day and inter-day studies. Concentration for the developed chromatogram was measured and statistical analysis was done for the inter-day and intra-day variation. The measurement of the concentration at six different concentration levels showed low

values of % RSD. Maximum RSD (%) for intra-day were found to be 1.73, while for inter-day variation, these were found to be 1.90 for charantin, which suggested a good precision of the method (Table 2).

#### 3.2.3. Accuracy

The accuracy of the proposed method was calculated by recovery studies, which afforded the recovery of 98.68-100.20 %, after spiking the additional standard analyte to the previously analysed

samples. The values of percent recovery are shown in Table 3 that indicated that the assay method was highly accurate.

**Table 2:** Precision of the proposed method

Conc. (ng/band)	Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Mean area $\pm$ SD	SEM	% RSD	Mean area $\pm$ SD	SEM	% RSD
100	1165.66 $\pm$ 11.01	4.49	0.94	996 $\pm$ 19.01	7.76	1.90
200	2119.66 $\pm$ 28.14	11.49	1.34	2111 $\pm$ 15.36	6.27	0.72
300	2829.33 $\pm$ 48.99	20.01	1.73	2688.66 $\pm$ 29.69	12.12	1.10

n= 6, SD= standard deviation RSD= relative standard deviation, SEM= standard error of mean,

**Table 3:** Accuracy of the proposed method

Excess drug added to analyte (%)	Theoretical content (ng)	Concentration found (ng) $\pm$ SD	Recovery (%)	% RSD
0	100	99.00 $\pm$ 1.73	99.00	1.75
50	150	147.81 $\pm$ 4.28	100.20	1.41
100	200	199.84 $\pm$ 4.19	99.25	0.41
150	250	250.71 $\pm$ 3.60	98.68	0.66

n= 3, RSD= relative standard deviation, SD = standard deviation

### 3.2.4. Robustness

Table 4 describes the robustness of the proposed method. The RSD (%) of the peak areas was calculated for the change in mobile phase composition, mobile phase volume and saturation time of development chamber at concentration levels of 200 ng/band (in triplicate). Statistical parameters were observed for

charantin. The method was found to be robust (% RSD <2). Substantial variation in result was obtained when the composition of mobile phase and mobile phase volume were varied but not significant.

**Table 4:** Robustness of the proposed method

Method parameters	Mean area $\pm$ SD	SEM	% RSD	Mean $R_f$ $\pm$ SD
Mobile phase volume	4092.66 $\pm$ 76.44	31.21	1.86	0.69 $\pm$ 0.03
Mobile phase composition	4136.16 $\pm$ 28.37	24.86	0.68	0.71 $\pm$ 0.02
Saturation time	4242.5 $\pm$ 60.91	11.58	1.43	0.73 $\pm$ 0.01

n= 3, SD= standard deviation, RSD= relative standard deviation, SEM= standard error of mean

### 3.2.5. Limit of detection and limit of quantitation

LOD and LOQ of the proposed method were determined by the standard deviation method. The LOD and LOQ of the proposed method were found to be 32.3 and 91.2 ng/band, respectively, which indicated that the proposed method can be used in wide concentration range for the detection and quantification of analyte effectively.

### 3.2.6. Specificity

The specificity of proposed method was determined by comparing  $R_f$ , 3D chromatogram and UV spectra of peaks of sample with that of standard as depicted in Figure 2A and 2B. Three point

peak purity i.e. peak start, peak apex and peak end was compared and was found superimposed. This indicated that the standard and sample peaks were not merging with any other components or impurities.

### 3.3. Application of assay method

The developed method was successfully applied for the detection and quantification of charantin in samples. The in-house herbal formulation was found to contain  $0.361 \pm 0.014$  of charantin (% w/w dry weight). The charantin content in *M. charantia* fruit extract was  $1.053 \pm 0.032$  (% w/w dry weight).

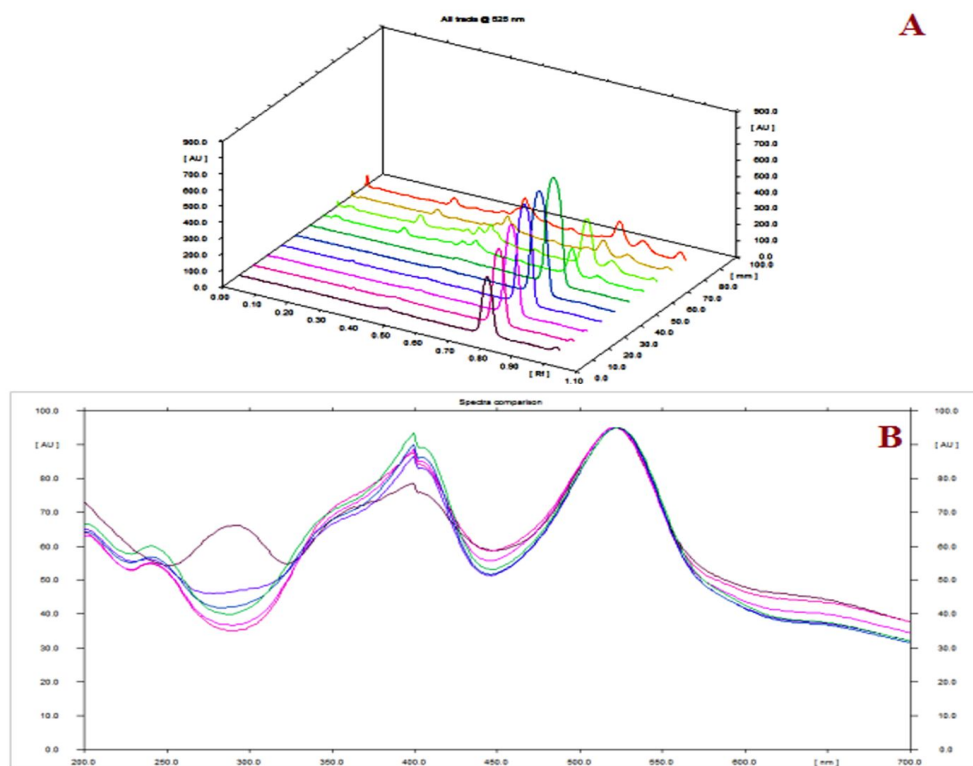


Fig 2: 3D chromatogram (A) and superimposed UV spectra of reference standard and test samples (B)

#### 4. Conclusion

UAE technique was developed for the fast and efficient extraction of charantin from the *M. charantia* fruits. A reproducible, robust, precise and accurate HPTLC densitometric method was developed and validated and applied for estimation of charantin in the extracts of *M. charantia* fruits and in-house formulation containing *M. charantia* as an ingredient. Therefore, this method can be successfully used for the routine analysis of charantin for standardization and quality control of agricultural and pharmaceutical products containing *M. charantia* as an ingredient.

#### 5. Acknowledgement

Authors gratefully acknowledge financial assistance from University Grants Commission (UGC), New Delhi, India; (Grant No. MRP-42-704/2013, SR).

#### 6. References

1. Taylor T. Technical data for bitter melon (*Momordica charantia*), herbal secrets of the rainforest, 2<sup>nd</sup> edition, Sage Press Inc. 2002.
2. Sarkar S, Pranava M, Marita R. Demonstration of the hypoglycemic action of *Momordica charantia* in a validated animal model of diabetes. *Pharmacol Res.* 1996; 33: 1-4.
3. Ahmed I, Adeghate E, Sharma AK, Pallot DJ, Singh J. Effects of *Momordica charantia* fruit juice on islet morphology in the pancreas of the streptozotocin-diabetic rat. *Diab Res Clin Pract.* 1998; 40(3): 145-51.
4. Sathishsekar D, Subramanian S. Beneficial effects of *Momordica charantia* seeds in the treatment of STZ-induced diabetes in experimental rats. *Biol Pharm Bull.* 2005; (6): 978-83.
5. Raman A, Lau C. Anti-diabetic properties and phytochemistry of *Momordica charantia* L. (Cucurbitaceae). *Phytomed.* 1996; 2(4): 349-362.
6. Celia G, Cummings E, David AP, Singh J. Beneficial effect and mechanism of action of *Momordica charantia* in the treatment of diabetes mellitus: a mini review. *Int J Diab Metab.* 2003; 11: 46-55.
7. Shih CC, Lin CH, Lin WL, Wu JB. Effect of *Momordica charantia* extract on insulin resistance and the skeletal muscle GLUT4 protein in fructose-fed rats. *J Ethnopharmacol.* 2009; 123(1): 82-90.
8. Shetty AK, Kumar GS, Sambaiah K, Salimath PV. Effect of bitter gourd (*Momordica charantia*) on glycaemic status in streptozotocin induced diabetic rats. *Plant Foods Hum Nutr.* 2005; 60(3): 109-112.
9. Lotlikar MM, Rajarama Rao MR. Pharmacology of a hypoglycaemic principle isolated from the fruits of *Momordica charantia* Linn. *Indian J Pharm.* 1966; 28(5): 129-33.
10. Sethi PD. HPTLC: High Performance Thin Layer Chromatography, CBS Publishers and Distributors, New Delhi, India, 1996.
11. Suthar AC, Banavalikar MM, Biyani MK, Priyadarshani I, Sudarshan, V, Mohan H. A high performance thin layer chromatography method for quantitative estimation of charantin in *Momordica charantia* Linn. *Indian Drugs.* 2001; 38(5): 230-35.
12. ICH. 2005. Validation of analytical procedures: text and methodology. ICH-Q2 (R1), International Conference on Harmonization: Geneva.