

METHOD DEVELOPMENT AND VALIDATION OF RICINOLEIC ACID BY HPTLC

Raghu Duraisamy*¹, Parthasarathy Mahadevan², Baskaran Siva³, Ramesh Jaya Rama Chandra Reddy⁴ and Gadevaru Sarathchandra⁵

Pharmacovigilance Laboratory for Animal Feed and Food Safety

Directorate of Centre for Animal Health Studies

Tamil Nadu Veterinary and Animal Sciences University

Madhavaram Milk Colony, Chennai – 600 051

ABSTRACT

*Ricinoleic acid, a toxic compound found in castor seeds (*Ricinus communis* L.), can be lethal when inhaled, ingested, or injected at doses of 5-10 µg/kg to human or animals. This study presents a validated, rapid, and precise high-performance thin-layer chromatographic (HPTLC) method for its quantification. Separation was achieved on silica gel 60 F₂₅₄ plates using a mobile phase of toluene, ethyl acetate and acetate acid (6:4:0.2 v/v/v). Densitometric scanning at 450 nm detected ricinoleic acid with an R_f value of 0.66 (±0.04). Linearity was observed in the range of 20-100 ng/spot with R = 0.998 ± 2.62%.*

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INTRODUCTION

The castor bean plant (*Ricinus communis* L.) is a perennial of the Euphorbiaceae family, originating from Africa and now cultivated globally in tropical and subtropical regions. Castor seeds and oil, have been used for medicinal purposes, such as treating infections and inflammation, as well as a laxative (Scarpa and Guerri, 1982). Castor seeds are also rich in protein, used as a supplement in animal feed (Diniz *et al.*,

2011), and the residual matter from castor oil production serves as organic fertilizer (Alexander *et al.*, 2008; Barnes *et al.*, 2009; Lima *et al.*, 2011). However, the use of castor bean residues is limited by toxic components, including ricin and ricinoleic acid, along with a less toxic alkaloid, ricinine (Lord and Spooner, 2011).

Ricinoleic acid, the primary component of castor oil (about 90%), is a 12-hydroxy-9-cis-octadecenoic unsaturated omega-9 fatty acid, also found in ergot (*Claviceps purpurea*). The chemical structure of ricinoleic acid is given in Fig. 1. Ricinoleic acid by ingestion or injection causes fatal poisoning in humans and animals with lethal doses of 5-10 µg/kg.

¹Assistant Professor,*Corresponding author E-mail: raghu_horti@rediffmail.com

²Technician

³Technician

⁴Professor and Head

⁵Former Professor and Head (Retd.)

The symptoms are burning sensations, nausea, abdominal pain, and diarrhea, progressing to tachycardia, hypotension, seizures, and circulatory collapse (Balint, 1974). Animals show varying sensitivities, with horses being the most affected; while, chickens are the least sensitive. Currently, there is no precise high-performance thin-layer chromatography (HPTLC) method existing for detecting ricinoleic acid. This study introduces a novel HPTLC method for its detection and quantification.

MATERIALS AND METHODS

Reagents and Thin Layer Chromatography (TLC) plate

In the present study, solvents used, were methanol, toluene, ethyl acetate and formic acid of chromatographic grade and purchased from Merck Limited, Mumbai, Maharashtra. The certified reference material (CRM) of Ricinoleic acid was procured from M/s. Sigma Aldrich, Bengaluru, Karnataka. TLC aluminium plates precoated with silica gel 60 F₂₅₄ (size 10 x 10 cm, layer thickness 0.2 mm, Supelco, Canada) were used as a stationary phase.

Preparation of standard

A stock standard solution of ricinoleic acid was prepared by accurately weighed 10 mg of ricinoleic acid and transferred into a 100 ml volumetric flask, dissolving in 50 ml of methanol. Then it was sonicated for 10 min and finally the volume was made up to 100 ml with methanol to obtain the concentration of

100 µg per ml. From this stock solution 5 ml was transferred into 50 ml volumetric flask and the volume was made up to 50 ml of methanol to obtain the working standard solution with the concentration of 10 µg per ml. Aliquots (3, 6, 9, 12, 15, 18 and 21 µl) of working standard were spotted on silica gel precoated TLC plate by Linomat applicator under N₂ stream for linearity and precision studies.

HPTLC apparatus

HPTLC system (CAMAG, Switzerland) comprising a CAMAG Linomat-5 applicator, a CAMAG TLC Scanner 3, a CAMAG twin-trough chamber (20 x 10 cm), CAMAG VisionCat software and separate Hamilton syringe for standard and sample, 25 and 100 µl respectively was used in this study.

Chromatographic condition

The HPTLC conditions were optimized to quantify ricinoleic acid using a TLC silica gel plate with a mobile phase of toluene: ethyl acetate: formic acid (6:4:0.2 v/v/v). Standard and sample extracts were applied as 8 mm wide bands, 4 mm apart, using an automatic Linomat-5 TLC applicator with N₂ gas flow at 7 psi. The chromatographic chamber was saturated with the mobile phase for 20-30 minutes before the plate's linear ascending development, allowing a 70 mm migration distance. Densitometric scanning was performed using a CAMAG scanner 3 with fluorescence at 366 nm, a slit dimension of 6 x 0.45 mm, and a scanning speed of 20 mm/s.

Calibration curve of ricinoleic acid

The ricinoleic acid content was determined using a calibration curve with a standard concentration range of 30 to 210 ng per spot. Different aliquots (3, 6, 9, 12, 15, 18, and 21 μ l) of the working standard solution were spotted on a precoated TLC plate using a CAMAG Linomat-5 applicator under a nitrogen stream. The plate was then dried with a hair dryer. Development and scanning followed the chromatographic conditions. A standard plot was created by plotting peak area against the concentration (ng/ml) of the spotted standard.

Validation of HPTLC method

The validation of the analytical method developed for precision, instrumental or system precision, repeatability or intra-day precision, intermediate precision or inter-day precision and accuracy were followed as per the ICH guidelines. The instrumental precision of the method was assessed by six times spotting of 3 μ l (30 ng per spot) of standard solution on a TLC plate and measured the peak area for 6 spots. The intra-day precision was analyzed using standard solution in the concentration ranging from 30 ng to 210 ng per spot for 5 times on the same day. Whereas inter-day precision was determined using the corresponding standard solution daily for 5 days in a week. All the precision types were evaluated by analysis of same concentration of ricinoleic acid with replicates ($n = 5$).

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were evaluated by applying different concentrations of the standard solutions of ricinoleic acid along with methanol as a blank and signal to noise ratio (3:1 for LOD and 10:1 for LOQ) was determined. LOD and LOQ were calculated based on the standard deviation of response (σ) and the slope of calibration plot (S), using the formula $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$.

RESULTS AND DISCUSSION

Method development

A simple, accurate, and precise HPTLC method has been developed. Methanol, due to its high solubility for ricinoleic acid, was chosen as the extraction solvent. A mobile phase of toluene: ethyl acetate: formic acid (6:4:0.2 v/v/v) provided a dense, compact spot with an R_f value of 0.66, ideal for quantification. Hassam and Hetta (2019) studied ricinoleic acid in castor oil and observed peak at R_f 0.67. Figure 2 shows the typical HPTLC densitometric chromatogram.

Validation of HPTLC method

Validation of TLC densitometric method for ricinoleic acid was done in terms of instrumental precision, intra-day precision, inter-day precision and accuracy. The linearity range for detection of ricinoleic acid was 30-210 ng per spot with the correlation coefficient (R) 0.998 and coefficient of variation (CV) 2.62%. The linearity results were given in the table 1 and figure 3. The mean area and

Table 1. Linearity of ricinoleic acid

S. No.	Concentration (ng/spot)	Area			
		A1	A2	A3	Mean peak area \pm RSD
1	30	0.0006	0.0006	0.00062	0.0006 \pm 1.90
2	60	0.0014	0.00145	0.0014	0.0014 \pm 2.03
3	90	0.0020	0.0019	0.0020	0.0019 \pm 2.93
4	120	0.0028	0.0029	0.0030	0.0029 \pm 3.44
5	150	0.0038	0.0037	0.0045	0.0036 \pm 4.16
6	180	0.00475	0.0047	0.0047	0.0047 \pm 0.61
7	210	0.0053	0.0052	0.0055	0.0053 \pm 2.86

Table 2. Instrument precision of ricinoleic acid

S.No.	Concentration (ng /spot)	Peak area
1	30	0.0007
2	30	0.0007
3	30	0.0007
4	30	0.0006
5	30	0.0007
6	30	0.0007
7	30	0.0005
Mean peak area 0.0006 ; RSD 11.97		

Table 3. Intra-day precision of ricinoleic acid

S. No.	Concentration (ng/spot)	Peak area					Mean \pm RSD
		A1	A2	A3	A4	A5	
1	30	0.0006	0.0005	0.0006	0.0006	0.0006	0.0006 \pm 7.71
2	60	0.0013	0.0012	0.0012	0.0013	0.0013	0.0013 \pm 4.34
3	90	0.0019	0.0018	0.0019	0.0018	0.0019	0.0019 \pm 2.94
4	120	0.0025	0.0025	0.0025	0.0025	0.0027	0.0025 \pm 3.52
5	150	0.003	0.0031	0.003	0.0031	0.0032	0.0031 \pm 2.71
6	180	0.0032	0.0034	0.0032	0.0034	0.0034	0.0033 \pm 3.29
7	210	0.0038	0.0041	0.0038	0.0044	0.0037	0.0040 \pm 7.27

Table 4. Inter-day precision of ricinoleic acid

S. No.	Concentration (ng/spot)	Peak area					Mean \pm RSD
		A1	A2	A3	A4	A5	
1	30	0.0006	0.0006	0.0006	0.0005	0.0006	0.0006 \pm 7.71
2	60	0.0013	0.0013	0.0013	0.0014	0.0012	0.0013 \pm 5.43
3	90	0.0018	0.0017	0.0018	0.0018	0.0017	0.0018 \pm 3.11
4	120	0.0023	0.0022	0.0025	0.0023	0.0023	0.0023 \pm 4.72
5	150	0.0034	0.0034	0.0032	0.0034	0.0028	0.0032 \pm 8.04
6	180	0.0038	0.0039	0.0039	0.0039	0.0037	0.0038 \pm 2.32
7	210	0.0045	0.0042	0.0045	0.0046	0.0046	0.0045 \pm 3.66

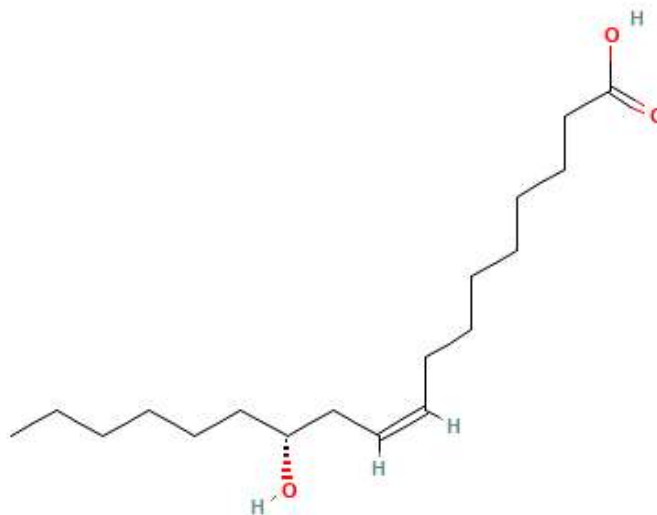


Fig 1. Chemical structure of ricinoleic acid (Source: Pub Chem)

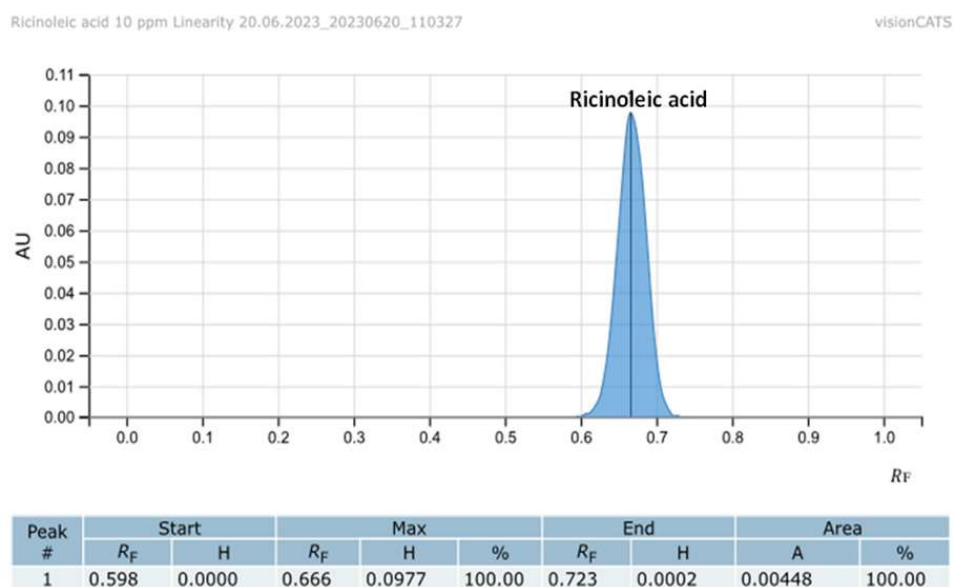


Fig 2. A typical HPTLC densitometric chromatogram of ricinoleic acid at 450 nm with a R_f 0.666.

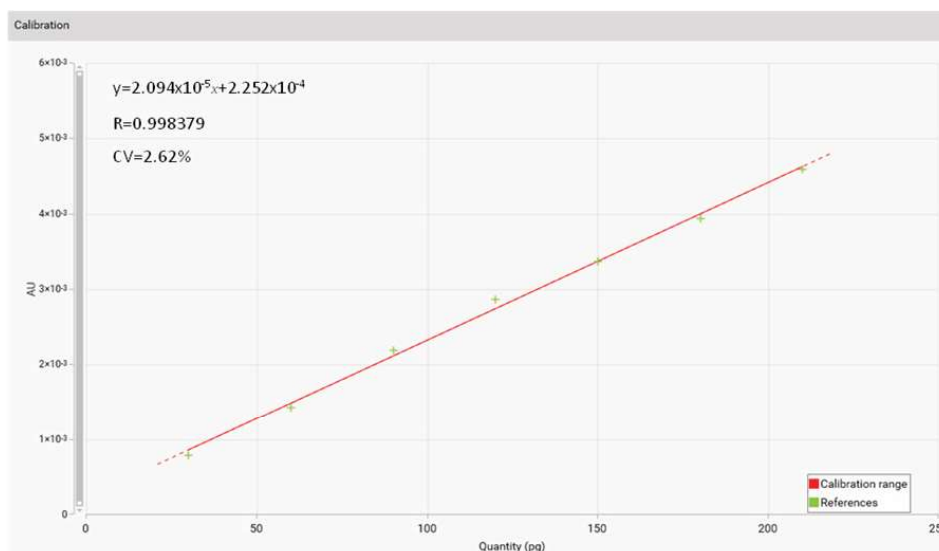


Fig 3. Calibration curve (linearity) of ricinoleic acid standard at 450 nm with correlation coefficient (R) 0.998 and coefficient of variation (CV) 2.62%.

relative standard deviation (RSD) was found 0.0006 and 11.97 respectively for instrument precision (Table 2).

The relative standard deviation (RSD) was ranged from 2.71 to 7.71 for intra-day and 2.32 to 7.71 for inter-day variation for different concentrations of ricinoleic acid which revealed an excellent precision and repeatability or reproducibility of the method (Table 3 and Table 4). The limits of detection (LOD) and quantification (LOQ) were found to be 15 and 45 ng respectively which represents the adequate sensitivity of the method.

CONCLUSION

Specific HPTLC method was developed and validated for the quantitative determination of ricinoleic acid with more precision and accuracy. Statistical analysis

confirmed the method's high reproducibility and selectivity, with good peak shape and resolution. This method is economical, rapid, simple, and sensitive, making it suitable for routine qualitative and quantitative analysis. It may also be useful for standardization purposes. Further standardization is needed for quantitative analysis of ricinoleic acid in animal feed and feed ingredients.

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