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Article

Development and Validation of a Stability-Indicating Method for Determination of Free Sterols in the Asian Medicinal Leech *Hirudo manillensis*

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A rapid, simple, sensitive, selective, precise and robust thin-layer chromatography densitometric method for the determination of free sterols in leech was developed and validated on silica gel layer using carbon tetrachloride–methanol–formic acid (9.5:1.5:0.55, v/v/v). Spectrodensitometric scanning was carried using a Camag TLC scanner III at 366 nm after spraying 2% methanolic sulphuric acid, which gave compact spots for cholesterol ($R_F = 0.35 \pm 0.02$). The regression analysis data for calibration plot implied a good linear relationship ($r^2 = 0.99958$) between response and concentration over the range 100–600 ng per spot with respect to peak area. The limits of detection and quantification were found to be 13.8 ± 0.51 and 45.01 ± 1.29 ng per spot, respectively. Validation was in accordance to the International Conference on Harmonization guidelines. Cholesterol was subjected to forced stress conditions of oxidation, hydrolysis and heat. Degradation products resulting from the forced stress did not interfere with detection because the degradant peaks were well separated from the cholesterol peak. The densitometric method can be regarded as stability-indicating and can be used for quality control assay of cholesterol in leech extract.

Introduction

Hirudinaria manillensis (Asian medicinal leech) from the family Hirudinidae, is an amphibious freshwater animal found in India, Indonesia, Malaysia, Sri Lanka, Thailand, Vietnam and China. Leeches are traditionally used as a cure in asthma, cough, epilepsy, colic, urinary complaints, headaches, acute laryngitis, nephritis, nephralgia, subacute ovaritis, epistaxis, swollen testicles, ophthalmia and brain congestion. (1, 2, 3) Recently, there has been a revival of interest in leeches used in surgical practice to alleviate venous congestion following limb or organ implantation (4), or for providing hirudin, the alternate anti-coagulant to heparin-related drugs (5).

Revival of interest in leeches was initiated by two Slovenian surgeons, M. Derganc and F. Zdravic, who described their use to prevent venous congestion of skin-flap transplants in an article in the British Journal of Plastic Surgery in 1960 (6). The two surgeons attribute their own use of leeches to a Parisian surgeon, Philippe-Frédéric, who used leeches to restore circulation following reconstruction of a nose in 1836.

Hirudo medicinalis Linnaeus, 1758 (European medicinal leech) is most commonly referenced for its use in medicine, although a recent study (7, 8) found that the leech used in most hospitals of developed countries is *Hirudo verbana* Carena, not *H. medicinalis*.

The leech is rich in enzymes and contains various biologically active substances like hirudin (which has anticoagulant property), bufrudin (which is a thrombin inhibitor) (9, 10), calin (which inhibits blood coagulation) or saratin (which inhibits collagen-mediated platelet aggregation), as well as several other prostaglandins (10, 11). It also contains histamine-like substances, which increase inflow of blood at bite sites, anesthetic-like substances, triglycerides, cholesterol (Figure 1) and cholesterol esterases (12, 13, 14, 15).

An increased interest in the clinical use of leeches as an adjunct to modern medical and surgical therapy has created a need to determine various methods of analysis of bio-molecules such as lipids in *H. manillensis*. Several chromatographic studies have been published for the determination of bio-molecules in the medicinal leech, based on gas–liquid chromatography (GLC) (16, 17, 18, 19), high-performance liquid chromatography (9, 11), high-performance thin-layer chromatography (21, 22, 23), thin-layer chromatography (TLC) (20) and mass spectroscopy (16, 19, 20); however, there are no reported methods regarding stability-indicating analysis of free sterols in the Asian medicinal leech, *H. manillensis*. Hence, in the present investigation, we have proposed a stability-indicating validated analytical methodology for the estimation of lipids with reference to cholesterol in the extract of Asian medicinal leech (*H. manillensis*).

Zipser *et al.* used TLC, one-dimensional and two-dimensional nuclear magnetic resonance spectroscopy (double quantum filtered-correlated spectroscopy and heteronuclear multiple quantum coherence), gas chromatography–mass spectroscopy and fast-atom-bombardment mass spectrometry (FABMS) to prove that cholesterol is the major free sterol present in *H. medicinalis* (16, 19). Additionally, TLC analysis by Rabinowitz determined free fatty acids and phophatidic acid as the largest groups of lipids (20). In other GLC studies, cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol have been identified as the major lipids in *H. medicinalis* (16, 18, 21).

Indian traditional medicine such as Ayurveda and Unani mention the use of leech extract obtained in various media for a number of ailments, including inflammation of joints, gout and varicose veins, and in the prevention of thrombophlebitis, and as an effective antioxidant or anticoagulant (2, 3). In this manuscript, we have tried to explore the use of mustard oil as a carrier for leech extract and to provide a rapid stability-indicating method of chromatographic analysis of leech extract.

Stability analysis or forced stress testing is carried out to investigate the effect of a variety of stressed conditions; for

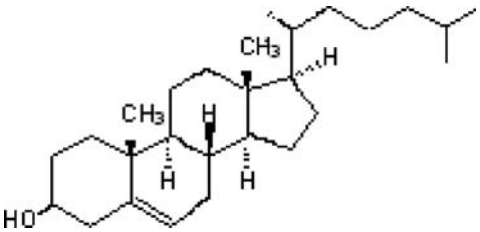

Figure 1. Chemical Structure of Cholesterol.

example, increased temperature, humidity and light, on the quality of a drug substance or drug product with time. Stability studies are used in the development of manufacturing processes, selection of proper packaging and storage conditions, and determination of product shelf-life.

Experimental

Materials

Standard cholesterol (98% purity) was purchased from Sigma-Aldrich Chemicals (New Delhi, India). Asian medicinal leeches (*Hirudo manillensis*) were purchased from Nitin Biologicals (New Delhi, India). All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals (Mumbai, India).

Maintenance of leeches and preparation of extract and sample

The study was approved by Institutional Animal Ethics Committee of Hamdard University, New Delhi, India [vide Committee for the Purpose of Control and Supervision Of Experiments on Animals (CPCSEA) Application 372/173/CPCSEA, 31/01/2007].

Leeches were maintained in aerated mason jars that contained 10 leeches and 1,000 mL of deionized water at 25 ± 5°C. The leeches were maintained in the laboratory without exogenous food and were used within 10 days of receipt. The water in the mason jars was changed every day.

Briefly, 10 gm of leeches were homogenized in 1 L mustard oil using a homogenizer (Hi-Tech Machineries; Ahmedabad, India). The resultant extract was strained, centrifuged (5,000 rpm for 15 min) and the supernatant was used.

For preparation of sample, 300 μL of leech extract was dissolved in 1,200 μL of dichloromethane.

TLC instrumentation and chromatography

Precoated silica gel 60F-254 alumunium plates (20 × 10 cm) with 200 μm thickness (E. Merck, Germany) were activated by heating at 100°C for 30 min after prewashing with methanol. Samples were spotted in the form of 4 mm bands with 5 mm inter-band space using a Camag microliter syringe attached to a Camag Linomat V (Switzerland) sample applicator. The rate of application was 150 nL/s with slit dimension and scanning speed at 5 × 0.45 mm and 20 mm/s, respectively. Linear ascending development was carried out in a twin trough glass chamber from Camag (Switzerland) and chromatograms were run up to 80 mm. The optimized mobile phase was carbon tetrachloride–methanol–formic acid (9.5:1.5:0.55, v/v/v). Chamber saturation time for mobile phase was optimized at 30 min at room temperature (25°C ± 2) at a relative humidity of 50 ± 5%. Following the development of TLC plates, they were dried in a current of air with the help of an air-dryer and derivatized using a 2% methanolic sulphuric acid solution. Densitometric scanning was performed on a Camag TLC scanner III in the absorbance mode at 366 nm operated by Win CATS software (Version 1.2.0) after heating it in a hot air oven at 110°C for 10 min. The source of radiation was a tungsten lamp emitting a continuous ultraviolet (UV) spectrum.

Calibration curve of cholesterol

A stock solution of cholesterol (100 μg/mL) was prepared in dichloromethane. Different volumes of stock solution, 1, 2, 3, 5 and 6 μL, were spotted on the TLC plate in triplicate to obtain concentrations of 100, 200, 300, 500 and 600 ng/spot of cholesterol, respectively. Linearity was determined by plotting peak areas versus the corresponding concentrations and subsequently treated by least-square regression analysis using WinCats Software. The same procedure was repeated five times to check the statistical significance and the average results were reported.

Method validation

The proposed method was validated as per International Conference on Harmonization (ICH) guidelines (24) and compared to similar methods reported by other laboratories (25–30).

Precision

The repeatability of the sample application was determined in accordance with ICH guidelines by using six replicates of the same concentration (200 ng/spot). Peak area was established and repeatability was expressed in terms of percent relative standard deviation (%RSD) and standard error (SE). The intra-day and inter-day variation for the determination of cholesterol was carried at three different concentration levels of 200, 300 and 400 ng/spot.

Accuracy

The accuracy of the proposed method was established using the standard addition method. Samples were spiked with an extra 50, 100 and 150% of the standard cholesterol and the mixtures were reanalyzed. The experiment was repeated in triplicate.

Specificity

The specificity of the method was verified by comparing the developed chromatogram of standard drug with that of the extract. The presence and position of cholesterol in the extract was confirmed by comparing the R_F values and spectra of the spot with those of the standard. Peak areas were compared to give the quantitative difference between the two samples. Peak purity was assessed by comparing the spectra at three different levels,namely, peak start (S), peak apex (A) and peak end (E).

Robustness of the method

Small changes in the mobile phase composition, mobile phase volume, duration of mobile phase saturation and activation of prewashed TLC plates were incorporated and the effects on the results were examined. The robustness of the method was studied in triplicate at a concentration level of 200 ng/spot and the %RSD and SE of peak areas were calculated.

Ruggedness

A solution with the concentration 1,000 ng/spot was prepared and analyzed on Day 0 and after 6, 12, 24, 48 and 72 h. Data were treated for %RSD to assess the ruggedness of the method.

Limit of detection and limit of quantification

Limit of detection (LOD) was defined as the lowest concentration of the analyte in a sample that could be detected and limit of quantitation (LOQ) was defined as the lowest concentration of the analyte in a sample that could be determined with acceptable precision and accuracy under the stated experimental conditions. The LOQ and LOD were determined based on the signal-to-noise ratio technique. The concentration of the sample that had a signal-to-noise ratio of three was fixed as the LOD. The concentration of the sample that had a signal-to-noise ratio of ten was fixed as LOQ.

Analysis of cholesterol in leech extract

The leech extract sample was prepared by dissolving 300 μL of leech extract in 1,200 μL of dichloromethane; 2 μL was applied on TLC plates and analyzed as described previously.

Forced degradation of cholesterol

Forced degradation studies provide an indication of the stability and specificity of the proposed analytical method. Stress conditions of acid and base hydrolysis, peroxide oxidation, UV and dry heat degradation were imposed upon standard cholesterol to evaluate the stability of the analytical method in accordance with the ICH guidelines (25–30).

Preparation of stock solution

A stock solution of cholesterol was prepared by dissolving 100 mg cholesterol in 5 mL chloroform and making the volume 100 mL by adding methanol. This stock (1,000 mg/mL) was used to study the degradation of cholesterol under various stress conditions.

Acid and base-induced degradation studies

The stock solution of cholesterol (25 mL) was separately refluxed with 25 mL 2M HCl and 2M NaOH (for acid and base degradation studies, respectively) in methanol at 80°C for 2 h.

Hydrogen peroxide induced degradation

The stock solution of cholesterol (25 mL) was added to 25 mL hydrogen peroxide (30% v/v H_2O_2) in a conical flask, heated in a boiling water bath for 10 min to completely remove the excess of hydrogen peroxide, and then refluxed for 2 h at 80°C.

UV degradation

The stock solution of cholesterol (25 mL) was exposed to UV radiation at 254 nm for 24 h to study its stability against UV radiation.

Dry heat degradation

The standard cholesterol powder (10 mg) was stored at 100°C for 3 h under dry heat conditions to study the inherent stability of the internal standard. Methanolic stock solution of this dry heat-exposed drug was prepared as described previously and 25 mL was removed.

The volumes of all the previously described solutions were made up to 50 mL in volumetric flasks and the resultant solutions (50 μg/mL) were used for application on TLC plates (2 μL each) for analysis.

Result and Discussion

Selection and optimization of mobile phase

The mobile phase composition was optimized using various solvents of nonpolar nature. Initially, carbon tetrachloride–formic acid in varying ratios was tried, but this led to band shouldering and peak widening. Addition of methanol to the mobile phase; that is, carbon tetrachloride–methanol–formic acid (9.5:1:0.55, v/v/v) resulted in good resolution with $R_F = 0.35$ for cholesterol because it did not give a sharp peak. Finally, the mobile phase consisting of carbon tetrachloride–methanol–formic acid (9.5:1.5:0.55, v/v/v) gave a sharp and well-defined peak at $R_F = 0.35$ (Figure 2). Chamber saturation with the mobile phase for 30 min at room temperature gave a reproducible, well-defined purple band after spraying the plate with 2% methanolic sulphuric acid solution and heating it at 110°C for 10 min.

Calibration curve

A good correlation coefficient, ($r^2 = 0.99958 \pm 0.02$) in the range of 100–600 ng/spot (Table I) with respect to the peak area is indicative of good linearity. The regression equation was found to be $y = 116.52x + 1488.388$; mean value (±SD) of slope and intercept were 116.52 ± 1.43 and 1488.388 ± 16.2, respectively. There was no significant difference in the slopes of standard curves [analysis of variance (ANOVA), $P > 0.05$].

Method validation

Precision

The repeatability of sample application was verified by %RSD and found to be 0.25 for six replicates of 200 ng/spot. The peak area for three different concentration levels at six replicates showed low values of SE and %RSD (0.18–0.51%) for inter-day and intra-day variation, which indicates the excellent precision of the method (Table II).

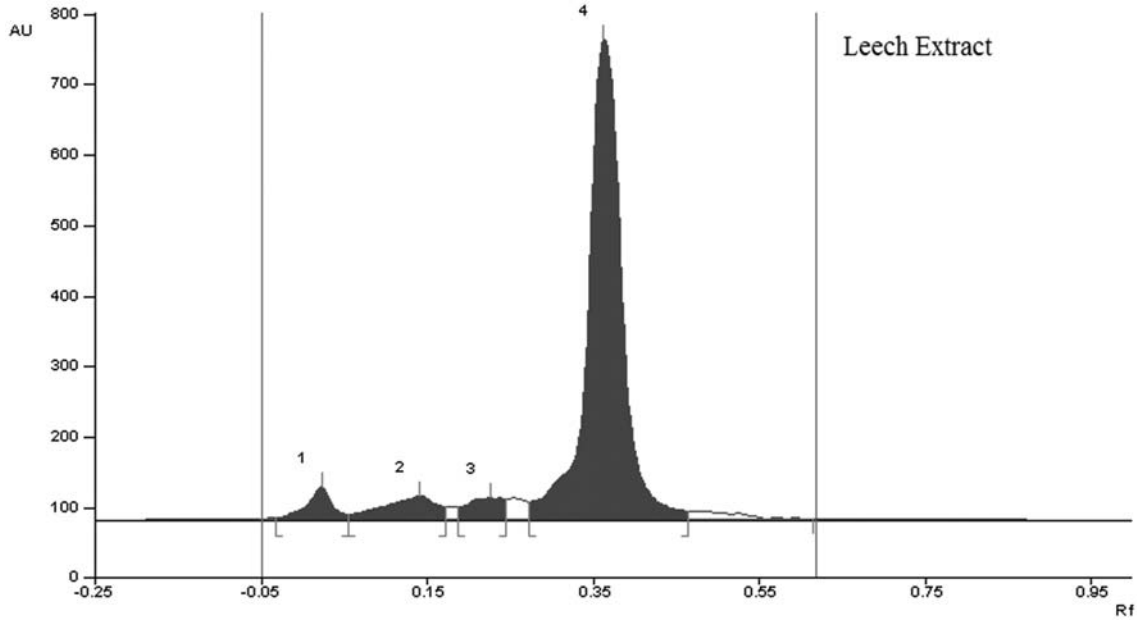

Figure 2. HPTLC chromatogram of Standard Cholesterol (300 ng per spot^{-1}, peak 3, $R_f = 0.35 \pm 0.02$) mobile phase: carbon tetra chloride: methanol: formic acid (9.5:1.5:0.5, v/v/v).

Table I
Calibration Plot Data for Cholesterol ($n = 3$)

Data	Cholesterol
Linearity range (ng)	100–600
Regression equation	$y = 116.52x + 1488.388$
Correlation coefficient	0.99958
Slope ± SD	116.52 ± 1.43
Intercept ± SD	1488.388 ± 16.2

Table II
Inter-Day and Intra-Day Precision of the HPTLC Method ($n = 6$)

Amount (ng per spot)	Mean area	SD	%RSD
Intra-day			
100	452.25	2.4	0.18
200	918.74	2.1	0.25
300	1,301.48	2.0	0.37
Inter-day			
100	469.73	2.25	0.26
200	898.04	2.3	0.33
300	1,295.3	1.97	0.51

Robustness of the method

The robustness of the method was verified by the low values of SD and %RSD obtained after introducing small deliberate variations in the parameters of the developed HPTLC method (Table III). The ruggedness of the method is illustrated by the low %RSD (0.2985) between peak areas of the chromatograms, indicating the stability of the biomarker during analysis and evaluation.

Recovery studies

The recovery of cholesterol from the samples was studied by the standard addition method. The recovery was found to be

Table III
Robustness of the HPTLC Method (n = 3, 200 ng/spot)

Parameter	SD of peak area	%RSD
Mobile phase composition (CCl_4–CH_3OH–HCOOH)	1.56	0.286
Mobile phase volume (10, 15 and 20 mL)	1.94	0.332
Duration of saturation (10, 20 and 30 min)	1.83	0.378
Activation of pre-washed TLC plates(10, 20 and 30 min)	1.72	0.198

Table IV
Recovery Studies ($n = 3$)

Excess drug added to analyte (%)	Theoretical content (ng)	Amount found (ng)	Recovery (%)	%RSD	SE
0	200	201.09	100.54	0.233	1.1
50	300	299.06	99.69	0.184	1.25
100	400	400.2	100.05	0.49	1.68
150	500	498.75	99.75	0.15	1.33

excellent (99.69–100.54%) with low %RSD and SE (Table IV). Thus, the method is accurate. Overlapping the spectra of the standard with that of the extract showed a good correlation ($r^2 = 0.9956 \pm 0.74$), signifying the specificity of the method.

LOD and LOQ

The LOD was found to be 13.8 ± 0.51 ng per spot, whereas the LOQ was found to be 45.01 ± 1.29 ng per spot, indicating appropriate sensitivity.

Analysis of cholesterol in leech extract

A single well-resolved spot was observed at the retention time of cholesterol during analysis of the leech extract (Figure 3).

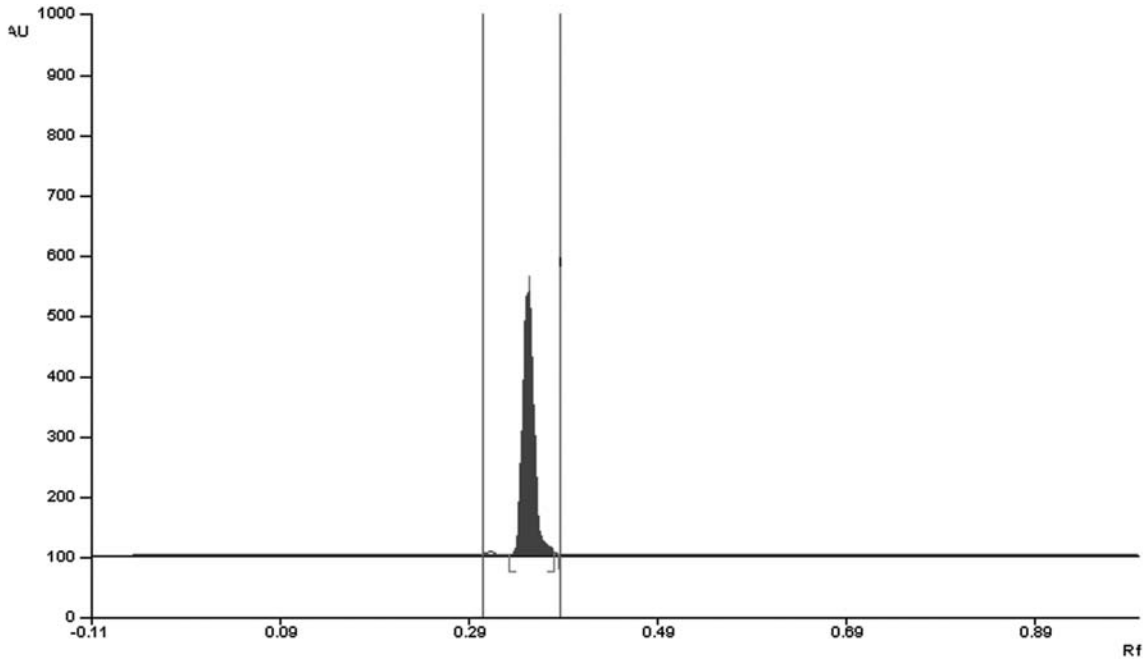

Figure 3. HPTLC Chromatogram of Leech extract (200 ng per spot^{-1}); peaks 1-3 belong to other components present in the extract. Peak 4 is that of cholesterol ($R_f = 0.35 \pm 0.1$); mobile phase: carbon tetra chloride: methanol: formic acid (9.5:1.5:0.5, v/v/v).

There was no interference from other components present in the extract, which appear at significantly different R_F values. The total content of cholesterol in the leech extract was found to be 0.65% (v/v).

Forced degradation of cholesterol

Cholesterol samples degraded by treatment with acid, base, hydrogen peroxide, dry heat and UV light were exemplified by well-separated spots of the pure cholesterol ($R_F = 0.35$) and some additional peaks at different RF values, as shown in the chromatograms (Figures 4–8). The degradant products are evident by their separate peaks (Table V), and hence, it can be safely concluded that the newly developed method has a stability-indicating nature. The method is highly specific for cholesterol because all the degradation products can easily be discerned from the biomarker compound.

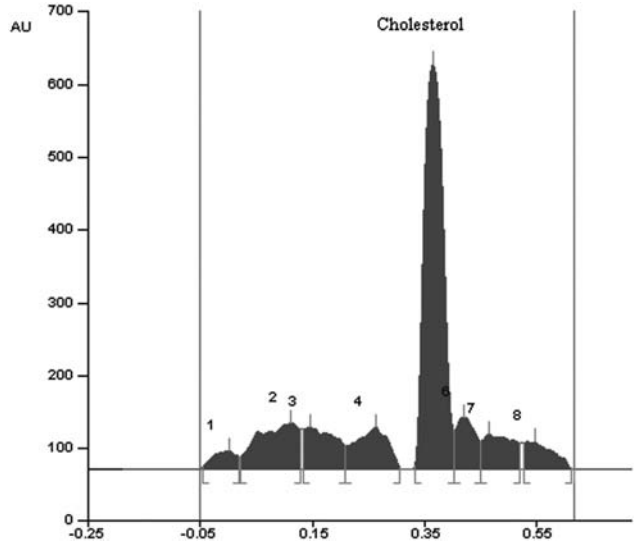

Figure 4. Acidic stress of Cholesterol, 2M HCl, refluxed at 80°C for 2 hours gave 7 degradation product peaks; degradant peak 1 ($R_f = 0.02$); degradant peak 2 ($R_f = 0.12$); degradant peak 3 ($R_f = 0.15$); degradant peak 4 ($R_f = 0.20$); degradant peak 5 ($R_f = 0.40$); degradant peak 6 ($R_f = 0.45$); degradant peak 7 ($R_f = 0.56$).

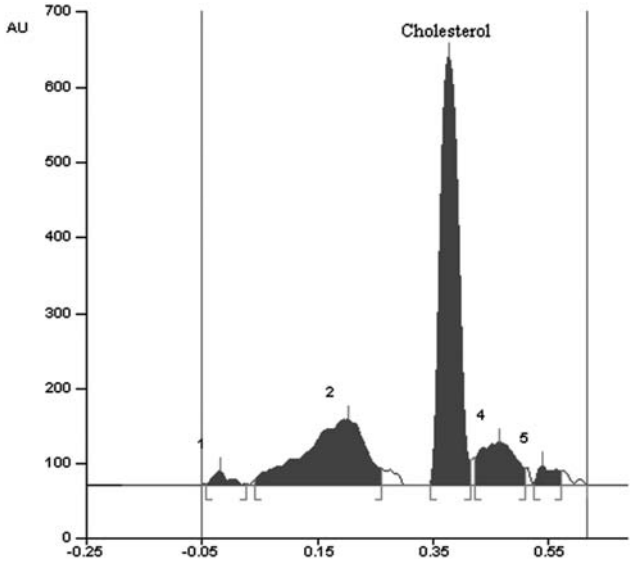

Figure 5. Basic stress of Cholesterol, 2M NaOH, refluxed at 80°C for 2 hours gave 4 degradation product peaks; degradant peak 1 ($R_f = 0.01$); degradant peak 2 ($R_f = 0.20$); degradant peak 3 ($R_f = 0.40$); degradant peak 4 ($R_f = 0.51$)

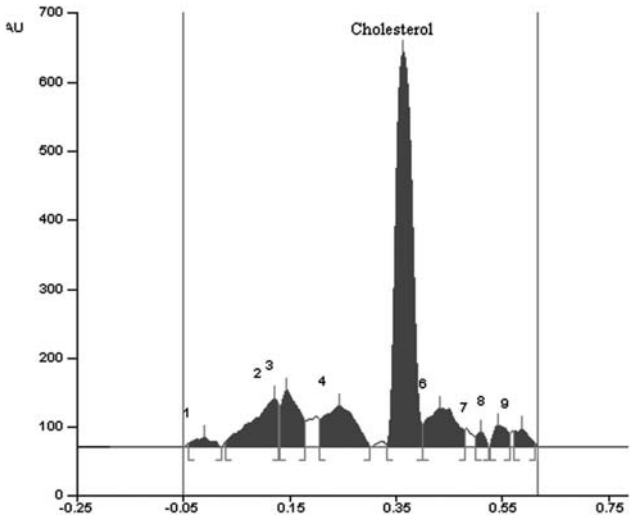

Figure 6. Peroxide stress of Cholesterol, 30% peroxide, refluxed at 80°C for 2 hours gave 8 degradation product peaks; degradant peak 1 ($R_f = 0.01$); degradant peak 2 ($R_f = 0.10$); degradant peak 3 ($R_f = 0.15$); degradant peak 4 ($R_f = 0.20$); degradant peak 5 ($R_f = 0.40$); degradant peak 6 ($R_f = 0.49$); degradant peak 7 ($R_f = 0.55$); degradant peak 8 ($R_f = 0.60$).

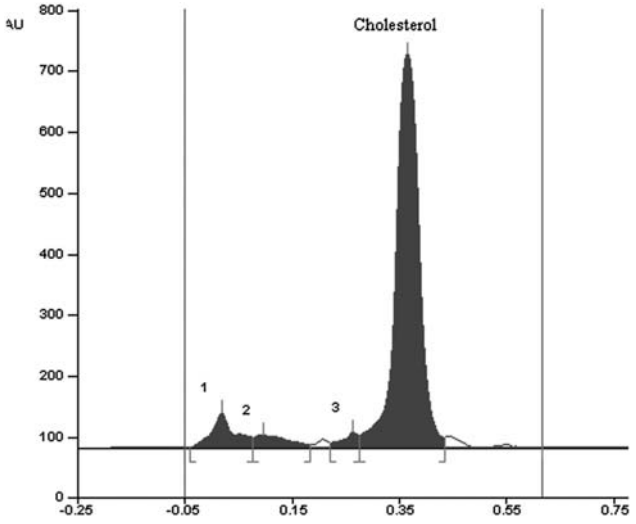

Figure 7. UV stress of Cholesterol gave 3 degradation product peaks; degradant peak 1 ($R_f = 0.05$); degradant peak 2 ($R_f = 0.10$); degradant peak 3 ($R_f = 0.25$)

Conclusion

A validated stability-indicating HPTLC densitometric method was developed for analysis of free sterols in *H. manillensis*. It is simple, rapid, economical, accurate, stability-indicating and robust. The method has been shown to be reproducible and selective by statistical analysis. A short analysis time of 8.15 min leads to the immediate and quick determination of cholesterol. The method can be used for qualitative and quantitative analysis of cholesterol and lipids in leech extract with minimal steps and instruments to give reproducible and accurate results. Moreover, because the method separates the degradant products of leech extract under different stress conditions, it may be used to study degradation kinetics in drug products.

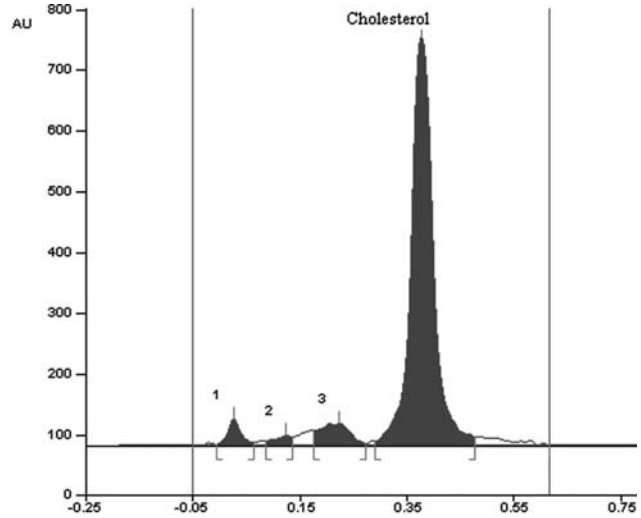

Figure 8. Dry heat stress of Cholesterol, stored at 100°C for 3 hours gave 3 degradation product peaks; degradant peak 1 ($R_f = 0.05$); degradant peak 2 ($R_f = 0.12$); degradant peak 3 ($R_f = 0.20$)

Table V
Forced Degradation of Cholesterol

Stress condition	Number of degradation products (R_F values)	Cholesterol (R_f)
Acid (2M HCl)	7 (0.02, 0.12, 0.15, 0.20,0.40, 0.45, 0.56)	0.37
Alkali (2M NaOH)	4 (0.01, 0.20, 0.40, 0.51)	0.35
Peroxide (30% H_2O_2)	8 (0.01, 0.10, 0.15, 0.20, 0.40, 0.49,0.55,0.60)	0.35
UV degradation	3 (0.05, 0.10, 0.25)	0.35
Dry heat	3 (0.05, 0.12, 0.20)	0.35

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