

Estimation of Andrographolide in *Andrographis paniculata* Herb, Extracts and Dosage forms

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Abstract: Andrographolide is an anti-inflammatory, antipyretic, anti-viral, immunostimulatory, anti-hyperglycemic, and anti-oxidant agent. High performance liquid chromatographic (HPLC) and high performance thin layer chromatographic (HPTLC) methods for quantitative determination of andrographolide were developed and validated. The methods showed satisfactory linearity, precision, good recovery and appropriate limits of detection (LOD) and quantification (LOQ). The content of andrographolide was determined and the results obtained by HPLC and HPTLC methods were in good agreement. The methods developed are suitable for the quality control applications in *Andrographis paniculata* plant, extracts and dosage forms.

Keywords: Andrographolide; *Andrographis paniculata*; dosage forms; HPLC; HPTLC.

1. Introduction

Andrographolide (3 - (2 - (Decahydro - 6 - hydroxyl - 5 - (hydroxymethyl) - 5, 8a - dimethyl - 2 - methylenenaphthyl)ethylidene) dihydro - 4 - hydroxyfuran - 2 (3H) - one) is the active principle of *Andrographis paniculata* extracts. *Andrographis paniculata* Nees (Family Acanthaceae) is available abundantly in India, Pakistan and Srilanka, growing in hot and shade places. It is cultivated in certain parts of India, East and West Indies and Mauritius. It is known by various names such as Kalmegh, Kalupnath, Kiriath and Mahatila meaning the “King of bitters”. *Andrographis paniculata* or kalmegh is one of the most widely used plants in Ayurvedic formulations [1].

Andrographis paniculata was recommended in Charaka Samhita dating to 175 BC for

treatment of Jaundice along with other plants in multi plant preparations [2]. It has also been used traditionally for sluggish liver as antidote in case of colic dysentery and dyspepsia [3]. It is used as bitter tonic, antispasmodic, antiperistaltic, stomachic and also an antihelmintic. It has been employed with benefit in case of general debility in convalescence after fevers, disorders of liver and advanced stages of dysentery [4]. The juice of fresh leaves is a domestic remedy in the treatment of colic pain, loss of appetite, irregular stools and diarrhea [5]. Long known in traditional Asian medicine as an immune system booster, *Andrographis* has demonstrated significant activity in fighting common cold, flu and upper respiratory infections [6, 7]. The pharmacological studies suggest

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anti-inflammatory [8,9], antipyretic [10], anti-viral [11], immunostimulatory [12], potential cancer therapeutic agent [13], anti-hyperglycemic [14] and anti-oxidant [15] properties.

In recent years focus on use of non-traditional approaches to treat diseases has been revived world wide. The evidence collected till now shows immense potential of medicine plants used in traditional systems. The herb, *Andrographis paniculata* is the main source of the bitter principle.

Various methods have been suggested for quantitative estimation of andrographolide in *Andrographis paniculata*. The gravimetric method described in the Indian pharmacopoeia was found to give high values [16]. This is due to some yellow coloring substance other than andrographolide which is also soluble in ethyl acetate.

The spectrophotometric method proposed by Maiti et.al [17] suffers from the disadvantage that the red colour formed with the addition of alcoholic potassium hydroxide to the solution of andrographolide is unstable and fades away quickly. Gained et.al [18] proposed a spectrophotometric method by extracting pure andrographolide from kalmegh measuring its absorbance at 226 nm but the extraction process was very tedious. Subbarao [19] has suggested a chemical method involving a lactone titration but the method has been reported to be not suitable for detecting minute quantities. High performance liquid chromatographic methods were reported for estimation of andrographolide in *Andrographis paniculata* [20-24] and in rabbit serum [25].

Thin layer chromatographic methods were also described for estimation of andrographolide in *Andrographis paniculata* extracts [22, 26] and also reported by using capillary electrophoresis chromatography (CE) [27-29].

The methods described above have several limitations like preparation of samples for estimation of andrographolide. In the present study, accurate, simple, specific and reproducible HPLC and HPTLC methods have

been developed and validated [30] for the determination of andrographolide in *A. paniculata* herb, extracts and dosage forms.

2. Experimental

2.1. Materials

Standard andrographolide was procured from M/s ChromaDex USA (purity 99.5%). Methanol, acetonitrile were HPLC grade, analytical grade phosphoric acid, toluene, ethyl acetate and formic acid were purchased from M/s Qualigens (Mumbai, India). Ultrapure water generated by the Barnstead Nanopure System Model 3750 was used. Methanol was used as a solvent for the preparation of standards and samples, acetonitrile and 0.1% (v/v) phosphoric acid in water (40: 60, v/v) were used as mobile phase for HPLC analysis. Methanol used as a solvent for preparation of standards and samples, toluene, ethyl acetate and formic acid (5.0: 3.5:1.5) used as a mobile phase for HPTLC analysis. All solutions used for HPLC analysis were filtered through 0.45 μ m membrane filter using Millipore Swinnex type filtration unit. *Andrographis paniculata* plants and extracts were provided by M/s Laila Impex, Vijayawada, India and dosage forms capsules and tablets containing andrographolide were procured from local market.

2.2. Sample preparation for HPLC & HPTLC analysis

2.2.1. *Andrographis paniculata* herb:

Weighed about 2-5 gm of *Andrographis paniculata* plant powder into a round bottom flask added about 30 ml of methanol and refluxed on a water bath for 30 min. The same operation was repeated twice with methanol (2 \times 30 ml) and combined all the methanolic extracts and made up to 100 ml with methanol and filtered through 0.45 μ m membrane filter.

2.2.2. *Andrographis paniculata* extracts:

About 100 mg of dry sample was dissolved in 75 ml of methanol, sonicated for 10 min and diluted to 100 ml with methanol.

2.2.3. Dosage forms:

Three different dosage forms were chosen for estimation of andrographolide. Of these two are tablets and one is capsules. The average weight of the tablets was determined by weighing 20 tablets. In the case of capsules the average weight determined by weighing 20 capsules. Hard gelatin shells were removed and finely powdered 200 -1000 mg of powdered samples were weighed and dissolved in about 75 ml of methanol, sonicated for 10 min and diluted to 100 ml with methanol. All the sample solutions were filtered through 0.45 μm membrane filter using Millipore Swinnex type filtration unit before injected into the HPLC.

2.3. Calibration curve of standard andrographolide – HPLC method

5 mg/ml andrographolide standard solution was prepared in methanol (stock solution). Standard working solutions were prepared by diluting standard stock solution with methanol in the concentration range 20.0 – 600 $\mu\text{g/ml}$. 20 μl from each working standard solution was injected in six replicates. Calibration curve was generated by linear regression based on peak areas.

2.4. Calibration curve of standard andrographolide – HPTLC method

Standard working solutions were prepared by diluting standard stock solution with methanol in the concentration range 50 - 1200 $\mu\text{g/ml}$. A 10 μl of working standard solution was spotted six times on the TLC plate. Calibration curve was generated by linear regression based on the peak areas.

2.5. HPLC instrumentation

The HPLC system, Supplied by M/s Shimadzu comprising LC-10AT VP pumps, SCL-10A VP auto injector and Phenomenex Luna C₁₈, 5 μm , (250 X 4.6 mm) column was used at ambient temperature. Isocratic elution was carried out with acetonitrile : 0.1% (v/v) phosphoric acid in water (40 : 60, v/v) at a flow rate of 1 ml / min, detection was at 223 nm using SPD – M10 AVP photodiode array detector. Class VP software was used for integration and calibration. Evaluation was via peak areas with linear regression.

2.6. HPTLC instrumentation

The samples were spotted in the form of bands of width 5 mm with a Camag microlitre syringe on pre-coated silica gel aluminum plate 60F-254 (20 cm \times 10 cm with 250 μm thicknesses (Merck, Darmstadt, Germany) using a Camag Linomat IV applicator (Switzerland). The plates were pre-washed by methanol and activated at 60° C for 5 min prior to chromatography. A constant application rate of 0.1 μl per second was employed and space between two bands was 5 mm. The slit dimension was kept at 5 mm \times 0.45 mm and 10 mm per second scanning speed was employed. The monochromatic band width was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene: ethyl acetate: formic acid (5.0: 3.5: 1.5) and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm \times 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at ambient temperature. The length of chromatogram run was 8 cm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air drier. Scanning was performed on Camag TLC scanner III in the reflectance-absorbance

mode at 223 nm and operated by Cats software 4.03. Evaluation was via peak areas with linear regression.

2.7. Estimation of andrographolide

To estimate the content of *Andrographis paniculata* plant and extracts samples, 10 μ l aliquots of sample were subjected to HPTLC and 20 μ l aliquots of sample were injected in to HPLC. The HPTLC plates were developed to a distance of 8 cm from the point of application, dried and scanned at 223 nm. The HPLC analysis was continued for 10 min, since the retention time of the andrographolide was 5.5 ± 0.4 min. The content of andrographolide was calculated by linear regression and mean percentages were calculated from six replicate experiments. In case of dosage forms, mean mg/capsule or tablet calculated from six replicate experiments.

3. Results and discussion

Because of the complexity of the chemical composition of herbal extracts, quality of the herbal extracts can only be assured by the use of validated analytical methods for identification and quantification of the active ingredients. The HPLC and HPTLC methods for the quantitative estimation of andrographolide were validated with regard to their specificity, precision, accuracy and linearity.

3.1. HPLC method validation

The development of suitable mobile phase is an important step in devising an analytical procedure. The composition of the HPLC mobile phase was optimized to achieve good resolution. The best resolution and peak shape was obtained by acetonitrile: 0.1% (v/v) phosphoric acid in water (40:60, v/v) as mobile phase. The compound with a retention time 5.8 min was identified as andrographolide (Figure 1). Specificity can be ascertained by comparing the standard and sample

peak purity. The peak corresponding to andrographolide in the sample was confirmed by comparing the spectrum obtained by photodiode array detector, which was completely in agreement with the standard (Figure 2).

Precision is a measure of either reproducibility or repeatability of the analytical method. Intermediate precision express the laboratory variations, by intra- and inter-day variation. Six determinations of three concentrations of standard andrographolide on the same day (intra-day) and on different days (inter-day) were carried out and expressed as percent relative standard deviation (% RSD) or co-efficient of variation (CV). The results noted in Table 1, reveal that no significant intra- and inter-day variations. The % RSD for intra- and inter-day analysis was found to be in the range 0.56 – 1.41 which are less than 2%. The accuracy of the method was determined from recovery studies. The pre-analyzed sample was spiked with three different concentrations of standard andrographolide and the mixtures were analyzed by the proposed method. The recoveries are in the range of 99.09 – 100.19% reported in the Table 2. The average recovery percentage value was found to be $99.78 \pm 0.60\%$.

The linearity of the standard curve was evaluated by injecting six standard working solutions containing 0.40 – 12.00 μ g/20 μ l andrographolide. Peak area and concentrations were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. Linearity was obtained over a concentration range of 0.40 – 12.0 μ g/20 μ l with a correlation coefficient of 0.9993 ± 0.0006 . The linearity of calibration graph and adherence of the system to Beer's law was validated by high value of correlation coefficient.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated to check the sensitivity of the method. The LOD and LOQ were calculated based on the responses at the signal to noise ratios 3:1 and 10:1 respectively. LOD and LOQ were experimen-

tally verified by diluting the known concentration of andrographolide until the average responses were approximately three or ten times the standard deviation of the responses

for six replicate determinations. The LOD and LOQ were found to be 0.001 and 0.004 $\mu\text{g}/20\mu\text{l}$, respectively.

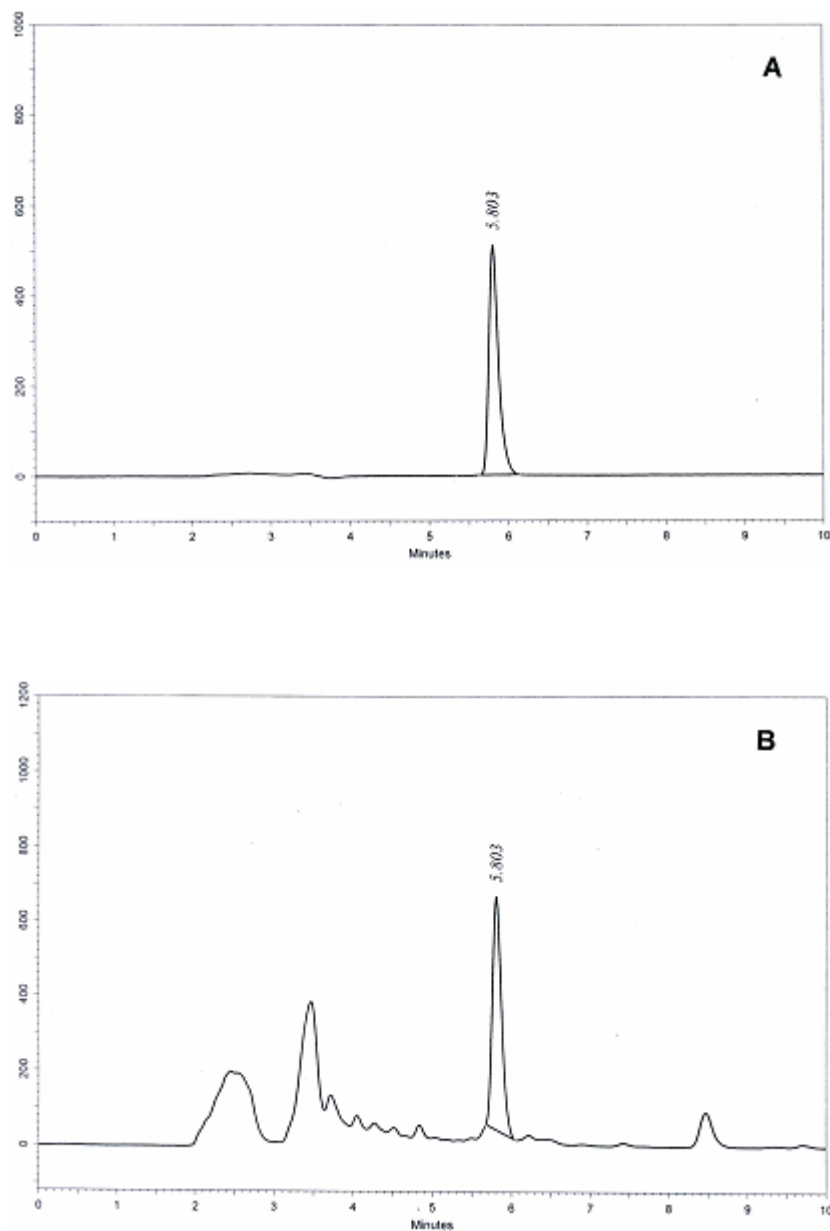


Figure 1. HPLC chromatograms of A) andrographolide standard B) *Andrographis paniculata* plant extract.

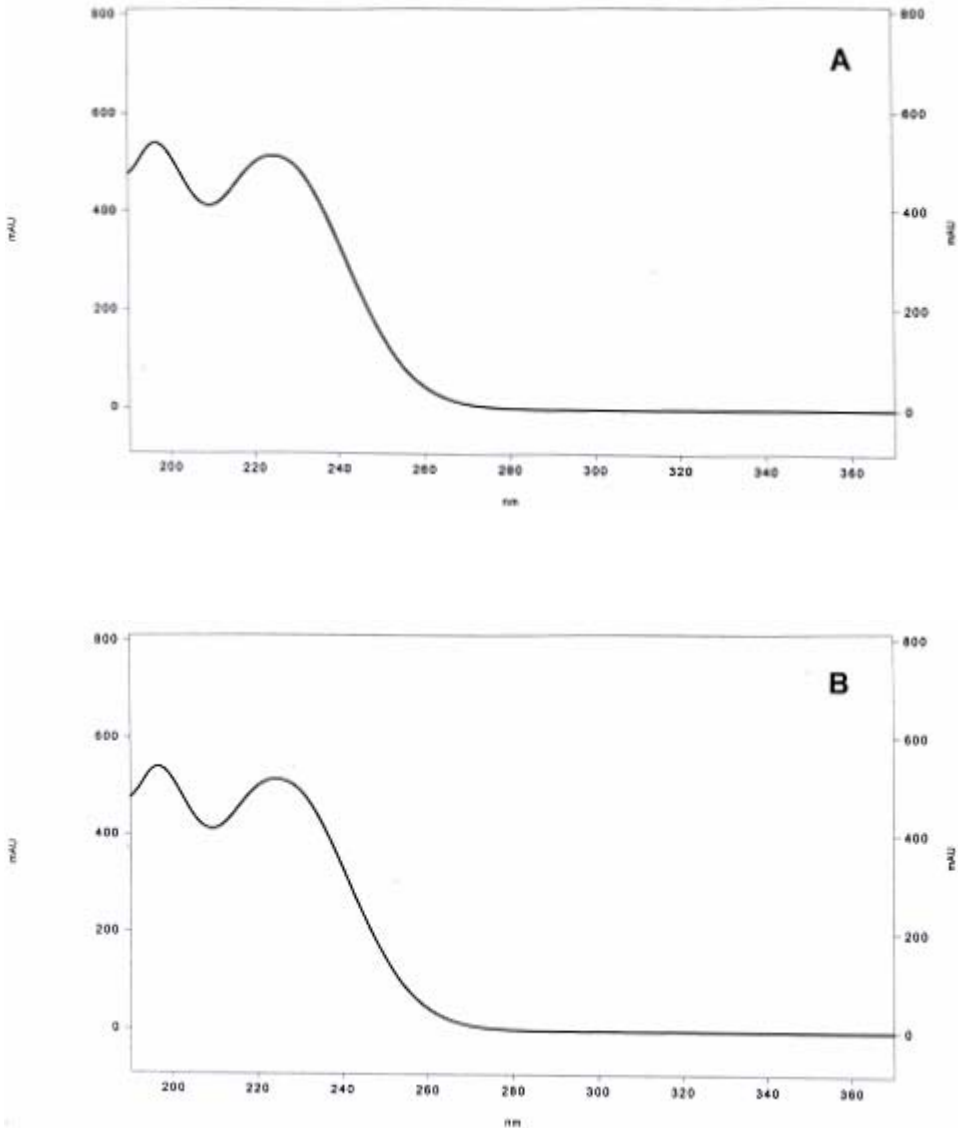


Figure 2. Spectra of andrographolide obtained by photodiode array detector A) andrographolide standard B) Andrographis paniculata plant extract.

Table 1. Intra- and Inter-day precision of HPLC and HPTLC methods (n=6)

HPLC Method				
Andrographolide (µg)	Intra-day		Inter-day	
	Andrographolide (µg)	% RSD	Andrographolide (µg)	% RSD
2.0000	2.0496 ± 0.0120	0.59	1.9998± 0.0130	0.65
6.0000	5.9950 ± 0.0337	0.56	6.0175 ± 0.0849	1.41
12.0000	12.0051±0.1476	1.23	11.9898±0.1055	0.88
HPTLC Method				
2.0000	1.9578± 0.0035	0.18	2.0155 ± 0.0044	0.22
6.0000	6.0050 ± 0.0030	0.05	6.0750 ± 0.0230	0.38
12.0000	12.0015± 0.0024	0.02	12.0105 ± 0.0336	0.28

Table 2. Recovery study of Andrographolide(n = 6)

HPLC Method		
Amount of Andrographolide added (mg)	Amount of Andrographolide recovered (mg)	% Recovery
1.0	1.0019	100.19
15.0	14.8635	99.09
25.0	25.0150	100.06
HPTLC Method		
0.50	0.5024	100.48
15.0	14.9580	99.72
25.0	25.1700	100.68

3.2. HPTLC method validation

The composition of the mobile phase for TLC was optimized by testing different solvent mixtures of varying polarity. The best results were obtained using toluene: ethyl

acetate: formic acid (5.0: 3.5: 1.5). The selected mobile phase produced highly symmetrical peaks showing good resolution (Figure 3). The compound with a R_f value of 0.44 ± 0.03 was identified as andrographolide.

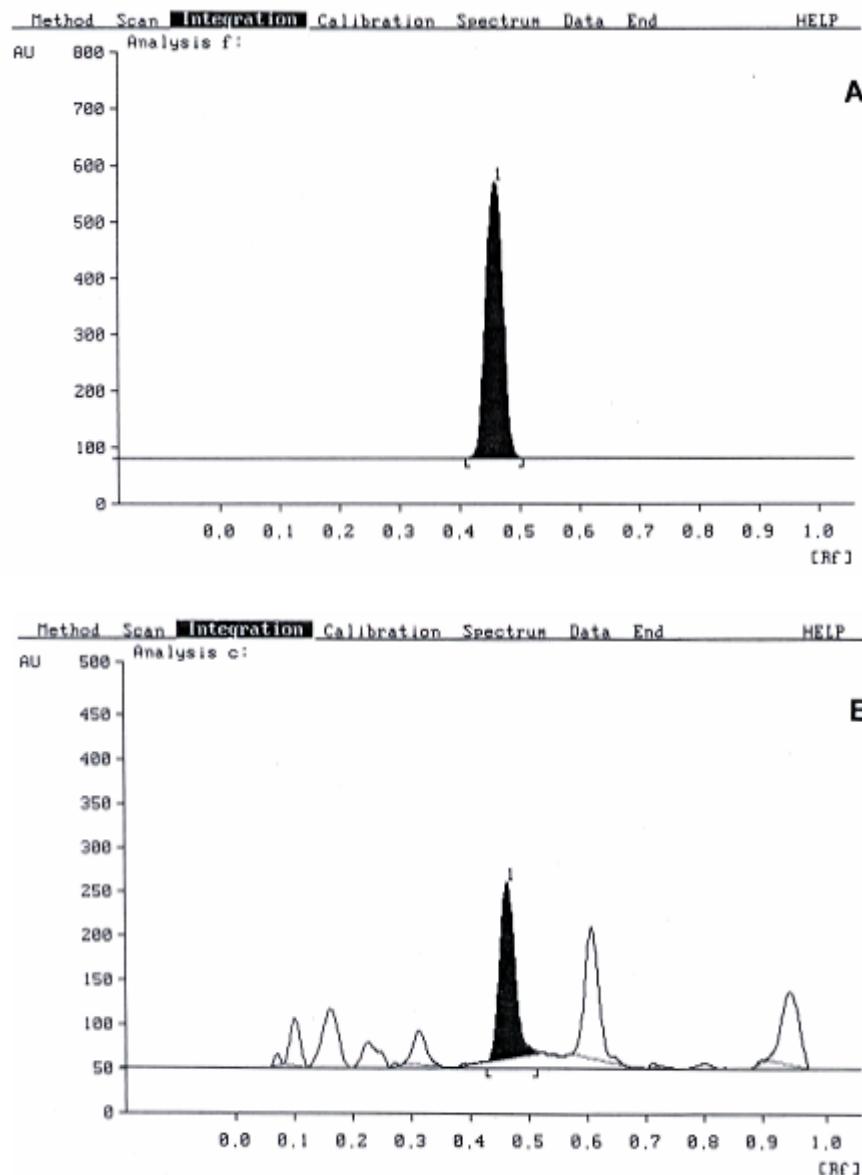


Figure 3. HPTLC chromatograms of A) andrographolide standard B) *Andrographis paniculata* plant extract.

The specificity of the method was ascertained by analyzing standards and samples. The spot for andrographolide in the sample was confirmed by comparing the R_f value. Spectral studies revealed that the peaks ob-

tained from both standard andrographolide and test samples were identical, because they had similar pattern as shown in Figure 4.

The precision of the method was studied by applying six replicates of the three different

concentration of the standard. Table 1 showed that the % RSD for intra- and inter- day analysis was found to be in the range 0.02 – 0.38, that is less than 2%.

The accuracy of the method was determined from recovery studies. A known but varying amount of standards of andrographolide was added to the pre-analyzed sample and analyzed according to the procedure. The results reported in Table 2. The recoveries are in the range of 99.72 - 100.68% and the average recovery percentage value was found to be $100.29 \pm 0.51\%$.

Linearity was evaluated by spotting six standard working concentrations containing 0.50 – 12.00 $\mu\text{g}/10\mu\text{l}$ of andrographolide. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. Linearity was found over the concentration range 0.50 – 12.00 $\mu\text{g} / 10\mu\text{l}$ with a correlation coefficient of 0.9999 ± 0.0001 . The linearity of the calibration curve and adherence to the system to Beer's law was validated by a high value correlation coefficient.

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank, methanol was spotted six times. LOD and LOQ were determined based on the standard deviation of the response of the blank and slope estimated from the calibration curve of the andrographolide. The LOD and LOQ were found to be 0.001 and 0.003 μg per spot for andrographolide.

3.3 Application of HPLC and HPTLC methods

The HPLC method developed was applied for the estimation of andrographolide in four different *Andrographis paniculata* herb samples, three different extracts and three different dosage forms, two tablets and one capsule. The obtained results were presented in Table 3. The %RSD values have been found to be less than 2% which shows the precision of

the method is reasonably good. The interference studies revealed that the presence of commonly used excipients like starch, talc, gelatin, malto dextrin, yellow dextrin, colloidal silicon dioxide, magnesium stearate and the plant extracts, Triphala, *Tinospora carifolia*, *Eclipta alba*, *Boerhavia diffusa* does not interfere in the estimation of andrographolide.

The HPTLC method developed was applied for the estimation of andrographolide in four different *Andrographis paniculata* herb samples, three different extracts and three different dosage forms, two tablets and one capsule. The results obtained were presented in Table 3. The %RSD values have been found to be less than 2% which shows the precision of the method is reasonably good. The interference studies revealed that the presence of commonly used excipients like starch, talc, gelatin, malto dextrin, yellow dextrin, colloidal silicon dioxide, magnesium stearate and the plant extracts, Triphala, *Tinospora carifolia*, *Eclipta alba*, *Boerhavia diffusa* does not interfere in the estimation of andrographolide.

The results obtained for both HPLC and HPTLC methods are almost similar and found to be very much suitable for the andrographolide estimation in plants, extracts and dosage forms.

4. Conclusions

The developed HPLC method can be utilized for the quantitative determination of andrographolide in *Andrographis paniculata* herb samples, extracts and dosage forms. HPTLC method also can be utilized for quantitative determination of andrographolide in *Andrographis paniculata* herb samples, extracts and dosage forms. The methods developed are simple, sensitive and statistically validated for linearity, accuracy and precision.

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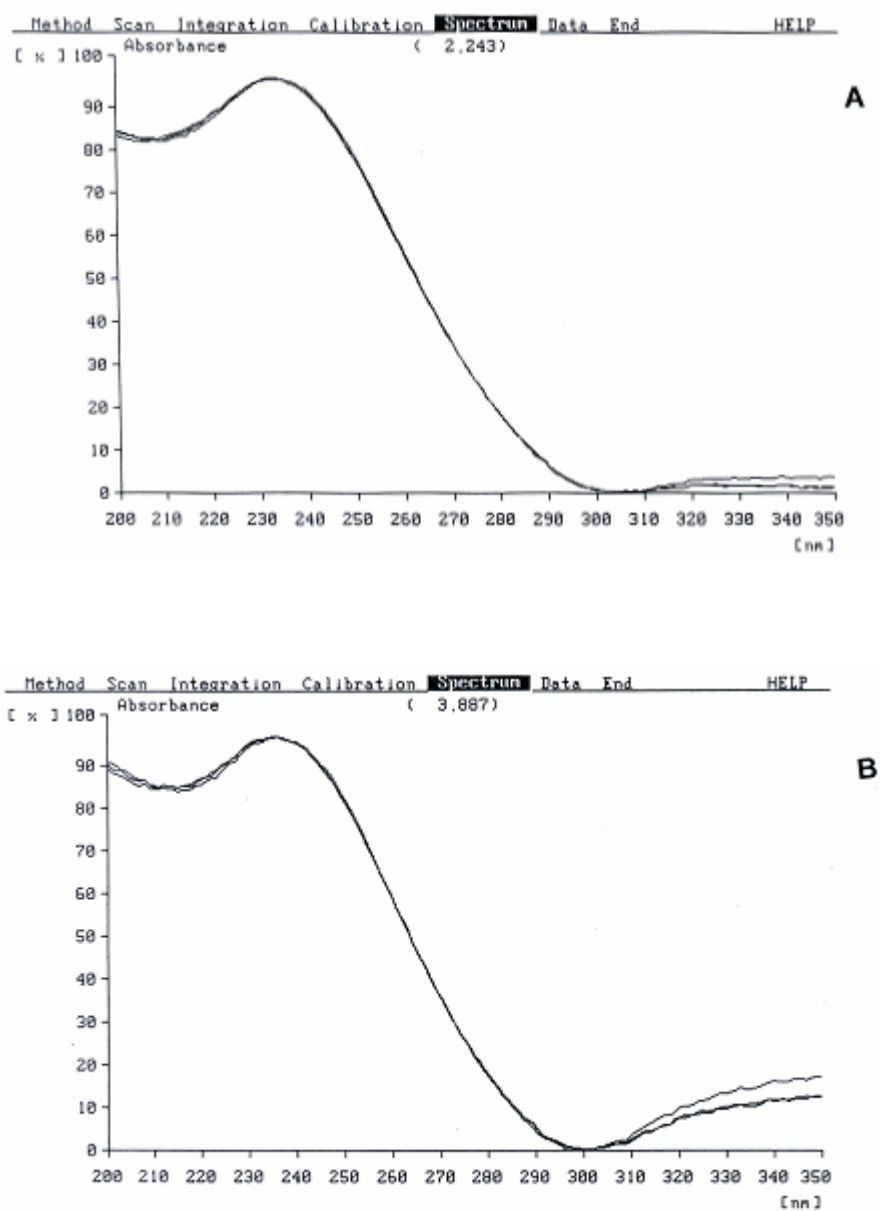


Figure 4. Over lay spectra of A) andrographolide standard B) Sample at peak start, peak maximum and peak end in absorbance mode in the UV range, taken on the Camag TLC scanner III.

Table 3. Estimation of andrographolide by HPLC and HPTLC methods (n = 6)

Sample	HPLC method		HPTLC method	
	Estimated amount	% RSD	Estimated amount	% RSD
RM-1	0.56 ^a	1.35	0.58 ^a	0.70
RM-2	0.44 ^a	0.93	0.47 ^a	1.72
RM-3	1.70 ^a	0.71	1.67 ^a	0.73
RM-4	0.68 ^a	0.93	0.87 ^a	0.73
EM-1	86.60 ^a	0.12	85.88 ^a	0.19
EM-2	41.44 ^a	0.38	41.35 ^a	0.38
EM-3	21.06 ^a	0.90	21.33 ^a	0.64
FM-1	1.2296 ^b	0.44	1.2046 ^b	0.36
FM-2	0.0492 ^b	2.00	0.3541 ^b	0.73
FM-3	0.5000 ^b	0.75	0.4597 ^b	0.74

RM: *Andrographis paniculata* plant.

EM: *Andrographis paniculata* plant extract

FM- 1: Amlycure D. S. Capsules, Aimil Pharmaceuticals (India) Ltd. A-13/2, Naraina Indl. Area, Phase, New Delhi-28.

FM- 2: Vasuliv Tablet, Vasu Pharmaceuticals Pvt. Ltd, 896/A, Makarpura, Vaddara-390010, India.

FM- 3: Heptin Forte, Alopa Herbal Health, No.9/1 Tank Road, Bangalore-560042, At No: 7, Industrial Suburb, Yeshwanthpur, Bangalore-560022.

a: Expressed as mg/100 mg

b: Expressed as mg/ Capsule or Tablet

References

- [1] Hooker, J. D. 1885. "*Flora of British India*", vol: IV, L. Reeve & CO. LTD, Ashford, Kent.
- [2] Sharma, P. V. 1983. "*Charka Samhita*", Ed. Chankhambhia Orientalia, Vol.II, Varanasi.
- [3] Handa, S. S., and Sharma, A. 1990. Hepatoprotective activity of andrographolide from *Andrographis paniculata* against carbon tetrachloride. *Indian Journal of Medical Research* B 92: 276-283.
- [4] Dastur, J. F. 1959. "*Medicinal plants of India and Pakistan*", Meyer Books.
- [5] Saxena, S., Jain, D. C., Bhakuni, R. S., and Sharma, R. P. 1998. Chemistry and pharmacology of *Andrographis* species. *Indian Drugs*, 35: 458-467.

- [6] Melchior, J., Spasov, A. A., and Ostrovskij, O. V. 2000. Double blind placebo controlled pilot and phase III study of activity of standardized *Andrographis paniculata* Herba Nees extract fixed combination (Kan jang) in the treatment of uncomplicated upper respiratory tract infection. *Phytomedicine*, 7:341-350.
- [7] Coon, J. T., and Ernst, E. 2004. *Andrographis paniculata* in the treatment of upper respiratory tract infections: A systematic review of safety and efficacy *Planta medica*, 70: 293-298.
- [8] Shen, Y. C., Chen, C. F., and Chiou, W. F. 2002. Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti - inflammatory effect. *British Journal of Pharmacology*, 135: 399-406.
- [9] Amroyan, E., Gabrielian, E., Panossian, A., Wikman, G. and Wagner, H. 1999. Inhibitory effect of andrographolide from *Andrographis paniculata* on PAF induced platelet aggregation. *Phytomedicine*, 6: 27-31.
- [10] Madav, S., Tripathi, H. C., Tandan, S. K., and Mishra, S. 1995. Analgesic, antipyretic and antiulcerogenic effect of andrographolide. *Indian Journal of Pharmaceutical Sciences*, 57: 121-125.
- [11] Chang, R. S., Ding, L., Chen, G. Q., Pan, Q. C., Zhao, Z. L., and Snith, K. M.1991. Dehydrographolide succinic acid monoester as an inhibitor against the human immunodeficiency virus. *Proceedings of Society of Experimental Biology and Medicine*, 197: 59-66.
- [12] Puri, A., Saxena, R., Saxena, R. P., Saxena, K. C., Srivastava, V., and Tandon, J. S. 1993. Immunostimulant agents from *Andrographis paniculata*. *Journal of Natural Products*, 56: 995-999.
- [13] Rajagopal, S., Kumar, R. A., Deevi, D. S., Satyanarayana, C., and Rajagopalan, R. 2003. Andrographolide, a potential cancer therapeutic agent isolated from *Andrographis paniculata*. *Journal of Experimental and Therapeutic Oncology*, 3: 147-158.
- [14] Bu-Chin, Y., Chen-Road, H., Wang-Chuan, C. and Juei-Tang, C. 2003. Antihyperglycemic effect of andrographolide in streptozotocin- induced diabetic rats. *Planta Medica*, 69: 1075-1079.
- [15] Zhang, X. F., and Tan, B. K. 2000. Anti-hyperglycaemic and anti-oxidant properties of *Andrographis paniculata* in normal and diabetic rats. *Clinical Experimental Pharmacology and Physiology*, 27: 358-363.
- [16] "Pharmacopia of India, 1955". Government of India Press, Calcutta.
- [17] Maiti, P. C., Kanji, S. K., and Chatterjee, R. 1959. Studies in Kalmegh extract. *Indian Journal of Pharmacy*, 21: 169-171.
- [18] Gaiind, K. N., Dar, R. N., and Kaul, R. N. 1963. Spectrophotometric estimation of andrographolide in Kalmegh. *Indian Journal of Pharmacy*, 25: 225-226.
- [19] Bhat, V. S., and Nanavati, D. D. 1978. *Andrographis paniculata* (Burm) Wall ex Nees (Kalmegh). *Indian Drugs*, July: 187-190.
- [20] Sharma, A., Lal, K., and Handa, S. S. 1992. Standardization of the Indian crude drug Kalmegh by high pressure liquid chromatographic determination of andrographolide. *Phytochemical Analysis*, 3:129-131.
- [21] Pholphana, N., Rangkadilok, N., Thongnest, S., Ruchirawat, S., Ruchirawat, M., and Satyavivad, J. 2004. Determination and variation of three active diterpenoids in *Andrographis paniculata* (Burm.f.) Nees. *Phytochemical Analysis*, 15:365-371.
- [22] Srivastava, A., Misra, H., Verma, R. K., and Gupta, M. M. 2004. Chemical finger printing of *Andrographis paniculata* using HPLC, HPTLC and densitometry. *Phytochemical Analysis*, 15: 280-285.
- [23] Du, Q., Jerz, G. and Wiinterhalter, P. 2003. Separation of andrographolide and

- neoandrographolide from the leaves of *Andrographis paniculata* by micellar electrokinetic chromatography. *Journal of Chromatography A*, 984:147-151.
- [24] Chen, L., Jin, H., Ding, L., Zhang, H., Wang, X., Wang, Z., Li, J., Qu, C., Wang, Y., and Zhang, H. 2007. On-line coupling of dynamic microwave – assisted extraction with high performance liquid chromatography for determination of andrographolide and dehydro andrographolide in *Andrographis paniculata* Nees. *Journal of Chromatography A*, 1140: 71-77.
- [25] Kumaran, K. S., Thirugnanasambantham, P., Viswanathan, S., and Sree Ramamurthy, M. 2003. An HPLC method for the estimation of andrographolide in rabbit serum. *Indian Journal of Pharmacology*, 35: 109-112.
- [26] Saxena, S., Jain, D. C., Gupta, M. M., Bhakuni, R. S., Mishra, H. O., and Sharma, R. P. 2000. High-performance thin layer chromatographic analysis of hepatoprotective diterpenoids from *Andrographis paniculata*. *Phytochemical Analysis*, 11:34-36.
- [27] Cheung, H. Y., Cheung, C. S., and Kong, C. K. 2001. Determination of bioactive diterpenoids from *Andrographis paniculata* by micellar electrokinetic chromatography. *Journal of Chromatography A*, 930: 171-176.
- [28] Zhao, Y., Ming, Y., Zhang, H., Luo, X., Chen, L., and Li, Y. 2005. Rapid determination of diterpenoids in *Andrographis paniculata* by microemulsion electrokinetic capillary chromatography with short-end injection. *Chromatographia*, 62: 611-615.
- [29] Yanfang, Z., Xingping, L., Zongde, Z., Liren, C., and Yongmin, L. 2006. Simultaneous determination of andrographolide and dehydroandrographolide in *Andrographis paniculata* and Chinese medicinal preparations by microemulsion electrokinetic chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, 40:157-161.
- [30] “*ICH Topic Q2B Validation of Analytical Procedures: Methodology*”, London, 1996.

