HPLC Determination of Andrographolide, Neoandrographolide and Dehydroandrographolide in Biological Fluids

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Abstract : A method has been developed for determining the major constituents of the plant commonly known in Thailand as "fah-talai-joan", *Andrographis paniculata* (Burm.f) Wall. ex Nees by high-performance liquid chromatography (HPLC); its constituents are andrographolide, neoandrographolide and 14-deoxy-11, 12-didehydroandrographolide. The reverse phase HPLC system consisted of a Lichrosorb R_8 column, and acetonotrile/H_2O/ ethanol (21/56/20, v/v) as the mobile phase. The column effluent was monitored by a UV-detector at the 205 nm wave-length. The correlation of calibration curve of the lactones was r=0.9998, whereas the reproducibility study gave the percent C.V. of about 5 per cent. With this analytical method, pharmacokinetics study of fah-talai-joan in both in situ and in vivo models was undertaken. It was found that 14-deoxy-11, 12-didehydroandro-grapholide was rapidly absorbed and neoandrographolide was the poorest absorption compound. Andrographolide was administered orally to rats, the drug plasma concentration showed absorption with a rate constant of 0.0078 $min.^{-1}$ Maximum drug plasma level of 1.76 μg/ml was observed at 2.04 hours after oral administration. The elimination half-life in rats was 1.34 hours.

The developed analytical procedure as well as the pharmacokinetic parameters obtained from this investigation provide valuable information for future clinical efficacy studies of fah-talai-joan.

เรื่องย่อ : **วิเคราะห์ Andrographolide, Neoandrographolide และ Dehydroandrographolide ในชีววัตถุโดยเครื่องแยกสารระบบ โครมาโตกราฟ ชนิดแรงดันสูง**

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สารศิริราช ๒๕๓๔; ๔๓:๗๖๐-๗๖๘.

ได้พัฒนาระบบที่เหมาะสมสำหรับตรวจวิเคราะห์สารสำคัญของฟ้าทะลายโจรซึ่งประกอบด้วย Andrographolide, Neoandrographolide และ 14-Deoxy-11, 12-didehydroandrographolide (dehydroandrographolide), โดยใช้เครื่องแยกสารระบบ โครมาโตกราฟ ชนิดแรงดันสูง (high performance liquid chromatography), โดยใช้คอลัมน์ Lichrosorb R_8 และ mobile phase ประกอบด้วยส่วนผสมของ อะซิโตไนไตรล์, น้ำ และ เอธิล์แอลกอฮอล์ ในอัตราส่วน ๒๔/๕๖/๒๐ ตรวจวัดสารแบบดูดกลืนแสง อัลตราไวโอเลต ที่ความยาวคลื่น ๒๐๕ นาโนเมตร, พบว่าค่าสหสัมพันธ์ที่ ๐.๙๙๙๘ และมีค่าสัมประ-

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สิทธิความแปรปรวน (% CV) ของระบบที่ ๕ เปอร์เซนต์. จากนั้นนำเอาระบบที่ได้พัฒนามาศึกษาการดูดซึมของสารในหนูขาวทั้ง in situ และ in vivo study พบว่า Dehydroandrographolide สามารถดูดซึมได้เร็วที่สุดและ Neoandrographolide ดูดซึมได้ช้าที่สุด. เมื่อให้สาร Andrographolide ในหนู พบว่าอัตราการดูดซึมของสารนี้เท่ากับ ๐.๐๐๗๘ นาที$^{-1}$, ให้ความเข้มข้นของยาสูงสุดในเลือดเท่ากับ ๑.๗๖ ไมโครกรัม/มิลลิลิตร ที่เวลา ๒.๐๔ ชั่วโมง, มีเวลาครึ่งชีพของยาที่ ๑.๓๔ ชั่วโมง. ระบบที่ได้พัฒนานี้จะเป็นประโยชน์ในการศึกษาทางคลินิกของสมุนไพรฟ้าทะลายโจรต่อไป.

Andrographis paniculata (Burm.f) Wall. ex Nees commonly known in Thailand as "fah-talai-joan" is a plant in the Acanthaceae family. The plant has long been used for the treatment of inflammation, diarrhoea, fever and as a bitter tonic.[1,2] Analysis of the plant has revealed that the aerial part of *Andrographis paniculata* contains the highest concentration of lactone compound, andrographolide (A_1), neoandrographolide (A_2) and 14-deoxy 11, 12 didehydroandrographolide or dehydroandrographolide (A_3), which are the principle chemical constituents of the plant[3] (Figure 1).

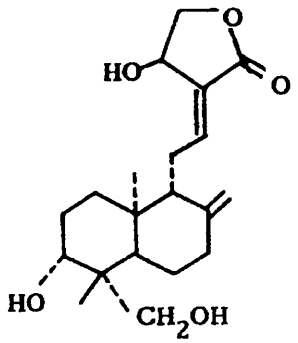
It has long been reported that this plant demonstrates antibacterial properties.[4-6] Pharmacological studies[7] showed that an 85 per cent ethanolic extract of this herb exhibited antipyretic activity when given orally to rabbits previously treated with typhoid vaccine extract dose = 2.5 gm per kg of body weight. In addition, while a water extract given intraperitoneally showed anti-inflammatory activity in a carageenan-induced rat hind-paw oedema model, the 85 per cent ethanolic extract produced an antispasmodic effect in isolated guinea-pig ileum.[8] Furthermore, a crude alcoholic extract (dose 500 mg per kg body weight could inhibit leukocyte migration by using cotton pellet implantation equal to prednisolone at 5 mg per kg and ibuprofen at 10 mg per kg.[9] A recent report[10] suggested that andrographolide or dehydroandrographolide may be responsible for the antispasmodic activity of this extract. Moreover, andrographolide has been found to provide the excellent antisecretory activity against all types of *E. coli* enterotoxins and was as good as loperamide at 1 mg dosage.[10]

The plant fah-talai-joan demonstrated no acute toxic effect in mice (LD_{50}=15g per kg) and produced no abnormalities in body growth, behavior, haematology, blood chemistry or histology of internal organs in rats.[11] Owing to a lack of apparent toxicity and excellent history of safety, fah-talai-joan was developed in the form of capsules for clinical trails. With regard to quality control of fah-talai-joan the active constitutent were reported as total lactones not less than 65 per cent.[12] In Thailand, total lactones of fah-talai-joan were reported in the range of 3.91-10.29 per cent.[13]

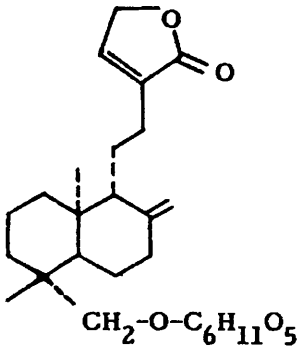
Due to the fact that the active constituents of fah-talai-joan have not been fully characterized, an analytical method to detect all three principal components of fah-talai-joan in the same sample should be sensitive, specific as well as highly reliable, HPLC is widely considered to be the technique of choice of biomedical research aimed at detecting small amounts of a drug. In this study, we have developed an HPLC technique to assay the principal constituents of fah-talai-joan in biological afluids, which enables the monitoring of the drug level in plasma. Hence, pharmacokinetic parameters of these major constituents in both *in situ* and *in vivo* models can be determined.

Experimental

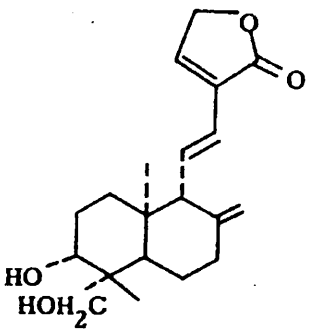
Instrumental: The HPLC system used consisted of a Waters 820 Chromatography Data Station, a Waters 510 HPLC Pump, a waters 590 Solvent Delivery System, a Waters 710 WISP™ System Processor and a Waters 490 Programmable Multiwavelength Detector. System development was carried out on a 39 × 150 mm μBONDAPAK CN (10 μm), a 39 × 150 mm μBONDAPAK phenyl (10 μm), a 39 × 150 mm NOVAPAK C_{18} (5 μm) and a 39 × 250 mm Li-



Andrographolide [A_1]



Neoandrographolide [A_2]



Dehydroandrographolide [A_3]

Figure 1. The structure of andrographolide (A_1), neoandrographolide (A_2) and dehydroandrographolide (A_3).

chrosorb R_8 (10 μm) as the analytical column and Upright HPLC precolumn (Upchurch Scientific) packed with Spherisorb 10 ODS_1 (Phenomenex).

Chemicals and Reagents: 85 per cent ethanolic extracts of fah-talai-joan, andrographolide (A_1), neoandrographolide (A_2), and dehydroandrographolide (A_3) were kindly provided by the Phytochemical Section, Division of Medicinal Plant Research and Development, Department of Medical Sciences, HPLC grade acetronitrile, ethanol and methanol were obtained commercially from Carlo Erba. Purified water was obtained from a Pureline system (Hitec, Yamato). All other chemicals were of analytical grade.

HPLC conditions: The HPLC system was operated at ambient temperature with detection at the 205 nm wave-length. The mobile phase consisted of acetonitrile/water/ethanol (24/56/20, v/v), which was filtered through a Nylon-66 membrane (0.45 μm pore size, Rainin) Pripor to use. The flow rate was 1.0 ml per min.

Drug: Fah-talai-joan emulsion was prepared from 85 per cent ethanolic extract using 2 per cent tween 80 and 4 per cent span80 as the emulsifying agents. Andrographolide emulsion was prepared in 0.67 per cent methycellulose.

Drug transfer from Rat Intestine

In situ study: drug absorption was investigated by the *in situ* intestinal loop technique of Duluisio et al. with slight modification.[14] Seven millilitres of fah-talai-joan emulsion was instilled into the lumen of the small intestine of rats. Samples of 0.2 ml from the intestinal loop were taken at 0, 2, 5, 10, 20 and 30 minutes for the analysis of A_1, A_2 and A_3. The volume of drug solution in the intestinal loop was kept constant by adding 0.9 per cent sodium chloride before the removal of each sample. The percentage of drug remaining in the lumen of the intestinal segment was determined as a function of time for a period of 30 minutes.

In vivo study: a group of eight rats (1 control and 7 drug treated) was used to obtain one set of data. Fifteen groups of eight rats each were

used in this study, hence 15 sets of data were obtained. The emulsions of fah-talai-joan and andrographolide were orally administered to the rats using an intubation tube at doses o¥ 500 mg per kg and 16 mg per kg, The animals were anesthetized with urethane at 1.25 gm per kg intraperitoneally before blood sampling. Blood was drawn from the controls prior to dosing and from the drug treated rats 30, 45, 60, 90, 120, 240 and 360 minutes post-administration, one rat at each time interval, by heart puncture. The plasma was separated and stored at -20°C pending analysis.

Sample preparation

In situ study: A1, A2 and A3 in the intestinal fluid were filtered through a C18 cartridge (MilliporeR) prior to processing as follows:

- Pipette 200 µl of intesitnal into a Sep-pak C_{18} cartridge;
- Elute by 5 ml HPLC mobile phase into a pear-shaped flask;
- Evaporate to dryness under reduced pressure; and
- Redissolve with 200 µl of HPLC mobile phase and transfer to WISP™ sample processor vials.

In vivo study: A_1, A_2 and A_3 in the plasma were determined by HPLC as follows:

- Add 500 µl plasma sample to 700 µl extractant (mobile phase: acetonitrile, 2:5, v/v);
- Vortex 2 minutes, centrifuge at 4,000 rpm for 5 minutes
- Remove the aqueous phase and evaporate under reduced pressure; and
- Redissolve with 200 µl of mobile phase and inject 500 µl into the HPLC.

Standard curve: Serum containing 1.5, 3.0, 15, 30 and 150 µg per ml of A_1, A_2 and A_3 were prepares. The serum standards were prepared and chromatographed using the same employed for the *in vivo* study. Standard curves were constructed from peak areas and amount of A_1, A_2 and A_3.

Recovery experiments: Analytical recovery was determined by preparing a standard solution of lactones (A_1, A_2 and A_3) at concentrations of 1.5, 3.0, 15 and 30 µg per ml in blank plasma. These spiked plasma standard solutions were then chromatographed. The peak area of lactones were then used to calculate the "measured" concentration of each lactone precent by comparing the peak area to previously prepared calibration curves. The percentage recovered was then calculated as follows:

$$\text{percent Recovery} = \frac{\text{measured concentration of lactone} \times 100}{\text{theoretical (spiked) concentration oflactone}}$$

Data analyses

Pharmacokinetic analysis and individual plasma andrographolide profiles from each treatment were analyzed according to one-compartment open model with first-order absorption and elimination using the PCNONLIN non-linear estimation program. Data were expressed as means ± standard error deviation mean and analyzed using the statistical computerized programme SPS.

RESULTS AND DISCUSSION

The major components A_1, A_2 and A_3 were detected at wavelength 205, 205 and 250 nm,[12] respectively. The mobile phase used in this HPLC system was limited by the absorption of A_1, A_2 and A_3 in the low UV region. Acetonitrile/water was chosen as the mobile phase owing to its UV-cut-off at 190 nm. When NOVAPAK C_{18} column was used at a flow rate of 1 ml per min. With UV detection at 205 nm, the initial condition of the mobile phase (30 per cent acetonitrile (v/v) was found to be insufficient, since only one peak, which belonged to A1, was observed. Increasing the mobile phase flow rate as well as the percentage of acetonitrile did not improve the elution of the other major components.

An attempt was made to improve the elution of the major component by using µBONDAPAK phenyl, µBONDAPAK CN and a Lichrosorb C_8 column instead of NOVAPAK C_{18}, It was found that when using the Lichrosorb R_8

column eluted with 30 per cent acetonitrile, all three major components, A_1, A_2 and A_3, could be eluted with an impractically long retention time broad peaks were obtained.

Lactones dissolved better in ethanol than methanol; hence, ethanol was the solvent of choice for improving chromatography. When we varied the composition of ethanol from 0 per cent, ETOH with 30 per cent ACN to 20 per cent ETOH, with 24 per ACN, while keeping H_2O at 56 per cent, the total run-time decreased to less than 20 mins. However, sensitivity also decreased because of the UV-cut-off at 225 nm. A typical chromatogram obtained from a sample of the active components is shown in 2 (a). A_1, A_2 and A_3 were separated within 18 minutes, with retention times (RT) of 6, 10.5 and 12.5 minutes, respectively.

Table 1 shows the percentage recovery of the three active constituents of fah-talai-joan with various extractants. Acetonotrile was found to be a good extractant compared with the others. The recovery valuves of A_1, A_2 and A_3 in serum at various concentrations are shown in Table 2. The mean recovery for the three lactones ranged from 78.3 to 91.3 per cent, with no apperent concentration-related effects. A linear relationship (with r = 0.999) was observed for all three active constituents over the range study (range 0.5-3.75 μg of A_1 and A_2, 0.5-8 μg of A_3). The detection limits for A_1 and A_2 were found to be below 100 ng and 300 ng for A_3, respectively.

Table 1. Per cent recovery of there lactones with various extractants.

Extractants	Per cent Recovery (Mean ± S.D)		
	A_1	A_2	A_3
Acetonitrile	92.43	80.16	85.20
	(4.3)	(5.8)	(4.8)
$ZnSO_4$/Ethanol	51.89	31.94	39.64
	(5.1)	(4.3)	(4.8)
Trichloroacetic acid	53.41	32.18	40.37
	(3.6)	(3.0)	(2.9)

This procedure was used in the pharmacokinetic study of fah-talai-joan. Figures 2b, 2c and 2d illustrate the cleaned chromatogram of 85 per cent ethanol fah-talai-joan extract in intestinal juice, in blank plasma and in spiked plasma, respectively. Pharmacokinetic studies of A_1, A_2 and A_3 in an *in situ* model are presented in the transfer rate constant of the three active constituents shown in Figure 3, as a plot of the percentage of A_1, A_2 and A_3 remaining in the intestine against time. The apparent first order transfer rate constant was determined for individual rats from the slope of the line of semilogarithymic plots of percentage of A_1, A_2 and A_3 remaining in the intestine versus time. The mean transfer rate constants of A_1, A_2 and A_3 were 0.0254, 0.0129 and 0.0499 $min.^{-1}$ corresponding to a half-life of 29.02, 55.24 and 14.17 min, respectively, as shown in Table 3. It was found that about 55.5 per cent of A_1, 25.5 per cent of A_2, and 77.5 per cent of A_3 had been absorbed in 30 minutes.

In vivo study: after oral administration of 85 per cent ethanolic extract to rats at a dose of 500 mg per kg weight, it was found that only andrographolide was detected in the plasma. Unfortunately, we could not prepare an emulsion of 85 per cent ethanol extract for oral administra-

Table 2. Assay Recovery of A1, A2, and A3 from plasma

Lactones Concentration (μg/ml)	Per cent Recovery (Mean ± S.D.)		
	A_1	A_2	A_3
1.0	104.11	80.24	87.32
	(9.3)	(8.4)	(8.7)
5.0	90.22	84.47	85.16
	(9.4)	(7.7)	(8.4)
10.0	91.30	78.32	82.63
	(7.3)	(7.3)	(6.9)
20.0	89.70	80.11	83.33
	(3.6)	(6.8)	(5.1)
50.0	90.10	78.22	84.76
	(3.8)	(4.2)	(3.2)

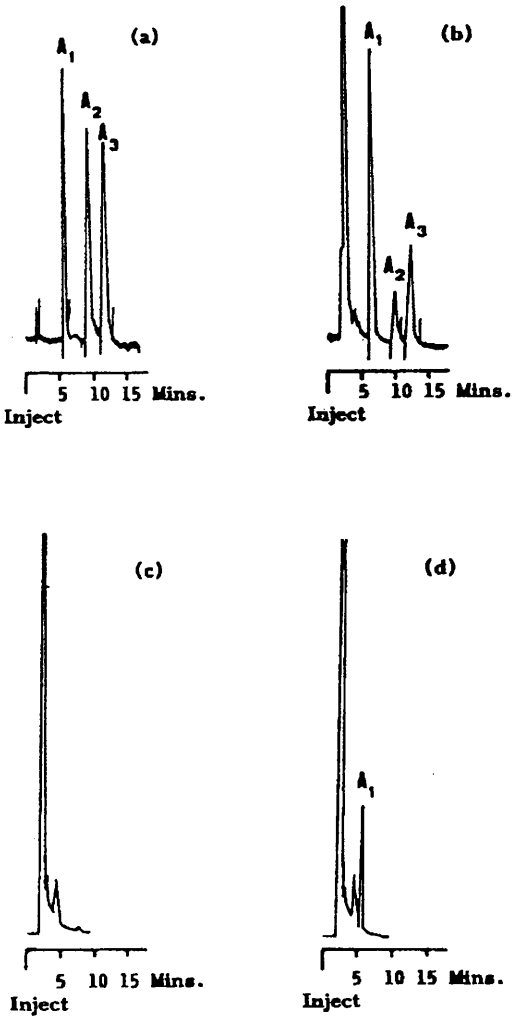


Figure 2. Typical chromatographms of
a) standard andrographolide (A_1), neoandrographolide (A_2) and dehydroandrographolide (A_3) and dehydroandrographolide (A_3)
b) extract of intestinal fluid spiked with 85 percent ethanolic extract of fah-talai-joan
c) extract of blank serum
d) serum spiked with andrographolide.

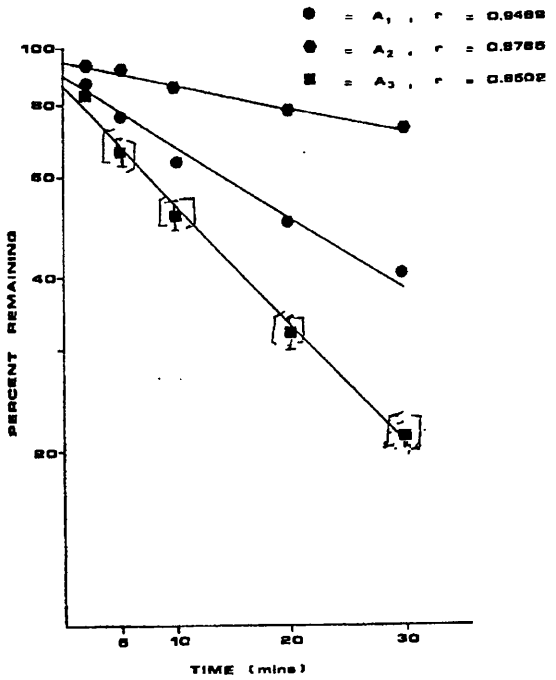


Figure 3. Percentage of andrographolide (A_1), neoandrographolide (A_2) and dehydroandrographolide (A_3) remaining in the intestinal as a function of time (each point represents the average results of 15 rats).

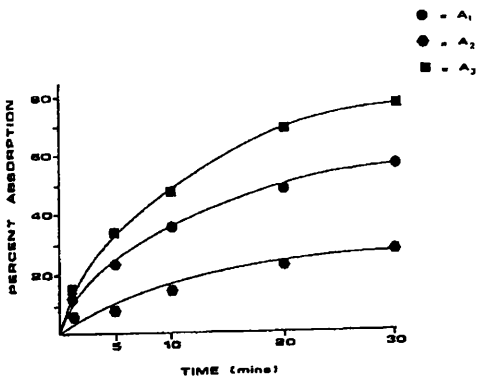


Figure 4. Cumulative percentage of andrographolide (A_1), neoandrographolide (A_2) and dehydroandrographolide (A_3) absorption a function of time (each point represents the average result of 15 rats).

tion at a dose greater than 800 mg per kg. A_1 is an interesting substance for its excellent antidiarrhoeal activity.[10] Therefore, we used a pure compound of A_1 for the *in vivo* study. Andro-

Table 3. Transfer rate constant and half-life of andrographolide (A_1), neoandrographolide (A_2) and dehydroandrographolide (A_3) from intestinal loop of anesthetized rats after fah-talai-joan emulsion was instilled into the cannulated segment of small intestine.

	Pharmacokinetic Value (Mean* ± SEM)		
	A_1	A_2	A_3
Transfer Rate Constant (mm^{-1})	0.0254 ± 0.002	0.0129 ± 0.001	0.0499 ± 0.002
Half-life of Transfer (min)	29.02 ± 1.80	55.24 ± 4.16	14.17 ± 0.56

$n = 15$

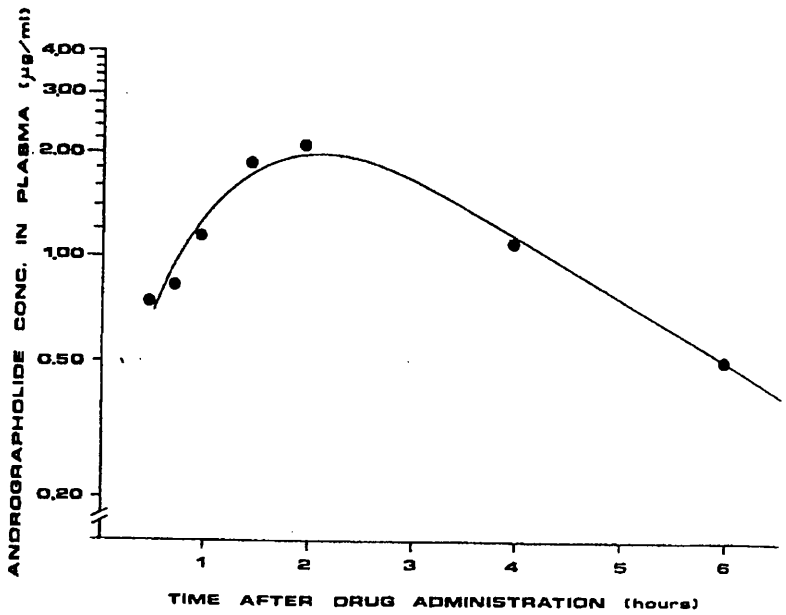


Figure 5. Semilogarithmic plot of average plasma concentration of andrographolide in rats versus time after oral administration of emulsion at a dose of 16 mg per kg.

Table 4. Summary of pharmacokinetic parameters of Andrographolide in rats after of oral administration at dose of 16 mg/kg

Pharmacokinetic Parameters	Mean* ± SEM
Absorption Rate Constant (Ka), min^{-1}	0.0078 ± 0.0004
Half-life of Absorption ($T_{1/2}$, a), h	1.5257 ± 0.1085
Elimination Rate Constant (Ke), h^{-1}	0.5157 ± 0.0172
Elimination Half-life ($T_{1/2}$, e), h	1.34 ± 0.05
Time to Maximum Concentration (T_{max}), h	2.04 ± 0.07
Maximum Concentration (C_{max}), $\mu g.ml^{-1}$	1.76 ± 0.19
Area under Curve (AUC), $\mu g.\ h.ml^{-1}$	9.66 ± 0.88

$n = 15$

grapholide at a dose of 66.7 mg per kg body weight was orally administered to rats. The semilogarithmic plot of concentrations of A_1 in the plasma as a function of time are shown in figure 5. The data points are the average of n=15 groups of eight rats each. By analyzing individual groups of data, using a one-compartment open model, pharmacokinetic parameters were obtained. Mean values of the absorption rate constant, elimination rate constant and elimination half-life were found to be 0.0078 min^{-1}, 0.5157 h^{-7} and 1.34 h^{-1}, respectively. At 2.04 hours after drug administration, the maximum plasma level of 1.76 μg per ml was observed. The area under the curve was calculated to be 9.66 $\mu g.h.ml.^{-1}$ All these parameter are summarized in Table 4.

CONCLUSION

The newly developed HPLC method provided good separation, with high selectivity and sensitivity, of andrographolide, neoandrographolide and dehydroandrographolide in biological fluids without interference from impurities. The detection limits of A_1, A_2 and A_3 were as low as microgram levels; the recover of extraction were found to be more than 87 per cent. The HPLC chromatogram showed that A_1, A_2 was A_3 were completely separated with retention time values of 6.7, 10.2 and 13.5 minutes, respectively. This procedure were be beneficial for future clinical studies of andrographolides.

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