PHARMACOKINETIC STUDY OF *ANDROHRAPHIS PANICULATA* CHLOROFORM EXTRACT IN RATS

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Summary

The aim of the present study is to develop an automated blood sampling method to evaluate the oral bioavailability of *Andrographis paniculata* chloroform extract (APCE) in conscious freely moving rat. The blood samples were collected from tail vein after APCE was administered orally at a single dose of 1 g/kg. Acetonitrile was used to extract the AGN and DDA from plasma samples after centrifugation 3000 rpm for 10 min at 5 ºC. A 20 µl aliquot of supernant was passed through a membrane filter before injection into an HPLC/UV system. The analytes were separated by Nucleosil C18 (250 × 4.6 mm i.d., particle size 5µm; Phenomenex) at ambient temperature and eluted with methanol: water (65: 35 v/v) pH 2.8 adjusted H_3PO_4) with a flow rate of 1.0 mL/min. The UV detector was set at 210 nm. The limit of detection of DA and DDA was 10 ng/mL in rat plasma. The concentration–response relationship from the present method indicated linearity over a concentration range of 10 to 30 000 ng/mL. Intra- and inter-assay precision and accuracy of DA and DDA in the biological fluids fell well within the predefined limits of acceptability (<15%). Biological fluids were thereby sampled following a dose of evodiamine (1 g/kg, p.o.). The oral bioavailability was estimated to be about 0.1% in the conscious rat system.

Keywords: *Andrographis paniculata*, Andrographolide, 14-deoxy-11, 12-didehydroandrographolide, Bioavailability.

Introduction

Andrographis paniculata (AP) Nees (Acanthaceae) is a medicinal plant commonly used in India, China and Southeast Asia for the treatment of a large variety of illness, which include meningitis, acute hepatitis, anti-inflammatory, antimicrobial and anticancer and against HIV infections. (1-5). The main objective was to develop and determine the oral bioavailability of ANG and 14-deoxy - 11, 12-didehydroandrographolide (DDA) from *Andrographis paniculata* chloroform extract (APCE) in rat plasma.

Several methods have been reported on the determination of ANG and 14-deoxyandrographolide in AP, including high-performance liquid chromatography (HPLC) coupled with on-line solid phase extraction (SPE) and ultraviolet (UV) detection was developed for determining ANG and dehydroandrographolide in rabbit plasma (6). Plasma samples (100 μL) were injected directly into a C18 SPE column and the biological matrix was washed out for six minutes using 15% aqueous methanol. By rotation of the switching valve, ANG and dehydroandrographolide were eluted in the back-flush mode and transferred to the analytical column by the chromatographic mobile phase consisted of methanol: acetonitrile (ACN): water (50: 10: 40; v/v). The UV detection was performed at 225 nm. Besides (7) also studied oral bioavailability of Kan Jang tablets (fixed combination of extracts from AP and *Eleutherococcus senticosus*) of warfarin rats did not produce significant effects on the pharmacokinetics of warfarin and practically no effect on its pharmacodynamics.

However the determination of ANG in biological matrix, especially in human plasma, is necessary for clinical pharmacokinetics. In this respect, the method of HPLC, GC-MS, and capillary electrophoresis has been reported by Panossian et al., (8). AP extract containing ANG and 14-deoxy-11, 12-didehydroandrographolide (DDA) were analyzed to determine their pharmacokinetics characteristics in rat plasma. The absorption of ANG and DDA was slow (T_{max} 3 hr) their C_{max} indicates good absorption. In this study we developed a simpler and slightly modified HPLC method (9) for determination of ANG and DDA in rat plasma.

Materials and methods

The fresh green aerial parts of the plant AP were collected from the cultivated nurseries of Malaysian Agriculture Development Institute, Kelantan, Malaysia. The leaves were dried at room temperature under shade for five days and milled into coarse powder using milling machine and subsequently subjected to the extraction by means of soxhlet apparatus (France).

Preparation of plant extracts:

The dried blended powder (895 gm) was first extracted with petroleum ether (60-80 °C) for a period of 48 h after which the powder was dried and successive extraction of the marc was repeated with chloroform and methanol for a period of 48 h respectively. All the three extracts were evaporated to dryness using Büchi Rotavapor (Switzerland) and ultimately dried in an oven. The following quantities, calculated as percentage yield, were obtained: 7.1 % (w/w) of petroleum ether extract, 9.66 % (w/w) of chloroform dried extract (APCE) and finally 11 % (w/w) methanol extract.

Chemicals and drugs:
Andrographolide was purchased from Sigma Chemicals Company. Methanol, Acetonitrile and water (HPLC grade) was acidified to pH 2.8 with phosphoric acid, obtained from Merck (Germany). All other solvents were analytical grade or HPLC grade.

Instruments
HPLC-UV analysis was performed on a Shimadzu LC-10AT (Shimadzu Corporation, Kyoto, Japan) system with a LC-6A solvent delivery pump equipped with a SPD-10A UV/VIS detector. Data were acquired and processed by Class-VP Chromato software. The analytical column used was Nucleosil C18 (250 x 4.6 mm i.d., 5µm; Phenomenex) along with a guard column C18 (10x 4.0 mm i.d; Phenomenex) used at ambient temperature for the elution of analyte.

Experimental animals:
Male Sprague-Dawley (SD), rats are obtained from Universiti Sains Malaysia (USM), animal house holding unit. All the experiments were performed according to the guidelines issued by the USM animal ethics committee. Age of animals about 10-12 weeks weighed between 260-310 g, was maintained under controlled conditions provided with normal water *ad libitum*.

Dosing of *Andrographis paniculata* chloroform extract (APCE):
The APCE first suspended in 10% v/v Tween 80 and made up to the required volume with distilled water. Food and water were withheld 12 hours before administration of the extract to rats. The dose was administered orally with a single dose of 1 g/kg and control rats were given with 10% v/v Tween 80 in distilled water.

Sampling of blood:
Six overnight fasted SD rats were administered the suspension of the extract orally. Approximately 0.5 to 0.7 mL of blood samples were collected from the tail vein (10) using a disposable syringe at 0 min (predose) 30, 60, 120, 240, 480, 720, 960 min, and 24 hrs after dosing. The rats were restrained in a rat holder during blood sampling. The initial blood sample was taken by clipping the end of the rat's tail while the subsequent blood samples were collected by removing the clotted blood with cotton rinsed with 100 IU of heparin solution. After each blood sampling, the wound was monitored for approximately 3 min to ensure there was no excessive bleeding. Blood samples were immediately transferred to a heparinized micro centrifuge tube and centrifuged at 3000 rpm for 10 min at 5 ºC. The resulting plasma samples (0.2 mL supernant) was transferred into 1.5 mL eppondroff tubes and stored at –80 ºC until HPLC analysis.

Chromatographic condition and sample preparation:
Plasma (0.2ml) was added to 0.3 mL of acetonitrile and centrifuged at 3000 rpm for 10 min at 5 ºC. The supernatant was passed through membrane filter (pore size 0.5 µm) prior to HPLC analysis. The chromatographic conditions used were Nucleosil C18 (250 x 4.6 mm i.d., 5µm; Phenomenex) along with a Guard Column C18 (10x 4.0mm i.d; Phenomenex) used at ambient temperature for the elution of analyte. The mobile phase consisting of methanol: water (65: 35, v/v) (pH = 2.8) was prepared and filtered through 0.45 µm nylon-membrane filter (Millipore Corporation, MA, USA) under vacuum before use. Methanol (JT Baker Co, USA) used as mobile phase was of HPLC-grade. The analysis was run at a flow rate of 1 mL/min. The UV detection was set at a wavelength of 210 nm.

Validation of HPLC method

Linearity:
The linearity of the responses was determined for seven concentrations (in plasma) by three injections. The contents of the reference standards were calculated using regression parameters obtained from the standard curve for blank plasma samples respectively. The limit of quantification (LOQ) was established at signal to noise ratio (S/N) of 10.

Precision and accuracy:
Intra- and inter-day precision of the assay were determined five times on the same day and continuously for 3 days at the QCs of the reference standards. Accuracy was determined by recovery studies. Recoveries of marker from plasma were estimated by comparing the area obtained from injections of standard solutions to those of standards in acetonitrile.

Calculation of other kinetic parameters:
The elimination rate constant K_{el} (hr^{-1}) were calculated by log/linear regression using terminal phase of the plasma concentration time plot. The maximum plasma concentration C_{max} (µg/mL) was taken directly from the data; time of maximum plasma concentration time curve T_{max} (h) time to achieve C_{max}; was also obtained directly from the data. Total area under plasma concentration time curve ($AUC_{0-\infty}$) was calculated by adding the total area under the curve from the time zero to the last measurable concentration (AUC_{0-t}), to the area from the last area from the last measurable measurable concentration to time infinity ($AUC_{0-\infty}$). (AUC_{0-t}) was calculated using the trapezoidal formula. The half-life, $T_{1/2}$, (h) was calculated by dividing the elimination rate constatant. ($T_{1/2} = 0.693/K_{el}$).

Statistical Analysis
Data were expressed as the mean and S.D. for each group.

Results and Discussion

Development and validation of HPLC method for determination of ANG and DDA in rat plasma

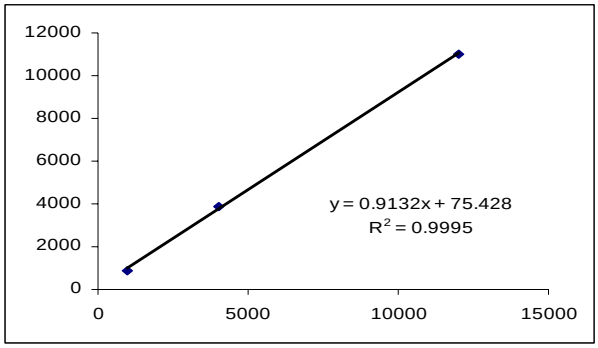
The ANG and DDA content of the freeze dried APCE determined prior to oral administration to the animals were 4.68 and 3.02% respectively. The rat plasma spiked with ANG, DDA show similar results. Typical chromatograms resulting from HPLC analysis of the acetonitrile (ACN) precipitate rat plasma is presented in Fig. 7. Blank plasma did not showed any interference peaks.

The linear regression analysis of ANG and DDA in plasma shown in the (Table 1). The correlation coefficient was more than 0.999 in standard curves (Fig.1 and 2). The calibration curves of ANG and DDA were linear over the concentration range of 10 to 30 ng/mL. Linearity was evaluated by determining six working concentrations in triplicate (Fig.1 and 2). The results showed that there was an excellent correlation between peak area and concentration of ANG and DDA in the concentration range tested. The limit of quantification of ANG and DDA determined in blank rat plasma is shown in Table 1. The LOQ of ANG and DDA 15 and 16 ng in plasma respectively.

The three different concentrations, low (50 ng/mL), medium (500 ng/mL) and high (25 000 ng/mL) of ANG and DDA recoveries were between 91.10 to 96.03 % and 91.42 to 94.81 % respectively. The results of intra-day and inter-day (n = 6) variations of ANG and DDA are shown in table 3. Acceptable precision was achieved with the method as revealed by the R.S.D. data, which did not exceed 10%. The compounds were stable at -20 and 4 ºC in plasma. No peaks corresponding to degradation products were observed. Though there was a decrease in peak areas at the third day but the change was not significant from the initial value when plasma and ACN precipitated plasma were kept at room temperature.

Table.1. Regression equations, correlation coefficient and linearity range of ANG and DDA in rat plasma.

Analyte	Plasma linear range (ng/mL)	Linear equations	r^2	LOQ	tR^a
ANG	10-30 000	Y= 0.91328x + 75.428	0.999	15	4.55
DDA	10-30 000	Y= 0.9127x + 75.490	0.999	16	7.61



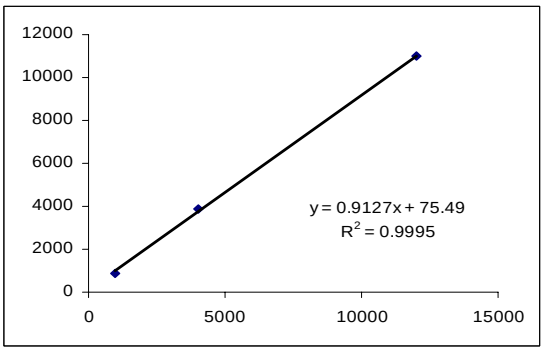


Figure. 1 & 2. Plasma calibration curve of andrographolide (ANG) and 14-deoxy-11, 12-didehydroandrographolide (DDA)

Table. 2. Recovery (n=3) of the ANG and DDA in rat plasma

Plasma	Amount added (ng/mL)	Amount measured (ng/mL)	Recovery (%)	RSD (%)
ANG	50	45.55	91.10	5.75
	500	481.66	96.33	1.05
	25 000	24009.66	96.03	3.53
DDA	50	45.71	91.42	6.32
	500	482.50	96.50	1.24
	25 000	23 704.33	94.81	5.90

Table. 3. Precision (n=6) of the ANG and DDA in rat plasma

Plasma	Amount added (ng/mL)	Intraday amount found (ng/mL)	RSD (%)	Interday Amount found (ng/mL)	RSD (%)
ANG	50	46.37	7.72	45.76	10.40
	500	485.83	5.97	483.53	0.95
	25 000	24693.75	2.22	24489.00	2.53
DDA	50	46.69	6.72	45.76	8.27
	500	486.56	1.40	482.60	0.85
	25 000	24750.50	2.06	24877.50	2.20

Pharmacokinetic application

This analytical method was developed for the application in pharmacokinetic study of ANG and 14-deoxy-11, 12-didehydroandrographolide (DDA) in a freely moving rat. Fig: 3 and 4 illustrates the concentration versus time profile of ANG and DDA with single oral dose (1 g/kg) administration to six individual rats in each group. A rapid increase of the plasma concentration of ANG and DDA was observed from 30 min to 2 hr after oral administration of the APCE and then declined from the 3 h. Fig 3 and 4. The method described above was successfully applied to a preliminary PK study in which the plasma concentration of andrographide and DDA. This analytical method was developed for the application in pharmacokinetic study of APCE in a freely moving rat. Fig. 3 and 4 illustrates the concentration versus time profiles of APCE with a single oral dose administration to six individual rats. The area under the concentration versus time curves for ANG and DDA were (AUC_{0-t}) 444 µg/mL and 212 µg/mL $AUC_{0-\infty}$ 462 µg/mL, 220 µg/mL half life is 16.38, 23.89 hrs, C_{max} 33.2, 16.60 time required to reach peak plasma concentration is 1 h, elimination rate constant was 0.042, 0.029 clearance were 36.1, 75.9 (mL/min), and volume of distribution was 51, 157 liters respectively for (1g/kg) for APCE.

Table. 5. Concentration profile of ANG, DA in rat serum samples (n = 6)

Sampling time (h)	Mean±SD (µg/mL) ANG	Mean±SD (µg/mL) DDA
0	0	0
0.5	23.37 ± 7.36	12.61 ± 0.40
1	33.24 ± 2.69	16.56 ± 0.42
2	29.94 ± 3.43	14.15 ± 0.38
4	25.32 ± 5.96	12.54 ± 0.49
8	21.37 ± 2.71	10.12 ± 0.39
12	17.18 ± 3.58	8.50 ± 0.45
16	13.09 ± 1.38	6.54 ± 0.26
24	12.55 ± 3.42	4.88 ± 0.29

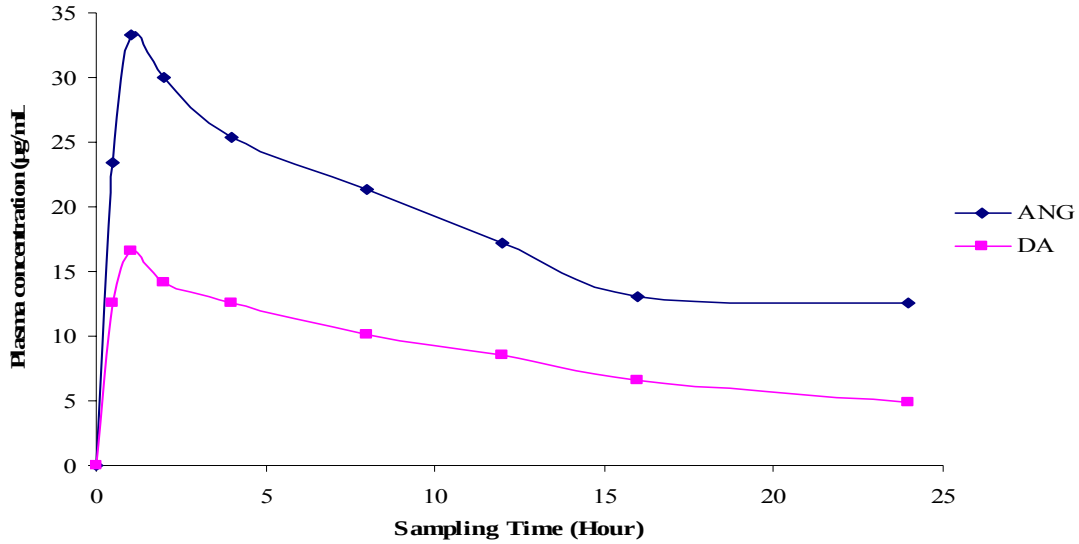


Figure .3 Plasma concentration ANG and DA versus time profiles determined after application of a single oral dose (1 g/kg) of *Andrographis paniculata* chloroform extract (APCE) in rats. Each point represents mean ± S.D. (n=6).

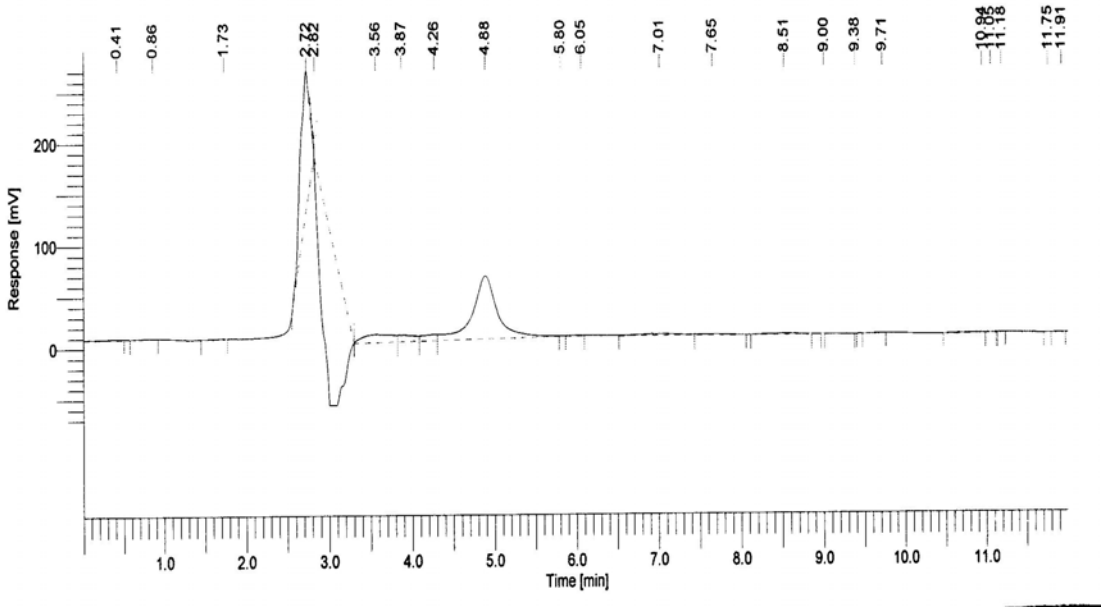


Figure. 4. HPLC chromatogram of rat plasma spiked with ANG

Table. 6. Pharmacokinetic parameters following oral administration of APCE extract single dose (1 g/kg).

Parameters	ANG	DDA
AUC $_{0-\infty}$	444	212
AUC $_{t-\infty}$	18.10	7
AUC $_{0-\infty}$	462	220
$t_{1/2}$	16.38	23.89
C_{max}	33.2	16.6
T_{max}	1	1
Ke	0.042	0.029
Clearance (mL/min)	36.1	75.9
Vd (L)	51	157
Extra %	3.92	3.21

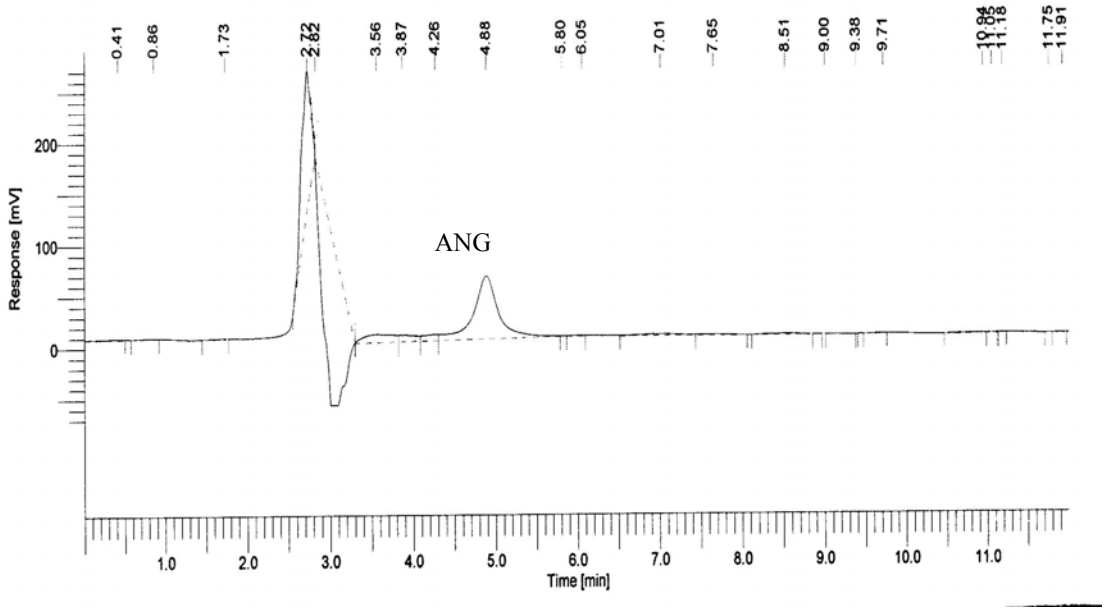
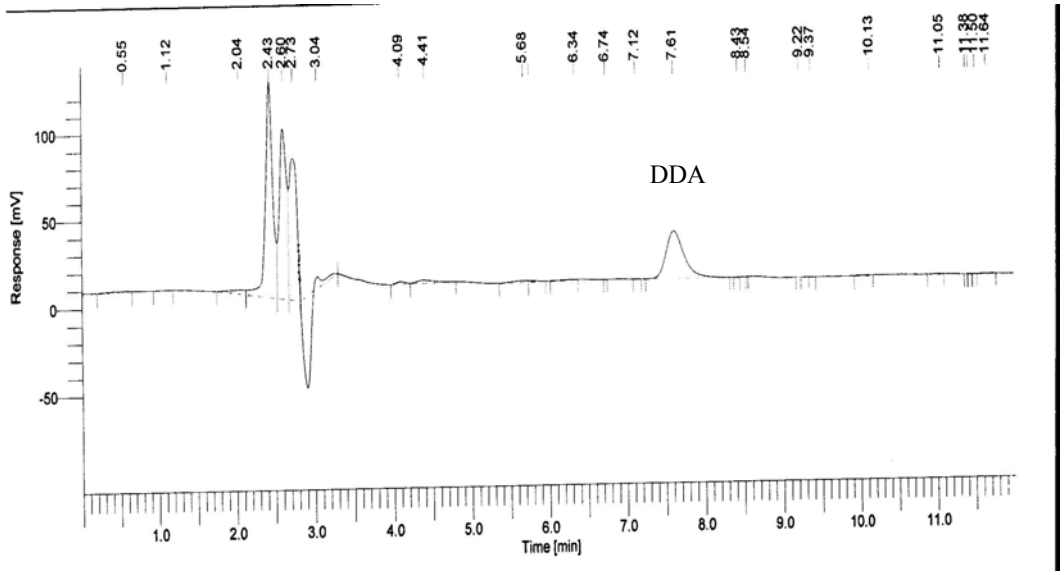
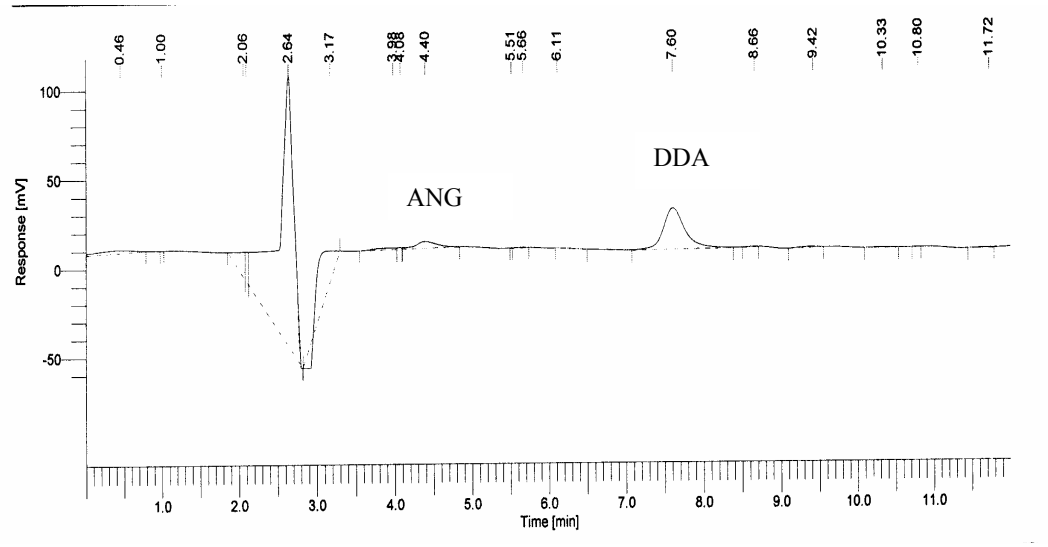


Figure. 5. HPLC chromatogram of rat plasma spiked with ANG



(a)



(b)

Figure.6a & 6b. HPLC chromatograms of DA and DDA spiked in rat plasma with, Rt = 7.61 min for DA; and, Rt = 7.60 min for DDA.

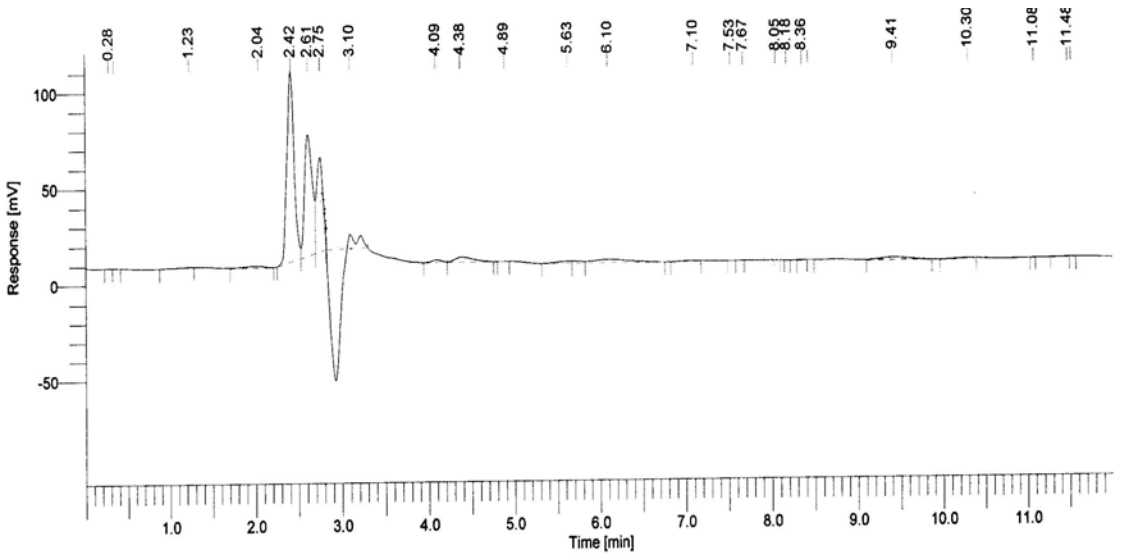


Figure.7. HPLC chromatograms of blank rat plasma

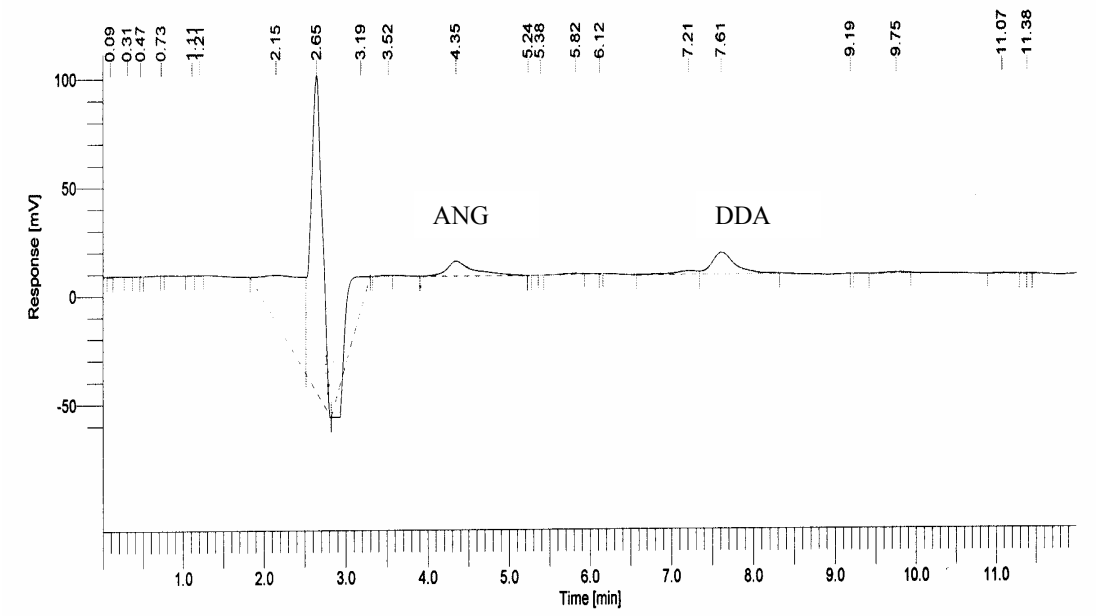


Figure. 8. HPLC chromatograms of APCE in rat plasma with ANG and dehydroandrographolide. 1, ANG, Rt = 4.35 min; 2, dehydroandrographolide, Rt = 7.61 min.

Conclusion

An HPLC bio analytical method for determination of ANG in APCE was developed and validated in rat plasma. The method was sensitive enough to determine the concentration of ANG and DDA on rat plasma. A comparatively good chromatogram obtained though a complex herb medicine plasma sample was injected. The method was validated for linearity, specificity, accuracy, precision, and recovery and good results were obtained.

The results of present study indicated that APCE showed non-linear pharmacokinetics in rats in the studied dose (1 g/kg). The mechanism is still ambiguous and further profound researches are required to elucidate some more compounds in complex herbal mixture. The pharmacokinetic results are useful for the further study of the clinical applications of the diterpene lactones from *Andrographis paniculata*.

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