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Developing of the methods of analysis of andrographolide

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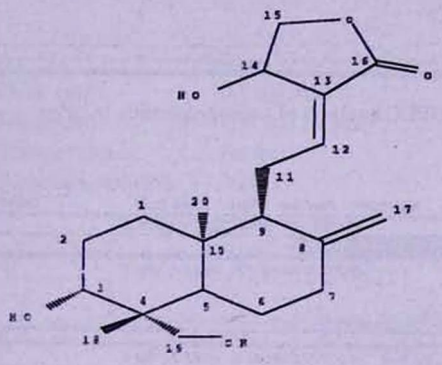

Fig. 1. Andrographolide

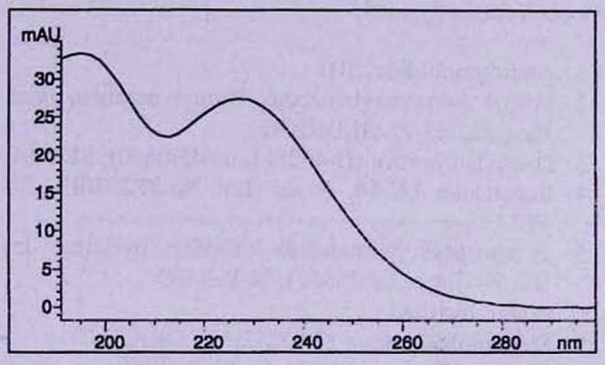

Fig. 2. UV-spectrum of andrographolide

Andrographolide (AND) is diterpene lactone (fig.1), an active component of *Andrographis paniculata* Nees., known as "alui" in China and "kalmegh" in India. Andrographis paniculata is used in Asia as a potent healer for a variety of diseases [1,3,4,13, 14]. It has been shown in several animal studies that extracts of Andrographis paniculata and its constituents, have anti-inflammatory [6,3], antiallergic [7], immunostimulatory [12] and antiviral activities [8]. In recent years the role of Andrographis paniculata in the management of the common cold [2,9] has received a lot of attention. Extract of Andrographis paniculata, standardized for its content of andrographolide and deoxyandrographolide and called "Kan Jang", has been used extensively in Scandinavia for the last 20 years in treating the common cold, acute uncomplicated sinusitis and upper respiratory diseases like the viral flu. Awarded "Product of the Year" by the Swedish Association of Health Food Producers, "Kan Jang" acts naturally and has been proven effective at reducing headache, fever, irritation, congestion, and fatigue quickly and without toxic side effects.

However, oral bioavailability and pharmacokinetic data of andrographolide have not yet been studied in either animals or humans. These data are extremely important not only for determining the right dosage regime, but also for eliminating the possibility of side effects due to overdosing. The exact measurement of plasma and urine levels of AND is an effective way to study pharmacokinetic data.

The amount of AND in plasma and urine is limited and the analysis of low concentration levels, and the complexity of the matrix in biological fluids demand efficient separation methods and instrumentation, giving selective and sensitive detection. There is only one publication on analysis of andrographolide in the blood plasma of rabbits after oral administration of the water extract of andrographis paniculata [15]. This method is based on oxidizing the hydroxyl group of andrographolide with hydrogen peroxide, coupling it with luminol – hydrogen peroxide – cobalt ion chemiluminescences reaction into an organic whole to realize the quantitative monitoring of andrographolide in blood.

This article details effective extraction procedures and the validated chromatographic conditions for the determination of AND in plasma and urine.

Every chromatographic method needs to be validated before first routine use. Awarded Good Laboratory Practice (GLP) method validation is the process for establishing if the performance characteristics of the analytical method are suitable for the intended application [11].

Materials and Methods

Pure andrographolide supplied by Swedish Herbal Institute was used as an analytical reference substance and analyzed for purity by HPLC, GC-MS, HPCE, 1H-NMR and

13C-NMR.

Quality grades and listed below reagents and materials have been currently used.

1. Andrographolide, SHI
2. Propyl 4-hydroxybenzoate, Propyl paraben, (Cat. No.: P5,335-7), ALDRICH
3. Diethylstilbestrol (D-4628, Lot 64H0690), SIGMA
4. Supelclean LC-18, 3 *ml* (Lot No.SP2030F), SU PELCO
5. N-trimethylsilylimidazole (TMSI): pyridine, 1:4 33159-U, Lot.LA76687), SUPELCO
6. Water, distilled
7. Methanol (Cat.No.: 17,933-7), ALDRICH
8. Acetone (Cat.No.:17,997-3), ALDRICH
9. Ethyl acetate (Art 6784.2), ROTH
10. Borate buffer 20 mM, pH 9.3 (Part No.:8500-6782), HEWLETT PACKARD

Results and Discussion

We analyzed the concentration level of AND in body fluids of rats using the BECKMAN HPLC GOLD system, which included the double pump module and UV-detector. The separation was run on LiChrospher100 RP-18 column with mobile phase consisting of methanol-water 60:40(v/v) mixture. Detector wavelength was 229 nm, at the maximum of UV-absorption of AND (fig. 2). The use of propyl 4-hydroxybenzoate as an internal standard (IS) corrects for extraction variability and allows accurate and precise quantitation of the AND. We used the chromatographic conditions listed below to obtain the chromatogram shown in the fig. 3 and 4. Retention times of AND and IS are respectively 3.58 and 6.94 min. Selectivity of the method is characterized by the relative retention time of AND to IS (3.58 / 6.94) which is equal to 0.515. Coefficient of variation (n=6) is less than 1%.

Linearity, extraction recovery, accuracy and intraday reproducibility studies were carried out. We evaluated the linearity between peak areas ratio of AND to IS and amounts ratio. In the range from 0.5 to 250 mg/ml of AND response was linear (r2 = 0.9991). Limit of detection was below 0.5 mg/ml of AND.

The recovery rate from urine was 90.8 %, from plasma -76 %. The main steps in the extraction of urine and plasma are illustrated in fig. 3 and 4 respectively.

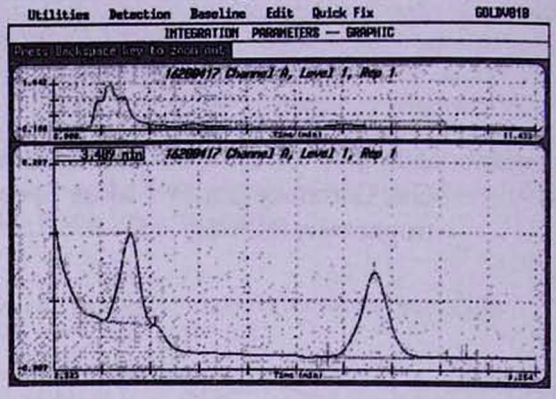

Fig. 3. HPLC analysis of andrographolide in urine

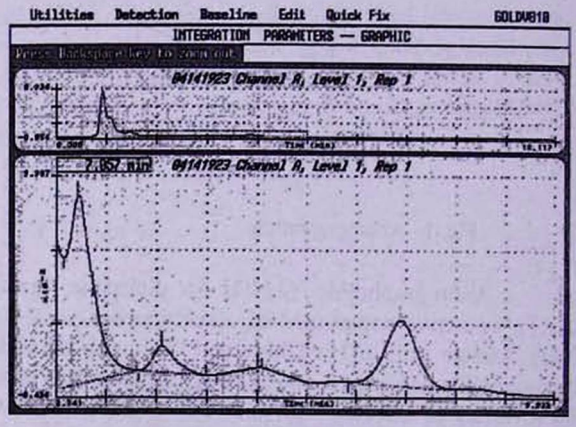

Fig. 4. HPLC analysis of andrographolide in plasma

Repeatability (intraday reproducibility) coefficient of variation was 1.16 % (n=5).

The precision of the method was established by injection of samples (n=5), which had been separately prepared under the conditions described in the method. The relative standard deviation (RSD) was 5.06 %.

The accuracy of the method was established by injection of samples obtained by addition of varying known amount of AND to urine and plasma. Overall accuracy was 102.02 %.

INSTRUMENTATION

BECKMAN HPLC GOLD system consisting of:

Detector:	BECKMAN UV-Detector Module 166
Pump:	BECKMAN Double pump Programmable Solvent Module 125
Injection Valve:	Rheodyne mod. 7725I with 20 ml loop.
Data Collection:	PS/1 Computer 486 DX-33 with management software supplied by Beckman; Epson FX - 800 printer.
Statistical analysis:	Prism software, version 2.0, GraphPad Software Inc.USA, 1996

Experimental conditions

Column	LiChroCART 125x4 *mm* HPLC cartridge with LiChrospher100 RP-18 (5*Mm*) (Merck, Darmstadt)
Mobile phase	Pump A-Water; Pump B-Methanol
Gradient	*Min.* — *%B*: 00 — 60; 00 – 10 — 60 – 60; 10 – 15 — 60 – 100; 15 – 20 — 100 – 100; 20 – 21 — 100 – 60; 21 – 25 — 60
Flow rate	0.7 *ml/min*
Detection	UV = 229 *nm*
Temperature	Ambient
Injection volume	20*Ml*

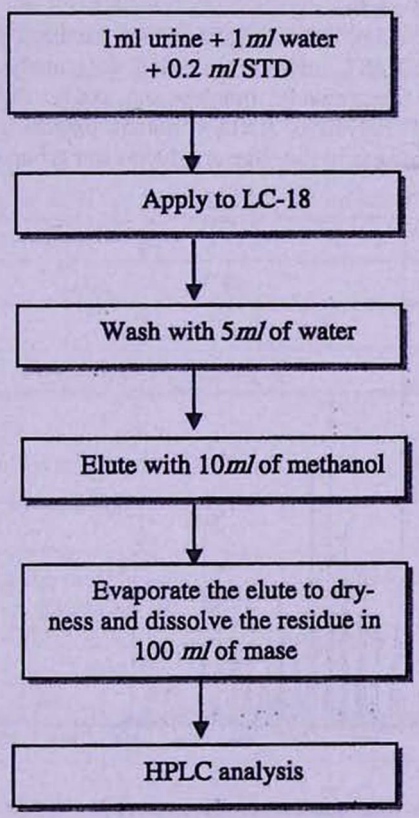

Fig. 5. Andrographolide sample preparation from urine for HPLC analysis

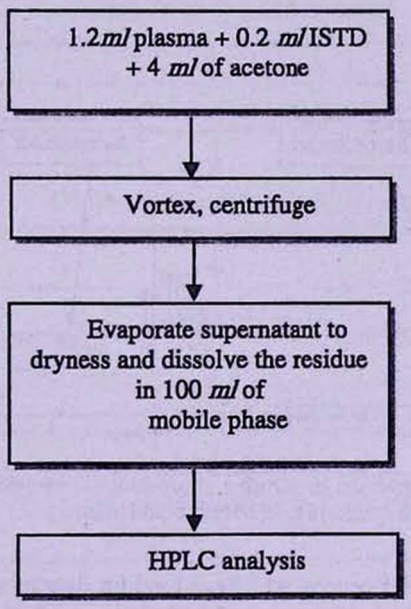

Fig. 6. Andrographolide sample preparation from plasma for HPLC analysis

Pre-assay preparation of urine samples

Urine was collected in collectors, and transferred into the measuring glass tubes. Then equal volume of distilled water and 0.2 *ml* of IS solution (propyl 4-hydroxybenzoate in methanol, 100 *Mg/ml*) was added to each sample. Samples were applied to Supelclean LC-18 tubes (pre-washed with 10 *ml* of methanol and 10 *ml* of water) followed by elution with 5 *ml* of water and 8 *ml* of methanol (separately). The methanolic solution was evaporated to dryness by a vacuum rotary evaporator, the residue dissolved in 100 *Ml* of HPLC mobile phase, centrifuged and subjected to HPLC.

Pre-assay preparation of blood samples

To 1.2±0.5 *ml* of blood plasma, 200 *Ml* of internal standard solution (propyl 4-hydroxybenzoate in methanol, 100 *Mg/ml*) and 4 *ml* of acetone were added, vortex, proteins precipitated at 4°C during 5 *min* and removed by centrifugation at 3000 *rpm* for 15 *min*. Supernatant was evaporated to dryness by vacuum evaporation and the residue dissolved in 100 *Ml* of HPLC mobile phase, centrifuged and subjected to HPLC.

Compared to HPLC GC/MSD system is highly sensitive and provides analyte specificity with the mass spectral information. The scan sensitivity specification for the

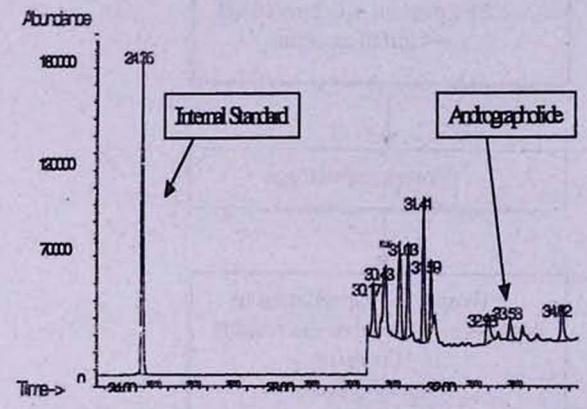

Fig. 7. Selective ion monitoring chromatogram showing the analysis of andrographolide in human blood plasma

HP GC/MSD system, which we used for determination of AND in human plasma is set with 1 picogram of octafluoronaphthalene. In selective ion monitoring mode the detection limit for AND is well below 50 *ng/ml*. We used a pulsed splitless injection, which transfers all of the sample to the column. By injecting larger samples lower concentration of AND can be detected successfully.

Data acquisition and analysis was performed in selected ion monitoring (SIM) mode in order to reduce sample matrix interferences. Appropriate ions are selected from full scan spectra. Several ions are selected for each compound. The ions were chosen very carefully in order to minimize sample matrix interferences; especially those from closely eluting targeted compounds. Dwell time was the same for all compounds to maintain a constant cycle time across a GC peak. Sample results were quantitated automatically at the end of a run. Quantitation was based on the intensity of target ions and confirmed by the detection of qualifying ions with known ratios to the target ion.

Andrographolide trimethylsilyl ester fragment ions with m/z 386 (M+-2 TMSOH), 296 (M+-3 TMSOH), 281 and diethylstilbestrol TMS derivative's (IS) fragment ions with m/z 412 (M+), 397, 383 were used for quantitation in SIM mode. The mass spectrum of AND is shown in fig. 8.

To construct the calibration curve, the following concentrations were used: 100, 250, 500, 750, 1000 *ng/ml* (AND) and 100 *ng/ml* (ST) ($r2 > 0.99$).

Six injections were used to calculate percent relative standard deviation. Overall accuracy and precision of the method are good: accuracy > 95%, precision < 10%. Retention times (Rt) of the peaks of AND and IS were 33.53 and 24.35 *min* correspondingly. Relative retention time of AND to IS (Rt AND / Rt IS) was 1.38. Coefficient of variation was 0.1%.

Andrographolide triacetates fragment ions m/z 133, 296, 356, and diethylstilbestrol dictates fragment ions with m/z 268, 310, 352 were also used for quantification in SIM mode.

By using HP's EasyID, Quick Levels Update, QEdit, and DOLIST software modules, data analysis was completed interactively, quickly and easily. Fig. 7 shows a typical analysis of AND in human plasma in SIM mode. The changes in baseline are due to ion group changes with time.

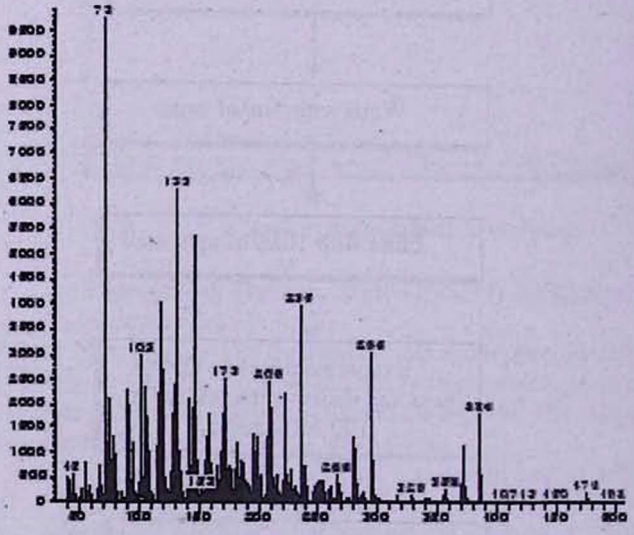

Fig. 8. Mass spectra of andrographolide – TMS ester

INSTRUMENTATION
HEWLETT PACKARD GC/MSD System:

Gas Chromatograph:	HP 6890 Plus Series
Injection Port:	Split/Splitless Inlet with EPC, Merlin Microseal TM, Single – taper liner
Sample Introduction:	HP 6890 series Automatic Liquid Sampler
Detector:	HP 5973 Mas Selective Detector
Data Collection:	ChemStation and HP Vectra VE with HP LaserJet 1100 Printer
Statistical analysis:	Prism software, version 2.0, GraphPad Software Inc.USA, 1996

Experimental conditions
Samples are analyzed according to the conditions mentioned below:

Column	HP-5MS Cross-linked 5% Phenyl Methyl Siloxane, 30.0*m* Length x 0.25*mm* Column ID x 0.25*um* Film thickness; max. temperature - 320°C (Part No. HP 19091S-433);
Carrier Gas	Helium, 44 *cm/sec*, 30*psi* at 160°C with EPC
Constant Flow	1.3*ml/min*
Oven	160°C(hold 10.0 *min*, linear gradient to 3000C at 5°C/*min*, hold 10.0 *min*.)
Injection	2*Ml*, Pulsed Splitless
Detector	Electron impact ionization, 70 *eV*, Selective Ion Monitoring mode, Maximum sensitivity autotune +300
Zones temperatures	Inlet - 230°C; Interface -250°C; Ion Source - 230°C;Quadrupole - 150°C.

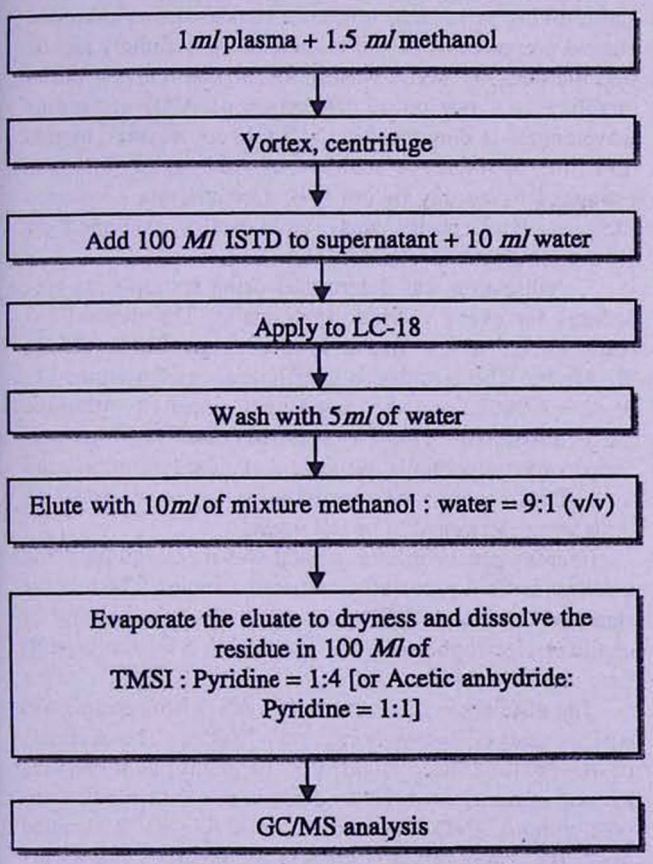

Fig. 9. Andrographolide samplepreparation from plasma for GC/MS analysis

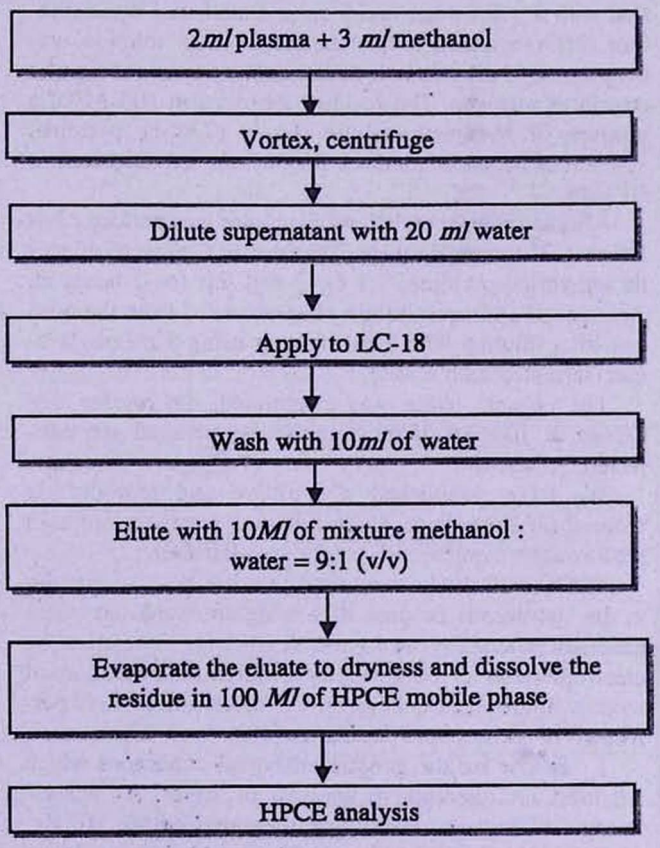

Fig. 10. Andrographolide sample preparation from plasma for HPCE analysis

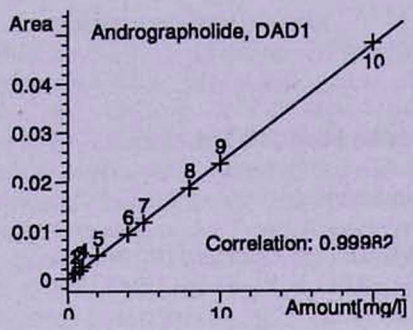

Fig. 11. Linearity of peak area ratio (Y) with amount of andrographolide (X)

To 1 *ml* of blood plasma, 1.5 *ml* of methanol was added, vortex and proteins precipitated at 4°C during 5 *min* by centrifugation at 3000 *rpm* for 15 *min*.100 *Ml* of internal standard solution (Diethylstilbestrol in methanol, 1.0 *Mg/ml*) was added to supernatant, which was removed and diluted with 10 *ml* of water. Samples were purified by solid phase extraction using LC-18 tubes. A sample was applied to Supelclean LC-18 tubes (3 *ml*, pre-washed with 10 *ml* of methanol and 10 *ml* of water) followed by elution with 5 *ml* of water and 5 *ml* of a methanol-water mixture, 9:1 (separately). The methanol-water solution was evaporated to dryness in a vacuum rotary evaporator and a stream of nitrogen. The residue dissolved in 100 *Ml* of a mixture of N-trimethylsilylimidazole (TMSI): pyridine, 1:4, left at room temperature for 30 *min* and subjected to GC/MS.

Alternatively, acetylation of samples was performed as follows. The samples were dissolved in a mixture of acetic anhydride-pyridine, 1:1 (v/v) and left for 2 hours at. Acetates of andrographolide were extracted from the mixture after dilution with 2 *ml* of water using 5 *ml* ethylacetate (saturated with water).

The organic phase was evaporated, the residue dissolved in 100 *Ml* of ethylacetate, centrifuged and subjected to GC/MS.

We have established a sensitive and reproducible method for analysis of AND in human plasma using high performance capillary electrophoresis (HPCE).

HPCE with diode-array detection has become popular in the last decade because it is straightforward and offers excellent selectivity and good sensitivity. Separation by electrophoresis is obtained by differential migration of solutes in an electric field. In CE electrophoresis is performed in narrow-bore capillaries [10].

Below are the general analytical conditions which we used for quantitative analysis of AND. An analyte mixture of human plasma was separated on the HP Extended Light Path Capillary (with "bubble" at the detection window) which improved sensitivity 3-fold over standard capillary. The capillary was filled with mixture of borate buffer and methanol (90:10,v/v). To find the optimum buffer pH and ionic strength, a series of injections of a standard solution was made. The best response was achieved when the pH of mobile phase was alkaline. Using the methanol in the concentration of 10% as an organic modifier increased the selectivity of the separation AND from plasma components.

Separations were performed under isocratic conditions at 15 *kV* at 30.0°C. A thermostating mechanism was included in order to control the column temperature during the run, which is important to determine the proper selectivity of the separation and for reproducibility of migration time of AND (tab. 1). Samples were injected hydrodynamically directly onto the capillary using a pressure of 50*mbar* for 4 *sec.*

A wavelength of 229 nm with reference wavelength of 500 *nm* was used for on-line acquisition of spectra for peak of AND. We used the capability of diode – array detector to monitor a sample at multiple detection wavelengths, and found it useful for AND peak identification as well as for providing reference peak in the electrophoregram. Using a three dimensional diode-array spectrograms at target (signal 229/8 *nm*, reference 500/100 *nm*) and qualifying (signal 195/8 *nm*, reference 500/100 nm, signal 229/8 *nm*, reference 271/20 *nm*) wavelengths and utilizing as internal reference standards two unknown plasma components A and B (tab. 1) we definitely identified the peak of AND. Absorption of non-relevant peaks therefore was minimized. Detection of AND at various wavelengths is illustrated in the figure 13. Besides, migration time of reference standard of Andrographolide was estimated every day before and after analysis of plasma samples. Peak purity was automatically examined by comparison with spectra of AND.

Validation was determined using six replicate procedures for every validation parameter. The method has been shown to be linear in the range from 0.25 to 20 *Mg/ml*. The correlation coefficient was determined to be r2 = 0.99962 and showing a good linear fit within the range tested (fig. 11). The detection limit test was performed by sequentially diluting a standard solution until the signal-to-noise ratio approached 3:1.A lower detection limit was determined to be 0.2 *μg/ml*.

Interday precision of a method was measured injecting a series (n=6) of repeatedly prepared samples. The relative standard deviation (RSD) was 14,15 %. Repeatability of capillary electrophoresis was obtained in a relatively short time (RSD= 1,54%).

The efficiency of the extraction AND from the plasma was measured. Recovery rate was 77.85%. The accuracy of the method was established by analysis of varying known amounts of AND to a series (n=6) of blood samples without AND. The content of AND was determined under the conditions described in the method. The resulting data were compared to the theoretical true value and used to calculate the statistical parameters. Overall accuracy was 99,49% with 4,45% of RSD.

Having set up the complete analytical method, we analyzed AND concentration in human plasma. The samples were prepared according to fig. 10. Fig. 12 represents a typical electrophoregram of separation of AND in human plasma.

Amounts of AND were calculated from standard calibration curve and based on the peak areas.

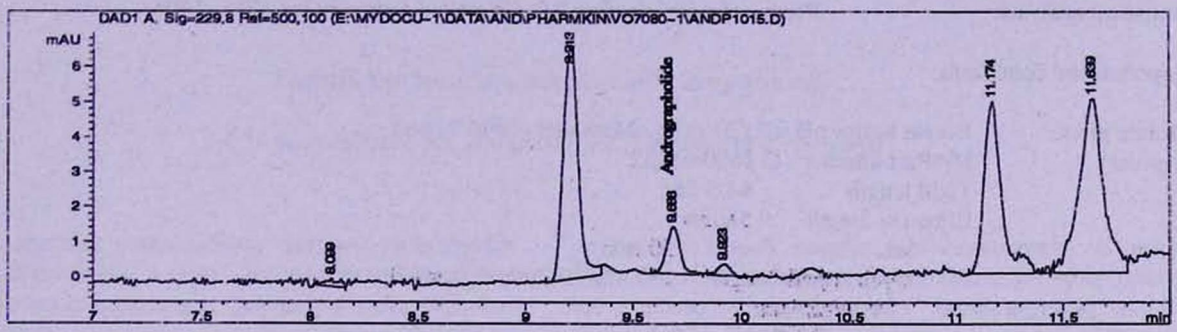

Fig. 12. HPCE separation of andrographolide in human plasma

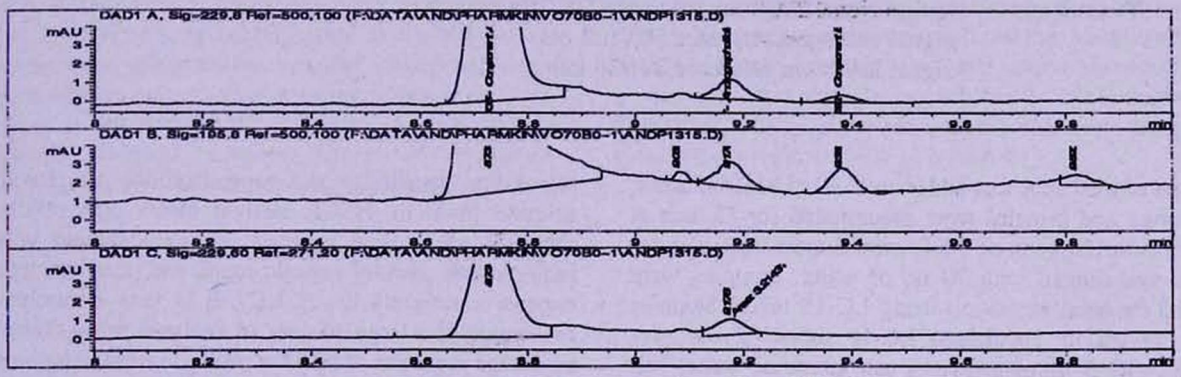

Fig. 13. Monitoring of andrographolide in human plasma at multiple detection wavelength

Table 1

Migration time and relative migration times precision of andrographolide

N	Run #	Migration time of component A , Min , M_A	Migration time of component B , Min , M_B	Migration time of andrographolide, *min* M_{AND}	Relative migration time (compound A) $RA=M_{AND}/M_A$	Relative migration time (compound B) $RB=M_{AND}/M_B$
1	37	8.673	9.215	9.053	1.044	0.982
2	82	8.979	9.756	9.535	1.062	0.977
3	103	9.223	9.934	9.706	1.052	0.982
4	125	8.992	9.674	9.461	1.052	0.977
5	141	8.735	9.382	9.177	1.051	0.978
6	167	9.402	10.113	9.888	1.052	0.978
Mean				9.470	1.052	0.979
Relative standard deviation (%)				3.32	0.55	0.24

INSTRUMENTATION
HEWLETT PACKARD Capillary Electrophoresis System analyzes samples:

Instrumentation:	HP High Performance Capillary Electrophoresis System, consisting of HP3DCE , HP KAYAK XA, HP Laser Jet 4000 printer.
Data Collection:	HP ChemStation for CE systems
Statistical analysis:	Prism software, version 2.0, GraphPad Software Inc.USA, 1996

Experimental conditions

Mobile phase	Borate buffer pH 9.3 (20 *mM*) / Methanol = 90/10 (v/v)
Capillary	HP-Part number - G 1600-61232
	Total length 64.5 *cm*
	Effective length 56 *cm*
	i.d. 50 *Mm*
	Optical path length 150 *Mm* (Bubble factor – 3)
Injection	50 *mbar/4 sec*
Temperature	30°C
Voltage	15 *kV*
Detection	target signal 229/8 *nm*, reference 500/100 *nm*
	signal 195/8 *nm*, reference 500/100 *nm*
	signal 229/8 *nm*, reference 271/20 *nm*

3 *ml* of methanol was added to 2 *ml* of blood plasma; and vortex and proteins were precipitated for 15 *min* at 4°C by centrifugation at 3000 *rpm*. Supernatant was removed and diluted with 20 *ml* of water. Samples were purified by solid extraction using LC-18 tubes. Samples were applied to Supelclean LC-18 tubes (3 *ml*, prewashed with 5 *ml* of methanol and 10 *ml* of water) followed by elution separately with 10 *ml* of water and 10 *ml* of methanol-water mixture, 9:1 (separately). The methanol-water solution was evaporated to dryness using a vacuum rotary evaporator. The residue was dissolved in 100 *Ml* of mobile phase: 20 *mM* borate buffer pH 9.3 - methanol, 90:10, v/v and used fresh for injection to HPCE.

Thus, the outcomes of conducted researches have shown that, for pharmacokinetic study of the AND the most convenient method is the method capillary electrophoresis. The given method has a right combination of selectivity, sensitivity and reproducibility to provide a solution problem. HPCE method offers high efficiency (N>105), short time analysis, minimal sample volume requirements, lack of organic waste and does not demand expensive reagents (as HPLC). It is very important for realization of a large number of analyses for optimization of dosage regiment of AND at multiple administrations.

The methods for the analysis of andrographolide presented in this article are prospective. The GC/MS method can be recommended for study of biotransformation of AND and when doing trace analysis. The method of HPLC is useful for analysis of AND in the animal plasma and urine.

On the whole, the offered method is easy to perform and provides accurate quantitative analysis of andrographolide.

Անդրոգրաֆոլիդի անալիզի մեթոդների մշակումը

Գ. Մամիկոնյան, Ա.Փանոսյան, Է.Գաբրիելյան, Գ.Վիկման

Մշակված են անդրոգրաֆոլիդի անալիզի նոր մեթոդներ արյան մեջ և մեզում: Andrographis paniculata N.- ի հիմնական քիմիական և կենսաբանորեն ակտիվ կոմպոնենտի՝ անդրոգրաֆոլիդի քանակական որոշման համար մշակվել են բարձրարդյունավետ մազանոթային էլեկտրաֆորեզի, բարձրարդյունավետ հեղուկային քրոմատագրության և քրոմատա-մասս-սպեկտրաչափման մեթոդներ:

Նկարագրված մեթոդները ենթարկվել են թեստավորման ըստ առավել կիրառելի չափաբանական բնութագրերի՝ ընտրողականության, ճշտության, ճշգրտության, գծայնության, աշխատանքային դիապազոնի, հայտնաբերման սահմանի: Առաջարկված մեթոդները ապահովում են անդրո-

գրաֆոլիդի անալիզի ճշտությունը և վերարտադրելիությունը և կարող են կիրառվել Andrographis paniculata N. ոգեթուրմ պարունակող պատրաստուկների ֆարմակոկինետիկայի ուսումնասիրության համար: Մշակված մազանոթային էլեկտրաֆորեզի մեթոդը գերադասելի է արյան մեջ անդրոգրաֆոլիդի քանակական որոշման համար: Քրոմատա-մասսսպեկտրաչափման և բարձրարդյունավետ հեղուկային քրոմատագրության մեթոդները կարող են օգտագործվել ԱՆԴ-ի մետաբոլիզմի և դուրսբերման կինետիկան ուսումնասիրելու համար:

Разработка методов анализа андрографолида

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Разработаны новые методы анализа андрографолида в плазме крови и моче. Для количественного определения андрографолида, основного химического и биологически активного компонента Andrographis paniculata N., использовались методы высокоэффективного капиллярного электрофореза, высокоэффективной жидкостной хроматографии и хромато-масс-спектрометрии. Описанные методы протестированы на ряд наиболее используемых метрологических параметров – избирательность, точность, достоверность, линейность, рабочий диапазон, предел обнаружения. Предложенные методы обеспечивают правильный и воспроизводимый анализ андрографолида и могут быть использованы для исследования фармакокинетики препаратов, содержащих экстракт Andrographis paniculata N. При этом вследствие высокой эффективности и экономичности наиболее удобным для количественного определения андрографолида в плазме крови является разработанный метод капиллярного электрофореза. Методы хромато-масс-спектрометрии и высокоэффективной жидкостной хроматографии приемлемы для изучения метаболизма и кинетики выделения андрографолида с мочой.

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