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A NOVEL, SIMPLE AND ACCURATE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-UV SPECTROMETRY METHOD FOR THE QUANTITATION OF COLCHICINE IN HUMAN PLASMA

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The present work describes development and validation of a new, simple and accurate method of colchicine determination in human plasma by liquid chromatography with photodiode array detection. Isocratic reserved-phase separation was carried out on a C18 column (Nucleosil, Macherey-Nagel, 250×4.6 *mm*, 3*μm*) employing acetonitrile-water (40:60 *v/v*) with flow rate 1 *ml/min* as a mobile phase. Detection was performed at 300 *nm*. Colchicine and proposed new internal standard alprazolam were extracted from the matrix using pH=8.0 phosphate buffer and dichloromethane. Retention times were 5 *min* and 13 *min* for colchicine and alprazolam, respectively. Calibration curves were linear in concentration range 0.1-10 *μg/ml*. The lower limit of quantification for colchicine was found to be 0.1 *μg/ml*. Recovery was estimated to be reasonable with the result equal to 91.88%. Method revealed satisfactory results concerning selectivity, as absence of interference was observed with endogenous components of plasma. Precision (as relative standard deviation, RSD %) and accuracy (as relative error, RE %) were in range of 1.23-4.99% and 1.7-4.92%, respectively. Colchicine was found to be stable after three freeze-thaw cycles in the dark, as the final concentrations of colchicine in the stability samples were 94.7-98.9% of the initial value.

Developed method was successfully applied in colchicine assay in plasma of healthy volunteers, as well as in target cells such as mononuclear and polymorphonuclear leucocytes of the blood.

Familial Mediterranean fever is a genetically inherited disorder which is common among populations living around Southern and Eastern costs of

Mediterranean Sea: Armenians, Jews, Turks and Arabs. Amyloidosis is the main complication of disease. Eventually, it leads to chronic renal failure and renal transplant.

Since 1972, the drug of choice for prophylaxis of amyloidosis and FMF attacks is colchicine [1]. Despite the fact that colchicine is the only drug for treatment FMF, 15% of the patients do not respond to the colchicine therapy [2], and there is no other effective drug for FMF treatment yet. Taking into consideration actuality and seriousness of problem for population of Armenia, it is necessary to explore this phenomenon and find out the reason of colchicine resistance.

The aim of this study is to develop and validate a novel, simple and accurate method of identification and assay colchicine in human plasma and blood cells. There are several methods of colchicine quantification in human fluids, including Liquid chromatography with tandem mass spectrometry (LC-MS/MS) [3], Liquid chromatography with ion spray ionization tandem mass spectrometry [4, 5], Liquid chromatography coupled with Diode array detector (DAD) [6] or UV [7] detector , Gas chromatography with mass spectrometric detection [8]. Each of mentioned methods has its drawbacks. Low reproducibility is the main drawback of GC method. MS methods are expensive and require highly qualified experts to work with the instrument. Therefore, it's advisable to use HPLC-UV or HPLC-DAD method to quantify colchicine in human plasma.

Chemicals and Reagents

Colchicine (95% HPLC, Fig.1) was supplied by Sigma Aldrich, USA, Alprazolam (100%, European pharmacopoeia reference standard, Fig.2) used as internal standard was supplied by European Directorate for the Quality of Medicines. Organic solvents acetonitrile (AppliChem, Germany) and dichloromethane (Carl Roth, Germany) were HPLC grade. All solutions were prepared using highly purified water (Milli–Q water purification system, Millipore, France). Di-ammonium hydrogen phosphate (Carl Roth, Germany) was analytical grade. Donated blood and plasma stabilized with sodium citrate were kindly provided by medical centre "Arabkir", Armenia.

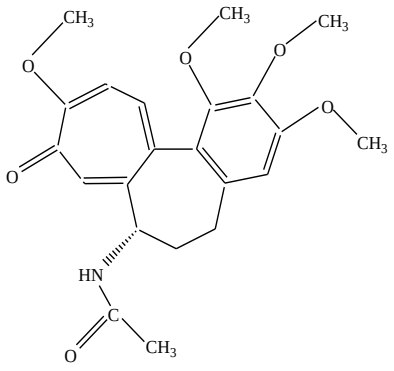

Fig. 1. Chemical structure of colchicine.

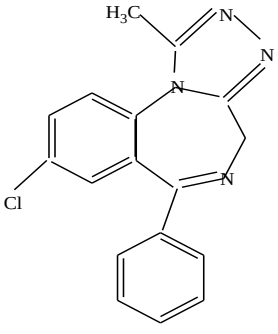

Fig. 2. Chemical structure of alprazolam.

Instruments and chromatographic conditions

An HPLC PLATIN Blue system (Knauer, Germany) with PDA detector set at 300 *nm* was used for quantitative determination of colchicine and alprazolam in all samples. The analytical column employed was Nucleosil C18, 250×4.6, 3 *μm* (Macherey-Nagel, Germany), which was thermostated at 30^0C. Elution was performed isocratically, with the flow rate 1 *ml/min.* The mobile phase consisted of acetonitrile and water (40:60 *v/v*) was filtered through Porafil MV 0.45 *μm* membrane (Macherey-Nagel, Germany).

Preparation of standard solutions

Stock standard solutions of colchicine (1000 *μg/ml*) and internal standard alprazolam (100 *μg/ml*) were prepared by dissolving corresponding amounts of both compounds in mobile phase and stored at 4^oC. Taking into consideration light sensibility of colchicine all working standard solutions were prepared before use in amber glass volumetric flasks at concentrations 1-100 *μg/ml*. Working standard solution of the internal standard was prepared by dilution of stock standard solution of alprazolam to 10 *μg/ml* using mobile phase as a solvent.

Preparation of sample solutions

In order to prepare sample solutions for every validation criteria appropriate amounts of colchicine standard solutions and constant 100 *μl* of standard solution of alprazolam (10 *μg/ml*) were added to 1 *ml* of plasma. After addition of 1.5 *ml* of phosphate buffer pH=8.0 obtained liquids were mixed 1 *min.* by vortex mixer. So as to extract colchicine and internal standard from the matrix 4.5 *ml* of dichloromethane was added, then mixtures were shaken vigorously in horizontal direction during 10 *min.*. After centrifugation for 10 *min.* at 3500 *rpm*, the clear organic layer was removed to 10 *ml* tubes and evaporated to dryness under the vacuum. Dry residue was reconstituted using 200 *μl* of mobile phase, mixed by vortex mixer for 5 *min.*, centrifuged at 3500 *rpm* for 5 *min.* and filtered by syringe nylon membrane filter 0.22 *μm* (Macherey-Nagel). 20 *μl* of this filtrate was injected into the column.

Method validation

Proposed method of colchicine assay in human plasma was validated by estimation of fundamental criteria of bioanalytical method validation such as selectivity, accuracy, precision, recovery, calibration curve and stability of analyte in spiked samples [9, 10]. Representative chromatograms of standard solutions of colchicine and alprazolam are shown in Fig 3. (A, B). Representative chromatogram of spiked sample solution is shown in Fig. 4.

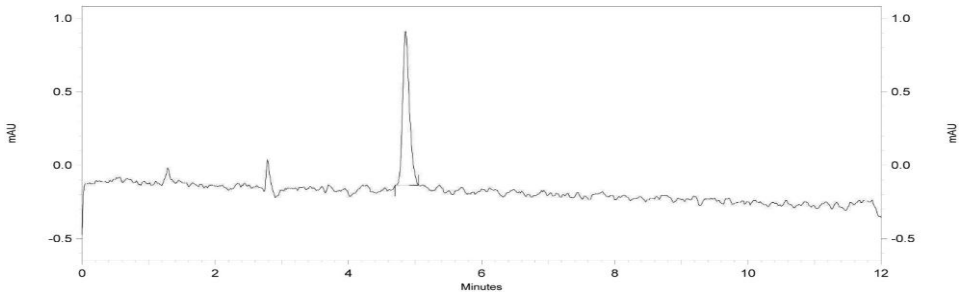

Fig 3A. Chromatogram of colchicine standard solution.

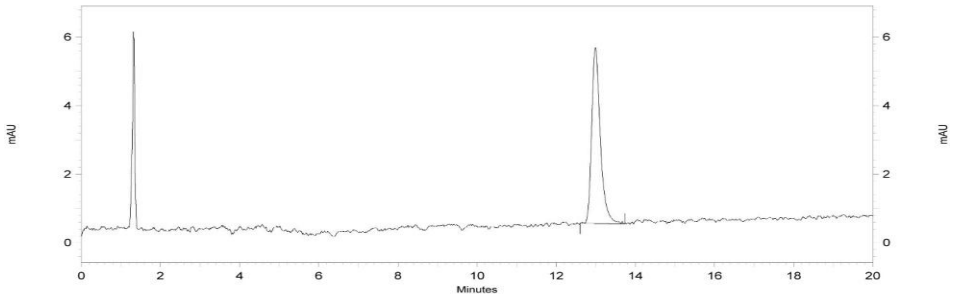

Fig. 3B. Chromatogram of alprazolam standard solution.

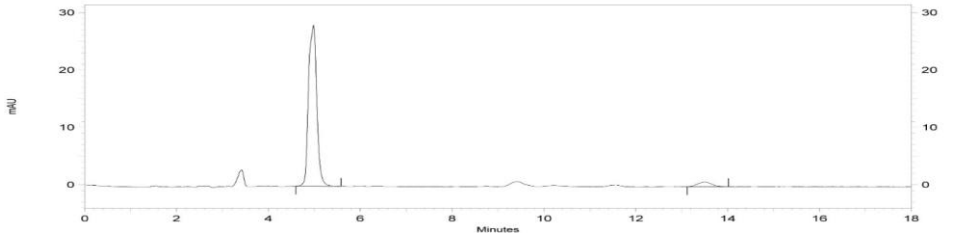

Fig. 4. Chromatogram of spiked sample solution.

Selectivity of the method was explored by comparison of the chromatograms obtained from plasma blank samples and plasma samples spiked with the colchicine. For reaching this goal 6 samples with the concentration of colchicine equal to 0.1 *μg/ml* in 6 different plasmas and their blank samples were prepared.

Recovery or limit of extraction of colchicine from the plasma was assessed at low (0.2 *μg/ml*), medium (1 *μg/ml*) and high (10 *μg/ml*) concentration of the analyte. In order to calculate extraction limit of colchicines ratio of pure standard solution of it were prepared with the same concentrations. Areas of sample and standard solutions were compared.

Samples for calibration curve were constructed using 6 concentration levels in the range 0.1-10 *μg/ml* by plotting colchicine peak areas versus its concentrations. The lowest concentration level of calibration curve is lower limit of quantification (LLOQ) which is defined as the lowest concentration of analyte that can be

determined with acceptable precision of RSD= ±20% and accuracy RE= ±20%. 6 samples with concentration 0.1 *μg/ml* were prepared to establish LLOQ value.

Accuracy and precision of were assessed at low 0.2 *μg/ml*, medium 1 *μg/ml* and high 10 *μg/ml* concentration levels. For that purpose 5 series of each concentration were prepared.

Stability of analyte in biological matrix at intended storage temperatures is established by carrying out freeze-thaw cycles at low 0.2 *μg/ml* and high 10 *μg/ml* concentration levels. 5 series of plasma samples spiked with colchicine were frozen at -20^{o}C, then leave to thaw at room temperature. This cycle was repeated 2 more times. After the last cycle samples were assayed.

Method application

Developed method was applied in colchicine assay in plasma of healthy volunteers, as well as in colchicine's target cells mononuclear and polymorphonuclear leucocytes of the blood. So as to carry out this experiment blood samples of male and female healthy volunteers were spiked with the colchicine to obtain concentration equal to 10 *μg/ml*. Then samples were kept in thermostat for 15, 30, 45 minutes, then centrifuged to separate plasma and fractions of leucocytes. Obtained samples were treated as described in sample solution preparation and assayed afterwards.

Results and discussion

Selectivity

Selectivity is the ability of a bioanalytical method to differentiate and quantify the analyte in the presence of other components in the sample.In this study potential interfering substances are endogenous plasma components. On the chromatograms of the selectivity samples no any interference was found with other substances peaks. Colchicine and internal standard were properly resolved. This indicates high selectivity of the method and its correspondence to acceptance criteria of bioanalytical method selectivity parameter. Chromatograms of plasma blank sample and plasma spiked sample are shown in Fig. 5 (A, B).

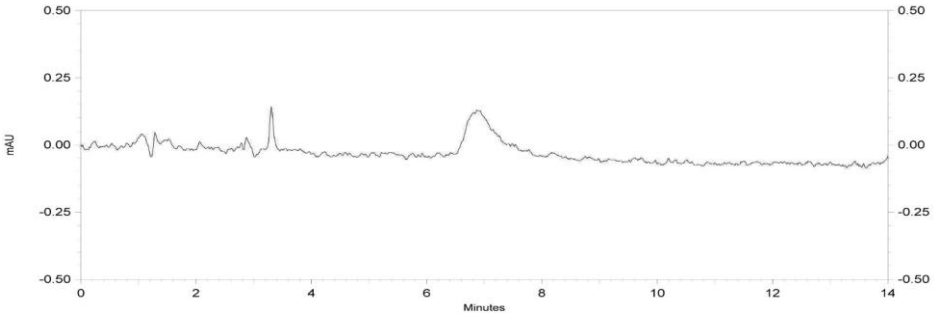

Fig. 5A. Chromatogram of plasma blank sample.

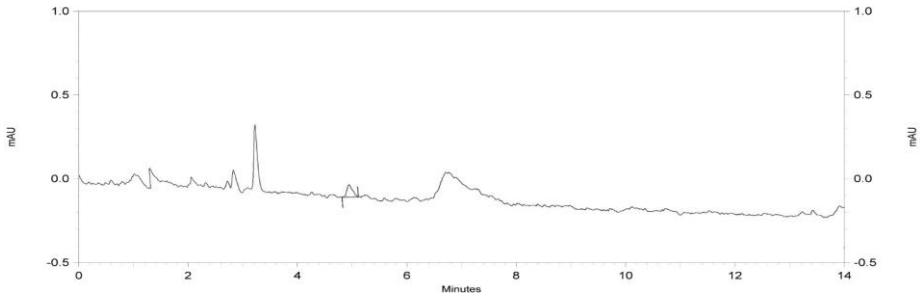

Fig. 5B. Chromatogram of spiked plasma sample.

Recovery

Recovery of an analyte is its limit of extraction from the matrix. Recovery at low, medium and high concentrations was examined using five series of spiked samples with corresponding concentrations. Mean recoveries at three concentrations were 89.09 % (0.2*µg/ml*), 92.32 % (1 *µg/ml*) and 94.24 % (10 *µg/ml*) samples. Average result of recovery at these three concentrations is 91.88%. So, extraction of colchicine from the matrix was estimate to be satisfactory.

Linearity and lower limit of quantification

Calibration curve is the relationship between instrument response and known concentration of the analyte. It should be linear in the range of working concentrations. Calibration curve was linear over the range 0.1-10 *µg/ml* with a regression coefficient of 0.9998. Calibration curve is shown in Fig. 6.

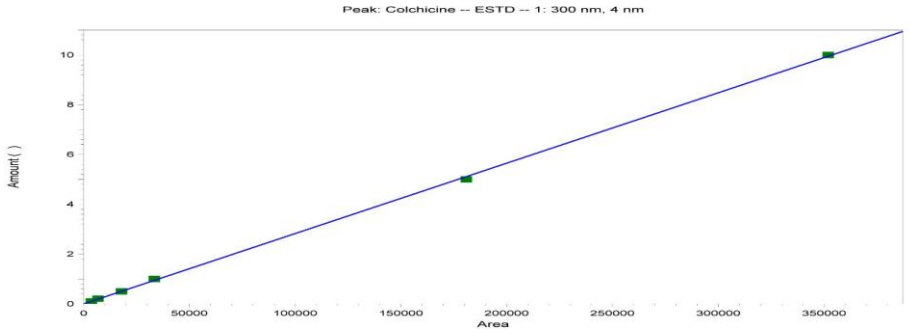

Fig.6. Calibration curve for Colchicine quantitation.

The lowest concentration level of the calibration curve is 0.1 *µg/ml*. It was established as the lower limit of quantification. Colchicine peak at this concentration was identifiable, discrete and reproducible.Concentrations of colchicine in 6 LLOQ plasma samples were determined with acceptable accuracy (RE=-5.67%) and precision (RSD=4.0 %). Results are shown in Table 1.

Table 1

Lower limit of quantification

Sample	Peak area		Determined concentration, *μg/ml*	RE, ± 20%
	Blank of plasma	LLOQ 0.1 *μg/ml*		
LLOQ 1	0	3451	0.095	-5
LLOQ 2	0	3601	0.099	-1
LLOQ 3	0	3561	0.098	-2
LLOQ 4	0	3340	0.092	-8
LLOQ 5	0	3232	0.089	-11
LLOQ 6	0	3391	0.093	-7
Mean	0	3429.333	0.094333	-5.67
SD		138.3223	0.003777	–
RSD, ±20%		4.033505	4.004019	–

Accuracy and precision

Criterion of accuracy is defined as closeness of obtained concentration of the analyte using proposed method to the true value. Calibration curve was used to appraise concentration of colchicine in quality control samples of accuracy. Results of accuracy were evaluated by calculating RE for each concentration level.

Precision of the method describes degree of closeness of obtained results to each other for the same concentration level. It is assessed by determining RSD between obtained concentrations.

Proposed method revealed satisfactory results concerning criteria accuracy and precision. 5 series of samples at low (0.2 *μg/ml*), medium (1 *μg/ml*) and high (10 *μg/ml*) concentration level were assayed for assessing this criteria. Result of accuracy and precision are shown in the Table 2. Method precision (as RSD %) and accuracy (as RE %) were in range 1.23-4.99% and 1.7-4.92%, respectively.

Table 2

Accuracy and Precision

QC sample	**Recovery, μg/ml**		
	0.2 *μg/ml*	**1 *μg/ml***	**10 *μg/ml***
1	0.204	1.069	11.026
2	0.204	1.003	9.996
3	0.207	1.070	10.484
4	0.201	1.087	11.008
5	0.201	1.003	9.947
N	5	5	5
Mean	0.2034	1.0464	10.4922
SD	0.00251	0.040259	0.523096
RSD, ± 15%	**1.234012**	**3.847397**	**4.985568**
RE, ± 15%	**1.7**	**4.64**	**4.92**

Stability

Stability research is performed to prove that storage conditions of the samples do not change concentration of the analyte in biological matrix. The aim of this study was to develop a method to quantify concentration of colchicine in human plasma. Usually, plasma samples are stored in deeply frozen condition. Therefore, it's rational to test stability by performing freeze/thaw cycles of spiked plasma samples. In order to reach this goal 5 series of spiked plasma samples with colchicine at low and high concentration levels were undergone 3 cycles of freeze/thaw as described above. The results of the stability study are presented in Table 3. Colchicine was found to be stable after three freeze-thaw cycles in the dark, as the final concentrations of colchicine in the stability samples were 94.7-98.9% of the initial value.

Table 3

Data of stability study

Sample		Area of the peak			
		QC L 0.2 *μg/ml*		QC H 10 *μg/ml*	
		QC sample (QC)	Reference sample (QC-ref)	QC sample (QC)	Reference sample (QC-ref)
Stability	1	6349	6775	339565	351821
	2	6401	6717	345187	350133
	3	6373	6693	347213	349905
	4	6383	6771	346554	351139
	5	6359	6695	354201	348995
N		5	5	5	5
Mean		6373	6730	346544	350399
SD		20.347	40.214	5235.103	1101.8
RSD, %		0.319	0.598	1.51	0.314
QC/QC_{ref}·100%		94.7		98.9	

Application of the method

Validated method was consecutively applied to assay colchicine in the plasma and leucocytes of healthy volunteers. Obtained plasma concentrations of colchicine were in the range 4,7-13,582 *μg/ml*. Content of colchicine in mononuclear and polymorphonuclear leucocytes of the blood was in the ranges 1,52-120,4 *ng/10^6 cells* and 0,63-32,29 *ng/10^6 cells*, respectively. Obviously, concentration of colchicine in mononuclear leucocytes is higher than in polymorphonuclear leucocytes. Obtained results of this in vitro experiment coincide with literature data [9]. Literature data are obtained from experiments using blood of patients with FMF, who are taking tablets of colchicine. Plasma concentration of colchicine in that case is in nanogram level. Table 4 represents comparison of the data of in vitro and in vivo experiments [2], [9]. Motioned data show that concentration of colchicine in all studied fractions of the blood is higher 1000 times in in vitro experiment, but tendency of distribution of colchicine is the same. This result prove that in vitro experiment accurately

represents in vivo processes and it can be used to model distribution of colchicine in the blood in easily handled in vitro experiments.

Table 4

Data of in vivo and in vitro experiments results

Obtained and literature data	Plasma	Polymorphonuclears	Mononuclears
Obtained date of application of the method In vitro experiment	4,7-13,58 μg/ml	0,63-32,29 ng/10^6cells	1,52-120,4 ng/10^6 cells
Chappey O. and co-authors In vivo experiment [2]	0,13-1,75 ng/ml	4-64 ng/10^9 cells	11,4-57,6 ng/10^9 cells
Lidar M. and co-authors In vivo experiment [11]	0,33-1,71 ng/ml	25,9-253,1 ng/10^9 cells	65-403 ng/10^9 cells

Conclusion

A novel and simple method of quantitative determination of colchicine in human plasma was developed, which used alprazolam as internal standard for the first time. The method was validated according to all requirements of method validation international guidelines. Validation revealed high degree of selectivity, linearity, accuracy, precision and stability and was successfully applied to in vitro assay of colchicine in plasma of healthy volunteers, as well as in target cells such as mononuclear and polymorphonuclear leucocytes of the blood. Data obtained from this method application proved that it can be used to model distribution of colchicine in the blood in easily handled in vitro experiments. Due to the usage of easily handled HPLC instrument and simple extraction procedure the method is rather practical and can be used in pharmacokinetic studies of colchicine, or in determination of colchicine in other biological specimens such urine, saliva and milk after making appropriate adjustments.

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This article could not be written to its fullest without Professor Tigran K. Davtyan PhD, DSc, Director of "Analytical laboratory" branch of Scientific Centre of Drug and Medical Technology Expertise CJSC who served as my supervisor and encouraged me throughout whole time spent doing this research.

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ԲԼՉՔ-ՈՒՄ ԵՂԱՆԱԿՈՎ ՄԱՐԴՈՒ ՊԼԱԶՄԱՅՈՒՄ ԿՈԼԽԻՑԻՆԻ ՔԱՆԱԿԱԿԱՆ ՈՐՈՇՄԱՆ ՆՈՐ, ՊԱՐԶ ԵՎ ՃՇԳՐԻՏ ՄԵԹՈԴ

Լ. Ռ. ՂՈՉԻԿՅԱՆ

Սույն աշխատանքում նկարագրված է լուսաղիոդային դետեկտմամբ բարձր արդյունավետության հեղուկային քրոմատոգրման եղանակով մարդու արյան պլազմայում կոլխիցինի քանակական որոշման նոր, պարզ և ճշգրիտ մեթոդի մշակումը և վերջինիս վալիդացիան: Իզոկրատիկ չրջված փազային բաժանումն իրականացվել է C18 աշտարակով

Nucleosil, Macherey-Nagel, 250×4.6 mm, 3 μm)՝ որպես շարժուն փազ կիրառելով ացետոնիտրիլ-ջուր լուծույթը (40:60 ծ/ծ) 1 մլ/րոպե հոսքի արագությամբ: Դետեկտման ալիքի երկարությունը ընտրվել է 300 նմ: Կոլխիցինը և ընտրված նոր ներքին ստանդարտ ալպրազոլամը լուծահանվել են՝ օգտագործելով pH=8.0 ֆոսֆատային բուֆեր և դիքլորմեթան: Կոլխիցինի և ալպրազոլամի զոլման ժամանակները համապատասխանաբար կազմում են 5 և 13 րոպե: Ստուգաչափման կորը գծային է 0,1-10 մկգ/մլ կոնցենտրացիայի տիրույթում: Կոլխիցինի քանակական որոշման ստորին սահման ընտրվել է 0,1 մկգ/մլ: Կոլխիցինի վերականգնումը գնահատվել է ընդունելի՝ 91.88% արդյունքով: Մեթոդը ընտրողականության վերաբերյալ ցույց է տվել բավարար արդյունքներ, ինչպես նաև չի նկատվել պլազմայում պարունակվող էնդոգեն միացությունների հետ որևէ ինտերֆերենցիա: Ճշգրտությունը (որպես հարաբերական ստանդարտ շեղում, %) և ճշտությունը (որպես հարաբերական սխալ, %) կազմում են համապատասխանաբար 1.23-4.99 և 1.7-4.92%: Կոլխիցինը լույսից պաշտպանված պայմաններում ցուցաբերել է կայունություն երեք սառեցում-հալեցում ցիկլերից հետո, կայունության նմուշներում կոլխիցինի վերջնական կոնցենտրացիաները կազմել են 94.7-98.9% ելային արժեքների համեմատ:

Մշակված մեթոդը հաջողությամբ կիրառվել է առողջ կամավորների պլազմայում, ինչպես նաև թիրախային բջիջներ հանդիսացող արյան մոնոնուկլեար և պոլիմորֆոնուկլեար լեյկոցիտներում կոլխիցինի քանակական որոշման համար:

НОВЫЙ, ПРОСТОЙ И ТОЧНЫЙ ВЭЖХ-УФ МЕТОД КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ КОЛХИЦИНА В ПЛАЗМЕ ЧЕЛОВЕКА

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Настоящая работа описывает разработку и валидацию нового, простого и точного метода определения колхицина в человеческой плазме с помощью высокоэффективной жидкостной хроматографии с диодно-матричным детектированием. Изократичное обращенно-фазовое разделение проводилось на колонке C18 (Nucleosil, Macherey-Nagel, 250×4,6 *мм*, 3 *мкм*) с использованием смеси ацетонитрил-вода (40:60 *об/об*) со скоростью потока 1 *мл/мин* в качестве подвижной фазы. Обнаружение проводилось при 300 *нм*. Колхицин и предлагаемый новый внутренний стандарт альпразолам были извлечены из матрицы, используя pH 8,0 фосфатный буфер и дихлорметан. Время удерживания – 5 и 13 *мин*. для колхицина и альпразолама, соответственно. Калибровочное кривое было линейным в интервале концентраций 0,1-10 *мкг/мл*. Концентрация 1 *мкг/мл* была установлена как нижний предел количественного определения колхицина. Возврат был оценен разумным с результатом, равным 91.88%. Метод показал удовлетворительные результаты в отношении селективности, т. к. не наблюдалось интерференции с эндогенными компонентами плазмы. Точность (как относительное стандартное отклонение, RSD %) и правильность (как относительная ошибка, % RE) были в

диапазоне 1.23-4.99 и 1.7-4.92%, соответственно. Было установлено, что колхицин в плазме остался стабильным после трех циклов замораживания-оттаивания в темноте, т. к. конечные концентрации колхицина в образцах стабильности были равны 94.7-98.9 % от исходного значения.

Разработанная методика была успешно применена в анализе колхицина в плазме здоровых добровольцев, а также в клетках-мишенях, таких, как одноядерные и полиморфноядерные лейкоциты крови.

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