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Development and Validation of Valganciclovir and its Active Metabolite Ganciclovir Determination in Human Plasma by HPLC-MS/MS Method

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Abstract

Introduction. Currently, physicochemical methods of quantification are actively used to determine the content of drugs in biological fluids. High-performance liquid chromatography with various detection methods is particularly widespread. One of the most difficult practical tasks is the chromatographic separation of so-called poorly retained compounds – drug substances poorly retained on the chromatographic column. Valganciclovir and Ganciclovir are among such substances.

Aim. The aim of this study is to develop a method for valganciclovir and ganciclovir in human plasma by high performance liquid chromatography with tandem mass-spectrometry (HPLC-MS/MS) for pharmacokinetic studies.

Materials and methods. Determination of valganciclovir and ganciclovir in plasma by HPLC-MS/MS. The samples were processed by acetonitrile protein precipitation.

Results and discussion. This method was validated by next parameters: selectivity, matrix effect, calibration curve, accuracy, precision, recovery, lower limit of quantification, carry-over and stability.

Conclusion. The method of the determination of valganciclovir and ganciclovir in human plasma was developed and validated by HPLC-MS/MS. The linearity in plasma sample was achieved in the concentration range of 5.00–1000.00 ng/ml for valganciclovir and 50.00–10000.00 ng/ml for ganciclovir. Method could be applied to valganciclovir and ganciclovir determination in plasma for PK and BE studies.

Keywords: valganciclovir, ganciclovir, plasma, HPLC-MS/MS, validation, bioequivalence.

Conflict of interest: no conflict of interest.

Contribution of the authors. Timofey N. Komarov, Margarita A. Tokareva, Olga A. Archakova, Dana S. Bogdanova, Alexandra A. Aleshina, Veronika V. Davydanova have developed and validated an analytical method. Natalia S. Bagaeva carried out statistical processing of the obtained results. Igor E. Shohin carried out the organization of work in this direction. All the above authors participated in the discussion of the results in the format of scientific discussion.

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Определение валганцикловира и ганцикловира в плазме крови человека методом ВЭЖХ-МС/МС

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Резюме

Введение. В настоящее время физико-химические методы анализа активно используются для определения содержания лекарственных веществ в биологических жидкостях. Особенно широкое распространение приобрела высокоэффективная жидкостная хроматография с различными способами детектирования. Одной из наиболее сложных практических задач является хроматографическое разделение так называемых слабоудерживаемых соединений – лекарственных веществ, плохо удерживаемых на хроматографической колонке. Одними из таких веществ являются валганцикловир и ганцикловир.

Цель. Целью исследования является разработка и валидация методики определения валганцикловира и ганцикловира в плазме крови человека методом высокоэффективной жидкостной хроматографии с тандемным масс-селективным детектированием (ВЭЖХ-МС/МС) для проведения фармакокинетических исследований.
Материалы и методы. Определение валганцикловира и ганцикловира в плазме крови человека проводили методом ВЭЖХ-МС/МС. В качестве пробоподготовки использован способ осаждения ацетонитрилом.
Результаты и обсуждение. Разработанная методика была валидирована по следующим валидационным параметрам: селективность, эффект матрицы, калибровочная кривая, точность, прецизионность, степень извлечения, нижний предел количественного определения, перенос пробы, стабильность.
Заключение. Разработана и валидирована методика определения валганцикловира и ганцикловира в плазме крови человека методом ВЭЖХ-МС/МС. Подтвержденные аналитические диапазоны методики составили 5,00–1000,00 нг/мл для валганцикловира и 50,00–10000,00 нг/мл для ганцикловира в плазме крови. Аналитические диапазоны позволяют применять разработанную методику для проведения исследований фармакокинетики препаратов валганцикловира и ганцикловира.

Ключевые слова: валганцикловир, ганцикловир, плазма, ВЭЖХ-МС/МС, валидация, биоэквивалентность.

Конфликт интересов: конфликта интересов нет.

Вклад авторов. Т. Н. Комаров, М. А. Токарева, О. А. Арчакова, Д. С. Богданова, А. В. Алешина, В. В. Давыданова участвовали в разработке и валидации биоаналитической методики. Н. С. Багаева проводила статистическую обработку данных. И. Е. Шохин отвечал за организационную часть исследования. Все вышеуказанные авторы участвовали в обсуждении полученных результатов в форме научной дискуссии.

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INTRODUCTION

Nowadays, physical and chemical test methods are actively used for determination of drug substance levels in biological fluids. High performance liquid chromatography (HPLC) with various detection methods has become most common. Depending on chromatographic conditions and used detector, it is possible to separate and determine substances with various properties which is especially effective for quantification of structurally related substances. However, chromatographic separation of so-called weakly retained compounds – drug substances poorly retained on a chromatographic column – is one the most difficult practical tasks. Various analytical approaches are used for the purpose: hydrophilic columns (e.g., HILIC type), ion-exchange columns, ion-pair reagents as eluents. However, the approaches may be not always used and rational for a mass spectrometry detector and for determination of drug substances in complex biological matrixes. Visually, the task can be considered as evidence by valganciclovir (VAL) (figure 1) and ganciclovir (GAN) (figure 2).

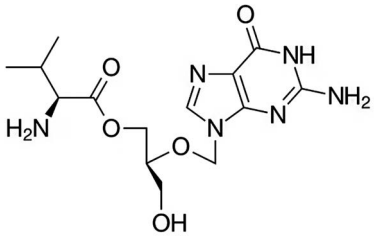

Figure 1. Chemical structure of valganciclovir

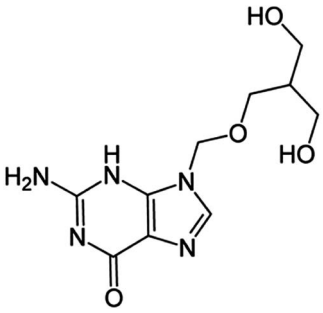

Figure 2. Chemical structure of ganciclovir

Ganciclovir was developed as a drug for cytomegalovirus infection, subsequently, the inhibition *in vitro* of other herpes viruses including type 1 and 2 human herpes virus, as well as, Epstein-Barr virus, chicken pox, type 6 herpes virus and monkey herpes B virus [1] was found-Initially, ganciclovir was used in pharmacotherapy, however its low bioavailability required intravenous administration. A pro-drug – *valganiclovir* tur-

ning to *ganciclovir* with a pharmacological action while being metabolized [2, 3] was developed for the increase of bioavailability.

However, valganciclovir and ganciclovir, as many antiviral drugs, have rather strong hydrophilic properties, which is shown by octanol – water partition coefficient values (log P) for the substances (table 1).

Table 1. Log P and pKa for valganciclovir, ganciclovir and acyclovir

	Valganciclovir	Ganciclovir	Acyclovir (internal standard)
Log P	–0.81	–1.66	–0.95
pKa	10.16	8.71	11.98
Reference	[4]	[5]	[6]

Based on chemical properties of the substances, particularities of chromatographic behavior, detection of substances and sample preparation should be considered. The literature sources provide various methods for determination of test substances in biological fluids with HPLC method combined with sedimentation by 6 % aqueous solution of perchloric acid and detection with the ultraviolet (UV) detector (UF) [7], sedimentation by 50 % aqueous solution of trifluoroacetic acid with UV-detection [8] for ganciclovir; sedimentation by acetonitrile and mass-spectrometry detection (MS) [9], and sedimentation by acetonitrile with diode array detection [10] for valganciclovir. However, it should be noted that the methods were provided for determination of only one substance: either valganciclovir, or ganciclovir. Earlier we had developed the method for joint determination for valganciclovir and ganciclovir in blood plasma with HPLC [11] method. It was the sole method among the ones described in scientific literature which allowed to determine valganciclovir and ganciclovvir simultaneously with the above-mentioned method. However, additional difficulties occurred during the tests that required the use of a specific chromatographic column YMC-Pack Polyamine II and establishment of eluent flow at the level of 2 ml/min which considerably increased reagent consumption. Moreover, for the achievement of separation of the test substances and the internal standard, the analysis of one sample lasted for 26 minutes which considerably increased the test time, in general.

MS detection allowed to reach an optimal chromatographic separation and at the same time to decrease eluent flow rate, and considerably to reduce test time for one sample that allows to reduce expenses. In the study, the development and validation of the method for determination of valganciclovir and ganciclovir in human blood plasma with HPLC-MS/MS method was given. As sample preparation, acetonitrile sedimentation was selected.

MATERIALS AND METHODS

Equipment

The chromatographic separation and detection were performed on high performance liquid chromatograph Nexera XR equipped with a gradient pump, column and sample thermostat, a decontaminator, an autosampler and a tandem mass-spectrometry detector (triple quadrupole). Source data was processed with software Lab Solutions (Ver. 5.91), Shimadzu Corporation, Japan.

Reagents and solutions

The following reagents were used in the work: methanol ("UHPLC-grade" class, J.T.Baker, Netherlands); acetonitrile ("LC-MS grade" class, Biosolve, France/the Netherlands/Israel); formic acid ("98 % pure" class, PanReac, Spain); ammonia hydrate ("for analysis" class, PanReac, Spain); Milli-Q water. For preparation of stock working solutions, reference standard samples of valganciclovir hydrochloride (USP reference standard, assay of 99.2 %), ganciclovir (USP reference standard, assay 97.5 %) and aciclovir were used (USP reference standard, assay of 94.6 %).

Stock reference solutions of valganciclovir, ganciclovir and internal standard (IS) (aciclovir (ACI) were prepared by dissolution of substance weighs in methanol; mixed working reference solutions of valganciclovir and ganciclovir and IS working solution of aciclovir were prepared by dilution of stock solutions with the same solvent up to necessary concentrations in blood plasma corresponding to levels 1–9, as well as, LLOQ levels (lower limit of quantification), L (low), M (middle) and H (high) (table 2).

Stock and working reference solutions were kept in the freezer at temperature of –45 °C. Samples of intact blood plasma were kept in plasma freezer at temperature of –45 °C.

Sample preparation

To 200 µl of a calibration sample placed to Eppendorf centrifuge microtest tubes 2 ml, 10 µl of aciclovir IS working solution was added, then 600 µl of acetonitrile was added, mixed on a vortex for 10 seconds, then centrifuged for 15 min. with a rate of 13500 RPM.

Then the supernatant was transferred to chromatographic vials and placed to the chromatograph autosampler.

Table 2. **Concentrations of analytes at calibration levels**

Level	Analityte concentration, ng/ml		IS concentration, ng/ml
	VAL	GAN	ACI
1	5.00	50.00	1000.00
2	10.00	100.00	1000.00
3	25.00	250.00	1000.00
4	50.00	500.00	1000.00
5	100.00	1000.00	1000.00
6	250.00	2500.00	1000.00
7	400.00	4000.00	1000.00
8	750.00	7500.00	1000.00
9	1000.00	10000.00	1000.00
LLOQ	5.00	50.00	1000.00
L	15.00	150.00	1000.00
M	500.00	5000.00	1000.00
H	800.00	8000.00	1000.00

Chromatographic separation and detection Conditions

- Chromatographic column: Shim-pack GWS C18, 150 × 4.6 mm, 5 µm.
- Thermostat temperature: 40 °C.
- Mobile phase: eluent A: 0.1 % formic acid solution in Milli-Q water with addition of 0.08 % ammonia (by volume); eluent B: 0.1 % formic acid solution, 10 % solution in Milli-Q water with addition of 0.08 % ammonia in acetonitrile (by volume).
- Gradient by composition of the mobile phase (MP) is given in table 3.

Table 3. **Gradient elution**

Time, min	Eluent A, %	Eluent B, %	Mobile phase flow rate, ml/min
0.00	85.00	15.00	1.00
0.70	85.00	15.00	
3.50	90.00	10.00	
4.00	0.00	100.00	
5.50	0.00	100.00	
5.70	85.00	15.00	
7.00	85.00	15.00	

- Injection volume: 5 µl.
- Run-time of the mass-spectrometry detector: 0.00–7.00 min.
- Ionization source parameters (electrospray): spraying gas 3 l/min, drying gas 20 l/min, heating block 400 °C, desolvation line 200 °C, capillary pressure 5.0 kV.
- Ionization mode: positive.
- Conditions for valganciclovir detection: 355.20 m/z → 152.05 m/z.
- Conditions for ganciclovir detection: 255.80 m/z → 152.00 m/z; 255.80 m/z → 134.90 m/z.
- Conditions for aciclovir detection: 226.00 m/z → 152.00 m/z, 226.00 m/z → 134.95 m/z.

RESULTS AND DISCUSSION

Method development

The particularity of the development of the method for chromatographic separation of substances is their weak retention on "conventional" columns with octadecylsilica gel. The use of a column with grafted amino groups also did not lead to good separation and appropriate separation of the substances. Columns containing octadecylsilica gel but various levels of total carbon (table 4) were also tested.

Table 4. **Some properties of chromatographic columns used in the study**

Chromatographic column	Total carbon content, %	Surface area, m^2/g
Phenomenex Luna C18(2) 50 × 2 mm, 5 µm	18.2	393
Phenomenex Luna NH2 50 × 3 mm, 5 µm	10.2	420
Waters XBridge C18 50 × 4.6 mm, 3.5 µm	18.0	178
YMC Hydrosphere C18 100 × 2 mm, 3 µm	12.2	330
Shim-pack GWS 150 × 4.6 mm, 5 µm	9.5	450

The best results were obtained when column Shim-Pack GWS with the lowest levels of total carbon was used. Despite negligible retention of the test substances on test column, nevertheless chromatographic peaks are separated from "dead" volume, and the method meets the requirements of normative documentation on validation parameters including selectivity parameter.

Method validation

The validation of the bioanalytical method was performed on the basis of the guidance on drug expertise, volume I [12] and FDA [13] and EMA guidelines [14] on the following parameters: selectivity, matrix effect, a standard curve, accuracy (at intracycle, intercycle levels), precision (at intracycle, intercycle levels), recovery, the lower limit of quantification, sample transfer, stability [stability of stock and working reference analyte and IS solutions; short-term stability ("bench-top" and "post-preparative" stability); stability in triple freeze-thaw cycles; long-term analyte stability in a matrix].

Selectivity

The analysis of 6 samples of intact blood plasma obtained from different sources, as well as samples of intact blood plasma with addition of working reference solutions up to concentration of valganciclovir of 5.00 ng/ml and ganciclovir of 50.00 ng/ml was performed. The analysis of hemolyzed intact blood plasma and samples with the increased lipid contents was separately performed. On the chromatograms of intact blood plasma samples, the peak signals with corresponding retention times of the substances and IS do not exceed 20 % of a signal at the level of the lower limit of quantification (LLQD) and 5 % of IS signal, respectively. The corresponding chromatogram is given below in figure 3.

Matrix effect

For evaluation of matrix effect, samples were analyzed with addition of the mixed working reference working solutions of valganciclovir, ganciclovir and IS working solution of aciclovir not being influenced by a biological matrix, as well as, samples prepared on intact plasma without regards to recovery of valganciclovir, ganciclovir and aciclovir from the biological matrix.

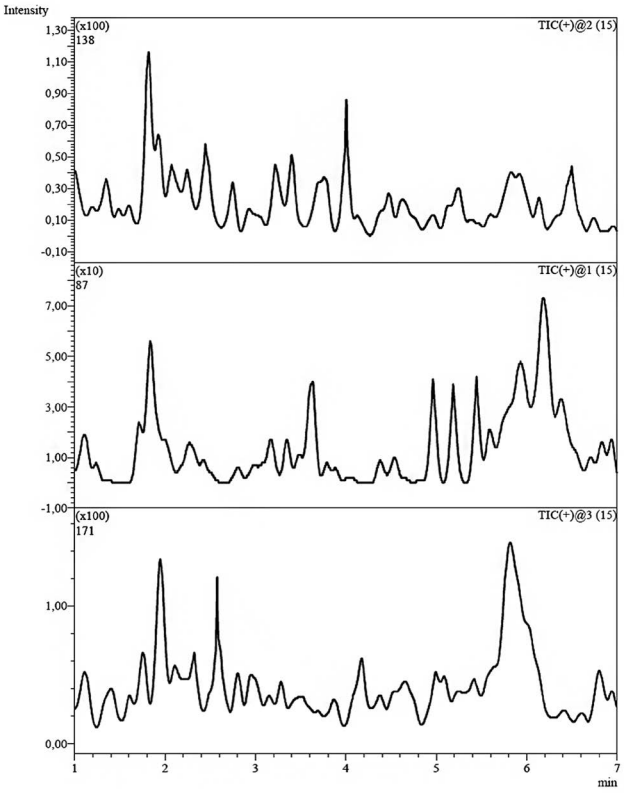

Figure 3. **Blank plasma sample chromatogram**

The matrix effect was assessed at low (level L) and high (level H) levels of analytical ranges of valganciclovir and ganciclovir concentrations (table 2).

Table 5. **The matrix factor of valganciclovir calculations, normalized by the IS matrix factor**

№	Mf of VAL (level L)	Mf of ACI (level L)	Normalised Mf (level L)	Mf of VAL (level H)	Mf of ACI (level H)	Normalised Mf (level H)
1	0.91	1.00	0.90	1.00	0.99	1.01
2	0.87	1.00	0.87	0.99	1.02	0.97
3	0.91	1.01	0.90	0.98	0.99	0.99
4	0.92	1.02	0.91	1.01	1.01	1.00
5	0.90	0.99	0.91	0.99	1.02	0.97
6	0.94	1.01	0.93	1.01	1.02	0.99
Average			0.90	Average		0.99
CV, %			2.18	CV, %		1.50

Table 6. **The matrix factor of ganciclovir calculations, normalized by the IS matrix factor**

№	Mf of GAN (level L)	Mf of ACI (level L)	Normalised Mf (level L)	Mf of GAN (level H)	Mf of ACI (level H)	Normalised Mf (level H)
1	1.18	1.00	1.17	1.04	0.99	1.04
2	0.98	1.00	0.98	0.82	1.02	0.81
3	0.97	1.01	0.97	0.84	0.99	0.85
4	1.14	1.02	1.12	0.99	1.01	0.98
5	1.29	0.99	1.30	0.85	1.02	0.84
6	1.21	1.01	1.19	1.00	1.02	0.98
Average			1.12	Average		0.92
CV, %			11.56	CV, %		10.58

The matrix effect for aciclovir IS was calculated at the level of 1000.00 ng/ml. The data is provided in tables 5 and 6

Calibration curve

The analysis of nine samples of intact blood plasma with addition of aciclovir IS working solution and working reference solutions of valganciclovir and ganciclovir up to the concentration specified

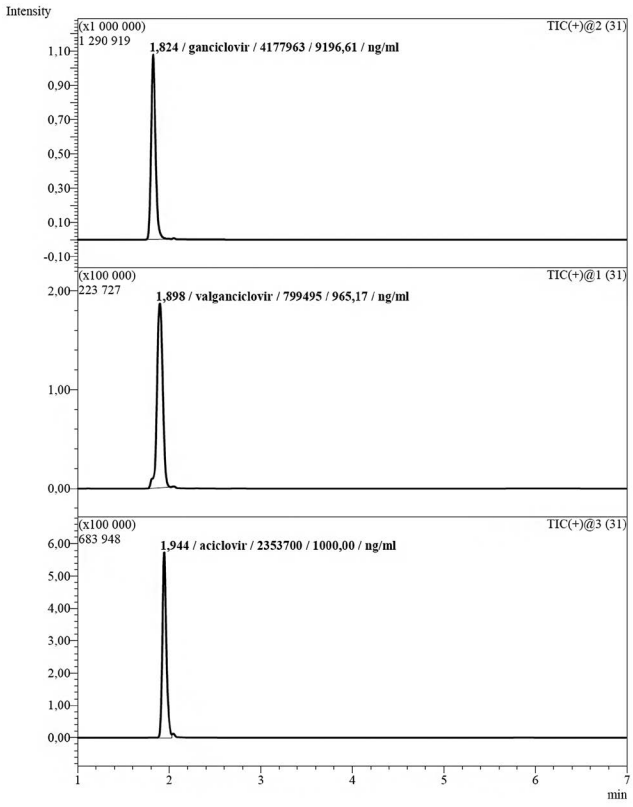

Figure 4. **Level 9 plasma sample chromatogram**

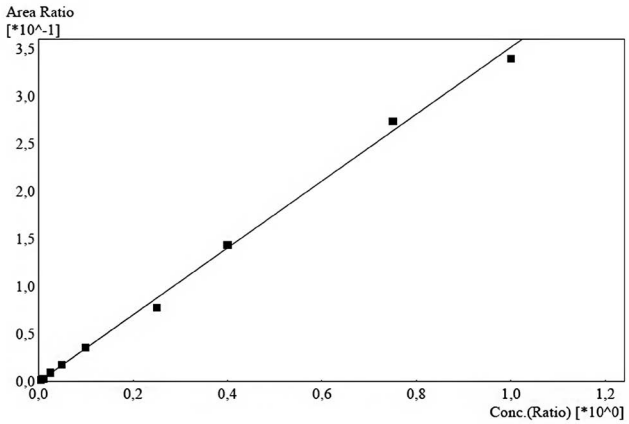

Figure 5. **The calibration curve representing dependence of the ratio area peak of valganciclovir to acyclovir on the concentration ratio of valganciclovir to the acyclovir in plasma**

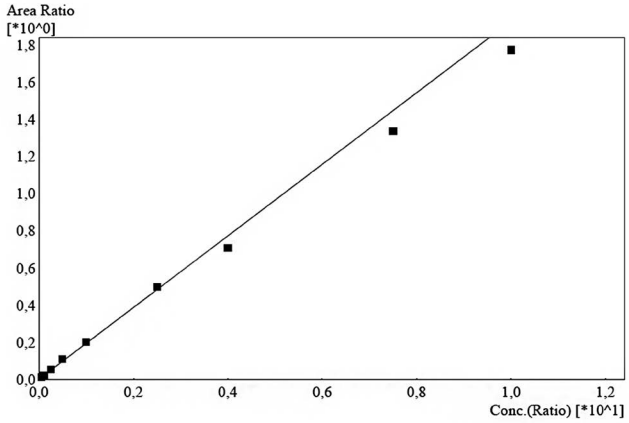

Figure 6. **The calibration curve representing dependence of the ratio area peak of ganciclovir to the acyclovir on the concentration ratio of ganciclovir to the acyclovir in plasma**

in table 2 (levels 1–9) was performed. Based on the obtained values, the calibration plot of "peak area of valganciclovir ratio to the peak area of aciclovir" from the ratio of valganciclovir concentration to aciclovir concentration in blood plasma (figure 5), as well as, the calibration plot of "peak area of ganciclovir to the peak area aciclovir from the ratio of ganciclovir concentration to aciclovir concentration of in blood plasma were constructed (figure 6). The obtained correlation coefficients meet the normal (not less than 0.99). The sample chromatogram at the level of 9 (table 2) is presented on figure 4.

Accuracy and precision

Blood plasma samples corresponding to the levels of LLOQ, L, M and H were analyzed (table 2). The analysis was performed in 3 sequences of 5 samples for each level. Accuracy and precision were evaluated within the cycle, between two cycles and between three cycles, as shown in table 7.

The obtained values of relative standard deviation (precision) and relative error (accuracy) correspond to the norms (not more than 20 % at LLQ level, not more than 15 % for the remaining points).

Recovery

To evaluate recovery (SI), 3 samples prepared from intact plasma were analyzed without recovery effect at levels L, M, and H (table 2), as well as quality control samples for recovery evaluation. Separately, hemolyzed intact blood plasma samples containing high lipid levels were analyzed. The data is shown in table 8.

Table 8. Calculation of valganciclovir and ganciclovir recovery at L, M, H levels from different biological matrix

	Recovery (level L), %	Recovery (level M), %	Recovery (level H), %
Valganciclovir			
Average	88.57	98.94	101.80
SD	6.78	2.76	2.48
RSD	7.66	2.79	2.43
Ganciclovir			
Average	93.98	90.95	98.01
SD	8.64	9.83	4.44
RSD	9.20	10.80	4.53

Recovery should not be equal to 100 %, but efficient and reproducible extraction of substances from the biological matrix should be ensured. The relative

Table 7. Accuracy and precision of the method

Injected (ng/ml)	Average found, ng/ml			SD			RSD, %			*E*, %		
	(*n* = 5)	(*n* = 10)	(*n* = 15)	(*n* = 5)	(*n* = 10)	(*n* = 15)	(*n* = 5)	(*n* = 10)	(*n* = 15)	(*n* = 5)	(*n* = 10)	(*n* = 15)
Valganciclovir												
5.00	5.80	5.45	5.29	0.08	0.62	0.56	1.37	11.35	10.53	16.08	8.96	5.89
15.00	14.46	14.08	13.72	0.62	0.75	0.80	4.26	5.31	5.86	–3.63	–6.11	–8.52
500.00	550.73	561.04	563.16	5.21	12.53	10.69	0.95	2.23	1.90	10.15	12.21	12.63
800.00	893.66	901.00	900.88	12.41	13.06	12.59	1.39	1.45	1.40	11.71	12.62	12.61
Ganciclovir												
50.00	48.32	47.39	47.18	1.13	2.33	2.06	2.33	4.91	4.37	–3.35	–5.21	–5.65
150.00	145.73	143.35	141.91	6.62	6.30	5.73	4.27	4.39	4.04	–2.85	–4.44	–5.39
5000.00	4740.25	4808.83	4892.22	77.26	104.66	153.94	1.63	2.18	3.15	–5.19	–3.82	–2.16
8000.00	7198.32	7372.03	7482.18	146.29	225.72	252.41	2.03	3.06	3.37	–10.02	–7.85	–6.47

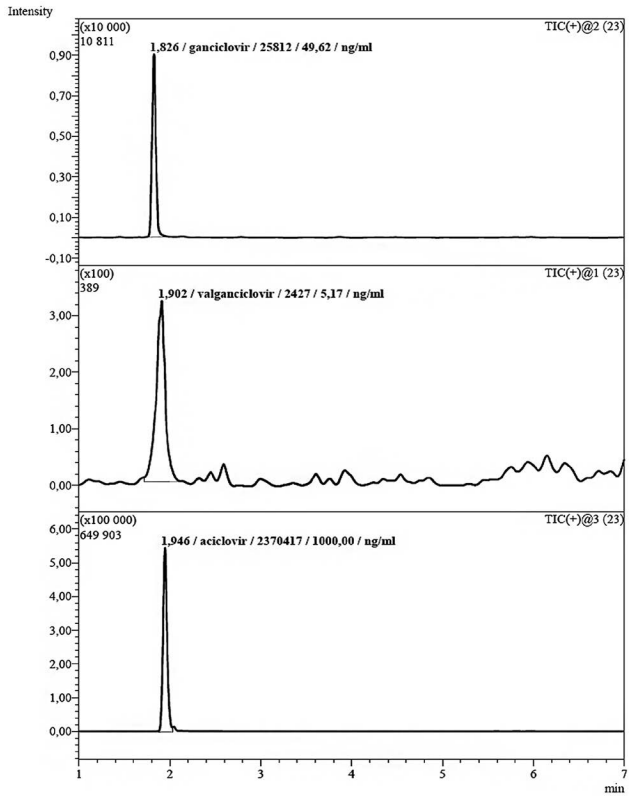

Figure 7. LLOQ plasma sample chromatogram

standard deviation of the calculated values of analyte recovery from biological matrices should not exceed 15 %.

Lower limit of quantification

LOQ methods were determined based on linearity, accuracy and precision data. As the method LLOQ, the minimum plasma concentration of valganciclovir and ganciclovir in blood in the analytical ranges for which valganciclovir and ganciclovir may be quantified with RSD and E values not more than 20 %, was taken LLOQ was 5.00 ng/ml for valganciclovir and 50.00 ng/ml for ganciclovir. The chromatograms of blood plasma containing valganciclovir and ganciclovir at LLOQ level are shown in figure 7. The detection limit of valganciclovir for the method was about 0.93 ng/ml, and the detection limit of ganciclovir was 0.73 ng/ml (signal-to-noise ratio 3.0).

Stability

The shot-term ("top-bench" and "postoperative") stability in triple freeze-thaw cycles, stability of reference solutions (when stored for 30 days at –45 °C), long-term stability (when stored for 59 days at –45 °C) of the test substances at lower and upper concentration levels. was confirmed.

Sample transfer

In the sequential analysis of the calibration sample with the highest concentration and the intact blood plasma sample on the chromatogram of the intact blood plasma sample, there were no peaks corresponding to the retention times of the peaks of the test substances and IS.

CONCLUSION

The method of determination of valganciclovir, ganciclovir in human blood plasma by HPLC-MS/MS was developed and validated. The confirmed analytical ranges of the procedure were 5.00–1000.00 ng/ml for valganciclovir and 50.00–10000.00 ng/ml for ganciclovir in human blood plasma. The obtained analytical ranges allow to use the developed procedure for the analytical part of pharmacokinetic studies of valganciclovir and ganciclovir.

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