ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Development of Methodology of Quantitative Determination Of Protein Kinase Inhibitor In Human Blood Plasma By Method of Highly Effective Liquid Chromatography With Mass-Spectrometric Detection.

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ABSTRACT

The method of quantitative determination on protein kinase inhibitor - imatinib in human blood plasma by means of highly effective liquid chromatography with tandem mass-spectrometric detection is developed. Chromatographic separation was achieved on column of 50x2.1 mm in size, filled by reversed-phase adsorbent C18 with particle size 1.7 mkm (ACQUITY UPLC BEH C18) at temperature of 45˚C. Mobile phase: methanol:0.01M solution of ammonia acetate in water (1:1). Method linearity was estableshedin range of concentrations of 0.03 to 3.83 mkg/ml in blood plasma. Method was successfully applied for studying of biological equivalence of reproduced medicine preparation which contains imatinib as active pharmaceutical ingredient, in comparison to reference one.

Keywords: imatinib; human blood plasma; highly effectivel iquid chromatography; validation.

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ISSN: 0975-8585

INTRODUCTION

Imatinib mesilate is an inhibitor of protein tyrosine kinase (Fig. 1). In the basic of clinical effectiveness of imatinib lies inhibition of BCR-ADL kinase. At first stages of pre-clinical and clinical tests was proved the effectiveness of pharmaceutical substance, and later of reference material too, for treatment of Ph-positive chronic mieloid leucosis at any phase. Later the preparation was approved for treatment of patients with KIT (CG117)-positive non-operable and / or metastatic gastrointestinal stromal tumors [1, 2]. At present time FDA approved a new indication to application of preparation Glivek – for treatment of children with detected for the first time acute lymphoblastic leucosis with positive Philadelphia chromosome (Ph +) [3]. In regard of clinical significance of considered preparation, the development and introduction in pharmaceutical market of reproduced medicine preparations containing imatinib, is actual.

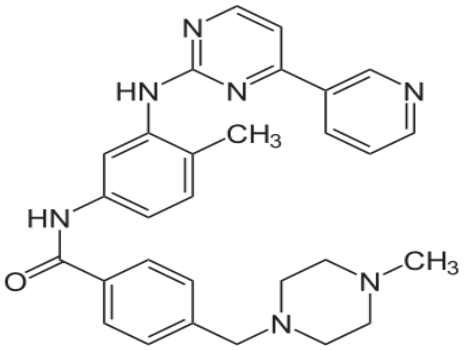

Fig. 1. Imatinib

At present time the method of highly effective liquid chromatography with mass-spectrometer detection (HELC-MS/MS) is a standard one for qualitative and quantitative estimation of analyte in plasma at conduction of researches of biological equivalence of medicine preparations.

In regard to stated above, the objective of this work is the development and validation of method of quantitative determination of imatinib by method of highly effective liquid chromatography with tandemmass-spectormeter detection.

Experimental part

In work were used the following reagents: imatinib, dasatinib (Sigma company), ammonia acetate (Merk company), methanol (Merk company) acetonitrile for gradient chromatography (Merk company), waster distilled and deionized by "Gene Pure" system (Thermo Scientific, USA).

Determination of imatinib in biological liquid (human blood plasma) was conducted on liqui chromatograph UltiMate 3000 RS LC (Thermo Fisher Scientifica, USA), equipped by vacuum degasser, gradient pump. auto-sampler, columns' thermostat. Detection of analyte was conducted on mass-spectrometer Velos Pro (Thermo Scientific, USA) with ionization in heated electrical spray (H-ESI-II). Development and validation of method was conducted in accordance to major requirements, set for bio-analytical methods [4, 5, 6, 7].

The study was supported by grant of the President of the Russian Federation № MD-4711.2015.7.

Samples preparation

With application of standard samples of dasatibin and imatinib (Sigma company) were prepared their initial solutions in 20% solution of acetonitrile in 0.1% formic acid in concentrations 0.0016% and 0.04% respectively. By series dilution of initial solution of imatinib were received its working solutions for construction of calibrating curve (in concentrations of 0.03 mkg/ml to 3.83 mkg/ml in plasma) and samples of quality control 0.1 mkg/ml; 1.8 mkg/ml and 3 mku/ml in plasma).

In order to prepare stated solutions to 600 mkg of plasma were added by 50 mkl 1% of water solution of formic acid, 50 mkl of internal standard solution, 50 mkl of respective initial solution of imatinib and 700 mkl

of acetonitrile. For preparation of blank into plasma was introduced 50 mkl of 1% formic acid water solution and 700 mkl of acetonitrile.

HELC-MS/MS

Chromatographic separation was achieved on column of 50x2.1 mm in size, filled by reversed-phase adsorbent C18 with particle size 1.7 mkm (ACQUITY UPLC BEH C18) at temperature of 45˚C. Chromatographic analysis as conducted with application of system UltiMate 3000 RS LC connected to mass-spectrometer detector in isocratic mode at the following chromatographic conditions:

HELC parameters	
Column:	ACQUITY UPLC BEH of 50x2.1 mm in size, filled by reversed-phase adsorbent C18 with particle size 1.7 mkm
Column temperature (˚C):	45
Sample amount (mkl):	2 mkl
Mobile phase:	methanol:0.01M solution of ammonia acetate in water (1:1)
Flow rate (ml/min):	0.4
Orientation retention times (min):	Imatinib – 2; Dasatinib – 3.
Injection time (min):	6

Studied analytes in plasma was detected with application ofmass-spectrometric detector Velos Pro – dual-chamber linear quadrupole ionic trap of low and high pressure with ionization in heated electrical spray (H-ESI-II) with technology of dual desolvation zone. Scanning was performed by selective chosen ions (SIM). Parameters of detector work are presented below:

Parameters of mass-spectrometer:	
Instrument:	Velos Pro (Thermo Scientific, USA)
Ionization type:	H-ESI
Polarity:	Imatinib "+"; Dasatinib "+".
Trasfer of masses:	Imatinib 494.3 →394.3; Dasatinib 488.0→401.2.
Collision energy:	Imatinib – 29; Dasatinib – 35.
Voltage on source (V):	Imatinib – 4500; Dasatinib – 5500.
Source temperature (°C):	Imatinib – 300 ; Dasatinib – 300.
Capillare temperature (°C):	Imatinib – 350; Dasatinib – 350.
Sheath gas pressure (Arb):	Imatinib – 40; Dasatinib – 40.
Aux gas pressure (Arb):	Imatinib – 15; Dasatinib – 15.
S-lens RF level (%):	Imatinib – 63.5; Dasatinib – 60.6.

RESULTS AND DISCUSSION

Application of column ACQUITY UPLC BEH of 50x2.1 mm in size, filled by reversed-phase adsorbent C18 with particle size 1.7 mkm in method allowed to receive satisfactory form of imatinib and dasatinib peaks. Chromatographic scheme was considered suitable at conduction of the following criteria: relative standard deviation of relation of squares of determined peaks to the peak of internal standard, calculated by six sequential chromatograms of one concentration solution – no more than 7%; factor of asymmetry of peaks of imatinib and dasatinib – no more than 2.2; effectiveness of chromatographic column, calculated by peak of imatinib – at least 1000 theoretical plates; signal/noise proportion for solution at level of lower limit of quantitative determination (LLQD) at least 10:1. Chromatograms of dasatinib (internal standard) and imatinib with concentration in plasma on level of LLQD (0.03 mkg/ml in plasma) are presented in Fig. 2.

ISSN: 0975-8585

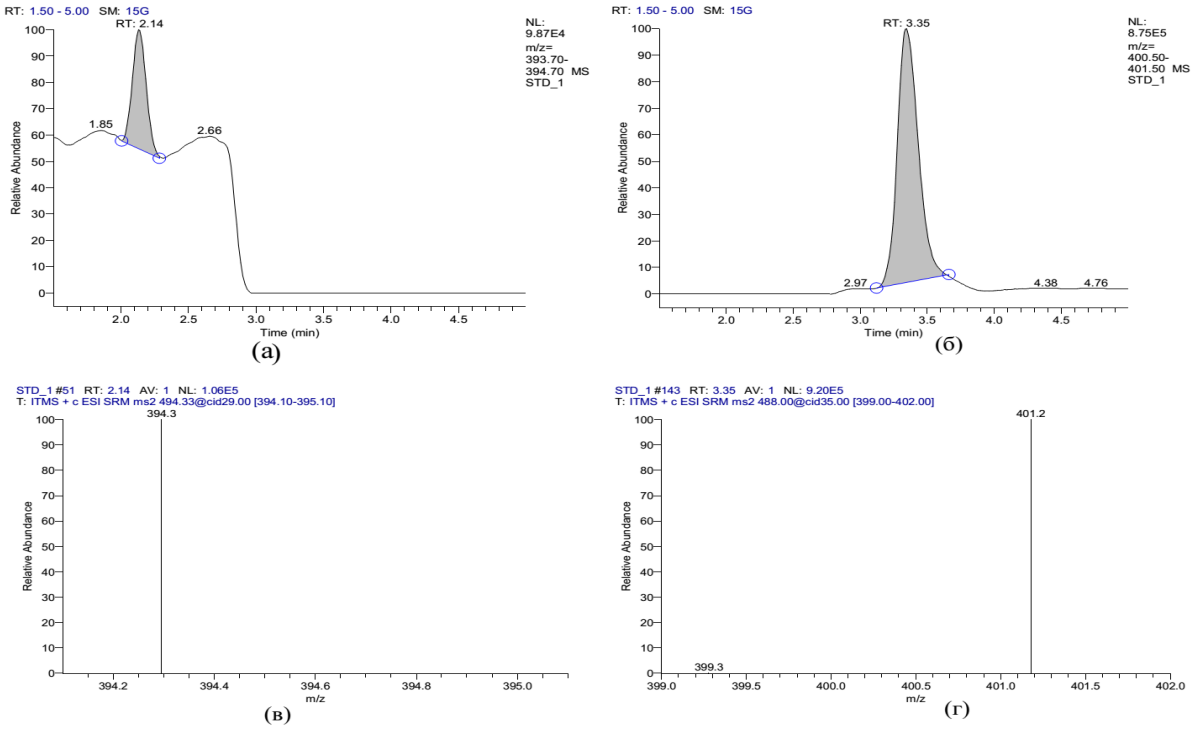

Fig. 2 - Chromatograms and mass-specter of imatinib (Fig. 2a and 2c) and dasatinib (fig. 2b and 2d) in plasma.

validation of analytical method was conducted in accordance to international requirements set for bioanalytical methods by the following indexes: selectivity, matrix effect, linearity, lower limit of quantitative determination, linearity, specificity, precision, reproductivity [4].

In order to avoid violation of linearity of gradual dependence related to impact of components of biological matrix on ionization in mass-spectrometry, one of important stages at development and validation of methods in blood plasma is the study of matrix effect. The stated index was researched on six samples f biological matrix of different donors. Values of coefficient of variations (CV) of normalized matrix factors for six different matrix on lower and upper levels of concentrations did not exceed 15% and were 12.8% and 5.1% respectively.

For construction of calibration curve were used the working solutions on seven levels of concentrations in tree repeats, blank (pure matrix) and blank with internal standard (matrix without introduction of analyte, but with addition of internal standard). Extracted standards of imatinib from plasma allowed to receive the linear calibration curve in dynamic range of 0.0.3 to 3.83 mkg in 1 ml of biological object with coefficient R^2 equal to 0.9997. None on mistakes did not exceed permissible thresholds – no more than 20% for LLQD solution and no more than 15% for remaining concentrations.

Correctness of method was estimated on samples of biological matrix with addition of known quantities of analyte on levels of concentrations: LLQD, lower quality control (LQC), average quality control (AQC) and upper quality control (UQC) that were received independently of solutions prepared for confirmation of method's linearity. Accuracy was expressed in per cents of nominal imatinib value. Results are represented in Table 1.

ISSN: 0975-8585

Table 1: Accuracy of determination of imatinib in plasma

Level of solution concentration		Cycle 1		Cycle 2		Cycle 3		Cycle 4	
	C_{intr}, mkg/ml	C_{found}, mkg/ml	CV, %	C_{found}, mkg/ml	CV, %	C_{found}, mkg/ml	CV, %	C_{found}, mkg/ml	CV, %
LLQD	0.030	0.0277	9.7	0.0304	9.0	0.0270	5.0	0.0332	2.0
		0.0316		0.0311		0.0302		0.0339	
		0.0300		0.0258		0.0292		0.0349	
		0.0333		0.0295		0.0278		0.0333	
		0.0260		0.0331		0.0303		0.0338	
LQC	0.100	0.1054	8.4	0.0968	3.9	0.0885	1.8	0.0934	3.1
		0.0919		0.0895		0.0884		0.0948	
		0.1132		0.0870		0.0885		0.0931	
		0.0946		0.0918		0.0902		0.0998	
		0.1008		0.0916		0.0922		0.0930	
AQC	1.80	1.8385	2.2	1.9570	3.6	1.8740	3.1	1.7862	2.2
		1.7426		1.7764		1.9424		1.7001	
		1.8330		1.8527		1.9452		1.7612	
		1.7980		1.8449		1.9052		1.7258	
		1.7811		1.8252		1.8051		1.7847	
UQC	3.00	2.9017	2.8	2.9674	4.8	2.7971	3.8	2.8513	2.7
		2.9098		3.0135		2.7798		2.9256	
		2.7624		2.7706		2.6126		2.7188	
		2.7584		2.6888		2.6625		2.8653	
		2.9072		2.9214		2.8663		2.8373	

In order to conduct the precision test was conducted six repeated measures of concentration (LLQD, LQC, AQC, IQC) of imatinib in the same sample by method described above in four different cycles. All results were close to each other (Table 2).

Table 2: Precision of method of determination of imatinib in plasma

Solution	$C_{found\ ave}$, mkg/ml	$C_{found\ ave}$, mkg/ml	$C_{found\ ave}$, mkg/ml	$C_{found\ ave}$, mkg/ml	CV, %	Criterion of acceptance
	day 1	day 2	day 3	day 4		
LLQD	0.0297	0.0300	0.0289	0.0338	7.2	no more than 20%
LQC	0.1012	0.0914	0.0896	0.0948	5.4	no more than 15 %
AQC	1.7986	1.8513	1.8944	1.7516	3.4	no more than 15 %
UQC	2.8479	2.8723	2.7437	2.8397	2.0	no more than 15 %

Therefore, the developed method of quantitative determination of imatinib in human blood plasma by method of highly effective liquid chromatography with mass-spectrometer detection is simple in performance, accords to requirements of validation characteristics and accurately allows to determine imatinib in blood plasma in concentrations of 0.03 mkg/ml to 3.83 mkg/ml.

ISSN: 0975-8585

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