A New and Sensitive LC-MS/MS Method for the Determination of Clopidogrel in Human Plasma

NELA BIBIRE[1], MADALINA VIERIU[1], GLADIOLA TANTARU[1]*, MIHAI APOSTU[1], LUMINITA AGOROAEI[1], ALINA DIANA PANAINTE[1], ALEXANDRU ZNAGOVAN[2], AUREL VLASE[3]

[1]"Grigore T. Popa" University of Medicine and Pharmacy of Iasi, Faculty of Pharmacy, 16 University Str, 700115, Iasi, Romania
[2]Nicolae Testemitanu" State University of Medicine and Pharmacy, 165 Stefan cel Mare si Sfînt Blv., Chisinău, Moldova
[3]Center for Drug Evaluation, S.C. Antibiotice S.A., Valea Lupului, 1 Str., 707410, Iasi, Romania

A high throughput and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the determination of clopidogrel in human plasma, using clopidogrel-d3 as internal standard. Detection was performed by positive ion electrospray tandem mass spectrometry on a HPLC Agilent 1200 Triple Ouad 6410 System. The mass transition of the ion-pair was followed for m/z 322.1→212.1, for clopidogrel and m/z 327→217, for clopidogrel-d3, in multiple reaction monitoring mode (MRM). The plasma samples were buffered (pH 2.0), extracted using a mixture hexan-ethyl acetate and then 10μL of the sample extract was injected into the LC-MS/MS system. Clopidogrel and the internal standard were separated on a Zorbax SB-C8 column using a mobile phase consisting of 50:50 (v/v) mixture of acetonitrile and 0.1% formic acid aqueous solution, with a flow rate of 1 mL/min. The proposed method has been validated for a linear range of 10-10000 mg/mL and the value of the regression coefficient was ≥0.99. The precision (CV%) and accuracy (%) were lower than 6.1 % for intra- and inter-days assays. The overall recoveries for clopidogrel and clopidogrel-d3 were 81 and 84%, respectively. The overall time of the analysis was 7 min. The method is sensitive, specific and reproducible and can be used for the therapeutic drug monitoring of clopidogrel.

Keywords: clopidogrel, HPLC, bioanalytical method validation

Clopidogrel is a thienopyridine derivative: [methyl(+)-(S)-α-(2-chloro-phenyl)-6,7-dihydrothienol[3,2-*c*]pyridin-5(4H)-acetate hydrogen sulfate] (fig. 1). It is a potent antiplatelet drug prescribed for the prevention of vascular thrombotic events. Following oral administration, clopidogrel is rapidly absorbed and undergoes extensive hepatic metabolism [1, 2].

Several assay methods are available for determination of clopidogrel, including HPLC-UV [3-7].

The HPLC assays are not sensitive enough to measure clopidogrel levels in biological fluids after oral administration of therapeutic doses. Recently, LC-MS/MS has been increasingly utilized as it can analyze test samples regardless of their purity in biological substances and measure the blood drug concentrations with high sensitivity and selectivity [8-12].

The present study reports a simple and sensitive LC-MS/MS method for the determination of clopidogrel in human plasma. This assay can be used for the therapeutic drug monitoring of clopidogrel.

The parameters usually examined in the validation process are selectivity/specificity, linearity, limit of quantification, accuracy and precision [13].

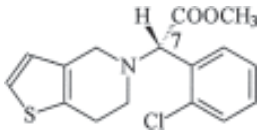


Fig. 1. Chemical structure of clopidogrel

Experimental part

All analyses were performed using the Agilent 1200 Triple Quad 6410 System. The system components included the Agilent 1200 Degasser, Agilent 1200 Binary Pump, Agilent 1200 Autosampler, Agilent 1200 Mass Selective Detector. The Agilent MassHunter software was used for system control and data acquisition. An analytical balance Mettler-Toledo XP56, a Sigma 2-16 K centrifuge and a Vibramax 110 shaker were used for the sample preparation. The separation was performed using a reverse phase column Zorbax SB-C8, supplied by Agilent, USA.

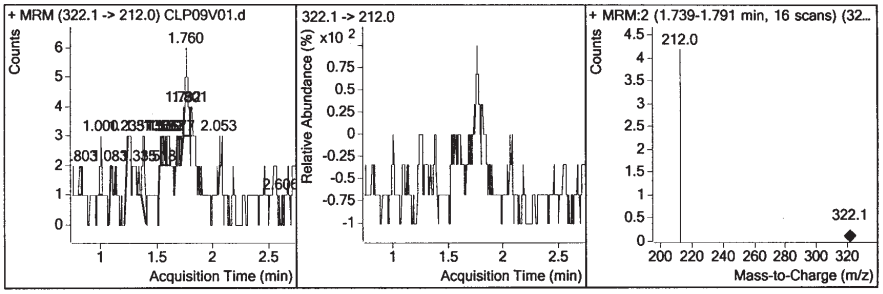
The standard clopidogrel hydrogen sulfate used in this study was supplied by U.S. Pharmacopoeia, with a purity of 98.8% as clopidogrel. The standard clopidogrel-d3 hydrogensulfate was supplied by SynFine Research. All solvents and other chemicals were HPLC grade provided by Merck, Germany. The human plasma (anticoagulant K3-EDTA) was obtained from SCIPAC, U.K.

The mobile phase consists in a mixture of 0.1% formic acid aqueous solution and acetonitrile (50-50, v/v), at flow rate of 1 mL/min.

A stock solution of clopidogrel with a concentration of 0.2 mg/mL was prepared by dissolving the appropriate quantity of clopidogrel bisulfate reference substance in 10 mL acetonitrile. A stock solution of clopidogrel-d3 with a concentration of 0.2 mg/mL was prepared by dissolving an appropriate quantity of clopidogrel-d3 hydrogensulfate reference substance in 10 mL acetonitrile. These solutions were stored at -25°C. It was established that they were stable for at least 95 days, in those conditions.

Nine solutions of different concentration were prepared for the study of the linearity response in human plasma,

*email: gtantaru2@yahoo.com



ISTD Compound *Clopidogrel-d3*

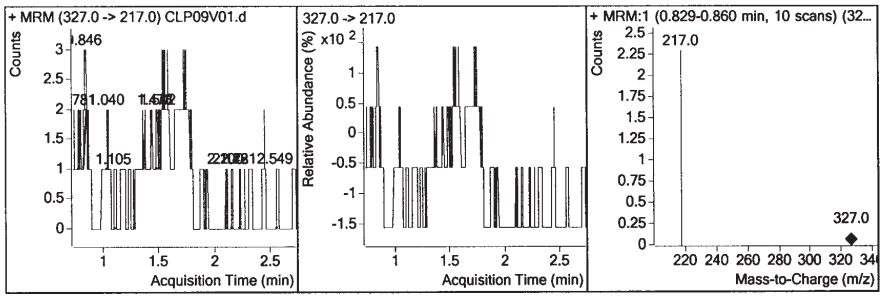


Fig. 2. Chromatogram recorded for blank sample

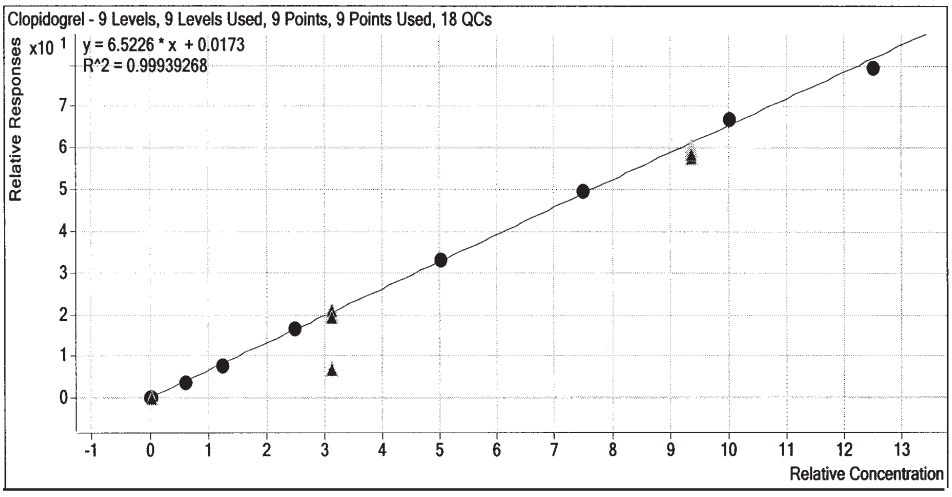


Fig. 3. The clopidogrel calibration curve obtained for plasma samples

covering the range of concentrations 10-10000 pg/mL. The theoretical concentrations of clopidogrel calibration standards were 10; 20; 500; 1000; 2000; 4000; 6000; 8000; 10000 pg/mL.

The quality control samples of clopidogrel with theoretical concentrations of 30, 2500 and 7500 pg /mL were considered to be appropriate for used in the validation of the analytical method.

The internal standard solution was added to each plasma sample in borosilicate glass tube, and those were used as plasma sample. The plasma samples were buffered (*p*H 2), and then they were extracted using a mixture hexan-ethyl acetate on a vortex mixer for 5 min. The upper organic layer was transferred to clean test tubes and evaporated at 40^{0}C under nitrogen. The residue was dissolved in a mixture water-acetonitrile (50:50,v/v). 10 µL of this solution was injected into the chromatographic system.

Results and discussions

The study of Selectivity/Specificity: The method described in this paper has been tested for possible interferences from other plasma factors. No overlapping peaks were detected at clopidogrel and internal standard retention time, 1.7 min (fig. 2). The bioanalytical method proved to be selective.

The linearity was investigated for theoretic concentration between 10-10000 pg/mL clopidogrel and the calibration curve was derived by plotting the peak-height ratio of the analyte and the internal standard against the concentration of clopidogrel, using linear regression analysis. The least-square linear regression revealed that the relationship was linear in the investigated domain, with a regression coefficient of 0.99993, meeting the acceptance criteria - $r^2 \geq 0.990$ (fig. 3).

The lower limit of quantification, i.e. the lowest standard level with a coefficient of variation less than 20%, is for clopidogrel 10 pg/mL. The bioanalytical method proved to be sensitive, allowing a precise quantification of concentrations as low as 10 pg/mL (fig. 4). Results are presented in table 1.

Table 1
LOWER LIMIT OF QUANTIFICATION

Analyte Concentration: 10 pg/mL		
	Calculated Concentration (pg/mL)	Accuracy %
	10.674	106.74
	11.052	110.52
	10.249	102.49
	9.508	95.08
	7.853	78.53
	7.527	75.27
N	6	6
Mean	*9.477*	*94.772*
SD (±)	*1.480*	
CV(%)	*15.615*	

Acceptance criteria: 4 out of 6 LLQC must be within 100±20% nominal value. Mean % Nominal 100±20%. CV (%)≤20%

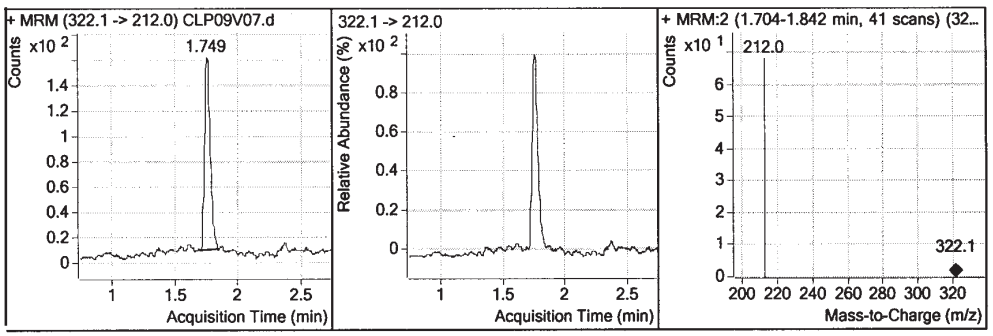
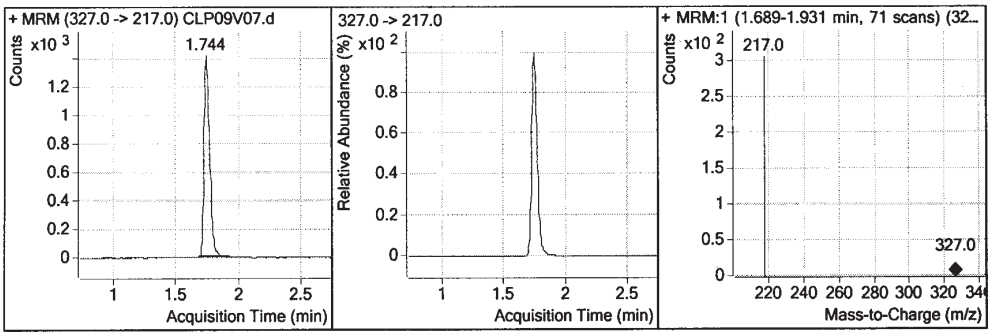


Fig. 4. Chromatogram recorded for sample containing 10 pg/mL clopidogrel



	Theoretical concentration - QC1 (30 pg/mL)		Theoretical concentration - QC2 (2500 pg/mL)		Theoretical concentration - QC3 (7500 pg/mL)	
No.	Calculated Conc. (pg/mL)	Accuracy %	Calculated Conc. (pg/mL)	Accuracy %	Calculated Conc. (pg/mL)	Accuracy %
1.	29.428	98.027	2540.724	101.578	7759.080	103.403
2.	28.132	93.712	2564.092	102.512	7828.556	104.329
3.	30.819	102.662	2488.762	99.501	7710.387	102.754
4.	27.122	90.345	2644.044	105.709	7848.035	104.588
5.	28.557	95.128	2560.978	102.388	8040.836	107.158
6.	25.855	86.127	2662.261	106.437	8087.979	107.786
N	6	6	6	6	6	6
Mean	*28.319*	*94.396*	*2576.810*	*103.072*	*7879.146*	*105.055*
SD (±)	*1.736*		*65.252*		*152.432*	
CV(%)	*6.132*		*2.532*		*1.935*	

Table 2
INTRA-DAY PRECISION AND ACCURACY FOR CLOPIDOGREL QUALITY CONTROL SAMPLES

Acceptance criteria: 67% Total QCs must be 100±15% nominal values.50% QCs per level must be 100±15% nominal values. Mean % nominal 100±15%; CV(%) ≤15%

	Theoretical concentration - QC1 (30 pg/mL)		Theoretical concentration - QC2 (2500 pg/mL)		Theoretical concentration - QC3 (7500 pg/mL)	
No.	Calculated Conc. (pg/mL)	Accuracy %	Calculated Conc. (pg/mL)	Accuracy %	Calculated Conc. (pg/mL)	Accuracy %
1.	32.853	109.510	2431.698	97.268	7727.130	103.028
2.	31.685	105.617	2455.572	98.223	7412.419	98.832
3.	29.323	97.743	2450.892	98.036	7557.611	100.768
4.	29.097	96.990	2386.714	95.469	7876.531	105.020
5.	28.763	95.877	2438.067	97.523	7321.038	97.614
6.	29.858	99.527	2496.953	99.878	7424.827	98.998
N	6	6	6	6	6	6
Mean	*30.263*	*100.877*	*2443.316*	*97.733*	*7553.259*	*100.710*
SD (±)	*1.636*		*35.919*		*212.138*	
CV(%)	*5.407*		*1.470*		*2.809*	

Table 3
INTER-DAY PRECISION AND ACCURACY FOR CLOPIDOGREL SPIKED QUALITY CONTROL SAMPLES

Acceptance criteria: 67% Total QCs must be 100±15% nominal values. 50% QCs per level must be 100±15% nominal values. Mean % nominal 100±15%. CV(%) ≤ 15%.

The accuracy and the precision of the method were calculated for three concentration levels of clopidogrel in human plasma. Six clopidogrel samples with theoretical concentration levels of 30 pg/mL (QC1), 2500 pg/mL (QC2) and 7500 pg/mL (QC3) were repeatedly injected into the system. Table 2 summarizes the results obtained for the intra-day parameters. The inter-day precision and accuracy were evaluated also, using six aliquots for each quality control sample concentration, prepared and analyzed in six different days. The results are presented in table 3.

Conclusions

A liquid chromatography-tandem mass spectrometry method has been developed and validated for the determination of clopidogrel in human plasma samples. This chromatographic assay fulfilled all the requirements of accuracy and precision, linearity, selectivity/specificity.

The assay has proven to be sensitive, specific and reproducible and it can be used for therapeutic drug monitoring of clopidogrel.

References

1. MIHORIANU, M., MANGALAGIU, I.I., JONES, P., DANILIUC, C.G., FRANZ, H., NEDA, I., Rev. Roum. Chim., **55**, 2010, p. 689.
2. FODOR, E., MAFTEI, C., MANGALAGIU, I.I., JONES, P., DANILIUC, C.G., FRANZ, H., NEDA, I., Rev. Roum. Chim., **55**, 2010, p. 559.
3. MITAKOS, A., PANDERI, I., J. Pharm. Biomed. Anal., **28**, 2002, p. 431.
4. TAKAHASHI, M., PANG, H., KAWABATA, K., FARID, N.A., KURIHARA, A., J. Pharm. Biomed. Anal., **48**, no. 4, 2008, p. 1219.
5. BEOM, S.S., SUN, D.Y., Biomed. Chromatogr., **21**, no. 9, 2007, p. 883.
6. RAMAKRISHNA, N., VISHWOTTAM, K., MANOJ, S., KOTESHWARA, M., SANTOSH, M., RAVIKUMAR, B., Rapid. Commun. Mass. Sp., **20**, no. 11, 2006, p. 1695.
7. ZOU, J.J., FAN, H.W., GUO, D.Q., LI, Y.B., SONG, L., ZHU, Y.B., YU, C.X., JIE, Z., LIU, J.H., HU, Y.F., Chromatographia, **70**, no. 11-12, 2009, p. 1581.
8. BIBIRE, N., TANTARU, G., APOSTU, M., AGOROAEI, L., VIERIU, M., PANAINTE, A.D., VLASE, A., Rev. Chim. (Bucharest), **64**, no. 6, 2013, p. 587.
9. ENACHE, C., FILIP, P., RAZUS, A., Rev. Chim. (Bucharest), **61,** no. 6, 2010, p. 546.
10. PEER, C.J., SPENCER, S.D., VANDENBERG, D.A., PACANOWSKI, M.A., HORENSTEIN, R.B., FIGG, W.D., J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., **880**, no. 1, 2012, p. 132.
11. KARAZNIEWICZ-ŁADA, M., DANIELAK, D., TE YK, A., ZABA, C., TUFFAL, G., GŁÓWKA, F., J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **911**, 2012, p. 105.
12. ENACHE, C., BIRZAN, L., RAZUS, A., Rev. Chim. (Bucharest), **63**, no. 6, 2012, p. 636.
13. *** Guidance for Industry: Bioanalytical Method Validation. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001

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