Development and Validation of a Bioanalytical Method for the Quantification of Clopidogrel in Human Plasma by LC-MS/MS using the Accuracy Profiles

Choukri El Khabbaz[†], Aimen El Orche*[‡], Mustapha Bouatia[§], Amine Cheikh[ǁ], My El Abbes Faouzi[ǂ], Yahia Cherrah[ǁ], Ratiba Boussen[†], Houda Bouchafra[#]

[†] Laboratory of Materials Nanotechnologies and Environment, Faculty of Science, Mohammed V University, Rabat, Morocco;
[‡] Team of Analytical & Computational Chemistry, Nanotechnology and Environment, Faculty of Science and Techniques, University of Sultan Moulay, Slimane, Beni Mellal-Morocco; *e-mail: aimen.elorche@gmail.com
[§] Laboratory of Analytical Chemistry, Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco;
[ǁ] Department of Pharmacy, Faculty of Pharmacy, Abulcasis University, Rabat, Morocco;
[ǂ] Laboratory of Pharmacology and Toxicology, Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco;
[#] Laboratory of Drug Sciences, Biomedical and Technological Research, Faculty of Medicine and Pharmacy, Hassane II University, Casablanca, Morocco;

Recieved: January 27, 2022; Accepted: August 09, 2022
DOI: 10.17721/moca.2022.153-161

A basic bio-analytical method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed for the determination of clopidogrel in human plasma. Clopidogrel-d3 was used as an internal standard (IS). Protein precipitation with acetonitrile for extraction of clopidogrel and it IS was used. Chromatographic separation of clopidogrel and IS was performed on a Synergi reverse phase C18 column (150 mm × 2 mm, 4 µm) using an isocratic mode. The mobile phase contained a mixture of 0.1% formic acid and acetonitrile (25:75) applying a flow rate of 0.4 mL/min. Ionization was performed by an electrospray ionization source (ESI), in positive MRM (Multiple Reaction Monitoring) mode. This method was validated by accuracy profiles approach using tolerance intervals limits. The calibration curve ranged from 0.0156 ng/mL to 8 ng/mL. This method can be employed effectively in bioequivalence and/or pharmacokinetics. In this method we have avoided the use of methanol in all steps of bioanalysis of clopidogrel to avert the back-conversion of clopidogrel metabolite.

Keywords: tandem mass spectrometry, clopidogrel, accuracy profiles

Clopidogrel hydrogen sulfate (d-methyl [2-chlorophenyl]-5-[4,5,6,7-tetrahydrothieno] [3,2-c pyridinyl] acetate hydrogensulfate) is a prodrug of the thienopyridine class, which inhibits platelet aggregation. Following metabolism, a thiol compound is formed by the oxidation of clopidogrel to 2-oxoclopidogrel [1]. This active metabolite, that selectively and irreversibly inhibits platelet aggregation, i.e., prevents binding of adenosine diphosphate to the platelet P2YI2 receptor [2,3]. This metabolite has not been detected in plasma based on EMA (European Medicines Agency more informations) recommendation [1].

Clopidogrel is poorly quantifiable in plasma, however the choice of chromatographic conditions is very important in order to quantify clopidogrel for pharmacokinetic study. We reported the different chromatographic conditions that are used by different researchers. The extensive literature survey revealed that the bioanalysis of clopidogrel and clopidogrel carboxylic acid is primarily performed by LC-MS/MS [4-18], except a study reported using HPLC-UV method for the detection of clopidogrel carboxylic acid in human serum [19].

Most researchers have used formic acid in water [11-18] and acetonitrile [5-8,10-11,13,15-19]. The others used ammonium formate with different concentration [5-8], and a lot of researchers have worked with methanol as an organic solvent in the mobile phase [12,14]. The elution mode of the mobile phase was either isocratic [5-11,13-14,16-17] or gradient [12,15,18] different type of column was used C8 [5-8,12-13] and C18 [10-11,14-15,19]. The length of the columns is between 50 and 100 mm, and the particle size is between 2.1 and 5 µm.

Various internal standards were used in the literature: Phenytoin [19], Ticlopidine [6,8,10,15], Repaglinide [11,14] Clopidogrel-d3/d4 [5,8,12], Clopidogrel-d4 [13,16], Nateglinide and Pioglitazone [11,14], Methaqualone [17], Piroxicam [18].

These internal standards have different physicochemical properties compared to clopidogrel. The most used IS (internal standard) are clopidogrel-d3 and Ticlopidine, they are the closest molecules of clopidogrel.

The main purpose of sample preparation is to

remove constituents from the biological matrix and make the sample more effective for analysis, various sample preparation of clopidogrel was used by researchers. Liquid-liquid extraction [4-8,10,14,17], protein precipitation [12,15,18] and solid phase extraction (SPE) [12,16].

The present paper describes a bioanalytical method that is simple and rapid for analysis of clopidogrel in human plasma. The LC-MSMS was used as an analytical method and the samples was prepared with protein precipitation. The aim through this study was to develop a method without methanol and which can quantify a lowest concentration of clopidogrel in human plasma so that it will be used easily in pharmacokinetics and bioequivalence trials.

Materials and Methods

Materials and Reagents

Clopidogrel was provided by Dr. Reddy's (Hyderabad, INDIA) and its internal standard (clopidogrel-d3) was delivered by Clearsynth (Telangana INDIA). LC-MS grade acetonitrile was purchased from VWR (Fantenay-sous-bois, France). The formic acid was purchased supplied from Merck (Darmstadt, Germany).

Instrument

Agilent UHPLC (LC/MS/MS) system (1290 infinity II) consist of quaternary pump solvent, auto sampler and column temperature oven. Mass spectrometry system triple quadrupole (6420) with electrospray ionisation ESI as an ionisation source.

Preparation of stock and working solutions

In order to prepare the stock solution of clopidogrel and its internal standard. Two stock solutions were prepared, with a concentration of 1mg/ml in acetonitrile. From the stock solution of Clopidogrel, working solutions were prepared to obtain concentrations range from 0.0156 to 8 ng/mL, and 5 ng/mL of Clopidogrel-d3.

Sample preparation

A sample preparation method by protein precipitation has been developed to extract clopidogrel and its internal standard from human plasma using acetonitrile as a precipitating agent.

The plasma samples were obtained by the medical biology laboratory of sheikh zaid foundation, with six different batches of plasma. 1ml of plasma sample was spiked with 100 µl of IS (0.05 µg/mL), then 2 ml of acetonitrile was added. After 1 min of vortex, at a temperature of 6°C, the sample was centrifuged for 10 min at 5000 r.p.m, the supernatant was filtered with a cellulose filter (0.2 µm), and then 10 µl of the solution was injected into the LC-MS/MS system .

Assay validation

Specificity and selectivity. Specificity is the capacity of a bioanalytical method to identify and differentiate the analyte from other substances, including its related substances. After injection of a blank plasma sample and a plasma sample spiked with the IS and the LLOQ (lower limit of quantification) concentration of the analyte. The response of the interfering peaks to the retention time of the analyte in the blank plasma should be less than or equal to 20 % of the response LLOQ concentration (ICH M10). The response of the interfering peaks to the retention time of the internal standard in the blank must be less than or equal to 5 % of the internal standard concentration. Analysing six distinct batches of human plasma to establish the lack of interference from endogenous plasma components is used to assess the method's specificity and selectivity.

Calibration and quality control samples. Eight point calibration curve was prepared by spiking 25 µL of working solution into 475 µL of blank plasma in propylene tube for obtaining final clopidogrel concentration range between 0.0156 – 8 ng/mL. The concentrations used for clopidogrel quality control were prepared with the same way to obtain final concentration: 0.0156 ng/mL as a LLOQ (lower limit of quantification), 0.062 ng/mL as a LQC (lower quality control), 3 ng/mL as a MQC (Medium quality control) and 6 ng/mL as a HQC (high quality control). With the exception of the LLOQ, which has a nominal percentage that does not exceed 20 %, all calculated concentrations must have a nominal percentage of 15.0 %. Calibration standards and the QCs should be prepared from separate stock solutions in order to avoid biased estimations which are not related to the analytical performance of the method (ICH).

Precision and trueness. The precision and accuracy will be assessed on the four quality control samples: lower limit of quantification, lower quality control, medium quality control and high quality control (LLOQ, LQC, MQC, and HQC). Over six repetitions of each QC concentration, intra-day accuracy and trueness are assessed.

Accuracy profile. The bioanalytical method was validated using the accuracy profile concept developed by the SFSTP (French Society of Pharmaceutical Sciences and Techniques) commissions. The accuracy profile is a simple choice tool based on total error with a guarantee that predicts future results when using an approved approach. Accuracy profile was acquired using tolerance intervals, calculating the upper and lower limits of the interval at each concentration level to construct a visual and easy-to-interpret graph.

Results and Discussion

Analysis conditions

For the detection of clopidogrel and IS, the LC-MS/MS conditions were extensively optimized in this work.

Using nitrogen as the drain gas, a quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source led to a flow rate of 1 ml/min, a nebulization pressure of 50 psi, and a gas drying temperature of 330 °C. The fragmentation voltage is 130V for Clopidogrel and 116V for Clopidogrel d3. LC-MS/MS was carried out in MRM (Multiple Reaction

Monitoring) mode using precursor and product ions [M + H] + m / z 322.1 → 212 and 184 for Clopidogrel with two collision energies of 14 eV and 22 eV respectively, [M + H] + m / z 325.09 → 215 for the Clopidogrel d3 (EI) with a collision energy of 14eV as shown in Figure 1.

The Agilent 1290 LC/MS/MS chromatographic chain is equipped with an auto-sampler kept at a temperature of 6 °C, a thermostatically controlled column oven set at 40 °C. A C18 (4 µm 150 × 2 mm) was used for analysis .The injection volume was set at 10 µL.

To conduct this analysis a mobile phase consisting of a mixture of 0.1 % Formic Acid: Acetonitrile (25 %: 75 %) in an isocratic mode was used.

Method validation

Specificity and selectivity

The specificity of a bioanalytical method was demonstrated by comparing the chromatograms of blank plasma and plasma spiked with the LLOQ concentration of clopidogrel and it IS. The performance of this test shows that the percentage of interference does not exceed 20 % for clopidogrel and 5 % for the IS in the six batches of plasma tested. The developed LC-MS/MS method exhibited good specificity, as the chromatograms of the extracted ions showed that there was no interference at the same retention time of clopidogrel (2.528 min) and IS (2.513 min). Representative chromatograms of multiple reaction monitoring (MRM) of blank plasma, blank plasma spiked with LLOQ of clopidogrel and it IS in Figure 2. These results confirm the high specificity and selectivity of this method as shown in Table 1.

Matrix effect and recovery

The recovery and the matrix effect were calculated at low, medium and high concentration levels. The recovery rates ranged from 81.66 % to 103.05 % and the matrix effects were in the range of 99.91 % to 114.64 %). Matrix effect does not exceed an interval of ±15 %, this results indicated that a significant matrix effect was absent as shown in Table 2.

The recovery obtained for all three concentrations is greater than 50 % with a coefficient of variation less than 15 % (RSD ≤ 15 %), the coefficient of variation of the matrix effect is less than 15 %.

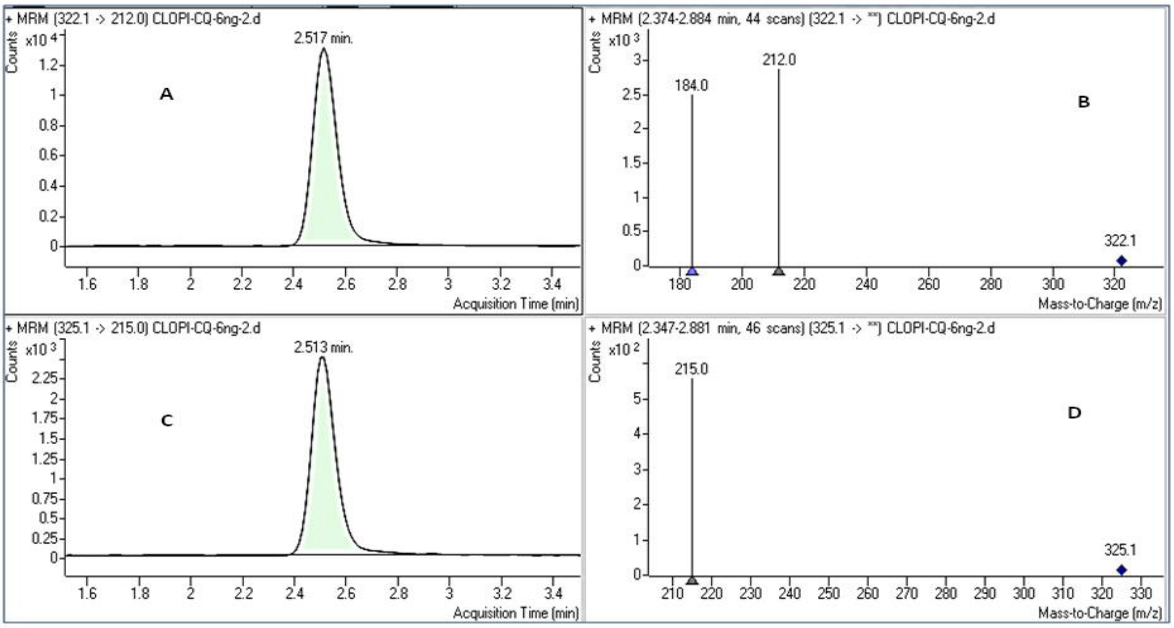

Figure 1. Chromatogram of clopidogrel (A), mass spectrum of clopidogrel (B), chromatogram of IS (C) and mass spectrum of IS (D).

Table 1. Interference percentage of clopidogrel and it IS with plasma blank.

	Clopidogrel		EI	
	LLOQ (0.0156 ng/mL)	Blanc-Plasma	EI (5 ng/mL)	Blanc-Plasma
Area	203	0	14209	0
Retention time (min)	2.52		2.52	
Percentage of interference	0 %		0 %	
Acceptance criterion	< 20 %		< 5%	

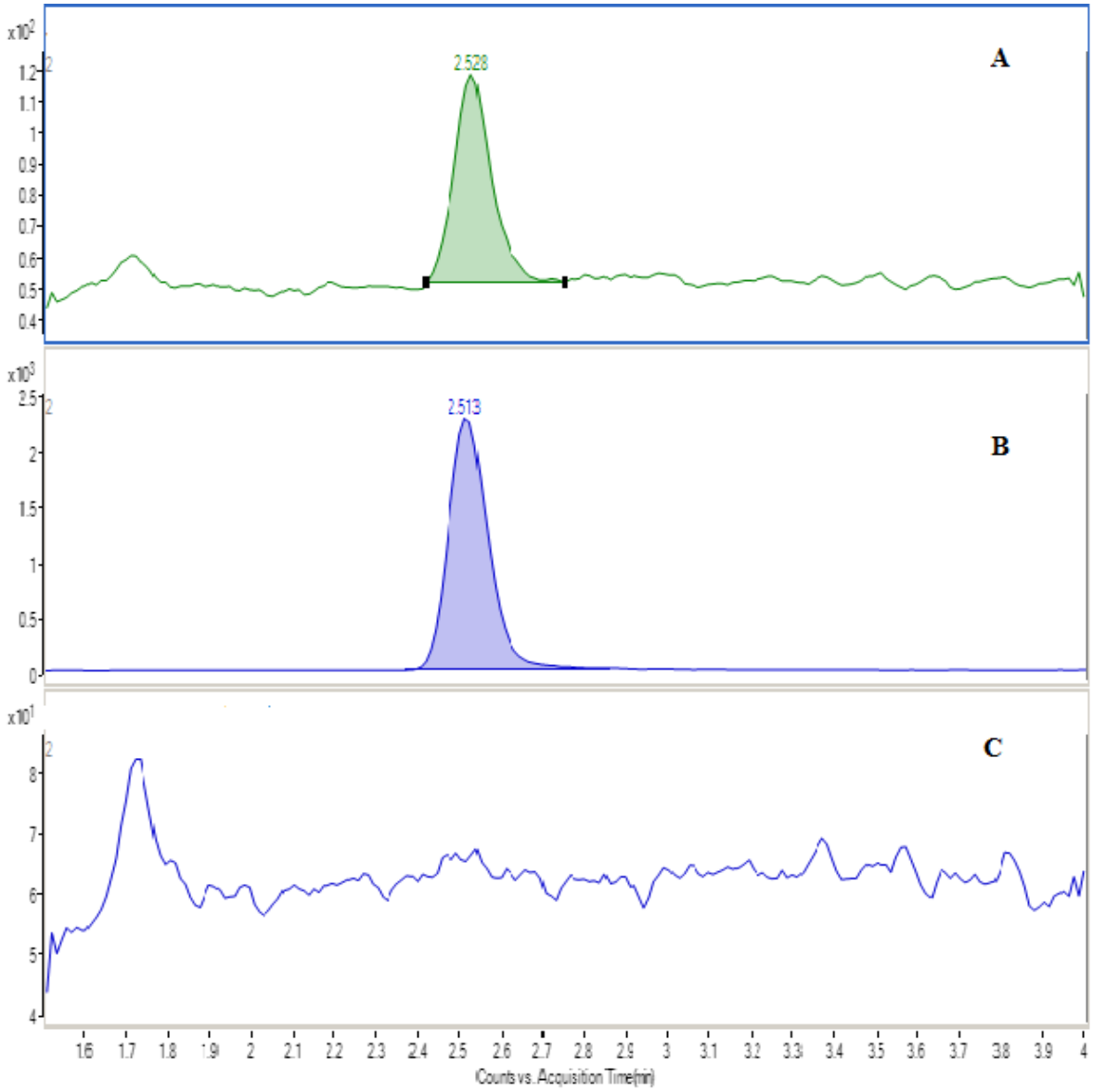

Figure 2. Representative MRM chromatogram of clopidogrel and IS in (A) blank human plasma, (B) human plasma spiked with clopidogrel, (C) blank human plasma spiked with IS.

Table 2. Extraction efficiency and matrix effect results.

	0.0156 ng/mL			0.5 ng/mL			8 ng/mL		
	Neat blank (in solution)	Post spiked	Pre-spiked	Neat blank (in solution)	Post spiked	Pre-spiked	Neat blank (in solution)	Post spiked	Pre-spiked
1	514	619	203	16872	17100	6121	302298	301943	98292
2	575	615	230	17013	17321	6152	304037	303019	101826
3	504	593	223	17348	17085	6023	308436	309051	98710
Average	531.176	608.956	218.965	17077.688	17168.850	6098.770	304923.666	304670.615	99609.564
Standard deviation	38.671	13.677	13.930	244.311	132.238	67.362	3163.922	3831.137	1930.916
RSD %	7.280	2.246	6.362	1.431	0.770	1.105	1.038	1.257	1.938
Matrix effect		114.643			100.534			99.917	
Recovery		103.057			89.280			81.668	

Calibration curve and response function

The calibration curve was examined throughout the concentration range 0.0156 ng/mL to 8 ng/mL. Several calibration models were explored to find the best regression model for this method. Linear regression with 1/X ponderation was found to be the most appropriate one.

A linear regression curve was fitted to the back-calculated concentrations of the validation calibrators to determine the linearity of the technique findings. Figure 3 shows the resulting equations and coefficients of determination. As seen in Figure 3, the regression performed adequately fit with a coefficient greater than 0.9997.

Precision trueness and accuracy profile

Precision trueness and accuracy were evaluated with the quality control concentrations LLOQ: 0.0156 ng/mL, LQC: 0.062 ng/mL, MQC: 3 ng/mL, HQC: 6 ng/mL on three different days.

The trueness of the method was expressed in terms of bias for the quality control concentrations. The results of the trueness were presented in the Table 3.

The precision was evaluated at intermediate precision and repeatability, the results were presented in terms of a relative standard deviation in the Table 3.

The upper and lower tolerance limits were calculated using β = 90 %. Table 1 shows the outcome in terms of relative value.

The accuracy profile is presented in Figure 2 by a β-expectation tolerance interval (β = 90 %) with an acceptance limit of ±15 % for the three concentration levels of LQC, MQC, HQC and ±20 % for the lower limit of quantification.

Table 3. Validation results of the LC-MSMS method to quantify clopidogrel in human plasma.

Concentration (ng/mL)	Repeatability (RSD %)	Intermediate precision (RSD %)	Bias (%)
LLOQ (0.0156 ng/mL)	8.599	8.823	1.100
LQC (0.062 ng/mL)	4.240	6.176	0.989
MQC (3 ng/mL)	1.496	1.969	11.952
HQC (6 ng/mL)	1.194	1.619	12.428

The precision of the method was determined by calculating the relative standard deviation for repeatability and intermediate precision at each validation level for clopidogrel.The result were reported in Table 1. As can be seen, the repeatability and intermediate precisions for clopidogrel ranged from 1.194-8.599 % and from 1.619-8.823 %, respectively.

The accuracy expressed in terms of relative bias (%), this criterion was evaluated from the validation quality controls of clopidogrel at four concentration levels. As can be seen in Table 1, these results show that the relative bias ranged from 0.989 % to 12.428 %, the results were acceptable according to the ICH (International Harmonization Council).

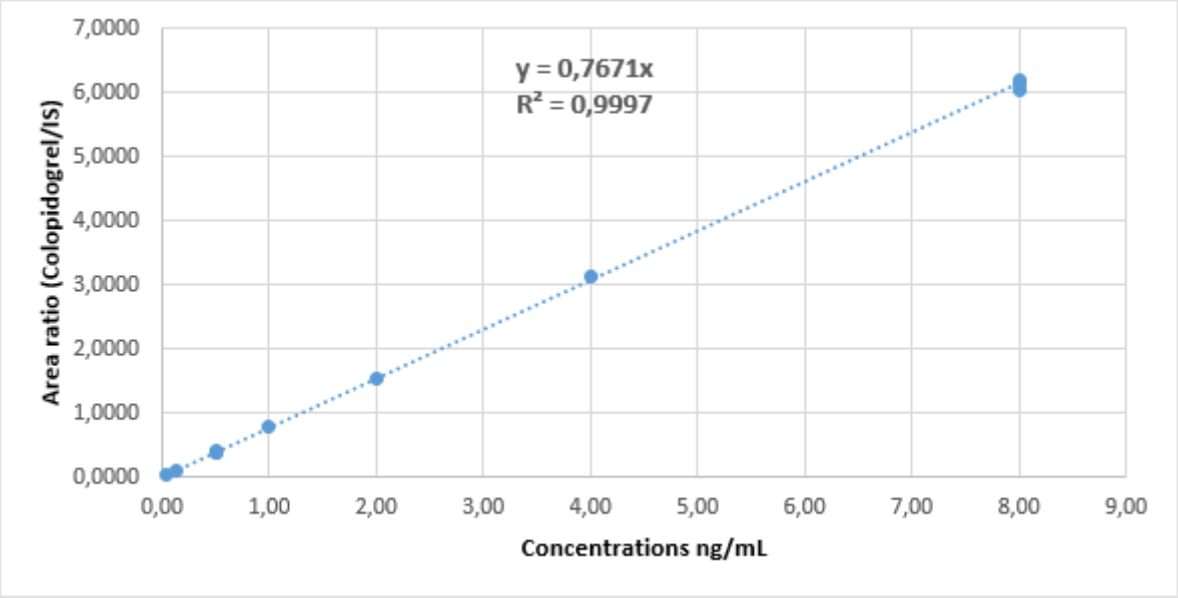

Figure 3. Response function of clopidogrel in the interval 0.0156 - 0.0312 - 0.125 - 0.5 - 1 - 2 - 4 and 8 ng/mL.

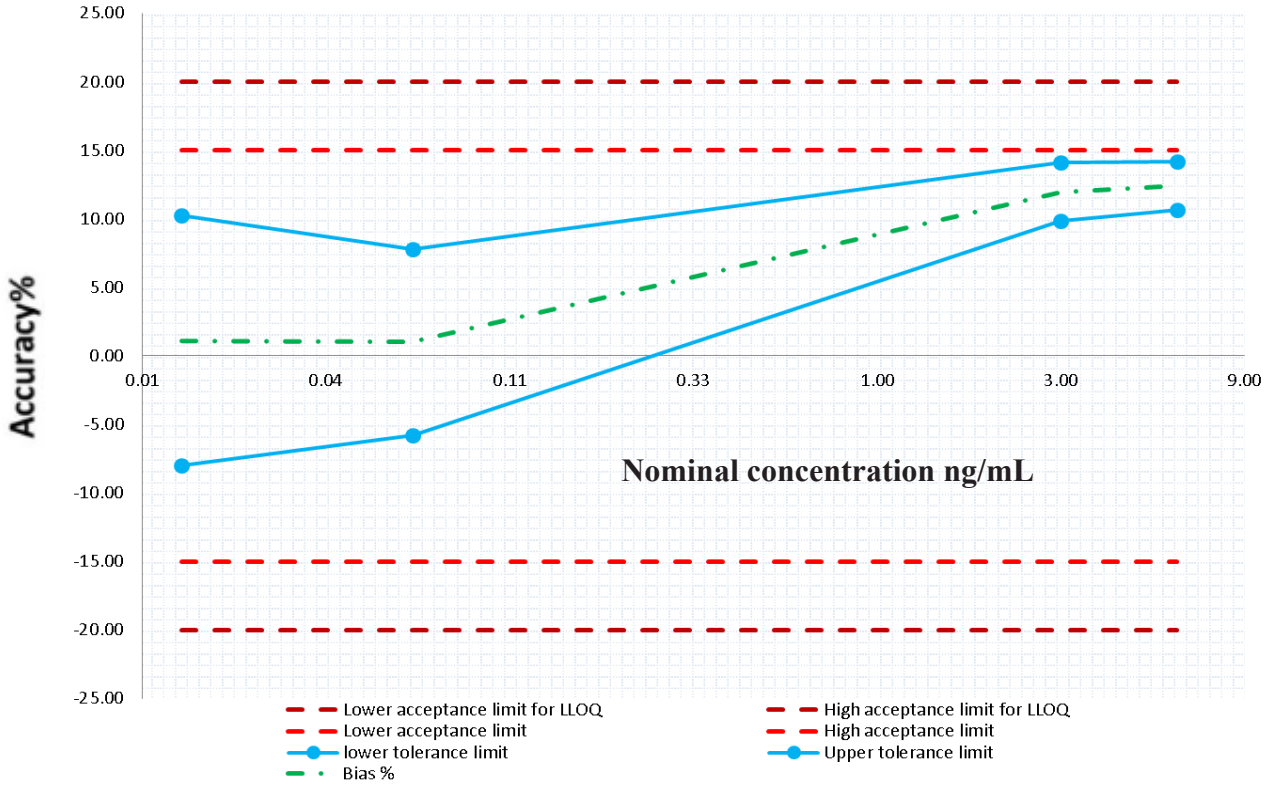

Figure 4. Accuracy profile for the validation of the LC-MS/MS analytical technique for the measurement of clopidogrel in human plasma using calibration curves based on a weighted 1/X linear regression.

The interpretation of the accuracy profile shown in Figure 4 is a straightforward. As long as the tolerance interval is within the acceptability limits, the probability that the difference between the values Z (the calculated concentration) found by the routine method and the reference value X (the nominal concentration) remains below the acceptability limit is greater than the chosen β value (proportion of future results typically 80 %): the method remains valid.

In the case of Figure 4, it was observed that the upper limit of the tolerance interval is within the upper limits of acceptability in all of the four concentration levels chosen for this study. This means that for a concentration between 0.0156 ng/ml and 6 ng/ml, the analyst can guarantee that the method consistently capable of producing an average β probability of acceptable results.

Uncertainty profile

This section aims to present a new strategy for evaluating the performance of quantitative bioanalytical procedures. Our main goal is to bring together two important concepts, namely validation and uncertainty, in order to develop a procedure to verify whether a method is well adapted to its objectives. To achieve this goal, we have introduced a simple and efficient graphical decision tool called uncertainty profile Figure 4. This tool uses the notion of uncertainty instead of the notion of total error to evaluate the performance of this bioanalytical method. In parallel, the proposed strategy allows a good estimation of the measurement uncertainty using the validation data without the need for additional experiments.

We have exploited the statistics of this technique to validate the method using the uncertainty profile. The results obtained by this approach are reported in Table 2.

The upper and lower uncertainty limits expressed as relative values (%) are presented in Figure 5 as a function of the introduced concentrations of clopidogrel. As can be seen from the results, the method was considered valid since the uncertainty intervals are included within the acceptance limits of ±20 % for LLOQ and 15 % for all other concentration levels.

Based on these validation results, it is clear how excellent and powerful the total error approach (the precision profile or uncertainty profile approach) is in evaluating the performance of the bioanalytical method proposed in this study. The decision, in the approaches proposed by ICH, is made on the basis of the classical statistical test based on the evaluation of RSD % and nominal percentage: bias and precision. In contrast, the Total Error approach allows the combination of errors to construct a bilateral interval that is expected to contain a proportion of future measurements included in the acceptance limit, which is consistent with the main objective of a bio-analytical method.

Table 4. Measurement uncertainty results and two uncertainty limits for the validation of the method for the determination of clopidogrel in human plasma.

Reference value in ng/mL	0.02	0.06	3.00	6.00
Compound standard uncertainty	0.002	0.004	0.089	0.161
Extended uncertainty	0.003	0.008	0.177	0.322
Relative uncertainty (%)	19.72	13.57	5.92	5.37
Low limit relative uncertainty (%)	80.28	86.43	94.08	94.63
High limit relative uncertainty (%)	119.72	113.57	105.92	105.37

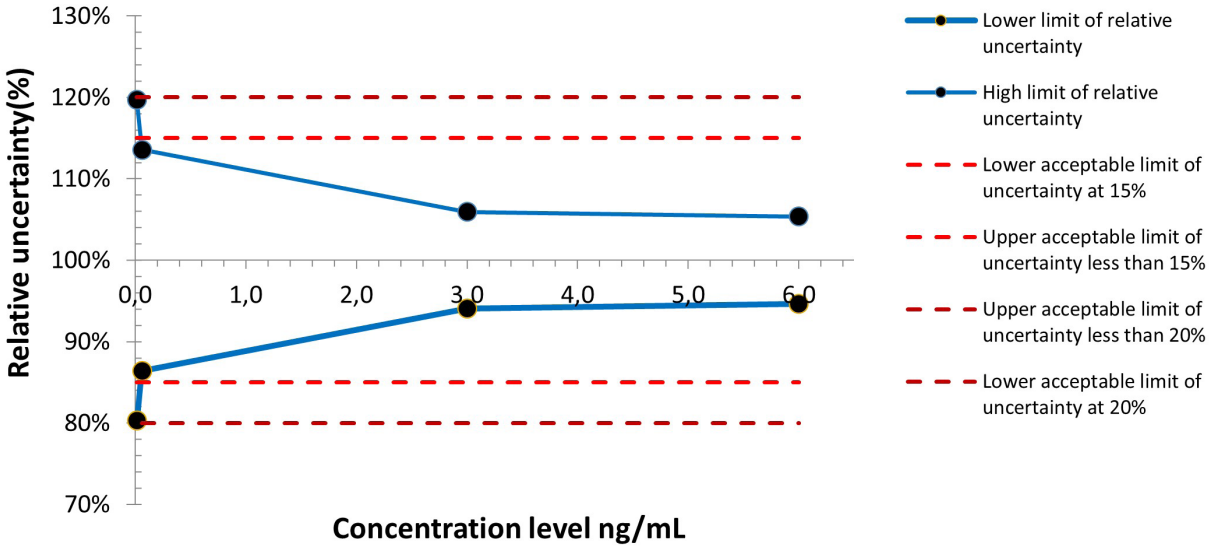

Figure 5. Uncertainty profile for the method validation of clopidogrel in human plasma.

In view of these results, we have shown that our strategy can easily be applied to evaluate the performance of bio-analytical methods, as well as to assess the uncertainty of measurements using information obtained from the validation process. As a result, the expression of uncertainty becomes a natural extension of method validation, which is, for analytical chemists, a much better understood concept than the classical approach and will therefore be adopted much more readily. Finally, we believe that bringing validation and uncertainty in the same approach allows the bio-analyst to control the risk of using the bio-analytical method in routine and to have a complete information on its performance.

On the other hand, we have compared the results obtained by the uncertainty profile, with the one calculated by the accuracy profile each time leads to the same decision. But, our preference goes to the uncertainty interval, because this type of strategy allows a better estimation of the measurement uncertainty compared to the total error interval.

Conclusion

This research was carried out in the context of developing and validating a simple and rapid bio analytical method that can be easily used for pharmacokinetic and bioequivalence studies.

The bioanalytical method was validated based on accuracy profile approach for its several advantages compared to classical approaches .First of all, this approach provides a very simple method of visual and graphical interpretation. Furthermore, it can minimize the risk of accepting a procedure that would not be sufficiently accurate or, conversely, rejecting a procedure that would be accurate .According to this validation approach, the range of validity corresponds to the concentrations for which the tolerance interval is within the limits of acceptability .The range of validity of the method is therefore between concentration levels 0.0156 ng/ml and 6 ng/ml.

In view of these results, we have shown that our strategy can easily be applied to evaluate the performance of bio-analytical methods, as well as

to assess the uncertainty of measurements using information obtained from the validation process. As a result, the expression of uncertainty becomes a natural extension of method validation, which is, for analytical chemists, a much better understood concept than the classical approach and will therefore be adopted much more readily. Finally, we believe that bringing validation and uncertainty in the same approach allows the bio-analyst to control the risk of using the bio-analytical method in routine and to have a complete information on its performance.

Acknowledgement

The authors would like to acknowledge the Sheikh Zaid Foundation for supporting and encouraging researcher.

Conflict of interest

The Authors declares that there is no conflict of interest.

References

1. Clopidogrel | European Medicines Agency https://www.ema.europa.eu/en/medicines/human/paediatric-investigation-plans/clopidogrel-0 (accessed 2021 -12 -12).

2. Clarke, T. A.; Waskell, L. A. The Metabolism of Clopidogrel Is Catalyzed by Human Cytochrome P450 3A and Is Inhibited by Atorvastatin. *Drug Metab. Dispos.* 2003, *31* (1), 53–59. https://doi.org/10.1124/dmd.31.1.53.

3. Savi, P.; Combalbert, J.; Gaich, C.; Rouchon, M. C.; Maffrand, J. P.; Berger, Y.; Herbert, J. M. The Antiaggregating Activity of Clopidogrel Is Due to a Metabolic Activation by the Hepatic Cytochrome P450-1A. *Thromb. Haemost.* 1994, *72* (2), 313–317.

4. Savi, P.; Pereillo, J. M.; Uzabiaga, M. F.; Combalbert, J.; Picard, C.; Maffrand, J. P.; Pascal, M.; Herbert, J. M. Identification and Biological Activity of the Active Metabolite of Clopidogrel. *Thromb. Haemost.* 2000, *84* (11), 891–896. https://doi.org/10.1055/s-0037-1614133.

5. Filipe, A.; Almeida, S.; Spínola, A. C. F.; Neves, R.; Tanguay, M.; Jiménez, C.; Shink, E. Single-Dose Randomized, Open-Label, 2-Way Crossover Bioequivalence Study of Clopidogrel 75 Mg Tablet in Healthy Volunteers under Fasting Conditions. *Int J. Clin. Pharmacol. Ther.* 2009, *47* (03), 187–194. https://doi.org/10.5414/CPP47187.

6. Di Girolamo, G.; Czerniuk, P.; Bertuola, R.; Keller, G. A. Bioequivalence of Two Tablet Formulations of Clopidogrel in Healthy Argentinian Volunteers: A Single-Dose, Randomized-Sequence, Open-Label Crossover Study. *Clin. Ther.* 2010, *32* (1), 161–170. https://doi.org/10.1016/j.clinthera.2010.01.010.

7. Lainesse, A.; Ozalp, Y.; Wong, H.; Alpan, R. Bioequivalence Study of Clopidogrel Bisulfate Film-Coated Tablets. *Arzneimittelforschung* 2011, *54* (09), 600–604. https://doi.org/10.1055/s-0031-1297056.

8. Brvar, N.; Lachance, S.; Lévesque, A.; Breznik, M.; Cvitkovič Marčič, L.; Merslavič, M.; Grabnar, I.; Mateovič-Rojnik, T. Comparative Bioavailability of Two Oral Formulations of Clopidogrel: Determination of Clopidogrel and Its Carboxylic Acid Metabolite (SR26334) under Fasting and Fed Conditions in Healthy Subjects. *Acta Pharm.* 2014, *64* (1), 45–62. https://doi.org/10.2478/acph-2014-0001.

9. Richter, W.; Erenmemisoglu, A.; Meer, M.; Emritte, N.; Emine, T.; Koytchev, R. Bioequivalence Study of Two Different Clopidogrel Bisulfate Film-Coated Tablets. *Arzneimittelforschung* 2011, *59* (06), 297–302. https://doi.org/10.1055/s-0031-1296400.

10. Savakula, S.; Pilli, N. R. Pharmacokinetic and bioequivalence comparison between clopidogrel bisulfate tablets 300mg: an open label, balanced, randomized-sequence, single- dose, two-period crossover study in healthy male volunteers. 9.

11. Setiawati, E.; Yunaidi, D.; Handayani, L.; Santoso, I.; Setiawati, A.; Tjandrawinata, R. Bioequivalence Study of Two Clopidogrel Film-Coated Tablet Formulations in Healthy Volunteers. *Arzneimittelforschung* 2012, *61* (12), 681–684. https://doi.org/10.1055/s-0031-1300587.

12. Silvestro, L.; Gheorghe, M.; Iordachescu, A.; Ciuca, V.; Tudoroniu, A.; Rizea Savu, S.; Tarcomnicu, I. Development and Validation of an HPLC–MS/MS Method to Quantify Clopidogrel Acyl Glucuronide, Clopidogrel Acid Metabolite, and Clopidogrel in Plasma Samples Avoiding Analyte Back-Conversion. *Anal. Bioanal. Chem.* 2011, *401* (3), 1023–1034. https://doi.org/10.1007/s00216-011-5147-4.

13. Zaid, A. N.; Al Ramahi, R.; Bustami, R.; Mousa, A.; Khasawneh, S. Comparative Fasting Bioavailability of Two Clopidogrel Formulations in Healthy Mediterranean Volunteers: An in Vitro–in Vivo Correlation. *Drug Des. Devel. Ther.* 2015, 2359. https://doi.org/10.2147/DDDT.S78658.

14. Zou, J.-J.; Fan, H.-W.; Guo, D.-Q.; Li, Y.-B.; Lin, S.; Zhu, Y.-B.; Yu, C.-X.; Zhou, J.; Liu, J.-H.; Hu, Y.-F. Simultaneous Determination of Clopidogrel and Its Carboxylic Acid Metabolite (SR26334) in Human Plasma by LC–ESI–MS–MS: Application to the Therapeutic Drug Monitoring of Clopidogrel. *Chromatographia* 2009, *70* (11–12), 1581–1586. https://doi.org/10.1365/s10337-009-1349-8.

15. Peer, C. J.; Spencer, S. D.; VanDenBerg, D. A. H.; Pacanowski, M. A.; Horenstein, R. B.; Figg, W. D. A Sensitive and Rapid Ultra HPLC–MS/MS Method for the Simultaneous Detection of Clopidogrel and Its Derivatized Active Thiol Metabolite in Human Plasma. *J. Chromatogr. B* 2012, *880*, 132–139. https://doi.org/10.1016/j.jchromb.2011.11.029.

16. Liu, G.; Dong, C.; Shen, W.; Lu, X.; Zhang, M.; Gui, Y.; Zhou, Q.; Yu, C. Development and Validation of an HPLC–MS/MS Method to Determine Clopidogrel

in Human Plasma. *Acta Pharm. Sin. B* 2016, *6* (1), 55–63. https://doi.org/10.1016/j.apsb.2015.11.001.

17. Kim, S.-D.; Kang, W.; Lee, H. W.; Park, D. J.; Ahn, J. H.; Kim, M. J.; Kim, E. Y.; Kim, S. W.; Nam, H. S.; Na, H. J.; Yoon, Y.-R. Bioequivalence and Tolerability of Two Clopidogrel Salt Preparations, Besylate and Bisulfate: A Randomized, Open-Label, Crossover Study in Healthy Korean Male Subjects. *Clin. Ther.* 2009, *31* (4), 793–803. https://doi.org/10.1016/j.clinthera.2009.04.017.

18. Karaźniewicz-Łada, M.; Danielak, D.; Teżyk, A.; Żaba, C.; Tuffal, G.; Główka, F. HPLC–MS/MS Method for the Simultaneous Determination of Clopidogrel, Its Carboxylic Acid Metabolite and Derivatized Isomers of Thiol Metabolite in Clinical Samples. *J. Chromatogr. B* 2012, *911*, 105–112. https://doi.org/10.1016/j.jchromb.2012.11.005.

19. Bahrami, G.; Mohammadi, B.; Sisakhtnezhad, S. High-Performance Liquid Chromatographic Determination of Inactive Carboxylic Acid Metabolite of Clopidogrel in Human Serum: Application to a Bioequivalence Study. *J. Chromatogr. B* 2008, *864* (1–2), 168–172. https://doi.org/10.1016/j.jchromb.2008.01.049.