

Development of a new HPLC method for simultaneous determination of clopidogrel and its major metabolite using a chemometric approach

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ABSTRACT: This paper presents the development and validation of a new HPLC-UV method for simultaneous quantitative determination of clopidogrel and its hydrolysis product clopidogrel carboxylic acid (CCA) from bulk material and dosage formulations. Development of the chromatographic method is based on a design of experiments (DOE) approach. A Box-Behnken experimental design was used to build the mathematical models and to choose the significant parameters for the optimization by simultaneously taking resolution, capacity factor and peak symmetry as responses. Derringer's desirability function was used for the selection of the optimum experimental conditions in terms of mobile phase composition, column temperature and flow rate. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision, robustness and system suitability. The method was further employed for the study of clopidogrel bisulfate hydrolysis kinetics under different pH conditions, with special emphasis on the acidic hydrolysis studies, since different clopidogrel salts are suffering pre-systemic metabolism through hydrolysis under the acidic pH of the stomach. This effect is generally difficult to quantify, since CCA is also the main circulating metabolite, and no differentiation between the pre-systemic and systemic CCA can be made. In the acidic environment created by a 0.1N HCl solution CLO degradation was slow at room or body temperature (25 and 37°C respectively), less than 5% of the initial CLO amount being hydrolyzed after 48h. Under forced conditions (85°C) however, 17.8% of CLO was transformed into CCA within 48 hours.

KEYWORDS: Clopidogrel, Clopidogrel carboxylic acid, HPLC, Design of Experiments, degradation study

Introduction

Clopidogrel (CLO) is a thienopyridine antiplatelet agent widely used in the management of different cardiovascular diseases, including atherothrombosis, unstable angina, or myocardial infarction [1]. The compound itself is a pro-drug which is inactive *in vitro*. *In vivo*, it selectively and irreversibly inhibits the binding of adenosine diphosphate (ADP) to its platelet receptors and the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex [2] following biotransformation to a thiolic active metabolite by means of CYP3A4 and CYP2C19 cytochrome P450 isoforms.[3].

Although CLO can be considered a 'blockbuster' drug – the CLO innovator product ranks second worldwide in the top of incomes generated by a drug [4], large interindividual variability in clopidogrel response has been reported [5], leading to 20-40% of patients presenting "resistance" to treatment, associated with a low protection rate against atherothrombotic events [6]. Despite its

widespread use, associated with significant "resistance" to treatment (for 20-40% of the patients). An important limitation in the study of CLO variability is the fact that until recently, no analytical methods were available for quantitative analysis of CLO and of its active thiol metabolite [7-10]. The study of CLO pharmacokinetics was based for many years on the determination of its major but inactive circulating metabolite, clopidogrel carboxylic acid (CCA), resulted *in vivo* via hydrolysis of CLO mediated by the hepatic esterases (Fig.1) [11].

However, CCA is at the same time the main CLO impurity, since CLO hydrolysis can occur in different conditions, such as the acidic gastric environment. In the EMA's "Questions & Answers: Positions on specific questions addressed to the pharmacokinetics working party" [12], the EMA experts underline the fact that under fasting conditions, the dissolution in the gastric media with a subsequent hydrolysis and formation of the inactive CCA metabolite is maximal, and the amount of CLO available for absorption is reduced. Since CCA formed in the

stomach is readily absorbable, no *in vivo* differentiation between CCA resulted from pre-systemic metabolism and the one resulting from

hepatic hydrolysis can be performed. Therefore, an *in vitro* evaluation of the CLO hydrolysis kinetics arises as a necessity.

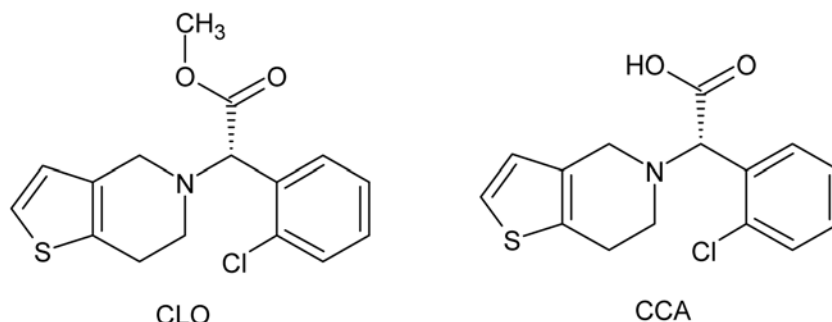


Fig.1. Chemical structure of CLO and of its hydrolytic degradation product and major *in vivo* metabolite, CCA

On the other hand, no HPLC method for simultaneous determination of CLO and CCA from bulk substance of pharmaceutical formulation is presented in literature. A literature survey revealed various assays for determining clopidogrel in pharmaceutical dosage forms, including spectrophotometry [13] thin-layer chromatography [14], high-performance thin-layer chromatography [15], HPLC-UV [16-22], LC-MS [23] or voltammetry [24]. CCA is determined usually from plasma samples, following its use as CLO “surrogate” in bioequivalence studies. Generally HPLC-UV [25], LC-MS [26-28] are used for separation and quantitative evaluation.

The present study aims to present the development and validation of a new HPLC-UV method for simultaneous quantitative evaluation of CLO and CCA from bulk material and pharmaceutical formulations, based on a design of experiments (DOE) approach.

The method is indented to quantitatively evaluate CLO degradation kinetics under different pH conditions, with special emphasis on CLO hydrolysis kinetics in simulated gastric fluid, in order to quantitatively evaluate the pre-systemic metabolism of CLO.

Material and Methods

Chemicals

Clopidogrel bisulfate standard was obtained from LGC Promochem GmbH (Germany). Clopidogrel Carboxylic Acid HCl was purchased from SynFine Research (Richmond Hill, Ontario, Canada).

HPLC grade acetonitrile was purchased from Merck KGaA (Darmstadt, Germany). Trifluoroacetic acid for HPLC was purchased

from Sigma-Aldrich (St Louis, MO, SUA). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced within the laboratory by means of a TKA GenPure system and used during the experiments. All other reagents were of analytical grade, purchased from different commercial suppliers and used without further purification.

Stock and working standard solutions

The stock solutions of CLO and CCA were prepared at a concentration of 500 µg/mL in acetonitrile. Working dilutions from stock solutions of the analytes were freshly prepared when needed. Spiked calibration standards in plasma have been prepared with both analytes together at the concentrations of: 0.125, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/mL.

The quality control samples used in the accuracy and precision evaluation (QCs) were spiked with the analytes at the following levels: 4, 20 and 80 µg/mL.

Quantitative HPLC analysis

The quantitative analysis of the selected compounds was carried out using a Waters liquid chromatographic system (Waters, Milford, MA, USA) consisting of consisting of a 600 E Multisolvant Delivery System, Waters AF in line degasser, 486 UV tunable absorbance detector and Waters 717 plus autosampler. The output signal was monitored and processed using Empower software (Waters, Milford, MA, USA). The chromatographic separation was achieved on a Hypersil Gold, 5-µm 150 x 4 mm column (Thermo Fisher Scientific, Waltham, MA, USA). The injection volume was 10 µl and the UV detection was performed at 210 nm. The elution was isocratic with mobile phase

consisting in mixtures of acetonitrile and trifluoroacetic acid. The exact composition of mobile phase, flow rate and column temperature were determined by means of experimental design.

Optimization of the HPLC method

Selection of the chromatographic column, as well as the aqueous component of the mobile phase was performed by using the one-factor-at-a-time (OFAT) approach. 0.1%, trifluoroacetic acid, 0.1% phosphoric acid, as well as 0.025M phosphate buffer solution with pH ranging between 3.0 and 7.0 were tested. The optimum results both in terms of resolution and peak symmetry were obtained with trifluoroacetic acid.

A Box-Behnken design approach of response surface methodology (RSM) was further employed in order to study the individual and combined effects of three independent factors, i.e. the column temperature (X_1), flow rate (X_2) and percent

of acetonitrile in the mobile phase (X_3) on chromatographic resolution (Y_1), asymmetry (Y_2), capacity factor - k' (Y_3), and number of theoretical plates - N (Y_4) of the CLO and CCA peaks.

The experimental design for the optimization along with statistical analysis of data was performed with Design-Expert 9.0 software, Trial Version (Stat Ease Stat-Ease, Inc., Minneapolis, MN, USA).

The design matrix for the Box-Behnken study was generated using three factors at three levels, including five replicates of the central point, resulting in a total of 17 analytical experiments. All experiments were performed in randomized order to minimize the effects of uncontrolled variables that may introduce a bias in the measurements.

The range and the levels of experimental variables investigated in this study are presented in Table 1.

Table 1. Factors and levels of Box-Behnken experimental design

Factors	Code	Range and levels		
		-1	0	+1
Column temperature (°C)	X_1	30	40	50
Flow (mL/min)	X_2	0.8	1	1.2
% organic modifier (acetonitrile)	X_3	24	28	32

Experimental data were fitted according to the following second-order polynomial equation calculated by multiple regression analysis:

$$Y = b_0 + \sum_{i=0}^n b_i X_i + \sum_{i=0}^n b_{ii} X_i^2 + \sum_{i < j} b_{ij} X_i X_j + \varepsilon$$

where Y represents the measured response, b are coefficients calculated by multiple regression analysis, X_i represent the main effects of the independent variables, $X_i X_j$ the interaction terms between variables, X_i^2 quadratic expressions of the independent variables (included into the model in order investigate nonlinearity) and ε represents the random error.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The quality of the fitted model was expressed by the coefficient of determination (R^2) and its statistical significance was checked by F -test and P -value test.

Validation of the HPLC method

The HPLC method was subjected to validation in accordance with the International Conference on Harmonization (ICH) regulations Q2(R1) [29] in terms of specificity, linearity, precision (repeatability and intermediate precision) and accuracy.

Assay specificity was evaluated in relation to interferences from the matrix components in the drug-free media.

The linearity assessment was performed for both CLO and CCA by using nine concentration levels, in the range 0.125-100 $\mu\text{g/mL}$. All analyses were performed in triplicate. The calibration curves and corresponding determination coefficients (R^2) were calculated by least squares linear regression analysis. Detection limit (LOD) and quantitation limit (LOQ) were evaluated based on the signal-to-noise ratio. The concentrations yielding to

signal-to-noise ratios of 3:1 and 10:1 were considered as LOD and LOQ, respectively.

Precision was evaluated for repeatability and intermediate reproducibility on spiked samples, at three different concentration levels (QC_{low} , QC_{medium} and QC_{high}). Precision was assessed by means of RSD% values computed for absolute peak areas resulting from interpolation on the corresponding calibration curves. Repeatability study was achieved by injection of 5 replicates from a single prepared spiked plasma sample within a single day experimental session, whereas intermediate reproducibility was tested

by means of five different samples processed in different experimental sessions for each concentration level.

The bias (%) between the concentration values determined for the QC samples and their nominal values was used as accuracy indicator.

Results and Discussion

According to BBD designs, a total of seventeen tests (including five replicates of the center point) were carried out in random order (Table 2).

Table 2. Box-Behnken experimental design matrix of the selected independent variables and studied responses

Run no.	X_1	X_2	X_3	Resolution (Y_1)	Tailing factor (Y_2)		k' (Y_3)		Plate Count (Y_4)	
					CCA	CLO	CCA	CLO	CCA	CLO
1	40	1	28	13.05	1.27	1.13	1.87	5.80	3328	4878
2	40	1.2	32	9.52	1.27	1.17	1.00	2.99	2551	4096
3	50	0.8	28	14.17	1.25	1.09	2.58	7.59	3928	5479
4	40	0.8	24	17.03	1.23	1.07	3.88	12.79	4286	5742
5	40	1	28	13.08	1.27	1.11	1.87	5.77	3326	4923
6	50	1.2	28	12.34	1.24	1.11	1.37	4.50	3030	4554
7	40	1.2	24	14.86	1.23	1.11	2.13	7.55	3270	4720
8	30	0.8	28	14.04	1.29	1.15	2.65	7.75	3804	5404
9	40	1	28	13.03	1.27	1.13	1.86	5.76	3291	4861
10	40	1	28	13.01	1.27	1.14	1.89	5.84	3315	4838
11	50	1	32	10.42	1.28	1.14	1.37	3.78	2968	4584
12	30	1	32	10.27	1.30	1.20	1.40	3.85	2915	4433
13	50	1	24	15.80	1.21	1.06	2.72	9.31	3771	5096
14	30	1.2	28	11.99	1.26	1.18	1.38	4.55	2878	4334
15	30	1	24	15.66	1.27	1.14	2.81	9.57	3612	5070
16	40	1	28	13.07	1.26	1.13	1.88	5.81	3205	4881
17	40	0.8	32	11.37	1.30	1.15	2.02	5.22	3455	5228

The importance of each term in the mathematical models was assessed using ANOVA. The magnitudes of the coefficients in the regression equations were utilized as the basis for judging statistical significance and illustrating the relative effects of linear, quadratic effects, as well as of the interactions between the parameters.

The values of R^2 shown in Table 2 indicate a good fitting, and an adequate capacity of the suggested model to represent the relation between factors and responses ($R^2 > 0.9$ in all

cases). The coefficient of determination values listed in the table represent the “adjusted R^2 ” values, obtained after the nonsignificant terms ($P > 0.05$) were removed from the models through the “backward elimination” process.

The relationship between the dependent and independent variables is further illustrated using the response surfaces, which enable the visual checking of the effects in the three dimensional space (Fig. 2-4). In all representations, one factor was kept constant at its center value.

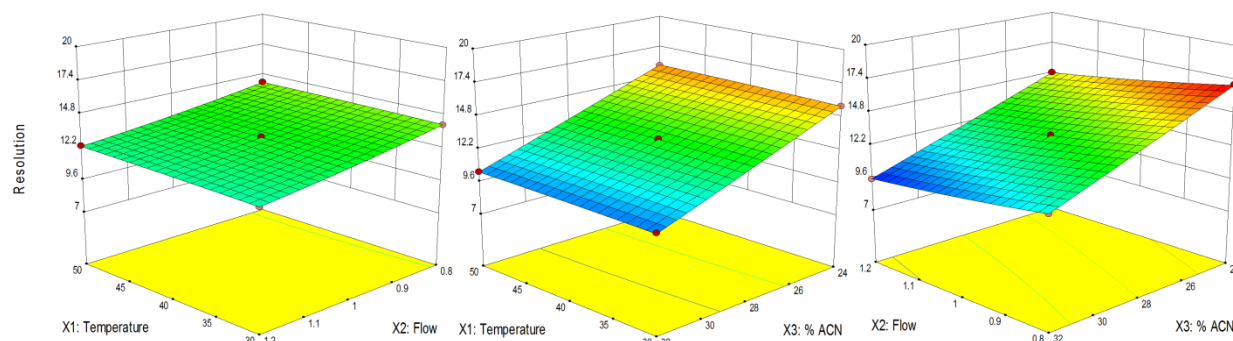


Fig.2. Response surface plots representing resolution of the chromatographic separation (Y_1) as a function column temperature (X_1), flow rate (X_2) and % organic modifier (X_3)

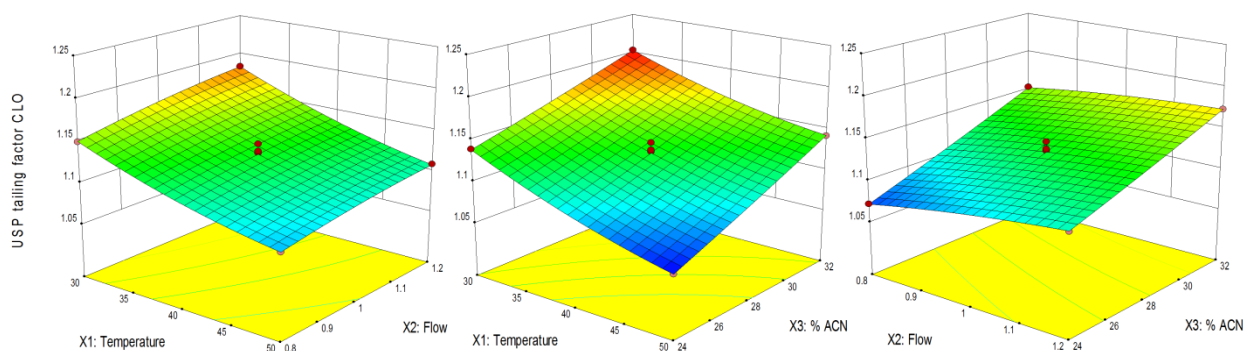


Fig.3. Response surface plots representing CLO peak asymmetry (Y_2) as a function column temperature (X_1), flow rate (X_2) and % organic modifier (X_3)

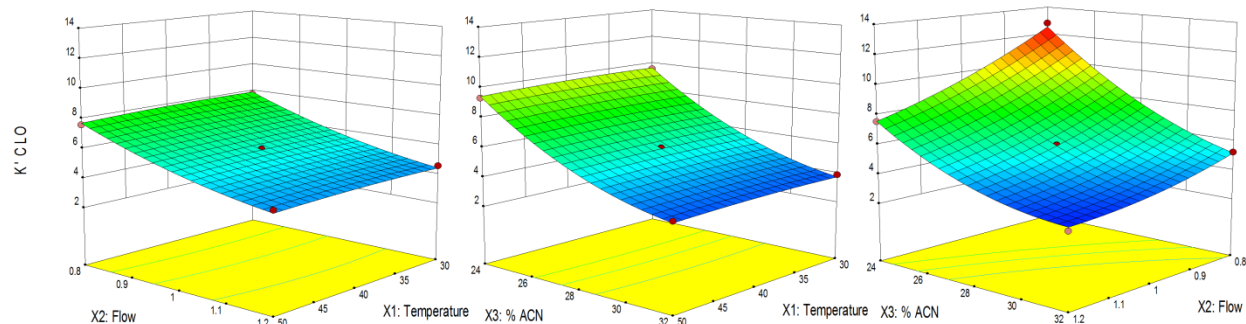


Fig.4. Response surface plots representing the capacity factor k' for the CLO peak (Y_3) as a function column temperature (X_1), flow rate (X_2) and % organic modifier (X_3)

The results suggest that independent variables have significant effect on all the selected responses, with the exception of temperature (X_1) not significantly influencing

the capacity factor for both CCA and CLO peak (Table 3). On the other hand, the interactive factors had only limited influence on the selected variables.

Table 3. Results of the regression analysis for the selected responses

Response	b_0	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{11}	b_{22}	b_{33}	R^2
Y_1	13.05	0.10	-0.99	-2.72	0.05	0.00	0.08	-0.04	0.12	0.03	0.9995
$p=$		0.00	0.00	0.00	0.06	0.95	0.01	0.18	0.00	0.31	
Y_2 CCA	1.27	-0.02	-0.01	0.03	0.00	0.01	-0.01	0.00	-0.01	-0.01	0.9324
$p=$		0.00	0.01	0.00	0.41	0.01	0.16	0.88	0.07	0.10	
Y_2 CLO	1.13	-0.04	0.01	0.04	0.00	0.01	0.00	0.01	0.00	0.00	0.9422
$p=$		0.00	0.00	0.00	0.57	0.35	0.46	0.16	0.51	0.81	
Y_3 CCA	1.87	-0.02	-0.66	-0.72	0.01	0.02	0.18	-0.03	0.15	0.23	0.9948
$p=$		0.22	0.00	0.00	0.61	0.57	0.00	0.26	0.00	0.00	
Y_3 CLO	5.80	-0.07	-1.72	-2.92	0.03	0.05	0.76	-0.10	0.41	0.94	0.9936
$p=$		0.36	0.00	0.00	0.79	0.65	0.00	0.33	0.00	0.00	
Y_4 CCA	3293.1	61.2	-468.0	-381.2	6.8	-26.3	27.7	21.4	95.5	2.1	0.9904
$p=$		0.01	0.00	0.00	0.76	0.26	0.23	0.34	0.00	0.92	
Y_4 CLO	4876.2	59.0	-518.6	-285.7	36.3	31.4	-27.4	-42.2	108.6	-38.4	0.9931
$p=$		0.00	0.00	0.00	0.08	0.12	0.17	0.05	0.00	0.06	

Legend: $p < .01$, $.01 \leq p < .05$, $.05 \leq p < .10$, $p \geq .10$

Peak retention (expressed as capacity factors) is negatively influenced by both flow rate and % of organic modifier, while temperature has no significant influence.

The chromatographic resolution is increased by increasing the column temperature, but is negatively influenced by the flow rate and ACN %.

The selected independent variables have antagonistic effect on CLO peak symmetry (Y_2) and number of theoretical plates (Y_4): higher temperature associated with lower flow rates and lower percentages of acetonitrile in the mobile phase are associated with both increased N values, and increased asymmetry. As asymmetry of the chromatographic peaks is an undesirable effect, for optimum separation a compromise must be achieved. However, the values for both plate count and USP tailing factor are within acceptable range in all cases (tailing factor < 1.2 and $N > 4000$ for CLO). Therefore, when optimizing the experimental conditions of the separation, mainly chromatographic resolution and capacity factors were considered.

Selection of the optimum experimental conditions of the HPLC analysis

When a simple response is being analyzed, the model analysis indicates areas in the design region where the process is likely to give the optimal results. However, most of the response surface problems involve the analysis of multiple responses. That complex problem

involves building an appropriate response surface model for each response followed by identifying a set of experimental conditions that optimizes all responses or at least keeps them in a desired range. One approach in that direction is by using the Derringer's desirability function, which allows for compromise among the various responses [30]. The function ranges between $d = 0$, for a completely undesirable response, to $d = 1$ for a fully desired response.

The criteria for the optimization of each individual response are shown in Fig.5. In order to shorten the analysis time while separating the first eluting peak from the solvent front, the capacity factor for CCA was targeted to 1.6; that is the immediate retention after the void time (t_o) of the column, whereas a capacity factor in the range 4-6 was targeted for the second eluting peak, CLO. Resolution of the chromatographic separation was set in the range 10-15, and a minimum N of 3000 was imposed to the CCA peak. No specific limitations were imposed to the tailing factor, as its value falls within the acceptable range in the samples of the experimental model. Using the polynomial equations describing the effect estimates on the dependent variables and the surface response methodology, an optimal formulation was developed. A column temperature of 30°C, associated with a flow rate of 0.988 mL/min and a percent of 29.44% acetonitrile were found to be the optimum experimental conditions (desirability value=0.988) (Fig.5).

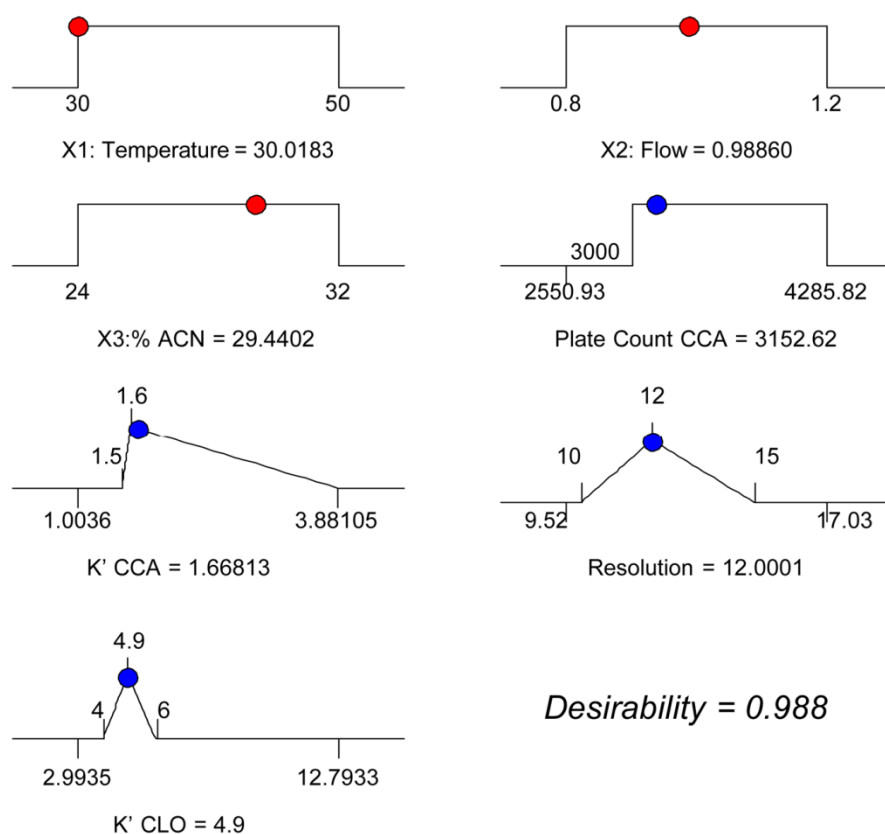


Fig.5. Optimization of the selected responses by means of the desirability function in ramp function graph representation. Optimum values of the independent variables are presented as red dots, whereas the predicted values of responses at the optimum factor levels are dotted in blue

Since factionary values for flow rate and %ACN are not practical, in the final optimized method the values were rounded to 1.0 mL/min

and 30% respectively. A representative chromatogram obtained under the optimized conditions is presented in Fig. 6.

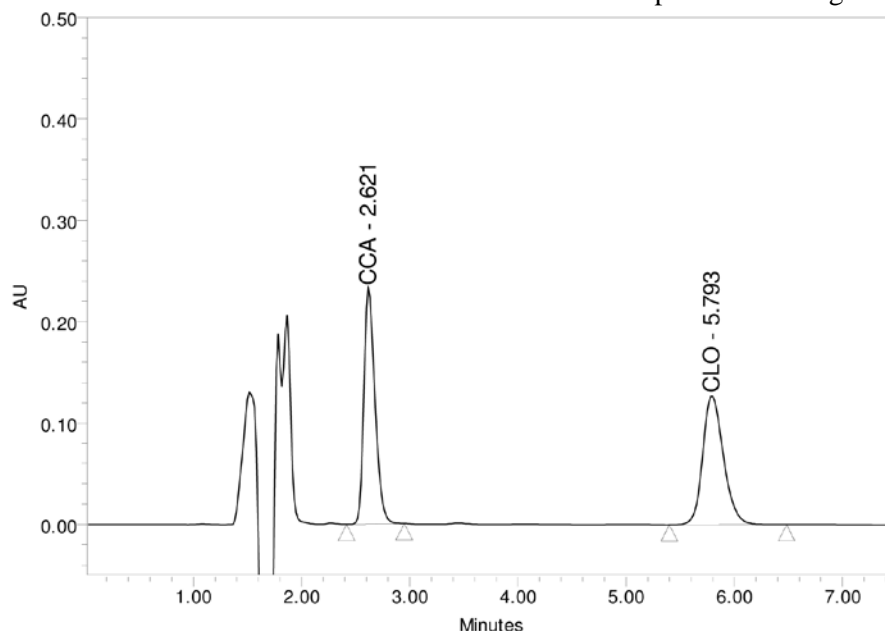


Fig.6. Representative chromatogram of a standard sample containing CLO and CCA obtained under the optimized chromatographic conditions (Flow = 1mL/min, Column temperature = 30°C and %ACN = 30%)

The corresponding values of the resolution of the chromatographic separation, capacity factors, asymmetry and plate count for both CLO and CCA were compared to the predicted values based on the polynomial equations

describing the effect estimates. The bias % ranged between -2.66 and 5%, suggesting a good predictive power of the experimental model (Table 4).

Table 4. Comparison of the predicted and experimental values for the model dependent variables

Parameter	Predicted	Experimental	Bias%
Resolution	11.64	11.87	2.00
K' CCA	1.57	1.62	3.03
K' CLO	4.57	4.79	4.99
USP tailing factor CCA	1.29	1.25	-2.60
USP tailing factor CLO	1.16	1.20	2.67
Plate Count CCA	3084	3214	4.20

Validation of the HPLC method

The HPLC method employed method for the simultaneous quantitative analysis of CLO and

CCA was found to be linear in the range 0.125–100 µg/mL, with a mean R^2 value of 0.9999 for both the tested compounds (Fig.7).

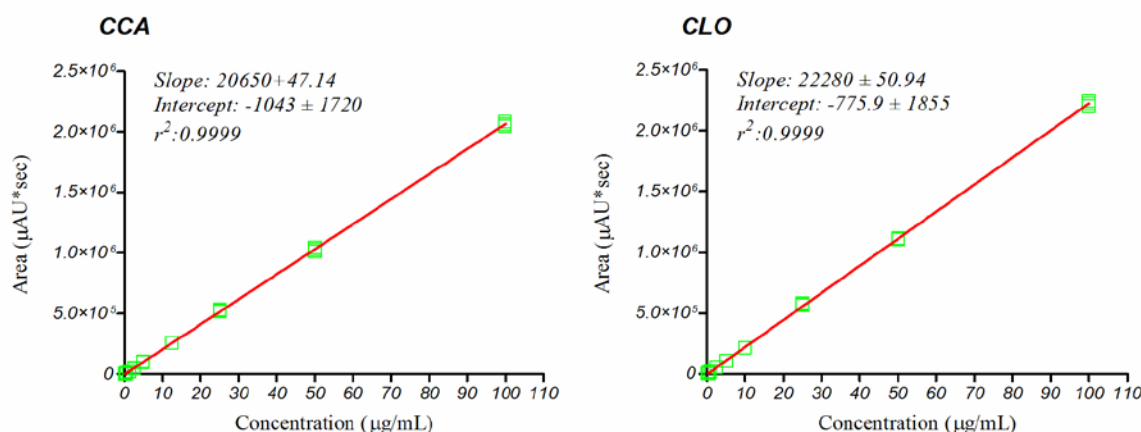


Fig.7. Calibration curves for CLO and CCA (n=3 replicates, range = 0.125-100 µg/mL)

The limit of quantification (LOQ), calculated based on a 10:1 signal-to-noise ratio was 0.016 µg/mL for CCA and 0.024 µg/mL for CLO. No endogenous peaks or other CLO degradation products interfered with the chromatographic separation.

Repeatability and intermediate reproducibility experiments resulted in RSD

values ranging from 0.14 to 2.36 %. The bias (%) between the experimental and nominal concentrations of the quality control samples did not exceed 3 %. All these results indicated that precision and accuracy of the assay are satisfactory (Tables 5 and 6).

Table 5: Accuracy and precision of the HPLC method – CCA peak

Conc (µg/mL)	Intra-day precision & accuracy			Inter-day precision & accuracy		
	Mean±SD	RSD (%)	Bias (%)	Mean±SD	RSD (%)	Bias (%)
4.0	4.055 ± 0.024	0.60	+1.59	3.930±0.093	2.36	-1.74
20.0	20.217 ± 0.029	0.14	+1.09	19.417±0.287	1.48	-2.91
80.0	81.275 ± 0.343	0.42	+1.39	80.175±1.086	1.35	+0.22

Table 6: Accuracy and precision of the HPLC method – CLO peak

Conc (µg/mL)	Intra-day precision & accuracy			Inter-day precision & accuracy		
	Mean±SD	RSD (%)	Bias (%)	Mean±SD	RSD (%)	Bias (%)
4.0	3.978±0.043	1.08	-0.56	3.928±0.069	1.77	-1.81
20.0	20.167±0.156	0.77	+0.83	19.786±0.320	1.62	-1.07
80.0	79.864±0.762	0.95	-0.17	79.964±0.946	1.18	-0.05

Application of the HPLC method for the study of hydrolytic degradation of CLO

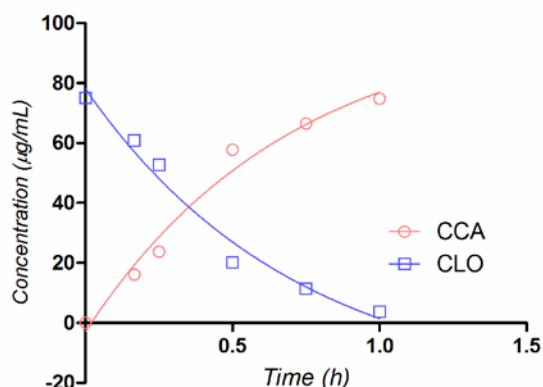


Fig.8. CLO hydrolysis under alkaline conditions (0.1N NaOH solution)

The hydrolysis of a 75 µg/mL standard solution of CLO was studied under acidic (0.1N HCl solution), neutral (0.05M phosphate buffer pH 6.8) and basic conditions (0.1N NaOH solution) over 48 hours. Clopidogrel bisulphate degrades under basic conditions at ambient

temperature and under acidic conditions at an elevated temperature resulting in a single major degradation product i.e. CCA, via hydrolysis of the ester group, as suggested by the mass balance evaluation. Under basic conditions, the hydrolysis is complete within 1 hour at room temperature (Fig.8).

In a neutral pH buffer solution, CLO was stable over 48 hours.

The acidic hydrolysis environment has increased biological relevance, since CLO could be degraded in the stomach to CCA. This in vivo reaction is very difficult to evaluate, since CCA is both the main degradation product and the main circulating metabolite of CLO.

In the acidic environment created by a 0.1N HCl solution CLO degradation is slow at room or body temperature (25 and 37°C respectively), less than 5% of the initial CLO amount being hydrolyzed after 48h. Under forced conditions (85°C) however, the acidic hydrolysis is significant: 17.8% of CLO is transformed in CCA within 48 hours. The reaction appears to follow a first order kinetics, with a half-life of about 108 hours (Fig.9).

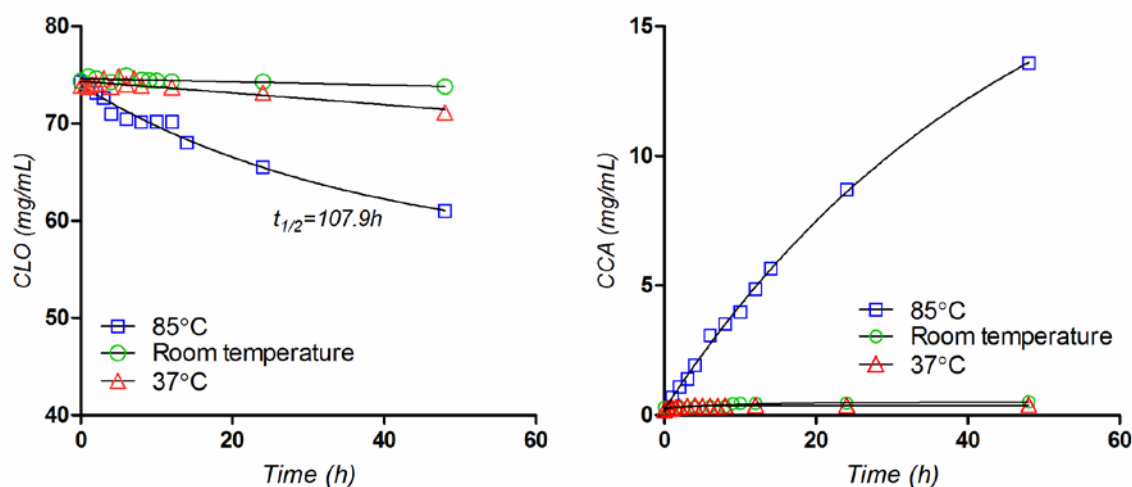


Fig.9. CLO hydrolysis under acidic conditions (0.1N HCl solution)

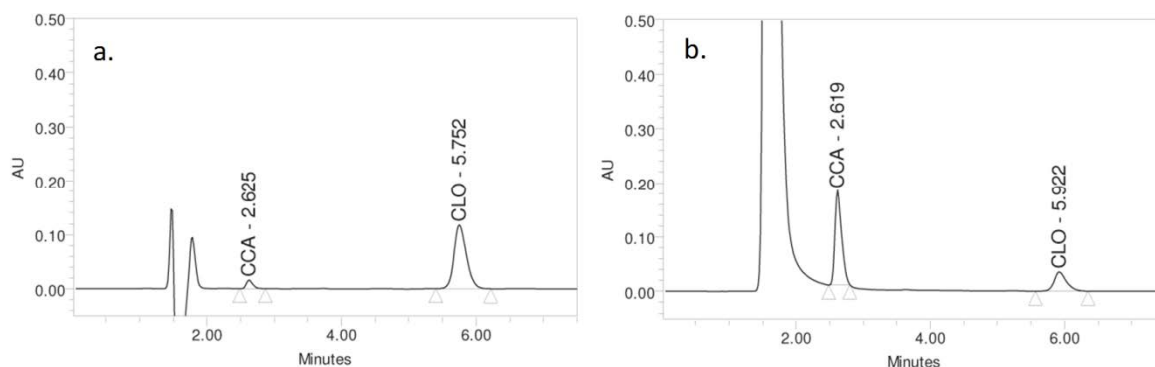


Fig.10. Representative chromatograms obtained after 12 hours of CLO acid hydrolysis at 85°C (a) and 0.5 hours of basic hydrolysis (b)

Representative chromatograms obtained after 12 hours of acid hydrolysis at 85°C and 0.5 hours of basic hydrolysis are presented in Fig.10.

Conclusion

This paper describes a novel method for simultaneous determination of CLO and its hydrolysis product CCA by HPLC. The development of the HPLC method was performed using a chemometric approach, by employing the Box-Behnken design approach and response surface methodology RSM. The optimized method was fully validated in accordance with the ICH guidelines, and used for the study of CLO hydrolytic forced degradation studies. The results suggested rapid degradation under basic conditions at ambient temperature and somewhat slower degradation under acidic conditions at an elevated temperature resulting in a single major degradation product i.e. CCA.

Acknowledgements

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References

1. Savi P, Herbert JM. Clopidogrel and ticlopidine: P2Y₁₂ adenosine diphosphate-receptor antagonists for the prevention of atherothrombosis, *Semin Thromb Hemost* 2005; 31(2): 174-83.
2. Savi P, Pereillo JM, Uzabiaga MF, et al. Identification and biological activity of the active metabolite of clopidogrel, *Thromb Haemost* 2000; 84(5): 891-6.
3. Kazui M, Nishiya Y, Ishizuka T, et al. Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite, *Drug Metab Dispos* 2010; 38(1): 92-9.
4. IMS Health. Top 10 leading brands by worldwide sales. [http://www.imshealth.com/ims/Global/North%20America/Canada/Home%20Page%20Content/Pharma%20Trends/Top10TherapeuticClassesWorldwideSales_En_11.pdf]. 2011 [cited 2014 Sep 8].
5. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, et al. Variability in Individual Responsiveness to Clopidogrel: Clinical Implications, Management, and Future Perspectives, *Journal of the American College of Cardiology* 2007; 49(14): 1505-16.
6. Dupont AG, Gabriel DA, Cohen MG. Antiplatelet therapies and the role of antiplatelet resistance in acute coronary syndrome, *Thrombosis Research* 124(1): 6-13.
7. Karazniewicz-Lada M, Danielak D, Rubis B, et al. The influence of genetic polymorphism of Cyp2c19 isoenzyme on the pharmacokinetics of clopidogrel and its metabolites in patients with cardiovascular diseases, *The Journal of Clinical Pharmacology* 2014; 54(8): 874-80.
8. Silvestro L, Gheorghe M, Iordachescu A, et al. 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): 1023-34.
9. Takahashi M, Pang H, Kawabata K, et al. Quantitative determination of clopidogrel active metabolite in human plasma by LC-MS/MS, *Journal of Pharmaceutical and Biomedical Analysis* 2008; 48(4): 1219-24.

10. von Beckerath, Taubert D, Pogatsa-Murray G, et al. Absorption, metabolism, and antiplatelet effects of 300-, 600-, and 900-mg loading doses of clopidogrel: results of the ISAR-CHOICE (Intracoronary Stenting and Antithrombotic Regimen: Choose Between 3 High Oral Doses for Immediate Clopidogrel Effect) Trial, *Circulation* 2005; 112(19): 2946-50.
11. Sangkuhl K, Klein TE, Altman RB. Clopidogrel pathway, *Pharmacogenet Genomics* 2010; 20(7): 463-5.
12. European Medicines Agency, Committee for Human Medicinal Products (CHMP). EMA/618604/2008 Rev.9 Questions & Answers: Positions on specific questions addressed to the pharmacokinetics working party. [http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002963.pdf]. 2014 [cited 2014 Oct 12].
13. Rajput S, George R, Ruikar D. Chemometric simultaneous estimation of clopidogrel bisulphate and aspirin from combined dosage form, 2008; 70(4): 450-4.
14. Antiç D, Filipiç S, Agbaba D. A simple and sensitive TLC method for determination of clopidogrel and its impurity SR 26334 in pharmaceutical products, *Acta Chromatographica* 2007; (18): 199-206.
15. Patel RB, Shankar MB, Patel MR, et al. Simultaneous estimation of acetylsalicylic acid and clopidogrel bisulfate in pure powder and tablet formulations by high-performance column liquid chromatography and high-performance thin-layer chromatography, 2008; 91(4): 750-5.
16. Gomez Y, Adams E, Hoogmartens J. Analysis of purity in 19 drug product tablets containing clopidogrel: 18 copies versus the original brand, 2004; 34(2): 341-8.
17. Alarfaj NA. Stability-indicating liquid chromatography for determination of clopidogrel bisulfate in tablets: Application to content uniformity testing, 2012; 16(1): 23-30.
18. Gosavi NP, Bhajane MU, Patil VV, et al. Development and validation of analytical and method for the simultaneous estimation of Clopidogrel Bisulphate and Atorvastatin calcium in bulk and in tablet, 2012; 3(3): 1065-71.
19. Al-Khayat MA, Haidar S, Mando H. Development and validation of RP-HPLC method for determination of clopidogrel in tablets, 2012; 14(2): 1-5.
20. Kulsum S, Ramya S, Snehalatha T, et al. Development and validation of RP-HPLC method for the simultaneous estimation of amlodipine besylate and clopidogrel in bulk and tablet dosage forms, *International Journal of Pharmacy and Technology* 2012; 4(2): 4337-49.
21. Panda SS. Ion-pairing RP-HPLC method for simultaneous determination of aspirin and clopidogrel bisulphate in tablet and capsule dosage form, *International Journal of PharmTech Research* 2010; 2(1): 269-73.
22. Javed MK, Iqbal Z, Khan A, et al. Development and validation of HPLC-UV method for the determination of clopidogrel in pharmaceutical dosage form and human plasma, 2011; 34(18): 2118-29.
23. Mitakos A, Panderi I. A validated LC method for the determination of clopidogrel in pharmaceutical preparations, 2002; 28(3-4): 431-8.
24. Dermis S, Aydogan E. Electrochemical study of the antiplatelet agent clopidogrel and its determination using differential pulse voltammetry in bulk form and pharmaceutical preparations with a glassy carbon electrode, *Pharmazie* 2010; 65(3): 175-81.
25. Souri E, Jalalizadeh H, Kebriaee-Zadeh A, et al. Validated HPLC method for determination of carboxylic acid metabolite of clopidogrel in human plasma and its application to a pharmacokinetic study, 2006; 20(12): 1309-14.
26. Ksycinska H, Rudzki P, Bukowska-Kiliszek M. Determination of clopidogrel metabolite (SR26334) in human plasma by LC-MS, *Journal of Pharmaceutical and Biomedical Analysis* 2006; 41(2): 533-9.
27. Singh SS, Sharma K, Barot D, et al. Estimation of carboxylic acid metabolite of clopidogrel in Wistar rat plasma by HPLC and its application to a pharmacokinetic study, 2005; 821(2): 173-80.
28. Patel NK, Subbaiah G, Shah H, et al. Rapid LC-ESI-MS-MS method for the simultaneous determination of clopidogrel and its carboxylic acid metabolite in human plasma, 2008; 46(10): 867-75.
29. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceutical for Human Use. ICH harmonized tripartite guideline validation of analytical procedures: text and methodology Q2 (R1). [http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf]. 2005 [cited 2014 May 10].
30. Montgomery DC. Design and Analysis of Experiments, 7th ed., 2009, John Wiley & Sons, Inc., New York.

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