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RP-HPLC-DAD Determination of Parent Clopidogrel in Plasma of Iraqi Patients with Chronic Coronary Syndrome: Analytical Application and Clinical Interpretation

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Abstract

Background: Clopidogrel is an oral antiplatelet prodrug used in coronary heart disease. Measurement of plasma parent clopidogrel provides an analytical marker of pharmacokinetic exposure, but it should not be interpreted as active metabolite concentration or direct platelet inhibition. This study applied an adapted RP-HPLC-DAD method to quantify plasma parent clopidogrel in Iraqi patients with chronic coronary syndrome.

Patients and Methods: This cross-sectional analytical-clinical study included 97 patients receiving clopidogrel therapy. Plasma parent clopidogrel was determined using isocratic RP-HPLC-DAD with 0.02 M potassium dihydrogen phosphate buffer pH 2.8: methanol (30:70, v/v), flow rate 1.5 mL/min, injection volume 100 µL, and detection at 220 nm. Calibration, system-suitability injections, and representative chromatograms supported the analytical application.

Results: The calibration curve was linear over the available range with equation Y = 93.98788X and correlation factor r = 0.9999534. Repeated 5 ppm standard injections showed stable retention time (4.564 ± 0.022 min; %RSD 0.491) and peak area (475.26 ± 3.30 mAU.s; %RSD 0.695). Mean plasma parent clopidogrel concentration was 7.72 ± 1.09 ng/mL, median 7.59 ng/mL (5.40-10.71). Age, duration of therapy, and urea were inversely correlated with concentration. Diabetic patients had lower concentrations than non-diabetic patients (7.41 ± 1.04 vs 7.96 ± 1.08 ng/mL, P = 0.012), and diabetes remained significant after adjustment for age and urea.

Conclusion: The adapted RP-HPLC-DAD method was feasible for estimating plasma parent clopidogrel exposure in this cohort. The endpoint represents parent-drug exposure, not active metabolite concentration or direct platelet P2Y12 inhibition.

تقدير تركيز الكلوبيدوغريل غير المتحول في بلازما المرضى العراقيين المصابين بالمتلازمة التاجية المزمنة باستخدام تقنية RP-HPLC-DAD :تطبيق تحليلي وتفسير سريري

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الخلاصة

المقدمة: ان دواء الكلوبيدوغريل من الادوية الأولية المضادة لتجمع الصفيحات الدموية لذا يستخدم عادة لمرضى أمراض القلب التاجية. ويمكن أن يوفر قياس تركيز الكلوبيدوغريل غير المتحول (غير المستقلب) في البلازما مؤشراً تحليلياً للتعرض الدوائي الحركي، مع ضرورة عدم تفسيره على أنه تركيز للمستقلب الفعال أو دليل مباشر على تثبيط الصفيحات.

المرضى وطرق العمل: تشمل هذه الدراسة على 97 مريضاً عراقياً يتناولون دواء الكلوبيدوغريل. تم قياس تركيز الدواء غير المتحول في البلازما باستخدام كروماتوغرافيا السائل عالية الأداء ذات الطور العكوس المقترنة بكاشف مصفوفة الثنائيات الضوئية (RP-HPLC-DAD). حيث استُخدم طور متحرك مكوّن من محلول فوسفات البوتاسيوم ثنائي الهيدروجين 0.02مولاري عند pH 2.8والميثانول بنسبة 30:70، مع حجم حقن 100 مايكروليتر والكشف عند 220نانومتر.

النتائج: أظهر منحنى المعايرة علاقة خطية ممتازة بمعادلة Y = 93.98788X ومعامل ارتباط r = 0.9999534. كما أظهرت الحقنات المعيارية المتكررة ثباتا جيدا في المدة الزمنية للاحتجاز ومساحة القمة. وقد بلغ متوسط تركيز الكلوبيدوغريل غير المتحول في بلازما المرضى 7.72 ± 1.09 ng/mL، وكان الوسيط 7.59 ng/mL ضمن مدى 5.40-10.71 ng/mL. وقد ارتبط العمر ومدة العلاج واليوريا ارتباطا عكسياً مع تركيز الدواء، إضافة الى ذلك أظهر مرضى السكري تراكيز أقل مقارنة بغير المصابين بالسكري، وبقي مرض السكر عاملاً مؤثراً في نموذج الانحدار المتعدد الاستكشافي.

الاستنتاج: يوفر تطبيق RP-HPLC-DAD أداة تحليلية مناسبة لتقدير التعرض للكلوبيدوغريل غير المتحول في بلازما المرضى، إلا أن هذا القياس لا يمثل تركيز المستقلب الفعال للدواء ولا يعد مؤشراً مباشراً على تثبيط الصفيحات الدموية عند المرضى

1. Introduction

The most common P2Y12 oral inhibitor used in patients with coronary heart disease is clopidogrel. Because of the nature of the prodrug, the clinical efficacy of clopidogrel depends on the metabolic activation of the drug to thiol active metabolites. On the contrary, the absorbed amount of clopidogrel is subject to metabolic inactivation via esterases. Therefore, the level of the intact clopidogrel molecule in the plasma sample is a measurement of the pharmacokinetics of the drug, rather than the drug's platelet inhibition or metabolism (Jiang et al., 2015; Lee et al., 2022; Pereira et al., 2024).

Even though the analysis of clopidogrel, 2-oxo-clopidogrel, and their active metabolites by LC-MS/MS method is highly sensitive, this method is not always available in every laboratory, particularly in scientific ones. In such case, the use of RP-HPLC with DAD detection can be considered as an alternative method for quantifying the parent drug under the consideration of its limitations (Peer et al., 2012; Xu et al., 2022).

Parent drug concentration should be analyzed with consideration of the time factor and may be affected by such factors as absorption, compliance, time of sampling, esterase and CYP-mediated metabolism, organ functions, diseases, and concomitant medications. Age and diabetes are clinically significant because of the possible impact on the disposition and efficacy of the antiplatelet treatment. Pharmacodynamic response and parent-drug exposure should be distinguished as two separate outcomes of treatment (Angiolillo et al., 2007; Pontis et al., 2022).

An RP-HPLC-DAD method for the determination of clopidogrel bisulfate was developed recently and uses the isocratic elution with potassium dihydrogen phosphate buffer pH 2.8 and methanol as the mobile phase with detection at 220 nm (Soliman et al., 2024). The current research applied the adapted RP-HPLC-DAD method for the quantitation of parent clopidogrel in plasma samples of Iraqi patients with chronic coronary syndrome and analysis of its analytical characteristics and clinical associations.

2. Materials and Methods

2.1. Study Subjects

This cross-sectional analytical-clinical study included 97 Iraqi patients with chronic coronary syndrome receiving oral clopidogrel therapy. Demographic and clinical information included age, sex, body mass index (BMI), smoking status, diabetes mellitus, duration of clopidogrel therapy, selected renal-function and liver-function tests, and selected concomitant medications when available.

The study was conducted after institutional scientific and ethical approval (reference number: HU9/2024), and informed consent was obtained before sample collection. The measured analytical endpoint was plasma parent clopidogrel concentration. It was interpreted as a pharmacokinetic exposure marker and not as a direct pharmacodynamic marker of platelet inhibition. No active thiol metabolite measurement, platelet aggregation assay, or VASP phosphorylation test was performed in this analytical application.

2.2. Apparatus and Software

The RP-HPLC analysis was performed on an HPLC system equipped with a column compartment, a quaternary pump, an auto-sampler injector and a diode array detector (DAD). Chromatographic data were obtained and peaks integrated where the chromatography software was available. Solution preparation and degassing was carried out in sonicator and pH of mobile phase was adjusted by pH meter. Instrumental records available were in accordance with a Dionex Ultimate 3000 RS HPLC platform (Thermo Scientific), operated on Chromeleon version 7.1.2.1479 software. UV-visible absorbance detection by DAD was used to monitor the clopidogrel peak at 220 nm.

2.3. Analytical Standard, Reagents, and Buffer Preparation

Lipid bisulfate reference standard (purity 99.90%) was used for calibration and quantification. The organic solvent used was HPLC-grade methanol. The aqueous phosphate buffer was prepared using potassium dihydrogen phosphate and the pH was adjusted using phosphoric acid. A 0.02 M phosphate buffer was prepared by dissolving 1.36 g potassium dihydrogen phosphate in distilled water in a 500 mL volumetric flask. The solution was filtered through a 0.45 µm membrane filter before use, adjusted to pH 2.8 with phosphoric acid, brought to volume and degassed by sonication after 15 min sonication.

2.4. Chromatographic Conditions and Calibration

The modified RP-HPLC-DAD was carried out by isocratic elution. The mobile phase was methanol (30:70 v/v) and 0.02 M potassium dihydrogen phosphate buffer (pH 2.8) was prepared. Injection volume was 100 µL (0.1 mL) and flow rate was 1.5 mL/min. The sample was detected by DAD at 220 nm. The chromatographic method was developed from the paper of Soliman et al. (2024) and was adapted to the quantification of plasma parent clopidogrel as opposed to dosage form analysis. Standard solutions of clopidogrel bisulfate were used to build the calibration curve. Method of quantification was peak-area response. Available calibration report used external-standard calibration of a linear curve with passing through the zero point. The points of calibration were recorded for 1, 3, 5, 7, and 9 ppm, regression equation was $Y = 93.98788 \times X$, the correlation factor was $r = 0.9999534$. The system suitability was evaluated by the means of repeated injection of 5 ppm standard solution with calculation of mean, standard deviation, and %RSD for retention time, peak area, and peak height.

2.5. Plasma-Sample Handling, Analytical Interpretation, and Data Handling

Patient blood samples were processed to obtain plasma according to the available routine laboratory procedure before chromatographic analysis. The final analytical worksheet values were recorded as plasma parent clopidogrel concentration in ng/mL and used for statistical analysis. When the chromatographic software or worksheet output already accounted for calculation and sample-preparation factors, no additional dilution factor was applied during final reporting.

Patient peaks were identified by comparison with the retention time of the clopidogrel bisulfate reference standard and the predefined retention window around the standard peak. The peak appearing around 4.5-4.6 min was used for quantification. Additional peaks observed in some patient chromatograms near 6.0 min were not assigned as parent clopidogrel and were not used for concentration calculation because no separate reference standard was available for their identification.

The analytical output was handled as clopidogrel-bisulfate-equivalent parent clopidogrel concentration. This work was classified as an adapted analytical application rather than a fully validated bioanalytical method-development study. Full plasma-matrix validation in compliance with current bioanalytical guidance (International Council for Harmonisation, 2022; International Council for Harmonisation, 2023) requires pre-defined assessments of matrix selectivity, recovery, carryover, lower limit of quantification, accuracy, precision, dilution integrity, matrix effect and analyte stability.

2.6. Statistical Analysis

The data were analyzed using standard descriptive and inferential statistics. Continuous variables were expressed as mean ± standard deviation and median (range) as appropriate. The primary endpoint was analyzed as a continuous pharmacokinetic exposure variable. Continuous variables were correlated using Pearson correlation. Categorical group comparisons were performed with independent-samples t-test or Welch t-test depending on the variance assumptions. Mann-Whitney U testing was used when applicable as a supporting non-parametric comparison. The dependent variable

was plasma parent clopidogrel concentration and the independent variables were clinically relevant predictors. Exploratory multivariable linear regression was performed A two-tailed P value of <0.05 was considered as statistically significant.

3. Results

3.1. Baseline Characteristics

Ninety-seven patients on clopidogrel were enrolled in the study. The cohort consisted of 60 (61.86%) males and mean age was 63.78 ± 8.82 years. Smoking was observed in 45 (46.39%) and diabetes mellitus in 43 (44.33%). The average body mass index (BMI) was 30.03 ± 3.57 kg/m2. Baseline characteristics are summarized in Table1.

Table1: Baseline Demographic and Clinical Characteristics Of The Study Cohort (N = 97).

Variable	Value
Age, years, mean ± SD	63.78 ± 8.82
Age, years, median (range)	64.00 (40.00-81.00)
BMI, kg/m², mean ± SD	30.03 ± 3.57
BMI, kg/m², median (range)	29.35 (22.23-38.29)
Male sex, n (%)	60 (61.86%)
Female sex, n (%)	37 (38.14%)
Smoking, n (%)	45 (46.39%)
Diabetes mellitus, n (%)	43 (44.33%)
Duration of clopidogrel therapy, months, mean ± SD	20.15 ± 13.06
Duration of clopidogrel therapy, months, median (range)	18.00 (3.00-60.00)
Creatinine, mg/dL, mean ± SD	0.86 ± 0.22
Urea, mg/dL, mean ± SD	32.66 ± 9.29
AST, U/L, mean ± SD	27.75 ± 6.84
ALT, U/L, mean ± SD	21.97 ± 8.51
ALP, U/L, mean ± SD	101.71 ± 29.13

3.2. RP-HPLC Analytical Application

Standard chromatogram available showed a distinct peak of clopidogrel at around 4.53-4.59 minutes. Calibration report showed identification of clopidogrel at about 4.50 minutes with retention time window of ± 0.20 minutes. Samples from patients showed a similar peak at 4.55-4.58 minutes indicating identification of parent clopidogrel based on retention time matching to that of the standard. Calibration graph showed good linearity within the range of 1-9 ppm available. Repetitive injections of 5 ppm standard solution also had a low % RSD of retention time and peak area, as in Table2, Table3 and Table4.

Table2: Summary of RP-HPLC Chromatographic Conditions For Plasma Parent Clopidogrel Determination.

Parameter	Condition
Chromatographic mode	Reversed-phase HPLC
Elution mode	Isocratic
Mobile phase	0.02 M potassium dihydrogen phosphate buffer pH 2.8:methanol
Mobile phase ratio	30:70, v/v
Flow rate	1.5 mL/min
Detector	Diode array detector (DAD; UV-visible absorbance detection)
Detection wavelength	220 nm
Column	ODS Hypersil C18 column, 250 × 4.6 mm, 5 µm
Injection volume	100 µL (0.1 mL)
Measured endpoint	Plasma parent clopidogrel concentration
Interpretation	Pharmacokinetic exposure marker, not a platelet inhibition marker

Table3: Calibration Data for Clopidogrel Standard Solutions.

Amount (ppm)	Peak-area response (mAU.s)	Response factor
1	98	0.0102
3	289	0.0104
5	472	0.0106
7	654	0.0107
9	845	0.0107

Table4: System Suitability Summary for Repeated Clopidogrel 5 Ppm Standard Injections (N = 9).

Parameter	Mean ± SD	%RSD	Observed range
Retention time (min)	4.564 ± 0.022	0.491	4.53-4.59
Peak area (mAU.s)	475.26 ± 3.30	0.695	470.14-480.25
Peak height (mAU)	625.45 ± 4.54	0.726	620.14-631.47
Peak width W05 (min)	0.25	0.000	0.25-0.25

The system-suitability results for the repeated injections of standard showed a narrow range of retention time and low variability of peak area. This ensured that the analytical application had sufficient short-term chromatographic repeatability. However, the results were not interpreted as a replacement for full plasma-matrix validation, as in Table5, Fig.1 and Fig.2.

Table5: Calibration Equation and Representative Chromatographic Characteristics

Item	Result
Calibration equation	Y = 93.98788 × X
Correlation factor	0.9999534
Response base	Peak area
Curve fit	Linear through origin
Calibration range available	1-9 ppm
Standard retention time	Approximately 4.53-4.59 min
Patient-sample target peak	Approximately 4.55-4.58 min
Additional sample peak	Approximately 6.00-6.09 min; not used for parent clopidogrel quantification

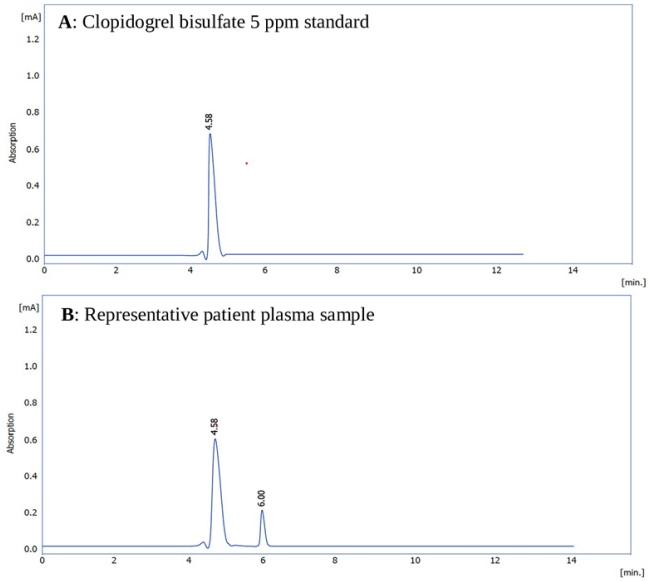

Figure1: Representative RP-HPLC-DAD Chromatograms of Clopidogrel Analysis. (A) Clopidogrel bisulfate 5 ppm standard solution showing the target peak at approximately 4.58 min. (B) Representative patient plasma sample showing a corresponding peak within the same retention window. Detection was performed at 220 nm with an injection volume of 100 µL.

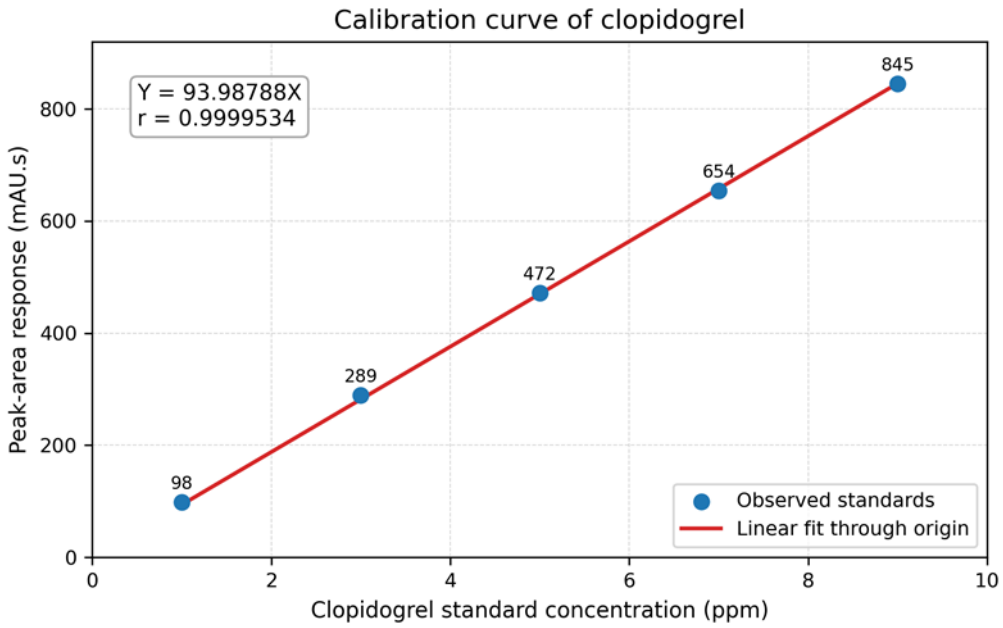

Figure2: Corrected Calibration Curve of Clopidogrel Showing Linear Peak-Area Response Across 1-9 Ppm with Equation Y = 93.98788X And Correlation Factor R = 0.9999534.

3.3. Plasma Parent Clopidogrel Concentration and Clinical Associations

Mean plasma parent clopidogrel concentration was 7.72 ± 1.09 ng/mL (median 7.59 ng/mL, range 5.40-10.71). A statistically significant inverse correlation was found between plasma parent clopidogrel concentration and age (r = -0.246, P = 0.015). A weaker inverse correlation was seen for urea (r = -0.228, P = 0.026) and an inverse correlation was seen for clopidogrel therapy duration (r = -0.275, P = 0.006). The parent clopidogrel concentration was not significantly correlated to creatinine, AST, ALT or ALP, whereas BMI was borderline inversely correlated (r = -0.193, P = 0.058), Table6.

Table6: Correlation Between Plasma Parent Clopidogrel Concentration and Selected Continuous Variables.

Variable	Pearson r	P value
Age	-0.246	0.015
BMI	-0.193	0.058
Duration of clopidogrel therapy	-0.275	0.006
Creatinine	-0.163	0.113
Urea	-0.228	0.026
AST	0.006	0.953
ALT	0.079	0.442
ALP	0.012	0.905

Diabetic patients had lower plasma parent clopidogrel concentrations than non-diabetic patients (7.41 ± 1.04 versus 7.96 ± 1.08 ng/mL, P = 0.012 by Welch t-test; supportive Mann-Whitney P = 0.0145). Sex and smoking status were not significantly associated with plasma parent clopidogrel concentration, Table7 and Table8.

Table7: Plasma Parent Clopidogrel Concentration According to Selected Categorical Variables

Variable	Group	n	Mean ± SD (ng/mL)	P value
Sex	Male	60	7.72 ± 1.10	0.947
	Female	37	7.71 ± 1.09	
Smoking	Yes	45	7.65 ± 1.00	0.584
	No	52	7.77 ± 1.18	
Diabetes Mellitus	Yes	43	7.41 ± 1.04	0.012
	No	54	7.96 ± 1.08	

Table8: Exploratory Multivariable Linear Regression Model for Predictors of Plasma Parent Clopidogrel Concentration. Model Summary: R^2 = 0.130; Adjusted R^2 = 0.102; F = 4.593; N = 96; P = 0.005.

Predictor	Unstandardized coefficient (B)	Standard Error	95% CI	P value
Age (years)	-0.0145	0.013	-0.040 to 0.011	0.268
Diabetes mellitus (yes vs no)	-0.4887	0.222	-0.930 to -0.047	0.031
Urea (mg/dL)	-0.0223	0.012	-0.046 to 0.001	0.060

3.4. Clinical determinants of plasma parent clopidogrel exposure

In this exploratory model, diabetes mellitus remained independently associated with lower plasma parent clopidogrel concentration after adjustment for age and urea. The model should be interpreted as exploratory because the study was cross-sectional and the available variables were not sufficient for a complete pharmacokinetic model. The model used 96 observations because one case had incomplete data for the variables included in the regression model, Fig3 A-C.

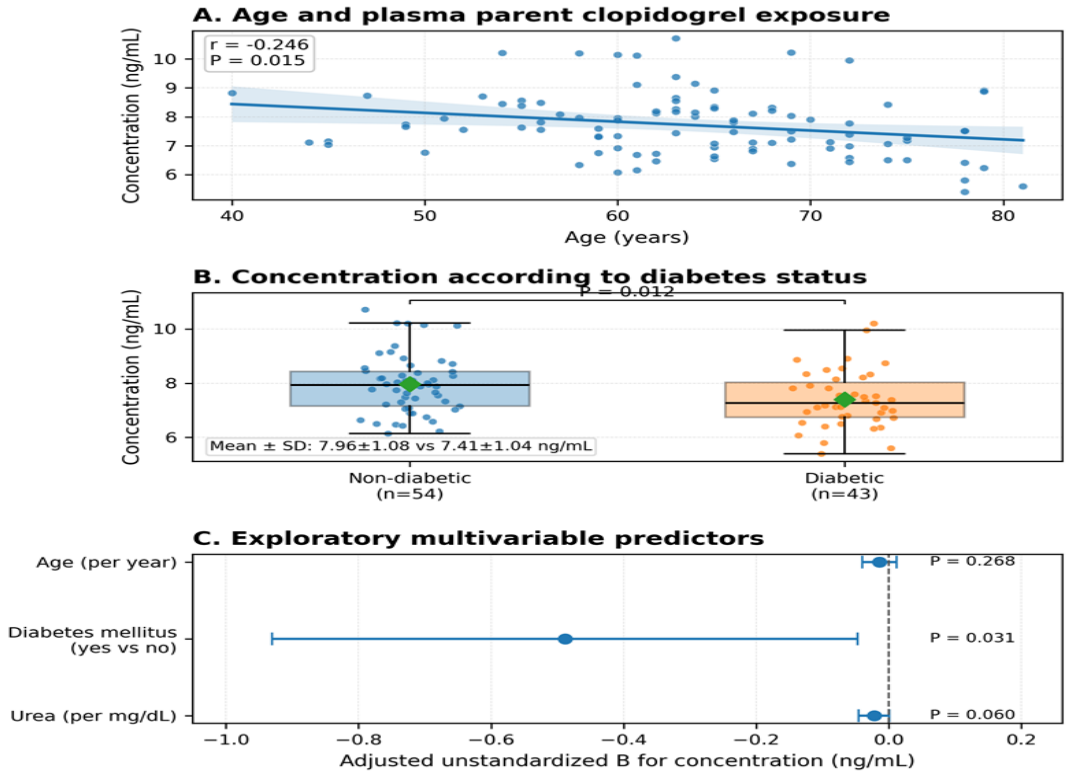

Figure3: Integrated Graphical Summary of Selected Clinical Determinants of Plasma Parent Clopidogrel Exposure. Panel A shows the relationship between age and plasma parent clopidogrel concentration with a fitted regression line and 95% confidence band. Panel B shows the distribution of plasma parent clopidogrel concentration according to diabetes status using a box-and-whisker plot with individual patient values. Panel C shows exploratory multivariable regression coefficients adjusted for age, diabetes mellitus, and urea. diabetes was coded as yes versus no. Model adjusted for age, diabetes mellitus, and urea; n= 96 due to one missing renal-function value.

4. Discussion

In the current study, an adapted RP-HPLC-DAD technique has been used to quantify the level of plasma parent clopidogrel concentration in Iraqi patients having chronic coronary syndrome. The current work does not provide for the complete bioanalytical method validation but rather for its practical application using accessible chromatography technique along with the calibration curve, repeated standard injections, and real-life patients' chromatograms.

The low %RSD values in regard to retention time and peak area can be seen as the proof of system repeatability in a short run. However, it should be emphasized that the results in question represent the system suitability rather than validation since no selectivity, sensitivity, accuracy, precision, stability, and other required experiments have been conducted. This differentiation is justified by current bioanalytical and analytical validation expectations (International Council for Harmonisation, 2022; International Council for Harmonisation, 2023).

It should be pointed out that the differences between the parent clopidogrel and active thiol metabolite are crucial. The level of parent clopidogrel concentration can be seen as the indicator of the drug concentration that is not metabolized by the time of blood sampling. Such concentration can be affected by many factors including absorption, metabolism, time, adherence, comorbidities, and medications (Jiang et al., 2015; Xu et al., 2022).

The inverse correlations between the levels of parent clopidogrel concentration and such parameters as age, duration of clopidogrel therapy, and urea can be interpreted as the evidence that some clinical or biochemical factors might play the role of interindividual variability determinants in terms of parent clopidogrel exposure. The correlation with urea should be considered cautiously as it does not reflect the renoclearance of clopidogrel since the latter is eliminated from the body unchanged to any significant extent.

The presence of diabetes mellitus correlated with lower levels of parent clopidogrel concentration independently of age and urea concentrations. It is important since patients with this pathology usually show considerable variation in their responses to clopidogrel in pharmacodynamic studies. Still, the current finding refers only to the level of parent drug concentration and cannot be considered as the evidence of decreased active metabolite concentration or decreased platelet aggregation since these parameters were not measured (Angiolillo et al., 2007; Lee et al., 2022; Pereira et al., 2024).

The strengths of this work include the usage of real patient plasma samples, standard injection, and repeated injections, as well as analytical and clinical interpretation in local Iraqi population. Limitations of this work include the lack of full plasma-matrix validation, blank plasma chromatograms, measurements of active metabolite concentration, and platelet function testing.

Conclusion

An adapted RP-HPLC-DAD method was applied for determination of plasma parent clopidogrel concentration in Iraqi patients with chronic coronary syndrome receiving clopidogrel therapy. The method showed excellent calibration linearity and acceptable short-run system repeatability based on repeated standard injections. Plasma parent clopidogrel concentration showed measurable interindividual variability and was associated with age, diabetes mellitus, duration of therapy, and urea in exploratory analyses. Diabetes remained associated with lower parent clopidogrel concentration after adjustment for age and urea. These findings support the usefulness of parent clopidogrel measurement as an accessible pharmacokinetic exposure marker, but they should not be interpreted as direct evidence of active metabolite exposure or platelet P2Y12 inhibition. Future study should include pharmacogenetic integration, active metabolite measurement, plasma-matrix validation, and platelet-function assessment. Also, samples should be timed.

Conflicts of Interest

The authors declare no conflict of interests.

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Ethical Approval

The study was conducted after approval by the relevant scientific and ethical committee (reference number: HU9/2024), and informed consent was obtained from participants before sample collection.

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