

UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

DOCTORAL THESIS

CHROMATOGRAPHIC ANALYSIS OF CLOPIDOGREL.PHARMACOKINETIC STUDIES

ABSTRACT

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I.INTRODUCTION

Chromatographic analysis of clopidogrel from biological samples emerged with the therapeutic strategies to reduce the risk of early and late occurring ischemic complications after platelet resistance and to improve survival.

This research provides qualitative and quantitative estimations of clopidogrel and its carboxylic acid metabolite from tablets and human plasma by high performance liquid chromatography. Further studies are therefore warranted to clarify the pharmacokinetic profile of clopidogrel and its carboxylic acid metabolite along with pantoprazole and atorvastatin in patients with acute coronary syndrome.

II.CURRENT STATE OF KNOWLEDGE

This part consists of the following three chapters which present data from literature survey (international guidelines recommendations, scientific articles).

The first chapter of the doctoral thesis describes the atherothrombotic risk assessment and outcomes, pathophysiology, epidemiology and clinical manifestations of atherothrombosis. Prevention of cardiovascular disease has become a lifelong approach with strategies and recommendations in nutrition, lifestyle and risk factors [1].

The second chapter summarizes the pharmacological profile of clopidogrel, describing the pharmacokinetic parameters and their consequences on clopidogrel pharmacodynamics, indications and administration, drug-drug interactions between clopidogrel - pantoprazole and clopidogrel-atorvastatin. The results of clinical trials and international guidelines recommendations for patients with acute coronary syndrome [2] and major cardiovascular risks are reported in tables.

The third chapter contains chromatographic methods from literature survey with their applications in bioequivalence, biodisponibility and pharmacokinetic studies of clopidogrel and its carboxylic acid metabolite.

Chromatographic analysis of clopidogrel was performed by HPLC-MS [3], HPLC-MS-MS [4-9], HPLC-UV [10-11]. The steps of chromatographic analysis are presented including sample preparation, clopidogrel extraction procedures from plasma, isocratic [12] and gradient [4] elution, mobile phase and pH optimization.

Current state of knowledge contains a list of chromatographic methods from literature survey implied in determination of clopidogrel and its carboxylic acid metabolite from human plasma [13].

III.PERSONAL CONTRIBUTIONS

III.1. Evidence of the chromatographic research

This chapter highlights the evidence of the chromatographic research in pharmacokinetic studies of clopidogrel-atorvastatin and clopidogrel-pantoprazole and the importance of plasmatic estimation of clopidogrel in patients with major cardiovascular risks.

III.2. Simultaneous Analysis of Clopidogrel Bisulfate, Acetylsalicylic Acid and Atorvastatin in Tablets by HPLC Method

This chapter presents preliminary tests during the method optimization , a CCF design, quadratic model , fitted with PLS was chosen to determine the optimum set of factors (acetonitrile and KH_2PO_4) and their effects on chromatographic parameters: resolution (R_s), retention time (t_R) and tailing factor (T_f). These effects were quantified with 11 experiments using MODDE 9.1 software.

Chromatographic analysis was carried out using a ThermoFinnigan chromatograph consisting of ternary solvent manager, a manual injector of 20 μL loop, PDA detector and a Thermo Finnigan Xcalibur software for data acquisition. The separation was achieved on an HDS Hypersil C_{18} analytical column (250x4.6mm, 5 μm particle size). The mobile phase consists in a mixture of 0.01 M KH_2PO_4 buffer (pH adjusted to 2.6 with phosphoric acid):acetonitrile:methanol 20:40:40 (v/v/v) at flow rate of 0.8 $\text{ml}\cdot\text{min}^{-1}$.

The linearity of the method was determined at six concentration levels ranging from 0.03-5 $\mu\text{g}\cdot\text{mL}^{-1}$ for clopidogrel bisulfate, 0.03-2 $\mu\text{g}\cdot\text{mL}^{-1}$ for acetylsalicylic acid and 0.04-1.25 $\mu\text{g}\cdot\text{mL}^{-1}$ for atorvastatin. The precision of the method was evaluated by carrying out six independent assays of clopidogrel bisulfate (0.05, 0.3, 1 $\mu\text{g}\cdot\text{mL}^{-1}$), acetylsalicylic acid (0.05, 0.5, 1 $\mu\text{g}\cdot\text{mL}^{-1}$) and atorvastatin (0.08, 0.1, 0.8 $\mu\text{g}\cdot\text{mL}^{-1}$) test samples against reference standard.

The simultaneous detection of clopidogrel, acetylsalicylic acid and atorvastatin in tablet dosage form is presented in figure 1 [14]. The retention times were: 3.138 min for acetylsalicylic acid, 4.133 min for atorvastatin and 6.833 min for clopidogrel bisulfate.

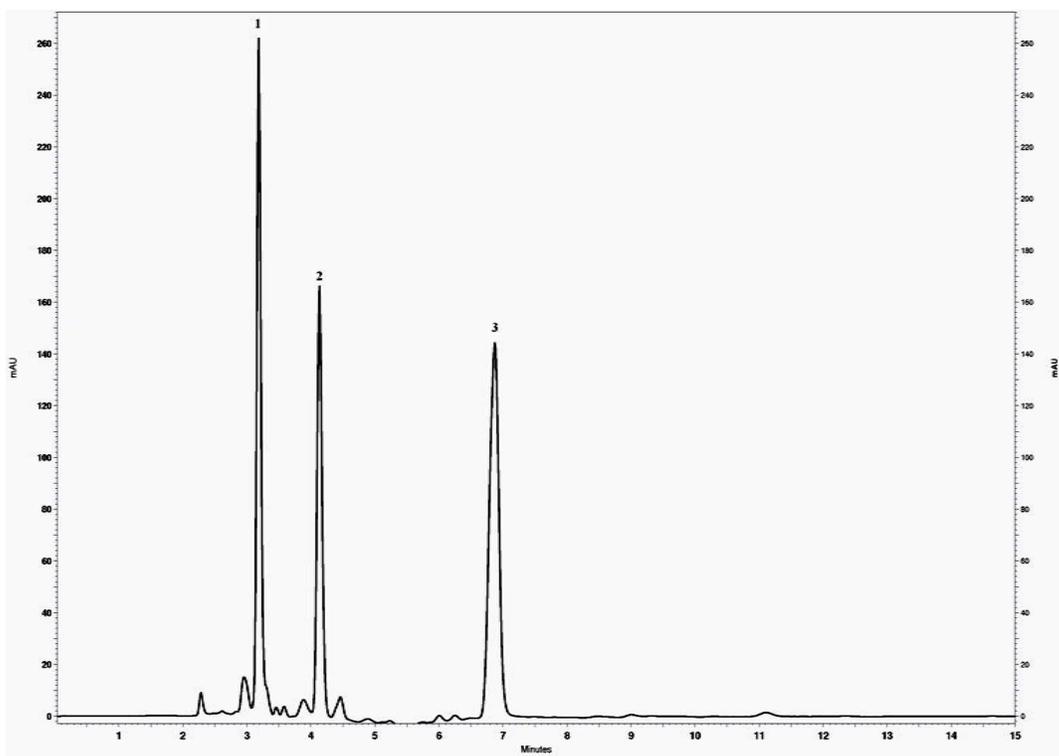


Figure 1 Chromatogram of acetylsalicylic acid (peak 1), atorvastatin (peak 2) and clopidogrel (peak 3) from tablets under optimized method with 0.01 M KH_2PO_4 buffer (pH adjusted to 2.6 with phosphoric acid):acetonitrile:methanol 20:40:40 (v/v/v) at flow rate of 0.8 $\text{ml}\cdot\text{min}^{-1}$

III.3. Contributions to High-Performance Liquid Chromatographic Determination of Clopidogrel Carboxylic Acid Metabolite and Pantoprazole in Human Plasma: A Pilot Study

A simple, sensitive and rapid reversed phase liquid chromatographic method is described for simultaneous estimation of clopidogrel carboxylic acid (CCA) metabolite and pantoprazole in human plasma. The chromatographic analysis was performed on the same „Thermo Finnigan Surveyor” chromatograph.

Chromatographic separation was performed on an HDS Hypersil C₁₈ column

(250x 4.6 mm; 5µm) via isocratic elution using water (pH=3 adjusted with orthophosphoric acid 85%): methanol: acetonitrile 36:10:54 (v/v/v) as mobile phase at flow rate of 1 mL·min⁻¹.

Protein precipitation with acetonitrile was the main procedure used for samples preparation. To 0.5 mL of plasma 25µL of IS (ibuprofen 100 µg·mL⁻¹) and 25µL working standard solutions were added. For protein precipitation 1 ml acetonitrile was added. It was further centrifugated at 4000 rpm for ten minutes. The supernatant was dried under a nitrogen current and the dry residue was prepared in methanol. 20 µL was injected in the chromatographic column.

The method was validated according to ICH [15] in terms of linearity, lower limits of quantification, precision, accuracy and specificity and successfully applied for simultaneous quantification of clopidogrel and pantoprazole in patients during their maintenance therapy with 75 mg clopidogrel and 20 mg pantoprazole. The calibration curves were linear in the concentration range of 0.03-5 µg·mL⁻¹ for carboxylic acid metabolite and 0.04-5 µg·mL⁻¹ for pantoprazole. LLOQ were 0.03 µg·mL⁻¹ for clopidogrel carboxylic acid metabolite and 0.04 µg·mL⁻¹ for pantoprazole respectively. Retention times for clopidogrel carboxylic acid metabolite, pantoprazole and ibuprofen (5µg·mL⁻¹) were: 4.083 , 4.550, 9.817 min.

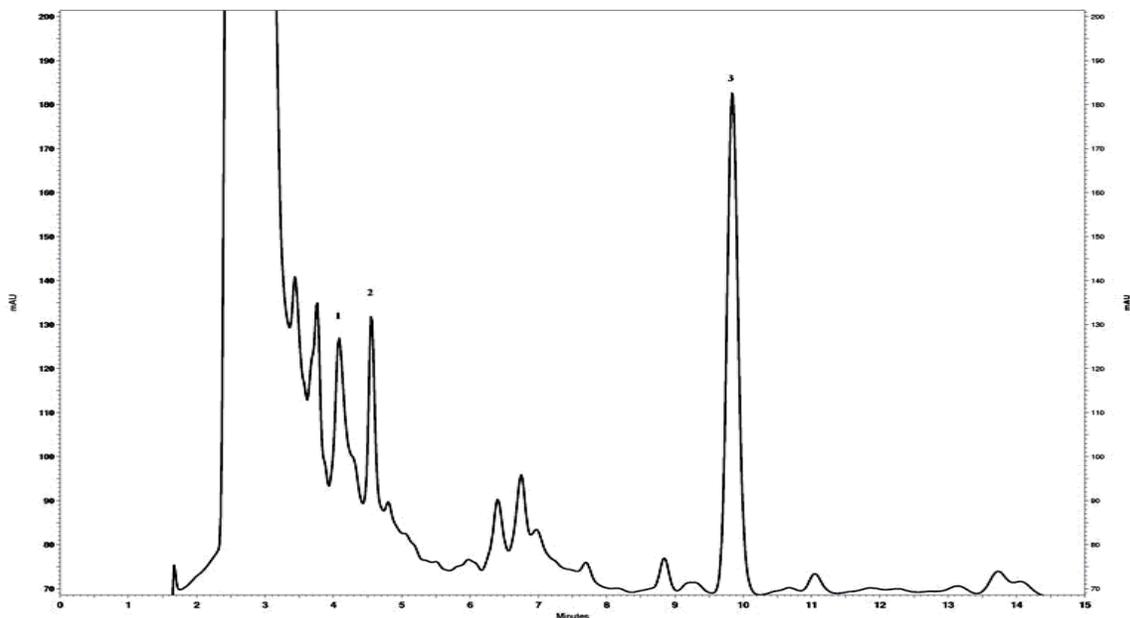


Figure 2 Chromatogram of plasma sample from a volunteer who received 75mg clopidogrel and 20 mg pantoprazole at 45 minutes post-dose under isocratic method at flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$ (peak 1- clopidogrel carboxylic acid metabolite, peak 2- pantoprazole, peak 3- ibuprofen)

III.4. Contributions to Development and Validation of an HPLC Method for Simultaneous Quantification of Clopidogrel Bisulfate, Its Carboxylic Acid Metabolite and Atorvastatin in Human Plasma: Application to a Pharmacokinetic Study

Numerous international guidelines provide evidence base recommendations of co-prescribing antiplatelets drugs and statins in secondary prevention of cardiovascular events in patients with atherothrombosis (acute coronary syndromes ACS, cerebrovascular disease and peripheral arterial disease PAD) [16].

The present chapter describes a selective gradient chromatographic method [17] for simultaneous determination of clopidogrel, its carboxylic acid metabolite and atorvastatin in human plasma and in vivo application in three patients following oral administration of clopidogrel and atorvastatin during their maintenance therapy.

Chromatographic analysis was carried out using the same „Thermo Finnigan Surveyor” chromatograph. A gradient method was proposed and described in table 1.

Table 1 Gradient program for simultaneous determination of clopidogrel, its carboxylic acid metabolite and atorvastatin

Time (min)	Solvent A (Buffer solution)	Solvent B (ACN)	Solvent C (CH ₃ OH)
0.01	90	10	0
2.00	90	10	0
3.00	60	40	0
4.00	30	50	20
8.00	30	50	20
10.00	30	40	30
18.00	30	50	20
19.00	90	10	0
20.00	90	10	0

All samples were identically treated as follows: to a 0.5 mL plasma samples, 20µL ibuprofen standard solution (50 µg·mL⁻¹), 20 µL working standard solutions of analytes and 200 µL of HCl solution (2 M) were added. Protein precipitation was obtained with 500µL of acetonitrile. The samples were shaken for 5 minutes and centrifugated for 10 minutes at 4500 rpm. Then, the supernatant was transferred into conical tubes and evaporated to dryness under gentle stream of nitrogen at ambient temperature. The residue was reconstituted in 500µL methanol and 20 µL was injected into chromatographic column.

The linearity of the method was determined at eight concentration levels ranging from 0.008-2 µg·mL⁻¹ for clopidogrel bisulfate, 0.01-4 µg·mL⁻¹ for its carboxylic acid metabolite and 0.005-2.5 µg·mL⁻¹ for atorvastatin. The calibration curves were determined from the lower limit of quantification (LLOQ) of 0.008 µg·mL⁻¹ for clopidogrel bisulfate, 0.01 µg·mL⁻¹ for clopidogrel carboxylic acid metabolite and 0.005 µg·mL⁻¹ for atorvastatin. Retention times for clopidogrel carboxylic acid metabolite, atorvastatin, ibuprofen 2 µg·mL⁻¹ (IS) and clopidogrel bisulfate were 9.663, 10.998, 11.802, 12.682 minutes. Forced degradation studies were performed for stability indicating method and

excipients interfering process.

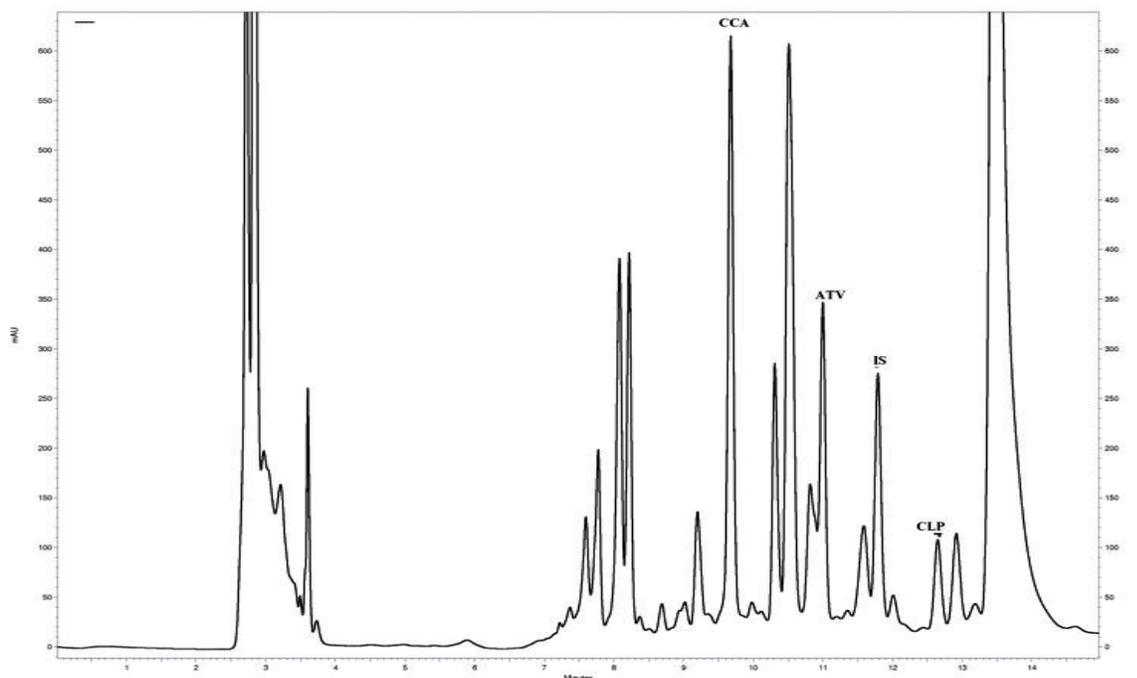


Figure 3 Chromatogram of *plasma sample from a volunteer who received 75mg clopidogrel and 40 mg atorvastatin at 40 minutes post-dose under optimized gradient method at flow rate of 1 mL·min⁻¹.*

The applicability of the method has been demonstrated in a pharmacokinetic study to patients undergoing antiplatelet therapy with 75 mg clopidogrel and 20, 40 and 80 mg atorvastatin. The results obtained for clopidogrel carboxylic acid metabolite were the followings: $C_{max}= 4.2 \mu\text{g}\cdot\text{mL}^{-1}$, $T_{max}=1 \text{ h}$, $t_{1/2}=4.51 \text{ h}$ for patient 1, $C_{max}= 4.77 \mu\text{g}\cdot\text{mL}^{-1}$, $T_{max}=1.24 \text{ h}$, $t_{1/2}=3 \text{ h}$ for patient 2, $C_{max}= 3.32 \mu\text{g}\cdot\text{mL}^{-1}$, $T_{max}=1 \text{ h}$, $t_{1/2}= 5.21 \text{ h}$ for patient 3. The pharmacokinetic parameters for atorvastatin were determined as followings: $C_{max}= 0.09 \mu\text{g}\cdot\text{mL}^{-1}$, $T_{max}=3 \text{ h}$, $t_{1/2}= 8.51 \text{ h}$ for patient 1, $C_{max}= 0.15 \mu\text{g}\cdot\text{mL}^{-1}$, $T_{max}= 2.52 \text{ h}$, $t_{1/2}= 8.37\text{h}$ for patient 2, $C_{max}= 0.19 \mu\text{g}\cdot\text{mL}^{-1}$, $T_{max}= 3.06 \text{ h}$, $t_{1/2}= 9.32 \text{ h}$ for patient 3. Effects of atorvastatin on clopidogrel pharmacokinetics were modest for the patients included in the study in order to stop their co-administration based on a drug-drug interaction.

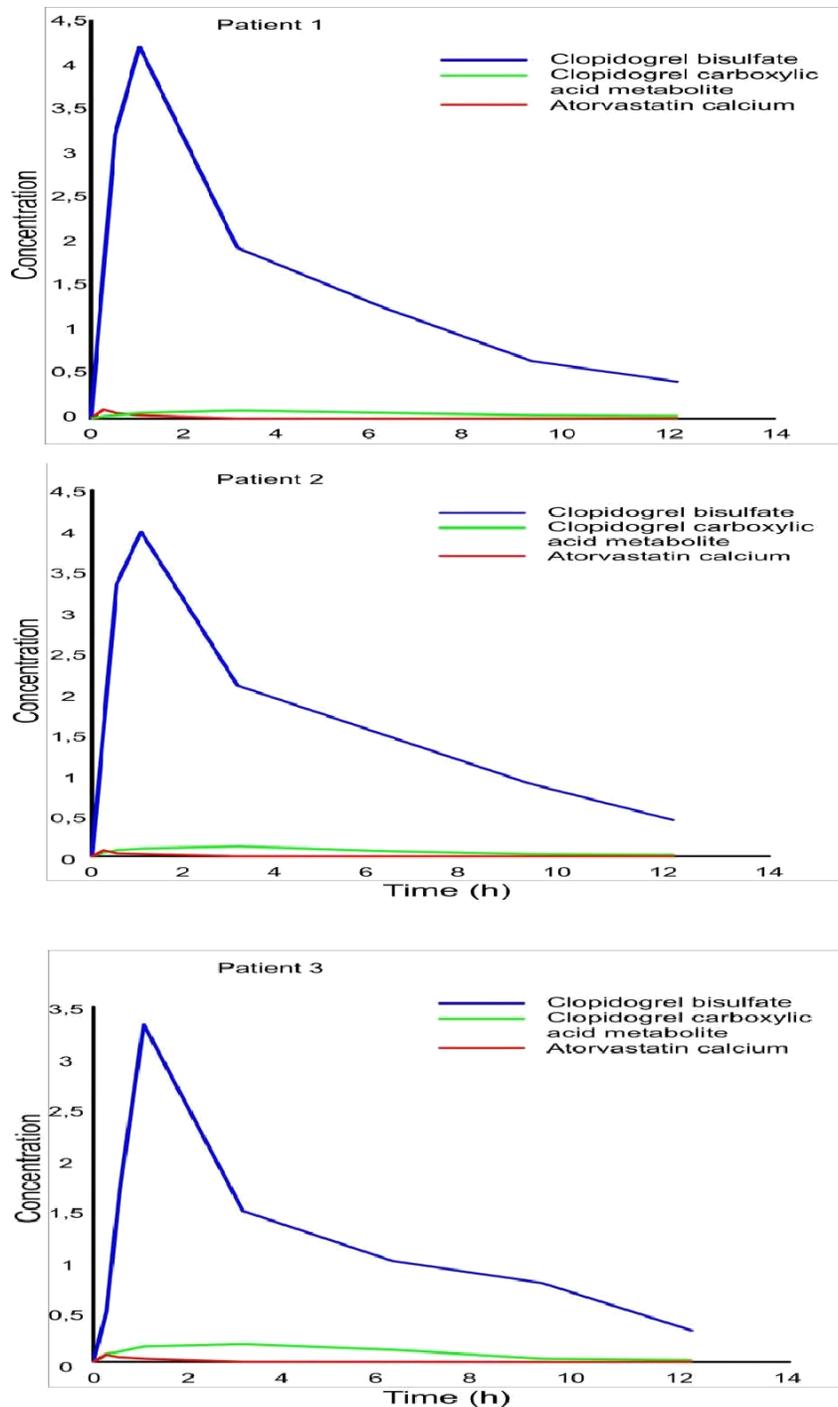


Figure 4 Plasma levels of clopidogrel, its carboxylic acid metabolite and atorvastatin versus time following administration of 75mg clopidogrel and 20 mg atorvastatin daily (patient 1), 75mg clopidogrel and 40 mg atorvastatin daily (patient 2), 75mg clopidogrel and 80 mg atorvastatin daily (patient 3)

IV. CONCLUSIONS

Preliminary tests during the method optimization were performed to determine the optimum set of factors (acetonitrile and KH_2PO_4) and their effects on chromatographic parameters: resolution (R_s), retention time (t_R) and tailing factor (T_f). Chromatographic analysis of clopidogrel from tablets was performed after the results from the study design, the mobile phase composition consisting in 0.01 M KH PO buffer (pH adjusted to 2.6 with phosphoric acid):acetonitrile:methanol 20:40:40 v/v/v at flow rate of $0.8 \text{ mL}\cdot\text{min}^{-1}$.

Chromatographic separation for clopidogrel carboxylic acid metabolite and pantoprazole from human plasma was performed via isocratic elution using water (pH=3 adjusted with orthophosphoric acid): methanol: acetonitrile 36:10:54 (v/v/v) as mobile phase at flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$. The method was successfully applied for simultaneous quantification of clopidogrel and pantoprazole in patients during their maintenance therapy with 75 mg clopidogrel and 20 mg pantoprazole.

The next step in the research was performed for development and validation of a gradient chromatographic method for simultaneous determination of clopidogrel bisulfate, clopidogrel carboxylic acid metabolite and atorvastatin in human plasma.

The applicability of the method has been demonstrated in a pharmacokinetic study to patients undergoing antiplatelet therapy with 75 mg clopidogrel and 20, 40 and 80 mg atorvastatin.

Chromatographic analysis of clopidogrel can be performed on patients with major cardiovascular risks and determine the possible drug-drug interactions from the multidrug therapy.

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