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Original Article

# High Performance Liquid Chromatographic Determination of Inactive Carboxylic Acid Metabolite of Clopidogrel in Human Serum: Application to a Bioequivalence Study

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## Abstract

A sensitive and rapid method is described for determination of clopidogrel carboxylic acid (CCA), the inactive metabolite of the antiplatelet agent clopidogrel in human serum. The analytical procedure involves liquid-liquid extraction of the analyte and an internal standard (phenytoin) with ethyl acetate. A mobile phase consisting of 0.05 M phosphate buffer containing triethylamine (0.5 ml/l; pH 5.7) and acetonitrile (56:44; v/v) was used and chromatographic separation was achieved using a $C_{18}$ analytical column at detector wavelength of 220 nm. The calibration curves were linear over a concentration range of 0.05-10 μg/ml of CCA in human serum. The total run time of analysis was 5.5 min. and the lower limits of detection (LOD) and quantification (LOQ) were 0.02 and 0.05 μg/ml, respectively. The method validation was carried out in terms of specificity, sensitivity, linearity, precision, accuracy and stability. The validated method was applied in a randomized cross-over bioequivalence study of two different clopidogrel preparations in 24 healthy volunteers.

*Keywords:* Bioequivalence study; Clopidogrel; HPLC; Metabolites.
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## 1. Introduction

Clopidogrel hydrogen sulfate, methyl (+)-(S)-α-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-acetate hydrogensulfate, is a thienopyridine derivative that irreversibly blocks ADP receptor. Clopidogrel is chemically related to ticlopidine with superior side effects profile and dosing requirements. The drug which reduces platelet aggregation is extensively used for prevention of thrombosis in patients undergoing placement of a coronary stent [1]. The drug is rapidly, but incompletely, absorbed after oral administration and extensively metabolized to an active metabolite. The parent drug or its active metabolite remains undetectable in plasma. The major circulating compound, however, is an inactive carboxylic derivative, which its blood concentration is used to document the pharmacokinetic profile of

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clopidogrel [2]. A few analytical methods including gas chromatography-mass spectrometry (GC-MS) [3], liquid chromatography-mass spectrometry (LC-MS) [4, 5] and high-performance liquid chromatography (HPLC) with UV detection [6, 7] have been published for determination of the inactive metabolite of clopidogrel in the biological fluids. In these methods, however, complex two steps extraction methods [3, 4] or long analytical run time (10 min. [4], 16 min. [6] and 12 min. [7]) are required. While limit of quantification (LOQ) of 0.01 μg/ml [5], 0.125 μg/ml [6] and 0.2 μg/ml [7] have been reported in published papers, in human single dose pharmacokinetic studies a LOQ of less than 0.1 μg/ml is required for measuring the analyte up to three half lives post-dose. The present paper which has been applied in a bioequivalence study of two different clopidogrel preparations, describes a simple and fast method for analysis of the inactive carboxylic metabolite of clopidogrel in human serum, with sensitivity of 0.05 μg/ml and analytical run time of 5.5 min.

## 2. Materials and methods

### *2.1. Chemicals and standard solutions*

Clopidogrel carboxylic acid (CCA) (purity 99.5%) was synthesized by Ind-Swift Laboratories Ltd. (Punjab, India). Phenytoin (I.S.) was from Sigma (St. Louis, MO, USA). All reagents used were of analytical grade except acetonitrile which was HPLC grade. Water was glass double-distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

A stock solution of CCA (1000 μg/ml) was prepared in methanol. Working standards of the drug (0.5-100 μg/ml) were prepared by serial dilution of the stock solution in methanol. Working standard solution of the I.S. (7.5 μg/ml) was prepared in methanol. A 2N hydrochloric acid solution was prepared

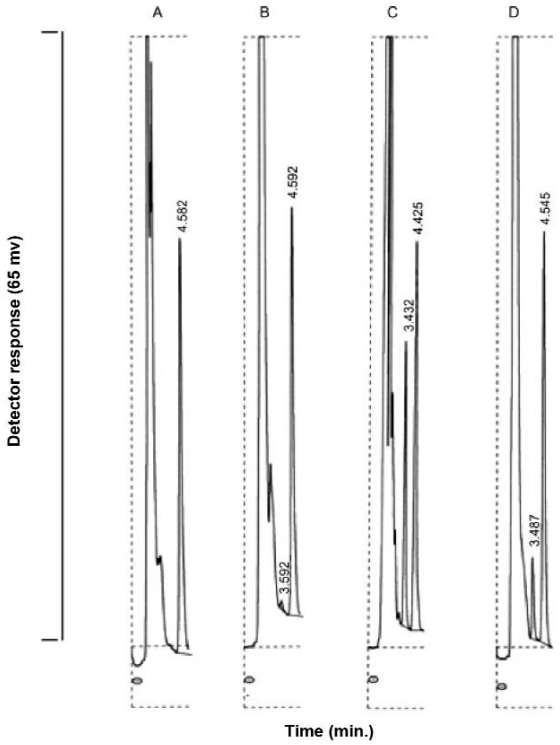


**Figure 1.** Typical chromatograms obtained from an extract of (A) human blank serum spiked with phenytoin as the I.S., (B) human blank serum spiked with 0.05 μg/mL CCA and phenytoin as the I.S., (C and D) serum samples obtained at 6 and 12 h after a single oral dose of 150 mg clopidogrel from a healthy volunteer containing 1.06 and 0.30 μg/mL of CCA, respectively. Peaks eluted at 3.4 and 4.5 min. correspond to CCA and the I.S., respectively.

**Table 1.** HPLC precision and accuracy results of the validation of clopidogrel carboxylic acid calibration curve.

| Known concentration (μg/ml) | Concentration found (mean±S.D.) | Coefficient of variation (%) | Accuracy (%mean deviation) |
|---|---|---|---|
| Inter-day | | | |
| 0.05 | 0.047±0.005 | 10.6 | -3.9 |
| 0.5 | 0.495±0.017 | 3.4 | -1.9 |
| 5 | 5.071±0.075 | 1.5 | 1.2 |
| Intra-day | | | |
| 0.05 | 0.051±0.006 | 11.7 | 1.1 |
| 0.5 | 0.512±0.016 | 3.1 | 1.8 |
| 5 | 5.135±0.082 | 1.6 | 1.4 |

Note: Accuracy has been calculated as a percentage of the nominal concentration.

in distilled water. All solutions were stored at 4 °C and were stable for at least 3 weeks.

*2.2. Chromatographic conditions*

The HPLC system used consisted of two pumps of Shimadzu LC-10A solvent delivery system, a system controller (SCL 10AD), a UV-Vis spectrophotometer detector (SPD-10AD) operated at wavelength of 220 nm, a column oven (CTO-10A), a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. The analytical column was a Shimpack CLC-ODS (Shimadzu, Kyoto, Japan), 150 mm×4.6 mm I.D., 5 μm particle size which was protected by a Shim-pack G-ODS guard column (1 cm×4.0 mm I.D., 5 μm particle size). A mixture of 0.05 M sodium phosphate buffer (pH 5.7; adjusted with phosphoric acid) and acetonitrile (56:44 v/v) was used as the mobile phase. The column oven temperature was set at 50 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 1.7 ml/min.

*2.3. Sample preparation*

Aliquots of blank, calibration standard or unknown human serum samples (1 ml) were pipetted into 100 mm×16 mm disposable glass tubes, containing 100 μl of the working internal standard solution. The samples were mixed with 200 μl of the HCl solution (2.0 N) and extracted with 5 ml of ethyl acetate. After vortex mixing for 30 s and centrifugation (5 min. at 6000×g) the organic phase was removed and evaporated to dryness under stream of nitrogen at 50 °C. The residue was reconstituted in 100 μl mobile phase and following brief mixing a volume of 20 μl was injected onto the HPLC system.

*2.4. Validation of the method*

*2.4.1. Calibration curve and linearity*

Calibration curves samples were prepared within the concentration range of 0.05-10 μg/ml using pooled human blank serum obtained from normal subjects. In disposable glass tubes (100 mm×16 mm), after evaporation of 100 μl from each working solutions of the analyte, under a gentle stream of nitrogen at 50 °C, the residues were reconstituted in 1 ml of drug-free human serum, mixed for 10 s on a vortex mixer and subjected to extraction and analysis as described above. The linearity of the method was checked in the same day ($n$=6) and in 6 consecutive days. Calibration curves (weighted regression line) were obtained by linear least-squares regression analysis of plots of peak-area ratio to I.S. versus drug concentrations.

*2.4.2. Accuracy, precision, sensitivity and specificity*

Quality control samples used in method validation were prepared with the drug working solutions to make low (0.05 μg/ml), medium (0.5 μg/ml) and high (5 μg/ml) concentrations of the analyte. Intra- and inter-day variations were calculated by repeated analysis ($n$=6) of different concentrations of CCA in a single analytical run and in ten

analytical runs performed on different days, respectively. Limit of detection (LOD) and LOQ were defined as the concentrations of the drug giving a signal-to-noise ratio of 3:1 and 10:1, respectively. The specificity of the method was examined by presence of disturbing endogenous peaks in 24 human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the internal standard (I.S.). Selectivity of the assay was examined by analysis of several potentially co-administrated drugs with clopidogrel.

*2.4.3. Recovery, selectivity and stability*

The extraction efficiencies of CCA at the above mentioned concentrations as well as the I.S. at applied concentration were calculated in replicates ($n$=5) by comparing the respective peak areas obtained by extraction of the samples from serum, with those obtained from the same amounts of un-extracted solutions in methanol. The stability of the analyte in blood specimen during sample handling was also verified by subjecting the samples to three freeze-thaw cycles and up to 60 days maintenance at -40 °C. Stability of the solutions of CCA and the I.S. were studied over a period of 3 weeks by comparing of the peak areas at different times.

*2.5. Application of the method*

The present method was applied in a randomized crossover bioequivalence study of two different clopidogrel preparations in 24 male healthy volunteers aged 24.8±6.5 years and weighing 75.3±4.2 kg with normal biochemical parameters. After an overnight fasting, all the subjects received a single oral dose of 150 mg clopidogrel from either Exir (Tehran, Iran) or Sanofi-Synthelabo (Plavix; Ambares, France) pharmaceutical companies on 2 working days separated by a wash-out period of 2 weeks. Blood sampling was carried out at suitable intervals up to 24 h using disposable glass tubes (100 mm×16 mm) without any additives. All the samples were allowed to clot at room temperature for 30 min. Serum was separated by centrifugation at 2000×g for 10 min. and were stored at -40 °C until analysis. Pharmacokinetic parameters including maximum concentration ($C_{max}$), area under the concentration time curve from zero to the time of last sampling ($AUC_{0-t}$) and area under the concentration time curve from zero to infinity ($AUC_{0-t}$) were compared. Student's t-test was used for statistical analysis of the data and statistical significance was defined at the level of $p$<0.05.

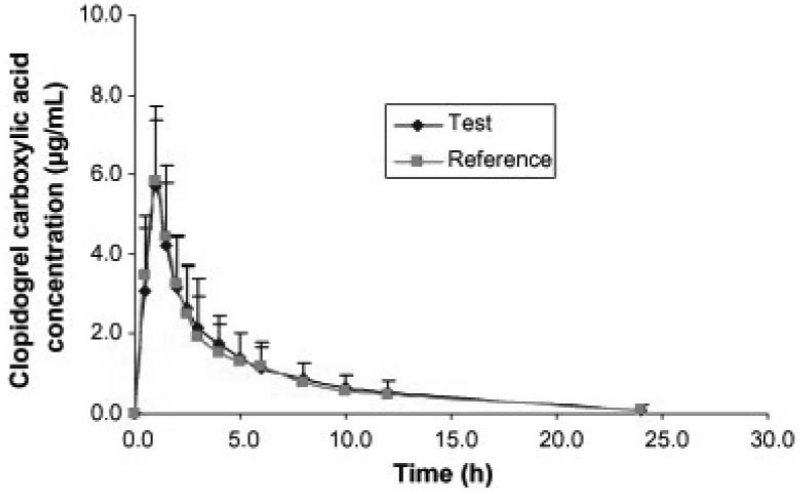


**Figure 2.** Mean serum concentrations-time profiles of clopidogrel carboxylic acid in 24 human volunteers following single 150 mg oral dose administration of two different clopidogrel preparations.

**Table 2.** Mean (±S.D.) pharmacokinetic parameters of CCA in 24 healthy volunteers after administration of a single 150 mg oral dose of clopidogrel in 24 healthy volunteers.

| Parameter prep. | Test | Reference | *p* value[a] |
|---|---|---|---|
| $T_{max}$ (h) | 1.35 (0.33) | 1.11 (0.42) | NS |
| $C_{max}$ (μg/mL) | 5.11 (1.85) | 5.46 (1.97) | NS |
| $AUC_{0-24}$ (μg h/mL) | 19.75 (9.08) | 20.62 (10.32) | NS |
| $AUC_{0-\infty}$ (μg h/mL) | 23.05 (9.4) | 24.70 (10.3) | NS |
| $T_{1/2}$ (h) | 8.84 (4.2) | 9.05 (4.75) | NS |

$T_{max}$, time to maximum concentration; $C_{max}$, maximum concentration; AUC, area under the concentration time curve; $T_{1/2}$, elimination half-life. NS, no significant difference. [a] $p<0.05$.

## 3. Results

### *3.1. Chromatography, sensitivity and linearity*

Under the chromatographic conditions described, no endogenous components from serum were found to interfere with the elution of CCA or the I.S. Representative chromatograms of human blank serum spiked with the I.S. and human blank serum spiked with the I.S. and CCA at the concentration of 0.05 μg/ml are shown in Figure 1A and 1B, respectively. Figure 1C and 1D show the chromatograms of serum samples obtained at 6 and 12 h after a single oral dose of 150 mg clopidogrel from a healthy volunteer, respectively. The calibration curves were linear over the concentration range of 0.05-10 μg/ml and the LOD and LOQ were found to be 0.02 and 0.05 μg/ml, respectively. The correlation coefficients for calibration curves were equal to or better than 0.9982. Intra- and inter-day reproducibility for calibration curves were determined on the same day in replicate ($n$=6) and on different days ($n$=6), respectively, using the same pooled serum sample. The intra-day average slope of the fitted straight lines was 12.85±0.542 μg/ml (CV=4.2%) and the mean intercept of the calibration curves was 1.97±0.188 (CV=9.5%). The corresponding mean (±SD) coefficient of the linear regression analysis was 0.9982±0.013 (CV=0.1%). For calibration curves prepared on different days ($n$=6), the mean±SD of results were as follows: Slope 13.64±0.521 μg/ml (CV=3.8%), coefficient of the linear regression analysis=0.9985±0.012 (CV=0.1%) and intercept=1.75±0.162 (CV=9.2%).

### *3.2. Recovery, accuracy, precision, selectivity and stability*

The inter- and intra-day accuracy and precision values of the assay method have been presented in Table 1. The coefficient variation values of both inter- and intra-day analysis were less than 11.7% whereas the percentage error was less than 3.9. The mean extraction efficacies of CCA and I.S. from serum were found to be 99±2% and 97±4%, respectively. The results of the selectivity study showed that using the same analytical conditions there were no interfering peaks from any of the following drugs: Acetaminophen, naproxen, indometacin, celecoxib, ibuprofen, diclofenac, theophylline, fursemide, amiodarone, cinnarizine, ticlopidine, glybenclamide, verapamil, propranolol diltiazem, aspirin, salicylic acid, omeprazole, cimetidine, codeine and caffeine. All of the drugs were tested at concentration range of 10-1000 ng/ml. The stock solutions of CCA and the I.S. were stable for at least 21 days when stored at 4 °C. The stability of the drug was found to be 100% from the initial value, after 60 days maintenance of the serum at -40 °C and following three thaw-freeze cycles.

### *3.3. Application of the method*

This method has been successfully used for the determination of serum concentrations of CCA in a randomized cross-over

bioequivalence study following single oral administration of two different clopidogrel preparations in 24 healthy volunteers. Typical serum concentration-time profiles of CCA have been shown in Figure 2 and resulted pharmacokinetic parameters have been summarized in Table 2.

## 4. Discussion

A few analytical methods have been published for determination of the inactive metabolite of clopidogrel in the biological matrix. A sensitive gas chromatography-mass spectrometric method with LOQ of 0.005 μg/ml has been published [3]. In this technique however, a complex two steps extraction method using both liquid-liquid and solid phase extraction procedures as well as derivatization of the analyte are required. Two LC-MS methods for the determination inactive metabolite of clopidogrel have been published [4, 5]. In the method described by Mitakos and Panderi [5] extraction of the analyte from the serum has been achieved using single step solid phase extraction, however, their method is not sensitive enough (LOQ 0.1 μg/ml) to be used in single-dose pharmacokinetic studies of the drug. In the other published LC-MS method [4] two steps time-consuming extraction procedure using both liquid-liquid and solid phase extraction techniques have been used however, the method is sensitive enough (LOQ 0.02 μg/ml) for measuring of the analyte up to at least three half lives post-dose. The inactive metabolite of the drug has been measured in Wistar rat plasma using HPLC and UV detection [6]. In this method, LOQ of 0.125 μg/ml has been reported using 50 μl injection. Furthermore, this method involves long run time of analysis (16 min.) and gradient elution of the mobile phase. In HPLC-UV technique described by Souri *et al*. [7] ticlopidine has been used as internal standard. To overcome close retention times of CCA and ticlopidine, they used a mobile phase with low flow rate (0.9 ml/min.) and high percent of aqueous phase which leads to long analytical run time (12 min.) and reduction of sensitivity (LOQ 0.2 μg/ml). To improve run time and sensitivity of the analysis, the flow rate should be increased and it is preferred to select a mobile phase with higher proportion of organic solvent. Thus, comparing to their method [7], we used a mobile phase with higher flow rate and more proportion of the organic solvent. Due to similar retention behavior of CCA and ticlopidine, a number of chemical agents were tested to choose I.S. and phenytoin was selected considering its UV spectrum, retention time, recovery and resolution from the drug and endogenous peaks. While their method has been applied in a human single dose study, poor sensitivity of the method, does not allow measurement of the analyte concentrations for more than 12 h following single dose administration of the drug. Furthermore, estimated elimination half-life of the inactive metabolite in their study is 2.25±0.84 h. This does not agree with reported values in the literature, using the same single dose of the drug [4, 8]. Determination of clopidogrel in human plasma by liquid chromatography/tandem mass spectrometry has been reported [9]. As low blood levels of the intact drug is achieved following single dose administration [8, 9] and considering difference in polarity between the drug and its inactive metabolite, like other published papers [3-7], our method failed to detect peak of clopidogrel in the samples. However, the major advantages of the present method are reduction in run time of analysis (5.5 min.) and improvement of sensitivity (LOQ 0.05 μg/ml) which allows determination of the analyte up to three half-lives post-dose more precisely, so that the extent of absorption until the last sampling time is more closer to its extrapolated value ($AUC_{0-\infty}$).

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