Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(8): 1023-1030

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Electrochemical and chromatographic studies of clopidogrel using cathodic stripping voltammetry and HPLC under new experimental conditions and its determination in the preparation tablet, urine and plasma samples

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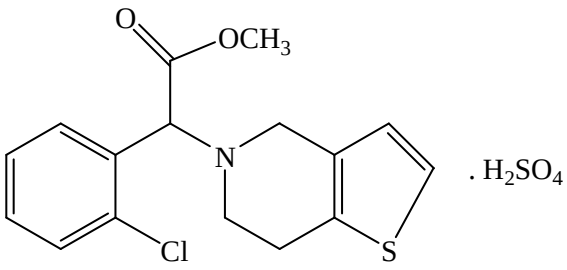
ABSTRACT

The voltammetric behavior of clopidogrel (CLP) drug have been studied by a cathodic stripping voltammetry technique in the carbonate buffer pH 10 on a surface of hanging mercury drop working electrode (HMDE) vs Ag/AgCl reference electrode and Pt auxiliary electrode. The experimental parameters of cathodic stripping voltammetry (CSV) have been studied and optimized; such as accumulation time and potential, scan rate, pulse amplitude and the convection rate for the analyzed CLP. The high performance liquid chromatographic (HPLC) technique was applied for the determination of CLP using acetonitrile, water and methanol as mobile phase and C-18 (5µm) with UV detector at 254nm. The sensitivity, accuracy, precision and selectivity of the used techniques were also evaluated. The used analytical methods were developed to determine CLP in the pharmaceutical and biological samples under the typical and optimum conditions. The statistical parameter (F-test) was calculated, which shown an insignificant systematic error between the CSV and HPLC methods.

Keywords: Clopidogrel, cathodic stripping voltammetry, HPLC, biological fluids, HMDE.

INTRODUCTION

Clopidogrel hydrogen sulfate (CLP) is chemically called methyl (ortho-chlorophenyl)-6,7-dihydrothieno[3,2] pyridin-5(4*H*)-acetate hydrogen sulfate (scheme 1). It is a novel thienopyridine derivative that irreversibly blocks adenosine diphosphate (ADP). Clopidogrel is related to ticlopidine with superior side effects profile and dosing requirements. The molecular formula of clopidogrel bisulfate is $C_{16}H_{16}ClNO_2S.H_2SO_4$. The molecule of clopidogrel contains an asymmetrical carbon at C-7 leading to the existence of two enantiomers (*R* and *S*)[1]. Clopidogrel is used alone or with aspirin to prevent serious or life-threatening problems with the heart and blood vessels in people who have had a stroke, heart attack, or severe chest pain. CLP is also used to prevent serious or life-threatening problems with the heart and blood vessels in people who have peripheral arterial disease (poor circulation in the blood vessels that supply blood to the legs). CLP is in a class of medications called anti-platelet medications. It works by preventing platelets (a type of blood cell) from collecting and forming clots that may cause a heart attack or stroke[2].



Scheme 1: Chemical structure of clopidogrel hydrogen sulfate (CLP)

Cathodic stripping voltammetry (CSV) is a sensitive electroanalytical method for the determination of trace amounts of pharmaceutical and chemical compounds. This is due to its waveform which measures the reduction current in pulses by taking two measurements and recording the difference as the potential is decreased. In this technique; the stripping step consists of a negative potential scan, creating cathodic current. In a cathodic stripping technique, the micro- working electrode described as anode during the deposition step and as cathode during stripping. The deposition step amounts to an electrochemical preconcentration of the analyte in the surface of the microelectrode is far greater than it is in the bluk solution[3].

Overall; many articles were published to investigate a cathodic stripping voltammetric behavior for determination of metals such as Cd^{II}, As^{III}, Se^{IV} Cu^{II}, Pb^{II} , Ni^{II}, Co^{II} and Zn^{II} [4-6] and the pharmaceutical compounds such as Timonacic[7], Cefoperazone[8], Heparin[9], Clotrimazole[10], Ciprofloxacin[11] and Spironolactone[12]. Clopidogrel has been determined using square wave voltammetry[13], cyclic voltammetry[14], potentiometry[15], spectrophotometry[16-18] and liquid chromatography[19-22].

On the other hand, the high performance liquid chromatography (HPLC) technique is a famous technique to analysis organic substances specially the pharmaceutical compounds[23-26]. The basic HPLC system consists of solvent reservoirs for containing the mobile phase, a special high-pressure pump for pumping the mobile phase through the column, a specially designed injection device for sample introduction, the column where the separation takes place, the detector for electronic sensing of the eluting mixture components, and a data system for acquiring and displaying the chromatogram[27].
Actually, no published articles were reported to analysis and determine of CLP using HPLC and CSV under the used and optimized experimental conditions as in this article. These studies have been applied to develop sensitive and an effective of the used techniques to determine CLP in the commercial drugs and biological samples.

EXPERIMENTAL SECTION

1. Apparatus

1.1. Cathodic stripping voltammetry

The cathodic stripping voltammetric measurement was applied by 797 AV computrace instrument (Metrohm, Switzerland Made) which controlled by (VA computrace 2.0) software. This instrument was based on three electrodes system included hanging drop mercury electrode (HMDE) as the working electrode, Ag/AgCl reference electrode and Pt auxiliary electrode. A Hanna instrument pH211 (Romanian Made) was used to obtain pH values over the range 2-12. Additionally, oxford adjustable micropipettes (Ireland) 100 - 1000 micro liter, were used to measure the volumes of the analyzed compounds. A labofuge 200 instrument; Heraeus Sepatech (German Made) was used to centrifuge urine and plasma samples.

1.2. High performance liquid chromatography

The high- performance liquid chromatographic instrument is called ultimate 3000, thermo scientific dionex (US) which used UV-Vis detector and auto sampler, with a 20 µL manual loop injector and C18 column 5 µm, connected computer dell optiplex 3010 (China). The chromatogram signals were printed via a hp laser jet pro 200 color M251n, Hewlett-packard development company (China). Oxford adjustable micropipette (Ireland) was used to inject micro-liter volumes of standard solutions of CLP and analysed samples.

2. Reagents and Chemicals

All reagents used were of analytical grade and the solutions were prepared with methanol and distilled water freshly dialy. Clopidogrel hydrogen sulfate standard was obtained from Qassim Pharmaceutical Plant Buraydah, Spimaco Addwaeih, Buraydah (Saudi Arabia) then a stock solution of 1×10^{-3}mol L^{-1} was prepared by dissolving the appropriate weight of CLP in methanol as solvent in a 50 ml volumetric flask. The CLP solutions should be fresh prepared and stored in the dark place. Other preparations in lower concentarations were done by diluting the stock solution with methanol. Commercial drug was purchased from the local pharmacy which called pedovex tablet containing75mg of CLP that manufactured by Tabuk pharmaceutical manufacturing company (Saudi Arabia). Britton-Robinson supporting buffer was prepared by dissolving 2.47 g of boric acid, 2.3 ml of glacial acetic acid and 2.7 ml of ortho-Phosphoric acid in 1.0 L distilled water. In addition, the carbonate supporting electrolyte was prepared by dissolving 10.6 g of sodium carbonate and 8.4 g of sodium hydrogen carbonate in 1.0 L distilled water.

3. Preparation of CLP tablet and biological samples

Ten CLP tablets were weighed (the content of one tablet is 75mg CLP) and become in bowder. The suitable weight of powder was dissolved in the solvent and by leaving 24 hours and convecting this solution, a stock solution was prepared. Thereafter, the diluted solutions were prepared by diluting stock solution with solvents. On the other hand, the biological samples such as urine and plasma were prepared by adding 1.0 ml of 5% $ZnSO_4.7\ H_2O$ solution, 0.1ml of NaOH and 1.0 ml of ethanol into 0.5 ml of human urine and plasma samples[28], then centrifuging of

solution for 15 min at 5000 rpm to separate out the insoluble and organic interferences. All voltammetric and chromatographic preparations and measurements were carried out at room temperature.

4. Procedures

4.1. Cathodic stripping voltammetric method

The procedure of CSV technique was applied to obtain cathodic voltammograms as following: 10 ml of carbonate buffer (pH 10) was pipetted in a dry and clean electrochemical cell. The studied concentration of analyte was added after stirring and purging the buffer solution with a nitrogen gas for 3 minutes almost. The deposition potential of -0.2 V vs. Ag/AgCl reference electrode was applied on HMDE while the solution of CLP was stirred and deposed for 30 seconds. The reduction scans were recorded and monitored over the range 0.0 – 1.5V.

4.2. High performance liquid chromatographic method

A 50: 40: 10 v/v/v % acetonitrile, methanol and water was used as mobile phase for the chromatographic measurnments of CLP. The instrumental HPLC conditions are: 2 ml/min flow rate, 254nm wavelength UV detector, 30^{o}C column oven temperature, 20 µL injection volume and C-18 (5µm). 10 µL of solutions were injected into the HPLC column. The HPLC running time was 5.0 min, resulting almost 3.143 min which was selected as optimum retention time for all chromatographic studies of CLP.

RESULTS AND DISCUSSION

1. Cathodic stripping voltammetric behavior of CLP

5×10^{-7} mol L^{-1} of CLP was voltammetric analysed using B-R, acetate, phosphate and carbonate buffers at different values of pH with solid and liquid working electrodes, just yielded a well defined reduction peak at -1.28 V onto the hanging drop mercury electrode surface by using carbonate buffer pH10 as shown in figure 1.

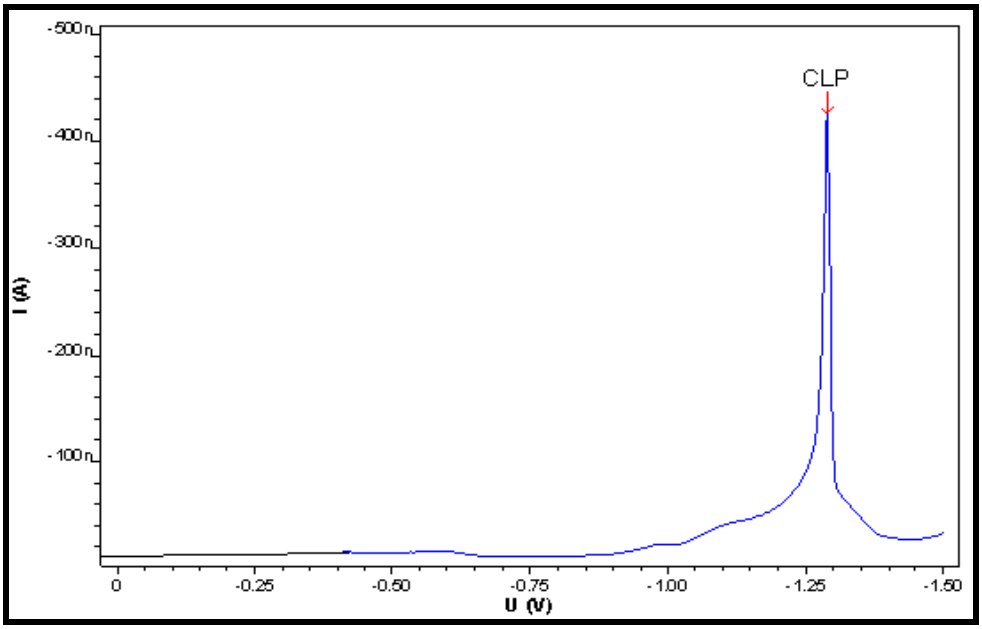
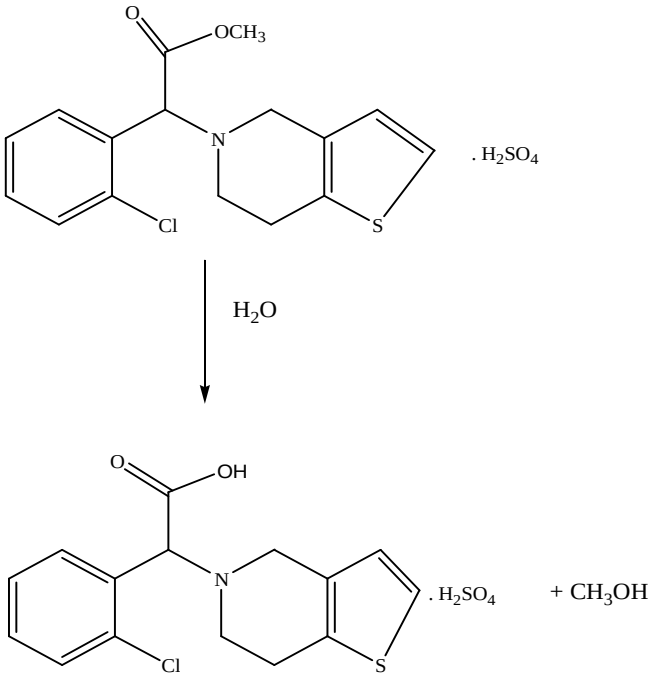


Figure 1: Cathodic stripping voltammogram of 5×10^{-7} mol L^{-1} CLP in carbonate buffer pH10

The cathodic stripping behavior was suggested a mechanism of CLP reduction process of carbonyl group in ester to result to alcohol as shown in scheme 2.



Scheme 2: Proposed mechanism for reduction process of CLP

2. HPLC behavior of CLP

5×10^{-4} mol L^{-1} of CLP was chromatographic analysed using 50: 40: 10 v/v/v % acetonitrile, methanol and water mobile phase, 254nm UV detector and 2 ml min^{-1} flow rate, yielded a well defined chromatographic signal at 3.143 min retention time and 82 mAU as shown in figure 2.

In case of using 1.5 ml min^{-1} flow rate, the HPLC signal was been changed to a long retention time as appeared in figure 3.

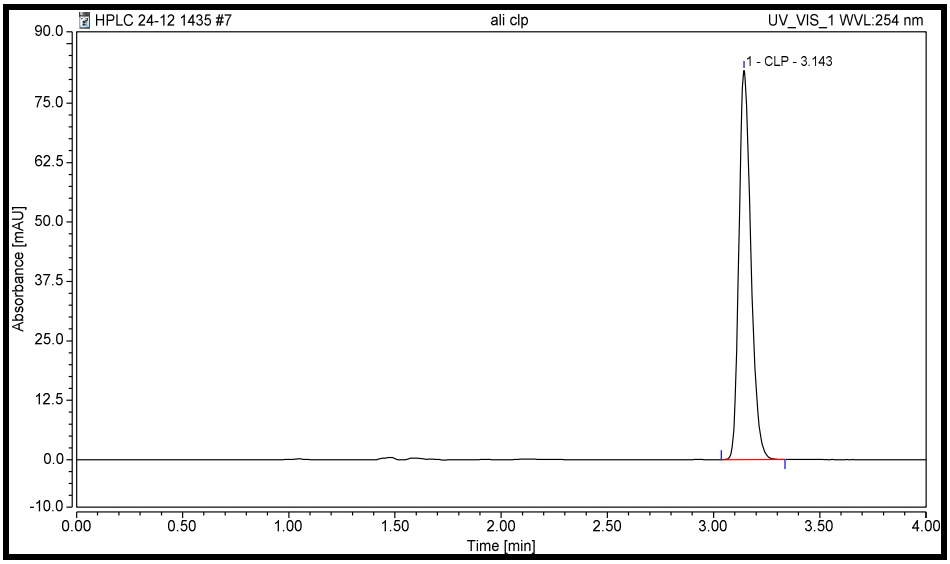


Figure 2: Chromatograph of 5×10^{-4} mol L^{-1} CLP under optimum conditions

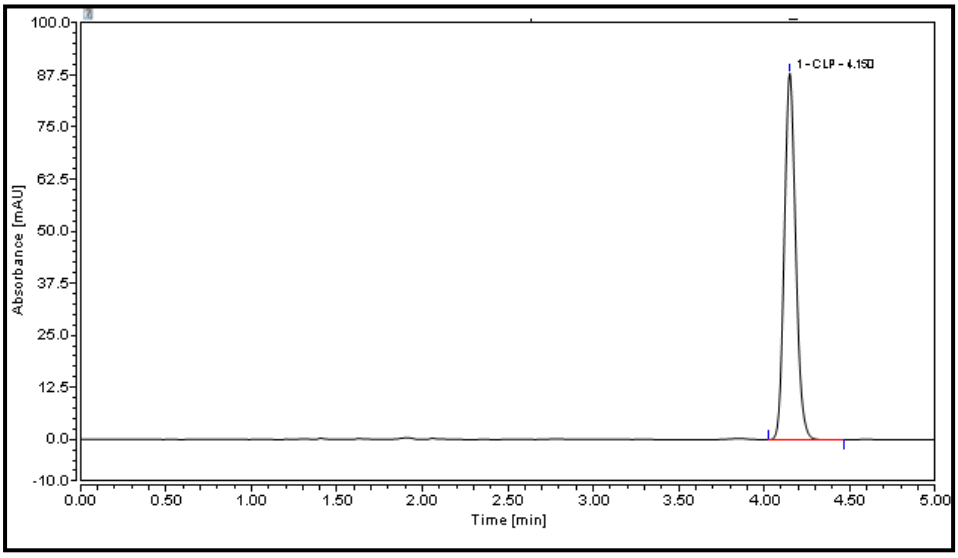


Figure 3: Chromatograph of 5×10^{-4} mol L^{-1} CLP using 1.5 ml min^{-1} flow rate

3. Optimization of voltammetric and Chromatographic conditions

In general, many voltammetric parameters were studied such as supporting electrolytes, pH, scan rates, accumulation potential and time, pulse amplitude, frequency, convection rates and area of electrode surface yielded, a well and sharp cathodic current was recorded at carbonate buffer pH10, -0.2 V accumulation potential, 30 sec accumulation time, 100 mVs^{-1} scan rate, 20 Hz frequency, 0.05 V pulse amplitude, 3000 rpm and 0.6 mm^2 surface area for 5×10^{-7} mol L^{-1} of CLP.

On the other hand, there were chromatographic parameters also studied such as flow rates, mobile phases, column temperatures and retention times to be obtained a well chromatographic signal. According to these studies, 2 ml min^{-1}, 50:40:10 v/v/v acetonitrile : methanol : water mobile phase, 30^0C and 3.143 min retention time were resulted to give a high chromatographic response for 5×10^{-4} mol L^{-1} of CLP. These voltammetric and chromatographic results were chosen as optimum values in the future experimental studies.

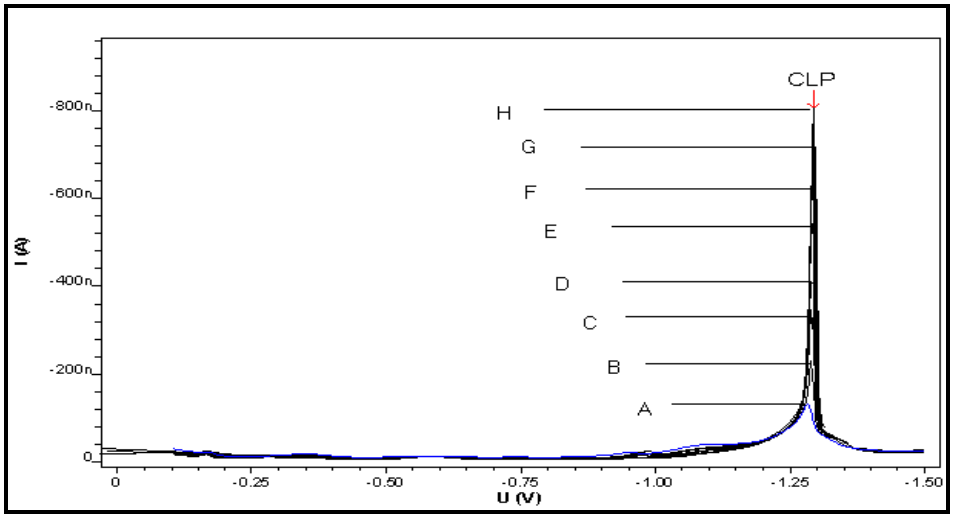


Figure 4: Voltammograms of CLP under optimum conditions (A: 1.0×10^{-7}, B: 2.0×10^{-7}, C: 4.0×10^{-7}, D: 6.0×10^{-7}, E: 1.0×10^{-6}, F: 1.5×10^{-6}, G: 2.0×10^{-6} , H: 2.5×10^{-6} mol L^{-1})

4. Quantitative Utility of voltammetric and chromatographic studies

4.1. Calibration curve and detection limit

Concerning cathodic stripping voltammetry, a linear relationship was observed between the current and the CLP concentrations over the range 1.0×10^{-7} – 2.5×0^{-6} mol L-1 under the optimum parameters as shown in figures 4 and 5. A linear least-square procedure of the calibration curve was given the following regression equation:

I_{ac} (nA) = 158.2 + 2.51 × 10^8 C r^2 =0.999, n = 8

Where; i_{ac} is the cathodi voltammetric current, C is the molarity concentration of CLP , r^2 is the correlation coefficient and n is the number of measurnments.

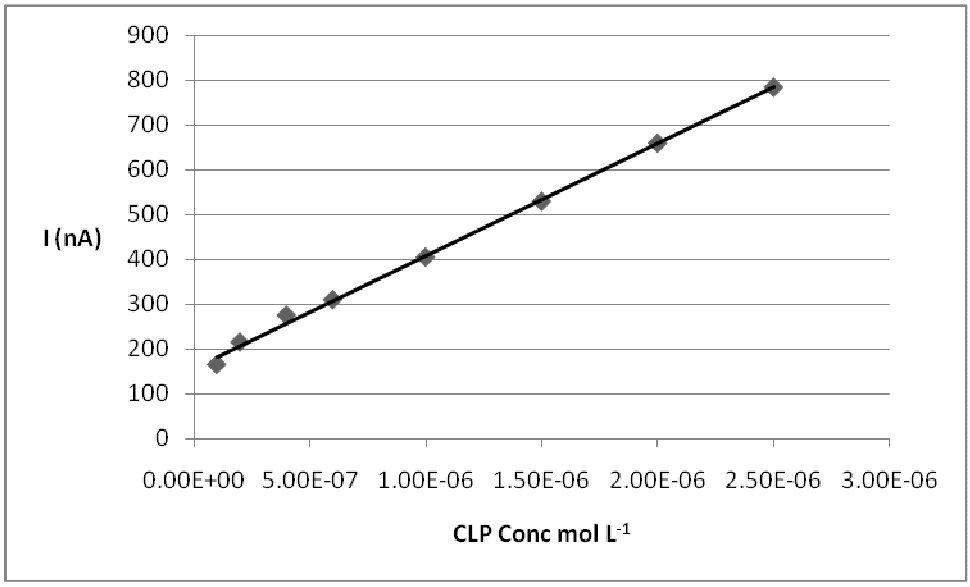


Figure 5: Calibration curve of CLP over 1.0 ×10^{-7} – 2.5 ×0^{-6} mol L-1

On the other hand, for HPLC analysis, a linear relationship was also observed between the chromatographic signal and the CLP concentrations over the range 1.0 ×10^{-4} – 1.0 ×0^{-3} mol L-1 under the optimum parameters (figure 6). A least-square procedure of the calibration curve was given the following regression equation:

Abs (mAU) = 3.43 + 1.45 × 10^5 C r^2 =0.99944, n = 6

Where; Abs is the chromatographic signal, C is the molarity concentration of CLP , r^2 is the correlation coefficient and n is the number of measurnments.

The detection limits (S/N=3) for the cathodic stripping voltammetric and HPLC methods were 9 × 10^{-9} and 6.41 ×10^{-6} mol L^{-1} respectively.

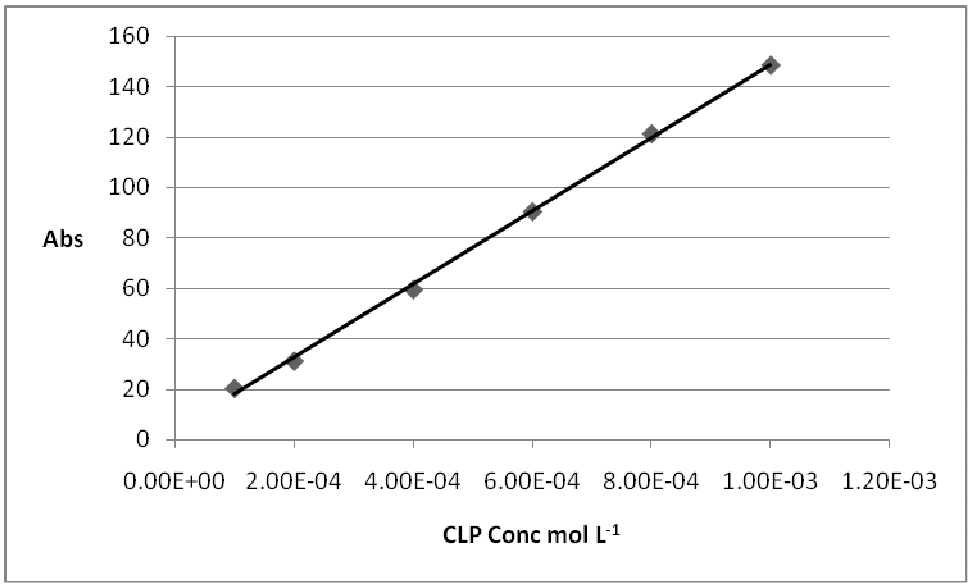


Figure 6: Chromatographic calibration curve of CLP over 1.0 ×10^{-4} – 1.0 ×0^{-3} mol L-1

4.2. Reproducibility and Stability

The reproducibility studies will be given more informatiom about the analytical performance of the CSV and HPLC methods for determination of CLP. In the analytical studies, the concentrations 7×10^{-7} and 5×10^{-4} mol L^{-1} of CLP were analysed by CSV and HPLC respectevily. These voltammetric and chromatographic measurnments were repeated ten times yeilded, relative standard deviations (RSD%) were 0.752% and 0.31%, respectevily, confirmed the precision of CSV and HPLC techniques.

On other hand, the stability of the analytical methods was evaluated by monitoring the voltammetric and chromatographic signals of CLP analysis for 60 min, yeilded, the voltammetric and chromatographic signals were approximitly fixed within the analytical period.

4.3. Effect of Interferences on the voltammetric study

Any commerical drug has usually substances as interferences which affected on the analyte signal. This study was carried out to evaluate a selectivity of the developed CSV. The interferences can be competed the analyte on the working electrode surface and adsorption sites resulting, increased or decreased in the voltammetric signals. The competitive interferences such as starch, sucrose and lactose which prepared at different concentrations (one, 5-times and 20-times) higher than CLP concentration, 5×10^{-7} mol L^{-1} were voltammetric studied to evaluate them effects on the cathodic current. The starch and lactose were negative affected on the CSV current at high concentrations. While the sucrose wasn't recorde any effect on the CSV signal.

5. Analytical applications

The voltammetric and chromatographic procedures were applied and devolped for determination of CLP in its pharmaceutical tablet and biological fluids. CLP content of commercial tablet (75mg CLP) was directly analysed by CSV and HPLC techniques afer dissolution and filtration steps for it. Electrochemically, CLP tablet was contained ester group, as mentioned in the suggested mechanism of CLP previouslly, which given the same cathodic reduction behavior in terms of potential and current. The developed CSV and HPLC methods were used to measure the recoveries of CLP content in its commercial tablets yielded, 93% ± 1.6 SD and 102% ± 0.71 SD, respectively as shown in table 1.

In addition, the used analytical methods; CSV and HPLC; were also applied to evaluate the recoveries of CLP in urine and plasma yeilded, 92% ± 1.22, 90% ± 0.71, 85% ± 0.63 and 80% ± 0.89, respectively as found in table 2.

Table 1: CSV and HPLC measurnments of CLP in its commercial tablets

	CSV		HPLC	
	Found (mg)	**% Recovery**	**Found (mg)**	**% Recovery**
Labeled Content CLP (75 mg)	68.25	91	76.5	102
	69	92	76.5	102
	70.5	94	76.5	102
	69.75	93	75.75	101
	71.25	95	77.25	103
	Mean	93	**Mean**	102
	SD	±1.6	**SD**	±0.71

Table 2: CSV and HPLC recoveries of CLP in biological samples

	CSV		HPLC	
	Urine CLP recovery %	**Plasma CLP recovery %**	**Urine CLP recovery %**	**Plasma CLP recovery %**
Added CLP (5×10^{-7} & 5×10^{-4} mol L^{-1})	90	89	85	80
	93	90	86	79
	93	90	85	81
	92	90	84	79
	92	91	85	81
	Mean= 92	90	85	80
	SD= ± 1.22	±0.71	±0.63	±0.89

According to the statistical evaluation (F-test) for the results of tablets analysis, there is no significant difference between the voltammetric results were obtained by CSV method and that obtained by HPLC. Using the equation $F = S_1^2 / S_2^2$, the calculated F value is 5.10 which was lessen than the critical value (6.59) at the 95% confidence level. It was conclused, no statistical evidence that the difference of the suggested method vary significantly from the difference of HPLC as a reference method. In addition, the statistical evaluation (F-test) for the results of urine and plasma analysis confirmed no significant difference between the voltammetric results were obtained by CSV and that obtained by HPLC. By the same way, the calculated F value is 3.75 and 1.60 for urine and plasma analysis

respectively, which were lessen than the critical value (6.59) at the 95% confidence level. It was also conclused, no statistical evidence that the difference of the suggested method vary significantly from the difference of HPLC as a reference method. In general, there are no difference at the precision of CSV and HPLC for analysis of tablet, urine and plasma.

CONCLUSION

The voltammetric and chromatographic studies of CLP were indicated the excelent selectivity, accuracy and precision of CSV and HPLC techniques for determination of CLP at pharmaceutical preparations and biological fluids under optimum new conditions. The detection limit values were confirmed a sensetivity of CSV is higher than HPLC. The statistical parameter (F-test) was evaluated for the used methods, yielded, there are no different in the precision of CSV and HPLC for CLP analysis.

Acknowledgement
The author greatly would like to thank the deanship of scientific research at Taibah University (KSA) for financial support to carry out this research at the Chemistry Department. He also thanks Mr. Awad alqarni and Ahmad alribdy at Qassim Pharmaceutical Plant Buraidah, Spimaco Addwayah, Saudi Arabia for their assistances concerning standard CLP drug.

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