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ORIGINAL ARTICLE

Voltammetric quantification of anti-hepatitis drug Adefovir in biological matrix and pharmaceutical formulation

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Abstract Electrochemical reduction behavior of Adefovir was studied using Hanging Mercury Drop Electrode (HMDE) in Britton–Robinson (BR) buffer solution. Voltammetric study showed one well-defined reduction peak in the potential range −1.2 to −1.4 V (vs. Ag/AgCl) due to reduction of C=N bond of the imidazole ring. Solid-phase extraction and protein-precipitation techniques were employed for extraction of Adefovir from human plasma. The proposed method allows quantification of Adefovir in human plasma over the concentration range 0.50–5.00 μg/mL with the detection limit 0.17 μg/mL, whereas in pharmaceutical formulation 0.25–2.25 μg/mL with the detection limit 0.08 μg/mL.

1. Introduction

Hepatitis B Virus (HBV) infection is a major global health problem. An estimated 2 billion people worldwide are infected with the HBV and about 350 million are chronically infected, which results in 0.6 million deaths every year [1]. Adefovir is an anti-hepatitis drug, which is found to be very effective against HBV. Adefovir dipivoxil (9-[2-[[Bis[(pivaloyloxy)-methoxy]phosphinyl]methoxy]ethyl]adenine) is a nucleotide analog, an oral prodrug of Adefovir (9-(2-phosphonomethoxy-ethyl)adenine) (Fig. 1), which rapidly converts into the parent drug in the body and suppresses the replication of HBV that is even resistant to other anti-HBV drugs such as lamivudine, emtricitabine and famciclovir [2–4].

The widespread use of this compound and the need for clinical and pharmacological studies warrant fast and sensitive analytical techniques for the assay of drug in pharmaceutical formulations, biological matrixes and also for toxicological and pharmacokinetic studies.

Several techniques have been explored for detection and quantification of Adefovir in pharmaceutical formulation and biological fluids such as spectrophotometry [5], high performance liquid chromatography [6,7], ion-pair liquid chromatography [8,9], hydrophilic interaction liquid chromatography–tandem mass spectrometry [10] and liquid chromatography–tandem mass spectrometry [11,12]. Although spectrophotometry and chromatography are the most commonly employed techniques, but these

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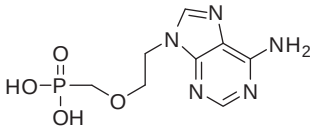

Figure 1 Chemical structure of Adefovir dipivoxil.

involve many derivatization steps and extraction purification approaches prior to final analysis. These techniques are very time consuming and the demand of expensive, sophisticated instrumentation and highly skilled personnel restrict their use in routine analysis. Since the last decade, electroanalytical techniques have been widely used in the field of pharmaceuticals analysis [13–18], as these techniques are inexpensive, rapid and do not require derivatization or the time consuming purification steps after extraction, when compared with other analytical techniques [8–12]. However, no electro-analytical methods have yet been reported for the quantification of Adefovir in pharmaceutical formulations and human plasma.

The purpose of the present study is to develop fast and sensitive voltammetric method for the detection and quantification of Adefovir in pharmaceutical formulations and human plasma using square-wave adsorptive stripping voltammetry and cyclic voltammetry due to higher sensitivity, simplicity and lower limit of detection.

2. Experimental

2.1. Instrumentation

Electrochemical measurements were performed using μ Autolab Type III potentiostat–galvanostat (Eco-Chemie B.V., Utrecht, Netherlands) with 757VA computrace software. The electrodes utilized in the study were hanging mercury drop electrode as working electrode, platinum wire as auxiliary electrode and Ag/AgCl (3 M KCl) as reference electrode. All pH measurements were obtained from digital pH meter (Decible DB-1011) fitted with a glass electrode and saturated calomel electrode as reference, which was previously standardized with standard buffer solutions of known pH.

2.2. Reagents and chemicals

Adefovir standard (99.43%) was obtained from Veeda Clinical Research Pvt. Ltd., India. Adefovir-containing tablets from different companies (labeled 10 mg Adefovir/Tablet) were obtained from commercial sources. Ultra pure water (Milli-Q purification system, Millipore Corp., Milford, MA, USA), was used throughout the studies. All chemicals employed in this work were of analytical reagent grade (Merck and Sigma Aldrich) and used without further purification.

2.3. Analytical procedure

Standard stock solution of Adefovir (1.0 mg/mL) was prepared by dissolving pure compound in methanol. Working solutions were prepared from stock solution using a mixture of methanol and water (50:50, v/v) as diluents. Drug free human blood plasma lots were procured from Radha Swami blood bank, Gwalior, India and sodium heparin was used as anticoagulant. These plasma lots were preserved at −20 °C in freezer and used after gentle thawing at room temperature. Plasma calibration and quality control samples were prepared by 5% spiking of respective working solutions in blank plasma. Analyte was extracted from spiked human plasma samples using solid-phase extraction (SPE) and protein precipitation (PP) techniques. For electrochemical measurements, a known volume of working solution was pipetted into a voltammetric cell and the total volume of 10 mL was obtained with BR buffer. Square wave and cyclic voltammogram were recorded after optimization of operational parameters.

2.4. Extraction procedure

SPE technique was used to extract Adefovir from human plasma samples. 500 μL of each calibrator and quality control samples were transferred to 1.5 mL eppendorf tubes. 250 μL BR buffer (pH 7.9) was then added to each sample and vortexed for 30 s. After vortexing, samples were centrifuged at 3500 rpm for 5 min. SPE was performed using Oasis HLB cartridges (30 mg/cc), a vacuum manifold device and a vacuum source. SPE cartridges were conditioned and equilibrated with 1.0 mL of methanol followed by 1.0 mL of water. Each sample solution was individually transferred to a solid-phase extraction cartridge and passed through the beds at a constant flow rate of 1.0 mL/min. The cartridges were washed with 1.0 mL of BR buffer (pH 7.9) and allowed to dry. The analytes were eluted with 1.0 mL of methanol and analysis was performed by aforementioned method.

3. Results and discussion

Reduction behavior of Adefovir was investigated on HMDE using Square-Wave Voltammetry (SWV), Square-Wave Cathodic Adsorptive Stripping Voltammetry (SWCAdSV) and Cyclic Voltammetry (CV). In all the electro-analytical techniques well-defined reduction peaks were obtained in the potential range of −1.2 to −1.4 V (vs. Ag/AgCl) due to reduction of the C=N bond of the imidazole ring, which was used for analytical measurements.

3.1. Effect of pH

The pH of supporting electrolyte is an important factor that affects the redox reaction of analyte. Hence, it is usually important to investigate the effect of pH in electro-analytical system. In order to optimize pH, the effect of pH on the reduction of Adefovir was studied in the range of 2.5–12. Fig. 2 shows the effect of pH on potential shift and current response. It revealed that the reduction peak current was the highest at pH 3.8 along with sharpest peak (Fig. 2, curve c). The peak potential was shifted towards more negative potential with increasing pH in the range 2.5–4.5 (Fig. 2, curves a–d), but after pH 4.5 no response was obtained. Therefore, BR buffer pH 3.8 was selected for the study purpose.

3.2. Selection of extraction technique

Two extraction techniques, PP and SPE were used for extraction of Adefovir from spiked human plasma. Both methanol and acetonitrile were used for protein precipitation. Better results were obtained from acetonitrile (Fig. 3A, curve

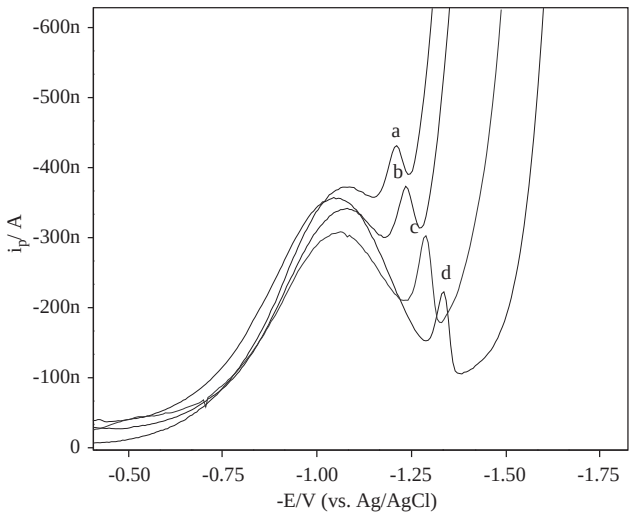

Figure 2 Effect of pH on the cathodic peak current (i_p/A) and potential shift of Adefovir (1.5 μg/mL) at hanging mercury drop electrode in BR buffer: a = 2.5 pH, b = 3.0 pH, c = 3.8 pH, d = 4.5 pH.

b) as against methanol (Fig. 3A, curve a). However, SPE technique gave a higher current response, lower background noise and sharper peak than PP technique (Fig. 3B). Thus, SPE technique was selected for extraction of Adefovir from spiked human plasma. Square wave voltammograms obtained through both extraction techniques are depicted in Fig. 3.

3.3. *Optimization of voltammetric conditions*

Reduction behavior of Adefovir was investigated at HMDE in BR buffer solution using square-wave and cyclic voltammetry. For best results both techniques require optimization of several instrumental and operational parameters. Instrumental variables such as frequency (f), accumulation time (t_{acc}), accumulation potential (E_{acc}), scan rate (Δs) pulse amplitude (ΔE_{sw}), etc. were examined.

Frequency was varied from 10–90 Hz. A linear relationship was obtained between the peak current and frequency of the signal. In order to improve the sensitivity without any distortion of the peak or the baseline, frequency 50 Hz was chosen.

Fig. 4 shows the overloaded cyclic voltammograms (Fig. 4A) and square-wave voltammograms (Fig. 4B) of Adefovir (1.50 μg/mL) at different accumulation time (t_{acc}). The peak height depends on the accumulation time, suggesting an effective adsorption of Adefovir on the HMDE. Peak current increased with increasing deposition time till 80 s but after 80 s it started decreasing. It was expected that this phenomenon may be related to the saturated adsorption of Adefovir on the HMDE surface. Accumulation time of 30 s was selected for the study purpose.

The effect of accumulation potential (E_{acc}) on the peak current (i_p/A) of Adefovir was examined over the potential range −0.1 to −0.9 V (vs. Ag/AgCl). At an accumulation potential of −0.1 V there was a slightly steep line, which increased gradually after −0.2 to −0.9 V. Thus, the accumulation potential was fixed at −0.9 V (vs. Ag/AgCl) and used throughout the study. Effect of scan increment (Δs) on the square-wave peak current of the drug was also studied. It revealed that the peak current increased linearly up to scan

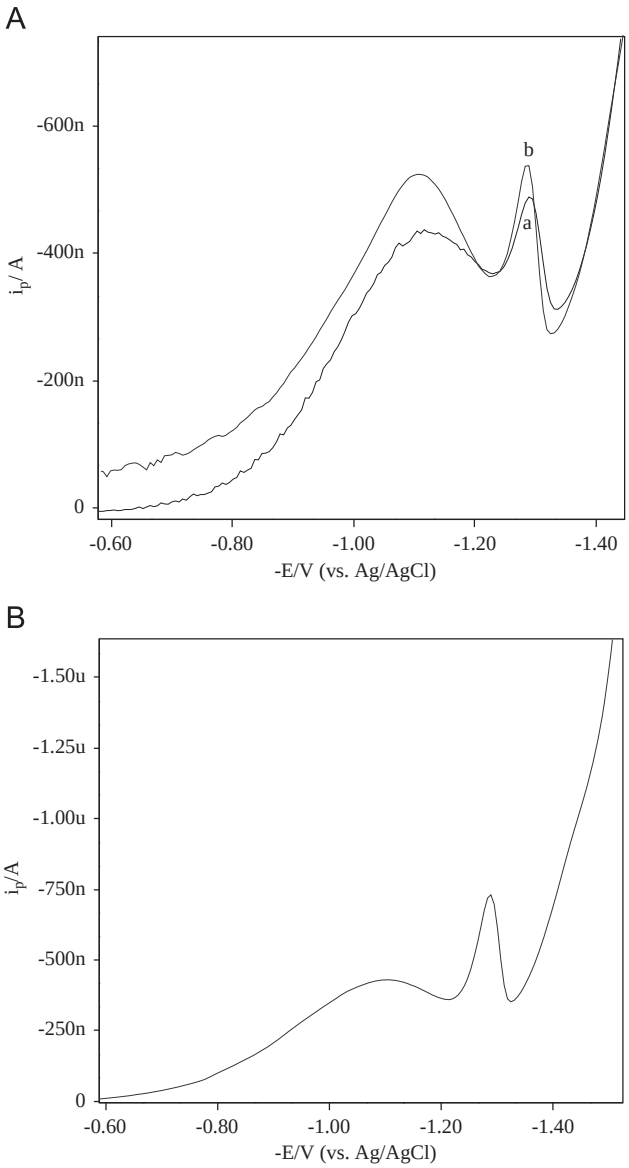

Figure 3 Square-wave voltammogram of Adefovir (1.5 μg/mL) extracted from human plasma, protein precipitation by methanol (A, curve a), protein precipitation by acetonitrile (A, curve b) and solid phase extraction (B).

increment of 2–10 mV/s. Thus, scan increment 10 mV/s was used for the study purpose. Pulse amplitude (E_{sw}) was examined at f 50 Hz and Δs 10 mV/s. The current increased linearly up to E_{sw} 10–50 mV/s after that peak distortion resulted in a poorer resolution. Thus, E_{sw} 50 mV/s was selected for the study purpose. Several operational parameters, which directly affect the voltammetric response, were also optimized such as purge time 25 s, stirring rate 2000 rpm, mercury drop size 4 cm^2, cleaning potential −0.2 V, cleaning time 10 s and equilibration time 10 s.

3.4. *Cyclic voltammetric behavior*

Cyclic voltammetric investigation of Adefovir was performed on surface of the HMDE in BR buffer solution (pH 3.8) at different potential sweep rates. Adefovir exhibited one well-

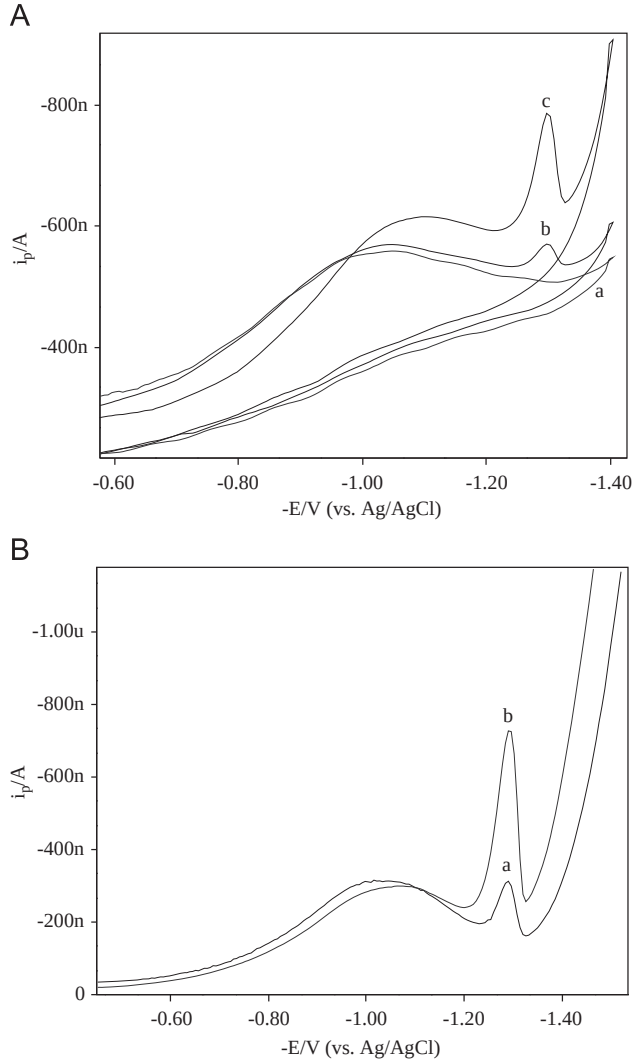

Figure 4 Cyclic voltammograms of Adefovir (1.50 μg/mL) at t_{acc} 0.0 s (A, curve b), at t_{acc} 30 s (A, curve c) and blank solution (A, curve a) and square-wave voltammograms at t_{acc} 0.0 s (B, curve a) and at t_{acc} 30 s (B, curve b).

defined reduction peak in the potential range −1.2 to −1.4 V (vs. Ag/AgCl), at all concentration levels. No peak could be observed in the anodic side of the reverse scan, indicating irreversible nature of the electrode process. The relation between the cathodic peak current ($i_p/\mu A$), the diffusion coefficient of the electroactive species, D_0 ($cm^2\ s^{-1}$) and the sweep rate, v (mV/s) is given by the following expression [19]:

$$i_p/\mu A = (2.99 \quad 10^5) n\alpha^{1/2} A C_0^* D_0^{1/2} v^{1/2} \qquad (1)$$

where n is the number of electrons exchange in the reduction process, α is the electron-transfer coefficient for an irreversible process, A is the surface area of the working electrode (cm^2), and C_0^* is the concentration of the electro active species.

Cathodic peak current showed linear variation on varying sweep rate 50–175 mV/s at a fixed concentration. The relationship between the reduction peak potential and sweep rate showed that the peak potential shifted negatively and peak current increased steadily with increasing sweep rate. This showed the irreversibility of electrode process at HMDE. The linear regression equation obtained for the cathodic peak current is $i_p/\mu A = 0.0388\ \nu\ (mV/s) + 0.0452$ with a correlation coefficient (r^2) of 0.9972. This result indicated that the electrochemical response of Adefovir at the hanging mercury drop electrode is an adsorption-controlled process.

3.5. Validation of proposed method

Validation was conducted as per ICH and USFDA guidelines for assay of Adefovir in pharmaceutical formulation and human plasma [20,21]. The proposed method was validated using the following criteria: system suitability, specificity, selectivity, linearity, recovery and robustness.

3.5.1. System suitability

The significance of system suitability experiment is to ensure that analytical system (including reagents, electrodes, instrument and analysts) is adequate for the intended analysis. Six replicate voltammetric readings of extracted plasma sample of Adefovir and Adefovir standard solution (1.50 μg/mL) were recorded and the relative standard deviation (% RSD) was calculated, 1.481% for extracted plasma sample and 1.582% for standard solution.

3.5.2. Speci city

Specificity is the ability of the analytical method to measure the analyte with acceptable accuracy and precision in the presence of other interferences. The specificity of the method for estimation of Adefovir dipivoxil was evaluated in presence of different concentrations of various excipients like pregelatinized starch, croscarmellose sodium, lactose monohydrate, talc and magnesium stearate. No interferences could be observed at reduction of Adefovir dipivoxil. The response of the analyte in the presence of excipients was compared with that of the pure Adefovir. It was found that assay result did not change.

3.5.3. Selectivity

In order to evaluate levels of endogenous compounds and to develop more sensitive and discriminating methods for quantification of analyte with less interference from other components, six different blank plasma lots were analyzed and these lots were also used to generate calibration curves. Six lower limit of quantification (LLOQ) level samples (0.50 μg/mL) and six blank samples one from each plasma lots were prepared. These plasma samples were extracted by SPE method and analyzed. In all plasma blanks, the response at the potential of Adefovir was less than 20% (acceptance criteria: ≤20%) of LLOQ response and no other endogenous peak was observed in voltammogram.

3.5.4. Linearity

Linearity for assay of Adefovir was performed by standard addition method. An excellent linearity was observed over a wide concentration range of 0.25–2.25 μg/mL (nine concentration levels) with a correlation coefficient (r^2) of 0.9977. The calibration plot resulted in a straight line, $i_p/\mu A = 0.4109 C_0^*(\mu g/mL) - 0.0041$. Limit of detection was found to be 0.08 μg/mL, lower than this, peak could not be resolved from background noise. The reduction peak current of the calibration standard was proportional to the concentrations of Adefovir over the testing range. In aqueous solution, accuracy of all calibration standards was within 85–115%, except LLOQ, where it was 80–120%.

Various statistical parameters for linear regression equation like slope, standard deviation, intercept, standard deviation,

Table 1 Square-wave voltammetric method validation parameters for standard and extracted plasma linearity.

Linearity parameters	Standard	Plasma
Slope	0.4109	0.2350
Standard deviation	0.0075	0.0039
Intercept	0.0041	−0.0395
Standard deviation	0.0105	0.0120
Correlation coefficient	0.9977	0.9984
Standard error of estimation	0.0145	0.0168
Sum of squares of regression	0.6332	1.0547
Sum of squares of residuals	0.0015	0.0017
Limit of detection (μg/mL)	0.08	0.17
Limit of quantification (μg/mL)	0.26	0.51

Table 2 Recovery of the Adefovir in human matrix and in pharmaceutical formulation.

Recovery level	Human matrix	Pharmaceutical formulation
Recovery level-1 [LQC][a] (%)	51.02	99.10
Recovery level-2 [MQC][a] (%)	50.51	99.00
Recovery level-3 [HQC][a] (%)	50.75	99.50
Mean recovery (%)	50.76	99.20
% CV of mean recovery	0.50	0.27

[a]Recovery at each level represents the average of six observations ($n=6$).

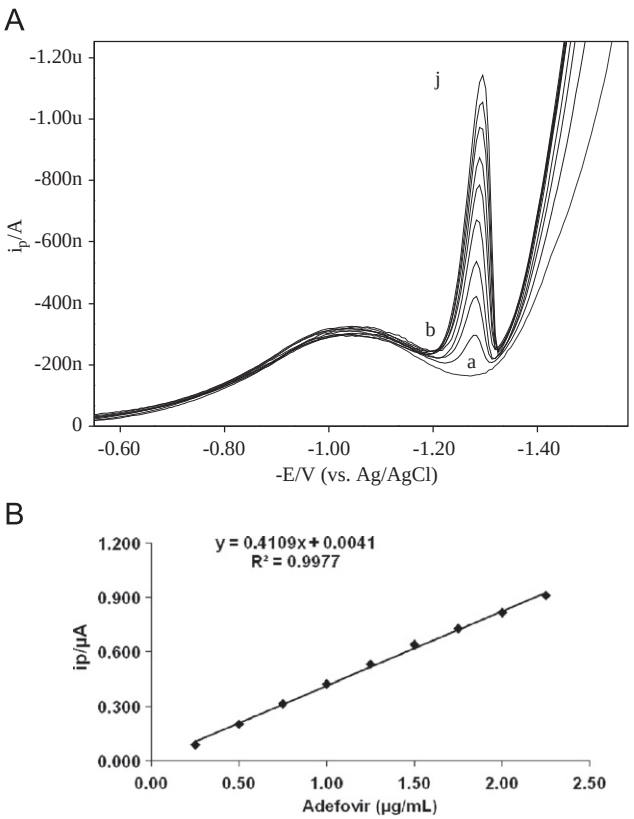

Figure 5 Square-wave voltammograms of Adefovir at different concentration levels, 0.25–2.25 μg/mL (A, curves b–j) and blank solution (A, curve a) and plot of concentration vs. current (B).

correlation coefficient, standard error of estimation, sum of square of regression and sum of square of residual were calculated and are listed in Table 1. Representative square-wave cathodic adsorptive voltammograms and plot of Adefovir concentration (μg/mL) vs. peak current ($i_p/\mu A$) are shown in Fig. 5.

3.5.5. Recovery

The recovery of an analyte is the detector response, which is obtained from a known amount of the analyte added to and extracted from the biological matrix by specific extraction method. Absolute recovery experiment was performed by comparing extracted quality control (QC) samples of three different concentration levels (LQC, MQC and HQC) in six replicates with un-extracted samples of the same levels. The results indicated that the recovery of Adefovir from human plasma was greater than 50% at all levels (Table 2).

Formulation product recovery experiment was carried out by standard addition method. For this, known concentration sample of pure Adefovir was mixed with definite amount of pre-analyzed formulations of the drug and the mixtures were analyzed as before at three different concentration levels in six replicates. The total amount of the drug was then determined and the amount of the added drug was calculated by the difference. The results of recovery studies were found to be quantitative, which are listed in Table 2. The effects of various tablets inactive ingredients such as pregelatinized starch, croscarmellose sodium, lactose monohydrate, magnesium stearate and talc were also investigated. It was noticed that none of them interfered in the determination at the levels normally found in dosage forms.

4. Analytical application

4.1. Analysis of spiked human plasma

In order to establish the analytical utility of the proposed method, spiked human plasma samples were analyzed. Prior to analysis samples were cleaned through solid-phase extraction technique. An excellent calibration curve was observed over a wide concentration range 0.50–5.0 μg/mL (eight concentration levels) with a correlation coefficient (r^2) of 0.9984. The calibration plot resulted in a straight line, $i_p/\mu A = 0.2350\ C_0^*(\mu g/mL) - 0.0395$. Representative square wave voltammograms are shown in Fig. 6 (A, curve b–i). No interference peaks were observed in the blank plasma samples within the studied potential range (Fig. 6A, curve a). Various statistical parameters for linear regression equation were also calculated and are listed in Table 1. Absolute recovery of Adefovir from plasma was calculated from the related linear regression equation, summarized in Table 2. Limit of detection (0.17 μg/mL) and limit of quantification (0.52 μg/mL) were achieved by means of square wave voltammetric methods, which proved the sensitivity of the method. The obtained mean recoveries and standard deviation indicated good accuracy and precision of the proposed method (Table 2).

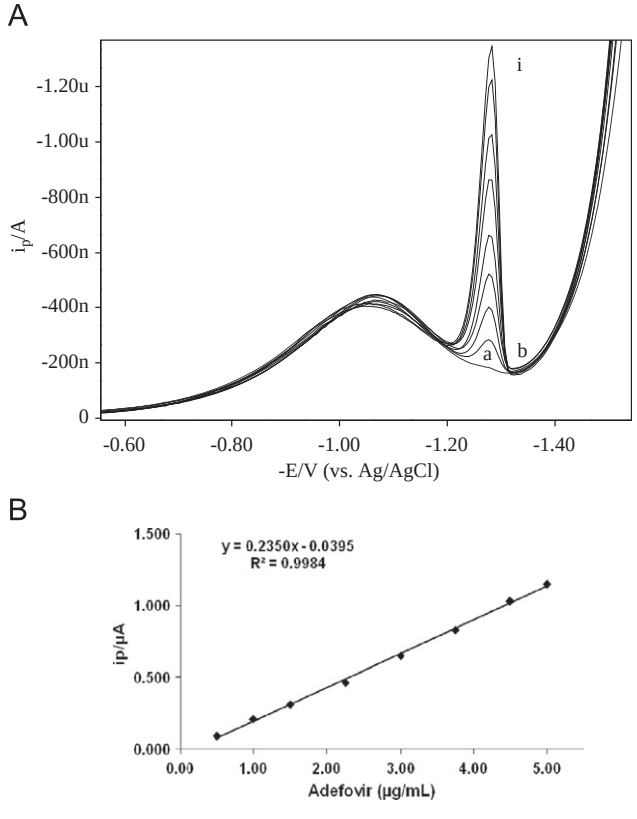

Figure 6 Square-wave voltammograms of Adefovir extracted from human plasma at different concentration levels, 0.5–5.0 µg/mL (A, curves b–i) and plasma blank (A, curve a) and calibration curve of Adefovir (B).

Table 3 Determination of Adefovir in different pharmaceutical formulations by proposed square wave voltammetric methods.

Tablet	Amount labeled (mg)	Amount found[a] (mg)	Error (%)
Adesera	10.00	10.07	+0.7
Adfovir	10.00	10.13	+1.3
Adheb	10.00	9.89	−1.1

[a]Amount found represents the average of six observations (n=6).

4.2. *Pharmaceutical analysis*

The proposed square-wave cathodic adsorptive stripping voltammetric method was successfully applied for determination of Adefovir in different pharmaceutical formulations without the necessity for sample pretreatment or time-consuming extraction steps prior to analysis. Adefovir contents were determined in three different formulations, i.e. Adesera (Mfd. by Cipla Ltd.), Adfovir (Mfd. by Sun Pharma Ltd.) and Adheb (Mfd. by Ranbaxy-Rexcel). Firstly, tablets were weighed and crushed by mortar pestle to a fine powder. An accurately weighed amount of powder was transferred to 10 mL volumetric flask containing 5 mL of methanol, to ensure complete solubility of the drug contents of the flask were sonicated for 5 min and its volume was made up to mark with methanol and centrifuged at 3500 rpm for 5 min. Clear supernatant liquid was withdrawn and diluted with BR buffer of pH 3.8, so that final concentration came under working range. The square-wave voltammograms were recorded under previously optimized conditions. The concentration of Adefovir obtained by the proposed method in different pharmaceutical formulations was compared with labeled claimed and is summarized in Table 3. Results show that Adefovir contents for all pharmaceutical formulations fall within claimed amount with error less than ± 2.0%, indicating that method could be applied for determination of Adefovir in pharmaceutical formulation.

5. Conclusion

The developed method provides a sensitive and selective way for determination of Adefovir in human plasma and pharmaceutical formulation. It was found that the developed method is more sensitive for determination of Adefovir dipivoxil in bulk and pharmaceutical formulation. This method is inexpensive, does not require large setup for pharmacokinetic studies, any additional step for extraction of drug from matrix like derivatization, further cleaning after extraction by dichloromethane and evaporation after extraction. Thus, this developed method can be preferred over conventional methods for quantification of Adefovir in pharmaceutical formulation and human plasma.

Acknowledgments

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