Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Der Pharmacia Sinica, 2013, 4(3):80-94

Der Pharmacia Sinica
ISSN: 0976-8688
CODEN (USA): PSHIBD

Electrochemical behavior and validated determination of the azathioprine in bulk form and body fluids

Gopal Lal Morya, Priyanka Sinha, Hemlata Sharma, K. K. Jhankal and D. K. Sharma*

Department of Chemistry, University of Rajasthan, Jaipur, India

---

ABSTRACT

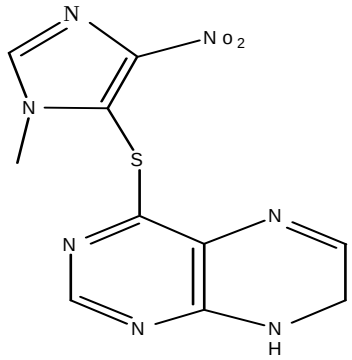
The electrochemical reduction and adsorption of Azathioprine were studied in a BR buffer under different pH conditions at a Glassy Carbon Electrode (GCE). The electrochemical behavior of AZA at glassy carbon electrode was studied by using cyclic voltammetry (CV), differential pulse cathodic adsorptive stripping voltammetry (DPCAdSV), and square-wave cathodic adsorptive stripping voltammetry (SWCAdSV). The voltammogram exhibited irreversible reduction peak in acidic media, corresponding to the reduction of nitro group in the drug. The number of electrons transferred in the reduction process was calculated and the probable reduction mechanism was proposed. A systemic study of the experimental parameters that affect the square-wave stripping response was carried out and experimental conditions were optimized. The electron-transfer coefficient (α) of the electrode reaction and the diffusion coefficient (D) of the reactant species were also determined. The process was successfully applied for assay of the drug in spiked human serum and spiked urine samples.

Keywords: Azathioprine, Square wave cathodic adsorptive stripping voltammetry, cyclic voltammetry, electron-transfer coefficient (α), diffusion coefficient (D)

INTRODUCTION

Azathioprine, 6-(1-methyl-4-nitroimidazole-5-yl) thiopurine (Scheme- 1), is an immunosuppressive agent. They are used in the chemotherapy of acute leukemia, for immunosuppressant after solid-organ transplantation, and increasingly for immunomodulation in autoimmune disease. Currently, Aza is standard treatment in patients with chronically active inflammatory bowel disease (IBD). Azathioprine acts on several modes in cellular immunity processes. It inhibits lymphocyte activation, lymphocyte differentiation, in vitro lymphocyte stimulation,and in vitro mixed lymphocyte reaction and it reduces the activity of natural killer lymphocytes. [1-5]

Azathioprine is a prodrug which is converted into 6-thiopurine and various methylnitroimidazole metabolites. Azathioprine has a nitroimidazole moiety and it has been shown that azathioprine is mutagenic in the Ames assay if anaerobic conditions are used. This suggests that mutation in the Ames assay is due to reduction of the nitro group by the bacteria. Nitroimidazoles have been shown to undergo nitro reduction with the formation of a hydroxylamine as the intermediate [6-9]. A few nitroimidazoles have been evaluated for carcinogenic activity, but in general, they have shown only weak tumorigenic activity. However, the nitroimidazoles that have been tested so far have been monocyclic and would not be anticipated to have strong carcinogenic activity, if any, In the evaluation by the International Agency for Research on Cancer of azathioprine it was determined that it was carcinogen in humans but that the evidence was not sufficient for animal experiments [10-14].



(Scheme- 1)

Therefore, the development of an analytical method sensitive and selective enough for determining azathioprine in both pharmaceutical and biological samples is of great importance. Several analytical methods have been developed to determine the concentrations of azathioprine in biological fluids and pharmaceutical preparations.

Most of the reported methods are micellar electrokinetic chromatography (MEKC), liquid chromatography, capillary electrophoresis and high performance liquid chromatography (HPLC) [15-17].

Although the selectivity and the detection limit have been improved in these methods, these are rather time-consuming methods and require large number of complicated steps to follow on for analysis. For this purpose, the desirable technique for the analysis of drugs should be rapid, simple, low cost, and of high sensitivity in analysis. In all available electrochemical methods, stripping voltammetric analysis is an extremely sensitive technique that utilizes a bulk electrolyte step to preconcentrate the analyte from the sample solution into or onto the working electrode [18-20].

Many organic compounds exhibit surface-active properties that are manifested by their adsorption from solution onto the surface of a solid phase. This phenomenon forms the basis for adsorptive stripping voltammetry (AdSV), where the species to be determined are accumulated on the electrode by adsorption. Adsorptive stripping voltammetry has been demonstrated as a sensitive analytical method for a wide range of pharmaceutical compounds adsorbing on the electrode surface [18-22].

The present work is concerned with a study of the voltammetric behavior of AZA at glassy carbon electrode. AZA could be adsorbed on the glassy carbon electrode and this phenomenon was put to analytical advantage in the design of adsorptive stripping voltammetric methods for the determination of AZA in pharmaceutical and human urine as well as serum samples.

MATERIALS AND METHODS

Materials

Azathioprine was obtained from RPG Life Science Ltd., Ankleswar, India, and was used as received. Tablets containing Azathioprine (Sensival) labeled 50 mg Azathioprine were obtained from commercial sources.

Reagents and solutions

A standard stock solution ($1x10^{-3}$ mol L^{-1})of bulk AZA was prepared by dissolving an accurate mass of the drug in an appropriate volume of double distilled water , which was then stored in the dark at 4 °C. More dilute solutions were prepared by accurate dilution just before use. The AZA solutions were stable and their concentrations did not change with time.

Britton–Robinson (BR) universal buffers of pH 2–10 (adjusted to the required pH with 0.1M sodium hydroxide solution and 0.1M hydrochloric acid) were prepared and used as supporting electrolytes. All chemicals used were of analytical reagent grade quality and were employed without further purification.

Analysis of Tablets

Ten tablets of the drug (AZA) were weighed and the average mass per tablet was determined and then ground to a homogeneous fine powder in a mortar. A portion of the finely ground material equivalent to 50mg of TAM double distilled water was accurately weighed and transferred into a 100 ml calibrated flask containing 60 ml double distilled water. The content of the flask was sonicated for about 10 min and then made up to volume with double distilled water. The solution was next filtered through a 0.45 (Whatman filter paper). The desired concentration was obtained by accurate dilution with double distilled water.

Analysis of Spiked Urine & Serum Samples

Drug-free human blood, obtained from healthy volunteers (after obtaining their written consent) was centrifuged (4000 rpm) for 30 min at room temperature, and separated serum samples were stored frozen until assay. An aliquot of serum sample was fortified with AZA dissolved in double distilled water to achieve a final concentration of 1x 10^{-3} M. Acetonitrile removes serum proteins effectively. After vortexing for 30sec., the mixture was then centrifuged for 10 min at 4000 rpm in order to eliminate serum protein residues. Appropriate volumes of this sample were transferred into the voltammetric cell and diluted up to the volume with Britton–Robinson (BR) universal buffer at pH 6.2 and subsequently analyzed according to the recommended in the general analytical procedure. A blank experiment was carried out adopting the above procedure. An aliquot of human urine sample was collected and analyzed in the same way as the serum samples.

Instrumentation

Model 1230A [SR 400] electrochemical analyzer (CHI Instrument, USA) was employed for electrochemical techniques, with a totally automated attached to a PC with proper CHI 100W version 2.3 software for total control of the experiments and data acquisition and treatment. A three-electrode cell system was used with activated glassy carbon electrode (ø = 3 mm, CHI) as working electrode, Ag/AgCl (3 M KCl) as the reference electrode and a platinum wire as the auxiliary electrode. A magnetic stirrer (CAT.NO-1250-2 LAB-LINE INSTRUMENT, INC. USA) and a stirring bar provided the convective transport during the preconcentration step. A digital pH-meter (CHINO- DB-1011) was used for measuring the pH values of the investigated solutions. The digital pH meter was fitted with a glass electrode and a saturated calomel electrode as the reference, which was previously standardized with buffers of known pH.

Pre-treatment of the glassy carbon electrode

The glassy carbon working electrode was polished to a mirror finish using a CHI polishing kit with alumina paste and thoroughly washed with double distilled water before measurements.

General analytical procedure

Britton–Robinson (BR) universal buffer of pH 6.2 (9 ml) and an appropriate volume of drug sample were introduced into the voltammetric cell, through which a pure nitrogen (oxygen free) stream was passed for 10 min before recording the voltammogram. An accumulation potential (vs. Ag/AgCl, KCl) was applied at the GCE for a selected time period while the solution was stirred at 400 rpm. At the end of the accumulation time period, the stirring was stopped and 10 sec. were allowed for the solution to become quiescent. Then, the voltammograms were recorded by scanning the potential toward the positive direction using the CV, DP and SWV waveform. All data were obtained at room temperature.

RESULTS AND DISCUSSION

The electrochemical behavior of AZA at GCE was studied by using cyclic voltammetry (CV), differential pulse cathodic adsorptive stripping voltammetry (DPCAdSV), and square-wave cathodic adsorptive stripping voltammetry (SWCAdSV). In all electrochemical methods, azathioprine gave one well-defined reduction peak in Britton–Robinson (BR) buffer, which is attributed to the reduction of the-NO_2 group of imidazole ring at the glassy carbon electrode.

Cyclic voltammetric studies

Typical cyclic voltammograms for AZA were recorded within the wide range (-100 to -1000 mV) of the potential at different pH, scan rate and concentration. The shape of cyclic voltammograms clearly indicated the irreversible nature of reduction [22-24].

Effect of pH

The reversibility of the reduction process was investigated by using CV. A Britton-Robinson buffer with different pH values was used as supporting electrolyte. The effect of the pH on the azathioprine peak was tested. No azathioprine peaks were observed at pH 1.8. At pH 4.5 one sharp peak at E=-0.292 V. In the case of pH range of 6-7.5 there are two peaks of azathioprine. The first sharp one appears at E=-0.46 V and the second, broad and low one, at E= -0.97 V. The sharp peak of azathioprine appearing at E = -0.46 V in Britton-Robinson buffer with pH 6.2 was chosen for further investigation as the most promising from the analytical point of view. The other possible choice, the sharp peak found at pH 4.5, was not only lower than the chosen one but also more difficult to analyze due to its position (E=-0.292 V) resulting in enhanced influence of residual urrent.[25].Other broad peaks were not suitable for analytical application.

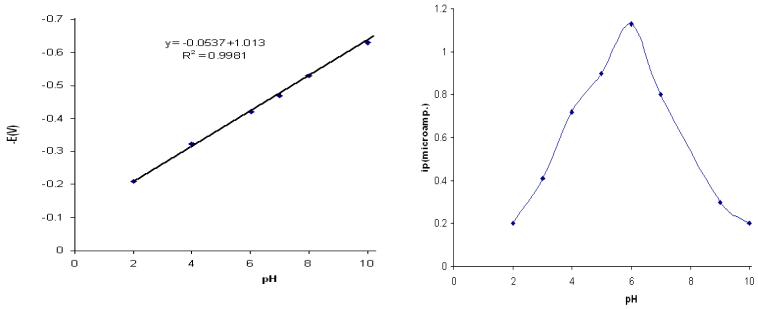


Fig.-1: (A). The linear relation between E_p and pH; (B). The plot of $i_p$vs pH

In 0.04 M Britton-Robinson buffer, azathioprine gives a linear relationship between peak potential (Ep) and pH. With increasing pH, the peak potential is shifted towards more negative values. That is expressed by the following equations:

For CV, pH 2-10: *E*p (V) = 0.0537 +1.101 pH, r^2 = 0.998

Linear pH dependence of the peak potential for reduction wave in the range of 2-10 shows that protonate participates directly in the reduction process. After pH 10, no significant displacement in peak potential was observed. The peak potential of the four electron wave is pH independent, because equilibrium is completely shifted to the left-hand side (i.e., pH<10), which indicates that proton-transfer occurs as a step consecutive to irreversible electrode processes .The linearity was observed in the pH range of 2-10, with a negative slope of 0.055 V per pH unit.

As shown in Figure 1 (curve), the height of the peak reaches a maximum at pH 6.2, and after that it decreases. Therefore, pH 6.2 was chosen as the optimum value for the determination of AZA.

This fact proves that it is the protonated form of azathioprine which undergoes reduction. The electrochemical reduction process could be presented by the following reaction; the observed irreversible peak at acid and neutral pH is due to the four-electron, four-proton reduction of the azathioprine nitroimidazole group to yield the hydroxylamine derivative according to the following overall reaction [25]

$$R\text{-}NO_2 + 4e^- + 4H^+ \longrightarrow R\text{-}NHOH + H_2O \quad (1)$$

In the same way, the equations describing the two new signals at alkaline pH correspond to

$$RNO_2 + e^- \longrightarrow RNO_2^{\cdot -} \quad (2)$$

$$RNO_2^{\cdot -} + + 3e^- + 4H^+ \longrightarrow R\text{-}NHOH + H_2O \quad (3)$$

Effect of Scan Rate

The effect of scan rate (ν) on the anodic peak current by using the solution of different concentration $1x10^{-6}$, $1.5x10^{-6}$, $2.0x10^{-6}$, $2.5x10^{-6}$ & $3.0x10^{-6}$ mole $L^{-1}$and recording CV's at 100, 200, 300, 400 and 500 $mVsec^{-1}$ scan rate. Table

1 summarizes voltammetric data for AZA in the neutral medium at different scan rates. Cyclic voltammograms showed one clearly defined reduction peak at -0.46V. No peaks were observed on the reverse scan, indicating the irreversibility of electrode processes.

The relation between the cathodic peak current i_{pc} (μA), the diffusion coefficient of the electro active species, D_o (cm^2 s^{-1}), and the scan rate, ν (mV s^{-1}), is given by (45)

i_{pc} = (2.99x10-5)n α½ A $C_o D_o^{½}$ ν½

Where n is the number of electrons exchanged in reduction, α is the transfer coefficient, A is the apparent surface area of the electrode (cm^2), and C_o is the concentration of the electro active species (m mole dm^{-3}). The transfer coefficient α for an irreversible process can be calculated from

$[E_{pc} – Epc/2] = 47.7/α$

Where Epc/2 is the potential at which the current equals one-half of the peak current. Careful inspection of data on the effect of scan rate reveals that the linearity of the relationship is realized up to scan rate. This indicates that the charge transfer is under the partial diffusion-controlled process and that adsorption of aggregates at the electrode surface is also possible.

The height of the peak decreased with repetitive scans, its potential not being shifted. The relationship between the peak potential (*E*p) and the scan rate (ν) is expressed as (46)

*E*p = (2.303RTα n*F*) log (RT/α *nF*) - (2.303RTα n*F*) log ν

A straight line is observed when *E*p (V) is plotted against log ν (Figs.3, curve) in pH 6.2 and can be expressed by the equation.

y(*E*p) = 0.047 (log ν) + 0.329(V), r^2 = 0.998

A value of αn = 0.59 was obtained. Accordingly, the number of electrons, *n*, transferred in the rate-determining step should equal four (*n* = 4).

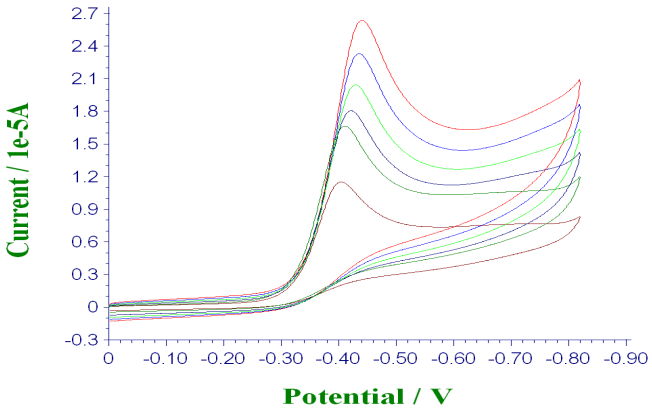


Fig 2: Effect of scan rate on cyclic voltammetric parameters of Azathioprine at pH 6.2 (concentration, $1x10^{-6}$ M)

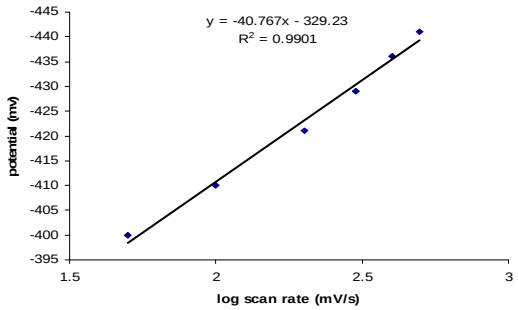


Fig.-3: Plot of –Ep versus log ν form voltammograms in Fig. 2 for AZA in concentration at BRB pH-6.2

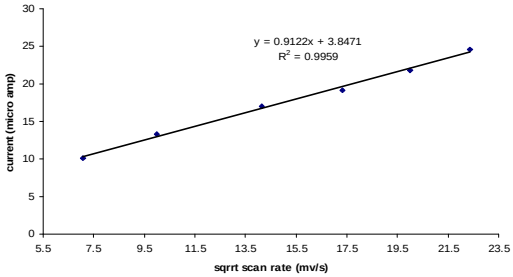


Fig.-4: Plot of i_p versus ν½ form voltammograms in Fig. 2 for AZA in 1x10^{-6} M concentration at BRB pH-6.2

According to the **Randles-Sevick** equation in a linear diffusion controlled process (i_{pc} α $\nu^{½}$), for the adsorptive process (i_{pc} α ν) (logi_{pc} α logν).The peak currents of AZA are plotted against the scan rate. The peak current (i_{pc}) increases linearly with increasing scan rate.A linear relationship was observed between the reduction peak current versus the square root of the scan rate (Figs.4, curve) with a significant correlation coefficient of 0.9975 indicating thereby that the electrode process is diffusion-controlled between the scan rate of 100 and 500 mV s^{-1}.Which may be expressed by the equation,

i_{pc} (μA) = 0.363 ν½ (mV/s)½ − 4.65, r^2 = 0.997

A slope close to theoretical value of 0.5 reported for an ideal reaction for the diffusion-controlled electrode process that the compound was diffusion at the electrode surface.

The adsorption process was also identified by a plot of log ip versus log ν (Figs.5 curve), giving a straight line, which can be expressed by the equation

log i_{pc} (μA) = 0.435 + 0.986 log ν(mV/s), r^2 = 0.999

A slope close to1.0 shows that the compound was adsorbed on the electrode surface (47).On the basis of above studies it is observed that reduction of AZA in the neutral medium is possible. Linear nature of i_{pc} / $\nu^{½}$ and almost constant values of αn clearly indicates that of these AZA is diffusion controlled.[26-27]

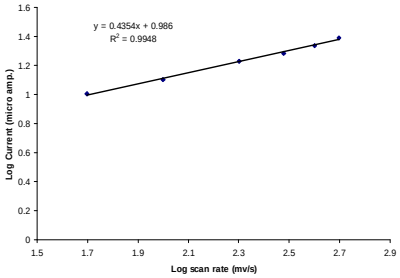


Fig.-5: Plot of log i_p versus log ν form voltammograms in Fig. 2 for AZA in1x10^{-6} M concentration at BRB pH-6.2

Effect of Concentration

Table-2 summarizes voltammetric data for AZA concentration in the neutral medium. Concentration likewise affected the magnitude of the peak current. This was seen by obtaining scans of $1x10^{-6}$, $1.5x10^{-6}$, $2.0x10^{-6}$, $2.5x10^{-6}$ & $3.0x10^{-6}$ mole L^{-1}desired AZA using a scan rate of 100mVsec^{-1} at pH 6.2. The effect of concentration for AZA on the appearance of the cyclic voltammograms can be seen in Figs.6.

The **Randles – Servick** equation also indicates that i_{pc} is directly proportional to concentration. A plot of this equation (i_{pc}/concentration) for AZA are shown in Figs.7 (curve), yields a straight line.

i_{pc} (μA)= 0.100xC (m mole L^{-1})+0.01, (r^2 = 0.998, n =5)

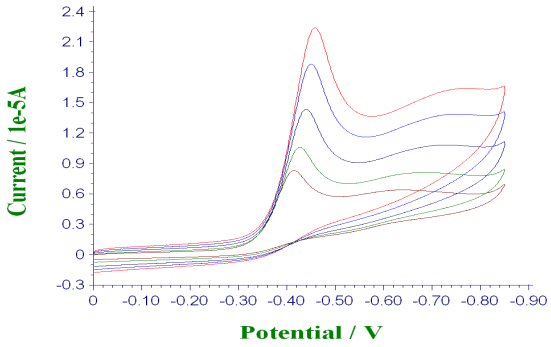


Fig.6: Cyclic voltammograms in pH 6.2 BRB solution on glassy carbon electrode at different concentrations of AZA (1-5): $1x10^{-6}$, $1.5x10^{-6}$, $2x10^{-6}$, $2.5x10^{-6}$, $3x10^{-6}$ M

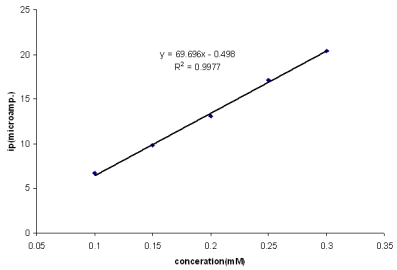


Fig.-7: Plot of i_p versus concentration from voltammograms in Fig. 6 for AZA at 100 mVs^{-1} SR and BRB pH-6.2

Stripping voltammetric studies

Stripping voltammetric methods were optimized for trace determination of AZA by pulse and square wave potential-waveforms. Stripping voltammograms of bulk AZA in the Britton Robinson (BR)universal buffer (pH 2 to 10) recorded by differential pulse and square wave voltammetry following its preconcentration onto the GCE by adsorptive accumulation for 15 sec. exhibited a well-defined single irreversible cathodic peak with a better enhanced peak current magnitude at pH 6.2. Therefore, a Britton Robinson (BR) universal buffer of pH 6.2 was chosen as a supporting electrolyte in the rest of study [27-30].

Differential pulse cathodic adsorptive stripping voltammetry (DP-CAdSV) method

The optimum operational conditions of pulse-height scan rate and preconcentration parameters for determination of bulk AZA applying differential pulse cathodic adsorptive stripping voltammetry (DP-CAdSV) at the GCE were identified. This was carried out by recording voltammograms of 2×10^{-8} mol L^{-1} bulk AZA in the Britton Robinson (BR)universal buffer of pH 6.2 under each of the following conditions: scan rate ν (10 mV s-1), pulse height a (5 to 50 mV), preconcentration potential and preconcentration time tacc (0 to 15 s). DP-CAdS voltammograms of various concentrations of AZA were recorded under the optimal operational conditions. A linear variation of the peak current (i_p) with concentration (C) of bulk AZA was obtained within the concentration range of 2×10^{-9} to 1×10^{-8} mol L^{-1}(Figure 8) following the regression equation: i_{pc} (μA) = 46.6 C (nM) + 0.3 (r = 0.998 and n = 5) (Curve 9.). A LOD of 1.32×10^{-6} mol L^{-1} and a LOQ of 4.38×10^{-6} mol L^{-1} bulk AZA were achieved (Table 3) applying the described DP-CAdSV method.[30-32]

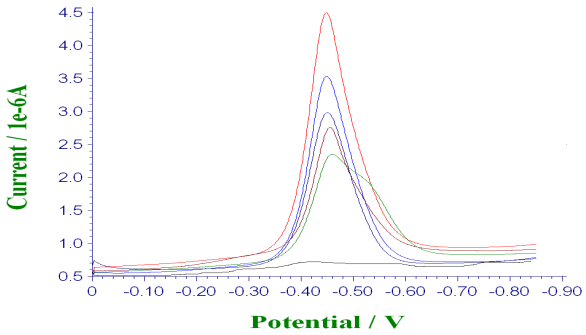


Fig. 8: The DPCAdS voltammograms for increased concentrations of azathioprine in bulk forms: (1) blank; (2) $2x10^{-9}$; (3) $4x10^{-9}$; (4) $6x10^{-9}$; (5) $8x10^{-9}$; and (6) $1x10^{-8}$; mol L^{-1}; Eacc.=0.0 V, tacc.=15 s, pulse amplitude 50 mV,pulse width 30 ms, and ΔE=10 mV and BR buffer(9mL) of pH 6.2

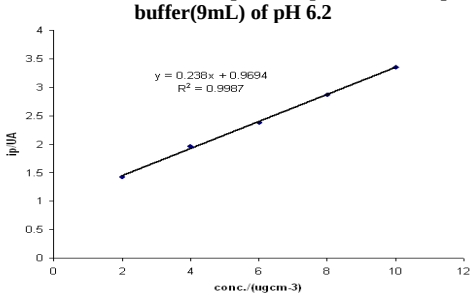


Fig.-9: Plot of i_p versus concentration from DPCAdS voltammograms in Fig. 8 for AZA in bulk forms

Square wave cathodic adsorptive stripping voltammetry (SW-CAdSV) method

Optimum operational conditions of both preconcentration and pulse-parameters for determination of bulk AZA applying square wave cathodic adsorptive stripping voltammetry (SW-CAdSV) were identified. This was curried out by studying the effect of changing of each of preconcentration potential ($E_{acc.}$), pulse-height a (5 to 50 mV), frequency f (20 Hz) and scan increment ΔEs (10 mV) on peak current magnitude of 1×10^{-9} mol L^{-1} bulk AZA in the Britton Robinson (BR)universal buffer of pH 6.2. SW-CAdS voltammograms of various concentrations of AZA were recorded under the optimal operational conditions. A linear variation of the peak current (i_p) with concentration (C) of bulk AZA was obtained within the concentration range of 1×10^{-9} to 1×10^{-8} mol L^{-1} (Figure 10) following the regression equation: i_{pc} (μA) = 76.36 C (nM)) + 0.176 (r = 0.997 and n = 7) (Curve 11). A LOD of 6.41×10^{-9} mol L^{-1} and a LOQ of 2.1×10^{-8} mol L^{-1} bulk AZA were achieved (Table 3) applying the described SW-CAdSV method. [33-34]

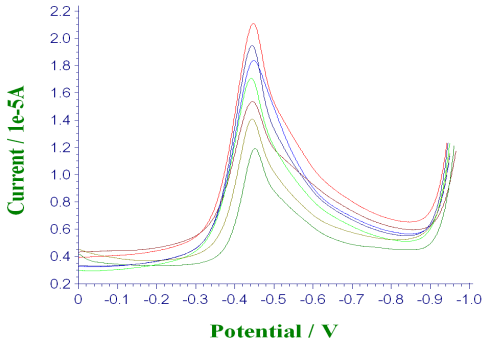


Fig.-10: The SWCAdS voltammograms for increased concentrations of azathioprine in bulk forms: (1) blank; (2) $1x10^{-9}$; (3) $1.5x10^{-9}$ (4) $2x10^{-9}$; (5) $4x10^{-9}$; (6) $6x10^{-9}$; (7) $8x10^{-9}$; and (8) $1x10^{-8}$; mol L^{-1}; Eacc.=0.0 V, tacc.=15 s, a=50 mV, f=20 Hz, ΔE=10 mV and BR buffer (9mL)of pH 6.2

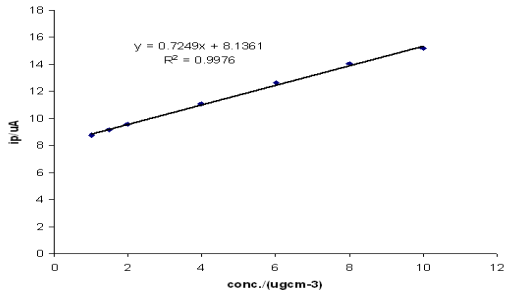


Fig -11: Plot of i_p versus concentration from SWCAdS voltammograms in Fig.10 for AZA in bulk forms

Validation of the Procedure

Validation of the proposed procedure for assay of the drug at trace levels was examined via evaluation of the limit of detection (LOD), limit of quantization (LOQ), reproducibility, recovery, selectivity, robustness and ruggedness. The Limits of detection (LOD) and quantification (LOQ) of AZA were calculated using the following equations:

LOD= 3s/b

LOQ=10s/b

Where s is the standard deviation of the intercept and b is the slope of the calibration curve reproducibility, accuracy and precision (51) of results applying the described stripping voltammetric methods were examined by performing five replicate analysis of standard solutions of bulk AZA. The mean percentage recovery (%R) had been calculated for the found concentrations as a percent of the nominal concentrations in the standard solutions. Accuracy was expressed as relative error (RE %) while precision was assessed from the relative standard deviation in percentage (RSD %) of the mean recovery. The obtained results confirmed the reliability of the described stripping voltammetric methods for assay of AZA [35-38].

Assay of AZA in spiked human urine

AZA in spiked human urine was successfully analyzed by the described voltammetric methods (DP-CAdSV and SW-CAdSV) without the necessity for extraction of the drug prior to the analysis. Representative DP-CAdSV and SW-CAdS voltammograms of AZA in spiked human urine recorded under the optimum operational conditions of the described stripping voltammetric methods are shown in Figure 12 and 14.

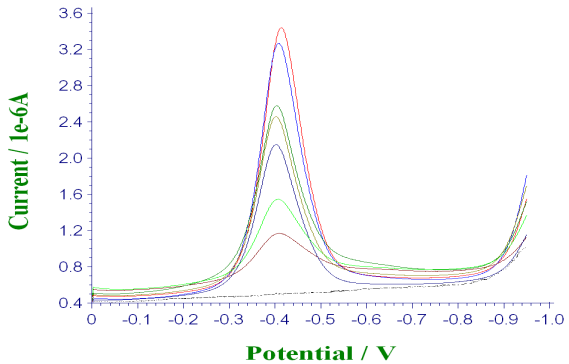


Fig.-12: The DPCAdS voltammograms for increased concentrations of azathioprine spiked in urine samples: (1) blank; (2) $1x10^{-9}$; (3) $3x10^{-9}$; (4) $5x10^{-9}$; (5) $7x10^{-9}$; (6) $9x10^{-9}$; (7)$1.5x10^{-8}$; (8) $2x10^{-8}$; mol L^{-1}; Eacc.=0.0 V, tacc.=15 s, pulse amplitude 50 mV and pulse width 30 ms, and ΔE=10 mV and BR buffer(9mL) of pH 6.2

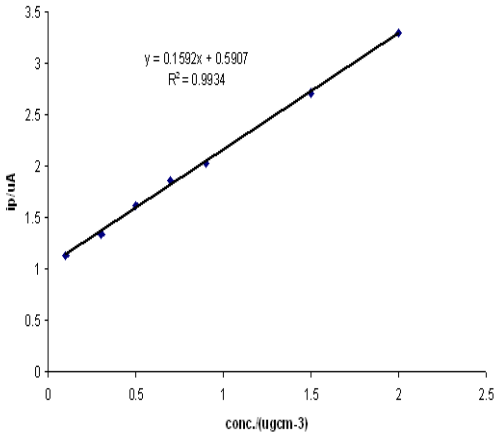


Fig.-13: Plot of i_p versus concentration from DPCAdS voltammograms in Fig.12 for AZA in urine sample

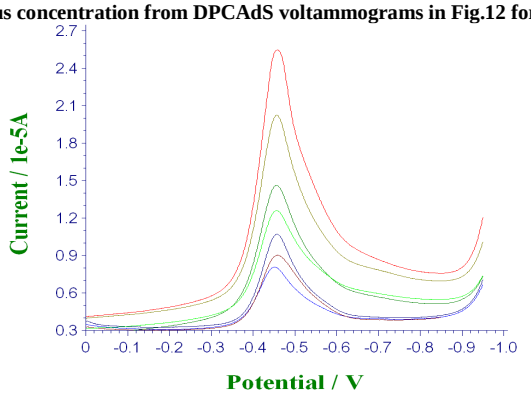


Fig.14: The SWCAdS voltammograms for increased concentrations of azathioprine spiked in urine samples: (1) blank; (2) $1x10^{-7}$; (3) $3x10^{-9}$; (4) $5x10^{-9}$; (5) $7x10^{-9}$; (6) $9x10^{-9}$; (7)$1.5x10^{-8}$; and (8) $2x10^{-8}$mol L–1; Eacc.=0.0 V, tacc.=15 s, a=50 mV, f=20 Hz, ΔE=10 mV and BR buffer (9mL)of pH 6.2

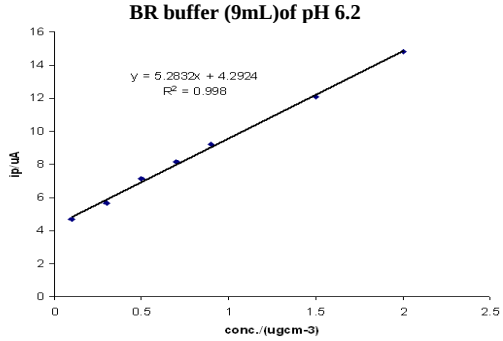


Fig-15: Plot of i_p versus concentration from SWCAdS voltammograms in Fig. 6for AZA in urine sample

As shown in Figure 13 and 15 (curve) respectively, no interfering peaks were observed in the blank human serum within the studied potential range. Linear variations of the peak current (i_p) with concentration of AZA in spiked human urine were obtained within the concentration ranges of 1×10^{-9} to 2×10^{-8} mol L^{-1} (DP-CAdSV) and 1×10^{-9} to 2×10^{-8} mol L^{-1} (SW-CAdSV) following the regression equations: (i_{pc} (µA) = 41.80 C (nmol L^{-1}) + 0.466; r = 0.993 and n = 7), and (i_{pc} (µA) = 55.5 C (nmol L^{-1}) + 0.169; r = 0.998 and n = 7), respectively. Detection limits of 5.38×10^{-9} and 1.6×10^{-9} mol L^{-1} and quantitation limits of 1.78×10^{-9} and 5×10^{-8} mol L^{-1} AZA were achieved by the described DP-CAdSV and SW-CAdSV methods (Table 3) respectively. Mean percentage recoveries and relative standard deviations of 100.36 ± 0.818 (DP-CAdSV) and 101.27 ± 2.30 (SW-CAdSV) were achieved based on replicate measurements of 1×10^{-9} mol L^{-1} (Table-4) AZA in spiked human urine. These results confirmed the reliability of the described stripping voltammetric methods for assay of AZA in human urine [39-40].

Assay of AZA in spiked human serum

AZA in spiked human serum was successfully analyzed by the described voltammetric methods (DP-CAdSV and SW-CAdSV) without the necessity for extraction of the drug prior to the analysis. Representative DP-CAdSV and SW-CAdS voltammograms of AZA in spiked human serum recorded under the optimum operational conditions of the described stripping voltammetric methods are shown in Figure 16 and 18.

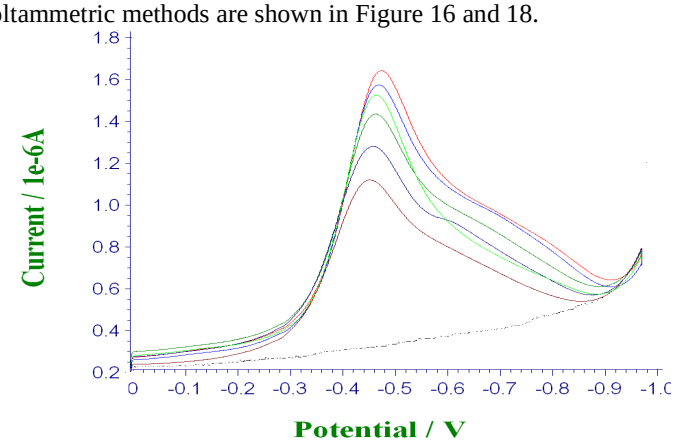


Fig.16: The DPCAdS voltammograms for increased concentrations of azathioprine spiked in serum samples: (1) blank; (2) $3x10^{-9}$; (3) $4x10^{-9}$; (4) $5.5x10^{-9}$; (5) $7.5x10^{-9}$; (6) $9.5x10^{-9}$; and (7) $1.5x10^{-8}$; mol L^{-1}; Eacc.=0.0 V, tacc.=15 s, pulse amplitude 50 mV, pulse width 30 ms, and ΔE=10 mV and BR buffer(9mL) of pH 6.2

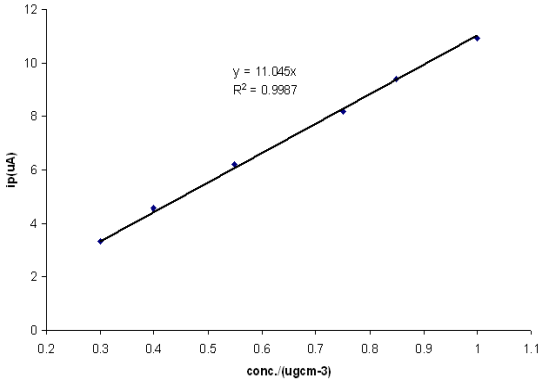


Fig.-17: Plot of i_p versus concentration from DPCAdS voltammograms in Fig.16 for AZA in serum sample

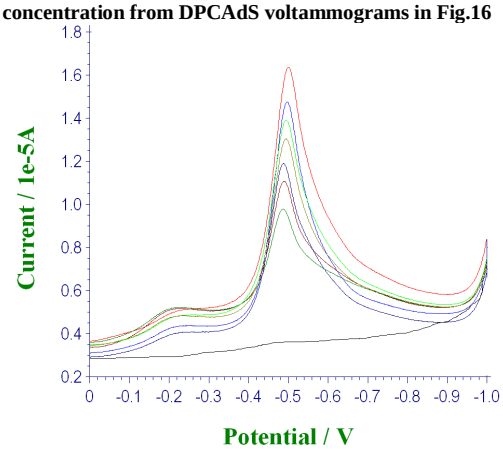


Fig.18: The SWCAdS voltammograms for increased concentrations of azathioprine spiked in serum samples: (1) blank; (2) $2x10^{-9}$; (3) $3x10^{-9}$; (4) $4x10^{-9}$; (5) $5.5x10^{-9}$; (6) $7.5x10^{-9}$; and (7) $9.5x10^{-9}$; and (8) $1.5x10^{-8}$ mol L^{-1}; Eacc.=0.0V, tacc.=15 s, a=50 mV, f=20 Hz, ΔE=10 mV and BR buffer (9mL)of pH 6.2

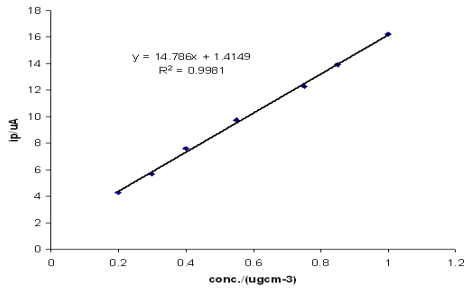


Fig- 19: Plot of i_p versus concentration from SWCAdS voltammograms in Fig.18 for AZA in serum sample

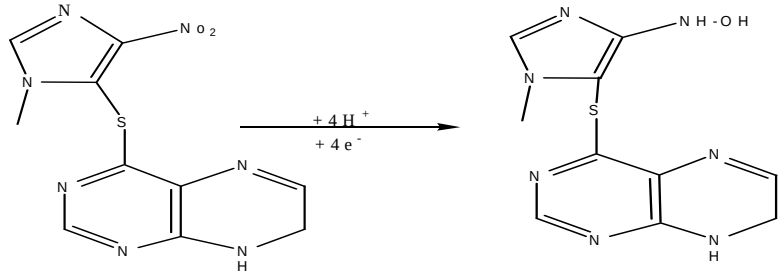
As shown in Figure 17 and 19 (curve) respectively, no interfering peaks were observed in the blank human serum within the studied potential range. Linear variations of the peak current (i_p) with concentration of AZA in spiked human serum were obtained within the concentration ranges of 3×10^{-9} to 1×10^{-8} mol L^{-1} (DP-CAdSV) and 2×10^{-9} to 1×10^{-8}mol L^{-1} (SW-CAdSV) following the regression equations: (i_{pc} (µA) = 41.80 C (nmol L^{-1}) + 0.466; r = 0.999 and n = 6), and (i_{pc} (µA) = 55.5 C (nmol L^{-1}) + 0.169; r = 0.998 and n = 7), respectively. Detection limits of 5.94×10^{-9} and 3.91×10^{-8} mol L^{-1} and quantization limits of 1.9×10^{-9} and 1.2×10^{-9} mol L^{-1}. AZA were achieved by the described DP-CAdSV and SW-CAdSV methods (Table-3), respectively. Mean percentage recoveries and relative standard deviations of 100.17 ± 0.390 (DP-CAdSV) and 100.40 ± 0.444 (SW-CAdSV) were achieved based on replicate measurements of 2×10^{-9} mol L^{-1} (Table-2) AZA in spiked human serum. These results confirmed the reliability of the described stripping voltammetric methods for assay of AZA in human serum.

Table-1: Stripping voltammetric determination of Azathioprine in various meduims using SWCAdSV and DPCAdSV Modes

Techniques	Bulk Form		Urine		Serum	
	SWCAdSV	DPCAdSV	SWCAdSV	DPCAdSV	SWCAdSV	DPCAdSV
Linearity range (µg)	$0.1x10^{-9}$ – $2x10^{-8}$	$0.1x10^{-9}$ – $2x10^{-8}$	$0.1x10^{-9}$ – $2x10^{-8}$	$0.1x10^{-9}$ – $2x10^{-8}$	$0.2x10^{-9}$ – $1x10^{-8}$	$0.3x10^{-9}$ – $1x10^{-8}$
Slope (µA/M)	$5.2x10^{-9}$	$1.59x10^{-9}$	$5.2x10^{-9}$	$1.59x10^{-9}$	$1.4x10^{-9}$	$1.07x10^{-9}$
Intercept (µA)	0.42	0.59	0.42	0.59	0.141	0.185
Correlation coefficient	0.998	0.993	0.998	0.993	0.998	0.999
t-test	0.28	0.25	0.28	0.25	0.28	0.43
Variance ratio (F)	0.98	0.92	0.98	0.92	1.01	1.96
LOD (M)	$1.6x10^{-9}$	$5.38x10^{-9}$	$1.6x10^{-9}$	$5.38x10^{-9}$	$3.91x10^{-9}$	$5.94x10^{-9}$
LOQ (M)	$5x10^{-8}$	$1.78x10^{-8}$	$5x10^{-8}$	$1.78x10^{-8}$	$1.2x10^{-8}$	$1.9x10^{-8}$
Repeatability of peak current (RSD%)	0.59	0.64	0.59	0.64	0.62	0.68
Repeatability of peak potential (RSD%)	0.21	0.23	0.21	0.23	0.19	0.22
Reproducibility of peak current (RSD%)	0.56	0.59	0.56	0.59	0.56	0.67
Reproducibility of peak potential (RSD%)	0.13	0.18	0.13	0.18	0.12	0.17

Controlled potential Electrolysis Behavior (CPE)

CPE was carried out on a graphite electrode in 0.04 M Britton-Robinson buffer at pH 6.2. A three-electrode circuit with an Ag/AgCl, electrode was used as reference and a platinum wire as a counter electrode.



(Scheme-2)

The number of electrons transferred, e values were calculated from the charge consumed by the desired concentration of azathioprine. The charge consumed was determined in neutral medium. For this purpose, 2mL solution of the electro active species was placed in the cell and electrolysis was carried out at a potential of -0.2 to -

0.95 V against Ag/AgCl reference electrode. During the electrolysis, solutions were continuously stirred and purged with nitrogen gas. Number of electrons e was calculated using the well-known Coulomb law.

$Q = nFe$ [1]

Where, Q = total net charge
n = mole number of electro active specie in solution
F = Faraday's constant, and e = the number of electrons.

The number of electrons was obtained after running at pH, the value is found to be 4 for cathodic peak of azathioprine. On the basis of CV, DPCAdSV, SWCAdSV, controlled potential electrolysis (CPE), and spectral studies, the following mechanism may be postulated for the reduction of azathioprine. (Scheme- 2)

Table2: Application of the stripping voltammetric determination of Azathioprine drug in various meduims using SWCAdSV and DPCAdSV Modes

	Bulk	**Form**	**Urine**		**Serum**	
Techniques	**SWCAdSV**	**DPCAdSV**	**SWCAdSV**	**DPCAdSV**	**SWCAdSV**	**DPCAdSV**
Added ($ng\ cm^{-3}$)	1 1.5 2 4 6 8 10	2 4 6 8 10	0.1 0.3 0.5 0.7 0.9 1.5 2	0.1 0.3 0.5 0.7 0.9 1.5 2	0.2 0.3 0.4 0.55 0.75 0.95 1.5	0.3 0.4 0.55 0.75 0.95 1.5
Found ($ng\ cm^{-3}$)	1.01 1.56 2.12 4.01 5.98 7.99 10.1	2.1 4.13 5.98 8.13 10.1	0.101 0.302 0.501 0.699 0.901 1.51 2.12	0.102 0.301 0.5.02 0.701 0.899 1.49 2.01	0.202 0.302 0.401 0.549 0.749 0.956 1.51	0.301 0.401 0.551 0.751 0.945 1.51
N	7	5	7	7	7	6
Average recovery %	101.00 104.00 106.00 100.25 99.66 98.87 100.10	105.00 103.25 99.66 101.62 100.10	101.00 100.60 100.20 99.85 100.11 100.66 106.50	102.00 100.33 100.40 100.14 99.88 99.33 100.50	101.00 100.66 100.25 099.81 099.86 100.60 100.66	100.33 100.25 100.18 100.13 099.47 100.66
Mean S.D RSD %	101.41 2.59 2.56	101.92 2.22 2.18	101.27 2.33 2.30	100.36 0.821 0.818	100.40 0.446 0.444	100.17 0.390 0.390
Bias %	-0.41	-0.92	-0.27	-0.36	-0.40	-0.17

Spectral studies

(I) Isolation

After electrolyses, the catholyte was cooled to 278 – 283K in ice and neutralized with sodium hydroxide solution. This solution was then filtered, checked by T.L.C. during the course of long term electrolysis, unreduced compound which is remained on filter paper and the product passed into the filtrate. The filtrate was treated with diethyl ether to extract the product and the same was washed with ice cold water to remove the base or salt (if any) and allowed to evaporate the ether. The product thus obtained was identified by usual physicochemical methods of analysis.

(II) Identification

Analysis of product had been carried out by usual physicochemical methods. The product was highly viscous and yellow color. Product was also characterized by spectrophotometric techniques as I.R. and N.M.R.

The following observations were made.

1. Solid was soluble in ether, acetone, and chloroform and partially soluble in water, and burnt with sooty flames showed aromatic nature of compound.
2. A single clear spot on silica gel-G plate was obtained in iodine chamber (80%C_6H_6+20%ethyl acetate medium) confirming that the product was a single compound and not a mixture.

3. Usual tests, for nitro group in the product, gave a negative test; however, test for hydroxyl amine gave positive results.
4. IR spectra were recorded in KBr on a SHIMADZU 400-50 infrared spectrophotometer (ν_{max} in cm^{-1}).
5. ^1H NMR spectra were recorded on JEOL AL 300 ^1H NMR spectrophotometer using $CDCl_3$ as solvent and TMS as an internal standard (chemical shift in δ ppm).

IR spectra
IR spectra of showed sharp one spikes peak of (-NH-OH) at 3350 cm^{-1} i.e. main characteristic peak which shows nitro group completely reduced in N-hydroxyl amine group.

NMR spectra
1. ^1H NMR of –NH-OH exhibited a broad singlet at δ 4.9 ppm.
2. A singlet appeared at 7.2 δ ppm due to (N-CH-C-) imidazole ring.
3. A borad singlet observed at δ 7.73 ppm by purine thioprine ring.
4. A singlet appeared at 2.42 δ ppm due to $–CH_3$ protons from imidazole ring.

CONCLUSION

The electrochemical study of azathioprine at the glassy carbon electrode and graphite electrode in Britton-Robinson buffer solution was studied and discussed, based on the adsorption behavior of azathioprine onto the glassy carbon electrode surface. The Cyclic voltammetric behavior gave well defined irreversible cathodic peak at -0.46V, so nitro group of azathioprine is converted into the N-hydroxylamine. A fully validated, simple, sensitive, selective, fast and low-cost differential pulse and square wave adsorptive cathodic stripping voltammetric methods were developed for determination of azathioprine in bulk form, in spiked human urine and serum. Since the proposed procedure allowed the achievement of a detection limit of the drug at the trace level in spiked human urine and serum by means of the described stripping voltammetric methods are low as well as they offer good possibilities for determination of drug in low-dosage bulk pharmaceutical preparations and in real biological fluids. The described methods could be recommended for use in trace analysis, quality control and clinical laboratories.

Acknowledgements
Authors are thankful to the University Grants Commission (U.G.C.), New Delhi, India for financial support for this work.

REFERENCES

[1] B. Elion, G. and G. H. Hitchings, A. G., Johns, D. G., Eds.; *Handbook of Experimental Pharmacology*; Sartorelli, Springer Verlag: Berlin, **1975**; 38, 404-425.
[2] D.C. Pearson, G. R. May, GH. Fick, LR. Sutherland. “Azathioprine and 6-mercaptopurine in Crohn disease. A meta-analysis” *Ann Intern Med*, 123:132-142, **1995**.
[3] D.C. Pearson, GR. May, GH. Fick, LR. Sutherland. “Azathioprine for maintenance of remission in Crohn’s disease” The Cochrane Database of Systematic Reviews, 4, CD000067, **1998**.
[4] W. Sandborn,, L. Sutherland, D. Pearson, G. May, R .Modigliani, C. Prantera. “Azathioprine or 6-mercaptopurine for induction of remission in Crohn’s disease” The Cochrane Database of Systematic Reviews, 3, CD000545, **1998**.
[5] A. Gorski, A. Korszak-Kowalska, A. Nowaczyk, G. Paczek, L. Gaciong. *Immunopharmacology 6*, 259-266, **1983**.
[6] J.D. Cook, W. Lai, B.McGrane. *Immunopharmacology*, *5*, 257-275, **1983**.
[7] A. Shafaati, B.J Clark, *Drug Dev. Ind. Pharm*., 26,267-273, **2000**
[8] H. Duclos, Maillot, M. C.; Galanaud, P. *Immunology*, *46*, 595-60, **1983**.
[9] H. T. Speck, H. S. Rosenkranz, *Mutagenicity of azathioprine Cancer* Res., 36:108-109, **1976**.
[10] G. F. Van Went, *MutÃ¢t.Res*. 68:153-162, **1979**.
[11] R.L. Koch, C. Rose, T. A. Rich and P.Goldman, *Btochem. Pharmacol*. 37: 411-414, **1982**.
[12] W. T. Speck, A. B. Stein, and H. S. Rosenkranz, *J. Nati. Cancer Inst.*, 56:283-284, **1976**.
[13] S. M.Cohen, E. Erturk, A. M Von Esch, A. J. Crovetti, and G. T. J. Bryan, *Nati. Cancer Inst.* 57: 403-417, **1973**.

[14] S. M. Cohen, "Toxicity and carcinogenicity of nitrofurans. in: Carcinogenssis: A Comprehensive Survey," G. T. Bryan (ed.), Vol. 4, pp. 171-231. New York: Raven Press, **1978**.
[15] Lyon, "Some antineoplastic and immune suppressive agents, in: IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemical to Man" *IARC*, 26, 47-78, **1981**
[16] E. Grdman, L.France, B.Bostrom, M. Canafax. *Biomed Chromatogr*, 4, 47-51, **1990**
[17] E.P. Tripodi, S.E. Lucangioli C.L barbaea, U.G.Rodriguez, C.N. Cardcci., *Chromatographia* 54, 93-98, **2001**
[18] P. Chaudhary, M. Swami, D.K. Sharma and R.V. Singh *J. App. Organomet. Chem.*, 23, 140-149, **2009**
[19] M. Kumari and D.K. Sharma *J. Korean Chem. Soc., 55 ,1, 50-56*, **2011**.
[20] S.Malik, M.Kumari, S.Chauhan and D.K.Sharma *Phosphorus, Sulfur and Silicon*, 185, 1759–1771, **2010**
[21] A. B. Mandal, B. U. Nair, *J. Phys. Chem.*, 95, 9008–9013,**1991**
[22] D.K. Sharma, S. Sharma, G. L. Mourya, and M. Kumari *J. Ind. Chem. Soc.*, 86, 225-232, **2009**.
[23] S. Sharma, Divya Wadhwa, Gopal Lal Mourya and Dr. D.K. Sharma *J. Ultra Chem. & phy. Sci.*, 20(1), 121, **2008**.
[24] J.G. Osteryoung, R. A. Osteryoung, *Anal. Chem.* 57: 101A-110A
[25] S. Sharma, M. Kumari and D.K. Sharma *Nature and Science*, 7 (3), 84-98, (**2009**).
[26] W. P. Wilson, S.A Benezra, "In Analytical Profiles of Drug Substances" Florey, K., E.d., Academic Press Inc., New York, 29, **1981**.
[27] R. Kalvoda, M. Kopanica, *Pure & Appl. Chem.*, 61, 1, 97-112, **1989**.
[28] J. Wang, "Analytical Electrochemistry," *VCH Publisher Inc. NY*, **1994**.
[29] R. Kalvoda, *Fresenius Journal of Analytical Chemistry*, 349, 8-9, **1994**.
[30] S. Sharma, Vidyottma and D.K. Sharma *Bull. Electrochem*, 23, 145-148 **2007**
[31] R. Jain, Dwivedi, A.; Mishra, *Langmuir*, 25(17), 10364–10369, **2009**.
[32] R. Jain, A. Dwivedi, R. Mishra, *J. Colloid Interface Sci*., 318, 296-201, **2007**.
[33] J. Wang, "In Stripping Analysis" *VCH Publishers*, Inc, 59, 1 **985**.
[34] J. G. Osteryoung, R. A. Osteryoung, *Anal. Chem.*, 57, 101–105, **1985**.
[35] J.C. Miller, J. N. Miller, "Statistics for Analytical Chemistry" 3rd ed., *Ellis Harwood Series, Prentice Hall: New York*, **1993**.
[36] M.E. Swatz, I. Krull, "Analytical Method Developmentand Validation" *Marcel Dekker, New York*. **1997**.
[37] Gopal L. Mourya, Krishna K. Jhankal, Pradeep Parashar and Digish K. Sharma *Der Pharm. Sinica*, 3(6), 708-714 (**2012**)
[38] C.M. Riley, T.W. Rosanske, Development and Validation of Analytical Methods, Elsevier Science Ltd, New York. **1996**.
[39] G. L. Mourya, K K Jhankal, Lathe A. Jones, Suresh K. Bhargava and D. K. Sharma, *Der Pharm. Lett.*, 4 (5), 1599-1606 (**2012**)
[40] Gunjan Varshney and D.K. Sharma, *Der Pharm. Lett*. 4 (6), 1742-1755 (**2012**).