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Determination of methylprednisolone acetate in biological fluids at gold nanoparticles modified ITO electrode

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Abstract

The electrochemical behavior of a corticosteroid methylprednisolone (MP), used for doping, has been studied at gold nanoparticles modified indium tin oxide (nanoAu/ITO) electrode. The nanoAu/ITO electrode exhibited an effective catalytic response towards its oxidation and lowered its oxidation potential by ~127 mV when compared with bare ITO electrode. Oxidation of MP has been carried out in phosphate containing electrolyte in the pH range 2.13–10.00 and a well-defined oxidation peak was noticed. Linear concentration curves are obtained over the concentration range 0.01–1.0 μM with a detection limit of 2.68×10^{-7} M at nanoAu/ITO electrode. A diffusion coefficient of 2.36×10^{-6} cm^2/s is calculated for MP using chronoamperometry. The proposed method is effectively applied to detect the concentration of MP in pharmaceutical formulations and human blood plasma and urine samples. A comparison of MP concentration determined in blood plasma and urine by the proposed method and GC/MS indicated that the results are essentially similar. It is believed that the method will be useful in determining this drug in case of doping.

Keywords: Indium tin oxide; Gold nanoparticles; Methylprednisolone; Blood plasma; Urine; Voltammetry

1. Introduction

Methylprednisolone (MP) [(6α,11β)-11,17,21-trihydroxy-6-methyl-pregna-1,4-diene-3,20-dione] is a synthetic corticosteroid drug typically used for its anti-inflammatory property [1]. MP(I) is used to achieve prompt suppression of inflammation in different parts of the body [2] and for the treatment of certain forms of arthritis, skin, blood, kidney (glomerulonephritis), eye (graves ophthalmopathy), thyroid and intestinal disorders (e.g., colitis) and asthma [3–10]. Certain types of cancer [11,12] have also been treated with methylprednisolone. MP is one of the commonly used medicines in acute lung injury caused by acid aspiration [13]. Lin et al. reported that MP is also effective in the cases of head injury [14]. Direct infusion of MP into perilymphatic space accelerates hearing recovery and reduces hair cell losses after impulse noise trauma [15]. Being a performance-enhancing drug, World Anti-Doping Agency (WADA) has banned the use of MP in sports when administered orally, rectally, intravenously or intramuscularly [16,17]. Its illicit use has prompted considerable interest during last few years in the development of methods for the testing of this drug in plasma and urine samples of athletes. MP(I) has been determined by relatively few techniques, such as high performance liquid chromatography (HPLC), HPLC with UV detection, spectrophotometry, gas chromatography–mass spectrometry and colorimetry [18–22]. Electrochemical methods have been widely applied for the determination of pharmaceutical formulations since they offer high sensitivity, low detection limit and use of simple instrumentation. However, there appears to be no electrochemical method reported for the determination of MP in literature till date.

Gold nanoparticles exhibit attractive properties in electrode modification by improving the electrode conductivity and enhancing the analytical sensitivity and selectivity [23]. This work aimed to study the voltammetric behavior of MP and its determination in pharmaceutical preparations as well as in

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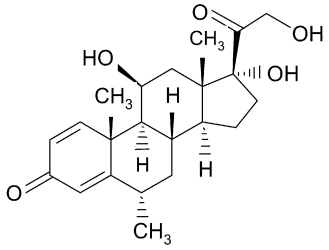
biological fluids, such as human blood plasma and urine employing differential pulse voltammetry (DPV) at gold nanoparticles modified ITO electrode. As electrochemical methods do not require costly instrumentation, it is expected that development of such a method will be highly beneficial due to its rapidity, sensitivity and cost effectiveness.



(I)

2. Experimental

2.1. Reagents

ITO spurted glass sheets of size 10 mm × 20 mm × 2 mm and resistivity 50 Ω cm^{-2} were obtained from CBC Optics Co. Ltd., Japan. Cetyltrimethylammonium bromide (CTAB), $HAuCl_4$ and $NaBH_4$ were purchased from Aldrich (USA). Methylprednisolone (MP) acetate injectable suspension USP (Depo-Medrol^{TM} sterile aqueous suspension) was purchased from Pharmacia India Private Limited, Mumbai, India. A new injection of MP was used each day for the experiments. According to The European Agency for the Evaluation of Medicinal Products, pharmacokinetic studies in humans showed that the esters of methylprednisolone, such as the acetate, sodium succinate, hemisuccinate and the phosphate, were rapidly converted *in vivo* to MP. Thus, the pharmacological activity of these esters is assigned to methylprednisolone.

2.2. Apparatus and procedure

To characterize the growth of gold nanoparticles on the ITO surface, JEOL JSM-7400 F field emission scanning electron microscopy instrument was used. The bare gold electrode (area 2.0 mm^2) was obtained from BAS, USA. The differential pulse voltammetric experiments were carried out at room temperature (27 ± 2 °C) using three electrode single compartment cell equipped with a platinum wire counter, Ag/AgCl (3 M NaCl) reference electrode (Model BAS MF-2052 RB-5B) and gold nanoparticles modified ITO (geometric area ∼0.0314 cm^2) as working electrode. Phosphate buffers in the pH range 2.13–10.00 (ionic strength, $\mu = 0.5$ M) were prepared according to the method reported by Christian and Purdy [24]. BAS (Bioanalytical Systems, West Lafayette, USA) CV-50W voltammetric analyzer controlled via a computer by its own software was used for the measurements. After recording each voltammogram, the surface of the modified electrode was cleaned by applying a potential of −0.3 V versus Ag/AgCl for 30–40 s to remove any adsorbed material. This resurfacing procedure resulted in reproducible peak currents with deviation of ±4%. The optimized differential pulse voltammetry (DPV) parameters used were: sweep rate 20 mV/s, pulse amplitude 5 mV, sample width 20 ms, pulse width 50 ms, pulse period 200 ms, quit time 2 s, sensitivity 10 μA/V, initial potential 0 mV and final potential 1000 mV. The concentration of MP in a clinical ampule was mentioned as 106 mM (40 mg/mL, total volume 2 mL) and was diluted with double distilled water to prepare stock solution of 2 mM.

GC–mass spectral studies (EI: 70 eV) for determination of methylprednisolone were carried out using Perkin-Elmer clarus 500 GC–mass spectrometer. The initial temperature of the column was 60 °C and the temperature was programmed upto 280 °C at a rate of 10 °C/min; this temperature being maintained for 5 min. The temperature of the injector was 250 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min.

2.3. Sample preparation

Urine and blood samples from the patients undergoing treatment with MP were obtained from the Civil Hospital of Roorkee. The samples were collected 2 days after the administration of 1.0 mL of a 40 mg/mL Depo-Medrol single dose. The blood with EDTA as anti-coagulant was ultracentrifuged (1000 rpm for 5 min) and the supernatant blood plasma was used to determine MP concentration using voltammetric technique at gold nanoparticles modified ITO electrode. Urine samples were diluted 10 times and blood plasma samples 25 times with buffer of pH 7.2 prior to use for analysis.

2.4. Fabrication of gold nanoparticles modified ITO electrode

Gold nanoparticles modified ITO electrodes were prepared by the method reported in literature [25]. In brief, nanoAu/ITO fabrication involved two-step process; in the first step, a sheet of ITO was washed by consecutive sonication in water, acetone, ethanol and finally again in distilled water. The dried ITO substrate was then immersed in seed solution (mixture of 0.5 mL of 10 mM $HAuCl_4$, 2 mL of 10 mM trisodiumcitrate and 0.5 mL of 0.1 mM $NaBH_4$ solution and 18 mL of distilled water). Such a treatment caused attachment of small nanogold particles of ca. 4 nm on the surface by physi-sorption. After the seeding procedure, the ITO sheet was thoroughly rinsed with distilled water and dried with nitrogen. The ITO substrate was then put into a growth solution (which is basically a mixture of cetyltrimethylammonium bromide (CTAB), $HAuCl_4$, ascorbic acid and NaOH solution) for further crystal growth of nanoparticles using Murphy's method [26]. Finally, after 24 h ITO substrate was taken out, flushed with distilled water and then dried with nitrogen. To use this nanogold modified ITO sheet as an attachment, the sheet was connected to a thin copper strip and molded between two pieces of scotch tape of size 50 mm × 12 mm. A 2 mm diameter hole was made on one side of tape for providing the contact with solution. The electrode is then ready for use. Thus, the exposed area of ∼3 mm^2 contacted the solution as working electrode and diffusion of species

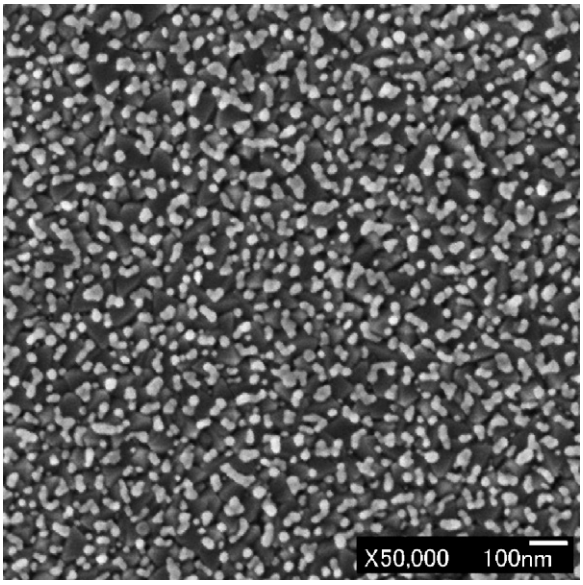


Fig. 1. A typical FE-SEM image of nanogold modified ITO surface at high resolutions.

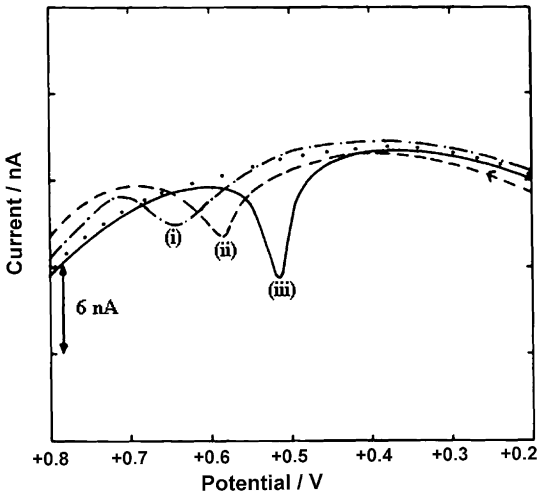


Fig. 2. A comparison of differential pulse voltammograms of 1.0 μM MP at pH 7.2 at (i) bare ITO electrode (–··–), (ii) bare Au electrode (- - -) and (iii) gold nanoparticles modified ITO electrode (—). The curve (iv) is for only 0.5 M phosphate buffer solution (background) at the modified electrode (●●●).

from the solution to the electrode surface will be chiefly linear. The prepared electrode was stored in air with contact side upwards.

To characterize the surface of modified ITO for the deposition of nanogold particles, field emission scanning electron micrographs were recorded at low and high magnification. It was found that spherical nanogold particles (diameter 50–60 nm) were evenly dispersed on the surface of ITO. A typical SEM image of the modified electrode at high resolution is shown in Fig. 1. The beneficial action of nanogold particles can also be observed by attaching them on other metal/non-metal surfaces, however, the advantage of using the ITO surface as substrate is its wide potential window and stable electrochemical and physical properties. The nanogold particles were attached quite strongly on the surface of ITO. This was confirmed by washing the electrode with running water followed by sonication of the electrode for 1 min. The FE-SEM image recorded after the sonication did not change and hence it was deduced that the nanogold particles attach to the ITO surface strongly. Further the modified electrode was also characterized [27] by recording a cyclic voltammogram of 1 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 0.1 M phosphate buffer of pH 7.2 at a sweep rate of 50 mV/s. A well-defined reversible redox couple with peak separation of 90 mV was observed which confirmed that the nanogold modified ITO electrode exhibits required characteristics and is suitable for electrochemical studies. According to the area of the reduction peak of the $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox couple, the deposited gold surface area is estimated to be 6.1×10^{-3} cm^2 for this gold nanoparticle-attached ITO, which is almost 20% of the geometric area of ITO. At many occasions it was noticed that an air bubble is formed at the working electrode on dipping the electrode in the solution which blocks the contact, however, such a bubble was easily removed by passing nitrogen through the solution.

3. Results and discussion

3.1. Voltammetric behavior of methylprednisolone

In order to illustrate the electrocatalytic effect of nanoAu/ITO electrode towards methylprednisolone, the differential pulse voltammograms of MP at three different working electrodes were recorded. Fig. 2 shows the differential pulse voltammograms obtained at the bare ITO, bare gold (Au) and gold nanoparticles modified indium tin oxide electrodes for 1.0 μM MP in 0.5 M phosphate buffer solution of pH 7.2. At bare ITO electrode surface, a broad oxidation peak with low current was observed at 642 mV (curve i). Under identical conditions, the oxidation peak of MP increases slightly at bare gold electrode (curve ii). However, the oxidation peak current of MP at the nanoAu/ITO electrode increases significantly and the peak potential shifts negatively from 642 to 515 mV, in comparison to that at the bare ITO electrode (curve iii). The remarkable enhancement in peak current response and the negative shift of the oxidation peak potential support the fact that the gold nanoparticles act as a very efficient promoter to enhance the kinetics of the electrochemical process. Thus, the nanoAu/ITO electrode shows electrocatalytic activity to the oxidation of MP.

The quantitative determination is based on the dependence of the peak current on concentration of MP. The current values are obtained by subtracting the background current at peak potential and are reported as an average of three replicate determinations. With increasing concentration of MP, the peak current (i_p) was found to increase in the range 0.01–1.0 μM at nanoAu/ITO electrode at pH 7.2. Fig. 3 depicts the differential pulse voltammograms with increasing concentration of methylprednisolone in 0.5 M phosphate buffer solution (pH 7.2). From the data generated during DPV studies, it was observed that the plot between i_p and concentration of MP at modified electrode was linear and passed through the origin. The dependence of peak current (after

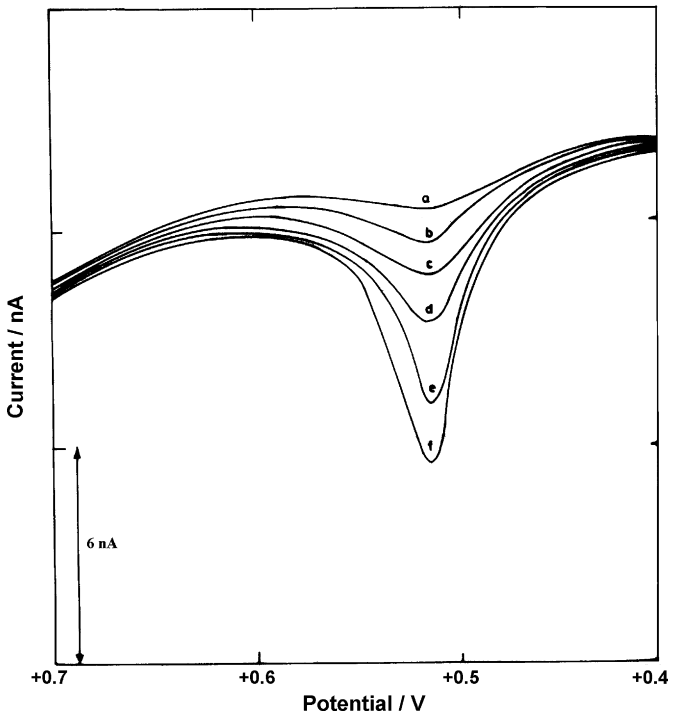


Fig. 3. Observed effect of increasing concentration of MP on differential pulse voltammograms. Curves were recorded at a = 0.05; b = 0.1, c = 0.3, d = 0.5, e = 0.75 and f = 1.0 μM concentration in phosphate buffer solution of pH 7.2.

background current correction in peak current) can be expressed by the equation

$$i_p(\times 10^{-7}\ \mathrm{A}) = 0.0783C$$

where C is the concentration (μM) having correlation coefficient of 0.9977 at nanoAu/ITO with sensitivity of 0.0078 μA μM^{-1}. This behavior indicated that MP can be safely estimated in the concentration range 0.01–1.0 μM at nanoAu/ITO electrode.

3.2. Effect of pH

DPV measurements were performed for MP oxidation at nanoAu/ITO electrode over the pH range 2.13–10.00 at a sweep rate of 20 mV/s and the relationship between pH value and the oxidation peak potential (E_p) was investigated. It was found that the E_p decreases linearly as the pH increases from 2.13 to 10.00 at Au/ITO electrode. The dependence of E_p on pH at Au/ITO electrode using linear regression analysis can be expressed by the relation:

$$E_p[2.13\text{–}10.00] = [1119.1\text{–}65.307\,\mathrm{pH}]\ \mathrm{mV\ versus\ Ag/AgCl}$$

having correlation coefficient ~0.9773. The observed slope of ~65 mV/pH clearly indicates that equal number of electrons and protons are involved in the electrode reaction. Though the pH studies were carried out to examine the effect of pH on peak potential, however, the subsequent detailed studies were carried out at pH 7.2 at which the buffer capacity was sufficient.

3.3. Effect of sweep rate

The effect of sweep rate (ν) on peak potential (E_p) and peak current (i_p) of MP was studied in the sweep range 5–25 mV/s at pH 7.2 at nanoAu/ITO electrode. It was observed that at MP concentration below 1.0 μM, the peak current increased with increase in sweep rate and the plot of i_p versus $\sqrt{\nu}$ was linear, whereas, at MP concentration greater than 1.0 μM, the current response varied linearly with the scan rate. This behavior indicated that at lower MP concentrations the electrode reaction is basically diffusion controlled whereas adsorption complications are associated at higher concentrations. It was also found that the peak potential shifted towards more positive potential as the sweep rate increased from 5 to 25 mV/s. The nature of the plot of E_p versus log ν was linear suggesting the nature of the electrode reaction as EC in which charge transfer is followed by irreversible chemical reactions. The dependence of E_p on log ν at nanoAu/ITO electrode can be expressed by the equation:

$$E_p(\mathrm{mV}) = 119.25\ \log \nu + 359.99$$

having correlation coefficient 0.991.

3.4. Chronoamperometric studies

Chronoamperometry was employed to study the catalytic oxidation of methylprednisolone as the electrode process was basically diffusion controlled at concentrations <1.0 μM. The primary requirement for applying Cottrell equation is that the diffusion should be linear [28] and since the nanogold modified electrode meets this requirement the diffusion coefficient of MP was determined. It is not unusual to observe diffusion controlled electrode process for oxidation of variety of compounds at lower concentrations and adsorption complications at higher concentrations [29,30]. Chronoamperometric measurements of different concentrations of MP at nanoAu/ITO electrode were carried out at pH 7.2. Fig. 4 shows the experimental plots of current (i) versus $t^{-1/2}$ with the best fits for different concentrations of MP used. The slopes of the resulting straight lines were then plotted against the MP concentration, from whose slope and using the Cottrell equation [28], the diffusion coefficient of MP was found to be 2.36×10^{-6} $\mathrm{cm^2/s}$.

3.5. Validation of the method

Validation of the optimized procedure for the quantitative assay of the drug was examined via evaluation of the detection limit, stability, recovery, specificity and precision. In accordance to IUPAC, the detection limit (DL) is described as $3s/b$, where s is the standard deviation of the replicate determination values under the same conditions as for a sample analysis in the absence of an analyte; b is the sensitivity, namely the slope of the calibration plot. The detection limit of MP at Au/ITO electrode is found to be 2.68×10^{-7} M.

The specificity of the optimized procedure for the assay of MP was investigated by observing any interference encountered from excipients usually found in the pharmaceutical tablets and formulations (e.g., starch, sodium chloride, lactose, sucrose and

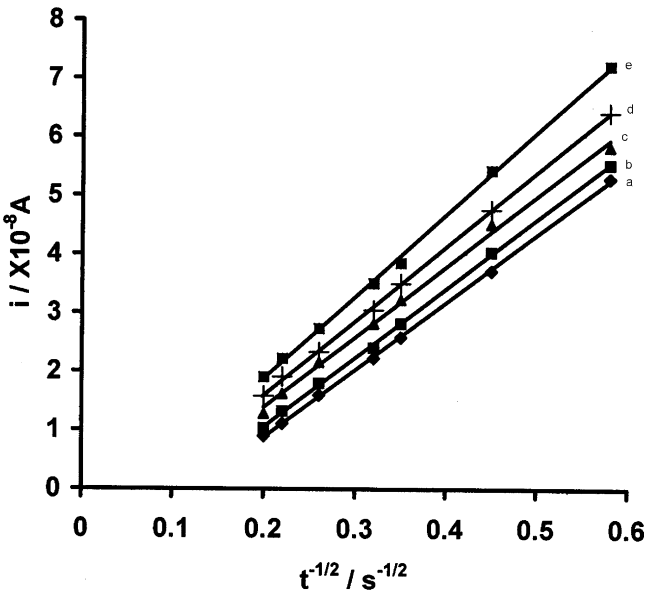


Fig. 4. Plots of current vs. $t^{-1/2}$ obtained for different concentrations of MP (a) 0.05, (b) 0.075, (c) 0.1, (d) 0.25, and (e) 0.50 μM in 0.5 M phosphate buffer of pH 7.2 using modified electrode. Inset shows plot of the slope of straight lines against the MP concentration.

benzyl alcohol). The tablet Medrol 4 had sodium chloride and sucrose in traces (actual amount not mentioned), whereas injection Solu-Medrol had benzyl alcohol (9.16 mg/mL) and traces of sodium chloride to adjust tonocity. Hence, it was considered desirable to elucidate the effect of these common excipients on the peak current of methylprednisolone. The effect of the excipients present in the dosage form was examined by carrying out the determination of 0.1 μM MP in the presence of different excipients in the concentration range 1 mg to 50 mg/mL. The study showed that none of the excipients caused a positive or a negative error greater than 2% indicating that the procedure was able to assay MP in the presence of excipients and hence the method can be considered specific.

The within-day precision of the electrode was evaluated through analysis of 0.1 μM MP solution. The relative standard deviation (R.S.D.) for six replicate determinations was 2.05%, thus indicating the high precision of the method. To evaluate the inter-day precision, the response of the modified electrode was examined for 6 consecutive days in 0.1 μM MP solution. The peak current showed a R.S.D. value of 2.65% indicating thereby that the proposed method has high accuracy. For five nanoAu/ITO electrodes constructed independently, the R.S.D. value is 1.92% for peak currents measurements. On the other hand, the modified electrode retains 92% of its initial response in 0.1 μM MP solution after 14 days of its first use, indicating an excellent stability of this electrode.

According to official validation guidelines, in cases where it is impossible to obtain samples of all drug product components, it may be acceptable to add known quantities of the analyte to the drug product for determining recovery. For this reason, in order to know whether the excipients in the tablets show any interference with the analysis, the recovery test was done by the standard addition method. Thus, the reliability of the voltammetric method was checked by recovery test. Several experiments were carried out by taking known concentrations of MP and their recovery was studied by the developed method. The recoveries were found to lie in the range from 96.0 to 104% (Table 1) and the relative standard deviation was 1.79%. The results reveal that the nanogold modified ITO electrode can be successfully applied to the determination of MP.

Table 1
Recovery test of MP at gold nanoparticles modified ITO electrode

Added (μM)	Found (μM)	Recovery (%)
0.050	0.052	104.0
0.075	0.072	96.0
0.100	0.097	97.0
0.200	0.196	98.0
0.300	0.302	100.7

3.6. Analytical applications

3.6.1. In human body fluids

To establish the usefulness of the developed method for the determination of MP in the urine and blood plasma samples of the patients undergoing treatment with MP, its concentration was determined in the samples after 2 days of administration of single dose of Depo-Medrol intramuscular injection. The gold nanoparticles modified ITO electrode has been utilized to detect MP content in two human blood plasma samples without pretreatment. Prior to their analysis, the samples were diluted 25 times with pH 7.2 phosphate buffer solution. A well-defined peak of MP at nanoAu/ITO electrode was observed at ~520 mV. A typical DPV of the plasma sample (sample no. 2) at nanoAu/ITO electrode is depicted in Fig. 5. Several other peaks at 350, 780, 810 mV are also observed in the DPV. These peaks are assigned to dopamine, ascorbic acid and uric acid on the basis of studies in our laboratory at nanogold/ITO electrode [31] as well as reported in the literature [27]. The peak of ascorbic acid and uric acid appear to merge with each other, however, were well separated with the peak of methylprednisolone. Thus, these compounds do not seem to interfere with the peak current response of methylprednisolone. The results obtained for determination of MP in blood and urine samples are compiled in Table 2.

As methylprednisolone has not been determined using voltammetric method to the best of our knowledge, it was considered worthwhile to cross validate the results of voltammetric determination with GC/MS analysis. For this purpose various concentrations of MP were also analyzed using GC/MS and a well-defined peak was obtained at $R_t \sim 22.75$ min. The peak area under the peak was calculated. The calibration curve was obtained by plotting the peak area ratio of the analyte peaks relative to that of the internal standard (3.7 μg/L) against the analyte concentrations. The resulting curve was linear. The diluted blood serum or urine was then analyzed after filtering with 5 micron Whatman filter paper. A 1.0 μL serum or urine was injected in GC/MS and the area under the peak was determined. Fig. 6 presents GC/MS chromatogram observed for blood plasma col-

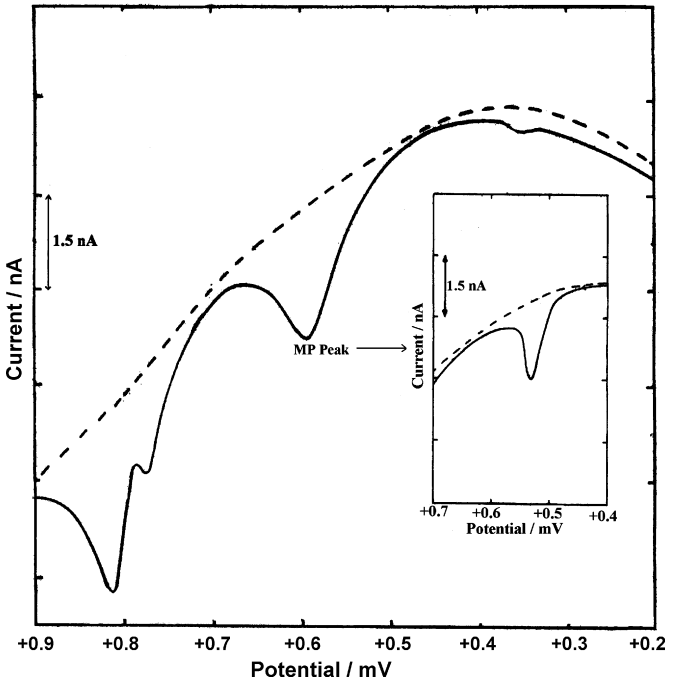


Fig. 5. Observed differential pulse voltammograms of (i) background (- - -) and (ii) human blood plasma sample no. 1 (—) at pH 7.2 at gold nanoparticles modified ITO electrode after 2 days of MP injection.

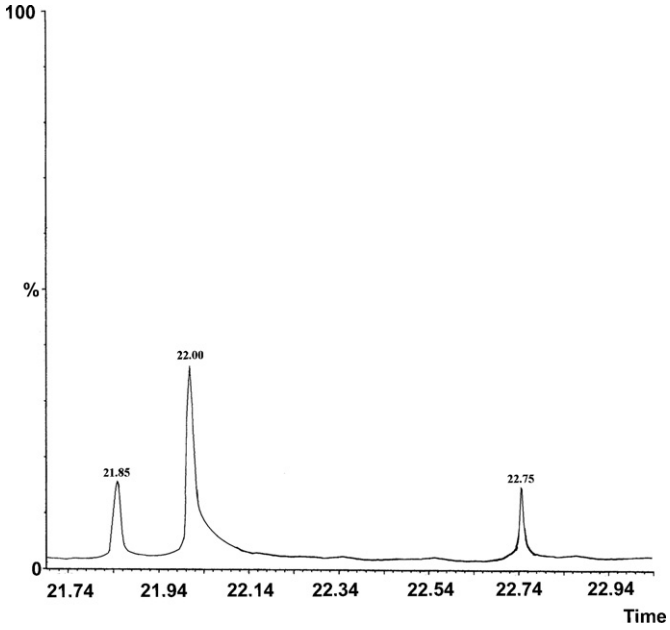


Fig. 6. A typical GC/MS chromatogram observed for blood plasma of patient undergoing treatment with methylprednisolone. The peak at $R_t \sim 22.75$ is due to methylprednisolone.

lected after 12 h of oral medication of methylprednisolone and a well-defined peak at retention time 22.75 min was noticed corresponding to MP. Several other peaks in chromatogram were also noticed at 19.09, 21.85, 22.00 min, however no attempt was made to characterize them. Using calibration curve, the concentration of MP was determined and summarized in Table 2. A comparison of the MP values obtained by GC/MS and proposed method clearly indicated that the results obtained by two methods are in good agreement.

The practical analytical application of the proposed method was further established by estimation of MP in human urine sample without pretreatment. Human urine samples were obtained from the patients and were diluted 10 times. The analysis was then carried out. The results obtained are listed in Table 2 and clearly indicate that MP can be easily determined in urine samples.

Table 2
A comparison of observed concentrations of MP in human blood plasma and urine after 2 days of MP injection at nanoAu/ITO modified electrode and by using GC/MS

Sample	Observed concentration (μM) as determined by			
	Nanogold electrode		GC/MS	
	Blood plasma	Urine	Blood plasma	Urine
1	0.271	–	0.278	–
2	0.192	–	0.198	–
3	0.321	–	0.330	–
4	–	0.335	–	0.341
5	–	0.690	–	0.698
6	–	0.734	–	0.704

4. Conclusions

The World Anti-Doping Agency has banned the use of all glucocorticosteroids in sports owing to their performance-enhancing effects. Hence, the use of methylprednisolone is forbidden in competitions thus making it a necessity in anti-doping control to determine MP in human body fluids. Methylprednisolone has no affinity for transcortin and binds only to albumin. Hence, its elimination half-life is ∼3 h and pharmacokinetics is linear with no dose dependency [32,33]. The proposed methodology provides a very sensitive and selective method of MP analysis using nanoAu/ITO electrode. Gold nanoparticles modified ITO electrode allowed the successful determination of MP with a detection limit of 2.68×10^{-7} M. The results obtained are promising and demonstrate the utility of the developed method for the determination of MP content in biological fluids as well as pharmaceutical preparations. The most probable site for the oxidation of MP seems to be the primary –OH group attached to the carbon at the 20th position. The primary alcoholic group would oxidize to aldehyde in a $2e^-, 2H^+$ process as reported in literature [34]. The air oxidation of several glucocorticosteroids has also been reported to give corresponding aldehydes in presence of cuperic ions [35]. It is expected that the proposed method will be effective for the determination of methylprednisolone in doping control in athletes.

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