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Fullerene–C_{60}–MWCNT composite film based ultrasensitive electrochemical sensing platform for the trace analysis of pyruvic acid in biological fluids

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ABSTRACT

We propose development of a novel electrochemical sensor based on fullerene-multi-walled carbon nanotubes composite film for the sensitive determination of the pyruvic acid in biological fluids. The developed sensor was characterized by cyclic voltammetry. The nanocomposite film of C_{60}–MWCNTs on GCE exhibits electrocatalytic activity towards pyruvic acid reduction and also decreases the reduction over-potential. The influence of the optimization parameters such as pH and effect of loading of composite mixture of C_{60} and MWCNTs on the electrochemical performance of the sensor were evaluated. Various kinetic parameters such as electron transfer number ($n=2$), proton transfer number ($m=2$) and charge transfer coefficient ($\alpha=0.56$) were also calculated. Under optimized conditions, the squarewave reduction peak current was linear over the concentration range of 2.0–55 nM with the detection and quantification limit of 0.1 nM and 0.8 nM respectively. The fabricated sensor was successfully applied to the detection of pyruvic acid in biological samples with good recovery ranging from 97.6% to 103.6%.

1. Introduction

Pyruvic acid is an important organic acid which is widely used in the chemical, drug and agro-chemical industries. It plays a central role in energy metabolism in living organism. Recent evidences suggest that pyruvic acid is the end-product of glucose oxidation and pyruvic acid is finally oxidized to CO_2 and H_2O through citric acid cycle or Krebs cycle. Thiamine acts as a coenzyme of the carboxylase which helps in oxidative decarboxylation of pyruvic acid. In the absence of thiamine, pyruvic acid fails to be broken down and hence, accumulates in blood and tissues. This metabolic disorders result in beri-beri and also heart becomes weak and enlarged which is obviously due to accumulation of pyruvic acid [1].

Literature survey reveals that various analytical methods, such as enzymatic fluorescence capillary analysis [2], capillary electrophoresis with amperometric detection [3], high performance liquid chromatography (HPLC) [4] and voltammetry/polarography [5,6] have been developed for the determination of pyruvic acid. Among them, electrochemical method has great potential for monitoring of pyruvic acid because of its inherent advantages such as fast response, ease of miniaturization, low cost, time saving, high sensitivity, excellent selectivity and in vivo real-time determination [7–17]. As per our knowledge no report is available for the detection of pyruvic acid based on C_{60}–MWCNTs composite film sensor. In this work we propose the fabrication of electrochemical sensor based on C_{60}–MWCNTs composite film and determination of pyruvic acid in biological fluids at very low concentration.

Recent investigations revealed that C_{60}-functionalized MWCNTs films (C_{60}–MWCNTs) were found to be more effective in facilitating the direct electron transfer of hemoglobin (Hb) than MWCNTs films. Moreover the heterogeneous electron transfer rate constant ks of Hb calculated on C_{60}–MWCNTs composite film was almost an order of magnitude larger than that on MWCNTs film [18]. Zhu et al. successfully constructed a C_{60}–MWCNTs composite film for the sensitive detection of dopamine in presence of ascorbic acid [19]. Thus in order to make most of the benefits of MWCNTs and fullerenes, we developed C_{60}–MWCNTs composite films very similar to those of C_{60} homogeneously dissolved in organic solutions but with overlaying redox features of MWCNTs in terms of a monotonic charge injection over the whole potential range [20].

Due to the electrochemical properties of fullerenes, their application as effective electrocatalysts for various chemical and

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biochemical reactions is progressing and the fabrication of electrochemical sensors based on fullerenes is under investigation. Electrochemical reduction of fullerene films is of interest, because it provides a straight forward and accurate way to control the reduction level of the material. Partially reduced fullerene–C_{60}-modified electrodes have been proved as an excellent working electrode having properties such as high electroactive surface area, excellent electronic conductivity and good biocompatibility [21]. Typically C_{60} films are deposited onto a suitable electrode substrate using the drop-dry or "casting" method from a suspension of fullerene in a volatile non-aqueous solvent, such as toluene or dichloromethane (DCM) [22–26].

In the present work, we have developed an electrochemical sensor based on C_{60}/MWCNTs composite film and compared the sensitivity with glassy carbon electrode (GCE) and multi-walled carbon nanotubes modified glassy carbon electrode (MWCNTs/GCE) for the detection of pyruvic acid in a solubilized system and biological fluids. The results showed that the developed sensor is more sensitive for the detection of pyruvic acid compared to bare GCE and MWCNTs/GCE.

2. Experimental

2.1. Chemicals and reagents

Fullerene (C_{60}) 98% pure, multi-walled carbon nanotubes (MWCNTs) of 99% purity, pyruvic standard were obtained from Sigma-Aldrich. A stock solution of 1.0 M pyruvic acid was prepared in distilled water. All chemicals used are of analytical reagent grade quality and were employed without further purification.

2.2. Apparatus

All voltammetric experiments were performed with Ω Metrohm model 797VA Computrace (Ion analyzer, Switzerland) through electrochemical software version 3.1. A three-electrode cell was employed incorporating a working C_{60}–MWCNTs–GCE, Ag/AgCl reference electrode and a platinum wire counter electrode. A Systronics digital μpH meter model-361 was used for pH measurements. All experiments were performed at room temperature.

2.3. Pretreatment of MWCNTs

Prior to modification of the GCE surface, the MWCNTs were pretreated as reported in the literature [27] in order to remove the probable amorphous carbons and metallic catalyst impurities. For this treatment 0.2 g of the MWCNTs was refluxed in a mixture of concentrated H_2SO_4 and HNO_3 for 4–5 h and then washed with doubly distilled water until the pH of the solution became neutral and then dried under an IR lamp.

2.4. Fabrication of fullerne–C_{60}–MWCNTs/GCE sensor

The sensor C_{60}–MWCNTs/GCE was fabricated as reported [27,28]. 1.0 mg pretreated MWCNTs and C_{60} (MWCNTs:C_{60}=2:1) were dispersed in 10 mL toluene in an ultrasonic bath for 30 min to give a suspension. Before the modification, GCE (2 mm in diameter) was polished with polish paper and alumina pastes of 0.5 and 0.1 μm and cleaned thoroughly in an ultrasonic cleaner with 1:1 nitric acid solution, alcohol and water, respectively. Then it was washed ultrasonically in double distilled water for 5 min and finally dried and stored at room temperature. 15 μL of the suspension was directly cast on a glassy carbon (GCE) electrode and the solvent was then dried under IR lamp. This modified glassy carbon electrode was subjected to potential scanning in acetonitrile (ACN) solution containing 0.1 M ammonia buffer as supporting electrolyte between 0.0 and −1.5 V (vs. Ag/AgCl) until reversible multistep electron transfer reaction was obtained. This modified glassy carbon electrode was characterized by cyclic voltammetry and then used for the determination of pyruvic acid solution containing 0.1 M ammonia buffer as supporting electrolyte between 0.0 and −1.5 V (vs. Ag/AgCl). The procedure for the fabrication of the sensor is shown in Scheme 1.

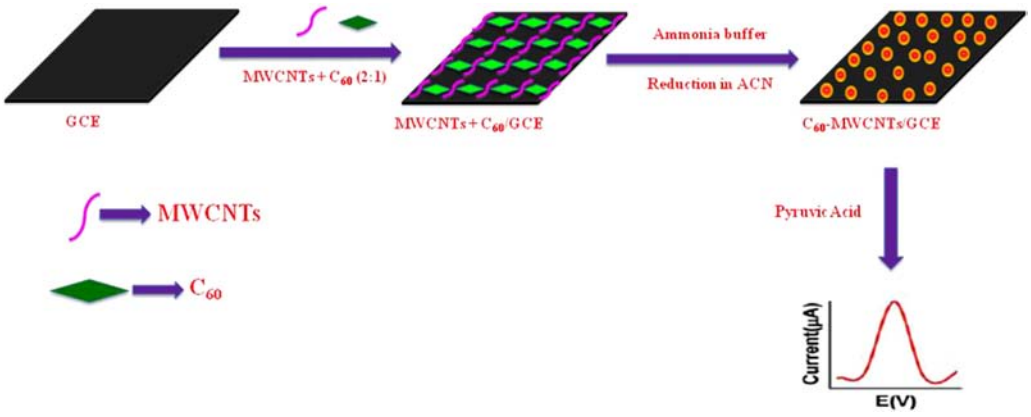
3. Results and discussion

3.1. Characterization of fabricated electrochemical sensor

The fabricated fullurene–C_{60} sensor was characterized and performed by cyclic voltammetry and square wave voltammetry (SWV). The cyclic voltammograms of 6.0 nM pyruvic acid in ammonia buffers (pH 8.2) at C_{60}–MWCNTs/GCE exhibit a single well defined cathodic peak in the chosen potential range. This cathodic peak may be assigned to the reduction of the C═O group. The electrocatalytic effect of the fabricated sensor C_{60}–MWCNTs/GCE for the reduction of pyruvic acid is shown in Fig. 1 which illustrates the voltammograms, CV (Fig. 1A) and SW (Fig. 1B) of pyruvic acid at a bare GCE, MWCNTs/GCE, and C_{60}–MWCNTs/GCE in pH 8.2. On the basis of these observations, it is clear that the fabricated sensor has significant catalytic effect on the pyruvic acid reduction leading to a decrease of the overpotential and an enhancement of the peak current than bare GCE and MWCNTs/GCE.

3.1.1. Electron transfer number (n) and proton transfer number

Electron (n) and proton (m) transfer numbers are the basic parameter of an electrode reaction. Bulk electrolysis with coulometry



Scheme 1. Schematic illustration of the stepwise electrochemical sensor fabrication process.

was used to determine the number of electrons involved in reduction of pyruvic acid at C_{60}–MWCNTs/GCE. The electron transfer number n can be obtained by Faraday equation [29].

$$N = \frac{\Delta Q}{FCV} \quad (1)$$

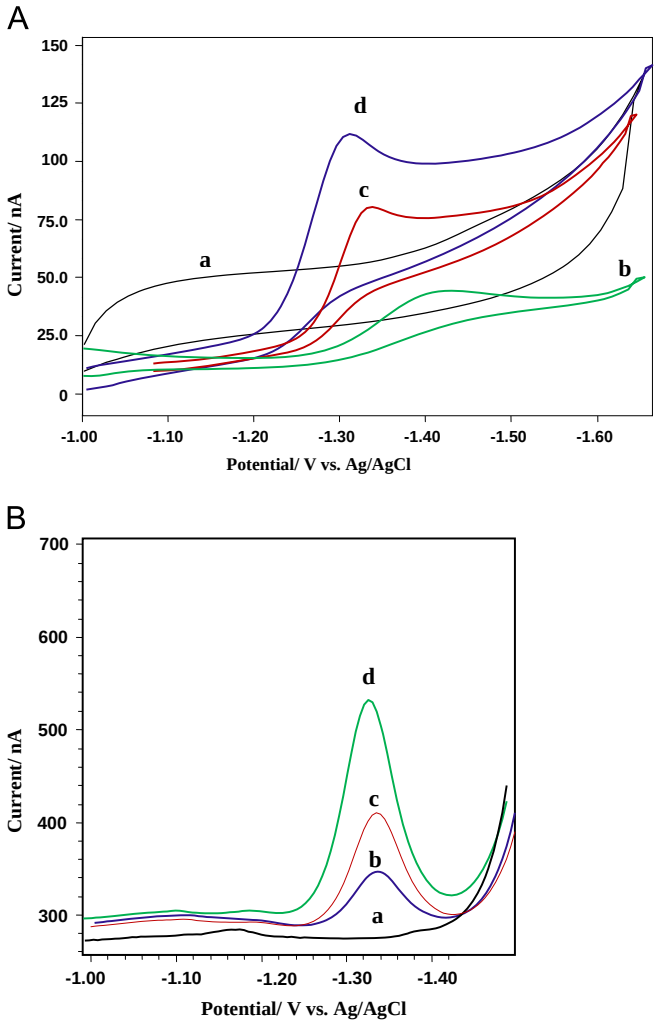


Fig. 1. Comparison of sensitivity of fabricated sensor towards the reduction of pyruvic acid. (A) Cyclic voltammograms, blank (curve a), 6.0 nM at GCE (curve b), at MWCNTs/GCE (curve c), and at C_{60}–MWCNTs/GCE (curve d), scan rate 30 mV s^{-1}. (B) Squarewave voltammograms, blank (curve a), 6.0 nM at GCE (curve b), at MWCNTs/GCE (curve c), and at C_{60}–MWCNTs/GCE (curve d).

where Q is the charge involved in the reaction ($\Delta Q = Q$ pyruvic acid $- Q$ blank), C is the concentration of pyruvic acid, V is the volume of electrolyte, F is the Faraday constant. The completion of the electrolysis is inferred from the current value which drops to the background current zero. From the result of bulk electrolysis with coulometry the electron transfer number n was calculated to be 2.1. From the studies of pH vs. peak potential (E_p), it was confirmed that equal number of electrons and protons are involved in the electrode process, thus the electrode process involves two protons (m) and two electrons (n) for the reduction of C=O bond of pyruvic acid.

3.1.2. Charge transfer coefficient

Pyruvic acid exhibited single well defined cathodic peak in the potential range -1.0 to -1.8 V, at all concentrations. No peak could be observed in the anodic direction of the reverse scans, suggesting the irreversible nature of the electrode process [30]. The peak potential shifted toward more negative values with increasing scan rate, confirming the irreversible nature of the reduction process. For a totally irreversible electrode process, the relationship between the peak potential (E_p) and scan rate (υ) is expressed as [31,32]

$$E_p = (2.303RT\ \alpha nF)\log\ (RTKf/\alpha nF) - (2.303RT\ \alpha nF)\log\ \upsilon \quad (2)$$

A straight line was obtained when E_p was plotted against $\log \upsilon$ at a particular concentration at pH 8.2 and can be expressed as

$$y(E_p) = 0.036\ (\log\ \upsilon) + 1.5596,\ r^2 = 0.99 \quad (3)$$

The effect of scan rate ($\upsilon^{1/2}$) on stripping peak current (i_p) was examined under the above experimental conditions (Fig. 2A). As the sweep rate was increased from 10 to 130 mV s^{-1} at a fixed concentration of pyruvic acid, (i) the peak potential shifted cathodically, (ii) the peak current increased steadily, and (iii) the peak current function, $i_p/AC\upsilon^{1/2}$, exhibited near-constancy.

A straight line was obtained when i_p is plotted against $\upsilon^{1/2}$ (Fig. 2A), which may be expressed by the equation

$$y(i_p) = 14.84\upsilon^{1/2}(\mathrm{mV/s}) - 38.98, r^2 = 0.998 \quad (4)$$

All these facts pointed toward the diffusion-controlled nature of the electrode process. The plot of $\log i_p$ of the peak current vs. $\log \upsilon$ in ammonia buffer of pH 8.2 (Fig. 2B) was straight line with slope 0.842, which is less than the theoretical value of 1.0 that is expected for an ideal reaction of surface species [32].

A straight line was obtained when log ip was plotted against $\log \upsilon$ at a particular concentration at pH 8.2 and can be expressed as

$$Y(\log\ i_p) = 0.842(\log\ \upsilon) + 0.356, r^2 = 0.997 \quad (5)$$

From Eqs. (1) and (2) we can obtain:

$$\frac{RT}{\alpha nF} = 0.036 \quad (6)$$

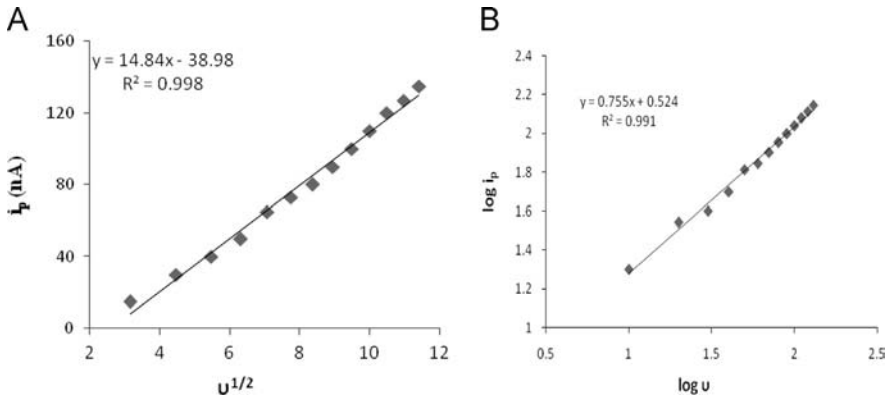


Fig. 2. Plot of (A) the square root of the scan rate vs. the peak current and (B) the logarithm of the scan rate vs. the logarithm of the peak current in 0.1 M ammonia buffer (pH = 8.2 ± 0.1).

Taking $T=298$, $R=8.314$, and $F=96{,}500$, the value of n calculated from Eq. (4) is 1.131. Since from the bulk electrolysis it is confirmed that two electrons are involved in the electrode process, then the value of charge transfer coefficient (α) is calculated to be 0.56 which is close to 0.5 also confirms irreversible nature of the electrode process.

3.2. Optimization of experimental conditions

3.2.1. Effect of pH

The shape and characteristics of all voltammograms were strongly dependent on various electrolyte and pH of the medium. Britton–Robinson (pH=2–10), acetate (pH=3.2–5.7), borate (pH=3–10), phosphate (pH=3–11) and ammonia buffer (pH=7.5–11) were used in the present study and the best results were obtained in ammonia buffer (0.1 M). Peak potential shifted toward more negative potential with increase in pH, indicating involvement of hydrogen ions in the electrode process. Variation of peak potential of pyruvic acid as a function of pH (7.5–11) can be expressed by the following equations:

$$\text{SWV; pH}7.5-11.0 : E_p(\text{V}) = -0.021-1.102\ \text{pH} : r^2 = 0.9912 \qquad (7)$$

The effect of pH on the reduction of pyruvic acid was studied by SWV. Fig. 3A clearly shows that reduction peak current increases gradually as the pH increases and the maximum current was achieved at pH 8.2. With further increase in pH, the reduction current conversely decreased. Therefore, pH 8.2 was optimized for the subsequent analytical experiments and was same pH as was reported for the detection of pyruvic acid.

3.2.2. Effect of varying C_{60}–MWCNTs dosages

As fullerene can facilitate the rate of electron transfer and thus improve the response of the electrochemical reduction of pyruvic acid, the impact of film thickness on reduction peak current was investigated. For this purpose several C_{60}–MWCNTs modified electrodes were prepared by putting different volumes of C_{60}–MWCNTs solution in the range 5–40 μL at GCE surface. Fig. 3B shows that the reduction peak current of pyruvic acid increases as C_{60}–MWCNTs dosage increases and the maximum peak current was achieved up to 20 μL and then the peak current decreases inversely which is probably due to more amount of fullerene which reduces the conductivity of electrode surface. So 20 μL of C_{60}–MWCNTs dosage is the optimized experimental condition for the detection of pyruvic acid.

3.3. Concentration study

The quantitative determination is based on the dependence of the peak current on concentration of pyruvic acid. The current values are obtained by subtracting the background current and are reported as an average of at least five replicate measurements. The peak current increases with increase in pyruvic acid concentration and a linear relationship between peak current and concentration is observed in the range 2.0–55 nM (Fig. 4).

The linear regression equation is

$$\text{SWV} : i_p(\mu A) = -0.029x + 0.010 \quad r^2 = 0.991 \qquad (8)$$

where C is the concentration of pyruvic acid. The correlation coefficient for the expression was 0.991 and the sensitivity of the proposed method was found to be 0.029 μA μM^{-1}. The detection limit of pyruvic acid at pH 8.2 ± 0.1 was calculated by using the formula $3k/b$, where k is the standard deviation of the blank and b is the slope of the calibration curve and was found as 0.1 nM.

3.4. Analysis of pyruvic acid in biological samples

In order to evaluate the applicability of the method to biological samples, pyruvic acid was determined in serum, plasma and urine samples under the same conditions as employed for the pure pyruvic acid by using standard addition method. Table 1 summarizes the results obtained for pyruvic acid in the corresponding biological samples together with SWV analysis. The content of pyruvic acid in serum, plasma and urine is calculated as 0.019, 0.017 and 0.020 mg/ml which are in accordance with that reported in literature [2,3]. The recoveries are in good agreement with the RSD values are less than 1%. Thus, the precision is very satisfactory for the analysis of biological samples. These results indicate that the content of pyruvic

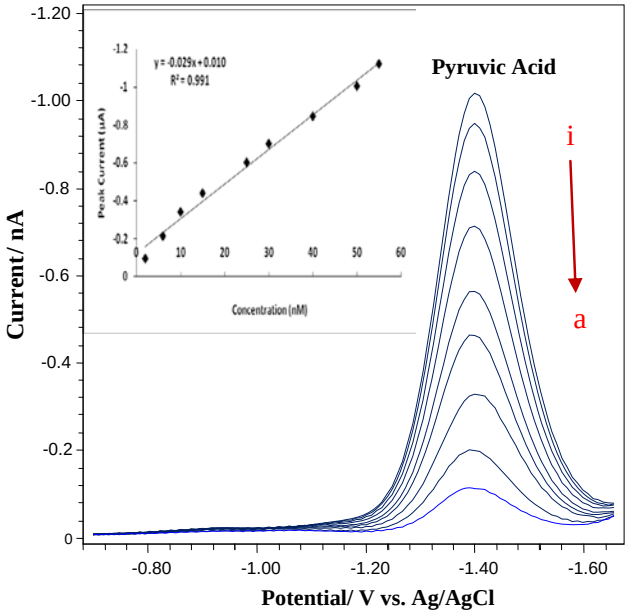


Fig. 4. Square-wave voltammetric peak current of different concentrations of peak current: (a) 2 nM, (b) 6 nM, (c) 10 nM, (d) 15 nM, (e) 25 nM (f) 30 nM (g) 40 nM (h) 50 nM and (i) 55 nM.

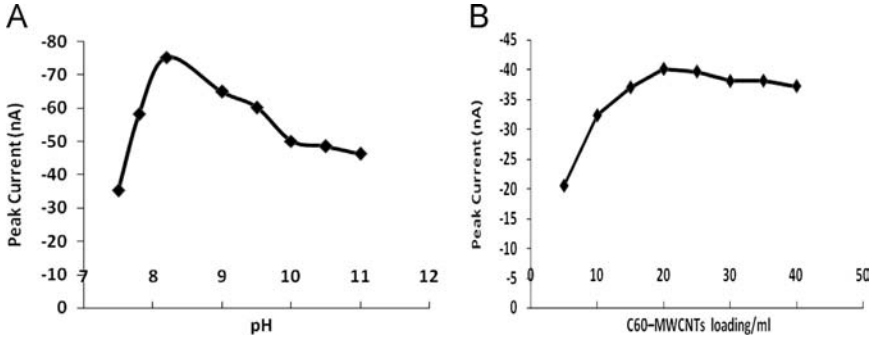


Fig. 3. (A) Influence of pH on the peak current response (using SWV) for 6.0 nM pyruvic acid in ammonia buffer (pH 7.5–11). (B) Effect of varying C_{60}–MWCNTs dosages.

Table 1
Analytical results for pyruvic acid in serum, plasma and urine.

Technique	Medium	[a]Added ng mL^{-1}	[a]Expected ng mL^{-1}	[a]Found ng mL^{-1}	% Recovery	RSD %
SWV	Serum	Nil	–	5.431	–	0.87
		5	10.431	10.381	99.67	0.95
		10	20.431	20.393	99.85	0.67
	Plasma	Nil	–	5.102	–	0.84
		5	10.102	10.942	98.94	0.79
		10	20.102	20.012	99.64	0.93
	Urine	Nil	–	6.420	–	0.94
		5	11.420	11.452	100.2	1.02
		10	21.420	21.414	99.97	0.86

[a] Average of five replicate measurements.

acid in the biological samples can be safely determined by using this method without interference from other substances in the blood samples. The recovery studies of standard additions to biological samples were carried out in order to provide further evidence of validity of the methods. It can be seen from this table that the mean recoveries and RSD values for SWV are in the range of 99.45–101.70%, which is good evidence of validity of method. As can be seen in Table 1, both SWV methods were applied to biological samples after a simple dilution step with direct measurements. Most ideal and suitable chemical conditions and instrumental parameters for the adsorptive stripping voltammetric determination were established, a calibration plot for the analyzed drug was recorded to estimate the analytical characteristics of the developed method.

3.5. Validation of the developed method

The proposed analytical method was validated with respect to parameters such as limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery, robustness and ruggedness.

3.5.1. Linearity

In order to determine the effect of concentration of pyruvic acid on stripping peak current, voltammograms of pyruvic acid are recorded at modified electrode. Under the optimum conditions, a very good linear correlation was obtained between the monitored voltammetric peak current and pyruvic acid concentration in the range 2.0–55 M. Least-square treatment of the calibration graph yielded the following regression equation:

$$\text{SWV}: i_p(\mu A) = -0.029x + 0.010 \quad r^2 = 0.991 \tag{9}$$

where i_p is the peak current, x is the analyzed drug concentration and r^2 is the correlation coefficient.

3.5.2. Detection and quantification limit

Detection limit is calculated by equation LOD = 3 s/m, where s is standard deviation of intercept and m is slope of the regression line. The calculated LOD value of pyruvic acid is 0.1 nM. The quantification limit (LOQ) is examined by the equation LOQ = 10 s/m. The calculated LOQ value is 0.8 nM. Both LOD and LOQ values confirmed the sensitivity of the proposed methods.

3.5.3. Accuracy and precision

The accuracy of the proposed method was checked by calculating the recovery of known amount of pyruvic acid (6.0 nM) added to ammonia buffer solution and analyzed via the optimized stripping voltammetric procedure. The value of the mean recovery obtained by the standard addition method was 100.9% with standard deviation of 1.2% (the analytical measurements repeated five times). The analytical precision of the developed method was verified from the reproducibility of 10 determinations of 6.0 nM pyruvic acid and the estimated relative standard deviation (R.S.D. %) was 1.07%.

3.5.4. Stability, reproducibility and interference studies

The stability of the fabricated sensor was estimated after 10 days period. Between the measurements the electrodes were kept in refrigerator for 10 days and then the sensor was used to detect the same concentration by SWV. The current response of the sensor was 95% after 10 days. The peak current did not show any obvious change in the peak current, demonstrating that the sensor had good stability for the detection of pyruvic acid. The reproducibility of the fabricated sensor was investigated by measuring the response of 6.0 nM pyruvic acid at 4 different electrodes prepared independently. The RSD value of 2.0% reveals that this fabrication method had good reproducibility.

3.5.5. Specificity/selectivity

Specificity is the ability of the method to measure the analyte response in the presence of all of the potential impurities. The selectivity of the optimized procedure for determination of pyruvic acid was examined in the presence of foreign species such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+}, glucose, valine, phenylalanine and lycine. Samples containing 6.0 nM bulk pyruvic acid and different concentrations of the excipient under evaluation were analyzed by means of the proposed method. The obtained mean percentage recoveries and RSD% values based on an average of five replicate measurements, 99.90 ± 0.54 to 100.10 ± 1.20, showed no significant interference from excipients. Thus, the proposed procedure can be considered to be specific.

3.5.6. Robustness

The robustness was examined by evaluating the influence of small variations of some of the most important procedure variables, including preconcentration potential and time and pH. The obtained results provided an indication of the reliability of the proposed procedure for the assay of pyruvic acid; hence, it can be considered as robust. The obtained mean percentage recoveries based on an average of five replicate measurements were not significantly affected within the studied range of variations of some operational parameters, and consequently the proposed procedure can be considered as robust.

3.5.7. Ruggedness

The ruggedness of the measurements is defined as the degree of reproducibility of results obtained by analysis of same sample under variety of normal test conditions such as different laboratories and different lot of reagents, under the same operational conditions at different elapsed time by two different analysts. The methods were found to be rugged with the results of variation coefficients 0.85% and 0.91% for SWAdSV, 1.3% and 0.94% for

Table 2
Comparison of various existing assays with square wave voltammetric analysis of pyruvic acid.

Method	Detection limit (μM)	Reference no.
Enzymic fluorescence capillary analysis	12	[2]
Capillary electrophoresis	0.388	[3]
Voltammetry/polarography	0.55	[6]
Square wave voltammetry	0.0001	Present work

DPAdSV methods for first and second analysts, respectively. The results show no statistical differences between different analysts.

3.6. Comparison of the sensitivity of the proposed method with previously reported methods

Table 2 compares the detection limit of the proposed method with the other reported methods. It is obvious that the sensitivity of the proposed method is superior to all previously reported methods. The data in the table reveal that the detection limit of the method is lower than all previously reported methods.

4. Conclusions

A sensitive electrochemical sensor was developed for the detection of pyruvic acid in biological samples and its analytical performance was systematically and comparatively studied with C_{60}–MWCNTs composite film based electrochemical sensor and bare GCE. The obtained results show that a nanocomposite film of C_{60}–MWCNTs provides significant advantages than GCE. This could be attributed to its larger specific surface area and greater electron transfer rate. A typical sample analysis took less than 5 min for the detection of pyruvic acid. Hence, an excellent approach towards the development of a novel C_{60}–MWCNTs sensor has been presented which would become a beneficial tool for convenient detection of pyruvic acid in biological fluids.

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