Electrochemical Assay of Heparin to Monitor Anticoagulation Action in Cardiovascular Patients

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Abstract Complications in anticoagulation therapy and long term consequences of the post thrombotic syndromes requires a fast and powerful therapy such as heparin therapy (anticoagulation) to minimize the thrombotic effects in patients. Thus, a simple approach via electrochemical method: Differential pulse polarography (DPP) has been developed for heparin analysis as a powerful clinical tool to monitor anticoagulation action in-patient undergoing heparin therapy. The method has been standardized for determination of heparin activity over the existing methods and a very well defined characteristic reduction peak at -1.25 V in 2 M NaOH was observed for heparin. A linear relation was observed with a regression equation as $y = 0.3117x + 0.8069$, for 0.1 to 2.0 units/ml heparin. The developed DPP method was observed with excellent precision, accuracy and recovery in human blood plasma samples and in pharmacological formulations. The limit of detection (LOD) and limit of quantification (LOQ) noticed to be 2.04 and 6.8 units/ml respectively. The DPP results compared with pharmacological screening through average thrombin time (TT) and applied to monitor invitro anticoagulation action of heparin in healthy human subjects. Statistical analysis done to validate developed DPP method for heparin analysis and its probable clinical use to monitor anticoagulation action to treat patients suffering from various cerebrovascular disorders (CVD) by proper dosing of heparin.

Keywords Heparin · DPP · CVD · Anticoagulation action · Citrated blood plasma · Average TT

Introduction

Thrombosis is one of the most common devastating cerebrovascular disorder (CVD), increases worldwide with modern changeable lifestyle, unbalance environment and some clinical shortcomings [1], which affect the arteries supplying blood to heart, brain and causing sudden death [2]. Several clinical trials have shown the benefit of anticoagulation (heparin therapy) in CVD patients. Heparin inhibits reactions that lead to the clotting of blood and the formation of fibrin clots both invitro and invivo [3]. Although heparin is effective anticoagulant, yet contraindications risk of bleeding, severe hypertension etc., [4], demand for proper monitoring of anticoagulation action in CVD patients. Beside, some certain clinical cases [5–9] also demands to regulate anticoagulation action of heparin to overcome the problem of overdose in CVD patients. Modern clinical tests commonly used to monitor heparin anticoagulation are non-specific and non-quantitative tests, which lead to improper heparin therapy. For this, alternate methods for heparin action are gaining considerable interest due to queries that have been raised as to the correlation between therapeutic effects versus proper anticoagulation monitoring of heparin in various CVD patients.

Polarography is electrochemical polarization of polarisable electrode (dropping mercury electrode DME) in connection with reference electrode, when successive increasing dc voltage applied externally to polarographic cell. The basic principle of polarography is the study of voltage–current relationship during electrolysis of the solution in a polarographic cell. In differential pulse

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polarography (DPP), each potential pulse fixed with amplitude (10–100 mV) at DME and superimposed on a slowly changing base potential. The current measured twice during the lifetime of each mercury drop: just before the application of pulse as well as just before the end of pulse. The difference between the current (differential current) measured at the two points for each pulse and plotted against the base potential to obtain a differential pulse polarogram (DPP). The electroactive species are either reduced or oxidised depending upon the polarity of the impressed potential at the characteristic potentials which is used to identify the species [10]. The DME keeps the interface always fresh between its constantly renewed surface and the solution, independent of the processes that were taking place at previous drops and thereby the measurements taken with it are perfectly reproducible. DPP method has been better ability to discriminate against capacitive current because it measures a difference current (helping to subtract any residual, capacitive current that remains prior to each step). From the DP polarogram, the peak potential is characteristic of electroactive material; basis of qualitative analysis, whereas differential current (equal to the peak height) is proportional to the amount of material in the solution; basis of quantitative polarographic analysis. DPP is a very satisfactory and widely used method for determination of many substances with a detection limit of about 10^{-8} M. The relative simplicity of the apparatus and the rapidity of gaining results attracted attention for analysis and remains an appreciated and trusted method in laboratories all over the world.

Looking at the demand, effectiveness and use of heparin therapy for CVD patients, the present paper reports more sensitive and accurate DPP heparin assay over existing methods [11, 12]. The importance of polarography in medicine, biochemistry and pharmacy [13] supports the utility and importance of the developed DPP method. Thus, polarographic determinations of heparin not only provide electrochemical analysis but also provide clinical utility for monitoring anticoagulation action of heparin in CVD patients undergoing heparin therapy.

Materials and Methods

Apparatus and Reagents

An Elico (India) μ p based pulse polarograph model CL-362 with attached deskjet 640C printer was used for DP polarographic measurements. The polarographic cell consisted of a degassing system and three electrodes assembly with dropping mercury electrode (DME) as a working electrode, calomel electrode as reference electrode and coiled platinum wire electrode as auxiliary electrode. DME used had a

capillary characteristics of $m^{1/2}t^{1/6} = 2.316 \text{ mg}^{2/3} \text{ s}^{-1/2}$ at 50 cm effective height of mercury column. The pH measurements made on a Systronic μ pH system 361. REMI RM-12C micro centrifuge used for centrifugation.

All the chemicals used were of A.R. grade. However, heparin sodium (150 units/mg) of CDH grade, New Delhi and two pharmacological samples hep-5 injection (5,000 units/5 ml) and enoxaparin sodium injection 40 mg/0.4 ml of Gland Pharma Limited, Hyderabad were used.

Fresh alkaline heparin solution of 10 units/ml was prepared in 0.2 M sodium hydroxide solution. Similarly fresh test analyte of pharmacological samples: hep-5 and enoxaparin of 10 units/ml were prepared in 0.2 M NaOH.

Healthy human blood samples from different age group (20–60 years) of both males and females subjects were collected to screen invitro monitoring anticoagulation action of heparin. Standard method was adopted to prepare citrated plasma [14].

Polarographic Screening

Heparin Analysis

Fresh alkaline heparin analyte was studied using DPP method in 0.2 M NaOH as supporting electrolyte. For this, experimental sets with 10 ml of 2 M NaOH and varying units (10–700 units) of heparin test analyte were separately prepared. Final volume was made up to 100 ml and the pH of each test solution was adjusted to 11.6 ± 0.1 using NaOH/HCl. The pH adjusted sample was transferred in a polarographic cell and purged with hydrogen gas for 10–15 min to remove the dissolve oxygen and polarograms were recorded using DPP parameters as: scan rate: 12 mV/s, sensitivity: 10 μ A, pulse amplitude: 100 mV, drop time: 1/2 s, scan type: forward and scan range from -0.8 to -1.8 V at control temperature (35°C).

To study the reproducibility, accuracy and precision of proposed method for heparin assay, five replicate DPP observations were performed under above-mentioned identical polarographic conditions on different days. For quantitative determinations, a calibration curve was plotted between the peak current (μ A) versus units/ml of heparin test sample. Beside, the analytical validation of developed heparin assay was examined through important statistical analytical parameters, LOD, LOQ, recovery etc.

Determination of Heparin in Pharmaceutical Formulations:

For high accuracy and precise analysis of samples, the comparative methods of polarography are more accurate than absolute methods [15]. Heparin is heterogeneous with respect to molecular size and is available in two forms as

unfractionated heparin (3,000–30,000 Daltons) and low molecular weight heparin [LMWH] (3,000–10,000 Daltons). The applicability of the developed polarographic heparin assay for quantitative and qualitative analysis was examined by using (1) hep-5 (unfractionated heparin) and (2) enoxaparin (LMWH) as pharmaceutical formulations. The DPP screening of hep-5 and enoxaparin were done similarly as heparin by using fresh alkaline test analyte of respective formulation (10 units/ml) under identical polarographic conditions for heparin assay. The polarograms were recorded using scan range from -0.7 to -1.7 V for hep-5, while -0.6 to -1.6 V in case of enoxaparin. For quantitative determinations, the method of external spiking through standard addition was adopted for each pharmaceutical formulation. The nominal content of drug determined by calibration curve or by regression equation developed for polarographic heparin assay and finally the percentage recovery was calculated.

Monitoring Anticoagulation Action of Heparin via Polarography:

The heparin therapy is individualised as heparin dose varies significantly among patients; the variance may be due to an acquired disorder and the effect of drug [16]. For this, the developed DPP heparin assay has been used for invitro monitoring anticoagulation action through quantifying heparin in human citrate plasma samples. Meanwhile pharmacological screening for invitro anticoagulation action were done through average thrombin time (TT) in seconds [17, 18]. The observed results for DPP and TT were compared and correlated for invitro anticoagulation action of heparin.

Beside, individual anticoagulation effect of heparin (heparin response) for each subject was also studied, which may help in designing the dose of heparin i.e., standard heparin nomograms for the proper management of heparin therapy [19].

For this, 2.0 ml of each normal and externally treated 10 units heparinised citrated plasma of same subject were incubated at 37°C for 6 h. 0.5 ml of incubated normal and heparinised citrated plasma samples were screened through DPP method separately under above developed polarographic conditions for heparin assay and final volume was kept as 25 ml. The average TT (three determinations) in seconds was determined for same incubated normal and heparinised citrated plasma samples. The heparin response for each subject was determined by using diffusion current obtained by DPP polarogram and regression equation developed for heparin assay. The above study has been done on ten healthy human blood samples (5 males and 5 females), who were free from any type of addictions (smoking, drinking, tobacco chewing or taking any type of drug).

The validation of monitoring anticoagulation action has been proved through by comparing analytical data obtained by DPP method and average TT. Beside, a comparative study has been done between human males and females for individualised heparin response.

Results and Discussion

Polarographic Assay of Heparin

The DP polarograms of fresh alkaline heparin repeatedly produced a very well defined characteristic polarographic

Fig. 1 DP polarogram of 5 units/ml heparin in 2 M NaOH

peak with E_p value as -1.25 V versus SCE in 2 M NaOH as supporting electrolyte, $\text{pH} = 11.6 \pm 0.1$ at the set polarographic conditions (Fig. 1). The cathodic DPP peak of heparin is due to reduction of active site at each moiety of acidic glycosaminoglycans of heparin structure responsible for its catalytic activity. Beside heparin analysis the developed heparin assay may also helpful in understanding more reactive sites of heparin in blood chemistry and hemostasis action which may help in modifying heparin structure to increase its potency power through molecular designing.

On recording heparin with varying units, the results obtained from polarograms (five replicate determinations for each slots of heparin units) revealed linear proportionality between peak current (i_d) and units of heparin from 10 to 600 units range and remain constant above 600 units. It was also observed from data in Table 1 that at lower range of heparin (below 200 units), there is a sharp increased in diffusion current as compared to steep increase at higher units (200–600 units), which indicates higher

sensitivity of developed method at lower units of heparin. On plotting a calibration curve between average peak current in μA (average wave height) and units of heparin/ml of polarographic solution (0.1–2.0 units), a linear curve with slope value of 0.3117 and intercept of 0.8069 was calculated at the optimum developed conditions for heparin assay.

Analytical Validation of Heparin Assay

Validation of the optimised DPP procedure for the quantitative assay of heparin was examined via LOD [20] and LOQ [21]. The regression equation observed for DPP heparin assay as $y = 0.3117x + 0.8069$ indicates high sensitivity. The low value of RSD for slope and intercept were 0.68 and 0.26, respectively with correlation coefficient (r) as 0.9809 revealed good precision. The calculated LOD and LOQ values as 2.04 and 6.8, respectively again confirmed the high sensitivity of heparin assay. Thus, the developed DPP heparin assay is more accurate, highly sensitive and low cost technique for qualitative and quantitative analysis for chemical, pathological and clinical purposes.

Heparin in Pharmacological Formulations

The DPP analysis of freshly prepared alkaline hep-5 test analyte showed a sharp peak at -1.25 V (Fig. 2a) and enoxaparin at -1.072 V (Fig. 2b), revealing the presence of heparin in hep-5 sample. The change in E_p value from -1.25 to -1.072 V in case of enoxaparin may be explained on the basis of matrix effect caused due to variation in molecular weight and active saccharide compositions (catalytic sites). Enoxaparin is LMWH, which is nearly one-third of unfractionated heparin size [22] and the mean molecular weight of heparin is 15,000 Daltons whereas LMWH have 4,500–5,000 Daltons.

The results obtained for varying units of hep-5 analyte revealed linear proportionality between peak current (i_d) and units of hep-5 from 0.1 to 2.0 units/ml. The linearity was found with slope value of 0.2072 and intercept as 0.8053 with correlation coefficient (r) as 0.9862. Beside, the standard deviation as 0.0636, standard error as 0.0112 and coefficient of variance (COV) with 0.0171 was observed for hep-5. While the result obtained for plot of diffusion current versus enoxaparin units/ml, showed a crossing effect with a characteristic of 0.5 units/ml (Fig. 3).

The observed results from Table 2 revealed average recovery percent for hep-5 and heparin as 95.69 and 104.6% with standard deviation as 1.01 and 0.92, respectively, validated the accuracy and applicability of developed DPP method through standard addition method. The

Table 1 Observed average results for developed DPP analysis of heparin

S. no.	Heparin analyte (units/ml)	Average diffusion current in μA^a	Optimum polarographic conditions developed for heparin assay
1	0	0.000 Blank	Supporting electrolyte = 2 M NaOH
2	0.1	0.819	$\text{pH} = 11.6 \pm 0.1$
3	0.25	0.854	Peak potential
4	0.5	0.92	$E_p = -1.25 \pm 0.1$ V
5	0.75	1.104	Test analyte = freshly prepared alkaline standard unfractionated heparin solution of 10 units/ml
6	1	1.165	Total polarographic cell volume = 100 ml
7	1.25	1.23	Temperature = 35°C
8	1.5	1.295	Conductivity water to make final volume during analysis
9	1.75	1.328	
10	2	1.384	
11	2.5	1.406	
12	3	1.421	
13	3.5	1.437	
14	4	1.452	
15	4.5	1.467	
16	5	1.473	
17	5.5	1.489	
18	6	1.501	
19	6.5	1.504	
20	7	1.505	

^a Average of five observations

Fig. 2 DP polarogram of 5 units/ml pharmaceutical formulations in 2 M NaOH pH 11.6 $\pm$ 0.1. **a** Hep-5, **b** Enoxaparin

results also confirmed that the developed DPP method is specific and sensitive method applicable only for unfractionated heparin and not for LMWH with best quantification for heparin in various types of unfractionated heparin pharmacological samples.

Monitoring Anticoagulation Action of Heparin through Polarography

Since sulfation of heparin is a major determinant of anticoagulation activity and sulfuric acid is link to hydroxyl group of sugar derivatives and amino group of glucosamine

[23], so DPP method has been used for monitoring anticoagulation action of heparin. The polarographic screening of heparinised citrated plasma samples produced a sharp cathodic peak with E_p value as -1.25 ± 0.02 V while normal human citrated plasma samples (non heparinised) showed a broad small peak at 0.62 V, under identical polarographic conditions developed for heparin assay. The observed DPP peak of heparinised plasma is due to presence of heparin in plasma samples, while slight shift in peak with ± 0.02 V may be due to matrix effect caused by interaction of heparin with plasma proteins [24]. Whereas the DPP peak of normal plasma may be due to various

Fig. 3 Crossing effect of enoxaparin (LMWH)

constituents of plasma. Beside, presence of strong anionic or other drugs in human plasma samples will not interfere in monitoring anticoagulation action of heparin by the developed polarographic assay. The drug may produce a separate peak in a DP polarogram (characteristic to drug) or may shift the peak of heparin on either side from -1.25 V due to interaction with heparin (matrix effect). Thus, monitoring anticoagulation action of heparin in patient can be successfully done by the polarographic heparin assay without interference of other drugs.

The observed diffusion current by DPP method and average TT represent the anticoagulation action of heparin in human plasma samples with individualised heparin response for ten human subjects are shown in Table 3. The average diffusion current i_d (x) was used to determine the heparin units (y) as heparin response for each subject through developed regression equation ($y = 0.3117x + 0.8069$). On comparing results observed by DPP method with pharmacological test (average TT), were found to be with similar anticoagulation action of heparin. The variation in results for heparin units (calculated from regression equation) for each sample indicates individualised action of heparin though each plasma was externally treated with the same dose of heparin (10 units/2 ml of plasma).

It was also observed Table 3 that in case of males both average TT and diffusion current (i_d) increased with age, which indicates the increase of anticoagulation action of heparin with increase of age. Whereas in case of females the results of average TT and diffusion current (i_d) were found to be random with increase of age. Meanwhile in females who had reached to menopause or near to reach needs special attention during anticoagulation therapy, as heparin response (anticoagulation action) for such cases was observed extremely low.

Beside, other used modern heparin assays such as protamine neutralization, chromogenic anti Xa, AT III activity, aPTT etc., may be influenced by other drugs. Since these assays depend upon the blood clotting factors, hypo/hyper conditions of any clotting factor of patient may alter the result and mislead the monitoring action for anticoagulation of heparin in patient. Thus, monitoring anticoagulation action through developed DPP method may be clinically useful and can be successfully use for any CVD patient undergoing heparin therapy of any age group.

Statistical Analysis for Monitoring Anticoagulation Action of Heparin in Human Plasma

The statistical analysis for invitro monitoring anticoagulation action through DPP method, average TT and heparin response in human subjects have been reported in Table 4. On correlating the observed results from table for standard deviation, standard error, RSD, indicates adequate precision and accuracy for invitro monitoring anticoagulation action of heparin in both human male and female subjects. The value of confidence and t -test at 95% confidence limit clearly indicate the superiority of DPP method over average TT for monitoring anticoagulation action. Though the results for heparin response varies i.e., heparin therapy is individualized yet a good precision was observed for measuring anticoagulation action through DPP method and thus heparin dose could be easily designed after DPP investigation of citrated plasma for CVD patients. The

Table 2 Drug recovery studies by DPP method

S. no.	Parameters	DPP results (μ A)	Calculated results (units)
1.	Labeled claim in hep-5 (units)	419.035	5,000 (units)
2.	Amount found in hep-5 (units)	400.985	4784.45 (units)
3.	Percentage recovery		95.69 (%)
4.	Percentage of standard deviation		1.01
5.	Amount of heparin added (units)	0.963	50 (units)
6.	Amount found (units)	0.970	52.3 (units)
7.	Percentage recovery		104.6(%)
8.	Percentage of standard deviation		0.92

Table 3 Invitro pharmacological and polarographic analysis of human heparinised plasma samples

S. no.	Age (years)	Thrombin time (s) ^a			DPP analysis of human citrated plasma samples	
		Normal plasma	Heparinised plasma	Net increase in T.T.	Average diffusion current (i_d) in μ A	Heparin response in units
1.	19	12.48	48.13	35.65	0.864	4.30
2.	21	12.97	49.41	36.44	0.952	4.41
3.	24	13.63	50.86	37.23	1.113	4.62
4.	32	14.71	52.18	37.47	1.149	4.66
5.	48	16.02	57.99	41.77	1.186	4.71

Study on citrated plasma samples of female subjects

1.	20	10.14	44.72	34.58	1.208	4.73
2.	22	12.93	41.65	28.72	0.917	4.37
3.	28	14.17	49.58	35.41	1.365	4.93
4.	34	15.74	47.92	32.18	1.139	4.65
5.	48	17.91	43.88	25.97	0.785	4.21

^a Average of three determinations**Table 4** Statistical analysis for anticoagulation activity of heparin in human subjects

S. no.	Methods	DPP analysis (μ A) ^a		Increased T.T. due to heparin (s) ^a		Subject response (heparin units) ^a	
		Male	Female	Male	Female	Male	Female
1.	SD ^b	0.138	0.232	2.378	3.983	0.176	0.288
2.	SE ^c	0.062	0.104	1.062	1.778	0.079	0.128
3.	RSD ^d	0.131	0.214	0.063	0.127	0.039	0.063
4.	Confidence ^e	0.121	0.203	2.084	3.491	0.154	0.252
5.	<i>t</i> -Test	1.698	7.911	1.769	2.778	2.509	4.258

^a Average of five determinations^b Standard deviation^c Standard error^d Relative standard deviation^e Theoretical value of *t*-test at 95% confidence limit ($n = 5$) is 2.776

repeatability of methods were determined from multiple measurements ($n = 5$) for both human males and females.

Conclusion

As such on the basis of observed analytical data and ongoing discussion it could be concluded that the developed DPP method provide a convenient and efficient method for heparin assay and also be used further for screening catalytic sites of heparin which may help in designing heparin molecule with high potency power. The developed DPP method is low cost, direct, rapid, highly selective, more sensitive and superior than other existing voltammetric and pharmacological methods. Moreover, because of its very low limit of detection and quantitation, the anticoagulation action of pharmacological formulations

could be tested by developed DPP method before using for patients. Thus, the developed DPP heparin assay may prove clinically fruitful to monitor anticoagulation action of heparin for invitro as well as favour invivo experiments for the patients who are undergoing heparin therapy.

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