Prothrombin Time (PT) for Human Plasma on QCM-D Platform: A Better Alternative to 'Gold Standard'

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Abstract

Measurements of hemostasis parameters such as Prothrombin time (PT) are substantial in various clinical cases of extensive surgery, dialysis or innate hemostasis disorders. This is the first study of recognition of PT assay for human plasma on quartz crystal microbalance with dissipation (QCM-D) platform. QCM-D technique has been compared in parallel with 'gold standard' mechanical coagulometer in the terms of anticoagulant monitoring in 20 plasma samples. In this report the shortest sample volume consumption of 2.66 µL of human plasma for PT has been demonstrated on QCM-D platform. This also demonstrates the shortest reagent (thromborel) volume consumption employed ever for PT. 'Gold standard' employs 38 folds higher plasma as well as reagent volume consumption. Additionally, QCM-D technique proved superior to 'gold standard' for monitoring of the whole process of plasma coagulation kinetics. It yielded total coagulation information from frequency and dissipation shifts, which are impossible on 'gold standard'. Furthermore, QCM-D platform offers the calculation of fibrinogen concentration along with PT and total coagulation monitoring in single set of measurements. Fibrinogen concentration can be calculated from calibration curves having R² values of 0.99. This is promising support for Point of Care (POC) settings in the perspective that it covers the extreme range of fibrinogen from 1.0 to 6 g/L. PT based QCM-D platform for plasma application proved better alternative to that of 'gold standard' and it paves the path towards routine laboratory method.

1 Introduction

Monitoring of hemostasis parameters like PT is substantial in many clinical settings, especially in cases of extensive surgery, dialysis or innate hemostasis disorders. Haemostasis monitoring also has primary significance in research perspectives such as in the cases of innate coagulation disorders and hemostatic effect of drugs with inflammatory potency. This is because the haemostasis parameters can drastically change within the lapse of a few minutes due to exposure of patients’ haemostatic system to large disturbances such as anticoagulation or haemo-dilution. Furthermore, undesired interactions with blood to artificial surfaces may lead to coagulation activation, blood platelet aggregation or proinflammatory effects. In these conditions frequent monitoring of the patients’ haemostasis status is often crucial for proper therapeutic directions and decisions. For gaining overview of the plasmatic coagulation system, PT test is clinical standard assay. PT assay can detect the extrinsic part of the plasmatic coagulation cascade such as coagulation factors VII, X, V and II. Accurate monitoring of hemostatic effects is a huge stress for healthcare providers. QCM is gaining popularity for pharmaceuticals and clinical studies by using suitable sensor thin films. It is popular due to its cost-effectiveness and robustness as compared with its counterparts. QCM-D has an outstanding potential for haemostasis in the perspectives of clinical POC settings. It could measure the complete coagulation process, starting from fibrin formation, giving PT information, yielding total coagulation point and kinetics after total coagulation. To date, literature of PT studies on QCM-D platform includes studies on whole blood samples rather than on plasma applications. But QCM-D platform as the better alternative to that of standard mechanical coagulometer’s ('gold standard') for PT...
application to human plasma samples has not been achieved\textsuperscript{16-19}. PT application to human plasma in the perspective of anticoagulant monitoring on QCM-D platform has not been investigated. This investigation is necessary for research and laboratory perspectives because such sorts of studies are usually done in plasma rather than in whole blood. This factor is substantial because of easiness, a possibility of storage of plasma samples for long time in frozen form and possibility of more research studies. Heparin is the most applied anti-coagulant in human surgery. Therefore, anticoagulant ‘heparin’ doses in plasma have been selected for PT studies on QCM-D platform. This is first report for recognition of PT for anticoagulant monitoring in plasma on QCM-D technique and it is compared in parallel with ‘gold standard’. This is first report that demonstrates that PT based QCM-D platform offers better alternative to ‘gold standard’. In this report the shortest sample volume consumption of 2.66 µl of human plasma sample (as well as the shortest reagent (thromborel) volume consumption) for PT on QCM-D platform has been demonstrated. On the other hand ‘gold standard’ employs 38 folds higher plasma as well as reagent volume consumption. QCM-D additionally offers the calculation of fibrinogen concentration along with PT in single set of measurements. Fibrinogen calculation in human plasma via PT on QCM-D platform is advantageous. Firstly, ‘gold standard’ coagulometer cannot yield fibrinogen information and PT in single set of measurements. It needs additional Claus or modified Claus method by employing different reagents for fibrinogen calculation. Secondly, R\textsuperscript{2} values of 0.99 have been achieved on calibration curves from QCM-D platform. PT application on QCM-D platform paves a path towards laboratory and clinical routine. This study is substantial in the perspectives of its robustness due to plasma method and its cost-effectiveness because of shortest sample (as well as reagent) volume consumption. This could be a promising support for POC settings for PT application for plasma on QCM-D platform. Furthermore, this study is a proof of principle for plasma application for PT assay on QCM-D technique and outstanding support for POC settings.

2 Materials and Methods

2.1 Reagents and chemicals

Thromborel S was purchased from Siemens Health care/Dade Behring, Germany. Thromborel S was reconstituted with the 10 mL deionized water and kept at 37 °C for 30 minutes prior to using it. It was stored according to the manufacturer directions. Heparin-Natrium-25000- (Ratiopharm) dilutions were made in 50 mM TRIS buffer (pH 7.4). Coagulation reference was purchased from Technoclone GmbH (Austria). 1-Vinyl-2-pyrrolidone (VP), Di vinyl benzene (DVB), N-methyl pyrrolidone, dimethylformamid (DMF), acetone and a,a’a-azobisisobutyronitrile (AIBN) at highest purity levels were purchased from Sigma-Aldrich. 50 mM TRIS buffer of pH 7.4 Hussain Prothrombin Time (PT) for Human Plasma on QCM-D Platform was prepared from Tris (2-hydroxy ethyl) amine hydrochloride (TRIS) (PAESEL+LORI GMBH & CO) and Sodium Chloride (VWR International BVBA).

2.2 Instruments, equipment and sensor chips

Mechanical coagulometer Merlin MC 1 (Merlin Medical, ABW Medizin und Technik, Lemgo, Germany), Spin-coater (Spin150-v3, Semiconductor Production Systems, Germany), UVACUBE 100 (λmax 350 nm, Hönle UV Technology Germany) and QCM-D (qCell T, 3T Analytik, Germany).

QCM-D (qCell T) uses 10 MHz, AT-cut-quartz QCM transducer. This piezoelectric transducer is coated with two different size gold electrodes, one electrode on each side with 8 mm diameter and 5 mm diameter respectively.

2.3 Polymer synthesis

70 µL DVB, 30 µL VP, 50 µL acetone, 50 µL DMF and 1.0 mg AIBN was mixed thoroughly in a reaction vial by using ultra-sonicator. This homogenous mixture was kept under UV lamp for 23 minutes. Afterwards, this transparent and homogenous polymer was further diluted with 700 µL acetone prior to spin coating onto the front electrode of each QCM.

2.4 QCM thin films

QCM electrodes were washed with N-methyl pyrrolidone followed by further cleaning with acetone. After cleaning of QCMs, 15 µl of diluted polymer was spin coated at 6000 rpm for 100 seconds onto the large electrode of each QCM. The thin film height (a 10 nm) on each QCM was controlled by QCM-D (qCell T). These QCMs (having thin films) were further kept under UV for 2.5 hours for achieving rigid sensor thin films for these measurements. Afterwards, these QCMs were either subjected to PT measurements on QCM-D or kept in the desiccator for storage prior to PT measurements on QCM-D.

2.5 Human plasma samples

For human plasma samples, whole blood samples from healthy human donors were received as the donation from the university hospital, Tuebingen, Germany. These fresh blood samples were gathered in tubes having 1.0 ml of 0.106 mol l-1 citrate. These samples were further centrifuged at 2500 x G for 15 minutes at room temperature to achieve platelets poor plasma (PPP) for present studies. For clarity, word ‘plasma’ or abbreviation PPP have been used throughout the manuscript rather than whole word ‘platelets poor plasma’.

2.6 PT QCM-D measurements

QCMs were carefully inserted into the measurement unit of QCM-D and were calibrated at 37 °C to achieve a stable baseline. Plasma
samples were induced with 0.00, 1.00 and 2.00 IU/mL doses of heparin. These doses of anticoagulant are important for laboratory and clinical studies. 4 µl of plasma sample was incubated at 37 °C in an Eppendorf for one minute in the incubation chamber of QCM-D. After incubation, 8 µl of thromborel (at 37 °C) was mixed into it by using a micropipette and 8 µl from the resultant mixture was injected immediately into the centre of incubated QCM. Here, 8 µl of the injected mixture contains 2.66 µl of plasma sample volume, which could be considered as the smallest plasma sample volume consumption in the history of PT to date. The reagent (thromborel) volume of 5.34 µl is also the shortest reagent volume consumption for PT assay. On the other hand, gold standard employs 38 folds higher plasma as well as reagent volume consumption as compared to those of QCM-D. This is crucial support for POC settings. After PT measurements on QCM-D, QCMs were disposed into wastage rather than reusing them, simply because today's clinic relies on disposals rather than reusable systems to avoid contamination.

2.7 PT mechanical coagulometer 'Gold Standard' measurements

PT measurements for plasma samples were carried out on mechanical coagulometer in parallel to QCM-D's by employing 100 µl of the according to plasma sample and 200 µl of thromborel with same incubation times and temperature.

3 Results and Discussions

QCM-D devices are gaining popularity due to their unique ultra-sensitivity towards mass, viscosity or density of wetting liquids. They produce sensor signals in the form of frequency and dissipation shifts. QCMs are also termed as Thickness Shear Mode Transducers because a standing mechanical shear wave within the quartz is produced on applying alternating voltage to the electrodes of QCM. This half wavelength is equal to the thickness of the quartz.

3.1 Surface morphology of sensor film

SEM image of sensor thin film on QCM demonstrated fine texture and flat surface morphology explained in recent report. Surface morphology is suitable for PT studies for plasma.

3.2 PT-QCM-D exemplary curves

After studies of surface morphology of thin films, the QCMs sensor thin films were subjected to human plasma samples for PT measurements on QCM-D platform according to the experimental section. PT exemplary measurement curves for human plasma coagulation along with negative controls on QCM-D platform have been displayed in figure 1.

An easy differentiation of PT coagulation can be demonstrated based on frequency (Δf (Hz)) and dissipation (damping) (ΔΓ (Hz)) curves comparison with negative control curves.

Hussain Prothrombin Time (PT) for Human Plasma on QCM-D Platform

Bandwidth (Δ Γ Hz) and dissipation (D) are same and they are related according to following equation.

\[ \Delta \Gamma (Hz) = 2D/n \]

\( fn \) is the resonance frequency of QCM at overtone n.

Figure 1: QCM-D-exemplary measurements of PT for plasma (from a healthy human donor). PT coagulation curve and two different negative controls i.e. negative control with plasma and without plasma. PT coagulation points and total coagulation points have been indicted by red star and black indicators in both cases of frequency and dissipation curves respectively.

PT coagulation and without coagulation measurement-curves are different in the terms of shapes and magnitudes of frequency and dissipation shifts. For better visualization, PT coagulation point has been indicated as 'red star' indicator. This is the start of falling (down lift) of frequency after stabilization of frequency signal. On the other side, an opposite behaviour can be depicted in dissipation curve. It is the start of uplift of dissipation after stabilization of the dissipation curve. Total coagulation is the end point of coagulation cascade and it has been demonstrated as 'black star' indicator. This can also be demonstrated in both cases of frequency and the dissipation curves respectively.

PT application for plasma coagulation in comparison to negative control measurements has been successful. In the next step, QCM-D exemplary signals PT of plasma of one healthy donor induced with heparin doses of 0.00, 1.00 and 2.00 IU/mL has been depicted in figure 2. These doses are clinically substantial both in laboratory and clinical perspectives.

UK J Pharm & Biosci, 2015: 3(6); 3
Hussain Prothrombin Time (PT) for Human Plasma on QCM-D Platform

3.3 PT-QCM-D vs PT-gold standard

Application of more real samples of human plasma samples for PT assay on QCM-D platform is substantial due to highly complex nature of human real plasma samples. Figure 3 depicts a plot for comparing tQCM (where "t" is PT on QCM) with tCoag (where "t" is PT on 'gold standard'). This plot contains the data of human plasma samples (n=20) with different doses of heparin. Correlation between the two techniques is promising and within analytical limits of deviations. A promising correlation line is passing through the origin. 0.00 - 1.00 IU/ml heparin doses in plasma samples yielded promising R² values of 0.97, while 2.00 IU/ml heparin yielded R² of 0.95.
produced a little spread but still data lies within analytical deviation limits. On combining the whole discussion presented above, PT assay on QCM-D platform is promising on comparing with 'gold standard' for plasma application in laboratory practice.

Hussain Prothrombin Time (PT) for Human Plasma on QCM-D Platform

dipped in coagulation mixture in a plastic cuvette. The stopping of rotation of this steel ball was measured by a magnetic field sensor and PT point was detected at one threshold point during viscosity change of the coagulation mixture.

Figure 4: The Bland Altman plot of PT measured on QCM-D and coagulometer. Plasma samples having different heparin doses have been indicated with appropriate symbols (n=20 plasma samples for each heparin dose)

3.5 Relative SD

Further statics of the puzzle is the %RSD data of tQCM-D in comparison to that of tCoag. Figure 5 demonstrates the %RSD data for different doses of heparin in plasma on both techniques.

PT on QCM-D platform for plasma samples with heparin doses of 0.00, 1.00 and 2.00 IU/mL yielded %RSD values of 12.45, 15.17 and 20.08 respectively. PT on ‘gold standard’ for plasma samples with heparin doses of 0.00, 1.00 and 2.00 IU/mL yielded %RSD values of 12.13, 15.45 and 20.56 respectively. Overall both techniques yielded %RSD values between 12 and 20 with slight fluctuations on both sides depending on heparin dose in plasma samples. Both techniques yielded the variability pattern in a similar way for same dose of anticoagulant. These variations are due to viscoelastic contribution from the VP-DVB sensor thin film used in QCM-D platform. The material of this thin film is different as compared plastic material of the cuvette used in ‘gold standard’ (no information from the provider). Additionally, both techniques are based on different fundamental working principles. Here, QCM-D viscoelastic measurements have been performed under static conditions. In contrast, ‘gold standard’ measurements are based on torque measurement of mechanical rotation of steel ball which is totally

Figure 5: %RSD data for tQCM-D and tCoag for plasma of healthy donors (n=20) with different doses of heparin

3.6 Fibrinogen concentration calculation

QCM-D offers the advantage of fibrinogen concentration calculation in addition to PT monitoring for human plasma from the measurement curve. For this purpose, standard reference platelets poor plasma (PPP) (without anticoagulant dose i.e. 0.00 IU/ml Heparin) with different concentrations of fibrinogen has been applied for achieving calibration curve. This calibration curve has been demonstrated in figure 6.

Calibration curve has been plotted by taking the frequency and dissipation shifts from PT point till the total coagulation point for reference plasma with different concentrations of fibrinogen. Frequency and dissipation shifts are directly proportional to the fibrinogen concentrations in reference standard plasma. Each case of frequency and dissipation shifts produced a linear curve with promising $R^2$ value of 0.99. This data is surprising in the perspective that it covers the extreme range of fibrinogen from 1.0 to 6 g/L. Fibrinogen calculation in human plasma via PT on QCM-D platform is advantageous. Firstly, ‘gold standard’ coagulometer cannot yield fibrinogen information and PT in single set of measurements. It needs additional Claus or modified Claus method by employing different reagents for fibrinogen calculation. Secondly as discussed above, $R^2$ values are outstanding on calibration curves. Figure 7 compares fibrinogen effect on PT in each case of QCM-D and ‘gold standard’.

UK J Pharm & Biosci, 2015: 3(6); 5
Hussain Prothrombin Time (PT) for Human Plasma on QCM-D Platform

because of mass (of fibrinogen) and viscoelastic effect of plasma to
the cuvette based on hydrophobic behaviour of plastic material.
QCM-D sensor thin film proved slight superior due to its non-
hydrophobic characteristics on comparing to that of ‘gold standard’.

Lastly, frequency and dissipation shifts from PT point till total
coagulation on QCM-D signals for 20 plasma sample probes (without
anticoagulant dose) have been depicted in figure 8. Deviations are
promising for a population of 20 plasma probes. Frequency to
dissipation ratio is 1.0, which can be observed from the plot. This
data of healthy donors agrees with the calibration curves presented
above, which have been calculated by applying standard reference
plasma.

Fibrinogen concentrations from 1.00 to 4.50 g/L yielded PT range of
18-19 seconds in each case of QCM-D and ‘gold standard’. The
higher concentration of 6.00 g/L fibrinogen yielded 37 seconds for PT
on QCM-D, while 41.5 seconds for ‘gold standard’. A slight higher
value of PT at the higher fibrinogen level on ‘gold standard’ is

Figure 6: Calibration curves for fibrinogen concentrations versus
frequency and dissipation shifts from PT point till total coagulation for
PPP (standard coagulation reference) on QCM-D. Each data point is
mean and ± SD of three measurements

Figure 7: Fibrinogen effect on PT for PPP (standard coagulation
reference). Each data point is the mean and ±SD of three measurements

Figure 8: Frequency and dissipation shifts calculated from PT point till
total coagulation on QCM-D measurement signals for plasma (n=20)
samples without anticoagulant dose

QCM-Ds are popular devices because of their mass\textsuperscript{26,27} and
viscoelastic sensitive properties. Mass and viscoelastic assessing of
the haemostasis at non-molecular levels are fundamental
requirements for detection of fibrin polymerization in coagulation
process, fibrinolysis and platelets fibrinogen interactions.
Furthermore, non-hydrophobic character of sensor thin film could
overcome unspecific effect from real samples of human plasma.
QCM-D platform could yield précised and accurate monitoring of
anticoagulant and whole haemostasis kinetic process. In this report
the shortest sample volume consumption of 2.66 µl of human plasma
sample (as well as the shortest reagent (thromborel) volume
consumption) for PT has been demonstrated. On the other hand
‘gold standard’ employs 38 folds higher plasma as well as reagent
volume consumption as compared to that of QCM-D. This could be a
promising support for POC settings for PT application for plasma on
UK J Pharm & Biosci, 2015: 3(6); 6
QCM-D platform. PT application on QCM-D platform paves a path towards laboratory and clinical routine. This study is substantial in the perspectives of its robustness due to plasma method and its cost-effectiveness because of shortest sample (as well as reagent) volume consumption.

4 Conclusions

Human plasma samples (n=20) containing different doses of heparin have been measured for PT assay application on QCM-D platform, and they are compared with ‘gold standard’ in parallel. QCM-D data has promising correlation with ‘gold standard’s’. The Bland-Altman plot demonstrated outstanding correlation of two techniques with in analytical deviation limits. Overall both techniques yielded % RSD values between 12 and 20 with slight fluctuations on both sides depending on heparin dose in plasma samples. Both techniques yielded variability pattern in a similar way for same dose of anticoagulant. Additionally, QCM-D technique proved superior to ‘gold standard’ for monitoring of the whole process of plasma coagulation kinetics. It yielded total coagulation information from frequency and dissipation shifts which are impossible on ‘gold standard’. QCM-D offers the advantage of fibrinogen concentration calculation in addition to PT monitoring for human plasma from the measurement curve. Each case of frequency and dissipation shifts produced a linear curve with promising R² value of 0.99. This data is astonishing in the perspective that it covers an extreme range of fibrinogen from 1.0 to 6 g/L. PT data for plasma studies on QCM-D platform presented above is promising for POC settings in laboratory and clinical applications. PT on QCM-D platform could be the potential candidate for routine laboratory method worldwide.

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6 Competing interests

Author declares no competing interests.

7 Author’s contributions

Present research was conceived, planned, carried out and written in the manuscript format by MH.

8 References


Hussain Prothrombin Time (PT) for Human Plasma on QCM-D Platform


