A Simultaneous Monitoring of Coagulation Time and Fibrinogen via PiCT on QCM-D

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Abstract

‘Prothrombinase induced Clotting Time’ (PiCT) has potential to detect all anticoagulants in clinics. In the present study, PiCT has been used as a tool for anticoagulant detection in human plasma on quartz crystal microbalance with dissipation (QCM-D) technique. QCM-D technique enables monitoring of PiCT point, total coagulation and fibrinogen concentration from frequency and dissipation curves in single set of measurements. This is impossible on mechanical coagulometer (which is considered as ‘gold standard’) technique, and it cannot yield coagulation and fibrinogen concentration from a single set of measurements. It requires additional Clauss method or modified Clauss method to calculate fibrinogen by employing different reagents and experimental setups. Additionally, the present report utilizes the lowest sample volume (and each reagent volume) consumptions of 1.66 µL. The sample/reagent volume consumption of 1.66 µL on QCM-D is 30 times lower in comparison with mechanical coagulometer’s that uses 50 µL for laboratory experiments for PiCT. This element is crucial for application of spot test via QCM-D in laboratory and clinics for Point of Care (POC) settings. Different doses of anticoagulant in 20 human plasma samples on QCM-D technique have been studied and compared in parallel to ‘gold standard’. PiCT on QCM-D technique yielded precise and accurate data. Additionally, both techniques produced % RSD values between 3 and 8.5 with slight fluctuations on both sides for PiCT points. The % RSD data for both techniques has lower variability for danaparoid. Furthermore, QCM-D technique enables monitoring of substantial fibrinogen concentrations (i.e. 1 - 6 g/L) with outstanding R² value of 0.99 on the calibration curve. PiCT-QCM-D technique proved superior at all concentrations of fibrinogen in standard reference plasma for PiCT range (precision) on comparing to that of ‘gold standard’.

1 Introduction

PiCT is a coagulation assay that uses a combination of a specific amount of FXa, phospholipids (a trypsin-like serine protease), and an enzyme which specifically activates FV (FV activator isolated from Russell’s viper venom) in the sample1. PiCT has potential to detect all types of anticoagulants such as unfractionated heparin, low molecular weight heparin, pentasaccharide, direct thrombin inhibitors and direct or indirect FXa inhibitors except vitamin K based e.g. Marcoumar or Warfarin. All anticoagulants used in today’s or tomorrow’s clinics act on the level of FXa (anti-Xa activity) or on the level of thrombin (anti-IIa activity), or on both levels simultaneously. Thus, PiCT is believed to be a universal test for monitoring all anticoagulants5.

After the lapse of 100 years of first coagulation test today’s clinic still required trained professional for this area. Practically, activated partial thromboplastin time (aPTT) is the most used coagulation assay. Its outcomes are extremely variable among different individuals, and it lacks precision and accuracy. Additionally, it is insensitive to drugs e.g. low molecular weight heparin and direct factor Xa inhibitors. The aPTT does not correlate with PiCT3, while PiCT has potential to reduce the drawbacks of aPTT5. To date, literature on PiCT includes the studies on the normal range of PiCT.
2 Materials and Methods

2.1 Chemicals and reagents

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1-Vinyl-2-pyrollidone (VP), N-methyl pyrrolidone, Di vinyl benzene (DVB), acetone, dimethylformamid (DMF), a,a’-azobisobutyronitrile (AIBN) and Danaparoid Sodium were purchased from Sigma-Aldrich. 50 mM TRIS buffer pH 7.4 was prepared from Sodium Chloride (WWR International BVBA) and Tris (2-hydroxy ethyl) amine hydrochloride (TRIS) (PAESSEL+LORI GMBH & CO). Danaparoid dilutions were made in 50 mM TRIS buffer (pH 7.4). PiCT reagents (PiCT activator and start reagent (25 mM CaCl₂)) i.e. pefakit PiCT controls UFH 505-22 were purchased from DSM Nutritional Products Ltd, Switzerland. Coagulation reference was purchased from Technoclone gmbh (Austria).

2.2 Equipment/Instrument

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

QCM-D uses 10 MHz quartz crystal transducer. QCM transducer is a piezoelectric AT-cut-quartz coated with two gold electrodes. Quartz crystal has an electrode on each side, 8 mm diameter and 5 mm diameter respectively.

2.3 Polymer synthesis

Synthesis of polymer along with surface morphology has been explained in detail recently. A mixture of 30 µL VP, 70 µL DVB, 50 µL DMF, 50 µL acetone and 1 mg AIBN was taken in a reaction vial. This mixture solution was kept under UV lamp for 23 minutes. The polymer was further diluted via addition of 700 µL acetone before the step of spin coating.

2.4 Thin films

Quartz crystals were cleaned with N-methyl pyrrolidone and followed by acetone. Afterwards, 12 µL of polymer was spin coated at 6000 rpm for 90 seconds onto quartz crystal electrode. QCM-D (qCell T) was used to control layer height (a 10 nm) of thin film on each quartz crystal as QCM-D works as a network-analyzer to detect layer height. The spin coated quartz crystals were kept under UV for 2.5 hours for complete hardening of thin films. Then, quartz crystals were subjected to PiCT measurements, or kept in the desiccator for storage prior to PiCT measurements on QCM-D.

2.5 Human plasma

Human whole blood samples from healthy volunteers were collected in syringes having 1.0 ml of 0.106 mol l⁻¹ citrate. These were received from the university hospital of Tuebingen, Germany. The
samples were centrifuged at 2500 x G for 15 minutes at room temperature to achieve platelets poor plasma (PPP).

2.6 PICT QCM-D measurements

Quartz crystals were mounted on QCM-D and were calibrated at 37 °C for obtaining stable baseline. PPP samples were induced with 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL doses of danaparoid. 5 µl of PICT activator was incubated at 37 °C for one minute in an Eppendorf and followed by 5 µl of PPP mixing. The mixture was re-incubated at 37 °C for three minutes in the incubation chamber of QCM-D. By using micropipette, 5 µl of PICT starter (CaCl₂) at 37 °C was completely mixed into the incubated mixture and immediately 5 µl from the end mixture was injected into the centre of incubated quartz crystal. 5 µl from the resultant mixture means 1.66 µl of human plasma (i.e. the lowest sample volume used for haemostasis). The lowest sample volume application is important for future automated-QCM-D-bioelectronics implementation in laboratory or clinics. After PICT-QCM-D measurements quartz crystals were disposed into wastage rather than reusing because today’s clinical set up is based on disposals rather than reusable systems to avoid contamination.

2.7 PICT mechanical coagulometer ('gold standard') measurements

PICT measurements were performed on mechanical coagulometer in parallel to QCM-D’s by using 50 µl volumes with same incubation times and mixing ratios.

3 Results and Discussions

Quartz crystal offers ultra-sensitivity towards mass, viscosity or density of wetting liquids via frequency and dissipation shifts.24,25,26

3.1 PICT-QCM-D exemplary curves

The QCMs having thin films were applied to PICT coagulation measurements by using set up demonstrated in experimental section. PICT exemplary measurement curves of coagulation for human real plasma inclusive negative controls are demonstrated in figure 1.

On comparing PICT coagulation measurements with negative controls, an easy differentiation of coagulation can be done in each case of frequency (Δf (Hz)) and dissipation (damping) (ΔΓ (Hz)) signals.

Bandwidth (expressed as ΔΓ Hz) and dissipation (expressed as D) are basically the same and are related to each other according to following equation.

ΔΓ (Hz) = 2D/ln

ln is the quartz crystal resonance frequency at overtone n. PICT coagulation and without coagulation signals vary from each other in shapes and magnitudes of both cases of frequency and dissipation shifts. PICT coagulation point has been represented as “red star”, is essentially the start of falling (down lift) of frequency after stability. An opposite behaviour can be seen in dissipation curve; it is the uplift of dissipation after attaining the stability. Total coagulation has been demonstrated as “black star” indicator, is the end of coagulation. This has been demonstrated in both cases of frequency and dissipation signals respectively.

PICT-QCM-D exemplary curves of plasma from one healthy donor induced with danaparoid doses of 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL have been demonstrated in figure 2.

Kinetic information of total coagulation monitoring on QCM-D signal is crucial for POC settings that made QCM-D technique unique.27 Kinetic information is impossible in standard coagulometer because it picks one threshold point during coagulation. Total coagulation point on frequency and dissipation signals is important for monitoring the mass effects from the mass directly attaching to the sensor thin film. Furthermore, danaparoid doses affect the frequency and dissipation shifts along with kinetics (in the form of PICT and total coagulation points). These effects are contributed from viscoelasticity and hydrophobicity of sensor thin film employed. Increase of the danaparoid dose within the same plasma sample of healthy donor yields longer kinetics of PICT and total coagulation points; and lowers frequency and dissipation shifts up to 15-25%.

3.2 PICT-QCM-D vs PICT-‘gold standard’

Further investigation into more real samples of human plasma is substantial because of extremely complex nature of human real blood/plasma samples.28,29 Figure 3 demonstrates a plot directly comparing tQCM (where “t” is PICT) of plasma samples (n=20 for each case) having different danaparoid doses with tCoag (where “t” is PICT on gold standard). An outstanding correlation line passing through the origin with in analytical limits of deviations can be demonstrated. Each dose of anticoagulant yielded the precise range which can be differentiated from counterpart doses. Lower doses of danaparoid yielded improved R² (i.e. 0.99) on comparing to R² (i.e. 0.97) of higher doses.

The data in all cases of plasma probes is within analytical deviation limits passing the ideal correlation line. This is important for POC settings of QCM-D technique, because of lowest sample volume consumption of 1.66 µL in comparison with ‘gold standard’ that uses 30 times higher sample volume of 50 µL for laboratory experiments for PICT. Plasma induced with 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL doses of danaparoid yielded precise PICT ranges of 30 ±4, 69 ±8, 120 ±12, 170 ±10 and 230 ±10 seconds respectively. Data could be further demonstrated in more real samples of human plasma.
regarded as precise and accurate for an anticoagulant recognition using a clotting assay.

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

3.3 Bland-Altman plot

In the next step, a Bland-Altman plot for t-QCM-D and tCoag is demonstrated in figure 4. For this study, Bland-Altman plot is substantial for comparison and the agreement of two techniques rather than correlation for a visual overview. The Bland-Altman plot for tQCM-D vs tCoag is a linear line within ±2SD agreement at 0.00 and 0.25 IU/mL doses of danaparoid, while it yielded spread within analytical deviation limits for higher doses of danaparoid (0.50 - 1.00 IU/mL). To shrink the silky thread of discussion, PICT-QCM-D vs ‘PICT-gold standard’ is promising in the perspectives of precision and accuracy for challenging anticoagulant sensing in laboratory and clinics.

3.4 Relative SD comparison

A further part of the story is the %RSD data of PICT-QCM-D for danaparoid in comparison to that of PICT-Coag. It has been demonstrated in figure 5. PICT on QCM-D platform for plasma having danaparoid 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL yielded %RSD of 5.98, 6.95, 8.64, 4.05 and 3.19 respectively. PICT on ‘gold standard’ for plasma having danaparoid 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL yielded %RSD of 5.95, 7.11, 8.32, 3.92 and 3.39 respectively. Overall both techniques yielded % RSD values between 3 and 8.5 with minor fluctuations on both sides. The relative standard deviation data for both techniques has demonstrated a lower variability for danaparoid.
3.5 Fibrinogen concentration calculations

QCM-D has the edge of fibrinogen calculation in addition to monitoring of PiCT coagulation point. In this purpose, reference standard platelets poor plasma (PPP) (at 0.00 IU/ml dose of anticoagulant) with different concentrations of fibrinogen has been subjected for achieving calibration curve. This has been demonstrated in figure 6.

Calibration curve has been achieved from the frequency and dissipation shifts, from PiCT point till the total coagulation point for reference standard plasma with different concentrations of fibrinogen. Frequency and dissipation shifts are directly related to the fibrinogen concentration in plasma. Both cases of frequency and dissipation shifts yielded linear curves having outstanding $R^2$ value of 0.99, which is astonishing because it covers an extremely wide range of fibrinogen from 1.0 to 6 g/L. Fibrinogen calculation in human plasma by using new born PiCT with QCM-D is advantageous.

Firstly, it is impossible via gold standard in single set of measurements. Secondly as discussed above, $R^2$ value is astonishing on the calibration curve. Thirdly, the dissipation to the frequency ratio is stable (i.e. 1.0) for reference plasma as well as in case of real samples induced with danaparoid.

Figure 7 compares fibrinogen effect on PiCT in both cases of QCM-D and ‘gold standard’.

Fibrinogen concentrations range 1.00-6.00 g/L yielded PiCT range of 28-35 seconds for QCM-D but a wider range 28-50 seconds for ‘gold standard’. The wider PiCT range at lower and higher fibrinogen levels in ‘gold standard’ is due to mass (of fibrinogen) and viscoelastic effect of plasma to the cuvette based on its hydrophobic behaviour of plastic material (no information from the provider). Quartz crystal-thin film proved superior because of its non-hydrophobic characteristics on comparing to that of ‘gold standard’.

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Figure 3: PiCT - tQCM-D of human plasma plotted against PiCT - (tCoag) obtained from a mechanical coagulometer (gold standard). Plasma samples with different danaparoid doses are given with appropriate symbols. (n=20 plasma samples for each danaparoid dose). Each data point is mean of three measurements with error bars ± SD of three measurements.

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

Figure 5: tQCM-D compared with coagulometer (tCoag); %RSD data for plasma of healthy donors (n=20) induced with different doses of danaparoid respectively.

Delta f or Delta T [From PiCT Point till Total Coagulation]

Figure 6: Calibration curve for fibrinogen versus frequency shifts for PPP (coagulation reference) on QCM-D. Each data point is mean and ± SD of three measurements.
QCMs are remarkable sophisticated devices for viscoelastic and mass sensitive properties. Viscoelastic and mass sensing of the haemostasis at non-molecular scales are the most important features for fibrin polymerization in coagulation process, platelets fibrinogen interactions and fibrinolysis. Further contribution from thin film's properties could help sensing of challenging supramolecular (i.e. anticoagulant), and precise and accurate of haemostasis kinetics monitoring. On considering PICT test in the perspectives of precision and accuracy, it paves a path towards a clinical laboratory routine test. Frequency and dissipation shifts on QCM-D additionally yield information of fibrinogen calculation providing calibration curves, which is impossible on ‘gold standard’. This factor replaces Clauss or modified Clauss methods required on ‘gold standard’. In the present study, QCM-D yielded precise and accurate anticoagulant (danaparoid) calculation, PICT, total coagulation and fibrinogen via frequency or dissipation shifts.

4 Conclusions

Plasma samples (n=20) induced with 0.00, 0.25, 0.50, 0.75 and 1.00 IU/ml doses of danaparoid yielded precise PICT ranges of 30 ±4, 69 ±8, 120 ±12, 170 ±10 and 230 ±10 seconds respectively. The %RSD data for both techniques has lower variability for danaparoid. Data could be regarded as precise and accurate for an anticoagulant recognition using a clotting assay. QCM-D technique yielded precise PICT range of 28-35 seconds for reference standard plasma having fibrinogen concentrations range from 1.00 to 6.00 g/L, but ‘gold standard’ yielded a wide range of 28-50 seconds. Additionally, fibrinogen concentrations in plasma (at 0.00 IU/ml dose of anticoagulant) can be calculated via monitoring frequency or dissipation shift from the measurement curve of QCM-D. PICT measurement from ‘gold standard’ cannot yield fibrinogen concentration information. Data of PICT-QCM-D presented above for haemostasis studies is promising for POC settings in clinical and laboratory applications, and could be a potential candidate for routine laboratory method worldwide. Further advantages of QCM-D include its cost-effectiveness, straightforwardness, simple instrumentation and possibility for miniaturization for a multi-channel measurement of all haemostasis parameters with other clinical or laboratory tests simultaneously.

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6 Competing interests

Author declares no competing interests.

7 Author’s contributions

MH conceived the study, planned, performed experiments and wrote the manuscript.

8 References

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