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(54) Titre : UTILISATION DU VENIN DE L'ACTIVITE FACTEUR Xa DU PLASMA PROVENANT DU VENIN DE VIPERE DE RUSSELL AFIN DE CONTROLER L'ACTIVITE DES INHIBITEURS FACTEUR Xa

(54) Title: USE OF RUSSELL'S VIPER VENOM-INDUCED PLASMA FACTOR Xa ACTIVITY TO MONITOR THE ACTIVITY OF FACTOR Xa INHIBITORS

(57) Abrégé/Abstract:
The invention relates to a method of monitoring the effect of a direct of indirect Factor Xa inhibitors comprising the steps of collecting a plasma sample from a patient, adding a solution of Russell's viper venom to the plasma sample and measuring the clotting time or the residual FXa activity chromogenically.

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(54) Title: MONITORING THE ACTIVITY OF FACTOR XA INHIBITORS

(57) Abstract: The invention relates to a method of monitoring the effect of a direct of indirect Factor Xa inhibitors comprising the steps of collecting a plasma sample from a patient, adding a solution of Russell's viper venom to the plasma sample and measuring the clotting time or the residual FXa activity chromogenically.

USE OF RUSSELL'S VIPER VENOM-INDUCED PLASMA FACTOR Xa ACTIVITY TO MONITOR THE ACTIVITY OF FACTOR Xa INHIBITORS

This invention is directed to measuring Factor Xa activity. Such activity can be measured using blood clotting time or chromogenic substrates. The instant invention concerns assay methods for measuring the activity of FXa inhibitors. More particularly, the present invention concerns the use of Russell's viper venom (hereinafter "RVV-X") to induce the individual's endogenous FXa activity and measure the effect of FXa inhibitors. The methods for measuring FXa activity in plasma are by clotting time and by chromogenic methodology.

The publication (Clinical Chemistry, 26/7, pages 885-890,1980) discloses an assay for factor X in which factor X is activated directly by Russel's viper venom. In Alcohol (Vol. 13, No. 6, pages 539-545, 1996) the effect of acetaldehyde upon factor X and factor Xa is described. In a case report an IgG is described which bounds to the light chain of intact factor X and thus inhibits the activation of factor X (Thrombosis & Haemostasis (72(3), pages 363-371, 1994).

Assays such as APTT and PT have been used before to measure blood clotting time. Such assays, however, are not sensitive to detect dosage differences in administration of direct Factor Xa (hereinafter "FXa") inhibitors. The present invention addresses the problem of monitoring the safety and efficacy of direct FXa inhibitors. The methods of the present invention can be used in all species for monitoring the safety and efficacy of intravenous or orally active FXa inhibitors. It is believed the methods of the present invention could also be used to measure the safety and efficacy of thrombin inhibitors and indirect FXa inhibitors such as anticoagulants for factors upstream of the coagulation cascade and heparin, more particularly low molecular weight heparin. The methods could be used for predicting the prothrombotic state of a patient. In the endeavor to provide improved assay methods for measuring the activity of FXa inhibitors, it has now been found that the same can be achieved by the use of Russell's viper venom (hereinafter "RVV-X") to measure prolongation of clotting time produced by the activity of FXa inhibitors.

The invention, as it is explained in the claims, achieves the object by a method of monitoring the effect of Factor Xa inhibitors comprising the steps of:

a) collecting a plasma sample from a patient, who has received a FXa inhibitor, an anticoagulant, an antithrombotic agent, or any combination thereof,

b) adding a solution of Russell's viper venom to the plasma sample and

c) measuring clotting time or a chromogenic change.

Another object of the invention is a method of monitoring the effect of Factor Xa inhibitors comprising the steps of:

a) collecting a plasma sample from a mammal,

b) providing Factor X deficient plasma sample of the same species to be used to make serial dilutions of the normal plasma sample,

c) adding a solution of Russell's viper venom to the plasma samples defined in a) and b),

d) comparing the clotting time measured for the plasma sample from a mammal with the clotting time measured for the plasma samples diluted with Factor X deficient plasma,

e) constructing a standard curve of % FXa activity (proportional to the normal plasma content) and measuring clotting time prolongation,

f) knowing the clotting time of an individual, who has received FXa inhibitor treatment, and obtaining the % residual FXa activity or % FXa inhibition from the standard curve.

Another object of the invention is a method of monitoring the effect of Factor Xa inhibitors comprising the steps of:

a) collecting a plasma sample from a mammal,

b) dividing said plasma sample into portions, saving one portion as the control normal plasma and adding serial dilutions of Factor Xa inhibitor to other portions,

c) adding a solution of Russell's viper venom to plasma samples defined in b),

d) comparing the clotting time measured for the plasma sample without Factor Xa inhibitor with the clotting time measured for the plasma samples with added Factor Xa inhibitor,

e) constructing a dose-dependent clotting time prolongation curve and determining the concentration of a FXa inhibitor required to prolong the clotting time twice longer than the control plasma clotting time.

A mammal is a human being or an animal such as cattle, sheep, rabbit, mouse or rat. A Factor Xa inhibitor is a compound which is an inhibitor of the blood clotting enzymes, especially factor Xa. The term "patient" means a human or a mammal.

Another object of the invention is a method of monitoring the effect of Factor Xa inhibitors by measuring the residual FXa activity chromogenically, comprising the steps of:

a) collecting a plasma sample from a mammal,

b) dividing said plasma sample into portions, saving one portion as the control normal plasma and adding serial dilutions of Factor Xa inhibitor to other portions,

c) adding a solution of Russell's viper venom to plasma samples defined in b),

d) comparing the FXa activity measured for the plasma sample without Factor Xa inhibitor with the residual FXa activity measured for the plasma samples with added Factor Xa inhibitor,

e) constructing a standard curve of dose-dependent inhibition of RVV-X induced by FXa inhibitor,

f) by using the standard curve the concentration of FXa inhibitor in patient at various time points during the FXa inhibitor treatment could be estimated.

Another object of the invention is to reduce or minimize variations in measurement of the clotting time due to the handling of the plasma samples. This is achieved by adding cephalin to the plasma samples. A preferred cephalin source is from rabbit brain.

A preferred chromogenic compound for determining the FXa activity is Spectrozyme Fxa ®. Other FXa chromogenic substrates can also be used. The method according to the invention is preferably performed in a buffer having a pH from 7 to 8. A preferred buffer is a tris(hydroxymethyl)aminomethan buffer (Tris), containing sodium chloride (NaCl) and polyethylene glycol-8000 (PEG-8000). Preferred concentrations are from 10

mM to 200 mM, from 20 mM to 600 mM, and from 0.02% to 1 % for Tris, NaCl, and PEG-8000, respectively.

Another object of the invention is to provide a kit for using the method according to the invention for a diagnostic assay for FXa inhibitor determination.

Another object of the invention is the use of the method according to the invention for measuring the activity of a Factor Xa inhibitor. A particularly preferred Fxa inhibitor is methyl-3-(4′-N-oxopyridylphenoyl)-3-methyl-2-(m-amidinobenzyl)-propionate.

In the examples it is shown that the RVVT protocol, is useful for measuring RVV-X induced FXa activity in plasma by clotting time. The second protocol, the RVVC protocol, is useful for measuring RVV-X induced FXa activity in plasma by chromogenic methodology. RVVT showed dose-dependent prolongation of clotting time by FXa inhibitors. RVVC showed dose-dependent FXa inhibition by FXa inhibitors. Both methods were successfully used to discriminate varying concentrations of FXa inhibitors in ex vivo plasma samples taken from human patients.

The method according to the invention is described in detail in the examples which follow

Example 1

Russell's viper venom-induced Clotting Time (RVVT) Protocol

Reagents:

FXa buffer:	0.05M Tris, 0.15M NaCl, 0.1% PEG-8000, pH7.5 add 6.06g Tris, 8.77g NaCl, 1.0g PEG-8000 to 800mL H_2O adjust pH to pH 7.5 using concentrated HCl. Fill up to 1L with H_2O
RVV-X:	Enzyme Research Lab. Dilute to 1mg/ml with FXa buffer then 1/400 in the same buffer.
RVV-X working solution (RVV-Ca):	2ml of 1/400 RVV-X + 18 ml of 0.0035 M $CaCl_2$. Concentration of RVV-X at this point is 0.25 µg/ml.

FX-deficient plasma: American Diagnostica Inc.

Procedure: All the reagents should be kept at 4°C (on ice-water mixture), before use.

Standard

1. Make 2-fold serial dilutions of normal pooled plasma or patient's plasma with FX-deficient plasma from 1 ~ 1/256
2. Add 0.2 ml FXa buffer, 0.1 ml plasma dilution to the cuvette then 0.1 ml of RVV-Ca (MLA-800 clotter add this reagent automatically).
3. Measure clotting time.
4. Construct standard curve.

Dose- dependent clotting time prolongation curve from the compound methyl-3-(4´-N-oxopyridylphenoyl)-3-methyl-2-(m-amidinobenzyl)-propionate (hereinafter "RPR") or the trifluoroacetate salt of RPR:

1. Add 0.1 ml FXa buffer, 0.1 ml serial dilution of RPR, 0.1 ml normal pooled or patient's plasma to the cuvette then 0.1 ml of RVV-Ca (MLA-800 clotter add this reagent automatically).
2. Measure clotting time.
3. Construct the dose-dependent clotting time prolongation curve.
4. Calculate concentration for 2xRVVT

Example 2

Russell's viper venom-induced FXa in plasma measured chromogenically (RVVC)

Protocol

RVVC ASSAY

RVV-induced FXa activity in plasma measured chromogenically.

A) Reagents

PEG-Ca++buffer: 0.05M Tris, 0.15M NaCl, 0.01 M $CaCl_2$, 0.1%

	PEG-8000, pH 7.50 Add 6.06g Tris, 8.77g NaCl, 1g PEG-8000, 1.47 g $CaCl_2$. 2 H_2O to 800ml H_2O adjust to pH 7.5 using concentrated HCl, fill up to 1 L with H_2O
RVV-X (Russell's' Viper Venom):	Add 44 µl of PEG-Ca^{++} buffer to 50 µl of RVV-X stock (Enzyme Research Labs, 1.88 mg/ml) to prepare 1mg/ml solution, keep on ice at all times. Dilute 1 mg/ml stock to 1/10 → 1/10→¼ (=1/400). Dilutions are made in PEG-CAE Buffer.
Substrate Spectrozyme FXa®:	Dissolve 50µ mole of Spectozyme FXa, American Diagnostic, in 5 ml sterile water to make a 10 mM stock solution.
Spectrozyme FXa® 1.6 mM working solution:	Add 0.8 ml of 10 mM stock to 4.2 ml of PEG-Ca buffer.

B) Procedure:

1. Add the reagents to the microtiter plates or test tubes in the following order:

	Reagent	Sample	Control 1	Control 2
1.	PEG-Ca^{++} buffer	120 µl	120 µl	145 µl
2.	control plasma	-	5 µl	5 µl
3.	clinical samples (plasma)	5 µl	-	-
4.	Spectrozyme FXa® 1.6 mM	50 µl	50 µl	50 µl
5.	RVV-X (1 mg/ml) 1/400 dilution	25 µl	25 µl	-

2. Follow the reaction kinetically for 5 min.

C) Calculation:

Measure the initial velocity, however, ignore the lag phase. In general measure the initial rate of the linear portion of the time course.

Use initial rates of clinical samples at different time points and then compared to the pre-dosing control to calculate % inhibition.

Various concentrations of the compound RPR spiked in plasma were assayed for PT, APTT and PVVT prolongation. The results in Table 1 suggest that RVVT is the most sensitive indicator among the three clotting time assays for the effect of the compound RPR, as a FXa inhibitor.

RVV-X induced clotting time (RVVT) assay could be conveniently used for monitoring the effect of RPR on patient's endogenous plasma FXa.

Table 1:

Test system	Effect of RPR on fold-change		
	Concentration of RPR (µg/ml)		
	50 µg/ml	166 µg/ml	322 µg/ml
RVVT	1,33	2,66	4,41
PT	1,08	1,16	1,25
APTT	1,08	1,41	1,5

Example 3

Russell's viper venom-induced Clotting Time (RVVT) Protocol (II)

Reagents:

FXa buffer: 0.05M Tris, 0.15M NaCl, 0.1% PEG-8000, pH7.5
add 6.06g Tris, 8.77g NaCl, 1.0g PEG-8000 to 800mL H_2O
adjust to pH 7.5 using ~ 2ml of concentrated HCl. fill up to 1L with H_2O

RVV-X: Enzyme Research Lab.
Dilute to 1mg/ml with FXa buffer then 1/200 in the same buffer.
2ml of 1/200 RVV-X + 18 ml of 0.035 M $CaCl_2$, final concentration of RVV-X at this point is 0.5 µg/ml.

Rabbit brain Cephalin	Centerchem
	Reconstitute with 3.125 ml of 0.035 M $CaCl_2$ in FXa buffer to a final concentration of 1.6 mg/ml.
RVV-X RB Cephalin working solution (RVV-Ca-Cephalin):	Equal volume of 0.5 µg/ml RVV-X mixed with equal volume of 1.6 mg/ml of rabbit brain Cephalin. The final concentrations of RVV-X and Cephalin in the reagent reservoir at this stage are 0.25 µg/ml and 0.8 mg/ml, respectively.
Procedure:	All the reagents should be kept at 4 ^{o} C (on ice-water mixture), before use.
Predose plasma normal RVVT	1. Add 0.2 ml FXa buffer, 0.1 ml predose plasma to the cuvette then 0.1 ml of RVV-Ca-Cephalin. (Final concentrations of RVV-X and Cephalin are 0.0625 µg/ml and 0.2 mg/ml, respectively) 2 Measure clotting time.
Result:	Calculate fold-change of RVVT

Effect of rabbit brain cephalin on RVVT

RVV-induced plasma (Pooled GK plasma and donors DN, RB, TD) clotting time with added dilutions of cephalin (n = 2)

Final concentration of rabbit brain cephalin (mg/ml)	Clotting time (sec)			
	pooled GeorgeKing plasma (# 99X1)	plasma DN	plasma RB	plasma TD
0	28.9	55.1	65.3	86.4
0.0049	28.4	40.9	44.3	52.5
0.0098	28.5	38.1	39.5	47.1
0.0195	27.1	33.4	33.2	39.1
0.039	25.5	29.9	28.9	33.9
0.078	24.7	27.7	26.4	31.6
0.156	25.1	27.4	25.3	29.8
0.312	27.2	30.4	26.1	31.6

Conclusion: RVVT variations due to the sample handling could be reduced by addition of ~0.2 mg/ml of rabbit brain cephalin in the reaction mixture.

WHAT IS CLAIMED IS:

1. A method of measuring the concentration of a Factor Xa (FXa) inhibitor when used in the treatment of a mammal comprising the steps of:

a) collecting a plasma sample from the mammal that has received the FXa inhibitor;

b) dividing said plasma sample into portions, saving one portion as the control plasma and adding serial dilutions of the FXa inhibitor to other portions;

c) adding a solution of Russell's viper venom (RVV-X) to plasma sample portions defined in b);

d) comparing FXa activity measured for the control plasma portion with the residual FXa activity measured for the plasma sample portions with added FXa inhibitor;

e) constructing a standard curve of a dose-dependent inhibition of RVV-X induced FXa activity by the FXa inhibitor; and

f) estimating the concentration of the FXa inhibitor at various points in time during the FXa inhibitor treatment by using the standard curve.

2. A method of measuring the concentration of a Factor Xa (FXa) inhibitor when used in the treatment of a mammal comprising the steps of:

a) collecting a plasma sample from the mammal that has received the FXa inhibitor;

b) dividing said plasma sample into portions, saving one portion as the control plasma and adding serial dilutions of the FXa inhibitor

to other portions;

c) adding a solution of Russell's viper venom to plasma sample portions defined in b);

d) comparing the clotting time measured for the control plasma portion with that of the plasma sample portions with added FXa inhibitor; and

e) constructing a dose-dependent clotting time prolongation curve and determining the concentration of the FXa inhibitor required to prolong the clotting time twice longer than the control plasma clotting time.

3. The method as claimed in claim 1, wherein the residual FXa activity is measured chromogenically.

4. The method as claimed in any one of claims 1 to 3, wherein cephalin is added to the plasma samples at step c).

5. The method as claimed in any one of claims 1 to 4, wherein the plasma samples are incubated in a buffer having a pH from 7 to 8 at step b).

6. The method as claimed in any one of claims 1 to 5, wherein the plasma sample is collected from a patient who has received an anticoagulant or an antithrombotic agent or a combination thereof in addition to the FXa inhibitor.

7. The method as claimed in any one of claims 1 to 6, wherein the method is used in a diagnostic assay kit for monitoring the level of FXa inhibition by an inhibitor in a mammal.

8. The method as claimed in any one of claims 1 to 6 for measuring the concentration of a direct or an indirect FXa inhibitor when used in the treatment of a mammal.

9. The method as claimed in claim 8, wherein the FXa inhibitor is methyl-3-(4'-N-oxopyridylphenoyl)-3-methyl-2-(m-amidinobenzyl)-propionate.