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Aniara Diagnostica LLC • 7768 Service Center Drive • West Chester, OH 45069 • Phone (513) 770-1991
Fax (513) 573-9241 • Email info@aniara com • www.aniara.com

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Dabigatran calibrator and control plasmas for drug measurement in plasma, when required

Amiral J.[1], Stangier J.[2], Peyrafitte M.[1]
[1]HYPHEN BioMed, research, Neuville sur Oise, France,
[2]Boehringer Ingelheim Pharma Gmbh & Co KG, Biberach an der Riss, Germany

Introduction

Dabigatran etexilate, an oral fixed-dose Direct Thrombin Inhibitor (DTI) prodrug, received approval for primary VTE prevention after hip and knee replacement surgery and is in development for other indications, e.g. stroke prevention in Atrial Fibrillation.

Although no routine anticoagulant monitoring is required for Dabigatran etexilate, in certain situations, eg in case of suspected overdose, it may be clinical helpful to assess the anticoagulant activity in patients. The **Hemoclot Thrombin Inhibitors (HTI)** kit (improved thrombin time "like" clotting assay) is reliable and accurate for this purpose (1, 2), and needs to be calibrated with specific dabigatran plasma calibrators at well defined concentrations. Ecarin clotting time and a chromogenic anti-IIa assay can also be used.

Aim

Preparation and validation of freeze dried plasma calibrators and controls (2 lots) supplemented with dabigatran (active moiety of the prodrug) at defined concentrations for dedicated assays, covering the expected plasma concentrations in usual Pradaxa® applications.

Assay principle

Plasma calibrators or diluted tested plasma (**dilution 1:8**) are mixed with a normal human plasma pool (**Reagent 1**). Clotting is then initiated by adding a constant and in excess amount of highly purified human α-thrombin (**Reagent 2**). The clotting time (CT) measured is directly related to the concentration of assayed Direct Thrombin Inhibitor (DTI) in plasma.

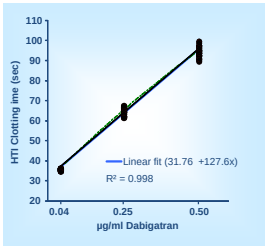
Materials and Methods

Preparation of Plasma Calibrators and controls:
Dabigatran was added to plasma at 3 concentrations (0.05, 0.25 and 0.50 µg/ml) for calibrators, and at 2 concentrations (0.10 and 0.30 µg/ml) for controls, and lyophilized.
Validation studies : These preparations were evaluated for :
- Linearity of calibration curves
- Stability following reconstitution
- Dabigatran concentration established against dabigatran spiked in various plasma pools using HTI assay, or by HPLC-MS/MS method (reference method) kindly run by Boehringer Ingelheim.
- Basic plasma values (without dabigatran) and matrix effect (recovery study).
- Standardization from lot to lot and comparison with HPLC results.

Results

Calibration curve

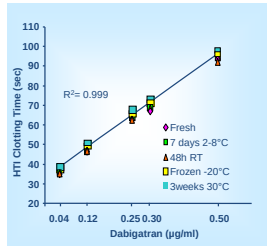
Calibration curve with Dabigatran Plasma Calibrators on STAR (6 independent series in duplicate)



A linear dose response curve is obtained with the HTI assay using the STAR, and similar results can be generated with various laboratory coagulation instruments **(r^2>0.99)**.
LLOQ is <0.05 µg/ml.

Stability

Stability of lyophilised calibrators and controls



The lyophilised system is highly stable after reconstitution (ΔC≤0.02µg/ml) :
- **7 days at 2-8°C**
- **48 hours at RT (18-25°C)**
- **At least 2 months frozen at -20°C or below**

Performances maintained in overheating studies (3 weeks at 30°C), and in real time follow up at 2-8°C (data not shown).

Basic values and recovery (matrix effect):

N=56 normals (untreated patients) were tested for basic clotting time, and **N=20 normals** spiked with two Dabigatran concentrations to evaluate matrix effect:

Normals (untreated, N=56)	
Mean CT	30 sec
SD	0.3 sec
Dab. Conc.	<0.02 µg/ml

Dabigatran added at (N=20):	+0.10 µg/ml	+0.25 µg/ml
Mean (µg/ml)	0.10	0.24
SD (µg/ml)	<0.01	0.01
Min-Max (µg/ml)	0.09-0.11	0.22-0.26
Recovery	100%	96%

Basic clotting times are very homogeneous, and dabigatran well recovered with no matrix effect.

Standardization of Dabigatran concentration and precision:

Dabigatran concentrations of prototype lots of lyophilised calibrators and controls were measured over multiple runs using HTI assay, or HPLC reference method:

Results for Lot 1	HTI assay Conc. (µg/ml) ±SD (N=16)	HPLC-MS/MS Conc. (µg/ml) ± SD (N=12)
(Cal. 0)	0	0
Cal.1	0.11 ± 0.01	0.10 ± 0.01
Cal.2	0.28 ± 0.02	0.24 ± 0.01
Cal.3	0.53 ± 0.03	0.48 ± 0.01
QC Low	0.14 ± 0.01	0.12 ± 0.01
QC High	0.34 ± 0.02	0.29 ± 0.01

When measured using HTI assay against a fresh reference curve, lyophilized reagents were 2 to 5 % lower than frozen plasma before lyophilization. Tested by HPLC, the concentrations were 10 to 15 % lower. This could be induced by the slightly higher volume obtained with the reconstituted lyophilized plasma.

Within run (inter vials, N=20) SD was ≤0.01µg/ml, and inter assay SD (N>10) was in the range 0.01-0.03 µg/ml for determined concentrations using HTI kit, validating excellent intra lot homogeneity, and robustness of the assay.

Both lots were evaluated and concentrations assigned by HPLC as the reference method, and homogeneous results were obtained with the HTI assay.

Lot 1 was then used as the reference material, using the concentrations measured by HPLC, and used for measuring lot 2.

Inter lots homogeneity and consistency with HPLC results:

When tested with HTI assay calibrated with lot 1 (STAR, considering HPLC reference values), concentrations measured for lot 2 fully matched with those obtained by HPLC:

	Dabigatran	Expected Conc. µg/ml	Measured Conc. µg/ml (HTI assay)	Measured Conc. µg/ml (HPLC)
Lot 2	Cal 1	0.05	**0.04**	**0.04**
	Cal 2	0.25	**0.26**	**0.25**
	Cal 3	0.50	**0.48**	**0.50**
	Ctrl Low	0.10	**0.13**	**0.12**
	Ctrl High	0.30	**0.31**	**0.30**
Lot 1	Ctrl Low	0.10	**0.12**	**0.12**
	Ctrl High	0.30	**0.29**	**0.29**

Measured values are consistent with HPLC results, and homogeneous from lot to lot. Similar results were obtained with a chromogenic assay (anti-IIa 2 stages, Biophen® DTI) (data not shown).

References

1. Measurement of the Pharmacodynamic Effect of Dabigatran: Thrombin Clotting Time, J. Stangier, K. Wetzel, W. Wienen, K. Rathgen, J. van Ryn, Boehringer Ingelheim Pharma GmbH & Co KG, Biberach, Germany, P615 -Boston ISTH 2009.
2. van RynJ., Stangier J., Haertter S.; Liesenfeld K.H., Wienen W., Feuring M, Clemens A., Dabigatran etexilate – a novel, reversible, oral direct thrombin inhibitor: interpretation of coagulation assays and reversal of anticoagulant activity. Thromb Haemost 2010; 103: 1116-1127

Conclusions

- **Lyophilized dabigatran plasmas:**
  - **can be used to calibrate and control the quality of both clotting and chromogenic assays.**
  - **can serve as primary standards based on the values assigned by HPLC-MS/MS.**
- **When required, the plasma of patients treated with Pradaxa® can be assessed with the described rapid, fully automatable and simple standardized system.**

ANIARA
7768 Service Center Drive • West Chester OH 45069
Phone: 513.770.1991 **Toll Free:** 866.783.3797 **Fax:** 513.573.9241 **Email:** info@aniara.com
www.aniara.com

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[1]HYPHEN BioMed, research, Neuville sur Oise, France, [2]Boehringer Ingelheim Pharma Gmbh & Co KG, Biberach an der Riss, Germany

Aim: Although no regular anticoagulant monitoring is required for Pradaxa (R), in certain situations in may be clinical helpful to assess the anticoagulant activity in patient. The Hemoclot Thrombin Inhibitors (HTI) kit (improved thrombin time "like" clotting assay) can be used and needs to be calibrated with specific dabigatran plasma calibrators at well defined concentrations. Ecarin clotting time and a chromogenic anti-IIa assay can also be used.

Method: Preparation and validation of freeze dried plasma calibrators and controls (2 lots) supplemented with dabigatran at defined concentrations for dedicated assays. Dabigatran was added to plasma at 3 concentrations (0.05, 0.25 and 0.50 µg/ml) for calibrators, and at 2 (0.10 and 0.30 µg/ml) for controls, and 1 ml aliquots filled and lyophilized in silikon vials. These preparations were tested for stability, over time or following reconstitution, and the exact dabigatran concentration established (against dabigatran spiked in various plasma pools, or by HPLC). Basic plasma values were measured without dabigatran.

Results: Dabigatran plasma calibrators (fresh or lyophilized) yielded a linear dose response curve with HTI assay (r^2>0.990, LLOQ of 0.02 µg/ml). Normals (N=31) were all below LLOQ. When measured against a fresh reference curve, lyophilized reagents were 2 to 5 % lower than frozen plasma before lyophilization (slightly higher volume after reconstitution). Tested by HPLC (N=20) the concentrations were 10 to 15 % lower. Both lots were evaluated by HPLC as the reference method. When tested with HTI assay and calibrated with lot 1 (HPLC values), concentrations measured for lot 2 matched with those obtained by HPLC. Similar results were obtained with the chromogenic assay (anti-IIa, 2 stages).

Conclusions: Lyophilized dabigatran plasmas can be proposed for calibration and quality control of dedicated assays, clotting or chromogenic, and using the values assigned by HPLC they can be used as primary standards.