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APPENDICES

Appendix A

Reagents preparation[66]

	Sterilization	Storage
Lysis buffer I	Autoclaving	4 degree C
109.54 g Sucrose		
10 ml Tris-HCl 1M		
5 ml MgCl ₂ 1M		
10 ml Pure Triton-X 100		
655 ml ddH ₂ O		
<u>1M Tris-HCl</u>	Autoclaving	room temp.
24.228 g Tris-base (121.14 g/mol)		
add 150 ml H ₂ O , adjust pH to 7.5 by conc HCl		
adjust to 200 ml with ddH ₂ O		
<u>1M MgCl₂ * 6H₂O</u>	Autoclaving	room temp.
20.33 g MgCl ₂ *6H ₂ O (203.30 g/mol)		
adjust to 100 ml with ddH ₂ O		
Lysis buffer II	Autoclaving	room temp.
15 ml 5.0 M NaCl		
48 ml 0.5 M EDTA		
937 ml ddH ₂ O		
<u>5M NaCl</u>	Autoclaving	
4.383 g NaCl (58.44 g/mol)		
0.5 M EDTA (pH 8.0)	Autoclaving	
Phenol-chloroform-IAA (25:24:1)	(dark glass bottle)	4 degree C
400 ml Phenol		
384 ml Chloroform		
16 ml Isoamyl alcohol		
7.5 M Ammonium acetate	filtration 0.2 micron	room temp.
57.81 g Ammonium acetate (7.708 g/mol)		
adjust to 100 ml with ddH ₂ O		
Proteinase K (20 mg/ml)		-20 degree C
100 mg + ddH ₂ O 5 ml		
10% SDS	(stock 30 ml)	room temp.
5 g SDS		
add 40ml H ₂ O, heat at 68 degree C then adjust to pH 7.2		
by conc. HCl , adjust to 50 ml with ddH ₂ O		

Appendix B

Genotyping analysis

Cytochrome P450 2C9 polymorphisms and VKORC1 genotypes were analyzed by Real time PCR, LightCycler® which is based on the principle of Fluorescence Resonance Energy Transfer (FRET)

The melting curve chart shows fluorescence (Y-axis) and temperature (X-axis) while the melting peak chart plots the first negative derivative of the fluorescence ($-d/dT$) versus temperature, and shows the melting temperature of each sample as a peak. Therefore, sample in different genotype will show in different peak.

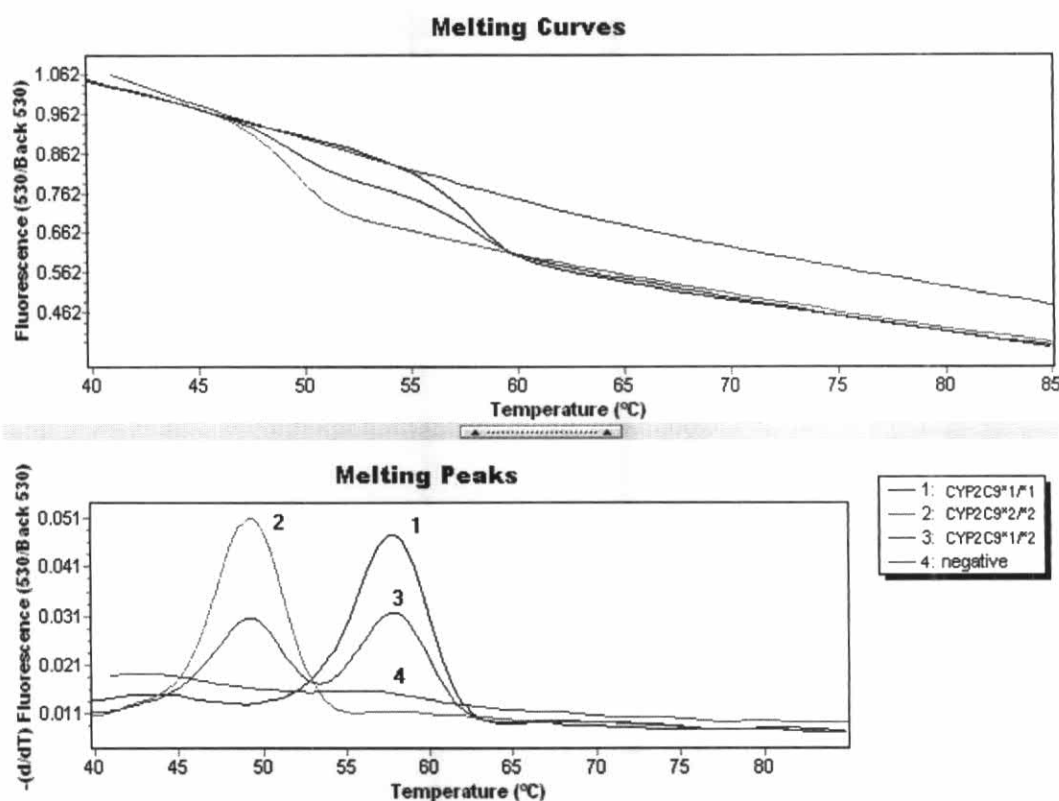


Figure 1 Melting curve and melting peaks chart of CYP2C9*2

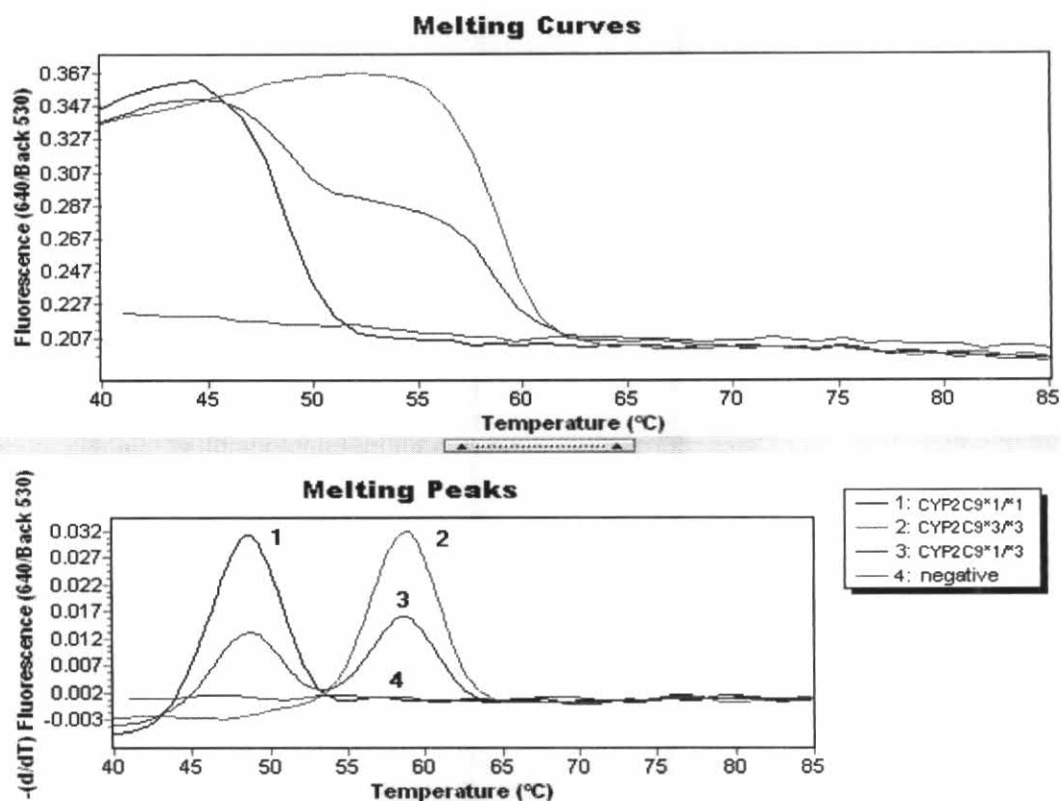


Figure 2 Melting curve and melting peaks chart of CYP2C9*3

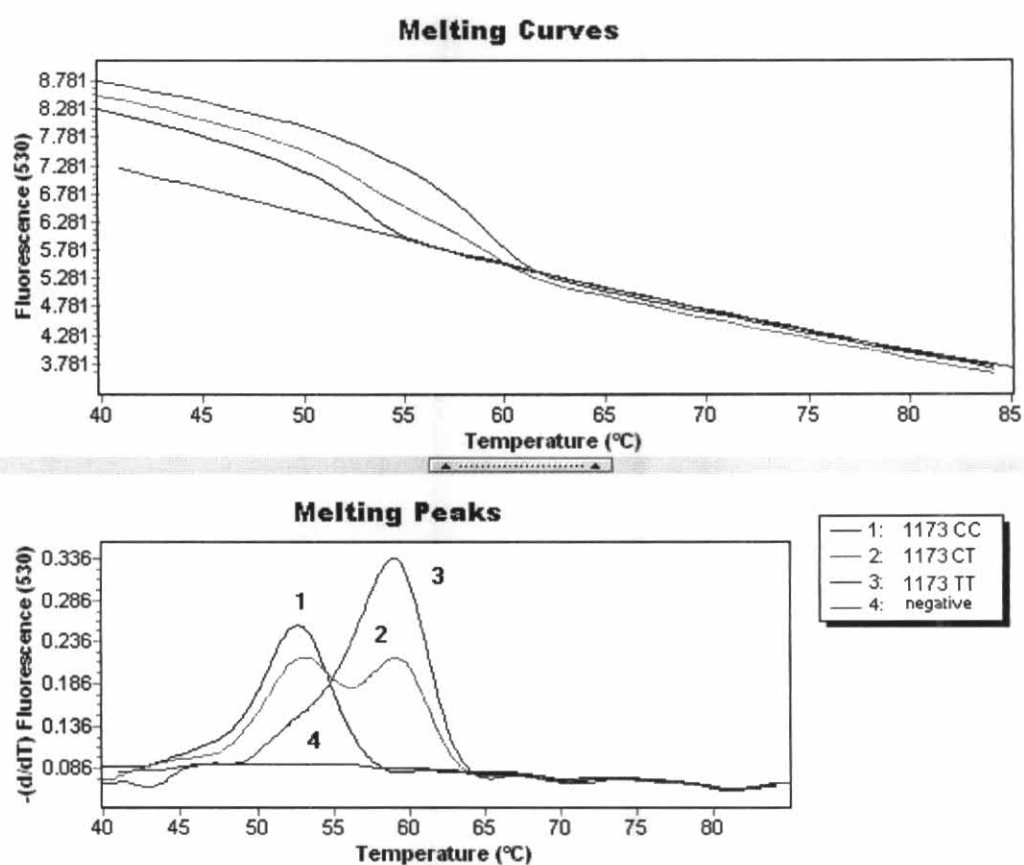


Figure 3 Melting curve and melting peaks chart of VKORC1 C1173T

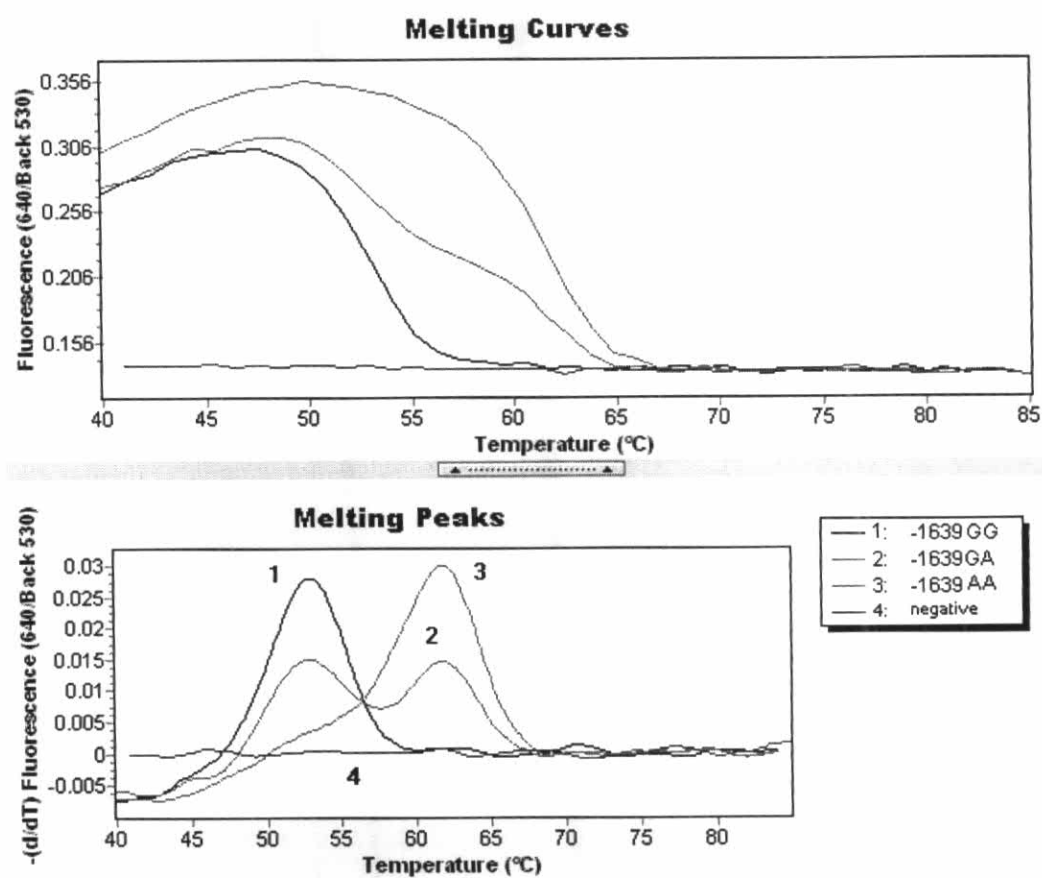


Figure 4 Melting curve and melting peaks chart of VKORC1 G-1639A

Appendix C

Validation of analytical method for warfarin concentration

Total plasma warfarin concentration was determined by using High-Performance Liquid Chromatography with UV detection.

1. Calibration curve and linearity

The linearity of calibration curve was determined from six standard plasma solutions (150, 200, 500, 1000, 1500 and 3000 ng/mL) of racemic warfarin. The calibration curve is shown in Figure 5. The warfarin concentration has been related to the peak height ratio and gave a better R^2 than those of peak area ratio. The least squares linear regression equation was

$$y = 0.0012x + 0.0463, R^2 = 0.9998$$

Where ;
 x = Plasma drug concentration
 y = Peak height ratio of drug to internal standard (IS)
 R^2 = Coefficient of determination

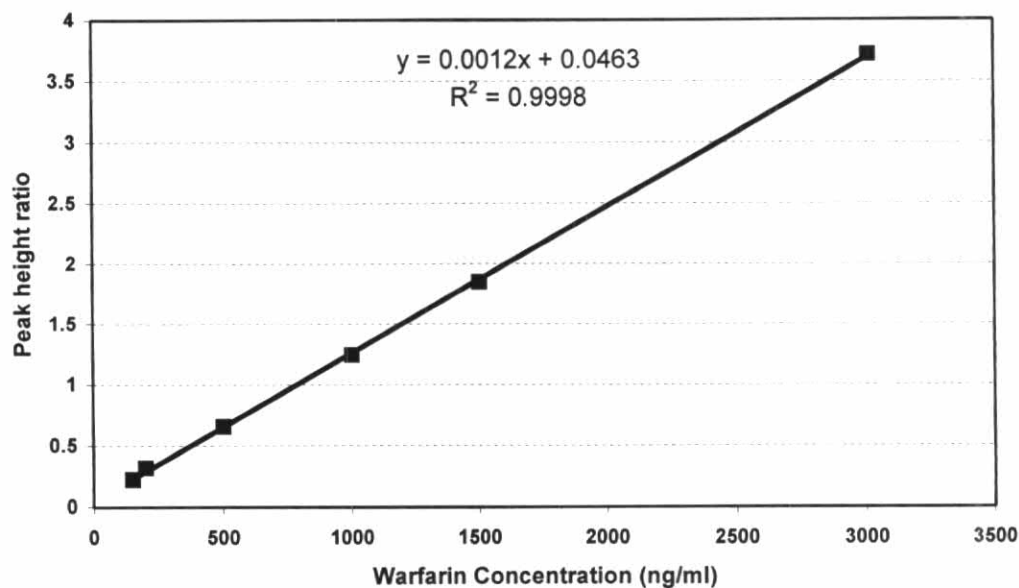


Figure 5 Calibration curve of warfarin

2. Specificity and selectivity

Chromatograms of drug-free plasma and spiked plasma are demonstrated in Figure 6. No interference from endogenous substances in plasma was observed.

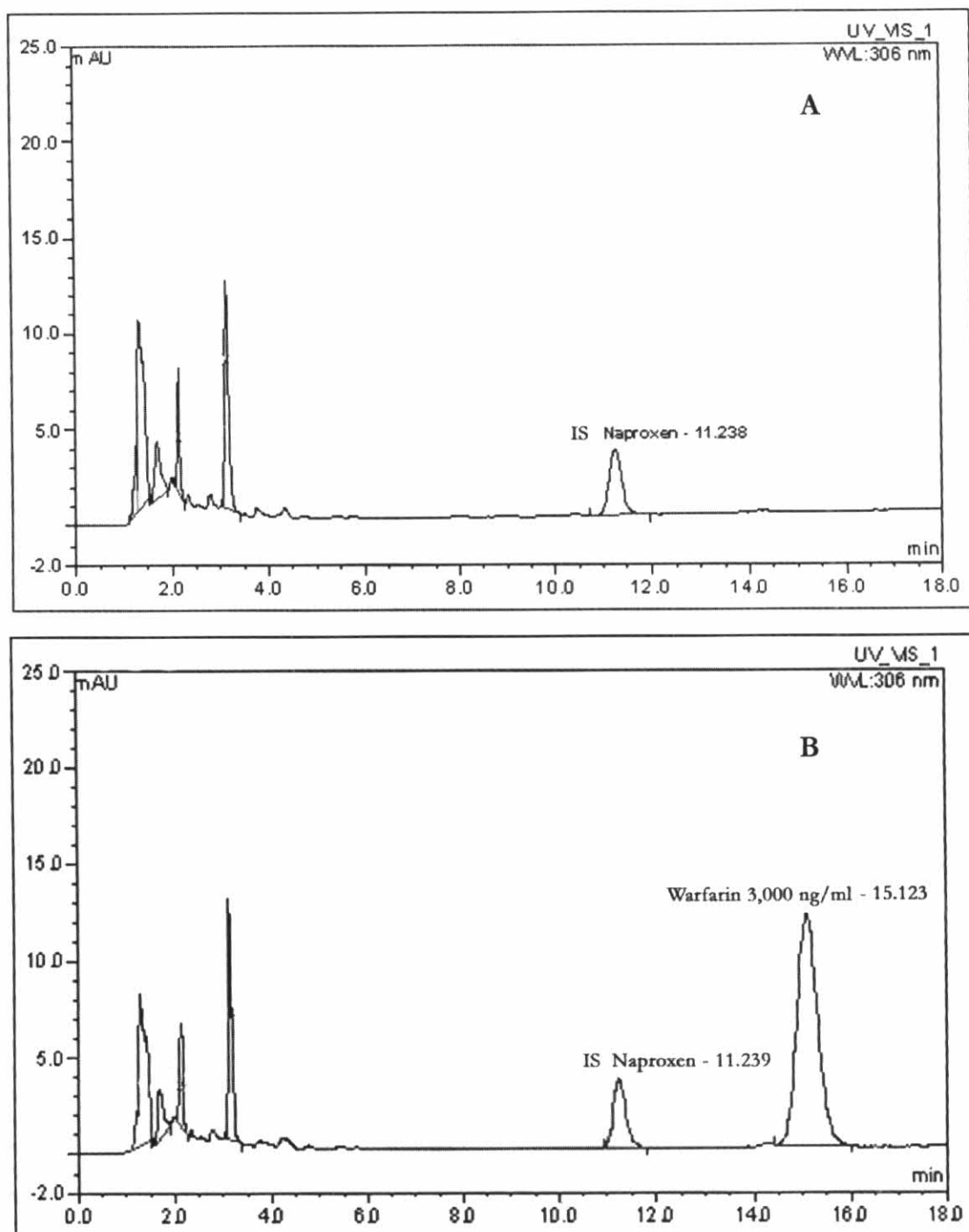


Figure 6 (A) Chromatogram of extracted blank plasma. (B) Chromatogram of Spiked plasma with warfarin 3,000 ng/mL and internal standard (IS).

3. Limit of quantification

Limit of quantification (LLOQ) was the lowest concentration on the calibration curve which had linear relationship with peak height ratio and had acceptable accuracy and precision within $\pm 20\%$ (%RD; percent deviation from the nominal concentration = 2.47% and %CV; coefficient of variation = 3.64 %). Chromatogram of spiked plasma for 150 ng/mL (LLOQ) is demonstrated in Figure 7.

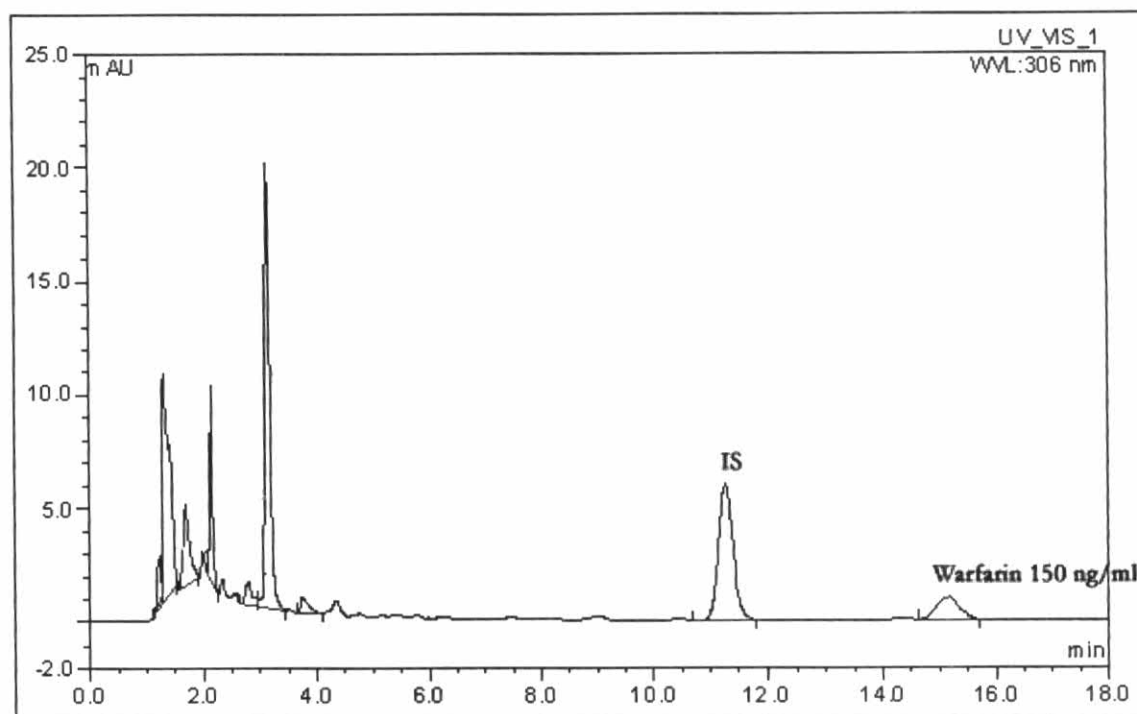


Figure 7 Chromatogram of spike plasma of IS (Naproxen) and Warfarin 150 ng/mL.

4. Accuracy, precision and recovery

The precision and accuracy of the assay procedure were evaluated from %CV and %RD, respectively. As shown in Table 1, the precision and accuracy of intra-day and inter-day were all in acceptable range (within $\pm 15\%$ for QCL, QCM and QCH, and $\pm 20\%$ for LLOQ). Recovery of extracted warfarin was approximately 65 %.

Table 1 Accuracy , precision and recovery of spiked plasma of warfarin

QCs	Acceptable limit ($\pm\%$)	Conc. (ng/mL)	Intraday (n=5)		Interday (n=15)		Recovery of drug* (%)	Recovery of IS* (%)
			Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)		
LLOQ	20	150	3.64	2.47	5.34	-1.98	-	-
QCL	15	300	3.69	-2.91	2.79	-1.57	57.058	64.811
QCM	15	800	6.35	3.55	6.18	0.68	66.596	68.247
QCH	15	2000	5.03	2.30	3.18	2.18	61.472	68.676

* compare with solution

5. Stability

The stability of warfarin in sample plasma and extracted samples at various conditions were determined (Table 2). The stability of warfarin of QCL and QCH were higher than 90% except in freeze-thaw stability of QCL. Acceptable warfarin stability was demonstrated for all phases of storage and processing.

Table 2 Stability of warfarin in sample plasma and final extracted

Condition	Stability (%)	
	QCL (300 ng/ml)	QCH (2,000 ng/ml)
Short – term stability		
- 10 hr at room temperature	102.54	96.70
Long -term stability		
- 3 months in -20°C	97.11	95.76
Final extraction stability		
- in autosampler for 6 hr	101.33	101.67
Freeze-thaw stability		
- 3 cycles	72.62	93.99

Appendix D

Data of individual patient

ID	Age (yo)	Wt (kg)	Gender	INR	Lastdose (mg)	Dose (mg/wk)	Conc. (ng/ml)	Time	Interval (hr.min)	<i>CYP2C9</i>	<i>VKORC1</i>	Clearance (ml/min/kg)	FII (%)	FVII (%)	Side effect
1	45	43.0	F	2.2	3.0	18.00	1912.000	morning	21.40	*1/*1	AA	0.0217	25.44	34.33	bruises, bleeding gum
2	55	67.0	M	1.6	5.0	35.00	592.401	morning	25.10	*1/*1	AA	0.0875	32.03	67.06	Never
3	61	61.0	F	1.7	3.0	25.50	854.824	morning	21.20	*1/*1	AA	0.0485	43.02	65.18	bruises
4	49	51.0	M	1.3	1.5	10.50	390.924	morning	23.20	*1/*1	AA	0.0522	47.03	66.28	GI bleeding, bruises
5	36	55.0	F	2.6	3.0	21.00	779.614	morning	24.45	*1/*1	AA	0.0486	21.29	12.70	leg pain
6	54	53.0	F	1.6	5.0	35.00	1282.947	evening	15.15	*1/*1	AB	0.0511	32.48	61.57	bruises
7	24	44.0	F	2.1	10.0	70.00	2312.322	morning	23.50	*1/*1	BB	0.0683	35.37	46.33	Never
8	66	50.0	M	1.6	3.0	13.50	556.969	morning	24.25	*1/*1	AA	0.0481	40.75	36.90	bruises
9	41	61.0	F	1.3	6.0	42.00	965.608	morning	24.00	*1/*1	BB	0.0707	68.47	99.87	bruises
10	55	73.0	M	2.8	1.5	10.50	577.460	morning	25.30	*1/*3	AA	0.0247	22.60	14.22	Never
11	38	60.0	M	2.4	4.0	28.00	745.296	morning	24.15	*1/*1	AA	0.0621	13.78	41.94	Never
12	36	59.0	F	2.3	7.5	52.50	1855.531	morning	23.45	*1/*1	BB	0.0476	28.13	38.47	bruises
13	35	70.0	M	3.1	3.0	21.00	821.454	morning	24.30	*1/*1	AA	0.0362	-	-	GI bleeding
14	47	55.0	F	1.8	1.5	16.50	560.056	morning	24.40	*1/*1	AA	0.0532	36.64	58.53	Aphasia
15	21	51.0	F	2.4	5.0	35.00	2,054.50	morning	25.10	*1/*1	BB	0.0331	24.16	33.40	Bleeding gum, ecchymosis

ID	Age (yo)	Wt (kg)	Gender	INR	Lastdose (mg)	Dose (mg/wk)	Conc. (ng/ml)	Time	Interval (hr.min)	CYP2C9	VKORC1	Clearance (ml/min/kg)	FII (%)	FVII (%)	Side effect
16	50	45.0	F	2.5	1.5	10.50	559.001	morning	23.40	*1/*1	AA	0.0414	12.26	28.58	Never
17	45	65.0	M	1.8	5.0	22.50	836.346	morning	24.25	*1/*1	AA	0.0410	35.89	38.51	bruises
18	46	54.0	F	2.4	2.5	26.25	1155.030	morning	23.25	*1/*1	AB	0.0418	21.02	38.58	red eye
19	57	82.0	M	1.9	3.0	21.00	653.975	bedtime	13.40	*1/*1	AA	0.0388	29.08	52.08	Never
20	49	52.0	F	2.6	2.5	17.50	804.183	morning	23.45	*1/*1	AA	0.0415	15.29	34.58	abnormal menstrual
21	44	55.0	F	2.3	4.0	28.00	909.05	morning	22.55	*1/*3	AB	0.0556	21.73	33.20	Never
22	56	60.0	F	2.3	2.5	17.50	726.870	morning	25.00	*1/*1	AA	0.0398	-	-	bruises, Bleeding
23	39	60.0	F	2.0	3.0	24.00	687.254	morning	23.45	*1/*1	AA	0.0578	27.98	39.62	bruises
24	57	60.0	F	1.4	3.0	15.00	1192.338	bedtime	13.10	*1/*1	AA	0.0208	45.92	65.50	Never
25	59	45.0	F	1.7	5.0	22.50	1031.747	morning	25.25	*1/*1	AB	0.0480	38.64	62.45	Never
26	33	52.0	M	1.6	5.0	35.00	1052.352	bedtime	8.10	*1/*1	AA	0.0635	40.26	71.49	Never
27	54	53.0	F	1.8	4.5	31.50	941.365	morning	23.25	*1/*1	AA	0.0626	44.84	45.39	bruises, bleeding gum
28	61	35.0	F	1.7	3.0	24.00	1116.904	morning	23.20	*1/*1	AB	0.0609	32.18	39.39	bruises
29	72	58.0	M	2.7	3.0	21.00	607.832	morning	22.15	*1/*1	AB	0.0591	26.23	22.75	bleeding from teeth
30	43	56.0	M	1.6	5.0	22.50	634.573	morning	24.25	*1/*1	AA	0.0627	33.88	49.04	bleeding stool, red eyes
31	69	52.0	M	2.2	3.0	26.25	1103.707	morning	23.55	*1/*1	AB	0.0454	26.74	30.67	bleeding gum
32	51	60.0	M	2.0	1.5	15.75	483.254	morning	20.45	*1/*1	AA	0.0539	-	-	GI bleeding
33	51	47.0	F	1.5	3.0	21.00	795.518	morning	24.00	*1/*1	AB	0.0557	-	-	bleeding gum
34	67	60.0	M	2.2	5.0	25.00	810.671	morning	24.50	*1/*1	AA	0.0510	15.02	33.76	tired
35	53	75.0	F	2.1	5.0	35.00	1424.759	bedtime	12.35	*1/*1	AB	0.0325	32.71	35.86	Never

ID	Age (yo)	Wt (kg)	Gender	INR	Lastdose (mg)	Dose (mg/wk)	Conc. (ng/ml)	Time	Interval (hr.min)	CYP2C9	VKORC1	Clearance (ml/min/kg)	FII (%)	FVII (%)	Side effect
36	22	53.0	F	1.4	3.0	21.00	1051.786	bedtime	11.00	*1/*1	AA	0.0374	61.13	111.38	bleeding gum
37	47	58.0	F	1.6	5.0	22.50	755.099	morning	24.30	*1/*1	AA	0.0509	32.94	68.99	bruises
38	44	70.0	M	1.9	6.0	42.00	1218.274	morning	24.20	*1/*1	AB	0.0489	26.74	33.40	bruises
39	59	56.0	M	2.2	4.0	28.00	634.185	morning	25.30	*1/*1	AA	0.0782	21.53	47.03	bleeding gum
40	47	57.0	F	2.4	2.5	17.50	907.970	morning	23.50	*1/*1	AA	0.0335	-	-	bruises
41	56	30.0	F	2.8	2.5	17.50	959.518	morning	20.15	*1/*1	AA	0.0603	26.23	11.17	bruises
42	40	59.0	F	2.7	3.0	21.00	617.710	morning	24.30	*1/*1	AA	0.0572	-	-	never
43	24	75.0	M	1.6	6.0	42.00	1249.497	bedtime	6.50	*1/*1	AB	0.0445	35.12	57.35	bruises
44	36	55.0	F	1.5	3.0	21.00	472.010	morning	21.55	*1/*1	AA	0.0802	56.95	48.16	bruises, hemorrhagic stroke
45	54	49.0	F	2.3	5.0	27.50	1089.409	morning	23.50	*1/*1	AB	0.0511	23.01	43.15	bruises, Epistaxis
46	57	48.0	F	1.9	5.0	25.00	759.624	morning	24.30	*1/*1	AA	0.0680	32.48	50.45	bruises, bleeding stool, ecchymosis
47	60	66.0	F	1.9	2.5	25.00	970.915	morning	24.00	-	-	0.0387	-	-	Never
48	47	87.0	F	2.1	4.5	31.50	1205.800	morning	20.55	*1/*1	AB	0.0298	-	-	bleeding gum, bruises
49	65	61.0	M	1.9	5.0	35.00	841.797	morning	24.30	*1/*1	AB	0.0676	31.81	45.03	Never
50	65	53.0	F	1.8	2.5	22.50	522.929	morning	23.30	*1/*1	AA	0.0804	30.32	34.36	bruises
51	31	59.0	M	2.7	2.5	17.50	823.431	morning	21.35	*1/*1	AA	0.0357	21.15	21.54	Epistaxis, bleeding gum
52	52	70.0	M	3.3	5.0	35.00	1146.356	morning	23.15	*1/*1	AA	0.0433	12.63	13.25	ecchymosis, bleeding gum
53	75	56.0	M	2.1	2.5	17.50	771.433	morning	2.00#	*1/*1	AA	0.0402	-	-	bruises, IM hematoma

Excluded because patient took warfarin before drawing blood

ID	Age (yo)	Wt (kg)	Gender	INR	Lastdose (mg)	Dose (mg/wk)	Conc. (ng/ml)	Time	Interval (hr.min)	CYP2C9	VKORC1	Clearance (ml/min/kg)	FII (%)	FVII (%)	Side effect
54	49	63.0	F	2.0	5.0	22.50	939.520	morning	22.00	*1/*1	AA	0.0377	-	-	bleeding gum, bruises
55	47	57.0	F	1.9	8.0	56.00	1783.196	bedtime	9.00	*1/*1	AB	0.0547	28.17	39.06	hematuria
56	55	51.0	M	2.9	2.5	17.50	797.133	morning	23.20	*1/*1	AA	0.0427	20.54	19.48	bleeding gum, arm& leg numb
57	36	67.0	M	1.9	3.0	24.00	310.888	morning	23.40	*1/*1	AA	0.1144	26.23	36.38	bleeding gum
58	49	47.0	F	1.5	5.0	22.50	557.390	morning	20.40	*1/*1	AA	0.0851	47.41	66.28	bruises, bleeding gum
59	40	61.0	F	2.6	5.0	35.00	1141.218	morning	22.10	*1/*1	AA	0.0499	17.96	17.86	bleeding gum, bruises
60	70	35.8	F	1.7	5.0	27.50	1356.629	morning	23.40	*1/*1	BB	0.0562	33.45	47.13	Never
61	40	50.0	F	4.2	5.0	35.00	1781.691	morning	24.20	*1/*1	AA	0.0390	8.26	21.53	bruises, Ischemic stroke
62	69	44.3	M	1.7	2.5	17.50	2432.800	morning	23.45	*1/*1	AA	0.0161	48.17	39.83	bruises, GI bleeding, Epistaxis, Cerebral infarction
63	70	66.0	M	3.3	5.0	35.00	1703.926	morning	24.20	*1/*1	AB	0.0309	18.34	21.29	no
64	48	45.0	F	3.0	3.0	21.00	1364.074	morning	22.10	*1/*1	AA	0.0339	9.97	14.92	ecchymosis, lots of menstruation, arm numb
65	26	100.0	F	1.4	2.5	27.50	842.597	bedtime	10.20	*1/*1	AA	0.0324	48.09	79.00	lots of menstruation, bruises
66	40	60.0	F	1.5	2.5	12.50	238.795	morning	47.30##	*1/*1	AA	0.0868	39.13	64.69	Epistaxis
67	43	78.0	M	1.4	2.5	25.00	747.198	morning	24.30	*1/*1	AB	0.0425	54.48	52.08	bleeding gum
68	41	60.0	F	1.7	2.5	17.50	676.712	morning	22.30	*1/*1	AA	0.0428	35.00	90.30	bruises
69	52	73.0	M	3.5	1.5	4.50	678.606	morning	22.30	*3/*3	AA	0.0090	4.97	15.42	bruises

Excluded because skip the dose

ID	Age (yo)	Wt (kg)	Gender	INR	Lastdose (mg)	Dose (mg/wk)	Conc. (ng/ml)	Time	Interval (hr.min)	CYP2C9	VKORC1	Clearance (ml/min/kg)	FII (%)	FVII (%)	Side effect
70	45	47.0	M	2.5	5.0	22.50	609.029	morning	23.50	*1/*1	AA	0.0779	18.28	25.01	Never
71	33	62.0	M	1.6	1.5	13.50	494.148	morning	22.20	*1/*1	AA	0.0437	43.08	44.83	arm fatigue
72	46	70.0	M	2.3	5.0	32.50	494.831	morning	21.00	*1/*1	AA	0.0930	17.95	37.94	bleeding gum, GI bleeding
73	30	48.0	M	1.8	3.0	21.00	1231.578	morning	24.50	*1/*1	AB	0.0352	35.00	39.23	Never
74	65	65.0	M	2.1	4.5	32.50	1477.164	evening	14.30	*1/*1	AB	0.0336	24.24	43.36	Never
75	32	57.0	F	1.4	5.0	35.00	2889.735	morning	25.10	*1/*1	AB	0.0211	62.08	41.94	bleeding gum
76	24	40.0	F	1.7	2.5	17.50	909.180	morning	21.40	*1/*1	AA	0.0477	44.82	48.72	bleeding gum, bruises, epistaxis
77	54	47.0	F	1.9	2.5	30.00	1529.097	morning	21.00	*1/*1	AB	0.0415	37.37	52.94	no
78	39	48.0	F	1.6	2.5	22.50	1121.416	morning	22.00	*1/*1	AB	0.0414	53.19	57.51	numb left side
79	44	60.0	F	1.3	5.0	35.00	1064.278	morning	22.30	*1/*1	AB	0.0544	80.92	75.49	no
80	64	67.0	F	2.2	1.5	13.50	876.850	morning	23.00	*1/*3	AA	0.0228	24.44	38.58	bruises
81	32	50.0	M	2.8	6.0	42.00	2488.135	morning	22.55	*1/*1	AA	0.0335	14.63	24.19	no
82	59	56.0	M	2.9	3.0	16.50	821.545	morning	23.30	*1/*1	AA	0.0356	17.57	31.06	hematoma of jejunum
83	37	59.0	M	1.3	3.0	21.00	466.922	morning	24.15	*1/*1	AB	0.0756	48.09	51.22	bleeding gum
84	23	57.0	M	1.8	3.0	21.00	611.222	morning	23.55	*1/*1	AA	0.0598	30.35	38.58	bleeding gum
85	48	60.0	M	2.7	7.5	52.50	1066.088	morning	27.15	*1/*1	AB	0.0814	13.87	21.53	no
86	49	56.0	F	2.1	2.5	17.50	764.718	morning	22.45	*1/*1	AA	0.0405	31.47	38.38	bruises, bleeding gum, hematuria
87	56	81.0	M	1.4	5.0	27.50	754.874	bedtime	13.50	*1/*1	AB	0.0446	60.55	97.19	GI bleeding

ID	Age (yo)	Wt (kg)	Gender	INR	Lastdose (mg)	Dose (mg/wk)	Conc. (ng/ml)	Time	Interval (hr.min)	CYP2C9	VKORC1	Clearance (ml/min/kg)	FII (%)	FVII (%)	Side effect
88	61	52.0	F	2.4	5.0	35.00	719.972	morning	23.50	*1/*1	AB	0.0927	22.28	66.20	no
89	45	55.0	F	2.5	5.0	40.00	1288.742	morning	24.55	*1/*1	BB	0.0559	24.26	51.67	bruises, GI bleed
90	39	44.0	F	2.0	3.0	21.00	824.621	bedtime	11.10	*1/*1	AA	0.0574	25.44	51.67	bleeding gum
91	78	59.0	M	2.3	2.5	17.50	571.828	morning	23.10	*1/*1	AA	0.0515	22.42	47.97	no
92	61	54.0	F	2.6	3.0	21.00	547.002	morning	24.10	*1/*1	BB	0.0705	19.05	44.53	hemoptysis
93	68	96.0	F	2.3	3.0	21.00	718.891	morning	24.25	*1/*1	AB	0.0302	27.06	31.48	no
94	43	61.0	F	2.4	5.0	35.00	1330.087	morning	24.30	-	-	0.0428	25.79	32.27	bruises
95	69	50.0	M	2.5	1.5	13.50	498.021	morning	24.15	*1/*1	AA	0.0538	19.76	36.07	no
96	63	68.0	M	1.2	1.5	13.50	249.676	morning	23.45	-	-	0.0789	57.75	82.73	bleeding from teeth
97	58	45.0	F	1.2	5.0	35.00	1353.077	morning	22.40	*1/*1	BB	0.0570	77.84	83.76	bruises, bleeding gum
98	31	56.0	F	2.3	2.5	17.50	839.796	morning	27.10	-	-	0.0369	29.68	34.76	bleeding gum
99	38	57.0	F	2.6	5.0	35.00	1445.195	morning	23.40	-	-	0.0422	9.51	21.17	bleeding gum
100	36	49.0	F	1.4	2.5	17.50	597.266	morning	23.10	*1/*1	AA	0.0593	57.75	84.81	no
101	33	58.0	M	3.3	2.5	30.00	1057.819	morning	21.50	*1/*1	AB	0.0486	17.21	22.81	bruises
102	45	59.0	F	1.5	5.0	35.00	964.530	morning	24.00	*1/*1	AB	0.0610	46.13	58.48	hand - foot numb
103	40	65.0	F	2.9	5.0	35.00	1005.487	morning	23.10	*1/*1	AA	0.0531	14.61	25.18	bruises
104	27	60.0	M	1.6	5.0	35.00	673.188	morning	25.10	*1/*1	AB	0.0860	39.06	54.97	no
105	55	56.0	M	1.8	5.0	35.00	598.819	morning	23.30	*1/*1	AB	0.1035	38.13	45.65	bleeding gum
106	54	56.0	M	1.5	2.5	27.50	443.320	morning	23.10	*1/*1	AB	0.1099	42.04	67.02	no
107	60	65.0	M	2.3	2.5	15.00	830.405	morning	23.00	*1/*1	AA	0.0275	28.63	41.86	bleeding gum

VITAE

Pol. Capt. Alisara Sangviroon was born on the 8th of February in 1978 at Nakornratchasima. She graduated with Bachelor degree in Pharmacy in 2000 from Faculty of Pharmacy, Chulalongkorn University. Her current position is a pharmacist at Police General Hospital.