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Fully automated LC-MS/MS analysis of anticoagulants using a novel reagent kit

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1. Introduction

Novel oral anticoagulants (NOACs) are, as an alternative therapy to vitamin K antagonists, used frequently to treat and prevent thromboembolism. Their precise quantitation is necessary to identify the presence/absence of an anticoagulant effect or to determine the concentration of drug that may be helpful for patient management.

Such analysis is mainly done by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). To streamline the workflow, we have developed a complete reagent kit including in-house stable isotope labeled standards for better precision and accuracy. Furthermore, we demonstrate here the use of a fully automated sample preparation system (CLAM-2000) coupled online with LC-MS/MS.

2. Method

To demonstrate that this multi-analyte approach, with a fully automated system LC-MS/MS, can be used as a walk-away unit, we have used a novel kit for anticoagulants analysis called DOSINACO™ (Alsachim SAS). The kit includes 9 analytes (Acenocoumarol, Apixaban, Argatroban, Betrixaban, Dabigatran, Edoxaban, Fluindione, Rivaroxaban and Warfarin) and their corresponding stable isotope labeled standards. CLAM-2000 (Shimadzu, Japan) was programmed to perform protein precipitation followed by filtration and sample collection. The sample is transported from CLAM-2000 to HPLC without human intervention for LC-MS/MS analysis in 7 min.

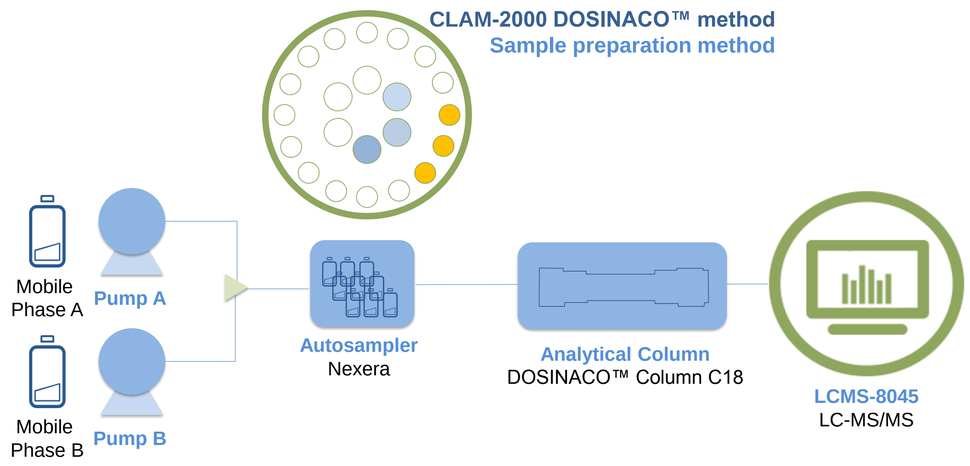


Figure 1. Schematic representation of the CLAM-2000 LC-MS/MS method for DOSINACO™

HPLC Conditions

Analytical column	: DOSINACO™ Column C18 2,1x50 mm, 5 µm
Pump A	: DOSINACO™ Mobile Phase A
Pump B	: DOSINACO™ Mobile Phase B
Rinse solution (Internal & External)	: (R0) DOSINACO™ System Cleaning Phase (R1) DOSINACO™ Mobile Phase B
Flow rate	: 0.5 mL/min
Oven temperature	: 50 °C

MS Conditions LCMS-8045

Ionization	: ESI Positive
DL temp.	: 200 °C
Heat Block temp.	: 400 °C
Interface temp.	: 400 °C
Nebulizer gas flow	: 3 L/min
Drying gas flow	: 5 L/min
Heating gas flow	: 15 L/min

Time program :

Time (min)	event	
0.00	Pump B conc.	2
0.50	Pump B conc.	2
2.50	Pump B conc.	50
3.00	Pump B conc.	98
5.00	Pump B conc.	98
5.01	Pump B conc.	2
7.00	Stop	

MRM transition :

Molecules	Transitions MRM (1)	Transitions MRM (2)
Acenocoumarol	354.10>163.10	354.10>296.10
Apixaban	460.20>443.20	460.20>199.10
Argatroban	509.20>384.20	509.20>70.00
Betrixaban	452.10>324.10	452.10>279.10
Dabigatran	472.20>289.20	472.20>144.20
Edoxaban	548.20>366.20	548.20>152.10
Fluindione	241.10>175.10	241.10>194.10
Rivaroxaban	436.10>145.10	436.10>231.10
Warfarin	309.10>251.10	309.10>163.10

Molecules	Transitions MRM (1)	Transitions MRM (2)
[$^{2}H_4$]-Acenocoumarol	358.10>167.10	358.10>300.10
[^{13}C, $^{2}H_8$]-Apixaban	469.20>452.20	469.20>199.10
[$^{13}C_6$]-Argatroban	515.20>390.20	515.20>70.00
[$^{13}C_6$]-Betrixaban	458.10>330.10	458.10>285.10
[$^{13}C_6$]-Dabigatran	478.20>295.20	478.20>144.20
[$^{2}H_6$]-Edoxaban	554.20>372.20	554.20>158.10
[$^{13}C_6$]-Fluindione	247.10>181.10	247.10>200.10
[$^{13}C_6$]-Rivaroxaban	442.10>145.10	442.10>237.10
[$^{2}H_6$]-Warfarin	315.10>257.10	315.10>163.10

Samples preparation for manual handling

1. Put 50 µL of samples/calibrators in 1.5 mL microtube
2. Add 25 µL of Internal Standard
3. Add 350 µL of Extraction buffer
4. Shake for 1 min
5. Centrifuge at 15,000 g for 7 min
6. Transfer 200 µL of supernatant to vial

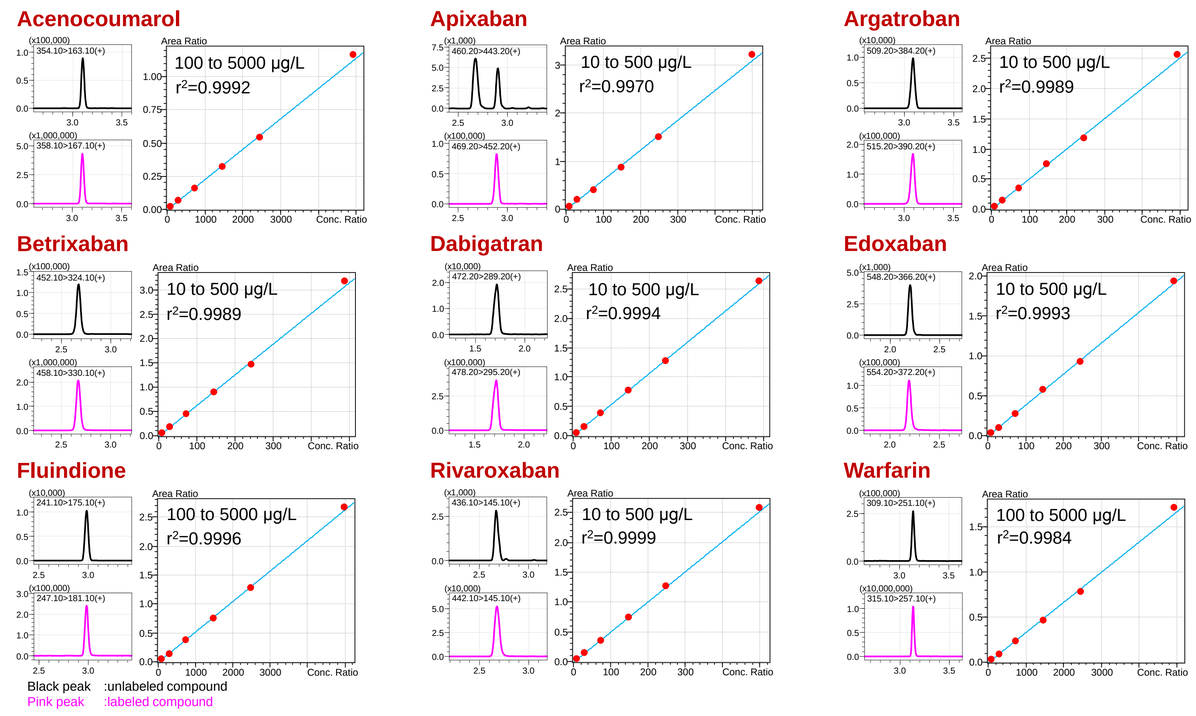
Samples preparation for CLAM-2000

1. Take 20 µL of Extraction buffer to sample cup
2. Add 20 µL of samples/calibrators
3. Add 155 µL of Extraction buffer
4. Add 12.5 µL of Internal Standard
5. Shake for 2 min at 1,900 rpm
6. Filtrate for 2 min

3. Result and discussion

A panel analysis of 9 anticoagulants using an automated sample preparation system, seamlessly integrated on-line with LC-MS/MS, and combined with the ready-to-use reagent kit DOSINACO™, demonstrates the capability to use a standardized platform for therapeutic drug monitoring even for non-expert users of Mass Spectrometry.

We carried out concurrent analysis over a range of concentrations in 10 µg/L to 500 µg/L for NOACs and in 100 µg/L to 5000 µg/L for old anticoagulants. The calibration curves that were generated had linear regression values of r^2 >0.99 for each curve. CV% values of concentration were within acceptable analytical ranges (<15%).



	Acenocoumarol				Apixaban				Argatroban			
Level	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
Average (µg/L)	185.9	858.3	1765.9	3536.8	17.8	79.0	162.3	323.3	17.2	81.8	172.6	333.6
CV (%)	1.8%	1.9%	1.7%	2.2%	10.6%	5.4%	6.0%	5.8%	5.5%	3.1%	3.2%	3.6%
Deviation (%)	0.6%	1.7%	2.9%	-0.4%	-1.0%	-4.2%	-4.7%	-4.3%	-2.9%	1.4%	0.9%	-4.6%

	Betrixaban				Dabigatran				Edoxaban			
Level	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
Average (µg/L)	18.4	81.6	167.3	338.2	17.8	82.6	168.7	336.4	17.5	84.6	174.3	361.3
CV (%)	3.8%	1.6%	1.6%	1.9%	2.4%	1.3%	1.2%	1.0%	8.7%	5.3%	4.7%	5.2%
Deviation (%)	3.3%	1.4%	-1.5%	-0.9%	-0.1%	1.6%	-0.1%	-2.0%	-6.2%	0.1%	0.0%	1.7%

	Fluindione				Rivaroxaban				Warfarin			
Level	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
Average (µg/L)	198.1	856.6	1795.8	3691.4	18.3	81.2	170.8	345.2	177.8	819.5	1692.1	3453.5
CV (%)	6.4%	2.9%	2.9%	4.2%	7.4%	5.6%	6.0%	8.1%	1.8%	1.4%	1.0%	2.2%
Deviation (%)	9.1%	0.1%	1.8%	-1.1%	-2.7%	10.3%	-0.5%	5.2%	-0.1%	2.2%	0.6%	-0.4%

N=9 (3 replicates per day during 3 days)

Figure 2. Calibration curves, MRM chromatograms (at calibrator level 1) and summary of 9 anticoagulants

Sample preparation and LC/MS/MS analysis can be performed in parallel to accelerate throughput using CLAM-2000.

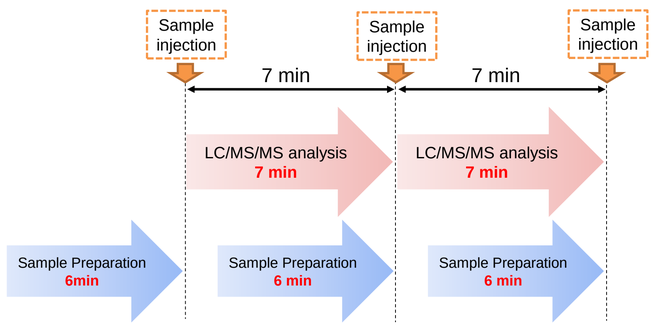


Figure 3. Analytical flow with parallel processing

4. Conclusion

The novel system workflow results in easier and safer operation for users without Chromatography and Mass Spectrometry experience, thus reducing risk of exposure and improving management of patients.