

DETERMINATION OF AMOXICILLIN/CLAVULANIC ACID IN HUMAN PLASMA BY LC-MS/MS WITH PURIFICATION BY ULTRACENTRIFUGATION. ADAPTATION TO THE ANALYSIS OF AMOXICILLIN IN ANIMAL TISSUES.

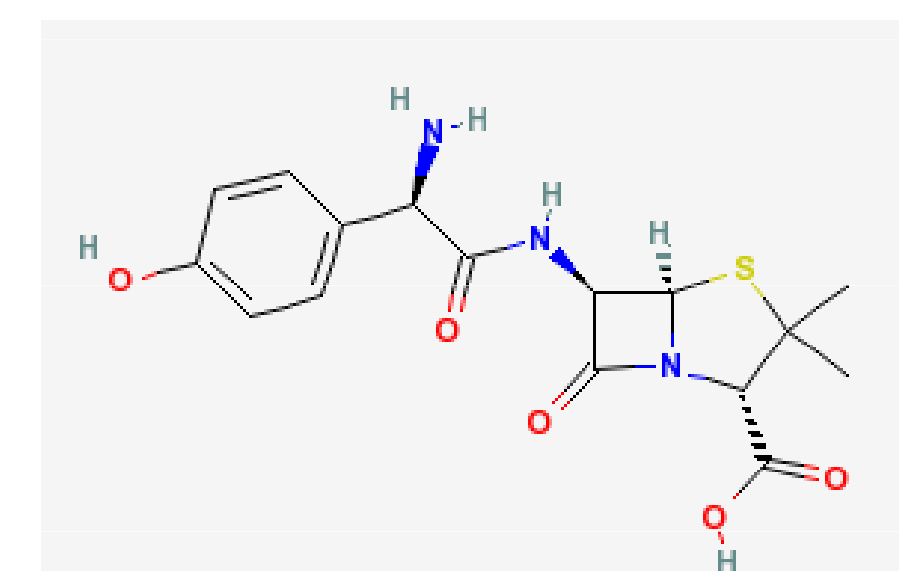
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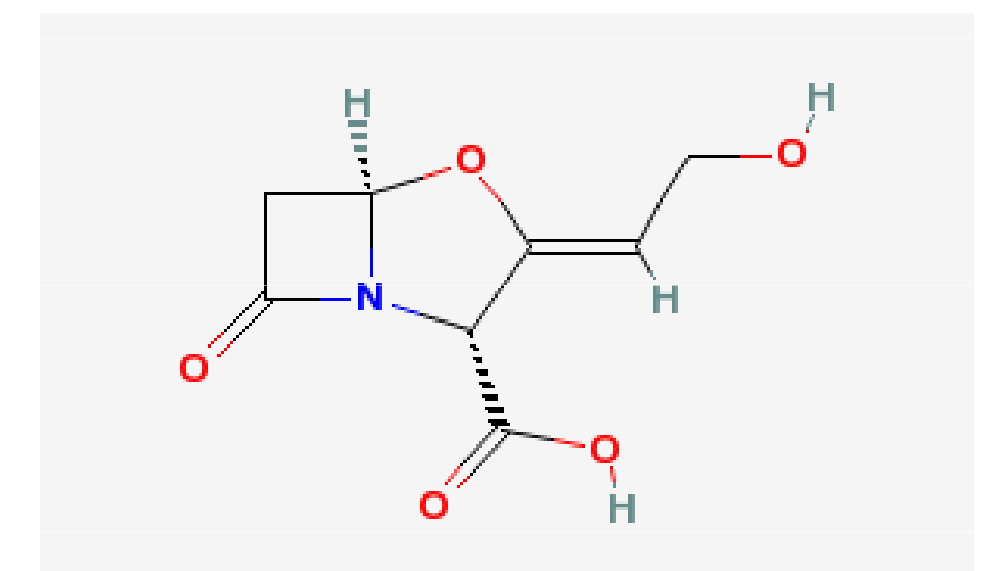
INTRODUCTION

Amoxicillin (alone or combined with clavulanic acid) is widely used in human and veterinary medicine because its broad spectrum and low cost. Although there are several articles published, its chemical analysis is not straightforward: its high polarity (also true for clavulanic acid) makes difficult to extract the drug from the biological matrices without endogenous interferences causing problems in the chromatography; and in addition, amoxicillin and clavulanic acid are unstable in several common laboratory conditions[1].

Amoxicillin
pKa: 2.4, 7.4, 9.0, 10.3
Log P: -2
Exact mass: 365.10



Clavulanic acid
pKa: 2.7, 12.2
Log P: -1.2
Exact mass: 199.04



HUMAN PLASMA METHOD DEVELOPMENT

The starting point was the method proposed by Reyns T. et al. [2] to clean up animal tissue extracts to determine amoxicillin using Microcon® filters and ultracentrifugation to separate the protein fraction. From these conditions we developed a method for analysing amoxicillin and clavulanic acid in human plasma samples.

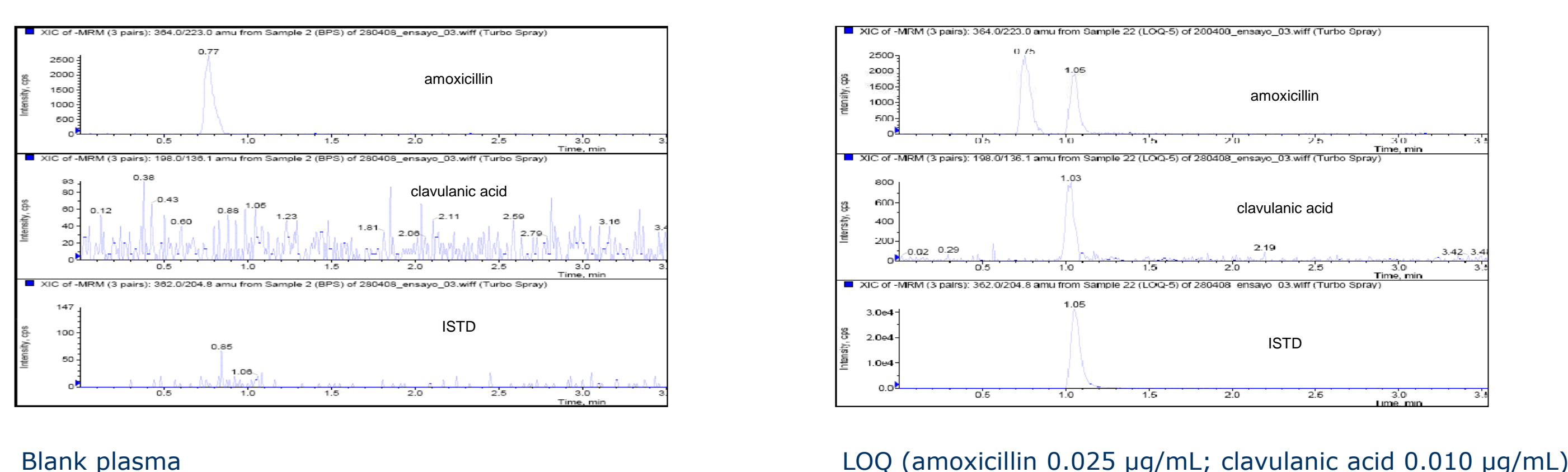
The main steps of the final developed method are:

- Addition of 100 µL of 50 mM ammonium acetate (pH 4.5) to 0.4 mL of plasma
- Addition of 50 µL of internal standard (Cefadroxil) working solution of 20 µg/mL
- Centrifugation 10 min at 14000 g (4°C)
- Transfer of 250 µL of the extract to eppendorf tubes with Microcon™ filters and centrifugation 30 min at 14000 g (4°C)
- Transfer 150 µL of the filtrate to a well plate

The instrumental conditions are:

LC		Agilent 1200	
Column	Atlantis™ T3, 50 × 2.1 mm, 3 µm (50°C)		
Flow	0.25 mL/min		
Gradient	A: 25 mM ammonium acetate (pH 4.5) B: Methanol : Acetonitrile (30:70)		
	Time	%B	
	0	0	
	0.5	0	
	0.6	10	
	1.4	10	
	1.5	40	
	3.0	0	
	6.0	0	
MS/MS		API 4000	
Ionisation	Electrospray (550°C)		
Detection	MRM (negative mode): Amoxicillin: m/z 363.98 → 223.03 Clavulanic acid: m/z 197.98 → 136.11 Cefadroxil (ISTD): m/z 362.01 → 204.84		

Examples of chromatograms obtained with human plasma:



ANIMAL TISSUE ANALYSIS

We have developed and validated (according to [3] requirements) four LC MS/MS methods for the analysis of amoxicillin in pig and calve muscle (injection site and surrounding areas), pig kidney and bovine milk.

The main steps of the methods for tissues are:

- Homogenization of 1.00g of tissue with 10 mL of NaH₂PO₄ 10 mM.
- Transfer 0.5 mL of the extract to eppendorf tubes with Microcon® Filter and centrifuge 30 min at 14000 g (4 °C).
- Transfer 0.4 mL of the filtrate to a well plate.

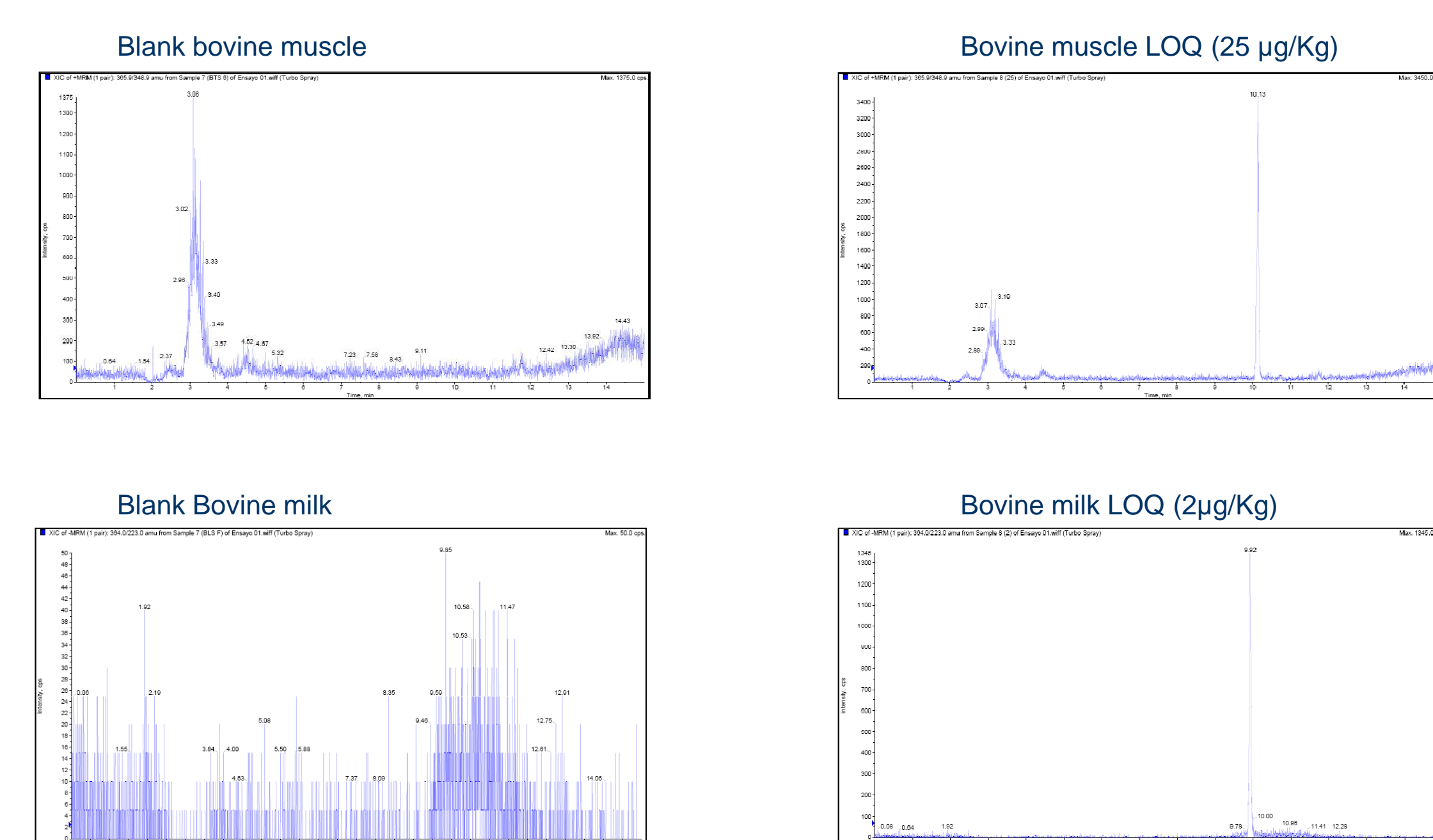
And for milk samples:

- Homogenization of 2.5mL of milk with 0.2 mL of acetic acid 10% and 2.5 mL of NaH₂PO₄ 10 mM.
- Transfer 0.5 mL of the extract to eppendorf tubes with Microcon® Filter and centrifuge 30 min at 14000 g (4 °C).
- Transfer 0.4 mL of the filtrate to a well plate.

The instrumental conditions are:

Pig muscle and kidney		Bovine muscle and milk	
LC	Agilent 1100	Agilent 1200	
Column	PLRP-S™ 100 Å 150x2.1 mm 3µm (30°C)		
Flow		0.20 mL/min	
Gradient		A: 0.1% formic acid in H ₂ O B: 0.1% formic acid in ACN	
		Time	%B
		0	98
		1.9	98
		2.0	80
		5.0	80
		5.1	50
		12.0	50
		12.1	98
		20.0	98
MS/MS	API 3000	API 4000	
Ionisation	Electrospray (450°C)	Electrospray (450°C)	
Detection	MRM in positive mode: m/z 365.90 → 348.90	MRM: -Muscle in positive mode: m/z 365.90 → 348.90 -Milk in negative mode: m/z 363.98 → 223.03	

The validated concentration ranges are 25-400 µg/Kg for tissues and 2-32 µg/Kg for milk. At present, we have analysed more than 640 tissue/milk samples from several depletion of residues studies.



HUMAN METHOD VALIDATION

The method was validated according to the FDA requirements:

Selectivity	No interferences were detected
Linearity	0.025-25 µg/mL (amoxicillin) 0.010-5 µg/mL (clavulanic acid)
Inter and intra-assay Accuracy and Precision	<15%
Recovery	> 85% for amoxicillin; >68% for clavulanic acid; >75% cefadroxil
Dilution effect	No dilution effect was observed (factor ¼)
Stability in plasma tested	4 h at room temperature After after 3 freeze/thaw cycles 199 days at -75°C
Stability in extracts (autosampler) tested	3 days at 4 °C
Stability in solution tested	6 h at room temperature and 26 days at -75°C

This method has been satisfactorily applied in two bioequivalence studies (aprox. 1500 samples)

CONCLUSIONS

The developed methods are very simple to carry out in a routine way and copes with the difficulties of other methods described in bibliography and/or used previously in our laboratory (unstability of the extracts, poor chromatography, very time-consuming sample preparation, etc). All they have been fully validated before using it for the analysis of incurred samples in GLP studies.

BIBLIOGRAPHY

- [1] de Baere S. et al, Anal. Chem. 74 (2002)
[2] Reyns T. et al, J. Chromatogr. B 861 (2008)
[3] Volume 8. Notice to applicants and Guideline (Veterinary medicinal products) Establishment of maximum residue limits (MRLs) for residues of veterinary medicinal products in foodstuffs of animal origin (October-2005).