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Research Article

Validation of a LC-MS Assay Applied to Synovial Fluid for Detection for Periprosthetic Joint Infection

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Abstract

The presence of Alpha defensin in synovial fluids are a potential biomarker for identification of periprosthetic infections: the identification method are based on proteomic test after enzymatic digestion of synovial fluid. The method can be used as screening method on all patients undergoing prosthetic review to distinguish cases of inflammation from infection with alternative methods (clinical or ELISA test).

The preliminary step of research it's the check of uniqueness of possible peptide derived from trypsin digestion of alpha denesin. This step of work was performed on real positive sample (compared with negative sample to confirm): after a sample preparation, the detection of peptide was carried out by liquid chromatography - time of flight mass spectrometry (LC-QTOF). The obtained data have been developed by proteomic software to identify the uniqueness biomarker peptide for alpha defensin and ensure that aren't any other human proteins able to generate identical or similar peptide after tryptic digestion.

The analytical method was subsequently validated according to the CLSI and FDA guidelines in order to verify the method specifications in accordance with the requirements for the application of the method in diagnostic protocols.

Keywords: *Synovial Fluid (SF); LC-MS Assay; Periprosthetic Joint Infection*

Introduction

Synovial fluid (SF), a highly viscous solution localized in the articular joints acts as physiological lubricant, due to its high viscosity, and represents a physical barrier against mechanical stresses [1]. SF may be considered an unconventional matrix for routine clinical laboratories. In fact, the presence of hyaluronic acid (sodium hyaluronate, HA), proteoglycan 4 and surface active phospholipids, is a challenge for handling and analysis, compared to other body fluids [2,3].

Several joint diseases are characterized by SF effusion, and in particular, the finding of a swollen joint in a patient with total joint arthroplasty may suggest either the presence of periprosthetic joint infection (PJI) or a prosthetic loosening (aseptic failure) [4,5].

The capacity to differentiate between septic and aseptic failure of the prosthesis is critical, since the treatment for PJI necessitates unique strategies to eradicate the infecting organism(s) and to restore joint function.

Currently, surgeons use a wide spectrum of tests in an attempt to diagnose PJI and the Musculoskeletal Infection Society (MSIS) recently published a definition of PJI, and suggested a diagnostic score based on a combination of clinical data and laboratory tests (MSIS criteria) such as: synovial fluid white blood cell (WBC) count and differential, synovial tissue histology, serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and bacterial isolation techniques [6]. Nevertheless, the diagnosis of PJI remains a serious clinical challenge and the need for improved diagnostic testing methods stimulated the research on synovial fluid biomarkers and led to the production of new assays, aimed to support decision-makers' judgments. However, it is recognized that the evaluation of a new biomarker is a multistep process [7], including assessing analytical performances and defining usefulness in terms of clinical outcome and economic impact.

The proposed work was planned to evaluate the ability of mass spectrometry (MS) systems to set up a test able to improve the therapeutic strategies against joint failures. The MS techniques make it possible a series of experiments that allow the deepest analysis of the SF samples, favoring the recognition of a possible target molecules. In order to carried out this study, it was proposed to follow a bottom-up proteomic approach. Therefore, the SF sample was undergo to the proteolytic digestion procedure before the MS analysis with different experimental conditions [8-20].

Materials and Methods

Chemicals and reagents

The chemicals used during the experiments were as follow: ammonium hydrogen carbonate, dithiothreitol, iodoacetamide, thiourea, tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), and urea as reagent grade (≥ 98%), trypsin (from bovine pancreas, > 7500 BAEE units/mg), hyaluronidase (Type I-S, lyophilized powder, 400 - 1000 units/mg) and acetonitrile (LC-MS grade). All the chemicals were purchased from Sigma-Aldrich Italia (Milan, Italy). The ultrapure water (LC-MS grade) was prepared using a in site device (Arium Pro System, ASTM Type 1) from Sartorius (Milan, Italy). The peptide ALNARKWYFF, used as Internal Standard (IS), was purchased from Peptide 2.0 (Chantilly, VA, USA). The synthetic peptide LWAFCC, used as standard for quantitative evaluation of SF samples, was purchased from DBA Italia (Milan, Italy). The denaturing solution was prepared by properly weighing of substances/chemicals to obtain a mixture of 320 mM DTT, 5M urea, 2.4M thiourea and 200 mM Tris HCl in ultrapure water.

Sample collection

Synovial fluids (SF) samples were collected from patients of IFCA Orthopedic Clinic (Florence, Italy), either before surgery or intraoperatively, according to standardized procedure. The blank SF (n = 15) were obtained from patients undergoing elective surgery for first knee arthroplasty. These samples were classified PJI negative by common tests currently used MSIS criteria (used as a control samples) and they employed as negative SF matrix to set up the LC-MS method. Leftover samples from EDTA tubes were kept at -30°C and transported to the MS facility according to temperature controlled procedure. Samples were kept frozen till further analysis.

Instrumental

The Agilent 1290 Infinity II coupled with 6545 QToF system (Agilent Technologies, Palo Alto, CA, USA) equipped to JetStream electrospray (ESI) source (LC-QToF) was used, in the preliminary studies, to perform the separation of the SF components in liquid chromatography (LC) and they detection in high resolution mass spectrometry (HRMS) and/or tandem mass spectrometry (MS/MS). The quantitative evaluation of SF samples was carried out in MS/MS acquisition with the Agilent 1290 Infinity II coupled with a 6495 Triple quadrupole system (LC-QqQ), equipped with JetStream ESI source (Agilent Technologies, Palo Alto, CA, USA).

Raw-data were collected and processed by MassHunter Qualitative Analysis software (vers. B.06.01) with Bioconfirm add-in. and Spectrum Mill software (vers. B.04.01) were used to elaborate the MS/MS data of hydrolyzed SF samples. Al the software employed were

purchased by Agilent Technologies (Palo Alto, CA, USA). Pubchem Protein database are used for identification of digest peptide after LC-QToF analysis (NCBInr 2017 database, subgroup Humans).

Thermostatic oven (Binder, BD 56 model) was used to maintain the samples at defined temperature during the digestion/hydrolysis processes.

Preparation of standards and quality control solutions

The stock solutions of LWAFCC and ALNARKWYFF (IS) peptides were prepared by weighing 1 mg of each analyte and dissolving them in 1 mL of ultrapure water. The internal standard solution (IS solution) was freshly prepared, directly from the its stock solution, by dilution up to 1 µg/ml in ultrapure water.

The calibration curve of LWAFCC (reference peptide) was obtained analyzing the standard solutions prepared at different concentration ranging from 0.1 to 100 µg/ml (0.1, 0.5, 1, 5, 10, 25, 50, 100 µg/ml) in SF blank matrix.

Sample preparation

An aliquot of 200 µl of SF sample was added with 20 µl of IS solution and 100 µl of 20 µg/mL hyaluronidase solution. The obtained mixture was incubated at 37°C for 2 hours to allow the hydrolysis of the hyaluronic acid. After cooling at room temperature, the sample was added by 100 µl of denaturing solution and kept at 50°C for 2 hours. The denatured protein solution was treated by 100 µl of 100 mM of ammonium bicarbonate and 50 µl of 500 mM of iodoacetamide. The obtained sample was protected from light for 2 hours at 37°C (Alkylation process). Finally, the alkylated sample was added with 25 µl of 200 mM DTT, 25 µl of 10 µg/ml of trypsin solutions and 400µl of ultrapure water. The obtained solution was incubated overnight at 37°C The final solution was filtered at 0.2 µm with regenerated cellulose immediately before LC-MS analysis.

LC-HRMS analysis

The preliminary study, aimed to identify the marker molecules of PJI condition, was conducted using the LC-QToF system equipped with the AdvancedBio Peptide Map column (Agilent Technologies, Palo Alto, CA, USA), 150 mm length, 2.1 mm internal diameter and 2.7µm of particle size, at constant flow of 0.25 mL min^{-1}, employing a binary mobile phases elution gradient. The solvents used were 5 mM formic acid in ultrapure water solution (solvent A) and 25 mM formic acid in acetonitrile (solvent B) according to the elution gradient as follows: initial at 95% solvent A for 2 minutes, then reduced at 45% A in 35 minutes, finally decreased at 5% A in 3 minutes, kept for 2.0 minutes, then returned to initial conditions in 0.1 minutes and maintained for 5.0 minutes for reconditioning, to a total run time of 50 minutes. The column temperature was maintained at 45°C and the injection volume was 5 µL. The ESI source was operated in positive ion mode, using the following setting: 4 kV capillary, 12 L/min gas flow,35 psi nebulizer gas, sheath gas 12 L/min at 250°C, 1000 V nozzle, 200 V fragmentor and 65 V skimmer.

The LC-QToF analysis was carried out by a data-dependent acquisition method (Auto-MS/MS) that allows the application of various MS acquisition experiments in different time segments. The Auto-MS/MS conditions, used during our study, were as follows: the selection of the five more intense ions, in the first time segment, then their submission to MS/MS experiment at variable collision energy, in the five next segment times. The cycle times of each data point (sum of the six time segments) was 735 ms. The detection of precursor ions and their respective product ions were performed in HRMS at resolution power of 45,000. Auto-MS experiment: this acquisition method allows to obtain HRMS data and at the same time MS-MS acquisition in HRMS mode.

In order to evaluate of the possible marker molecules of PJI condition, the LC-QToF analysis were performed on two synovial samples: the first was a control SF sample and the other was a PJI positive (according to MSIS criteria) for the presence of biomarker (in particular, alpha defensin).

LC-MS/MS analysis

The quantitative evaluation of SF samples were carried out in MS/MS mode with LC-QqQ system applying the chromatographic conditions used with LC-QToF previously described. The MS/MS acquisition parameters were the ESI source was operated in positive ion mode, using the following setting: 4 kV capillary, 12 L/min gas flow, 35 psi nebulizer gas, sheath gas 12 L/min at 250°C, 1000 V nozzle, MS/MS transition 856.3/339.1 at CE 35V, 856.3/300.2 at CE 32V, 856.3/371.2 at CE 36V.

Results of Method Validation

Identification of biomarker peptide

The isolation and identification of the marker peptide from alpha defensin was achieved by LC-MS analysis, after tryptic digestion of two synovial samples, one negative (id control sample) and one positive for the presence of alpha defensin (Positive according to MSIS criteria). The sequence of alpha defensin was obtained from the Pubchem Protein database. After preparation (as reported in Preparation of standards and quality control solutions), samples were run by LC-QTOF with Auto-MS experiment: this acquisition method allows to obtain HRMS data and at the same time MS-MS acquisition in HRMS mode. The acquisition software isolated the five more intense ions for each segment and produced a fragmentation resulting in HR MS-MS spectra. To verify the presence of alpha defensin, MassHunter Qualitative Analysis software (Agilent Technologies, Palo Alto, CA, USA) was used with Bioconfirm add-in (Agilent Technologies, Palo Alto, CA, USA). The software simulates the digestion of protein (including the modification with iodoacetamide) and it combines theoretical peptide structure data with acquired data, also using MS-MS fragmentation data.

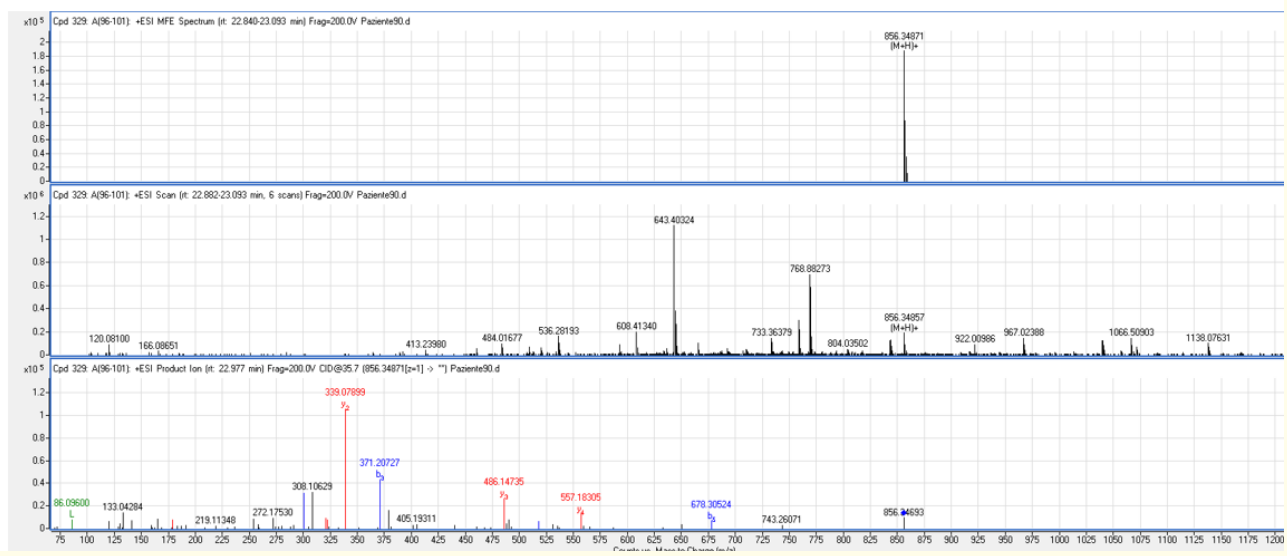

Figure 1: *Identification of marker peptide - Comment. MS spectra 1: EIC m/z 856.3487 (tolerance 5 ppm) of fragment LWAFCC peptide. MS spectra 2: HRMS spectra at 22.994 min. MS spectra 3: product ion at 35.7 V of m/z 856.3487 and identification of aminoacid fragments.*

After the identification of the putative fragment by Bioconfirm software, the raw data were elaborated with Spectrum Mill Software (Agilent Technologies, Palo Alto, CA, USA), to verify the uniqueness of marker peptide of alpha defensin. The software performs an extraction and deconvolution of the raw data and compares the MS/MS data with a protein database. The digested peptides are compared with

the update protein database (NBCI Protein Database – subgroup Human Protein). The peptide LWAFCC is identify as a specific marker for the presence of alpha defensin (see the MS Edman search results in following figure).

The peptide sequence used for the study is present in multiple isoforms of defensin (alpha-defensin isoform 1 (defensin HNP-1), isoform 2 (defensin HNP-2) and isoform 3 (defensin HNP-3).

MS Edman Search Results

Press stop on your browser if you wish to cancel this MS Edman search prematurely.

Sample ID (comment): **Enter_Comment**
Database searched: **NCBInr.uomo**
Number of database entries:**294727**
MS Edman search selects **3319** entries (results displayed for top **200** matches).

Peptide Masses are	Cysteines Modified by	Peptide N terminus	Peptide C terminus	Combinatorial Output	Max # AA Substitutions	Search Type	Regular Expression
monoisotopic	**carboxymethylation**	**Hydrogen (H)**	**Free Acid (O H)**	**off**	**2**	**Sequence Only**	**LWAFCC**

	Number of Substitutions	Matching Sequence	Peptide M+H	Protein MW (Da)/pI	Species	MS-Digest Index #	Accession #	Protein Name
1	0	(R)LWAFCC(-)	858.3161	10549.0/6.54	Homo sapiens	105333	4758146	neutrophil defensin 1 preproprotein
2	0	(R)LWAFCC(-)	858.3161	11336.0/7.58	Homo sapiens	181704	695271700	neutrophil defensin 1 isoform 1 preproprotein
3	0	(R)LWAFCC(-)	858.3161	11380.0/6.54	Homo sapiens	196582	767950128	PREDICTED: neutrophil defensin 3 isoform X1
4	0	(R)LWAFCC(-)	858.3161	10593.0/5.71	Homo sapiens	225043	4885179	neutrophil defensin 3 preproprotein
5	0	(R)LWAFCC(-)	858.3161	7521.4/6.76	Homo sapiens	269551	7702778	neutrophil peptide 3 precursor

Figure 2: *MS Edman search results. Qualification of uniqueness of LWAFCC to alpha defensin.*

Bioanalytical method validation

The validation of the method was performed in accordance to Bioanalytical method validation – guidance for industry (May 2018 revision) published by U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) end Center for Veterinary Medicine (CVM).

The method validation are performed by LC-MS/MS instrument since the quantitative determination of the samples has been optimized for this type of instrument.

All data and results of method validation are published in annex 1.

LOD and LOQ determination

To evaluate the method performance, LOD and LOQ are calculated with 60 replicates of negative samples, in accordance to CLSI EP17-A2:20012 [21]. LOD is calculated as 3SD of blank samples (use a negative matrix as a blank sample) and LOQ as 10SD of blank samples. Results are reported in following table 1.

	LC-MS/MS	LC-HRMS
LOD (µg/ml)	0.09	0.18
LOQ (µg/ml)	0.29	0.55

Table 1

Trueness and precision

In order to verify the method reproducibility in human samples (data on simulant not shown) in accordance to CLSI EP15-A3:2014 [21], a negative samples was spiked with 5 and 50 µg/ml of marker peptide: 25 samples for each level were prepared as reported in Preparation of standards and quality control solutions; results are shown following table 2.

	Mean	SD	CV%
5 µg/ml	4.98	0.41	8.23
50 µg/ml	50.12	0.36	0.72

Table 2

Discussion of the Results of the Clinical Study

The number of joint arthroplasties is steadily growing due to several factors, most notably the ageing of population and the incidence of obesity.

A painful joint arthroplasty is an alarming symptom that needs to be carefully evaluated, since it can be caused, mainly, by mechanical problems or by infection.

PJI in hip, knee and shoulder arthroplasty are a rare jet severe complication, draining financial and human resources, with specific surgical and therapeutic procedures to eliminate the infecting organism(s) and to reestablish joint function and patient wellbeing.

However, the ability to distinguish between septic and aseptic failure of the prosthesis is still a critical step and the criteria indicated by the Musculoskeletal Infection Society (MSIS), based on clinical data and laboratory tests, and does not appear to answer the clinical need in all patients' conditions.

The search for a molecule able to precociously reveal the onset of an infection, either serological or in SF, produced several putative biomarkers but alpha defensin appears to have gained the interest of scientists, manufacturers and surgeons [22,23].

During validation, biomarkers and the respective assays have to pass numerous steps: marker identification, technical assay validation, and, clinical validation, each step being foundation of the following one [24].

Only few markers pass all required validation steps. Those who have successfully passed the whole process will be reliable and robust markers for use in clinical routine.

Presently, Alfa defensin measure in SF is performed either by laboratory based alpha- defensing immunoassay or by Synovasure "quick test" (lateral flow test kit). Both methods claim high degree of accuracy in the diagnosis of PJI [25] and a recent meta-analysis compared the pooled clinical sensitivity and specificity of both methods [16], however, immunoassay are known to presents several limitations, such as: poor specificity, high day-to-day running costs, lot-to-lot variation in the antibody production and, specifically in ELISA methods, high sample volumes and long Turn Around Time (TAT).

Liquid Chromatography-Mass Spectrometry (LC-MS) offers the potential to be an alternative to immunoassays and offers the highest sensitivity and precision for the identification and detection of analytes, although it still requires high skilled professionals and may need a long TAT.

Alfa defensin has the potential to be the new paradigm for the diagnosis of periprosthetic joint infection, but in order to meet the expectations, studies on alpha defensin diagnostic accuracy should use a reliable analytical method and a sound protocol, to avoid over-estimation of the accuracy of the test.

When the IFCA Orthopedics Research group (Florence, Italy), a team of orthopedic surgeons dealing with 100 - 150 cases of PJI / year, requested the implementation of SF alpha defensin assay, these concepts where reviewed and shared with the IFCA Clinical Laboratory staff and a common strategy was settled. Briefly, the project consists in developing a new LC-MS assay, specifically designed to overcome

the peculiarity of the SF matrix and to evaluate clinical performance on a diagnostic accuracy study designed in accordance with STARD (Standards for Reporting of Diagnostic Accuracy) [26] and QUADAS (Quality Assessment of Diagnostic Accuracy Studies) [27] criterion lists. The first part of the project is reported in the present paper and was conducted by the Buzzi Lab (Prato, Italy), a public no profit organization. The protocol of the diagnostic accuracy study was written by IFCA Orthopedics Research group (Florence, Italy), approved by local ethical committee (Comitato Etico Area Vasta Centro Florence Italy) and the patients recruitment is presently ongoing.

The results of the assay validation show a robust method for qualitative and quantitative research of alpha defensin in synovial fluid. The reproducibility of both intra-day and inter-day method may be considered adequate, as well as LOD and LOQ [28].

Conclusion

To insert a new biomarker in the better position of a clinical cascade is of paramount importance for both patient outcome and resource use.

The efforts to clarify the role of SF alpha defensin in PJI clinical pathway are still ongoing. Its role as triage test, add on test or even as novel reference standard, depends on technical and clinical performances. We believe that our assay may contribute to the understanding of such methodological issues and to the economical and human resources optimization.

Annex 1

Data and Results of method validation according to bioanalytical method validation - Guidance for industry (rev. May 2018)

Items	Results	Hyperlink
Methodology	LC-MS/MS	BC0001
Method validation	Defensin-001	MIP_Def001
Biological matrix	Synovial fluid	MIP_Def001
Anticoagulant	EDTA	
Calibration curve range	0,1 - 100 ng/ml	Cal_range Sheet
Analyte of interest	Digest peptide - LWAFCC (C alckylated wit iodoacetamide)	NA
Internal standard	Digest peptide from ALNARKWYFF - monitored ALNAR	NA
Inter-run accuracy (for each QC concentration)	Spiked peptide in matrix at different concentration for QC level	A&P Sheet
Inter-run precision (for each QC concentration)		
Selectivity	Spiked peptide in matrix at concnetration of LLOQ level	Selectivity Sheet
Carryover	High QC control followed by Zero calibration	Carryover Sheet
Recovery	Included in Accuracy and Precision verification	A&P Sheet
Sensitivity	Spiked peptide in matrix at concnetration of LLOQ level	Sensitivity Sheet
Diluition	No data available at this step of work	
Stability	No data available at this step of work	
Incurred sample reanalysis (ISR)	NA	
Repeat analysis	Not permitted	

Table 3

Cal_range Sheet

Level of calibration	Theoretiacal Concentration (ng/ml)	Response (count)	Concentration from regression (ng/ml)	Difference (%)	Aceptance criteria (%)	Response
Blank	0,0					
Zero calibrator	0,0	58				
1	0,1	1846	0,108	-7,4%	±15%	Aceptable
2	0,5	12256	0,465	7,5%	±15%	Aceptable
3	1,0	39761	1,116	-10,4%	±15%	Aceptable
4	5,0	279075	4,489	11,4%	±15%	Aceptable
5	10,0	540129	9,916	0,8%	±15%	Aceptable
6	25,0	1144747	26,591	-6,0%	±15%	Aceptable
7	50,0	2254541	48,758	2,5%	±15%	Aceptable
8	100,0	6355093	100,151	-0,2%	±15%	Aceptable

Table 4

Selectivity sheet

Individual source	Response at retention time (count)	Concentration from regression (ng/ml)	Response at LLOQ (ng/ml)	LLOQ level (ng/ml)	Aceptance criteria (%)	Difference (%)	Response	IS response spike sample (count)	IS response calibrator (count)	Aceptance criteria (%)	Difference (%)	Response
Sample 1	55	0,00	0,526	0,5	±20%	5,2%	Aceptable	1357	1298	±5%	4,5%	Aceptable
Sample 2	48	0,00	0,536	0,5	±20%	7,2%	Aceptable	1265	1316	±5%	-3,9%	Aceptable
Sample 3	62	0,02	0,556	0,5	±20%	11,2%	Aceptable	1308	1356	±5%	-3,5%	Aceptable
Sample 4	54	0,00	0,436	0,5	±20%	-12,8%	Aceptable	1444	1446	±5%	-0,1%	Aceptable
Sample 5	50	0,00	0,458	0,5	±20%	-8,4%	Aceptable	1162	1147	±5%	1,3%	Aceptable
Sample 6	66	0,02	0,515	0,5	±20%	3,0%	Aceptable	1425	1435	±5%	-0,7%	Aceptable

Table 5

Carryover sheet

Samples	Response (count)	Concentration from regression (ng/ml)	LLOQ level (ng/ml)	Carryover at LLOQ (ng/ml)	Aceptance criteria (%)	Difference (%)	Response
Zero cal. - repl. 1	65	0,03	0,5	0,53	±20%	6,0%	Aceptable
Zero cal. - repl. 2	62	0,02	0,5	0,52	±20%	4,0%	Aceptable
Zero cal. - repl. 3	58	0,00	0,5	0,50	±20%	0,0%	Aceptable
Zero cal. - repl. 4	54	0,00	0,5	0,50	±20%	0,0%	Aceptable
Zero cal. - repl. 5	60	0,01	0,5	0,51	±20%	2,0%	Aceptable

Table 6

Zero calibration are injected after an injection of highest quality control calibration level (100 ng/ml).

Sensitivity sheet

Samples	Response Zero calibrator (count)	Response sample test 0,1 ng/ml (count)	Concentration from regression (ng/ml)	Nominal concentration (ng/ml)	Accuracy aceptance	Accuracy	Response	Precision aceptance	CV%	Response
Replicate 1	55	1850	0,100	0,1	±20%	0%	Aceptable	±20%	3%	Aceptable
Replicate 2	64	1912	0,104	0,1	±20%	4%	Aceptable			
Replicate 3	58	1806	0,098	0,1	±20%	-2%	Aceptable			
Replicate 4	69	1966	0,107	0,1	±20%	7%	Aceptable			
Replicate 5	71	1889	0,102	0,1	±20%	2%	Aceptable			
Replicate 6	54	1795	0,097	0,1	±20%	-3%	Aceptable	±20%	5%	Aceptable
Replicate 7	52	1823	0,099	0,1	±20%	-1%	Aceptable			
Replicate 8	61	1946	0,105	0,1	±20%	5%	Aceptable			
Replicate 9	54	1796	0,097	0,1	±20%	-3%	Aceptable			
Replicate 10	53	2013	0,109	0,1	±20%	9%	Aceptable			
Replicate 11	61	1937	0,105	0,1	±20%	5%	Aceptable	±20%	4%	Aceptable
Replicate 12	64	1749	0,095	0,1	±20%	-5%	Aceptable			
Replicate 13	58	1824	0,099	0,1	±20%	-1%	Aceptable			
Replicate 14	54	1846	0,100	0,1	±20%	0%	Aceptable			
Replicate 15	53	1921	0,104	0,1	±20%	4%	Aceptable			

Table 7

A&P Sheet

QC level LLOQ - 0,5 ng/ml

Samples	Nomimal concentration (ng/ml)	Concentration from regression (ng/ml)	Accuracy aceptance	Accuracy	Response	Precision aceptance	CV%	Response
Batch 1								
Replicate 1	1,50	1,489	±15%	-1%	Aceptable	±15%	4%	Aceptable
Replicate 2	1,50	1,476	±15%	-2%	Aceptable			
Replicate 3	1,50	1,563	±15%	4%	Aceptable			
Replicate 4	1,50	1,556	±15%	4%	Aceptable			
Replicate 5	1,50	1,489	±15%	-1%	Aceptable			
Replicate 6	1,50	1,640	±15%	9%	Aceptable			
Batch 2								
Replicate 1	1,50	1,473	±15%	-2%	Aceptable	±15%	5%	Aceptable
Replicate 2	1,50	1,445	±15%	-4%	Aceptable			
Replicate 3	1,50	1,346	±15%	-10%	Aceptable			
Replicate 4	1,50	1,565	±15%	4%	Aceptable			
Replicate 5	1,50	1,554	±15%	4%	Aceptable			
Replicate 6	1,50	1,532	±15%	2%	Aceptable			
Batch 3								
Replicate 1	1,50	1,645	±15%	10%	Aceptable	±15%	7%	Aceptable
Replicate 2	1,50	1,554	±15%	4%	Aceptable			
Replicate 3	1,50	1,457	±15%	-3%	Aceptable			
Replicate 4	1,50	1,347	±15%	-10%	Aceptable			
Replicate 5	1,50	1,348	±15%	-10%	Aceptable			
Replicate 6	1,50	1,547	±15%	3%	Aceptable			

Table 8

QC level L - 1,5 ng/ml

Samples	Nomimal concentration (ng/ml)	Concentration from regression (ng/ml)	Accuracy aceptance	Accuracy	Response	Precision aceptance	CV%	Response
Batch 1								
Replicate 1	1,50	1,489	±15%	-1%	Aceptable	±15%	4%	Aceptable
Replicate 2	1,50	1,476	±15%	-2%	Aceptable			
Replicate 3	1,50	1,563	±15%	4%	Aceptable			
Replicate 4	1,50	1,556	±15%	4%	Aceptable			
Replicate 5	1,50	1,489	±15%	-1%	Aceptable			
Replicate 6	1,50	1,640	±15%	9%	Aceptable			
Batch 2								
Replicate 1	1,50	1,473	±15%	-2%	Aceptable	±15%	5%	Aceptable
Replicate 2	1,50	1,445	±15%	-4%	Aceptable			
Replicate 3	1,50	1,346	±15%	-10%	Aceptable			
Replicate 4	1,50	1,565	±15%	4%	Aceptable			
Replicate 5	1,50	1,554	±15%	4%	Aceptable			
Replicate 6	1,50	1,532	±15%	2%	Aceptable			
Batch 3								
Replicate 1	1,50	1,645	±15%	10%	Aceptable	±15%	7%	Aceptable
Replicate 2	1,50	1,554	±15%	4%	Aceptable			
Replicate 3	1,50	1,457	±15%	-3%	Aceptable			
Replicate 4	1,50	1,347	±15%	-10%	Aceptable			
Replicate 5	1,50	1,348	±15%	-10%	Aceptable			
Replicate 6	1,50	1,547	±15%	3%	Aceptable			

Table 9

QC level M - 25 ng/ml

Samples	Nomimal concentration (ng/ml)	Concentration from regression (ng/ml)	Accuracy aceptance	Accuracy	Response	Precision aceptance	CV%	Response
Batch 1								
Replicate 1	25,00	26,789	±15%	7%	Aceptable	±15%	4%	Aceptable
Replicate 2	25,00	24,365	±15%	-3%	Aceptable			
Replicate 3	25,00	24,397	±15%	-2%	Aceptable			
Replicate 4	25,00	23,987	±15%	-4%	Aceptable			
Replicate 5	25,00	23,647	±15%	-5%	Aceptable			
Replicate 6	25,00	24,147	±15%	-3%	Aceptable			
Batch 2								
Replicate 1	25,00	26,795	±15%	7%	Aceptable	±15%	4%	Aceptable
Replicate 2	25,00	27,987	±15%	12%	Aceptable			
Replicate 3	25,00	26,467	±15%	6%	Aceptable			
Replicate 4	25,00	25,874	±15%	3%	Aceptable			
Replicate 5	25,00	24,997	±15%	0%	Aceptable			
Replicate 6	25,00	25,643	±15%	3%	Aceptable			
Batch 3								
Replicate 1	25,00	25,699	±15%	3%	Aceptable	±15%	7%	Aceptable
Replicate 2	25,00	23,124	±15%	-8%	Aceptable			
Replicate 3	25,00	23,123	±15%	-8%	Aceptable			
Replicate 4	25,00	26,951	±15%	8%	Aceptable			
Replicate 5	25,00	26,887	±15%	8%	Aceptable			
Replicate 6	25,00	23,612	±15%	-6%	Aceptable			

Table 10

QC level H - 25 ng/ml

Samples	Nomimal concentration (ng/ml)	Concentration from regression (ng/ml)	Accuracy aceptance	Accuracy	Response	Precision aceptance	CV%	Response
Batch 1								
Replicate 1	100,00	105,321	±15%	5%	Aceptable	±15%	4%	Aceptable
Replicate 2	100,00	106,951	±15%	7%	Aceptable			
Replicate 3	100,00	108,356	±15%	8%	Aceptable			
Replicate 4	100,00	104,236	±15%	4%	Aceptable			
Replicate 5	100,00	97,614	±15%	-2%	Aceptable			
Replicate 6	100,00	96,332	±15%	-4%	Aceptable			
Batch 2								
Replicate 1	100,00	96,346	±15%	-4%	Aceptable	±15%	2%	Aceptable
Replicate 2	100,00	95,328	±15%	-5%	Aceptable			
Replicate 3	100,00	94,668	±15%	-5%	Aceptable			
Replicate 4	100,00	92,187	±15%	-8%	Aceptable			
Replicate 5	100,00	94,579	±15%	-5%	Aceptable			
Replicate 6	100,00	99,125	±15%	-1%	Aceptable			
Batch 3								
Replicate 1	100,00	108,476	±15%	8%	Aceptable	±15%	5%	Aceptable
Replicate 2	100,00	105,693	±15%	6%	Aceptable			
Replicate 3	100,00	103,539	±15%	4%	Aceptable			
Replicate 4	100,00	101,663	±15%	2%	Aceptable			
Replicate 5	100,00	94,325	±15%	-6%	Aceptable			
Replicate 6	100,00	98,648	±15%	-1%	Aceptable			

Table 11

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