BSN
Biological Sales Network

FloMass Urinary Free Cortisol and Cortisone

Reagents for 200 assays

Instruction Manual

REF **EUM06200**

IVD **For *in vitro* diagnostic use**

CE

PROVIDE PERSONAL SOLUTIONS

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1 INTRODUCTION

1.1 IVD SYMBOLS

IVD	In vitro diagnostic medical device /Dispositif médical de diagnostique en vitro /In-Vitro-Diagnostikum / Producto sanitario para diagnóstico in vitro / Dispositivo medico-diagnostico in vitro / Dispositivo médico para in til in vitro diagnostik
LOT	Batch code / Code du lot / Chargenbezeichnung / Código de lote / Codice del lotto / Código do lote / Número do lote / Lotnummer
PN	Packing number / Numéro d'emballage / Packnummer / Número de envase / Numero confezioni / Número de embalagem / Número de embalagem / Emballagenummer
REF	Catalog number / Référence du catalogue / Bestellnummer / Número de catálogo / Numero di catalogo / Referência de catálogo / Código / Katalognummer
	Use by / Utiliser jusqu'au / Verwendbar bis / Fecha de caducidad / Utilizzare entro / Prazo de validade / Data limite de utilização / Holdbar til
	Temperature limitation / Limites de température / Temperaturbegrenzung / Limite de temperatura / Limiti di temperatura / Limites de temperatura / Limite de temperatura / Temperaturbegrænsning
	Add liquid / Ajout de liquide / Flüssigkeit zugeben / Añadir líquido / Aggiungi liquido / Adicionar líquido / Adicionar líquido / Tilføj væske
	Store in the dark / Conserver à l'abri de la lumière / Dunkel aufbewahren / Almacenar en ambiente oscuro / Conservare al buio / Armazenar no escuro / Guardar longe da luz / Opbevares mørkt
	Contains sufficient for <n> tests / Contenu suffisant pour "n" tests / Inhalt ausreichend für <n> Prüfungen / Contenido suficiente para <n> ensayos /Contenuto sufficiente per "n" saggi / Conteúdo suficiente para "n" ensaios / Conteúdo suficiente para <n> testes / Indeholder tilstrækkeligt til "n" test
	Consult instructions for use / Consulter les instructions d'utilisation / Gebrauchsanweisung beachten / Consulte las instrucciones de uso / Consultare le istruzioni per l'uso / Consulte as instruções de utilização / Consultar Instruções de uso / Se brugsanvisning
	Manufacturer / Fabricant / Hersteller / Fabricante / Fabbricante / Fabricante / Fabricado por / Producent
	This way up / Haut / Diese Seite oben / Este lado arriba / Questo lato in alto / Este lado para cima / Este lado para cima / Denne side op
	Recyclable / Recyclable / Recyclebar / Reciclable / Riciclabile / Reciclável / Reciclável / Genanvendeligt
	Brittle / Fragile / Zerbrechilich / Fragile / Fragil / Skrøbelig

1.2 ABBREVIATIONS

AME: Apparent Mineralocorticoids Excess
CAD: Collision Gas Pressure
CE: Collision Energy
CFU: Free Urinary Cortisol
CLSI: Clinical and Laboratory Standards Institute
CS: Cushing syndrome
CUR: Curtain Gas
CV: Coefficient of Variation
CXP: Collision Exit Potential
DP: Desolvation Potential
EP: Entrance Potential
ESI: Electrospray Ionization
GS1: Gas 1
GS2: Gas 2
HPLC-MS/MS: High Performance Liquid chromatography-tandem mass spectrometry
IS: Ion Spray Voltage
LLOD: Lower Limit of Detection
LLOQ: Lower Limit of Quantification
M/Z: Mass/Charge ratio
MPA: Mobile Phase A
MPB: Mobile Phase B
MRM: Multiple Reaction Monitoring
PP: Polypropylene
Q1: Quadrupole 1
Q3: Quadrupole 3
RT: Retention Time
S/N: Signal/Noise ratio
TEM: Source temperature

1.3 CLINICAL APPLICATION

FloMass Urinary Free Cortisol and Cortisol is an in vitro diagnostic kit intended for the quantitative determination of Cortisol and Cortisone in human urine samples using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).
The analysis of free urinary Cortisol (CFU) constitutes the first approach for the screening of endogenous Cushing syndrome (CS) at biochemical laboratory level. Endogenous Cushing syndrome is caused by prolonged exposure to high level of endogenous Cortisol. It can be caused by excessive production from one or both adrenal glands and by overproduction of adrenocorticotropic hormone that usually regulates the production of Cortisol [1-5].
Specific biochemical tests are necessary to diagnose CS because it usually manifests itself through nonspecific symptoms as hypertension, truncal obesity and mood disorders.

One of the first line tests for diagnosis is CFU that measured within 24 hours can be useful also in other clinical conditions characterized by a high level of Cortisol in serum, for example, pseudo Cushing syndrome, mental disorders, pathological obesity, poorly monitored diabetes mellitus and alcoholism.
Also, the apparent mineralocorticoid excess syndrome (AME) is due to an imbalance of Cortisol. 11ß-hydroxysteroid dehydrogenase 2 (11ß-HSD) regulates Cortisol, oxidizing it to Cortisone [1-3].
Cortisol is mainly secreted by adrenal glands, while Cortisone is mainly produced by 11ß-HSD 2 that converts bioactive Cortisol in inactive Cortisone preventing the activation of mineralocorticoid receptor caused by Cortisol [1].
Cortisol and Cortisone simultaneous determination is very important for the diagnosis of AME, CS but also congenital adrenal hyperplasia and adrenal insufficiency.
Immuno-enzymatic assays are widely used for measurement of urinary Cortisol, but it is now clear that these methods arise issues due to cross-reactions of the antibodies with endogenous steroids metabolites (Cortisone and synthetic glucocorticoids as prednisolone) [1,2].

2 PRINCIPLE OF THE METHOD

This kit is intended for the quantitative and simultaneous determination of free Cortisol and Cortisone in untreated human urine samples using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Analytes are isolated from urine samples thanks to a combined method of protein precipitation and dilution.
At the beginning of the preparatory phase, to normalize the sample preparation and the instrumental variability, the internal standard marked with stable isotopes is added (Table 1).

ANALYTE	INTERNAL STANDARD
Cortisol	Cortisol $^{2}H_{4}$
Cortisone	Cortisone $^{2}H_{8}$

Table 1: Analytes measured by kit EUM06200 and related internal standard

Once extracted, analytes are chromatographically separated by a specific reverse phase column. Subsequently, they enter into in ESI source where they are transferred to the gas phase and ionized. Then ions enter in the triple quadrupole mass spectrometer, where they are measured in MRM mode.
Thus, only selected ions with a defined mass/charge (m/z) ratio are isolated in first quadrupole and subsequently transferred in to the collision cell where they are fragmented by impact of an inert gas (nitrogen or argon). Among the fragments, only those with a defined m/z ratio are isolated in the third quadrupole for subsequent detection.
Measurement in MRM mode with HPLC separation ensures high selective and sensitive analytes identification and quantification.

3 COMPONENTS AND ACCESSORIES

3.1 KIT CONTENTS

Components for sample preparation included in the kit are shown in Table 2.

CATALOG NUMBER	DESCRIPTION	QUANTITY	STORAGE
EUM06011	Mobile Phase A	900 mL	Room temperature
EUM06012	Mobile Phase B	900 mL	Room temperature
EUM06021	Solution Precipitant	50 mL	Room temperature
EUM06031	Internal Standard	4.5 mL	-20°C

Table 2: Components, description, quantity and storage of kit EUM06200

The kit consists of reagents for 200 assays.
The expiry date of the intact kit is shown on the external product label. Follow the storage conditions given on the product label of each component of the kit and keep it away from light and/or heat.

3.2 KIT SUPPORT ACCESSORIES

CATALOG NUMBER	DESCRIPTION	QUANTITY	STORAGE
EUM06041	6-Levels Calibrators, lyophil.	2 x 6 x 1.0 mL	-20°C
EUM06051	3-Levels Controls, lyophil.	2 x 3 x 2.0 mL	-20°C
EUM00C06	Analytical Column	1 pc	Room temperature
EUM00A07	Holder + Precolumn	1 pc	Room temperature
EUM00A06	Precolumn	4 pcs	Room temperature

Table 3: Accessories, description, quantity and storage of kit EUM06200

3.3 CONTROLS AND CALIBRATION OF ANALYTICAL SYSTEM

Calibration should be done using 6-Levels Calibrators (EUM06041) containing the analytes. Calibrators should follow patient samples preparation (Chapter 7). A new calibrator series should be prepared for each analytical run.
BSN supplies quality control sets at three different concentration levels (EUM06051).
Lyophilized controls in urine matrix are useful to verify the accuracy and precision of analytical procedures and to determine the analytes in the matrix.
For the analyte concentrations, stability and accessories preparation, refer to package leaflet.

3.4 CHROMATOGRAPHIC SYSTEM

The kit has been validated using analytical column (EUM00C06) coupled to the precolumn (EUM00A06) and its holder (EUM00A07).
Stress tests on column showed that it is possible to carry out approximately 500 analysis in matrix with a single precolumn. It is recommended to perform some blank injections before each run and verify the backpressure values.

4 REQUIRED INSTRUMENTS

The kit requires a HPLC system with a tandem mass spectrometer and dedicated software. Triple quadrupole mass spectrometers should be of low or medium-high level, following two different sample preparations (Chapter 7).

4.1 REQUIRED HPLC MODULES

1. Binary pump able to support a backpressure of 600 bar or more
2. Autosampler with cooling function (10°C)
3. Column Heater (60°C)
4. Degasser

4.2 REQUIRED EQUIPMENT AND MATERIALS FOR SAMPLE PREPARATION

1. Centrifuge (10000-13000 rpm) for 1.5- or 2-mL vials
2. Vortex for vials
3. Nitrogen blow down evaporator or evaporator under vacuum/centrifuge for 1.5- or 2-mL vials, needed for protocol 2 (Paragraph 7.2)
4. Pipettes and tips
5. 1.5- or 2-mL PP vials
6. Autosampler vials with plastic adapter for 200 µL
7. Chemical hood

5 HPLC-MS/MS SYSTEM CONDITIONS

Ionization: ESI positive mode
MS/MS: specific MRM
Injection volume: 10 µL (variable according to instrumental sensitivity)
Running time: 6 min
Column Heater: 30°C

Chromatographic gradient

TIME (min)	%MPA	%MPB	FLOW (mL/min)
0.00	85	15	0.40
0.50	85	15	0.40
1.50	50	50	0.40
3.50	40	60	0.40
4.00	0	100	0.40
5.00	0	100	0.40
5.01	85	15	0.40
6.00	85	15	0.40
6.01	Stop	Stop	Stop

Table 4: Chromatographic gradient of kit EUM06200

Column conditioning: column should be conditioned for 5 min at chromatographic gradient initial condition. Then, run 3 blank injections (MPA only) using the gradient as above.

Backpressure: at a flow of 0.4 ml/min, chromatographic system backpressure should not exceed 600 bar.

Column storage: in order to preserve the column once detached from instrument, it is necessary to leave it in the to run a blank injection and insert it in the suitable package closing firmly with caps.

Example of chromatogram

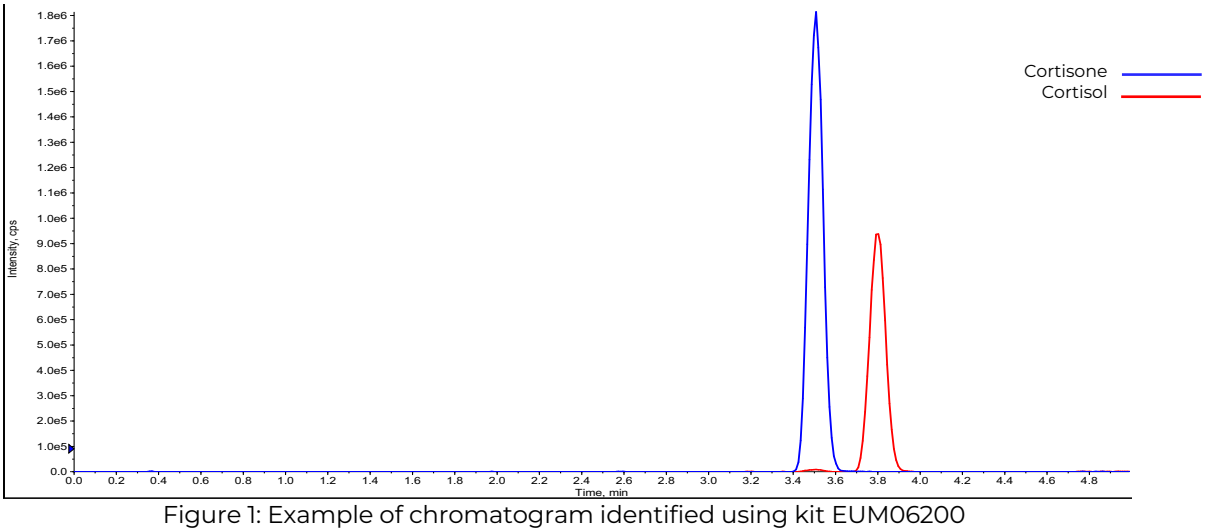

Figure 1: Example of chromatogram identified using kit EUM06200

6 SOURCE PARAMETERS AND TRANSITIONS

6.1 SOURCE PARAMETERS

Source parameters used in MS method of kit EUM06200 with a triple quadrupole mass spectrometers Sciex series X500 are shown below.

Curtain Gas (CUR): 30 psi
Collision Gas Pressure (CAD): Medium
Ion Spray Voltage (IS): 5500 V
Temperature (TEM): 450°C
Gas 1 (GS1): 40 psi
Gas 2 (GS2): 50 psi

6.2 TRANSITIONS

Monitored mass transitions and the MS parameters for each analyte using a HPLC Shimadzu Nexera combined with a Sciex series X500 triple quadrupole mass spectrometer are shown in Table 5. ESI positive mode.

ANALYTE	RT	Q1	Q3	DP	EP	CE	CXP
Cortisol 1	2.6	363.2	121	80	10	32	11
Cortisol 2	2.6	363.2	115.1	80	10	70	11
Cortisol IS	2.6	367.2	121.1	80	10	32	11
Cortisone 1	2.6	361.2	163.1	100	10	32	10
Cortisone 2	2.6	361.2	121.1	100	10	35	10
Cortisone IS	2.6	369.2	169.1	100	10	32	10

Table 5: Detected transitions, retention times and potentials using HPLC Shimadzu + Sciex mass spectrometer

Monitored mass transitions and the MS parameters for each analyte using a HPLC Shimadzu Nexera combined with an Ionics 3Q-120 triple quadrupole mass spectrometer are shown in Table 6. ESI positive source.

ANALYTE	RT	Q1	Q2	CE	CCL2	ED	EV
Cortisol 1	2.17	363.2	121.1	-33	-55	50	50
Cortisol 2	2.17	363.2	97.1	-35	-30	50	50
Cortisol IS	2.17	367.2	121.1	-33	-55	50	50
Cortisone 1	2.17	361.0	163.5	-36	-35	50	50
Cortisone 2	2.17	361.0	123.0	-45	-70	50	50
Cortisone IS	2.17	369.0	169.5	-36	-35	50	50

Table 6: Detected transitions, retention times and potentials using HPLC Shimadzu + Ionics mass spectrometer

7 SAMPLE PREPARATION

Preparation of Internal Standards Mix Solution: Transfer all the contents of Internal Standard vial (EUM06031) in Solution Precipitant bottle (EUM06021) and mix well.
This solution is stable for 12 months since the preparation date if kept at 2-8 °C.

Calibrators and quality controls follow the same sample preparation.

7.1 PROTOCOL 1 (FOR MEDIUM-HIGH SENSITIVITY INSTRUMENTS)

1. Pipette 200 µL of urine in a 1.5- or 2-mL PP vial
2. Add 200 µL of the Internal Standard Mix Solution
3. Vortex for 20-30 sec
4. Wait 5 min and then centrifuge for 5 min at 12000 rpm
5. Transfer 200 µL of supernatant in a plastic vial with low volume insert and analyze with HPLC-MS/MS technique

7.2 PROTOCOL 2 (FOR LOW SENSITIVITY INSTRUMENTS)

1. Pipette 200 µL of urine in a 1.5- or 2-mL PP vial
2. Add 200 µL of the Internal Standard Mix Solution
3. Vortex for 20-30 sec
4. Wait 5 min and then centrifuge for 5 min at 12000 rpm
5. Transfer 300 µL of supernatant in a new 1.5- or 2-mL PP vial
6. Dry the sample in a heater block (45°C) supplied with nitrogen flow (or use an evaporator under vacuum/centrifuge)
7. Resuspend pellet with 30 µL of a mix composed of MPA and MPB (50:50)
8. Vortex for 1 min
9. Centrifuge for 3 min at 12000 rpm
10. Take all the supernatant and pipette it in a plastic vial with low volume insert and analyze with HPLC-MS/MS technique

8 COLLECTION AND STORAGE OF THE SAMPLES

The kit is intended for the analysis of human urine samples collected following standard methods, such as those described in documents GP16-A3 of the Clinical and Laboratory Standards Institute (CLSI) [6]. The measurement should be performed on 24 hours' fresh urine samples, not treated with agent.

Stability of the samples: in non-treated urinary samples, Cortisol and Cortisone (with a pH value between 5 and 8) keep their values ±5% for 24 hours, if stored at 2-8°C [7].

8.1 EXPECTED VALUES AND RESULTS INTERPRETATION

Each laboratory should conduct a pilot study in order to determine the distribution of the analytes concentration in relation to its population. In order to establish the population dimension study, it is recommended to check CLSI document EP28-A3C [8].
Reference values and normal ranges are set according to the distribution.

8.2 REFERENCE RANGES

Cortisol and Cortisone reference ranges are listed in Table 7, classified by age range and gender of adults [9-12].

AGE RANGE	CORTISOL (µg/24 h)	CORTISONE (µg/24 h)
3 - 8	1.40 – 20.0	5.50 – 41.0
9 - 12	2.60 – 37.0	9.90 – 73.0
13 - 17	4.00 – 56.0	15.0 – 108
> 18 (males)	4.20 – 60.0	17.0 – 141
> 18 (females)	3.00 – 43.0	15.0 – 122

Table 7: Analytes reference values

Note: reference ranges are taken from selected and updated scientific literature. Their update corresponds to the date of revision of this document.
Reference ranges values are not a recommendation by the manufacturer, but they can be used as guideline for own reference ranges of each clinical laboratory.

9 VALIDATION DATA

Validation data have been obtained with a HPLC-MS/MS system consisting of a HPLC Shimadzu Nexera coupled to an Ionics 3Q-120 triple quadrupole mass spectrometer.
Refer to Paragraph 4.2 for the materials and equipment used in the sample preparation.

9.1 LINEARITY, DETECTION LIMITS AND QUANTIFICATION

A linear regression analysis of real values concentration has been completed in order to evaluate linearity of calibration curve for each analytic session.
Linearity range of acceptability corresponds to $R^2 \geq 0.98$. All values obtained are higher than the above-mentioned value.

Detection limit (LLOD) and quantification limit (LLOQ), which concentration provide a peak with S/N>3 and S/N>10 respectively, are reported in the table below (Table 8).

ANALYTE	LLOD (ng/mL)	LLOQ (ng/mL)	LINEARITY (ng/mL)
Cortisol	0.8	2.4	2.4 – 15000
Cortisone	0.7	2.1	2.1 - 15000

Table 8: LLOD, LLOQ and linearity

9.2 RECOVERY

Increasing amount of standard has been added to a pool of human urine (endogenous level) in order to evaluate analytical recovery characteristics. Three different levels of enriched urine (low, medium, high level) have been obtained.

ANALYTE	ENDO (ng/mL)	LOW (ng/mL)	MEDIUM (ng/mL)	HIGH (ng/mL)
Cortisol	17.4	29.6	41.4	114
Cortisone	56.9	75.7	90.3	197

Table 9: Concentrations in urine pools

Recovery = (Measured quantity on enriched matrix - Measured quantity on non-enriched matrix) / Added quantity

Average recovery range of acceptability = ±20%, all the values obtained are higher than the above-mentioned value

ANALYTE	AVERAGE RECOVERY (%)	MIN RECOVERY (%)	MAX RECOVERY (%)
Cortisol	97	87	106
Cortisone	94	80	112

Table 10: Average, minimum and maximum recovery values

9.3 PRECISION

Average concentration values (ng/mL) measured in the real urine pool (endogenous) and in 2 pools enriched with increasing concentrations of analytes (medium and high level) are reported in Table 11.
Precision has been evaluated as intra-assay, inter-assay and total coefficient of variation.

Intra-assay precision has been determined assaying 10 replicates of each sample (n = 10).
Inter-assay precision has been determined assaying 3 repetitions in 8 analytical series (n = 24) of each sample.

Total CV% = $(CV\%Intra^2 + CV\%Inter^2)^{1/2}$

Range of acceptability used for each variation coefficient are reported below.
Range of acceptability CV% Intra-assay = 10%
Range of acceptability CV% Inter-assay = 20%
Range of acceptability CV% Total = 20%

Obtained results respect the imposed ranges of acceptability.

ANALYTE	AVERAGE CONC. (ng/mL)			CV% INTRA			CV% INTER			CV% TOTAL		
	Endo	Medium	High	Endo	Medium	High	Endo	Medium	High	Endo	Medium	High
Cortisol	17.4	41.4	114	9.7%	2.2%	1.9%	2.2%	2.9%	3.5%	9.9%	3.6%	4.0%
Cortisone	56.9	90.4	197	3.8%	1.1%	1.3%	6.0%	6.5%	1.8%	7.2%	6.6%	2.2%

Table 11: Intra-assay, inter-assay and total precision

10 GENERAL LIMITATIONS

- Kit must be used with the calibrators and the internal standard indicated in the kit instructions. The use of other standards or materials with this kit has not been validated.
- The use of different mobile phases, solutions or reagents other than those indicated in Paragraph 3.1 “KIT CONTENTS” has not been validated.
- This kit has been validated with the configuration described in Chapter 9 “VALIDATION DATA”.
  The use of other triple quadrupole system, HPLC systems and columns, which may require further development of the method, has not been validated.
- Do not use the kit after the expiry date of its components.

11 REFERENCES

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ANNEX 1: EC DECLARATION OF CONFORMITY

B.S.N. Srl as Manufacturer and the only responsible for in-vitro diagnostic medical devices placed on the market under his own name, declares that these products meet all the provisions of the Legislative Decree n. 332 of the 8th September 2000, directive of in vitro diagnostic medical device 98/79/EC (in particular with regard to annex I) and subsequent amendments and additions. According to point 9 of Legislative Decree 332/2000 and subsequent amendments, the in vitro diagnostic medical device belongs to the fourth category of devices, that is GENERIC IN VITRO MEDICAL-DIAGNOSTIC DEVICES.

COMPONENT	CODE	CERTIFICATION
FloMass Urinary Free Cortisol/Cortisone	EUM06200	CE-IVD marked medical device according to Annex III
Mobile Phase A	EUM06011	CE-IVD marked medical device according to Annex III
Mobile Phase B	EUM06012	CE-IVD marked medical device according to Annex III
Solution Precipitant	EUM06021	CE-IVD marked medical device according to Annex III
Internal Standard	EUM06031	CE-IVD marked medical device according to Annex III
Calibrators for Cortisol/Cortisone in Urine, lyophil	EUM06041	CE-IVD marked medical device according to Annex III
Controls for Cortisol/Cortisone in Urine, lyophil.	EUM06051	CE-IVD marked medical device according to Annex III
Chromatographic Column	EUM00C06	CE-IVD marked medical device according to Annex III
Holder + Precolumns	EUM00A07	CE-IVD marked medical device according to Annex III
Precolumn	EUM00A06	CE-IVD marked medical device according to Annex III

Quality assurance system complying following directives:

- ✓ UNI CEI EN ISO 13485:2016,
- ✓ UNI EN ISO 9001:2015.

This declaration becomes invalid if modifications are introduced without B.S.N. Srl consent.
It is declared that the product is placed on the market in non-sterile package.
It is declared that B.S.N. Srl will keep all documents referred to the Annex III of the European Directive 98/79/EC at the disposal of the competent authorities for a 5-year period from the last date of production of the kit.
After the placing on the market of the products in question, it is declared that the Manufacturer has notified the competent authority of the application of post-market surveillance as requested from the European Directive 98/79/CE.
This declaration is valid five years from the date of issue.

Castelleone (CR), 29 April 2022 Director

ANNEX 2: INTERFERENCES

As reported in literature, in LC-UV method many interferences are known [3]. Despite better specificity, HPLC-MS/MS method is not free of interferences as well. In standard routine measurement, some interferences are reported, mainly caused by synthetic corticosteroids (Table 12) [10-13].

INTEFERING MOLECULE
Fenofibrate
Prednisone
Prednisolone
Tetrahydro-prednisolone
Dexamethasone
Beclomethasone
Fludrocortisone
11deoxy-cortisone (21Hydroxy-progesterone)
11deoxy-cortisol
11deoxy-corticosterone

Table 12: Possible interfering molecules