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HORIZONTAL - ORG

HORIZONTAL STANDARDS ON ORGANIC MICRO-POLLUTANTS FOR IMPLEMENTATION OF EU DIRECTIVES ON SLUDGE, SOIL AND TREATED BIO-WASTE

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DETERMINATION OF PHARMACEUTICAL PRODUCTS IN SLUDGE, SOILS AND SEDIMENTS.

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PU	Public	PU
PP	Restricted to other programme participants (including the Commission Services)	
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1. INTRODUCTION

Drugs absorbed by the organism after intake are subject to metabolic reactions, such hydroxylation or cleavage. However a significant amount of the original or metabolised substance leaves the organism via urine or feces. The contamination concerns ground and surface water but also wasted water and the solid matrices like sludge or soils.

Due to their polarity, persistence and water solubility, some drugs and metabolites are able to pass through the wastewater treatment plants. Their low adsorption on sludge and soils may cause the contamination of surface and ground water.

So it is necessary to analyse these molecules and this is why an analytical method by SPE-LC-MS/MS is presented here allowing to search 12 molecules belonging to the four predominant therapeutic classes in France which are analgesics/anti-inflammatories, lipid regulators, betablokers and anti-epileptics.

2. SCOPE

The purpose of this report is to present the development of an analytical method to analyse pharmaceutical compounds in solid matrices. Pharmaceuticals analysis has been carried out on a LC/MS-MS Quantum.

The main difficulty in this project is the lack of sample certified in researched analytes. Even with spiked solid matrices it is still delicate to verify correctly the impact of extraction step because it does not reproduce a real sample.

What is proposed here is an analytical method on pharmaceuticals products on sludge, soils and sediments.
This document provides a final protocol on extraction and purification tested on spiked sludge, soils and sediments with pharmaceutical compounds.

Material	Validated
Sludge	×
Soil	×
Sediment	×

3. NORMATIVE REFERENCE

Concerning pharmaceutical products normative reference does not exist.

4. TERMS AND DEFINITIONS

4.1 Analyte

The term of analyte is used to define each pharmaceutical compound searched in this project.
In the following table are presented the pharmaceutical compounds which are searched in France by this analytical method. But the operator may add other compounds according to the use in his country.
The surrogate standards are chosen according therapeutic use or practice restricted in France.

4.2 Calibration standard

A solution prepared from stock solutions of each pharmaceutical product is used to calibrate the response of the instrument with respect to analyte concentration.

4.3 Internal standard

The use of internal standard is advised. For example several internal standards are added to the sample before extraction. The internal standards are used to correct for losses during the analysis and are used for calculating the concentration of the analytes.

4.4 Sample

The sample undergoes a pre-treatment. This pre-treatment consists of homogenisation, grinding, sieving, drying, etc. The sample is ready for the chemical analysis.

5. PRINCIPLE

After pre-treatment the sample (freeze-dried) is extracted by ultrasonication with an appropriated solvent. Then the extract is purified on a suitable cartridge.
The extract is analyzed by high performance liquid chromatography (HPLC) on a C_{18} column and detected by mass spectrometry.
The identification is based on the retention times of the analytes and on the MS-detection. The detection is made with the mode MS/MS in order to avoid interferences and the problem of overquantification.

6. REAGENTS

6.1 Solvents

6.1.1 Methanol (HPLC grade)

6.1.2 Water (HPLC grade)

6.1.3 Acetonitrile (HPLC grade)

6.2 Pharmaceutical standards and internal standards for calibration

Pharmaceutical standards are of analytical grade (>90%).
For example, analysis can be undertaken with these compounds.

Therapeutic group	Name of the compound	CAS number
analgesics/anti-inflammatories	Paracetamol	103-90-2
	Paracetamol-d4	Not available
	Diclofénac	15307-79-6
	Diclofenac-d4	*Non dispo*
	Kétoprofen	22071-15-4
	Naproxen	Not available
	Ibuprofen	15687-27-1
	Phenazone	60-80-0
	Phenazone-d3	*65566-62-3*
betablokers	Métoprolol	56392-17-7
	Propranolol	318-98-9
	Propranolol-d7	*344298-99-3*
anti-epileptic	Carbamazepine	298-46-4
	Primidone	125-33-7
lipid-regulator	Bezafibrate	49562-28-9
	Gemfibrozil	25812-30-0
	Gemfibrozil-d6	*25812-30-0*

Note : It is preferable to get one internal standard per molecule. Yet if it is not possible the recovery must be checked.

6.3 Stocked solutions preparation

Stocked solution (solution 1) containing all pharmaceuticals at 100 mg/L is prepared in methanol. This solution is stored in dark glass bottles at –20°C for one month maximum.

All Deuterium-labelled substances are prepared in mix in methanol at a concentration of 100 mg/L and stored in dark glass bottles at –20°C (solution 2)

6.4 Working solutions preparation

The working solutions have to be prepared by dilution of stocked solutions each time that extractions have been done. Indeed some of pharmaceutical compounds can be deteriorated in solution in less than 24 hours (seeing in the ruggedness report n°2 sent in January 2006).

- The working solution at 1 mg/L (solution 3) is prepared by dilution of stocked solution (solution 1) in methanol for the pharmaceuticals.

- A mix of the internal standards at 100 µg/L (solution 4) is prepared by dilution of stocked solution (solution 2) in water/methanol (95/5).

Calibration standards are prepared with appropriate amounts of the working solution.

For example concentrations for the calibration can be between 5 and 500 ng/mL and concentrations for internal standards can be 100 ng.

A point of control is necessary to follow the performance of the chromatographic system. This solution, at 1 mg/L (solution 5), is prepared by dilution of stocked solution 1 in methanol. That can allow also to compare the value of internal standard in the control with the value of internal standard in the sample and to notice some possible losses. Indeed a low recovery in internal standard can not afford a good calculation for the compounds.

6.5 Others chemicals

- Sodium sulfite
- Ethylenediaminetetraaceticaciddisodium salt

6.6 Small materials

Cartridges:

- Cartridges of silica anion exchanger to eliminate interferences (cartridge 1)
- Cartridges having lipophilic and hydrophilic properties (cartridge 2). It is necessary to extract these compounds and to target the maximum of compounds

7. APPARATUS, CHROMATOGRAPHIC AND DETECTION PARAMETERS

7.1 Materials

- Ultrasonication tank
- Centrifuge
- Visiprep
- Evaporator under nitrogen

7.2 Analytical apparatus

7.2.1 Chromatographic separation

The HPLC apparatus consists of an autosampler, LC pump and a column oven. It is preferable to work with a column C_{18} with a guard column C_{18}. It is advised to maintain the column temperature at 25°C.
For example, the flow-rate is 0.2 mL/min and the injection volume is 35 µL.
For example the dimensions of the column are 150*2.1mm, 5µm and the dimensions of the guard column are 10*2.1 mm, 5 µm.
The chromatographic conditions have to be optimized in order to have good separation to not have co-elution between compounds.

7.2.2 Mass spectrometry detection

The detection is carried out using a tandem mass spectrometer.
The mode ESI is chosen to analyse the pharmaceutical compounds.
The Selected Reaction Monitoring (SRM) mode is chosen for quantification. It allows avoiding interferences.

For example, in this method, the spray voltage is fixed at 3500 V, the temperature of the ESI heater is set at 350°C and the pressure in the collision cell is set at 1.5 mTorr.

8. SAMPLING AND PRESERVATION OF SAMPLES

8.1 Sampling

Store the samples in a dark place at a temperature below 10°C, if possible in a refrigerator.
Note : Freeze-dried samples, if kept sealed, may be stored for a longer period at room temperature.

8.2 Sample pre-treatment

Before the extraction procedure, the sludge is freezed, lyophilised and grinded at 0.2 mm then the dry material is kept at room temperature in amber bottle till pre-treatment analysis.

9. PROCEDURE

9.1 Extraction of a dry sample

The extraction method is described below.
All types of solid matrices (sludge, soil, sediments and composts) are extracted following the same protocol.

a) Take 1 g of sample
b) Add 20 mL of acetonitrile with 0.1% NH_3
c) Add the internal standard and spike with the mix of pharmaceutical compounds in the medium of range
d) Put the flask in the sonication cuve and extract during 15 minutes
e) Put the flask in the centrifuge
f) Centrifuge during 10 minutes in 5822 rpm or 3600 g
g) Transfer the liquid phase in a tube and do the same operation two another times.

Note : if more sample is used, adjust the quantity of solvent. This modification has to be verified and tested.

9.2 Concentration

The total volume of extract (around 60 mL) is collected in a tube and the solvent is evaporated by the use of a gentle stream of nitrogen at room temperature until around 4-5 mL.

9.3 Clean-up

100 mL of water is added to the extract (volume of 4-5 mL). Then, the mixture is filtered and adjusted to acidic pH before the step of purification.
The clean up is carried out using cartridges 1 and 2 (the 1 is above the 2)
The two cartridges are conditioned and after loading the extract, the cartridge 1 is removed and the cartridge 2 is rinsed with different solvents. Then this cartridge is dried under nitrogen during approximately 30 minutes. The pharmaceuticals compounds are desorbed with 8 mL of methanol. The extract is reduced to dryness under nitrogen and reconstituted in 1 mL of water/methanol (95:5) prior to LC-MS/MS analysis.

10. CALIBRATION AND ANALYSIS OF SAMPLES

10.1 Blanks

10.1.1 Injection blank

Injection blank is necessary to verify the chromatographic system.
The background noise must be controlled and it must be lower than 30% of the limit of quantification. It allows verifying there are no interferences with calibrated compounds.

10.1.2 Extraction blank

The blank is obtained by doing all the protocol with solvent only.
The value of the blank must be lower than 50% of the Limit of Quantification.

10.2 Calibration

All the working solutions have to be prepared each time. They have prepared by dilution of stocked solutions. Indeed it was observed that some of the molecules can be deteriorated in solution in less than 24 hours so the calibration must be done each time (seeing in the ruggedness report n°2 sent in January 2006).

Calibration standards are prepared with appropriate amounts of the working solution (solutions 3 and 4) to achieve correct concentrations.
For example, for the range going from 5 to 500 ng/mL the quantity of internal standard can be 100 ng.

10.3 Control solution

This solution (solution 5) is prepared by dilution of stocked solution 1 in methanol for the pharmaceuticals. The value of the points of control can be located in the 20 and 80% of the range.

10.4 Analysis of samples

The background noise must be verified. The samples are controlled by calculating the control ratio which is defined below :

Control ratio R = area of internal standard in the sample/area of internal standard in the point of control

The targeted value is 1 ± 0.4.
If the control ratio is not in the range defined the operator must verify areas of internal standards in sample or in points of control in order to examine the origin of the problem.

11. CALCULATION AND EXPRESSION OF RESULTS

The method is based on internal standard calculations.

11.1 Calibration

From the chromatograms of the calibration standards we obtain a calibration curve by plotting the ratio of the mass concentrations against the ratio of the peak areas using equation (1) :

$$\frac{A_c}{A_{is,c}} = s. \frac{\rho_c}{\rho_{is,c}} + b$$

Where :
A_c is the response of analyte in the calibration standard
$A_{is,c}$ is the response of internal standard in the calibration standard
s is the slope of the calibration function
ρ_c is the mass concentration of analyte in the calibration standard solution in µg/mL
$\rho_{is,c}$ is the mass concentration of internal standard in the calibration standard solution in µg/mL
b is the intercept of the calibration curve with the ordinate

11.2 Calculation

From the chromatograms of the samples we calculate the mass concentrations of the analytes from the calibration curve using equation (2) :

$$\omega_s = \frac{(A_s / A_{is,s}) - b}{s \,.m.}$$

Where :
ω_s is the concentration of analyte found in the pre-treated sample in mg/kg/g freeze-dried sample
A_s is the response of analyte in the sample
$A_{is,s}$ is the response of internal standard in the sample
b is the intercept of the calibration curve with the ordinate

s is the slope of the calibration function
m is the mass of the test sample used for extraction in grams