Journal of Chromatography A, 1224 (2012) 19–26

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of glucocorticoids in sewage and river waters by ultra-high performance liquid chromatography–tandem mass spectrometry

P. Herrero, F. Borrull, E. Pocurull*, R.M. Marcé

Departament de Química Analítica i Química Orgànica, Universitat Rovira i Virgili Campus Sescelades, Marcel·lí Domingo, s/n, 43007 Tarragona, Spain

ARTICLE INFO

Article history:
Received 19 October 2011
Received in revised form
15 December 2011
Accepted 16 December 2011
Available online 23 December 2011

Keywords:
Glucocorticoids
Ultra-high performance liquid chromatography
Tandem mass spectrometry
Solid-phase extraction
Sewage
River water

ABSTRACT

In this paper we present a method based on ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) combined with a solid-phase extraction to determine nine glucocorticoids in river waters and sewage. In addition, we attempt the chromatographic separation of two glucocorticoid epimers (betamethasone and dexamethasone) which until now had not been determined simultaneously in environmental matrices. For SPE, we have tested three commercial polymeric polar/non-polar balanced sorbents. The recoveries were close to or above 90% in rivers and sewage influents and effluents. The repeatability expressed as relative standard deviation (%RSD, $n = 3$, 10 ng/L) was less than 8% in all cases. The method obtains LODs for glucocorticoids at low ng/L levels in aqueous environmental matrices (0.5–20 ng/L depending on the matrix and the analyte). The method was applied to determine these compounds in three Catalan rivers (Ebre, Ter and LLobregat) and two sewage treatment plants in the Tarragona area. Cortisone, cortisol, prednisone and prednisolone were frequently determined in influent sewage samples between 21 and 285 ng/L. Moreover, the two epimers were successfully determined below LOQs in some influent sewage samples.

1. Introduction

Pharmaceutical compounds are included in the group of so-called emerging organic contaminants and are frequently used for both human and veterinary use [1,2]. Synthetic glucocorticoids form an important group of these drugs and have a wide range of therapeutic applications, such as in the treatment of asthma or other inflammatory diseases. However, they also have a significant effect as growth promoters [3,4]. Due to bad farming practices around the world they were banned for use as growth promoters in the EU in 1990.

Extensive use of these compounds to treat humans and animals can lead to the transfer of these pollutants to the environment by different ways. Glucocorticoids are poorly absorbed by the organism, which means that around to 50–90% of these drugs are quickly excreted in urine and faeces. Human residues are placed directly into the urban sewer system and are carried to sewage treatment plants. Pharmaceutical residues excreted by animals can be directly filtered into the soil, or if it is used as a fertiliser, it can be scattered over a wide expanse of land. These compounds also get into the environment because they are very frequently administered with fodder (or administered directly into the water in the case of fish farms) and are therefore once more filtered directly into soil [5]. However, the amount introduced into the environment is probably small, and we have no evidence of veterinary drugs persisting in the environment [6]. Nevertheless, a continuous drip feed of these compounds over time may result a relatively high concentration or long term chronic exposure if they are not removed by sewage treatment plants. Glucocorticoids and other classes of steroid hormones are potentially endocrine disrupting chemicals and therefore, detection and identification of these compounds in environmental matrices can help to ensure human health, uncover bad farming practices, prevent hormonal disorders and other diseases in aquatic species, and determine the efficiency of sewage treatment plants in eliminating them.

Several studies have shown that current treatments used in sewage treatment plants (STPs) are not fully effective in eliminating these contaminants [7], which in turn may lead to environmental problems and a risk to human health.

Numerous studies have recently been published regarding the determination of these compounds in biological matrices [8–14] in which maximum residue levels (MRLs) have been established (UE n° 37/2010) [15]. Unfortunately, there are few studies on environmental matrices [1,5,16–23]; however, those that have been carried out confirm the presence of these drugs in the environment. Most studies have been carried out in China, and all of these have found residues of glucocorticoids in sewage influents and, to a lesser extent, effluent sewage [5,17] and sewage sludge [22].

* Corresponding author. Tel.: +34 977 55 84 92; fax: +34 977 55 84 46.
E-mail address: eva.pocurull@urv.cat (E. Pocurull).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.12.054

Studies in Japan [24] and France [19] also confirm the presence of these compounds in sewage. However, studies in river waters [17,25] show no significant presence of these drugs. Only studies in river water in China report a systematic occurrence at levels below ng/L [5,23]. A study in hospital wastewater in the Netherlands [21] reports quite high values for glucocorticoid residues.

Chromatographic methods are the most widely used to determine glucocorticoids in environmental waters. Liquid chromatography [24] and more recently ultra-high performance liquid chromatography [5], gas chromatography [26] and micellar electrokinetic chromatography [27] have been used to determine these compounds in a wide range of matrices. Liquid chromatographic techniques are the most suitable because glucocorticoids are non-volatile compounds which do not have ionisable groups. Consequently they cannot be directly analyzed using conventional GC or CE techniques, which means that it is necessary to apply complementary strategies such as derivatisation in gas chromatography or micelles in capillary electrophoresis. Tandem mass spectrometry [5], fluorescence [28] and UV–vis [29] are the most commonly used detection techniques in LC. LC–MS/MS with ESI or APCI interface are the preferred techniques due to their high sensitivity and selectivity [4]. Often, a solid-phase extraction pretreatment is necessary to determine glucocorticoids in environmental aqueous matrices because they are present at very low concentrations (low ng/L values). Several papers indicate that polar/non-polar balanced polymeric sorbents, such as Oasis HLB or Strata-X, are the most suitable for this purpose [25].

The complete chromatographic separation of glucocorticoids is not easy because they all have a very similar chemical structure. Betamethasone and dexamethasone are two epimers permitted under European legislation and they are used as anti-inflammatory and immunosuppressant. They do not possess selective fragmentation when subjected to tandem mass spectrometry and therefore it is necessary to separate them completely. However, chromatographic separation of epimers is usually omitted from the literature and, therefore, one of our priorities is the chromatographic discrimination between these two epimers and then evaluate their occurrence and fate in rivers and STPs, while the others common glucocorticoids such as cortisol, cortisone, prednisone and prednisolone besides others (methylprednisolone, flumethasone or triamcinolone acetonide) are also evaluated in the present study.

This paper focuses on the development of an analytical method based on an ultra-high performance liquid chromatography–(electrospray)–tandem mass spectrometry (UHPLC–(ESI)–MS/MS) with a previous SPE preconcentration step. The method is designed to simultaneously determine nine glucocorticoids used for both human and veterinary purposes in sewage and river water. The mass spectrometry fragmentation pathways of these compounds have also been studied. The method has been applied to water samples from two different sewage treatment plants and three Catalan rivers.

2. Experimental

2.1. Reagents and standards

The standards used were betamethasone, cortisol (hydrocortisone), cortisone, dexamethasone, dichlorisone acetate, flumethasone, methylprednisolone, prednisolone, prednisone and triamcinolone acetonide. All were purchased from Sigma–Aldrich (St. Louis, USA). The glucocorticoids' chemical structure is presented in Fig. 1. Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and storing it at −20 °C. Fresh stock solutions of 100 mg/L in methanol were prepared every month and stored at 4 °C. Working solutions were prepared daily by diluting these solutions with water/acetonitrile (4:1).

Ultrapure water was obtained using an ultrapure water purification system from Veolia waters (Sant Cugat del Vallés, Spain). Acetonitrile (ACN), methanol (MeOH) and ethyl acetate (EtOAc) were of HPLC grade from SDS (Peypin, France) and nitrogen gas was from Carburos Metálicos (Tarragona, Spain). Formic acid was purchased from Merck (Darmstadt, Germany) and used to adjust the pH of the mobile phase and the samples.

2.2. Sample preparation

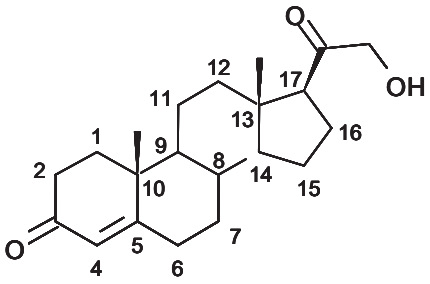
The river water samples were collected from three Catalan rivers (Ebre, Ter and Llobregat) and the sewage samples were collected from the influent and effluent of two urban sewage treatment plants (STPs) located in the area of Tarragona (STP1 and STP2), population is around to 140,000 hab. for STP1 and 107,000 hab. for STP2. The STPs receive urban sewages and some industrial discharges and use activated sludge biological treatment. The biological oxygen demand (BOD_5) for influent water is about 400 mg/L at both STPs and the average flow rate is 30,000 m^3/day for STP1 and 16,000 m^3/day for STP2. All samples were collected by using pre-cleaned amber glass bottles and filtered using a 1.2 μm glass fibre filter (Fisherbrand, Loughborough, UK) and a 0.45 μm nylon filter (Whatman, Maidstone, UK). The samples were then acidified to pH 3 with formic acid to prevent microbial growth and stored at 4 °C until analysis. Prior to analysis, the samples were allowed to reach room temperature.

2.3. UHPLC–(ESI)MS/MS conditions

The chromatographic instrument was an Agilent 1200 series (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). It was equipped with an automatic injector, a degasser, a binary pump, and a column oven. The chromatographic column was a Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm, 1.8 μm) (Agilent Technologies).

A ternary mobile phase with a gradient elution was used. Solvent A was water/acetonitrile (78:22 v/v) with formic acid (0.1%) and solvent B was methanol/acetonitrile (78:22 v/v) with formic acid (0.1%). The gradient was started at 0.8% of B, then increased to 5% in 5 min, 15% in 6.5 min, 50% in 0.5 min, kept constant for 1.5 min, increased to 99.9% in 0.5 min, kept constant for 0.5 min, and finally returned to 0.8% B in 0.5 min. All the compounds eluted within 13.5 min. The oven temperature was kept at 50 °C to reduce the backpressure and to decrease the retention factor of analytes. The flow rate was 1 mL/min and the injection volume was 50 μL.

A flow injection of a standard solution of each compound was used to find the optimum conditions for each compound in the ESI source. The average conditions selected for the optimum performance of the ESI in the negative mode were: nebuliser pressure 40 psi, drying gas (N_2) flow rate 12 L/min, drying gas temperature 350 °C, and capillary voltage 2000 V. Cone voltage and collision energies were optimised for each compound to obtain three MRM transitions, one for the quantification and two more for the confirmation of analytes. These are described in Table 1. The ratios between both transitions were used for confirmation purposes. Three time windows were used: 4–8 min (prednisone, prednisolone, hydrocortisone and cortisone), 8–12.5 min (methylprednisolone, betamethasone, flumethasone and dexamethasone) and 12.5–14 min (triamcinolone acetonide).



		1-2	6	9	11	16	17	16-17
Betamethasone	BMS	=		F	OH	CH_3	OH	
Cortisol (Hydrocortisone)	HCOR				OH		OH	
Cortisone	COR				=O		OH	
Dexamethasone	DMS	=		F	OH	CH_3	OH	
Flumethasone	FMS	=	F	F	OH	CH_3	OH	
Methylprednisolone	MPNL	=	CH_3		OH		OH	
Prednisolone	PNL	=			OH		OH	
Prednisone	PNS	=			=O		OH	
Triamcinolone acetonide	TACA	=		F	OH			O-C(CH_3)$_2$-O

Fig. 1. Chemical structure of glucocorticoids.

2.4. Solid-phase extraction

For the SPE procedure we tested a 200 mg Bond Elut Plexa (Varian, Agilent Technologies), a 500 mg Oasis HLB (Waters, Wexford, Ireland) or a 500 mg Isolute ENV+ (IST, Hengoed, UK) connected to a manifold (Teknokroma, San Cugat del Vallés, Spain) with a pump as a vacuum source.

The SPE cartridge used was Bond Elut Plexa (200 mg). It was preconditioned with 5 mL of MeOH followed by 5 mL of water. Volumes for STP influent and effluent water samples were 100 and 250 mL respectively, and 500 mL for river water samples. This sample volume was loaded into the cartridge at a flow-rate of 10–15 mL/min. Before the elution step, the sorbent was dried under vacuum. The retained analytes were eluted from the cartridge with 10 mL of MeOH. The eluate was concentrated under a flow of nitrogen gas to dryness and the residue was redissolved in 1 mL of water/acetonitrile (4:1) for river water and 3 mL of water/acetonitrile (4:1) for influent and effluent sewage.

3. Results and discussion

3.1. UHPLC–(ESI)MS/MS optimisation

Previous studies have reported that glucocorticoids form different precursor ions depending on the mobile phase and ionisation mode used [5,10]. In the present study, we have studied the formation of precursor ions and their daughter ions under the aforementioned chromatographic conditions. Because glucocorticoids do have not acid–base properties in the pH range, when a C_{18} reverse phase column is used, we chose formic acid at a concentration of 0.1% (v/v), in accordance with the literature, to maximise the ionisation of analyte in the electrospray interface ($[M+H]^+$ in positive mode or $[M+For]^-$ in negative mode).

Positive ionisation mode not only shows the $[M+H]^+$ ion, but also the intense formation of a sodium adduct $[M+Na]^+$ and the low intensity formation of a potassium adduct $[M+K]^+$. This results in a decrease in the formation of $[M+H]^+$ ion due to competition from the adduct ionisation pathways. Thus, we attempted maximise the sodium adduct formation by adding a small amount of sodium acetate (100 μM) as the mobile phase additive. This amount had to be small to prevent ion suppression caused by a high concentration of ions species in the electrospray interface. However, we needed to be able to ensure the optimal formation of the sodium adduct and its reproducibility, especially in environmental matrices.

Unfortunately, when we studied the fragmentation of these precursor ions at low collision energies, no fragmentation of the sodium adduct was observed. When these energies were increased, the *m*/*z* signal of the adduct ion disappeared as a neutral product and *m*/*z* signals were not detected. However, the precursor $[M+H]^+$ produced many fragments at low collision energies, resulting in less intense MRM transitions. Of particular note is the presence of other precursors such as $[M+H-H_2O]^+$ or $[M+H-HF]^+$, which also reduce the positive ionisation mode's suitability for these compounds because they lead to the formation of multiple precursors.

When we worked in the negative ionisation mode, the most abundantly formed precursor ion was $[M+For]^-$ [30], although chloride adducts $[M+Cl]^-$ also appeared. These adducts are less abundant than those generated by sodium in the positive ionisation mode. The precursor ion $[M+For]^-$ generates the highly abundant daughter ion $[M-H-CH_2O]^-$ for the quantification MRM transition, and other fragments for the confirmation MRM transitions. These fragmentation pathways were present in all the compounds studied except triamcinolone acetonide, whose most abundant daughter ion was $[M-H-HF]^-$. As Fig. 1 shows, triamcinolone acetonide has a slightly different structure from the other glucocorticoids that have been studied. The acetonide group ($-OC(CH_2)_2O-$) creates a heterocyclic ring between the carbons C16—C17 instead of the hydroxyl group in the C17 present in the rest of glucocorticoids. The ketone (C18) and hydroxyl groups (C17 and C19) are strongly related to the formation of adduct with formiate (see Fig. 2). Therefore the presence of the acetonide group between the carbons C16—C17 eliminates the interaction by hydrogen bond with the hydroxyl group at C17. Thus, it seems that the elimination of the (CH_2O) group is intrinsically linked to the elimination of the formiate.

Some doubt may arise as to whether the daughter ion observed is $[M-H-CH_2O]^-$ or $[M+For-C_2H_2O_2-H_2O]^-$ due to the fact that both have the same *m*/*z* ratio. However, the literature always reports that $[M-H-CH_2O]^-$ is the daughter ion and studies with acetic acid have obtained the same daughters ions from different

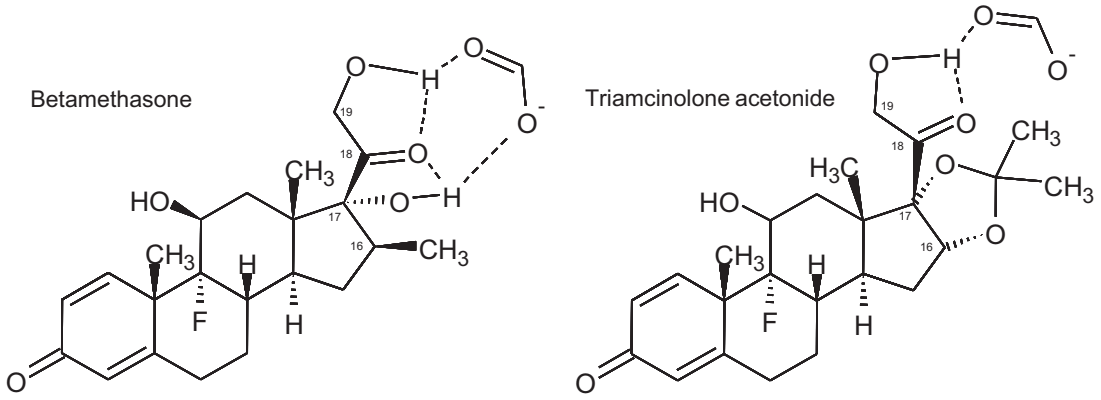


Fig. 2. Formiate adduct formation for betamethasone and triamcinolone acetonide.

parents ions $[M+AcO]^-$. This indicates that the acid is necessarily eliminated and that the daughter ion generated is the $[M-H-CH_2O]^-$. All of this confirms the hypothesis that the formiate (or acetate) and the (CH_2O) group are jointly eliminated because this neutral loss is not observed in triamcinolone acetonide, where the interaction between formiate and analyte is much more labile than in other glucocorticoids.

The parameters that affect the performance of the ESI interface in both modes were individually optimised for each compound. The variables optimised and the intervals tested were: nebuliser pressure (20–60 psi), drying gas temperature (150–350 °C), drying gas flow (5–13 L/min), capillary voltage (1500–6000 V), and cone voltage (0–200 V). The values that provided the best response were selected as the optimum values for each compound and are described in Section 2.4.

The mobile phase pH and temperature of the column have not been studied experimentally because these analytes do not show acid–base properties and therefore the pH will not have a significant effect on their chromatographic separation. The temperature did not affect the selectivity of these compounds because they have extremely similar structures. Only, an increase in column temperature decreases the retention factor of the analytes alike. Given that we were using an UHPLC column (1.8 μm), we chose a temperature of 50 °C to reduce the system backpressure. We also reduced the analysis time and did not submit the column to stress, in accordance with manufacturer's specifications. We studied between 0.1% and 0.3% (v/v) of formic acid to determine whether there had been any improvement in the ionisation of the analytes.

Using a ternary mobile phase provides many benefits in the separation of glucocorticoids. The epimers dexamethasone and betamethasone cannot be separated with methanol alone, but they can be separated with acetonitrile, as reported in the literature [31]. This has been confirmed experimentally, but unfortunately the use of acetonitrile does not provide sufficient selectivity to separate the set of more polar compounds (prednisone, prednisolone, cortisol and cortisone) or to differentiate between the flumethasone and betamethasone compounds. However, methanol does separate these compounds. We have used a quaternary pump to optimise the gradient elution and we are working with three solvents (water, methanol and acetonitrile) to find a composition of acetonitrile, which that remains constant throughout the separation and thus allows the use of a binary pump in the final method.

Separation of glucocorticoids shows a characteristic feature. All compounds show different ranges of retention times depending on similar slight variations in their structure. Thus, the first group which contains prednisone, prednisolone, hydrocortisone and cortisone compounds appears between 4.9 and 5.5 min. This set of compounds has a common structure (similar to cortisol) because all glucocorticoids are a derivates of cortisol. Methylprednisolone, betamethasone, flumethasone and dexamethasone elute between 9.9 and 11.1 min. These compounds have a methyl group linked to the carbons C6 or C9. Finally, triamcinolone acetonide shows a retention time of 13.1 min and this high retention factor is due to the presence of the acetonide group between the C16 and C17 carbons. This compound could be run faster, but if the percentage of organic phase increases more quickly also increases the ion suppression in this range. Because of this, it is necessary elute triamcinolone acetonide slowly in order to separate it from interfering substances that would not allow its determination.

3.2. SPE procedure

Since we expect to find the compounds under study at very low concentrations in the environment, it is necessary to apply a SPE procedure before chromatographic analysis. Different papers and our prior knowledge led us to choose polymeric sorbents as the most suitable for initiating the study of the SPE procedure [5,10,32,33]. Polar/non-polar balanced polymeric sorbents have good affinity with slightly polar and non-polar compounds, such are the majority of drugs. In this study we compared three commercial polymeric sorbents: Oasis HLB (vinylpirrolidone–divinylbenzene) and Bond Elut Plexa (Hydroxylated surface and polystyrene–divinylbenzene core) have a macroporous structure, and Isolute ENV+ (Hydroxylated polystyrene–divinylbenzene) has a hypercrosslinked structure. We started by testing Oasis HLB because it is the most commonly used. The pH was not expected to have any effect on the extraction of the analytes because they do not have any ionisable group, and this was corroborated by the results obtained in the two different pHs studied (3 and 7). However, the SPE procedure was done at pH 3 because the samples had been acidified to this pH with formic acid to prevent microbial growth.

The elution of the compounds was tested with two different solvents (MeOH and EtOAc) and different volumes (2–10 mL). The results in Table 2 show that MeOH is the best solvent for extracting glucocorticoids because ethyl acetate is unable to efficiently extract the compounds and only recoveries from 25 to 50% were obtained by the majority of studied glucocorticoids. Furthermore, 10 mL of methanol compared with smaller volumes slightly improved the glucocorticoid recoveries. Also, loading volumes of 500 and 1000 mL with ultrapure water were tested, but no breakthrough was found. Therefore, we decided to use a volume of 1000 mL for the subsequent tests.

The other polymeric sorbents (Bond Elut Plexa and Isolute ENV+) were tested under the conditions that had been previously optimised by the Oasis HLB sorbent in order to gauge their suitability.

Table 1
LC–MS/MS acquisition parameters in MRM mode.

Compound	tr (min)	C.V. (V)	Precursor ion (*m*/*z*)	Quantifier ion[a] (*m*/*z*)	Qualifier ion 1[a] (*m*/*z*)	Qualifier ion 2[a] (*m*/*z*)
PNS	4.9	100	403 $[M+For]^-$	327 (10) $[M-H-CH_2O]^-$	299 (15) $[M-H-CH_2O\text{-}CO]^-$	285 (30) $[M-H-CH_2O\text{-}CH_2CO]^-$
PNL	5.0	100	405 $[M+For]^-$	329 (10) $[M-H-CH_2O]^-$	280 (30) $[M-H-CH_2O-CH_4-H_2O-CH_3]^-$	295 (30) $[M-H-CH_2O-CH_4-H_2O]^-$
HCOR	5.4	100	407 $[M+For]^-$	331 (10) $[M-H-CH_2O]^-$	297 (35) $[M-H-CH_2O-CH_4-H_2O]^-$	282 (35) $[M-H-CH_2O-CH_4-H_2O-CH_3]^-$
COR	5.5	100	405 $[M+For]^-$	329 (10) $[M-H-CH_2O]^-$	301 (15) $[M-H-CH_2O-CO]^-$	311 (30) $[M-H-CH_2O-H_2O]^-$
MPNL	9.9	110	419 $[M+For]^-$	343 (10) $[M-H-CH_2O]^-$	294 (35) $[M-H-CH_2O-CH_4-H_2O-CH_3]^-$	309 (35) $[M-H-CH_2O-CH_4-H_2O]^-$
BMS	10.4	110	437 $[M+For]^-$	361 (15) $[M-H-CH_2O]^-$	307 (35) $[M-H-CH_2O-CH_4-HF-H_2O]^-$	292 (35) $[M-H-CH_2O-CH_4-HF-H_2O-CH_3]^-$
FMS	10.5	110	455 $[M+For]^-$	379 (15) $[M-H-CH_2O]^-$	305 (35) $[M-H-CH_2O-CH_4-2HF-H_2O]^-$	325 (30) $[M-H-CH_2O-CH_4-HF-H_2O]^-$
DMS	11.1	110	437 $[M+For]^-$	361 (10) $[M-H-CH_2O]^-$	307 (35) $[M-H-CH_2O-CH_4-HF-H_2O]^-$	292 (35) $[M-H-CH_2O-CH_4-HF-H_2O-CH_3]^-$
TACA	13.1	110	479 $[M+For]^-$	413 (15) $[M-H-HF]^-$	337 (20) $[M-H-C_2H_6CO-HF-H_2O]^-$	375 (10) $[M-H-C_2H_6CO]^-$

C.V. is cone voltage.
[a] Collision energies are in brackets (eV).

Table 2
Recoveries and RSDs (%, $n = 3$) for tested elution solvents.

	EtOAc		MeOH	
PNS	50	(3)	95.6	(0.9)
PNL	31	(10)	96.3	(0.7)
HCOR	32	(13)	92.5	(0.4)
COR	58	(8)	96	(1)
MPNL	32	(22)	101.1	(0.5)
BMS	25	(21)	102	(1)
FMS	28	(16)	100.6	(0.5)
DMS	27	(19)	102.8	(0.5)
TACA	82	(5)	110	(1)
DCSA	101	(5)	94	(3)

Sorbent: Oasis HLB 500 mg; load volume: 100 mL of ultrapure water spiked at 50 μg/L; and elution volume: 5 mL.

The results are shown in Table 3 which shows that both Bond Elut Plexa and Oasis HLB are the most suitable sorbents and have very similar recoveries (>90%) and repeatability (<1%) for glucocorticoids. Isolute ENV+ does not offer a good recovery (only 17%) for the most non-polar compound (dichlorisone acetate) and the recoveries obtained for the other glucocorticoids are lower (>70%) than the recoveries obtained with other sorbents under study. Therefore, both Bond Elut Plexa and Oasis HLB are suitable for carrying out an SPE procedure for glucocorticoids. Of these two sorbents, we chose the Bond Elut Plexa sorbent because less research has been conducted on it than on Oasis HLB. This meant that by studying it we could increase the range of possible SPE sorbents.

In addition, we also evaluated the evaporation to dryness of the extract and its redisolution in 1 mL of mobile phase to achieve a higher preconcentration factor and found no significant analyte losses.

Conversely, when working with environmental samples, it was likely that we would need to reduce the load volume sample depending on its origin (river water, influent or effluent sewage) because of the complexity of these matrices. We evaluated the load volume for different kinds of environmental samples and the results led us to choose a sample volume of 500 mL for river water, 250 mL for STP effluent and 100 mL for STP influent.

In first instance, the matrix effect was studied to determine whether we could use an external standard calibration curve to quantify the analytes. The matrix effect was evaluated in the three matrices (river water, STP influent and STP effluent) and was calculated by comparing the signal response obtained when spiking a sample blank after extraction with the signal response obtained from a standard solution at the same concentration. We were then able to see that all matrices cause ion suppression in all the analytes. In river water ion suppression was around 20%, but in sewage samples ion suppression increased to 65% for some analytes.

We would have used a deuterated surrogate as the internal standard to correct the error caused by ion suppression, but its low commercial availability and high price led us to reject this

Table 3
Recoveries and RSDs (%, $n = 3$) for tested sorbents.

	Oasis HLB		Bond Elut Plexa		Isolute ENV+	
PNS	93.4	(0.9)	98.8	(0.8)	77	(8)
PNL	94	(1)	97.6	(0.6)	95	(2)
HCOR	96	(0.7)	98.3	(0.3)	90	(6)
COR	97.1	(0.4)	97.3	(0.6)	70	(9)
MPNL	98	(1)	98.9	(0.1)	99.6	(0.4)
BMS	94.5	(0.8)	99.1	(0.7)	97.5	(0.3)
FMS	93	(2)	98.2	(0.9)	97.8	(0.7)
DMS	98.3	(1)	97.2	(0.5)	100.0	(0.3)
TACA	96	(4)	100.0	(0.1)	91	(2)
DCSA	99.7	(0.1)	100.1	(0.6)	16.9	(0.1)

Elution solvent: MeOH (10 mL); Load volume: 1000 mL of ultrapure water spiked at 50 μg/L.

Table 4
Validation values for the SPE-LC–MS/MS method.

	LOD (ng/L)	Linear range (ng/L)	Recovery[a] (%, $n = 3$)	Repeatability[a] (%RSD, $n = 3$)
Betamethasone	0.5[b]	1.5–100	94	2
	3.0[c]	10–600	92	4
	7.5[d]	20–1500	95	2
Cortisol (Hidrocortisone)	0.5	1.5–100	97	3
	3	10–600	96	3
	7.5	20–1500	97	3
Cortisone	0.5	1.5–100	91	3
	3	10–600	93	6
	7.5	20–1500	88	2
Dexamethasone	0.5	1.5–100	92	1
	3	10–600	90	3
	7.5	20–1500	91	6
Flumethasone	0.5	1.5–100	87	1
	3	10–600	94	5
	7.5	20–1500	91	7
Methylprednisolone	0.5	1.5–100	94	2
	3	10–600	93	7
	7.5	20–1500	93	5
Prednisolone	0.5	1.5–100	99	2
	3	10–600	90	8
	7.5	20–1500	96	3
Prednisone	0.5	1.5–100	82	4
	3	10–600	98	2
	7.5	20–1500	96	3
Triamcinolone acetonide	1.5	5–100	96	2
	9	20–600	90	5
	20	50–1500	97	4

[a] River water, effluent and influent sewage spiked at 10, 20 and 30 ng/L respectively.
[b] River water.
[c] Effluent sewage.
[d] Influent sewage.

possibility. Given that the aim of this paper is to study the presence of glucocorticoids in different kinds of waters and to evaluate their elimination in sewage treatment plants, we looked for a compound with similar structural and chemical properties. As result, we chose dichlorisone acetate as the internal standard in agreement with the literature [10]. Unfortunately, the chromatographic retention of this compound is much higher than the other glucocorticoids under study, which means that they are not suitable for use as a matrix effect corrector. In addition, their fragmentation pathways and signal responses are too different from those of the target compounds. All this resulted in the decision not to use the internal standard in our study, a decision supported by the high reproducibility obtained in the SPE procedure. We then used a matrix matched calibration curve to correct the ion suppression and recovery of the analytes.

Recovery of the analytes in environmental samples was evaluated by comparing them with a blank sample spiked before and after the SPE procedure at the same final concentration. The evaporated extracts were redissolved with a volume of 1 mL of mobile phase. In addition, a blank sample was evaluated in order to subtract the possible signal generated. The experimental results show that recoveries for all analytes exceeded 95% in all kinds of water samples (river and sewage).

We then decided to evaluate how to reduce the matrix effect. As has been discussed, the load sample volumes used do not affect the correct recovery of the analytes and only cause a signal loss due to ion suppression. Thus, an easy and quick solution would be the redisolution volume of the SPE extracts. To do this, we looked for a suitable dilution factor to reduce the matrix effect and achieve an appropriate preconcentration factor. As a result, we chose redisolution volumes of 1 mL for river samples and 3 mL for sewage waters. These volumes provided about 20% of the matrix effect in river water and sewage.

Table 5
Qualifier ion ratios in spiked samples and concentrations (ng/L) of glucocorticoids in STP1 sewages.

	Qualif. 1 ratio (%)	Qualif. 2 ratio (%)	October 2010			December 2010		February 2011		April 2011		
			Influent		Effluent	Influent	Effluent	Influent	Effluent	Influent		Effluent
PNS	20	9	<20		n.d	<20	<10	<20	<10	21	(24,11)	n.d
PNL	15	13	25	(17,12)	n.d	<20	n.d	<20	n.d	24	(16,13)	n.d
HCOR	15	16	190	(17,19)	<10	n.d	n.d	n.d	n.d	136	(18,16)	<10
COR	21	8	135	(24,9)	n.d	n.d	n.d	n.d	n.d	122	(23,7)	n.d
MPNL	20	21	<20		n.d	<20	n.d	n.d	n.d	n.d		n.d
BMS	24	18	<20		<10	<20	n.d	n.d	n.d	<20		n.d
DMS	19	14	<20		<10	<20	n.d	n.d	n.d	<20		n.d
TACA	98	78	<50		n.d	n.d	n.d	n.d	<20	n.d		<20

[a] Qualifier ion ratios (%) in samples are in brackets.

3.3. Method validation

In order to validate the method, we evaluated the linear range, the LOD, the LOQ, the repeatability (expressed as relative standard deviation) and the recoveries for glucocorticoids under optimised SPE conditions in the three kinds of water samples studied. River water, influent and effluent sewage were spiked at a level close to the limit of quantification. The spiked level was 10, 20 and 30 ng/L for river water, influent and effluent sewage respectively and analysis was carried out in triplicate.

The method was linear in the studied range (LOQ-100 ng/L for river, LOQ-600 ng/L for effluent and LOQ-1500 ng/L for influent). Limits of detection (LOD) and quantification (LOQ) were obtained experimentally. The analyte concentrations for the method LOD corresponded to an analyte signal of approximately 3 times when these was compared with the noise signal obtained in a region near to the analyte signal and the method LOQ corresponded at the lower point into the liner range of calibration curve. These limits were obtained from all three matrices and were equal for all the glucocorticoids (0.5 ng/L and 1.5 ng/L in river water, 3 ng/L and 10 ng/L in effluent sewage and 7.5 ng/L and 20 ng/L in influent sewage), except triamcinolone acetonide, whose limits were higher (1.5 ng/L and 5 ng/L in river water, 9 ng/L and 20 ng/L in effluent sewage and 20 ng/L and 50 ng/L in influent sewage). This is due to their different signal response, as discussed in Section 3.1. These limits are similar to those reported in previous methods [17,19,24,25]. Only methods described by Hong Chang et al. [5,23] achieved lower LOD and LOQ since their preconcentration factor is higher, but this method is more laborious because it includes several clean-up steps.

Analyte recoveries were calculated with a matrix calibration curve obtained from a set of blank samples fortified after the SPE procedure at different concentration levels. This operational mode ensured that the concentration required in the calibration curve was real and eliminated the signal differences between the standards and samples caused by the matrix effect. Also a blank sample was analyzed in order to subtract the possible signal of existing analytes (cortisone and hydrocortisone appeared at a significant level in the sample blanks). All recoveries exceeded 82% and in most cases were between 90 and 99%. Relative Standard Deviation (%RSD) was always less than 8% and was commonly found between 1 and 5%.

All validation parameters for the nine glucocorticoids are shown in Table 4.

4. Application to environmental samples

The SPE/UHPLC–MS/MS method developed was used to determine the nine glucocorticoids in various river waters and in influent and effluent sewages. These results show that the river waters did not contain glucocorticoid residues. However, glucocorticoids were frequently detected in the sewage samples, often between the LOD and LOQ. The results are shown in Tables 5 and 6.

In influent sewage four glucocorticoids were determined at 20–300 ng/L (prednisone, prednisolone, cortisol and cortisone) and four more were found above the LOD (methylprednisolone, betamethasone, dexamethasone and triamcinolone acetonide). In different samples of effluent sewage we found five glucocorticoids between the LOD and LOQ. Flumethasone was not detected in any sewage sample. In all cases, the presence of these compounds in the different samples was corroborated by two qualifier ion ratios and their retention time, as can be seen in Tables 5 and 6 under requirements of Commission Decision 2002/657/EC [34]. An example of the MRM chromatograms obtained for influent sewage is shown in Fig. 3.

Table 6
Concentrations (ng/L) of glucocorticoids in STP2 sewages.

	February 2011			April 2011		
	Influent		Effluent	Influent		Effluent
PNS	45	(22,11)	<10	23	(24,11)	n.d
PNL	33	(17,10)	n.d	25	(16,13)	n.d
HCOR	270	(17,20)	<10	136	(16,18)	<10
COR	285	(23,9)	n.d	122	(23,7)	n.d
MPNL	<20		n.d	n.d		n.d
BMS	<20		<10	<20		n.d
DMS	<20		<10	<20		n.d
TACA	n.d		n.d	n.d		<20

[a] Qualifier ion ratios (%) in samples are in brackets.
[b] Values in spiked samples are in Table 5.

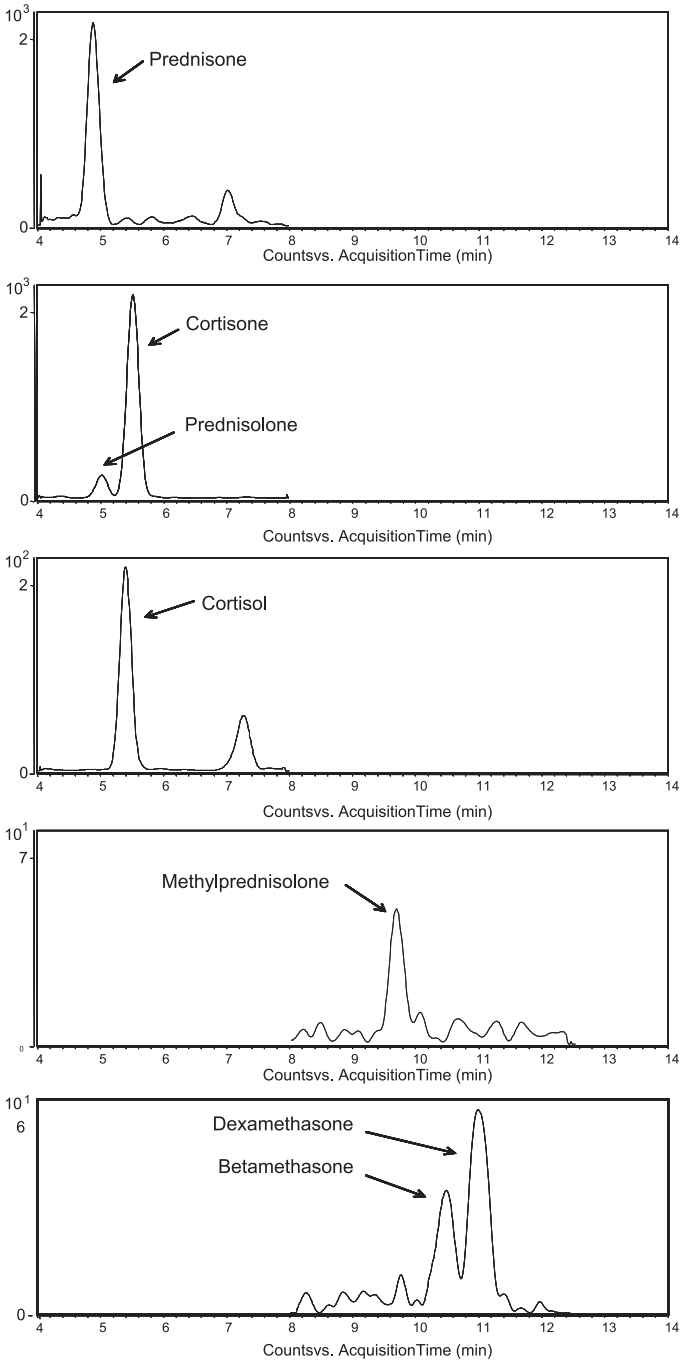


Fig. 3. MRM chromatogram obtained of influent sewage collected in STP 2 in February 2011.

The study of glucocorticoids residues in influent and effluent sewage showed that the treatments received in the two STPs under study (activated sludge biological treatment) are quite effective. The concentration found in influent sewage was reduced in effluent sewage to low ppt levels (<LOQ). However, these low levels do not allow an objective assessment of their elimination ratio and, unfortunately, on a couple of occasions glucocorticoid residues were still detected in effluent sewages at levels close to the LOD.

As expected, the highest levels of glucocorticoids corresponded to endogenous glucocorticoids (cortisol and cortisone). Nevertheless, two synthetic glucocorticoids (prednisone and prednisolone) were detected in influent sewage at significant concentrations when compared with the cortisol or cortisone results.

The results coincide with other studies in the EU and Asia, which indicates that the presence of glucocorticoid residues in river waters is negligible since only cortisone (up to 2.67 ng/L) and hydrocortisone (up to 1.9 ng/L) have been detected in rivers in China [17] and Hungary [25], respectively. Moreover, a study in urban rivers in China [23] demonstrated that river samples near to STPs discharging sites contain several glucocorticoids residues. A large number of glucocorticoid residues were detected at very low levels when sewage samples were analyzed in different countries, as is reported in the literature [5,17,19,22,24]. The most commonly detected glucocorticoids are cortisone and hydrocortisone in influent sewage. Nevertheless, they are reduced when they pass through to STPs.

5. Conclusions

The chromatographic separation of two epimers (betamethasone and dexamethasone) should be highlighted since both have been detected in certain influent and effluent sewages which had not been studied previously. This should help provide a better understanding of the presence of these compounds in environmental water samples.

We have successfully developed an analytical method based on SPE preconcentration and UHPLC–MS/MS to determine nine glucocorticoids in river waters and influent and effluent sewages at low ng/L levels in less than 13 min.

The recoveries for glucocorticoids in water samples were close to or above 90% at levels near to the LOQ in all the water types. The repeatability was less than 8% in terms of relative standard deviation.

The method was then used to determine these drugs in three rivers and two STPs in Catalonia. The most commonly found glucocorticoids were prednisone, prednisolone, hydrocortisone and cortisone in influent sewages, although other glucocorticoids were detected below the LOQ in some samples.

Acknowledgments

The authors wish to thank the personnel of the sewage treatment plants for their cooperation with all aspects of this study.

This study was financed by the General Research Directorate of the Ministry of Science and Technology of Spain (CTM2011-28765-C02-01) and the General Research Directorate of the Catalan Government (2009SGR223).

References

[1] S.C. Kim, K. Carlson, Water Res. 40 (2006) 2549.
[2] S.D. Richardson, Anal. Chem. 81 (2009) 4645.
[3] H.F. De Brabander, B. Le Bizec, G. Pinel, J.P. Antignac, K. Verheyden, V. Mortier, D. Courtheyn, H. Noppe, J. Mass Spectrom. 42 (2007) 983.
[4] Y. Yuan, C.L. Xu, C.F. Peng, Z.Y. Jin, W. Chen, L.Q. Liu, Crit. Rev. Anal. Chem. 38 (2008) 227.
[5] H. Chang, J.Y. Hu, B. Shao, Environ. Sci. Technol. 41 (2007) 3462.
[6] A. Martínez-Villalba, E. Moyano, M.T. Galceran, Rapid Commun. Mass Spectrom. 23 (2009) 1255.
[7] M. Pedrouzo, F. Borrull, R.M. Marcé, E. Pocurull, J. Chromatogr. A 1216 (2009) 6994.
[8] J.P. Noben, B. Gielen, E. Royackers, M. Missotten, A. Jacobs, J. Raus, Rapid Commun. Mass Spectrom. 16 (2002) 1590.
[9] R.L. Taylor, S.K. Grebe, R.J. Singh, Clin. Chem. 50 (2004) 2345.
[10] Y. Luo, C.E. Uboh, L.R. Soma, F.Y. Guan, J.A. Rudy, D.S. Tsang, Rapid Commun. Mass Spectrom. 19 (2005) 1245.
[11] X. Cui, B. Shao, R. Zhao, Y. Yang, J. Hu, X. Tu, Rapid Commun. Mass Spectrom. 20 (2006) 2355.
[12] B. Shao, X. Cui, Y. Yang, J. Zhang, Y. Wu, J. AOAC Int. 92 (2009) 604.
[13] O. Van den hauwe, F. Dumoulin, J.P. Antignac, M.P. Bouche, C. Elliott, C. Van Peteghem, Anal. Chim. Acta 473 (2002) 127.
[14] M. Mazzarino, F. Botre, Rapid Commun. Mass Spectrom. 20 (2006) 3465.
[15] T.E. Commission, Off. J. Eur. Commun. L15 (2010) 1.
[16] R.L. Gomes, M.D. Scrimshaw, J.N. Lester, Environ. Sci. Technol. 43 (2009) 3612.
[17] S. Liu, G.G. Ying, J.L. Zhao, F. Chen, B. Yang, L.J. Zhou, H.J. Lai, J. Chromatogr. A 1218 (2011) 1367.
[18] N. Gineys, B. Giroud, E. Vulliet, Anal. Bioanal. Chem. 397 (2010) 2295.
[19] A. Piram, A. Salvador, J.Y. Gauvrit, P. Lanteri, R. Faure, Talanta 74 (2008) 1463.
[20] E. Vulliet, J.B. Baugros, M.M. Flament-Waton, M.F. Grenier-Loustalot, Anal. Bioanal. Chem. 387 (2007) 2143.
[21] M. Schriks, J.A. van Leerdam, S.C. van der Linden, B. van der Burg, A.P. van Wezel, P. de Voogt, Environ. Sci. Technol. 44 (2010) 4766.
[22] Z. Fan, S. Wu, H. Chang, J. Hu, Environ. Sci. Technol. 45 (2011) 2725.
[23] H. Chang, Y. Wan, J. Hu, Environ. Sci. Technol. 43 (2009) 7691.
[24] Y. Kitaichi, A. Miyamoto, K. Uchikura, J. Health Sci. 56 (2010) 547.
[25] A. Tolgyesi, Z. Verebey, V.K. Sharma, L. Kovacsics, J. Fekete, Chemosphere 78 (2010) 972.
[26] L. Amendola, F. Garribba, F. Botrè, Anal. Chim. Acta 489 (2003) 233.
[27] L. Song, J. Bai, W. Zhou, Chromatographia 68 (2008) 287.
[28] N. Shibata, T. Hayakawa, K. Takada, N. Hoshino, T. Minouchi, A. Yamaji, J. Chromatogr. B 706 (1998) 191.
[29] I. Baranowska, B. Kowalski, Water Air Soil Pollut. 211 (2010) 417.
[30] O.J. Pozo, K. Deventer, P.V. Eenoo, F.T. Delbeke, Anal. Chem. 80 (2008) 1709.
[31] E.M. Malone, G. Dowling, C.T. Elliott, D.G. Kennedy, L. Regan, J. Chromatogr. A 1216 (2009) 8132.
[32] A. Tolgyesi, L. Tolgyesi, V.K. Sharma, M. Sohn, J. Fekete, J. Pharm. Biomed. Anal. 53 (2010) 919.
[33] Á. Tölgyesi, V.K. Sharma, L. Kovacsics, J. Fekete, J. Chromatogr. B 878 (2010) 1471.
[34] T.E. Commission, Off. J. Eur. Commun. L221 (2002) 8.