

Determination of natural and synthetic glucocorticoids in effluent of sewage treatment plants using ultrahigh performance liquid chromatography-tandem mass spectrometry

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Abstract A sensitive and comprehensive analytical method for glucocorticoids (GCs) in water samples was developed and applied to effluent of sewage treatment plants (STPs). In the present study, totally 10 natural and synthetic GCs, including cortisol, betamethasone valerate, clobetasol propionate, clobetasone butyrate, difluprednate, betamethasone, dexamethasone, betamethasone dipropionate, methylprednisolone, and prednisolone, were targeted. Analytes were extracted and concentrated using an OASIS HLB solid phase extraction cartridge. Chromatographic separation and quantification were achieved using an ultrahigh performance liquid chromatograph coupled with a tandem mass spectrometer (UHPLC-MS/MS). Method detection limits were 0.05 to 0.89 ng/L, which were 1–2 orders of magnitude more sensitive than in the previous reports. Cortisol was detected in more than half of (27 out of 50) analyzed effluent samples at concentrations in the range of ND–1.36 ng/L, indicating continuous discharge of natural GC via STP effluent. On the other hand, dexamethasone+betamethasone, prednisolone, betamethasone valerate, and clobetasol propionate were

detected in 25, 8, 20, and 9 samples among 50 effluent samples, respectively, suggesting not extreme but significant administration of synthetic GCs.

Keywords Glucocorticoids · Cortisol · Dexamethasone · Betamethasone · Betamethasone valerate · Prednisolone · Effluent of sewage treatment plant · LC-MS/MS

Introduction

The pollution of aquatic environment by endocrine disrupting chemicals has been of public concern for the past two decades. However, most of the studies on contamination status and mechanisms of adverse effects on reproductive health focused mainly on estrogen and androgen receptor agonists and antagonists, and only limited information is available on the other nuclear receptor ligands. Glucocorticoids (GCs) are a group of steroid hormones that play an important role as physiological regulators controlling cardiovascular, metabolic, immunologic, and homeostatic functions. GCs behave through binding to the glucocorticoid receptor (GR), which is a nuclear receptor expressed in almost all the vertebrate cells. Cortisol, which is the most predominant endogenous GC, is involved in gluconeogenesis, fat production/metabolism, and protein catabolism. Due to their immunosuppressive property, a lot of synthetic GCs have been synthesized and are commercially available for therapeutic use as anti-inflammatory pharmaceuticals against allergies, asthma, autoimmune diseases, etc. The synthetic GCs such as dexamethasone, prednisolone, and betamethasone are administered to livestock and companion animals as well as humans. Sometimes, their anti-inflammatory property leads to their application at high doses as anticarcinogens in lymphatic leukemia or myeloma patients. These medicines are excreted directly or as conjugates

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and then treated in sewage treatment plants (STP). However, some medicines may be discharged to the aquatic environment due to their low removal or deconjugation during sewage treatment processes. The adverse effect of dexamethasone on reproduction of fathead minnow was reported at a high exposure level (500 µg/L) (LaLone et al. 2012). In addition, relatively low levels of prednisolone and beclomethasone exposures resulted in increase in plasma glucose and decrease in peripheral blood leucocytes (Kugathas and Sumpter 2011). We also found increased concentrations of some amino acids in serum of common carp exposed to relatively low levels of GCs (Nakayama et al. 2014). As some of the synthetic GCs are reported to be more potent than natural cortisol, occurrence and potential risk must be investigated even if the concentrations are low. Therefore, a sensitive, reliable, robust, and comprehensive analytical method is required to understand the distribution and fate of natural and anthropogenic GCs in the aquatic environment. In recent years, some quantitative analytical methods were reported and synthetic GCs as well as endogenous cortisol were found in the aquatic environment, such as influent/effluent of STPs and river water (Chang et al. 2007; Herrero et al. 2012; Kitaichi et al. 2010; Liu et al. 2012; Schriks et al. 2010). In China, cortisol, cortisone, dexamethasone, and prednisolone were detected in the river water receiving the effluent of an STP at the maximum level of 1.9 ng/L (Chang et al. 2007). GR activity was determined in STP effluent and surface water in the Netherlands using GR CALUX bioassay (van der Linden et al. 2008). Recently, the same group reported that the main contributors to the GR agonist activity were triamcinolone acetonide, dexamethasone, and prednisolone (Schriks et al. 2010). To the best of our knowledge, there is only one report on the occurrence of GCs in the STP influent and effluent in Japan, although only betamethasone valerate was among the nine target compounds detected at a significant level in the effluent (Kitaichi et al. 2010). All the reports employed only cortisol-*d*₂ as a surrogate (or no surrogate) to correct recovery and the matrix effect. It is well known that quantitative analysis using LC-MS or LC-MS/MS encounters problems from suppression/enhancement of ionization efficiency, and the recent study recommended the use of both surrogates and internal standards (for recovery correction and response calibration, respectively) and employment of effective pretreatment steps to avoid the effect of matrices in the sample extract (Tomy et al. 2005). In the previous study, we succeeded in minimizing the matrix effect on signal intensity of hexabromocyclododecanes in LC-MS/MS using both ¹³C-labeled and deuterized compounds as the recovery correcting surrogates and response calibrating internal standards, respectively (Isobe et al. 2007). Therefore, both surrogate and internal standard correcting recovery and matrix effect are necessary for the reliable and robust quantitative analytical method using LC-MS/MS as a detector. In this regard, an analytical

method for determining GCs in aqueous samples using an ultrahigh performance liquid chromatograph coupled with a tandem mass spectrometer (UHPLC-MS/MS) was developed in the present study. We employed two surrogates and an internal standard to calibrate recoveries and responses, respectively. Additionally, the method developed was used to examine the diurnal variation of GC levels in the effluent of an STP.

Materials and methods

Chemicals and reagents

Ten GCs were selected as the target compounds for the present study; the selection was based on the production and import amount (Health Policy Bureau 2012). Information on target compounds including CAS number, chemical formula, prescribing pattern, and log K_{ow} calculated using KOWWINTM (USEPA) are summarized in Table 1. All the standards used were of the highest available purity. Cortisol, betamethasone valerate, clobetasol propionate, clobetasone butyrate, and difluprednate were purchased from Sigma-Aldrich (Tokyo, Japan). Betamethasone, dexamethasone, betamethasone dipropionate, methyl prednisolone, and prednisolone were obtained from Tokyo Chemical Industry (Tokyo, Japan). Cortisol-*d*₄ (Toronto Research Chemicals) and betamethasone dipropionate-*d*₁₀ (Tokyo Chemical Industry) were used as surrogates for correcting the loss during sample purification protocols. Dexamethasone-*d*₅ (Toronto Research Chemicals) and beclomethasone dipropionate (Sigma-Aldrich) were used as internal standards for correcting the suppression of ionization efficiency. Working standards (from 0.1 to 50 pg/µL) of GCs were prepared in methanol and stored in the dark at 4 °C.

Sample collection

To estimate the diurnal variation in GC concentrations in the effluent of a sewage treatment plant (STP), a total of 13 effluent samples were collected at a STP (Ehime, Japan) every 3 h from 6:00 May 22nd to 18:00 May 23rd, 2012. Sampling time, weather, water temperature, pH, and EC are summarized in Table 2. In addition, we conducted another series of sampling surveys to reveal the inter-plant and seasonal variations of GCs in effluents. Effluent samples were collected from 9 STPs located in Kyoto, Osaka, and Hyogo, in July and December, 2012. During the survey, 2–3 effluent samples were collected in the morning, afternoon, and evening from each location. Sewage treatment at the STPs consists of a conventional activated sludge process including primary settlement, aerobic reaction, and secondary sedimentation, and the plants are receiving mainly domestic wastewater (and partly effluents from small scale industries). Basic information on

Table 1 Information on target compounds and MRM conditions

Compound	CAS #	Chemical formula	MW	Prescribing pattern	log Kow ^a	Ionization mode (+/-)	Precursor ion (m/z)	Product ion (m/z)	DP (eV)	CE (eV)	CXP (eV)
Betamethasone dipropionate	5593-20-4	C ₂₈ H ₃₇ FO ₇	504.59	Ointment	3.66	+	527.2	453.0	56	31	36
Betamethasone valerate	2152-44-5	C ₂₇ H ₃₇ FO ₆	476.58	Ointment	3.94	+	499.1	397.1	96	23	22
Clobetasone butyrate	25122-57-0	C ₂₆ H ₃₂ ClFO ₅	478.99	Ointment	2.98	+	501.0	413.0	106	27	30
Difluprednate	23674-86-4	C ₂₈ H ₃₄ F ₂ O ₇	508.55	Ointment	3.11	+	531.0	443.1	106	35	28
Clobetasol propionate	25122-46-7	C ₂₅ H ₃₂ ClFO ₅	466.97	Ointment	2.98	+	489.1	415.1	156	25	10
Cortisol	50-23-7	C ₂₁ H ₃₀ O ₅	362.47		1.62	-	331.2	297.1	-145	-24	-15
Dexamethasone	50-02-2	C ₂₂ H ₂₉ FO ₅	392.47	Ointment	1.83	-	361.2	306.9	-190	-28	-19
Betamethasone	378-44-9	C ₂₂ H ₂₉ FO ₅	392.47	Tablet	1.94	-	361.2	306.9	-190	-28	-19
Methylprednisolone	83-43-2	C ₂₂ H ₃₀ O ₅	374.47	Injection solution	1.82	-	343.2	309.0	-175	-24	-7
Prednisolone	50-24-8	C ₂₁ H ₂₈ O ₅	360.50	Tablet	1.40	-	329.2	294.9	-190	-30	-21
Beclomethasone dipropionate	5534-09-8	C ₂₈ H ₃₇ ClO ₇	521.04	NA		+	543.1	433.0	161	31	28
Betamethasone dipropionate- <i>d</i> ₁₀						+	537.1	458.1	166	31	32
Cortisol- <i>d</i> ₄						-	335.2	301.1	-165	-30	-21
Dexamethasone- <i>d</i> ₅						-	364.3	310.1	-175	-30	-7

^a log Kow was calculated by KOWWIN (ver. 8)

DP declustering potential, CE collision energy, CXP cell exit potential

all the surveyed STPs including established year, sewage collecting area, population, residential time, and dominated sewer system are summarized in Table 3. Effluent samples were collected in 1 L amber glass bottles, which were prewashed with MeOH, transferred to the lab with ice pack and stored in the dark at -20 °C until extraction. Extraction was usually performed within 4 days after sampling to avoid any further degradation.

Solid phase extraction

Sample extraction was performed referring to the previous studies (Chang et al. 2007; Herrero et al. 2012; Kitaichi et al. 2010; Liu et al. 2012; Schriks et al. 2010). An aliquot (100 mL) of an effluent sample was spiked with surrogates, i.e., cortisol-*d*₄ and betamethasone dipropionate-*d*₁₀ (50 µL of 10 pg/µL each), filtrated through a glass fiber filter (GF/F,

Table 2 Sample description and concentrations of glucocorticoids (ng/L) in effluent samples from STP-EW

ID	Time	Weather	Tw (°C)	pH	EC (mS/m)	Cortisol	Dexamethasone/betamethasone	Prednisolone	Betamethasone valerate
EW-1	6:00	Fine	22.4	6.92	49.6	0.64	ND	ND	ND
EW-2	9:00	Fine	23.2	6.92	53.8	0.42	ND	ND	ND
EW-3	12:00	Fine	24.1	6.88	50.9	0.87	0.63	0.67	ND
EW-4	15:00	Fine	24.5	6.96	49.6	1.36	0.79	0.89	ND
EW-5	18:00	Fine	23.2	6.82	47.2	1.25	0.44	ND	ND
EW-6	21:00	Fine	22.9	6.86	50.4	0.69	0.32	ND	ND
EW-7	0:00	Fine	22.7	6.86	50.9	0.51	ND	ND	ND
EW-8	3:00	Fine	22.6	6.85	53.0	0.67	ND	ND	ND
EW-9	6:00	Fine	22.5	6.87	49.2	0.89	0.32	ND	ND
EW-10	9:00	Fine	23.7	6.84	48.8	0.55	ND	ND	ND
EW-11	12:00	Cloudy	23.8	6.82	49.6	0.76	ND	ND	3.13
EW-12	15:00	Cloudy	23.4	6.87	48.1	0.79	ND	ND	2.27
EW-13	18:00	Fine	23.3	6.8	47.0	0.62	ND	ND	2.70

ND below the detection limit

Table 3 Basic information on sewage treatment plants (STPs)

STP ID	Established year	Collecting area (km ²)	Sewage collecting population	Residential time (h)	Sewer system
EW	1962	26.8	207,783	NA	Separate/combined
KE-01	1989	47.5	419,300	11	Separate
KE-02	1973	19.3	146,800	12	Separate/combined
KE-03	1986	8.4	64,500	NA	Separate
KE-04	1939	81.5	779,600	13–17	Separate/combined
KE-05	1963	19.2	175,500	8–10	Separate/combined
KE-06	1996	68.4	287,800	17	Separate
KE-07	1970	62.6	461,700	19	Separate
KE-08	1960	18.7	292,500	NA	Combined
KE-09	1967	10.6	111,800	NA	Combined

NA not available

47 mm diameter, 0.7 µm pore size), and subjected to a SPE cartridge. An Oasis HLB plus (225 mg; Waters, Tokyo) cartridge was employed for SPE in the present study. The cartridge was preconditioned with 10 mL of MeOH and 10 mL of purified water prior to use. After passing the effluent sample, the cartridge was dried by vacuuming the air and the analytes were eluted with 5 mL of ethyl acetate. The eluate was evaporated under gentle stream of nitrogen gas, reconstituted with 450 µL of MeOH/H₂O (6:4, v/v), and transferred to a glass screw vial. The final extract was spiked with an internal standard solution including dexamethasone-*d*₅ and beclomethasone dipropionate (50 µL of 10 pg/µL each), and 10 µL of the extract was injected into an UHPLC-MS/MS. Beclomethasone dipropionate is not a labeled compound, but it is a pro-drug and was not detected during our preliminary survey of STP effluent, so we employed this compound as an internal standard.

Instrumentation

Identification and quantification were performed using an UHPLC (UHPLC-XR, Shimadzu, Kyoto, Japan) coupled with an MS/MS (Triple Quad 5500 Q-trap, ABSciex, Tokyo, Japan) operating in electrospray ionization (ESI). Chromatographic separation was achieved with an Ascentis Express F5 analytical column (100×2.1 mm, particle size 2.7 µm, Supelco, Bellefonte, USA) at a flow rate of 0.3 mL/min for both positive ionization (PI) and negative ionization (NI) mode analysis. Under the operating conditions and with this analytical column, dexamethasone and betamethasone co-eluted and was quantified from the one common peak. Although peak separation of these two compounds can be achieved under specific conditions (Herrero et al. 2012; Kitaichi et al. 2010), it would take a relatively long retention time. To reduce the run time, we chose the present chromatographic conditions with the run time less than 15 min. Mobile phases consisted of (A) 10 % acetonitrile in water (v/v) and (B)

MeOH. Gradient conditions were as follows: (A) 95 %, (B) 5 % for initial, and held for 1 min, then ramped to (A) 0 %, (B) 100 % at 10 min, and held for 2 min. Quantification was performed in multiple reaction monitoring (MRM) mode. The respective MS parameters were set as follows, curtain gas: 30 and 10 psi; collision gas: 9 and 7 psi; ion spray voltage: 5500 and -4500 V; temperature: 700 °C for both; ion source gas 1: 80 and 50 psi; and ion source gas 2: 70 and 60 psi for PI and NI mode, respectively. Analytes were identified with MRM transition (pair of precursor/product ions) and retention time (RT) of each peak. MRM transitions and other operating parameters were optimized for transmission of the [M-H]⁻ or [M+H]⁺ ions, as shown in Table 1. The mass spectrometer was controlled, and integration and quantification were performed using Analyst 1.5.1 software of AB Sciex.

Quality assurance and quality control

Linearity of the calibration curve was checked for every batch of samples by analyzing standard solutions at seven different concentrations ranging from 0.1 to 50 µg/L. The calibration curve was drawn using nominal concentration and peak area with weighed (1/X) least square regression for each target compound. Instrumental detection limit (IDL), which represents the sensitivity of an instrument, was defined as 3 times the standard deviation of seven replicate analysis of the lowest concentration standard solution (0.1 or 0.5 µg/L). Method detection limit (MDL) was calculated based on IDL and concentration factor of 200 since 100 mL of effluent was concentrated to 500 µL in this study. Recoveries and repeatability of the method were examined by five replicate analyses of Milli-Q water and effluent samples spiked with 25 ng (50 pg/µL×500 µL) of standards. Procedural blank was processed with every batch of analysis (7 samples) to check the contamination, and no significant concentration was found in any of the blank samples.

Results and discussion

Method validation

MRM chromatograms of target GCs (10 µg/L standard solution) are shown in Fig. 1. As seen from figure, good peak separation was achieved within 12 min under the conditions established in the present study. The determination coefficient (r^2) values for all the target compounds were higher than 0.99 (Table 4), which can be considered as good linearity. In the present study, all the IDLs, ranging from 0.1 to 1.8 pg as the injected amount (Table 4), were higher than the limit of quantification (LOQ) calculated from the signal-to-noise ratio of 10 (data not shown), which is adopted in many other studies. Therefore, we employed those IDLs to calculate the MDLs of the method. As a consequence, MDLs of analytes ranged from 0.05 to 0.89 ng/L and these values were, to our knowledge, at least 1–2 orders of magnitude more sensitive when compared to the reported methods (Herrero et al. 2012; Kitaichi et al. 2010). Recoveries of analytes spiked to Milli-Q water, and STP effluent were 64.6–127 and 73.5–155 %, respectively (Table 4), indicating that all the analytes were recovered quantitatively through the method and matrix effect

was corrected by internal standards. Recoveries for some compounds exceeded 100 %, which may lead underestimation of the concentrations. Recoveries were corrected with the response of internal standards. Responses of the analytes were affected by sample matrices and therefore recoveries could not be corrected perfectly using a limited number of labeled surrogates. As shown in the table, repeatability of the analysis was also evaluated using relative standard deviations (RSD). RSD values ranged from 1.0 to 7.2 % and from 2.5 to 7.4 % for Milli-Q water and STP effluent, respectively, indicating that the developed method can provide reliable results. We also performed a set of 5 replicated analysis of a STP effluent sample to estimate the repeatability, and RSDs were listed in Table 4. Although most of the analytes were not detectable, RSDs for betamethasone valerate, cortisol, and methylprednisolone were below 10 %, showing the reproducibility of the method. As mentioned above, dexamethasone and betamethasone were not separated and co-eluted in the present study. Therefore, we quantified as the total concentrations of dexamethasone and betamethasone (reported as dexamethasone+betamethasone in the following section) using a single co-eluted peak based on the response of mixed standard solution (1:1, w/w).

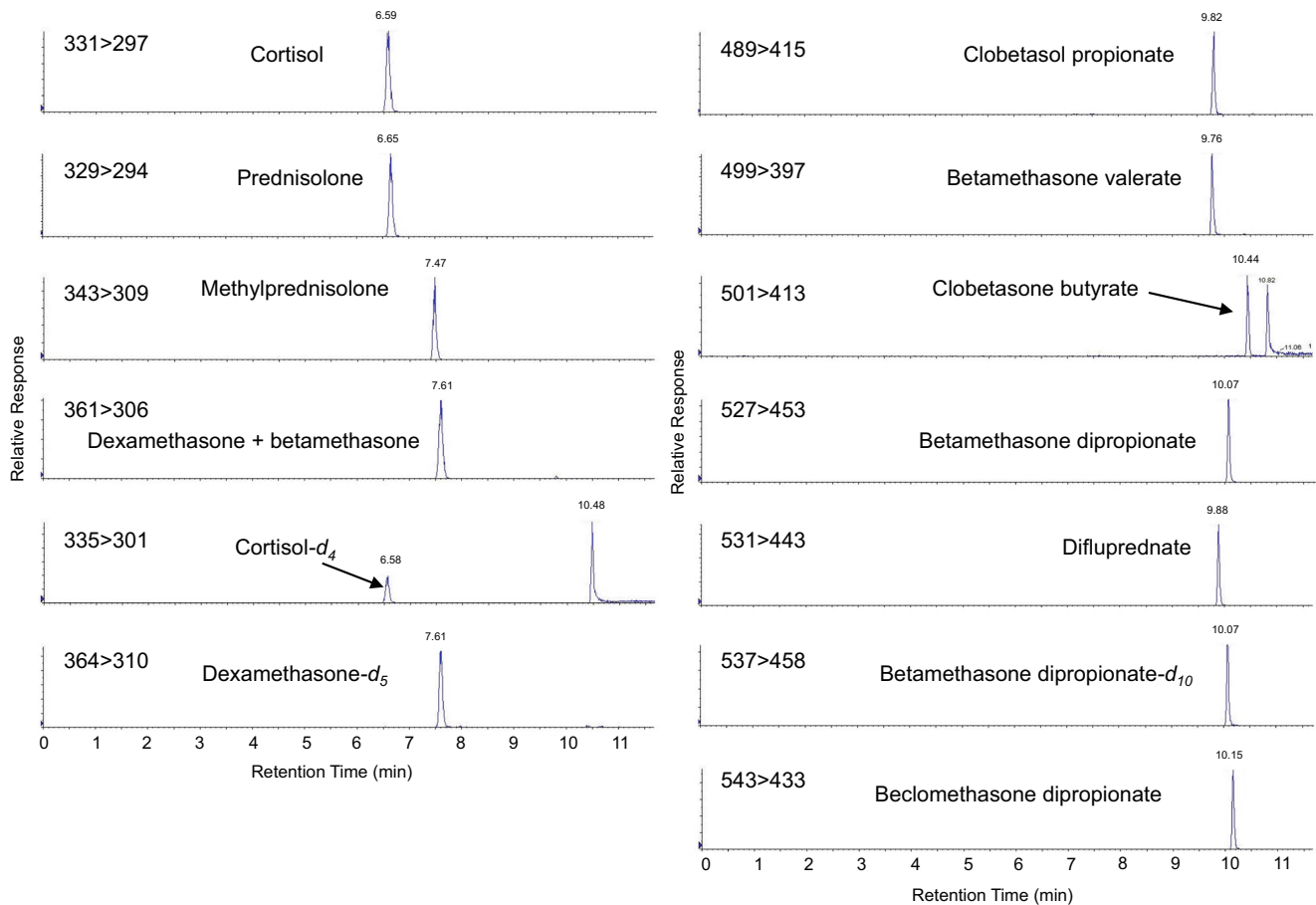


Fig. 1 MRM chromatogram of GC standard solution

Table 4 Linearity, IDL, MDL, recoveries, and repeatability of glucocorticoids

	R ²	IDL (pg)	MDL (ng/L)	Recovery (Milli-Q)		Recovery (effluent)		Repeatability
				Avg (%)	RSD (%)	Avg (%)	RSD (%)	RSD (%)
Betamethasone dipropionate	0.994	0.2	0.11	99.1	4.1	112	5.6	NA
Betamethasone valerate	0.996	0.4	0.21	127	1.0	130	6.1	4.1
Clobetasone butyrate	0.995	1.8	0.89	76.1	6.7	117	5.1	NA
Difluprednate	0.995	0.3	0.15	106	4.4	148	3.9	NA
Clobetasol propionate	0.997	0.4	0.21	126	7.2	155	5.5	NA
Progesterone	0.997	0.1	0.05	86.1	4.5	99.0	2.5	NA
Cortisol	0.993	0.1	0.05	73.9	1.1	94.8	3.3	5.4
Dexamethasone+betamethasone	0.994	0.2	0.08	114	4.6	99.6	3.1	NA
Methylprednisolone	0.995	0.3	0.13	86.2	5.6	98.9	4.1	3.8
Prednisolone	0.995	0.2	0.08	119	1.0	83.9	3.5	NA

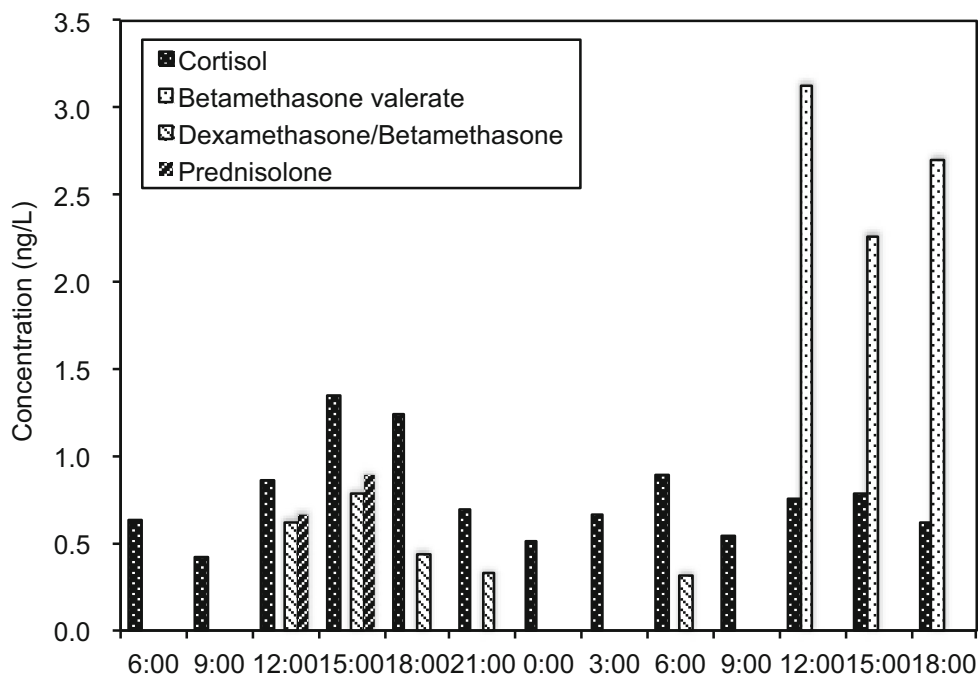
IDL instrumental detection limit, MDL method detection limit, RSD relative standard deviation, NA not available

Diurnal variation of GCs in STP effluent

Concentrations of the detected GCs during diurnal variation study (STP-EW) were summarized in Table 2 and Fig. 2. Among the 10 target GCs, only four groups of compounds including cortisol, dexamethasone+betamethasone, betamethasone valerate, and prednisolone were detected and the others were below MDLs. Because beclomethasone dipropionate is a pro-drug and difluprednate is an antedrug, these compounds could be metabolized immediately after administration and not found in wastewater. Cortisol, which was the most frequently detected compound, was found in all the analyzed effluent samples within

the range of 0.42–1.36 ng/L. Although diurnal variation in concentration was not substantial, cortisol levels were slightly higher in the afternoon samples than those collected in morning and night. During sampling period, physical/chemical characteristics (water temperature, pH, and conductivity) of effluent were fairly constant (Table 2), suggesting that wastewater treatment efficiency could be stable over time and diurnal variation in cortisol level might result from variation in levels in influent. This trend might be affected by our usual lifestyle pattern, although we could not get supporting evidence. Since normal residential time of treatment plant is 8 h, high levels of cortisol observed in the afternoon could

Fig. 2 Concentrations of cortisol, betamethasone valerate, dexamethasone/betamethasone, and prednisolone in the effluent of sewage treatment plant (STP-EW) during 6:00 22nd to 18:00 23rd May 2012



reflect the intensive use of shower and/or toilets in the morning. In contrast to cortisol, dexamethasone+betamethasone, betamethasone valerate and prednisolone were detected only in 5, 3, and 2 samples, respectively, among the 13 analyzed effluent samples. Detection patterns of those synthetic GCs were different among those three groups of compounds. Dexamethasone+betamethasone and prednisolone were detected mainly in the afternoon of day 1 (May 22nd). During

that period, cortisol levels were also relatively high, which may suggest the similar potential sources of those chemicals. On the other hand, betamethasone valerate was detected during the afternoon of day 2 (May 23rd) and not found in other samples. Since there is no information on daily fluctuation in the use of pharmaceuticals, we could not discuss these variations. Though the diurnal variations of synthetic GCs could not be explained in our study, our results indicate that there

Table 5 Concentrations of glucocorticoids (ng/L) in effluent samples from STP-KE-01-KE-09

	Year/month/day	Time	Cortisol	Dexamethasone/ betamethasone	Prednisolone	Betamethasone valerate	Clobetasol propionate
KE-01	2012/7/28	16:40	ND	ND	ND	3.7	ND
	2012/7/29	10:17	ND	0.32	ND	ND	ND
	2012/12/13	17:00	ND	0.57	ND	2.2	ND
	2012/12/14	7:20	ND	0.54	ND	2.3	ND
	2012/12/14	12:10	ND	0.85	ND	0.94	0.91
KE-02	2012/7/28	17:30	0.48	0.29	0.51	ND	ND
	2012/7/29	9:52	0.71	0.51	0.94	ND	ND
	2012/12/13	17:40	ND	0.59	0.79	ND	ND
	2012/12/14	8:00	0.73	1.3	1.6	ND	ND
	2012/12/14	12:30	1.0	ND	1.1	0.84	0.92
KE-03	2012/7/28	18:00	0.81	ND	ND	ND	ND
	2012/7/29	9:12	0.48	ND	ND	ND	ND
	2012/12/13	18:10	ND	ND	ND	ND	ND
	2012/12/14	8:30	0.42	ND	ND	ND	ND
	2012/12/14	13:00	ND	ND	ND	ND	ND
KE-04	2012/7/28	18:37	0.53	0.31	ND	ND	ND
	2012/7/29	8:38	0.48	ND	ND	ND	ND
	2012/12/13	19:00	ND	ND	ND	1.3	ND
	2012/12/14	9:30	ND	ND	ND	1.2	ND
	2012/12/14	13:30	ND	ND	ND	1.0	ND
KE-05	2012/7/28	15:15	ND	0.34	ND	ND	1.7
	2012/7/29	12:24	ND	1.1	0.69	ND	ND
	2012/12/14	18:40	ND	1.2	ND	0.82	1.4
	2012/12/15	7:00	ND	0.93	ND	0.90	2.4
	2012/12/15	12:10	ND	0.33	ND	0.99	ND
KE-06	2012/7/28	12:00	ND	0.34	ND	ND	ND
	2012/7/29	14:17	0.74	1.3	ND	ND	ND
	2012/12/14	19:30	1.3	ND	ND	4.7	2.5
	2012/12/15	7:40	0.53	ND	ND	4.3	3.0
	2012/12/15	13:30	0.47	ND	ND	2.8	2.9
KE-07	2012/12/15	10:00	ND	ND	ND	2.3	ND
	2012/12/15	14:00	ND	0.81	ND	3.6	1.3
KE-08	2012/7/28	10:00	ND	0.61	ND	ND	ND
	2012/7/29	15:20	ND	ND	ND	ND	ND
KE-09	2012/7/28	11:35	ND	0.48	ND	ND	ND
	2012/7/29	16:11	ND	0.37	ND	ND	ND
	2012/12/14	17:00	0.58	ND	ND	1.2	ND

ND below the detection limit

exists such variations for these chemicals and is reported for the first time. Levels of betamethasone valerate were relatively high among the target GCs, probably because of a high production volume of this compound in Japan (Health Policy Bureau 2012). The previous research reported that removal of betamethasone valerate during sewage treatment processes was low (Kitaichi et al. 2010). They investigated GCs in influent and effluent of sewage treatment plants, and betamethasone valerate was the only GC found in the effluent samples though some GCs were detectable in influent samples. In addition, another report showed that the removal of dexamethasone and prednisolone was higher than 99 % in sewage treatment plants (Public Works Research Institute 2011), indicating that these compounds could be removed effectively during treatment. Low removal of betamethasone valerate and high removal of dexamethasone and prednisolone during sewage treatment processes might result in relatively high level of betamethasone valerate found in the present study. Further research on the controlling factors for diurnal/daily variation in levels of these synthetic GCs is warranted to understand their fate in the aquatic environment.

Inter-plant variation in GC levels in STP effluent

Concentrations of the detected GCs during inter-plant variation study (STP-KE-01-KE-09) are summarized in Table 5. In addition to four GCs detected in STP-EW (i.e., cortisol, dexamethasone+betamethasone, betamethasone valerate, and prednisolone), clobetasol propionate was also detected in the 9 samples among the 37 analyzed samples. Although we could not perform the statistical analysis due to limited sample size, concentrations of betamethasone valerate (ND-4.7 ng/L) and clobetasol propionate (ND-3.0 ng/L) were higher than those of the other three compound groups. Only one GC was detected in STP-KE03 and 08 (cortisol and dexamethasone/betamethasone, respectively), while all the five compounds were found in STP-KE02. Cortisol was not detected in STP-KE-01, 05, 07, and 08, though it was constantly detected in STP-EW. On the other hand, no synthetic GCs were detected in KE-03. Since population, receiving capacity, residential time, and established year of treatment plants seemed not to have effect on this trend, we could not find any reason for the absence of synthetic GCs in this station. Those detected concentrations were in the range of reported concentrations in river waters or STP effluents from several countries (Chang et al. 2007; Herrero et al. 2012; Kitaichi et al. 2010; Liu et al. 2012; Schriks et al. 2010), indicating widespread GC contamination in the aquatic environment with certain geographical variations in pattern and use of synthetic GCs. Though the concentration ranges were almost same between samples in July and December, detection frequencies were higher in December than July. This may be because lower water temperature resulted in lower removal in December, so GCs were

detected more frequently compared to July. Another reason, though we could not get any evidence, could be intensive GC prescription for atopic dermatitis during winter, since winter is usually dry (i.e., less precipitation) on the Pacific side of Japan. Therefore, the seasonal variation in detection frequency/concentration of GCs should be monitored to reveal the controlling factors and potential adverse effects on the aquatic biota. In the present study, all the detected GC levels were less than reported Lowest Observable Adverse Effect Concentration (LOAEC) (Kugathas et al. 2013; Kugathas and Sumpter 2011; LaLone et al. 2012). However, further research on the toxicity of GCs in the aquatic environment is required.

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