



## UvA-DARE (Digital Academic Repository)

### High-resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in the Netherlands

Schriks, M.; van Leerdam, J.A.; van der Linden, S.C.; van der Burg, B.; van Wezel, A.P.; de Voogt, P.

**DOI**

[10.1021/es100013x](https://doi.org/10.1021/es100013x)

**Publication date**

2010

**Document Version**

Final published version

**Published in**

Environmental Science and Technology

[Link to publication](#)

**Citation for published version (APA):**

Schriks, M., van Leerdam, J. A., van der Linden, S. C., van der Burg, B., van Wezel, A. P., & de Voogt, P. (2010). High-resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in the Netherlands. *Environmental Science and Technology*, 44(12), 4766-4774. <https://doi.org/10.1021/es100013x>

**General rights**

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

**Disclaimer/Complaints regulations**

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

*UvA-DARE is a service provided by the library of the University of Amsterdam (<https://dare.uva.nl>)*

# High-Resolution Mass Spectrometric Identification and Quantification of Glucocorticoid Compounds in Various Wastewaters in The Netherlands

MERIJN SCHRIKS,<sup>\*,†</sup> JAN A. VAN LEERDAM,<sup>†</sup> SANDER C. VAN DER LINDEN,<sup>‡</sup> BART VAN DER BURG,<sup>‡</sup> ANNEMARIE P. VAN WEZEL,<sup>†</sup> AND PIM DE VOOGT<sup>†,§</sup>

KWR Watercycle Research Institute, Nieuwegein, The Netherlands, BioDetection Systems B.V., Amsterdam, The Netherlands, and Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands

Received January 4, 2010. Revised manuscript received May 3, 2010. Accepted May 11, 2010.

In the past two decades much research effort has focused on the occurrence, effects, and risks of estrogenic compounds. However, increasing emissions of new emerging compounds may also affect the action of hormonal pathways other than the estrogenic hormonal axis. Recently, a suite of novel CALUX bioassays has become available that enables looking further than estrogenic effects only. By employing these bioassays, we recently showed high glucocorticogenic activity in wastewaters collected at various sites in The Netherlands. However, since bioassays provide an integrated biological response, the identity of the responsible biological compounds remained unknown. Therefore, our current objective was to elucidate the chemical composition of the wastewater extracts used in our previous study by means of LC-high-resolution Orbitrap MS/MS and to determine if the compounds quantified could account for the observed glucocorticoid responsive (GR) CALUX bioassay response. The mass spectrometric analysis revealed the presence of various glucocorticoids in the range of 13–1900 ng/L. In extracts of hospital wastewater—collected prior to sewage treatment—several glucocorticoids were identified (cortisol 275–301 ng/L, cortisone 381–472 ng/L, prednisone 117–545 ng/L, prednisolone 315–1918 ng/L, and triamcinolone acetonide 14–41 ng/L) which are used to treat a great number of human pathologies. A potency balance calculation based on the instrumental analyses and relative potencies (REPs) of the individual glucocorticoids supports the conclusion that triamcinolone acetonide (REP = 1.3), dexamethasone (REP = 1), and prednisolone (REP = 0.2) are the main contributors to the glucocorticogenic activity in the investigated wastewater extracts. The action of these compounds is concentration additive and the overall

glucocorticogenic activity can be explained to a fairly large extent by their contribution.

## Introduction

The presence of xenobiotic compounds in our environment has become a topic of worldwide concern, especially since some of them may disrupt hormone-dependent (physiological) processes, such as vertebrate fetal development (1). Much attention has been directed to anthropogenic compounds that target the estrogen receptor such as 4-nonylphenol, bisphenol A, phthalate plasticizers (2, 3), and the natural and synthetic hormones 17 $\alpha$ -(ethynyl) estradiol, 17 $\beta$ -estradiol, estriol, and estrone (4, 5). In the last decades it has become clear that these compounds may enter the aquatic environment through effluents of sewage treatment plants (STPs) (6–8) and their presence has been linked to adverse population-level effects on aquatic vertebrates such as fish (9, 10). As such, during the last decades a large research effort is directed toward the occurrence, effects on physiological processes, and risks of (xeno)estrogenic compounds which have been extensively discussed elsewhere (2, 4, 11, 12). However, increasing emissions of new and unknown hormonally active compounds, such as pharmaceuticals and personal care products (13, 14), into the aquatic environment may also affect other important hormone-dependent physiological processes in humans and wildlife. Therefore, we recently carried out a comprehensive study on the occurrence of hormonally active compounds in various Dutch surface and waste waters using novel CALUX bioassays for estrogens, progestins, glucocorticoids, and androgens (15). We found significant levels of all activities, and particularly high levels of glucocorticogenic activity. However, since CALUX bioassays provide an integrated biological response, the identity of the responsible glucocorticogenic compounds remained to be established. A first indication of compound identity can be found in a report in which Chang and co-workers (16) demonstrate the occurrence of several natural and synthetic glucocorticoids (cortisol, cortisone, dexamethasone, 6 $\alpha$ -methylprednisolone, prednisolone, and prednisone) in sewage treatment plant effluents and receiving waters in China. The glucocorticoids most frequently detected in effluent were cortisol, cortisone, and prednisolone. Glucocorticoids are essential endocrine hormones involved in the regulation of nearly every physiological process and their effects are largely mediated through binding to the glucocorticoid receptors (GRs) (17, 18). They have been among the most frequently prescribed drugs and are applied against a great number of human pathologies including allergies, skin problems, asthma, and arthritis (19). Recently more attention has been drawn to interference of xenobiotics with targets of the glucocorticoid pathway such as the function of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) or disruption of glucocorticoid receptor (GR) function (20). Interference with these delicate mechanisms has been associated with, e.g., impaired gluconeogenesis, reproductive toxicity, hypertension, and altered vascular function (21). In addition, recent findings by Hillegass and co-workers (22), show that matrix metalloproteinase-13 is a target for dexamethasone leading to several developmental abnormalities in Zebra fish (*Danio rerio*). However, the environmental effects of glucocorticoid exposure (in the ng/L range) on other aquatic species are poorly understood at present and deserve more research attention.

Partially based on the results obtained by Chang and co-workers (16, 23), we hypothesize that the GR CALUX bioassay

\* Corresponding author phone: +31 (0)30 6069564; e-mail: merijn.schriks@kwrwater.nl.

<sup>†</sup> KWR Watercycle Research Institute.

<sup>‡</sup> BioDetection Systems B.V.

<sup>§</sup> University of Amsterdam.

response we have observed in wastewater effluents (reported in ref 15) could have been caused by (combinational) effects of such natural and synthetic glucocorticoids. Since chemical analysis was not performed in our previous study, the identity of the compounds responsible for the then-reported GR response remained unknown. Therefore, the objectives of the present research were (i) to identify and quantify the concentration of glucocorticoid compounds in wastewater extracts stored from our previous study and (ii) determine whether the compounds quantified could account for the magnitude of GR CALUX bioassay response observed. To this end, a chemical analysis of a selection of glucocorticoid compounds was performed in several potent wastewater extracts stored from our previous study on glucocorticogenic activity of wastewaters (15) by means of LC-high-resolution Orbitrap MS/MS. An inventory was made of existing glucocorticoid compounds/medicines; to this end scientific publications, pharmaceutical handbooks, and the Internet were searched and the availability of reference standards was assessed. Finally, a potency balance analysis was conducted and a mixture activity experiment was carried out to evaluate if the detected glucocorticoid compounds act in an additive or synergistic manner.

## Experimental Section

**Chemicals.** The inventory led to 18 relevant glucocorticoids for which reference standards were available. Aldosterone (CAS 52-39-1), amcinonide (CAS 51022-69-6), betamethasone (CAS 378-44-9), cortisone (CAS 53-06-5), dexamethasone (CAS 50-02-2), flunisolide (CAS 3385-03-3), fluorometholone (CAS 426-13-1), hydrocortisone (cortisol) (CAS 50-23-7), 6 $\alpha$ -methylprednisolone (CAS 83-43-2), prednisolone (CAS 50-24-8), prednisone (CAS 53-03-2), triamcinolone (CAS 83474-03-7), and triamcinolone acetonide (CAS 8054-16-8) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The compounds desoximetasone (CAS 382-67-2), prednicarbate (CAS 73771-04-7), rimexolone (CAS 49697-38-3), and triamcinolone hexacetonide (CAS 5611-51-8) were purchased from Chemos (Regenstauf, Germany). Paramethasone (CAS 53-33-8) was obtained from the Schering-Plough Corporation (Oss, The Netherlands). These standards represent compounds that are known to be present in drugs and have GR mediated activity. Glucocorticoid standards were purchased in the highest purity available (96% or higher). Dimethylsulfoxide (DMSO) was at least 99.7% pure and purchased from Merck (Darmstadt, Germany). The solvent ethyl acetate was obtained from Baker (Deventer, The Netherlands). Water used for LC-MS/MS analysis was generated from an ultra pure water system from Millipore (Bedford, MA) with a specific resistance of 18 M $\Omega$ ·cm. Ultra gradient acetonitrile (Mallinckrodt Baker, Deventer, The Netherlands) was used as an organic modifier. Chemicals to strip wastewater of glucocorticogenic compounds, including 4C Norit A charcoal and dextran T-70, were obtained from Sigma-Aldrich. Stock solutions of the individual glucocorticoid standards were prepared at 1 mM in DMSO and stored at -20 °C in brown glass vials (Thermo Electron corporation, Breda, The Netherlands) in the dark.

**Samples.** For the present study, various potent wastewater extracts in DMSO, stored from our previous study, were chemically analyzed (15). We selected an industrial wastewater (from a factory producing veterinary medicines), a hospital wastewater (further referred to as hospital wastewater 1), a paper mill effluent, and a municipal sewage treatment plant effluent. These extracts had been stored for approximately two years at -20 °C in conical glass vials (Omnilabo, Breda, The Netherlands) in the dark. Furthermore, a new batch of 25 L of hospital wastewater was collected on 8/19/2009 in an ultracleaned stainless steel drum to (i) get an impression of the identity/quantity of glucocorticoids

in hospital wastewater at a different point in time (further referred to as hospital wastewater 2), and (ii) provide a suitable wastewater matrix to perform recovery experiments of individual glucocorticoid standards. To obtain wastewater devoid of glucocorticoid compounds, hospital wastewater 2 was stripped with activated charcoal as described for the removal of steroids in fetal bovine serum (24). Briefly, freshly collected (hospital) wastewater was filtered over a 5  $\mu$ m Whatman grade no. 3 paper filter ('s Hertogenbosch, The Netherlands) and stirred overnight with 2.5 g of dextran coated activated charcoal (DCC) per liter of wastewater at 4 °C. Next, wastewater was centrifuged at 500g for 10 min to pellet the charcoal and subsequently the stripped wastewater was filtered over a 1  $\mu$ m Envirocheck HV cartridge (Pall Gelman Laboratory, Ann Arbor, MI) prior to 0.45  $\mu$ m filtration on cellulose nitrate filters (Sartorius). All glassware was extensively cleaned with sodium hydroxide in ethanol absolute and all materials were rinsed with distilled acetone and distilled petroleum ether prior to use. Finally, stripped wastewater was also utilized to perform an additional experiment to assess the combined action of glucocorticoids in a mixture. To this end, 6 glass bottles with 1 L of stripped wastewater were spiked in duplicate with stock solutions (1 mM in DMSO) of 5 individual glucocorticoid standards (cortisol, cortisone, prednisolone, prednisone, and triamcinolone acetonide) at concentration levels of 210, 290, 230, 90, and 30 ng/L, and with a mixture of the same set of glucocorticoid standards, respectively. The rationale for choosing these compounds is to mimic a true environmental sample being hospital wastewater 1 (cf. Table 2 for instrumentally detected concentration levels of glucocorticoids). After a 3-fold extraction with ethyl acetate (according to ref 15), the samples were redissolved in 50  $\mu$ L of DMSO followed by subsequent LC-MS/MS and GR CALUX bioassay analysis. Potency balance analyses were conducted by comparing the observed GR CALUX bioassay response magnitudes to those predicted based on the concentrations of known glucocorticoid standards present in the extract. Concentrations of individual glucocorticoids as determined by means of LC-MS/MS were multiplied by their GR CALUX bioassay specific relative potencies (REPs) (calculated by dividing the EC<sub>50</sub> of the reference compound dexamethasone by the EC<sub>50</sub> of compound x; (25)). The sum of the products for all target compounds present in an extract provides an estimate of the dexamethasone equivalents (dex EQs) in the extracts.

### Recovery Determination of Glucocorticoid Standards.

To determine the recovery of the extraction procedure for a set of 18 glucocorticoid standards, duplicate samples of 1 L of surface water and stripped (hospital) wastewater were spiked with a mixture of compounds at a concentration level of 1  $\mu$ g/L. An exception was made for cortisol, cortisone, prednisolone, prednisone, and triamcinolone acetonide which were spiked at realistic environmental concentration levels of 210, 290, 230, 90, and 30 ng/L, respectively. Spiked surface waters/stripped wastewaters were extracted thrice with ethyl acetate and extracts were evaporated to dryness and redissolved in 50  $\mu$ L of DMSO. The extracts were next diluted 1:9 (v:v) in ultrapure water and subsequently 1:1 (v:v) in acetonitrile (final volume 1 mL containing 5% DMSO) upon LC-MS/MS analysis.

**Mass Spectrometric Conditions.** The LC-LTQ-FT Orbitrap MS system consisted of a Surveyor autosampler model Plus, a Surveyor quaternary gradient LC pump, and an LTQ-FT Orbitrap mass spectrometer (Thermo Electron GmbH, Bremen, Germany). The linear ion trap (LTQ) part of the hybrid MS system was equipped with an Ion Max Electrospray Ionization (ESI) probe and operated in the positive ion mode. Full-scan accurate mass spectra (mass range from 50 to 600 Da) were obtained at high resolution (30,000 fwhm) and

**TABLE 1. Glucocorticoid Standards Investigated in This Study, and Characteristics of GR CALUX Bioassay and LC-MS/MS**

compound	CAS no.	elemental composition	GR CALUX EC50 (nM)	REP <sup>a</sup>	retention time on LC-MS/MS (min.)	m/z (theoretical) [M + H] <sup>+</sup>	product ion mass 1 (Da)	product ion mass 2 (Da) (relative abundance to product ion mass 1)
aldosterone	52-39-1	C21H28O5	112.2 ± 4.84	0.008 ± 0.06	20.97	361.20095	343	315 (25%)
amcinonide	51022-69-6	C28H35FO7	0.49 ± 0.04	1.7 ± 0.09	40.33	503.24396	483	399 (90%)
betamethasone <sup>b</sup>	378-44-9	C22H29FO5	1.02 ± 0.05	0.8 ± 0.06	25.71	393.20718	373	355 (20%)
cortisol	50-23-7	C21H30O5	11.4 ± 0.87	0.07 ± 0.08	22.56	363.21660	327	309 (70%)
cortisone	53-06-5	C21H28O5	>1000 <sup>c</sup>	<0.0008 ± 0.00006	23.19	361.20095	343	163 (80%)
desoximetasone	382-67-2	C22H29FO4	0.66 ± 0.03	1.3 ± 0.06	28.99	377.21226	357	339 (30%)
dexamethasone	50-02-2	C22H29FO5	0.84 ± 0.03	1 ± 0.05	25.94	393.20718	373	355 (30%)
flunisolide	3385-03-3	C24H31FO6	0.49 ± 0.03	1.7 ± 0.07	27.32	435.21774	321	397 (100%)
fluorometholone	426-13-1	C22H29FO4	0.59 ± 0.03	1.4 ± 0.06	29.14	377.21226	357	399 (50%)
6 $\alpha$ -methylprednisolone	83-43-2	C22H30O5	2.25 ± 0.14	0.4 ± 0.07	24.88	375.21660	357	339 (60%)
paramethasone <sup>b</sup>	53-33-8	C22H29FO5	1.14 ± 0.04	0.7 ± 0.05	25.71	393.20718	337	355 (90%)
prednicarbate	73771-04-7	C27H36O8	4.75 ± 0.20	0.2 ± 0.06	39.80	489.24829	381	471 (80%)
prednisolone	50-24-8	C21H28O5	3.68 ± 0.34	0.2 ± 0.1	22.40	361.20095	343	325 (60%)
prednisone	53-03-2	C21H26O5	>500 <sup>c</sup>	<0.002 ± 0.0004	22.68	359.18530	341	313 (50%)
rimexolone	49697-38-3	C24H34O3	0.83 ± 0.04	1 ± 0.06	41.58	371.25807	353	295 (20%)
triamcinolone	83474-03-7	C21H27FO6	5.67 ± 0.23	0.2 ± 0.05	19.37	395.18644	375	357 (30%)
triamcinolone acetone	8054-16-8	C24H31FO6	0.37 ± 0.01	2.3 ± 0.04	27.23	435.21774	415	397 (20%)
triamcinolone hexacetone	5611-51-8	C30H41FO7	3.40 ± 0.17	0.3 ± 0.06	46.94	533.29091	513	397 (90%)

<sup>a</sup> Relative potencies (REPs) expressed as EC50<sub>dexamethasone</sub>/EC50<sub>compound</sub> × <sup>b</sup> Similar molecular weight and retention time. <sup>c</sup> Estimated; maximum induction was not reached.

processed using Xcalibur v.2.0 software. The Electrospray source conditions were as follows: capillary voltage 4.5 kV, heated capillary temperature 275 °C, capillary voltage 30 V, and tube lens 65 V. The mass spectrometer was operated in a data-dependent-acquisition (DDA) mode in which both MS and MS<sup>2</sup> spectra were acquired. In this mode, the acquisition software probes the MS spectra in real-time on a scan-by-scan basis to select the most intense parent ions for MS<sup>2</sup> analysis. The instrument is initially set to operate in full-scan (“survey”) mode until an ion exceeds a preset threshold at which point the instrument switches into the product-ion mode (MS<sup>2</sup>). The products ions were generated in the LTQ trap at a normalized collision energy setting of 35% and using an isolation width of 2 Da. Five microliters of the final extract was injected into the LC system consisting of a 100 mm × 2.0 mm i.d. column packed with 3- $\mu$ m Omnisphere C<sub>18</sub> material (Varian-Chrompack, Middelburg, The Netherlands). The guard column was 4.0 × 2.0 mm i.d. packed with pellicular C<sub>18</sub> material (Phenomenex). The analytical column and the guard column were maintained at a temperature of 10 °C in a column thermostat. A linear gradient of acetonitrile (5 to 60%) and water with 0.05% formic acid was used in 40 min and increased to 100% acetonitrile in 5 min and held at this composition for an additional 10 min. The analytical column was re-equilibrated for 15 min between consecutive runs. The flow rate of the mobile phase was 0.3 mL/min (for further details, see ref 26).

**GR CALUX Bioassay and Determination of Relative Potencies (REPS).** Human U2OS osteosarcoma cells, stably transfected with a GR-controlled luciferase reporter gene construct were cultured as described previously (27). GR CALUX bioassays were performed as described by Van der Linden et al. (15). Briefly, cells were seeded into 96 wells plates with DF medium (without phenol red and supplemented with DCC stripped serum). After 24 h of incubation (37 °C, 7.5% CO<sub>2</sub>), the medium was replaced by medium containing sample extracts (max 0.4% DMSO) for activity testing. After 24 h of exposure in triplicate, the medium was removed and the cells were lysed in 30  $\mu$ L of triton-lysis buffer. The amount of luciferase activity was quantified using a

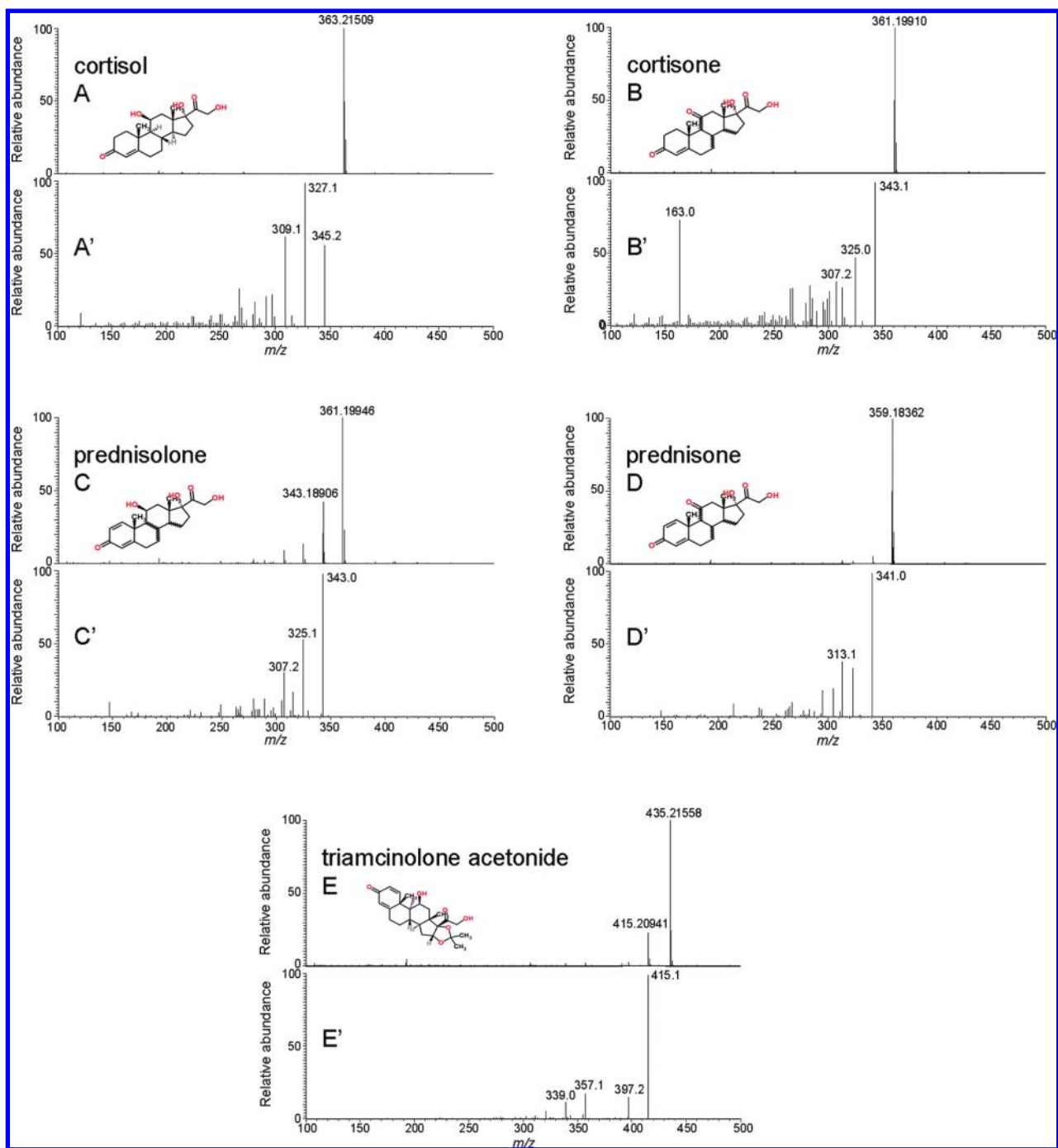
luminometer (Berthold, Germany). To rule out any confounding influences due to toxicity of the extracts, cells were monitored for signs of cytotoxicity by means of light microscopy. On all plates, a dose–response curve of the reference compound dexamethasone was included for adequate quantification of the response to nanograms of dexamethasone equivalents per liter (ng dex EQs/L). Dose response curves of all individual glucocorticoid standards and dexamethasone were fitted using a sigmoidal fit with variable hill slope (log(agonist) vs response – variable slope) in Graphpad Prism 5. Relative potencies (REPs) of all glucocorticoid standards were calculated as described.

## Results and Discussion

The DCC stripping of (hospital) wastewater as described resulted in a response < limit of detection (LOD) in both the GR CALUX bioassay and the LC-MS/MS procedure, respectively (data not shown). Hence, we conclude that the stripping procedure with activated charcoal does remove glucocorticoid compounds to a sufficient extent, enabling a proper assessment of individual compound recoveries.

**Characteristics of Instrumental and Biological Procedures for Glucocorticoid Analysis.** An overview of the analytical and GR CALUX bioassay detection characteristics of the 18 glucocorticoid standards is presented in Table 1. The six point calibration curves of glucocorticoid standards recorded by means of LC-MS/MS were fitted quadratically ( $R^2 > 0.999$ ) in the 10–2000 ng/L range (final water concentrations). All samples were concentrated or diluted in such a way that concentrations fell within this range. The estimated instrumental LOD was 10 ng/L for all glucocorticoid standards with the exception of aldosterone and prednisolone (20 ng/L). Glucocorticoid compounds could not be detected reliably below the given LODs.

For mass spectrometric conditions initially both positive and negative ionization modes were tested on their suitability for the types of wastewater sampled. In the positive ionization mode we obtained better signal-to-noise ratios/sensitivities and therefore this mode was used to produce the final data



**FIGURE 1.** High-resolution full scan MS spectra (A–E) and low-resolution MS product ion spectra (A’–E’) for a selection of 5 glucocorticoid standards.

as shown in the present study. Pseudomolecular ion accurate masses and product ion masses recorded in the Orbitrap MS/MS are presented in Table 1. Figure 1A–E shows the high-resolution MS spectra for a selection of 5 glucocorticoids. For confirmation purposes, MS<sup>2</sup> mass experiments (Figure 1A’–E’) were performed with the LTQ to construct low resolution product ion spectra with nominal masses.

The recoveries of the extraction procedure for 18 glucocorticoid standards spiked to surface water ranged between 55% ± 11% (aldosterone) and 114% ± 23% (flunisolide) with an overall average of 92% ± 17% (Figure 2). The recovery of glucocorticoid standards spiked to stripped wastewater was generally somewhat lower, ranging from 43% ± 5% (aldosterone) to 100% ± 6% (fluorometholone) with an overall average of 75% ± 18%. This might be the result of ion

suppression due to the more complex matrix of wastewater. To investigate the potential effect of the stripping method on the recovery of glucocorticoids standards, a nonstripped wastewater extract was spiked with glucocorticoid standards. Table S1 (Supporting Information) shows that the “spike” recovery of spiked glucocorticoid compounds to a nonstripped wastewater extract is sufficient (>50%) with the exception for triamcinolone hexacetonide. However, the latter compound is less relevant for the present study since it was not detected in any sample extract. The relative potencies (REPs) of all glucocorticoid standards tested are listed in Table 1. Figure 3 presents GR CALUX concentration response curves for six major glucocorticoids found in the various wastewater extracts. From the total number of 18 glucocorticoid standards tested, five (amcinonide, desoximetasone,

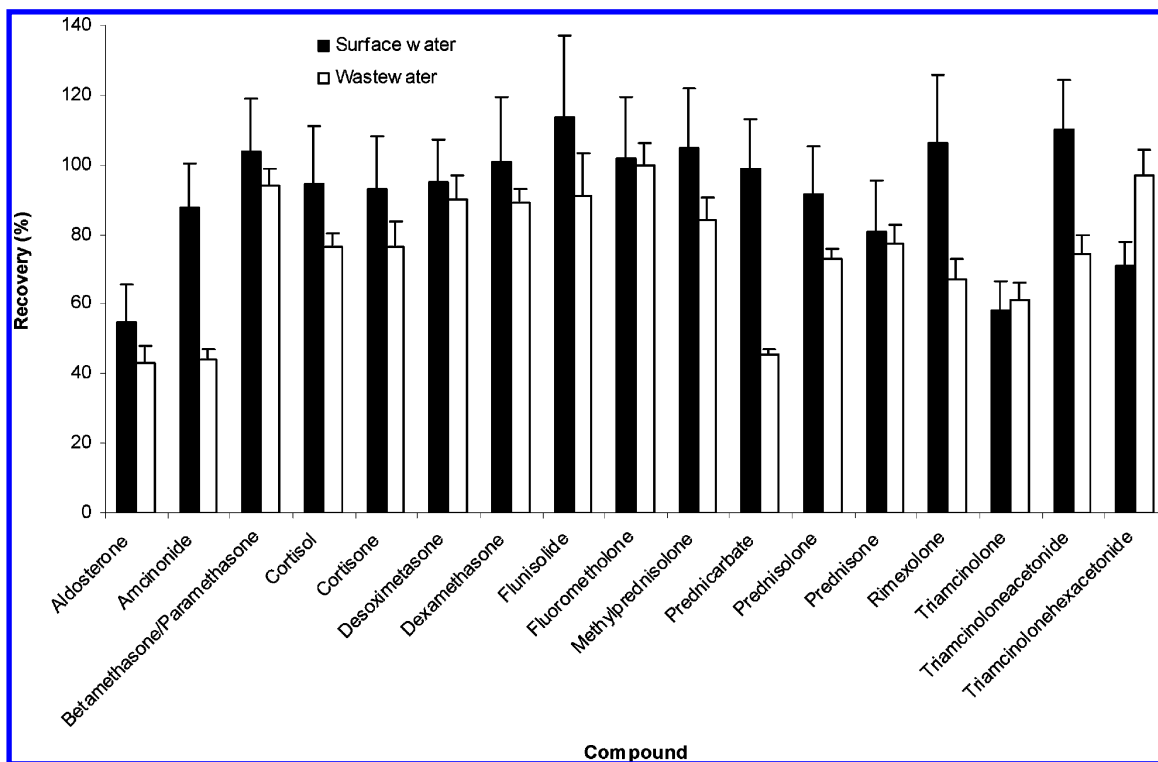


FIGURE 2. Recoveries of glucocorticoid standards spiked into surface water and stripped wastewater. Bars represent mean values  $\pm$  standard deviation of two independent extractions at a spiked compound concentration level of 1  $\mu\text{g/L}$ .

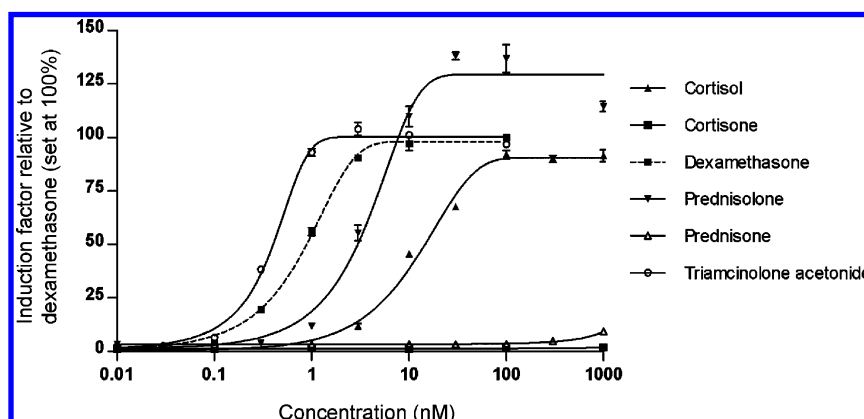


FIGURE 3. Concentration response curves for a selection of glucocorticoid standards obtained with the GR CALUX bioassay. The responses are expressed as induction factor relative to the reference agonist dexamethasone which is set at 100%. Each point represents a triplicate measurement  $\pm$  SD.

flunisolide, fluorometholone, and triamcinolone acetonide) were more potent than the reference agonist dexamethasone. The main reason for choosing the latter compound as a reference standard is to allow comparison of the results to historical data. The least potent glucocorticoid standards were prednisone and cortisol, with REPs of  $<0.002$  and  $<0.0008$ , respectively. For the latter glucocorticoid standards, the REP was estimated since maximum induction in the GR CALUX bioassay was not reached. The method LOD for the GR CALUX bioassay was 1.35 ng dex EQs/L.

#### Occurrence of Glucocorticoids in Wastewater Extracts.

In total, six different glucocorticoids were found in the various extracts (Table 2). Figure 4 shows a typical LC-high-resolution Orbitrap MS chromatogram of hospital wastewater 2 including extracted-ion chromatograms (positive-ion mode) for the detected glucocorticoids. Overall, the highest concentration ( $\sim 2 \mu\text{g/L}$ ) was observed for prednisolone in hospital wastewater 2, while this compound was also found at lower

concentrations in the extracts of industry and hospital wastewater 1 from our previous study (Table 2). The synthetic glucocorticoid triamcinolone acetonide was found at relatively low concentrations ( $<40 \text{ ng/L}$ ) in hospital wastewaters 1 and 2 and in sewage treatment plant effluent. The fact that this compound was the only one found in treated sewage treatment effluent at a concentration level equal to raw hospital wastewater may suggest that triamcinolone acetonide is poorly degraded or insufficiently removed during sewage treatment. However, this is an assumption and more research on the removal efficiency of this potent glucocorticoid compound is necessary.

Interestingly, it was observed that hospital wastewater 1 contained the same set of glucocorticoids as found in hospital wastewater 2. However, the concentration levels of prednisone and prednisolone determined in hospital wastewater 2 were much higher than those observed in hospital wastewater 1. It is unlikely that this discrepancy can be

**TABLE 2. Concentration of Hormones in Extracts of Various Wastewaters Collected in The Netherlands**

sample location (sampling date)	GR CALUX (ng dex EQs/L)	glucocorticoids	LC-MS/MS measured concentration <sup>a</sup> (ng/L)	relative potency (REP) <sup>b</sup>	predicted (total) dex EQs <sup>c</sup> (ng dex EQs/L)
Industry wastewater (12/05/2006; Van der Linden et al., 2008)	243 ± 32	cortisol	13 ± 1 (80%)	0.07 ± 0.08	0.92 ± 1.1
		cortisone	26 ± 3 (80%)	<0.0008 ± 0.00006	max. 0.02 ± 0.1
		dexamethasone	90 ± 9 (89%)	1 ± 0.05	90.0 ± 0.1
		prednisolone	247 ± 28 (73%)	0.2 ± 0.1	49.3 ± 0.5
		prednisone	N.D. (77%)	-	-
		triamcinolone acetonide	N.D. (74%)	-	-
					Σ 140.2 ± 1.3
hospital wastewater 1 (12/11/2006; Van der Linden et al., 2008)	96 ± 13	cortisol	275 ± 27 (80%)	0.07 ± 0.08	19.2 ± 1.1
		cortisone	381 ± 44 (80%)	<0.0008 ± 0.00006	max. 0.03 ± 0.8
		dexamethasone	N.D. (89%)	-	-
		prednisolone	315 ± 30 (73%)	0.2 ± 0.1	63.0 ± 0.5
		prednisone	117 ± 12 (77%)	<0.002 ± 0.0004	Max. 0.2 ± 0.2
		triamcinolone acetonide	41 ± 4 (74%)	2.3 ± 0.04	93.2 ± 0.1
					Σ 175.7 ± 1.5
hospital wastewater 2 (8/19/2009)	609 ± 79	cortisol	301 ± 29 (80%)	0.07 ± 0.08	21.1 ± 1.1
		cortisone	472 ± 54 (80%)	<0.0008 ± 0.00006	max. 0.4 ± 0.1
		dexamethasone	N.D. (89%)	-	-
		prednisolone	1918 ± 182 (73%)	0.2 ± 0.1	383.6 ± 0.5
		prednisone	545 ± 58 (77%)	<0.002 ± 0.0004	1.1 ± 0.2
		triamcinolone acetonide	14 ± 1 (74%)	2.3 ± 0.04	31.1 ± 0.1
					Σ 437.2 ± 1.3
paper mill treated wastewater (01/15/2007; Van der Linden et al., 2008)	11 ± 2	cortisol	N.D. (80%)	-	-
		cortisone	N.D. (80%)	-	-
		dexamethasone	N.D. (89%)	-	-
		prednisolone	N.D. (73%)	-	-
		prednisone	N.D. (77%)	-	-
		triamcinolone acetonide	N.D. (74%)	-	-
					0
sewage treatment plant effluent (11/15/2006; Van der Linden et al., 2008)	38 ± 13	cortisol	N.D. (80%)	-	-
		cortisone	N.D. (80%)	-	-
		dexamethasone	N.D. (89%)	-	-
		prednisolone	N.D. (73%)	-	-
		prednisone	N.D. (77%)	-	-
		triamcinolone acetonide	14 ± 1 (74%)	2.3 ± 0.04	31.1 ± 0.1
					Σ 31.1 ± 0.1

<sup>a</sup> Data were corrected for individual compound recoveries for wastewater and represent single measurements including estimated (instrumental) standard deviation (~10%). Data between parentheses show individual compound recoveries in wastewater (%) as presented in Figure 2. <sup>b</sup> Relative potencies (REPs) (cf. Table 1). <sup>c</sup> Compound-derived dexamethasone equivalents. Total dex-EQs = Σ (compound<sub>i</sub> REP × compound<sub>i</sub> concentration). N.D. not detected.

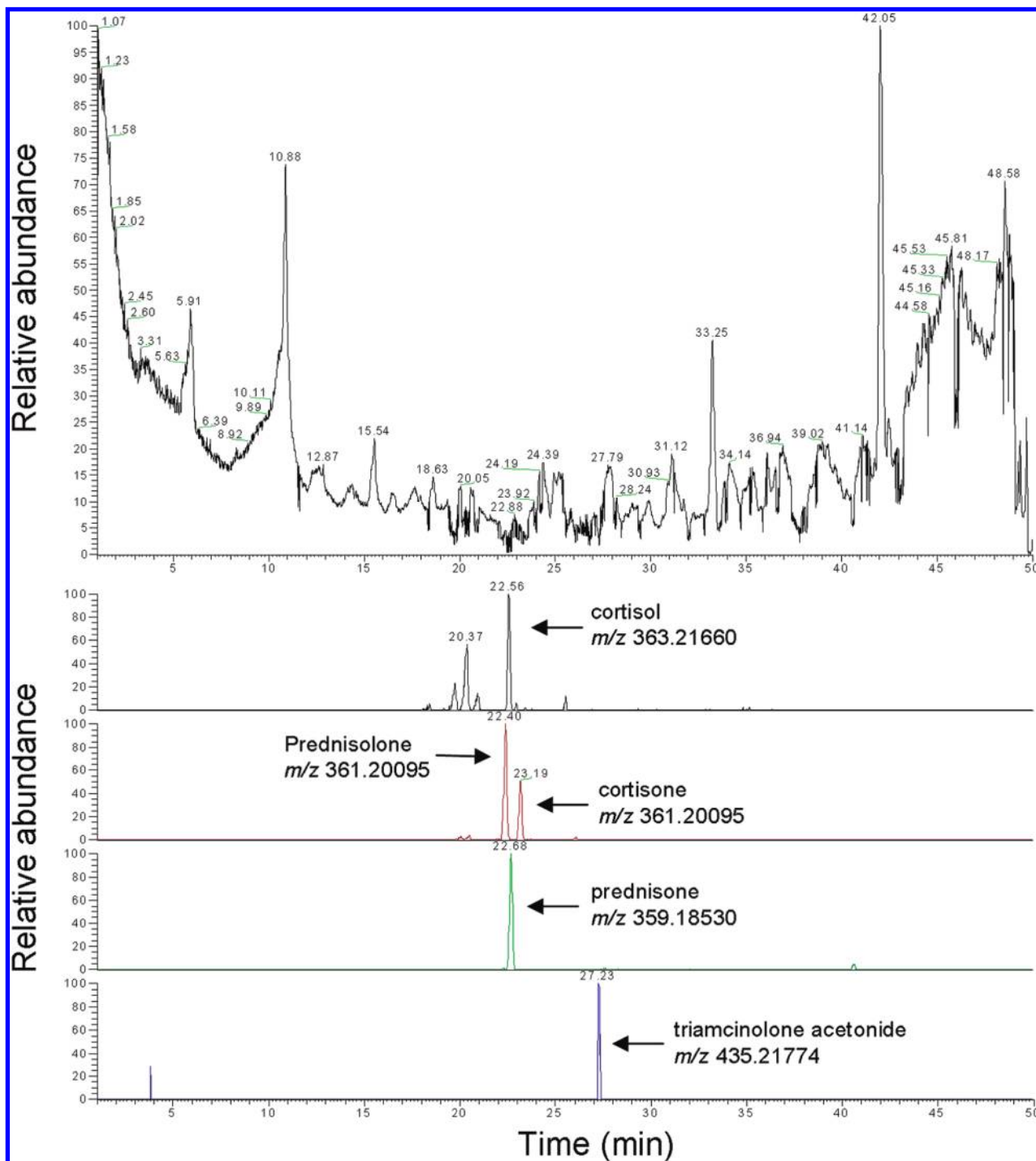
explained by degradation of glucocorticoid compounds during storage, since repeated measurements show that concentration levels of glucocorticoids standards stored during 1.5 y (at -18 °C) remain relatively constant (Supporting Information, Table S2). Possibly, a higher periodical prescription of glucocorticoid containing medicines to hospitalized patients during the time of collection of hospital wastewater 2 has led to the higher concentration levels observed.

The compounds prednisolone and prednisone belong to frequently applied glucocorticoids and their prescribed use in The Netherlands amounts to, respectively, 19.2 and 3.3 kg per 1,000,000 inhabitants in 2007 (Van der Aa, personal communication based on data obtained from the Foundation for Pharmaceutical Statistics, SFK). These data cover oral consumption and injections (prednisolone only) of sale quantities of prescribed drugs delivered by public pharmacies. Total consumption in The Netherlands will be higher, since sale quantities by hospitals are not included. For comparison, Besse and co-workers (28) present an estimated total annual consumption in France of 57.6 and 23.8 kg per 1,000,000 inhabitants for prednisolone and prednisone in 2004, respectively. These data cover sale quantities of all prescribed drugs delivered in France by both hospitals and pharmacies.

Considering the fact that glucocorticoids such as cortisol, cortisone, prednisone, and dexamethasone are relatively well removed during sewage treatment (% removal >98%) (16), only low concentrations of these compounds in surface

waters are expected. Chang and co-workers (16) indeed found low concentrations for a set of six glucocorticoids (cortisol, cortisone, prednisolone, prednisone, dexamethasone, and 6α-methylprednisolone) in the range of 0.02–4.2 ng/L in samples taken from the Tonghui and Qing rivers in China. This is further corroborated by the data we have reported earlier on GR activity measured in three Dutch surface waters which ranged from 0.39 to 1.3 ng dex EQs/L (15).

When chemically determined concentrations of the individual glucocorticoids present in the sample extracts are multiplied by their corresponding relative potencies (REPs) and subsequently summed (Figure 5A), it was found that for most extracts the so-called (chemically) “predicted” GR CALUX response was lower than the actually measured GR CALUX response. In extracts of industry wastewater, hospital wastewater 2, and sewage treatment plant effluent the predicted response explaining the experimentally obtained GR CALUX response was in the range of approximately 60%–80%. As hypothesized by others in the case of estrogenic activity of water samples, this may suggest the presence of unidentified agonists present in the extracts (29, 30). Indeed, several other glucocorticoids were identified (with unknown REPs) in the extracts by means of an accurate mass based screen (flucortin, fluprednidene, and hydrocortisone aceponate; results not shown), however compound identities could not be confirmed due to a lack of analytical standards. In addition, various glucocorticoid compounds could have been present in the extracts in concentration levels below the given



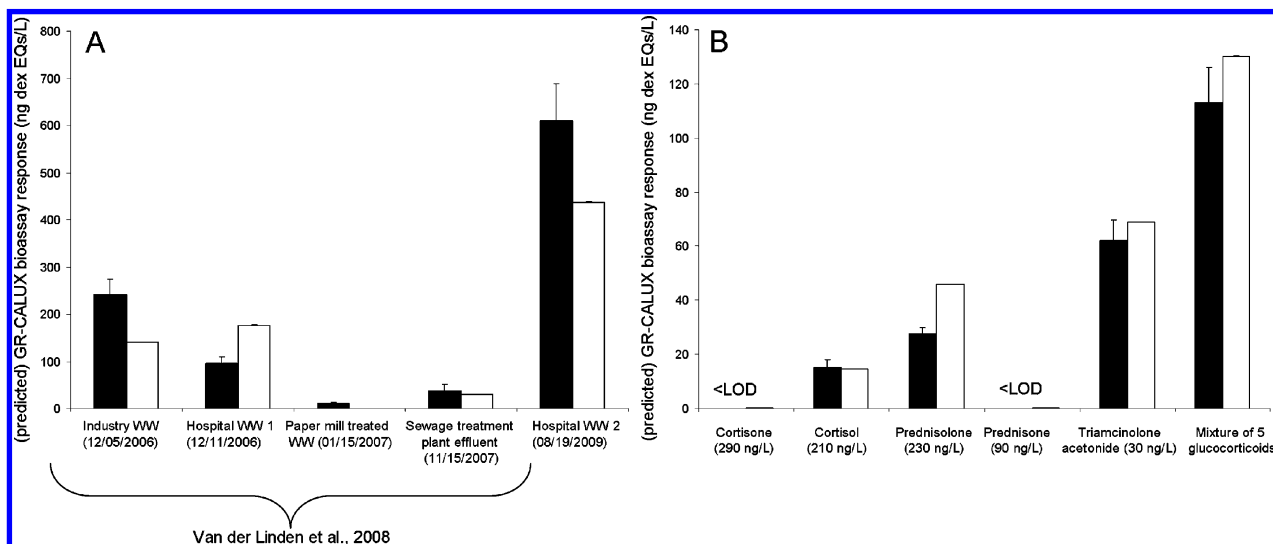
**FIGURE 4.** Full scan LC-Orbitrap MS chromatogram (top) of hospital wastewater 2 and extracted-ion chromatograms (positive-ion mode; bottom panels) for a selection of 5 glucocorticoids based on accurate mass with a mass window of 5 ppm.

LOD. However, at a level below 10 ng/L their contribution to the overall glucocorticoid activity in potent wastewater extracts such as hospital wastewater 1 and 2 would only be marginal, given their REPs.

In hospital wastewater 1 the predicted GR CALUX response amounted to approximately 170% of the experimentally obtained GR CALUX response. The reason for this observation is poorly understood at present, but an explanation may be sought in unknown antagonistic compounds present in the mixture. To investigate the combined activity of mixtures of glucocorticoids in the GR CALUX bioassay in more detail, stripped wastewater was spiked with five individual glucocorticoid standards and

a mixture of the same set of glucocorticoid standards followed by extraction and subsequent GR CALUX bioanalysis of the extracts. Figure 5B illustrates that low-potency glucocorticoids such as cortisol and prednisone did not contribute to the overall response of the mixture. Furthermore, it can be observed that the predicted GR CALUX bioassay response is in good agreement with the experimentally obtained GR CALUX bioassay response. Therefore, it can be concluded that the combined behavior of the set of selected glucocorticoids can be best described by the concept of concentration addition. For chemicals—such as (xeno)estrogens and androgens—that interact with well-defined molecular targets concentration additivity has been





**FIGURE 5.** Measured GR CALUX bioassay response (black bars) versus “predicted GR CALUX bioassay response” (white bars, corrected for individual compound recoveries in wastewater) elicited by (A) the various wastewater (WW) extracts and (B) by extracts of five individual glucocorticoid standards and a mixture (at the same concentration levels as the individual glucocorticoid standards). GR CALUX bioassay responses of the extracts were interpolated into a dexamethasone standard curve (0.03–100 nM) and response magnitude is presented as nanogram of dexamethasone equivalents/L (ng dex EQs/L).

observed before (25, 31–33). However, these results cannot explain the discrepancy between the predicted GR CALUX bioassay response and the experimental GR CALUX bioassay response that is observed in hospital wastewater 1. Therefore, alternative explanations must be sought for this observation, such as (i) interexperimental variation in the REP leading to a deviation in the predicted GR CALUX bioassay response (25), (ii) the presence of unidentified compounds that might have modulated the activity of the identified GR agonists, and finally (iii) the presence of unidentified agonists that were not taken into account while calculating the predicted GR CALUX bioassay response.

At present, there are insufficient data to predict the environmental concentrations of glucocorticoid compounds in surface waters. However, as shown by Chang and co-workers (16), glucocorticoids seem to be relatively well removed in sewage treatment plants. This is consistent with the fact that environmental concentrations of glucocorticogenic activity in surface waters measured so far were in the (low) ng/L range (15, 16). The significance for aquatic biota of (chronic) exposure to low concentrations of glucocorticoids is unknown at present and deserves more research attention. From a technological perspective, the removal efficiency of specific glucocorticoids in drinking water purification plants needs further attention. Studies addressing this issue are underway in our laboratory.

### Acknowledgments

Authors J.A.v.L. and S.C.v.d.L. contributed equally to this work. We thank Hans van Beveren, Pascal Kooij, and Dennis Vughs (KWR) for their excellent technical assistance. Dr. Wim Dokter of the Schering-Plough Corporation (Oss, The Netherlands) is acknowledged for provision of the glucocorticoid standard paramethasone. Thanks to Monique van der Aa of the National Institute for Public Health and the Environment, RIVM (Bilthoven, The Netherlands) for provision of SFK data on prescription of glucocorticoid containing drugs in The Netherlands. The critical comments of Dr. Minne B. Heringa (KWR) and Dr. Corine Houtman (HWL, The Netherlands) on the manuscript are highly appreciated. This study was financially supported by the Technau project (EU FP6, contract 018320) and

the Joint Research Programme of the Dutch water companies (BTO).

### Supporting Information Available

Additional tables of data. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

### Literature Cited

- Cooper, R. L.; Kavlock, R. J. Endocrine disruptors and reproductive development: a weight-of-evidence overview. *J. Endocrinol.* **1997**, *152*, 159–166.
- Sumpter, J. P. Xenoendocrine disruptors—environmental impacts. *Toxicol. Lett.* **1998**, *102–103*, 337–342.
- Legler, J.; van den Brink, C. E.; Brouwer, A.; Murk, A. J.; van der Saag, P. T.; Vethaak, A. D.; van der Burg, B. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicol. Sci.* **1999**, *48*, 55–66.
- Belfroid, A. C.; Van der Horst, A.; Vethaak, A. D.; Schafer, A. J.; Rijs, G. B.; Wegener, J.; Cofino, W. P. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci. Total Environ.* **1999**, *225*, 101–108.
- Ying, G.; Kookana, R. S.; Ru, Y. J. Occurrence and fate of hormone steroids in the environment. *Environ. Int.* **2002**, *28*, 545–551.
- Shore, L. S.; Shemesh, M. Naturally produced steroid hormones and their release into the environment. *Pure Appl. Chem.* **2003**, *75*, 1859–1871.
- De Voogt, P.; Belfroid, A. C.; de Boer, J.; Gerard, B. J.; Rijs, G. B. J. Efficacy of Wastewater Treatment Plants in the Netherlands for Removal of Estrogens and Xenoestrogens. In *Estrogens and Xenoestrogens in the Aquatic Environment: an Integrated Approach for Field Monitoring and Effect Assessment*; Vethaak, A. D., Schrap, S. M., de Voogt, P., Eds.; SETAC Press: Pensacola, FL, 2006.
- Ankley, G. T.; Brooks, B. W.; Huggett, D. B.; Sumpter, J. P. Repeating history: pharmaceuticals in the environment. *Environ. Sci. Technol.* **2007**, *41*, 8211–8217.
- Sumpter, J. P.; Johnson, A. C. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *Environ. Sci. Technol.* **2005**, *39*, 4321–4332.
- Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 8897–8901.

- (11) National Institute for Public Health and the Environment (RIVM). *Assessment of Human Health Risks for Oestrogenic Activity Detected in Water Samples, Using the ER-CALUX Assay*; RIVM: Bilthoven, The Netherlands, 2004.
- (12) Vethaak, A. D.; Lahr, J.; Schrap, S. M.; Belfroid, A. C.; Rijs, G. B. J.; Gerritsen, A.; de Boer, J.; Bulder, A. S.; Grinwis, G. C. M.; Kuiper, R. V.; Legler, J.; Murk, A. J.; Peijnenburg, W.; Verhaar, H. J. M.; de Voogt, P. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere* **2005**, *59*, 511–524.
- (13) Global Water Research Coalition. *Pharmaceuticals and Personal Care Products in the Water Cycle - Final Report*; GWRC/Kiwa Water Research/STOWA: London, UK, 2004.
- (14) Schwarzenbach, R. P.; Escher, B. I.; Fenner, K.; Hofstetter, T. B.; Johnson, C. A.; von Gunten, U.; Wehrli, B. The challenge of micropollutants in aquatic systems. *Science* **2006**, *313*, 1072–1077.
- (15) Van der Linden, S. C.; Heringa, M. B.; Man, H. Y.; Sonneveld, E.; Puijker, L. M.; Brouwer, A.; Van der Burg, B. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. *Environ. Sci. Technol.* **2008**, *42*, 5814–5820.
- (16) Chang, H.; Hu, J.; Shao, B. Occurrence of natural and synthetic glucocorticoids in sewage treatment plants and receiving river waters. *Environ. Sci. Technol.* **2007**, *41*, 3462–3468.
- (17) Rousseau, G. G.; Baxter, J. D.; Tomkins, G. M. Glucocorticoid receptors: Relations between steroid binding and biological effects. *J. Mol. Biol.* **1972**, *67*, 99–115.
- (18) Munck, A. Leung, K. Glucocorticoid receptors and mechanisms of action. In *Receptors and Mechanism of Action of Steroid Hormones, Part II*; Pasqualini, J. R., Ed.; Marcel Dekker: New York, 1977.
- (19) Lu, N. Z.; Wardell, S. E.; Burnstein, K. L.; Defranco, D.; Fuller, P. J.; Giguere, V.; Hochberg, R. B.; McKay, L.; Renoir, J. M.; Weigel, N. L.; Wilson, E. M.; McDonnell, D. P.; Cidlowski, J. A. International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. *Pharmacol. Rev.* **2006**, *58*, 782–797.
- (20) Odermatt, A.; Gumy, C.; Atanasov, A. G.; Dzyakanchuk, A. A. Disruption of glucocorticoid action by environmental chemicals: Potential mechanisms and relevance. *J. Steroid Biochem.* **2006**, *102*, 222–231.
- (21) Odermatt, A.; Gumy, C. Glucocorticoid and mineralocorticoid action: Why should we consider influences by environmental chemicals? *Biochem. Pharmacol.* **2008**, *76*, 1184–1193.
- (22) Hillegass, J. M.; Villano, C. M.; Cooper, K. R.; White, L. A. Matrix metalloproteinase-13 is required for zebra fish (*Danio rerio*) development and is a target for glucocorticoids. *Toxicol. Sci.* **2007**, *100*, 168–179.
- (23) Chang, H.; Wan, Y.; Hu, J. Determination and source apportionment of five classes of steroid hormones in urban rivers. *Environ. Sci. Technol.* **2009**, *43*, 7691–7698.
- (24) Procedure for removal of steroid hormones from serum. Available at [http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product\\_Information\\_Sheet/c6241pis.Par.0001.File.tmp/c6241pis.pdf](http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/c6241pis.Par.0001.File.tmp/c6241pis.pdf); accessed November 2009.
- (25) Houtman, C. J.; Sterk, S. S.; Van de Heijning, M. P. M.; Brouwer, A.; Stephany, R. W.; Van der Burg, B.; Sonneveld, E. Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays. *Anal. Chim. Acta* **2009**, *637*, 247–258.
- (26) Hogenboom, A. C.; van Leerdam, J. A.; de Voogt, P. Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry. *J. Chromatogr., A* **2009**, *1216* (3), 510–519.
- (27) Sonneveld, E.; Riteco, J. A.; Jansen, H. J.; Pieterse, B.; Brouwer, A.; Schoonen, W. G.; van der Burg, B. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. *Toxicol. Sci.* **2006**, *89*, 173–187.
- (28) Besse, J. P.; Garric, J. Human pharmaceuticals in surface waters. Implementation of a prioritization methodology and application to the French situation. *Toxicol. Lett.* **2008**, *176*, 104–123.
- (29) Snyder, S. A.; Villeneuve, D. L.; Snyder, E. M.; Giesy, J. P. Identification and quantification of estrogen receptor agonists in wastewater effluents. *Environ. Sci. Technol.* **2001**, *35*, 3620–3625.
- (30) Global Water Research Coalition. *Tools to Detect Estrogenicity in Environmental Waters - Final Report*; GWRC/Water Environment Research Foundation (WERF): Alexandria, VA, 2008.
- (31) Payne, J.; Rajapakse, N.; Wilkins, M.; Kortenkamp, A. Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environ. Health Perspect.* **2000**, *108*, 983–987.
- (32) Houtman, C. J.; Van Houten, Y. K.; Leonards, P. E. G.; Brouwer, A.; Lamoree, M. H.; Legler, J. Biological validation of a sample preparation method for ER-CALUX bioanalysis of estrogenic activity in sediment using mixtures of xeno-estrogens. *Environ. Sci. Technol.* **2006**, *40*, 2455–2461.
- (33) Brain, J. V.; Harris, C. A.; Scholze, M.; Backhaus, T.; Booy, P.; Lamoree, M.; Pojana, G.; Jonkers, N.; Runnalls, T.; Bonfà, A.; Marcomini, A.; Sumpter, J. P. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environ. Health Perspect.* **2005**, *113*, 721–728.

ES100013X