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Microwave-assisted enzymatic hydrolysis followed by extraction with restricted access nanocomposites for rapid analysis of glucocorticoids residues in liver tissue

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ABSTRACT

We developed a novel, simple and fast method for the determination of glucocorticoids residues in liver tissue by combining microwave-assisted enzymatic hydrolysis and restricted access matrix dispersive solid phase extraction (RAM-dSPE) followed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Firstly, microwave-assisted enzymatic hydrolysis was introduced in order to obtain a maximum amount of unconjugated parent drug in a short time (8 s), while the traditional method commonly needs 4–12 h. Secondly, further cleanup was carried out by RAM-dSPE based on the graphene@mSiO$_2$-C8 nanomaterials which were synthesized by coating mesoporous silica onto hydrophilic graphene nano-sheets through a surfactant-mediated co-condensation sol-gel process. The enzymatic hydrolysis influencing factors (pH of the buffer, microwave radiation power, incubation time) and the experimental conditions of RAM-dSPE (sorbents amount, type and volume of the elution solvent, adsorption and desorption time) were optimized. Three glucocorticoids (prednisolone (PREL), betamethasone (BE) and dexamethasone (DE)) were selected as models to evaluate the feasibility of the method. According to the results, the developed method provided low detection limit (S/N=3) of 0.01–0.05 μg kg^{-1} and good linearity range of 0.25–800 μg kg^{-1} ($R^2 > 0.996$) for glucocorticoids. The limit of quantification (S/N=10) range from 0.03 to 0.19 μg kg^{-1}. Compared with other traditional methods, the developed method could provide similar or even better results in a greatly reduced analysis time.
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1. Introduction

Glucocorticoids (Table 1) are medications that have been used in livestock production due to their pharmacological activities such as inhibition of inflammatory, allergic and immunological responses [1–3]. However, it has been observed that overdosed glucocorticoids are illegally added in animal fodder, aiming to improve feed intake and weight-gain [4]. Animal tissues with high glucocorticoids residues may be consumed by human which results in numerous side effects including hypertension, obesity and hyperglycemia [5]. In order to prevent the potential risks on human health [6,7], the European Union (EU) regulations have set maximum residue limits (MRLs) of dexamethasone, betamethasone and prednisolone in different animal species and in different tissues such as muscle, liver, fat, and kidney [8,9].

In order to determine the glucocorticoids residues in animal tissue, proper sample preparation procedures are necessary. The sample preparation procedures commonly consist of two parts: enzymatic hydrolysis and extraction. The enzymatic hydrolysis process is aimed to isolate small drug molecules from tissues after homogenization using *Helix pomatia* β-glucuronidase under thermostatic incubation. However, a substantial amount of time is needed to complete the enzymatic hydrolysis process (typically reported 4–12 h), which severely lengthens the whole analysis time [10–12]. Microwave-assisted enzymatic hydrolysis [13] has been reported to be a good option to reduce the hydrolysis time by employing the microwave irradiation in the enzymatic hydrolysis process. However, it has mainly been applied in tryptic digestion of proteins in proteomics study [14,15]. In this study, we initially investigated the feasibility of employing the microwave irradiation in the enzymatic hydrolysis process to completely release glucocorticoids from liver in a short time.

On the other hand, after unconjugated glucocorticoids released from liver tissue, the extraction of these small molecules out of the complex matrix is still perceived as the bottleneck and challenge to the analysts. Conventional extraction and clean-up approaches are liquid-liquid extraction (LLE) [16], solid-phase extraction (SPE)

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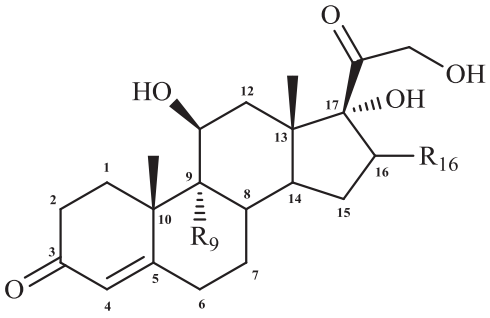
Table 1
Chemical structures of the targeted glucocorticoids.



Analytes molecule	Abbreviations	Double-bond position	R_9	R_{16}
Prednisolone	PREL	Δ^{1-4}	–F	–
Dexamethasone	DE	Δ^{1-4}	–F	$-CH_3(\alpha)$
Betamethasone	BE	Δ^{1-4}	–F	$-CH_3(\beta)$
Hydrocortisone	HC (I.S.)	–	–	–

[12,17–20], etc. However, the LLE methods have some disadvantages. For instance, they are laborious, time-consuming and non-environmentally friendly due to the consumption of large amount of organic solvent. The SPE methods are more convenient and economic but not capable of eliminating interferences caused by bio-macromolecules like proteins. Restricted access matrix dispersive solid phase extraction (RAM-dSPE) [21] emerged in recent years, and is of great significance in overcoming the obstacles. RAM-dSPE employs porous silica materials for the separation of low-molecular-weight analytes from matrix components by a combination of size exclusion and conventional adsorption chromatography. In our previous works [22], various graphene-based mesoporous silica materials were synthesized as the RAM-dSPE adsorbents. In this work, sandwich-structured graphene/mesoporous silica composites with C8-modified pore-walls (denoted as C) were successfully prepared via a simple route utilized to extract glucocorticoids released from liver tissues. The unique structure endowed the material with regularly aligned pores, large surface area, hydrophilic exterior surface, and hydrophobic interior pore-walls. Small molecules could enter into the pores and be reserved through hydrophobic interaction with C8 groups anchored in the pore-walls while macromolecules like proteins and polysaccharides were kept out by size exclusion effect.

The aim of the work was to provide a rapid and efficient method for analysis of glucocorticoids residues in tissue. Microwave-assisted enzymatic hydrolysis and RAM-dSPE were combined to achieve this goal. Liver was chosen as the research subject in this study since it usually suffers the highest drug accumulation. Three kinds of glucocorticoids, prednisolone (PREL), betamethasone (BE) and dexamethasone (DE) were selected as the model molecules to investigate the extraction ability of this method.

2. Experimental

2.1. Reagents and standards

Graphene was purchased from J&K chemical Corporation (Beijing, China). Tetraethylorthosilicate (TEOS), ethanol, ethylene glycol, concentrated nitric acid, sodium hydroxide, concentrated ammonia solution (28 wt%), and cetyltrimethyl ammonium bromide (CTAB) are of analytical grade and purchased from Shanghai Chemical Corp. n-Octyltriethoxysilane (abbreviated as C8, purity > 97.5%) was purchased from Sigma-Aldrich (St, Louis, MO, USA). The standards used were prednisolone (PREL), dexamethasone (DE), betamethasone (BE), and Hydrocortisone (HC, I.S.). All were purchased from Sigma-Aldrich (St, Louis, MO, USA). The glucocorticoids' chemical structure is presented in Table 1. *Helix pomatia* β-glucuronidase (113,400 unit mL^{-1} β-glucuronidase, Lot. No. SLBG8609V) was purchased from Sigma-Aldrich. Diethyl ether, methanol, acetone, hexane and dichloromethane, all HPLC grade, were obtained from TEDIA (Ohio, USA), Merck (Darmstadt, Germany), Fisher Scientific (New Jersey, USA). All of the other chemicals are of analytical grade and acquired from Sinopharm (Shanghai, China). Milli-Q water prepared by Milli-Q system (Millipore, Bedford, MA) was used in all experiments.

Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg L^{-1} and storing it at −20 °C. Fresh stock solutions of glucocorticoids (10^5 μg kg^{-1}) were prepared every month and stored at 4 °C in dark. Working solutions were prepared daily by diluting these solutions with water/acetonitrile (1:2).

2.2. Liver samples

Wistar male rats (weight about 150 ± 20 g, mean ± SD, Center of experimental animals, Fudan University, Shanghai, China) were treated simultaneously with corn oil solution of betamathasone and dexmethasone, receiving two separated injections at 0.075 mg kg^{-1} (i.p.). The rats were killed 1 h after injection. Liver samples were collected and stored in labeled plastic boxes at −80 °C until analyzed.

Pig liver samples were obtained from a local supermarket, minced by a homogenizer and frozen at −20 °C in 50 mL polypropylene centrifuge tubes. Each analysis was conducted in triplicate. These samples were analyzed and those found to contain

Table 2
Precision of the analytical method for PREL, DE and BE from liver measured with graphene@mSiO_2-C8 composites based on LC-MS/MS (0.5 MRL, 5 MRL and 50 MRL).

Analyte	Fortified concentration (μg kg^{-1})	Intra-day precision (n=5)		Inter-day precision (n=5)	
		Measured ± SD (μg kg^{-1})	RSD (%)	Measured ± SD (μg kg^{-1})	RSD (%)
PREL	5 (Low)	5.3 ± 0.4	5.9	5.4 ± 0.3	6.5
	50 (Medium)	51.8 ± 0.7	4.3	52.9 ± 0.6	5.5
	500 (High)	530.1 ± 12.2	5.2	528.4 ± 13.7	5.7
DE	1 (Low)	1.05 ± 0.04	3.9	1.07 ± 0.03	6.5
	10 (Medium)	10.42 ± 0.06	3.2	10.79 ± 0.02	4.0
	100 (High)	102.5 ± 3.8	4.5	105.8 ± 5.4	8.6
BE	1 (Low)	1.06 ± 0.02	3.1	1.06 ± 0.04	3.9
	10 (Medium)	10.05 ± 0.07	2.6	10.14 ± 0.05	3.4
	200 (High)	210.3 ± 8.9	3.8	213.6 ± 12.5	4.0
	10 (Medium)	10.05 ± 0.07	2.6	10.14 ± 0.08	3.4
	100 (High)	105.2 ± 4.45	3.8	104.8 ± 5.25	4.0

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