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Development of suitable method for large-scale urinary glucocorticoid analysis by liquid chromatography–mass spectrometry

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ABSTRACT

Levels of urinary glucocorticoids and their concentration ratios have been analyzed as potential markers for various pathological statuses. Large-scale studies may possibly accelerate the investigations; however, a suitable method needs to be established. Analytical conditions for measurement of urinary glucocorticoids with LCMS were examined. Electrospray ionization in the positive ion mode was applied for detection of cortisol (precursor > product ion: 363.3 > 121.0), cortisol-d4 (internal standard, IS, 367.4 > 121.1), and cortisone (361.2 > 163.2). To maximize ionization, acetic acid-ammonium acetate buffer (18 mM) at pH 5.3 was employed as eluent A. A C18 column (100 mm × 2.1 mm, 2.7 μm) at 50 °C was used for the 9.5 min binary gradient separation starting with 60% eluent A with methanol being eluent B. Linear correlations were observed between the concentrations and the peak areas in the concentration range of 1–300 ng/mL with correlation coefficients (r) of 0.998 and 0.997 for cortisol and cortisone, respectively, without IS adjustment, and 0.999 with IS adjustment for both cortisol and cortisone. Solid-phase extraction (SPE) using a 2 mL centrifuge column was performed for the urine samples, with the original and final volumes being 100 μL. The SPE of 12 urine specimens could be performed within 30 min. The effect of the sample matrix on the quantification of endogenous compounds present in the urine extract was limited (coefficient of variation (CV) of IS-adjusted matrix factor: 4.4–8.1%; urine extracts of 8 individuals); however, substantial peak reduction of cortisol was observed at low concentrations. Exogenous contaminants originating from the SPE centrifuge column seemed to be a main cause for this phenomenon because the pure-water extract showed similar peak reduction. A recovery of ~50% was obtained for both cortisol and cortisone. Adjustment with the IS improved the apparent recovery, with ~100% being obtained for both cortisol and cortisone. The recovery rate decreased when the urine samples were concentrated in the SPE step; the reduction was greater for cortisol than for cortisone. The lower limit of quantification (LLOQ) was set at 2.5 ng/mL when the injection volume was 10 μL, based on the reproducibility of the standards which were measured (CV of 12 repetitions: 10.1% for 0.5 ng/mL cortisol and 19.6% for 1 ng/mL cortisone), the matrix effect (−55% at 2 ng/mL concentrations of cortisol), and the recovery rate (~50%). Furthermore an alternative approach for preparation of the cortisol standards was required for low concentration range (2.5–20 ng/mL) because of the effect of the matrix. Degradation of original urine specimens at room temperature was minimal during the first 24 h. The extracted urine samples degraded over time; however, their concentrations were corrected with the IS, allowing for analysis up to 5 days after extraction. In conclusion, an analytical method for urinary glucocorticoids was established, which is fast, sensitive, and well suited for practical application to large-scale study.

1. Introduction

Glucocorticoids (Fig. 1) are secreted by zona fasciculata cells of the adrenal cortex, in response to physiological and psychological challenges through the hypothalamic-pituitary-adrenal (HPA) axis [1,2]. Recently, the association between increased levels of glucocorticoids and various kinds of diseases, such as psychiatric disorders, cardiovascular disease, and rheumatoid arthritis, has been the subject of numerous studies [1–5]. For example, levels of urinary glucocorticoids were reported to be higher in patients with bipolar disorder and schizophrenia than in healthy controls [6].

Secreted glucocorticoids are regulated in peripheral organs by 11β-hydroxysteroid dehydrogenase (11β-HSD) isozymes by switching between cortisol, the active form, and cortisone, the inactive form (Fig. 1).

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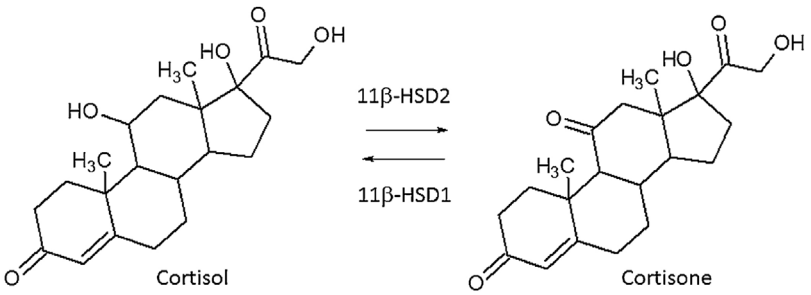

Fig 1. Structure of glucocorticoids. 11β-HSD: 11β-hydroxysteroid dehydrogenase.

Type 1 (11β-HSD1) functions as reductase, converting cortisone to cortisol, and type 2 (11β-HSD2) is a dehydrogenase, which converts cortisol to cortisone [7].

11β-HSD1 is reported to be expressed in the liver, adipose tissue, skeletal muscle, and brain [8]. The activity of 11β-HSD1 is associated with particular kinds of pathological statuses such as obesity, impaired glucose tolerance, and inflammation [9–12]. The 11β-HSD1 activity has been estimated by considering the ratio of total urinary cortisol (sum of free body and conjugates) to total urinary cortisone [11,13]. 11β-HSD2, on the other hand, is expressed in aldosterone target tissues, such as the kidney, to regulate blood pressure by inactivating cortisol, which has mineralocorticoid activity [14]. It is well established that the urinary free cortisol/cortisone ratio reflects the 11β-HSD2 activity [15–17]; for example, water loading immediately decreases the urinary free cortisol/cortisone ratio [16].

As such, urinary glucocorticoids are regulated by the HPA axis and the 11β-HSDs. Nowadays, urinary [6,11,17,18], salivary [19,20], and blood [18,21] glucocorticoids and their concentration ratios are analyzed by immunoassay [20,22], gas chromatography–mass spectrometry [11,13,17], high-performance liquid chromatography (HPLC) [18,23], and liquid chromatography–mass spectrometry (LCMS) [21,24]; however, the significance of the markers has not been fully uncovered. For further investigations, large-scale study is necessary. The methods currently used are not sufficiently suitable for processing and analyzing large amounts of samples in terms of the required sample volume, processing time, cost, and accuracy. The present study is aimed at developing a fast, simple, sensitive, accurate, and economical LCMS method for urinary glucocorticoid analysis.

2. Materials and methods

2.1. Reagents

LCMS-grade methanol, cortisol-d4 (9, 11, 12, 12-d4) solution (0.1 mg/mL solutions in methanol), ammonium formate and cortisone were obtained from Sigma-Aldrich (St. Louis, MO). LCMS-grade acetic acid, cortisol, and creatinine were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Mass spectrometric conditions

Ionization was carried out in the positive mode. The operating conditions were as follows: nebulizer gas: 3 L/min; drying gas: 15 L/min; interface heater: 350 °C; temperature of desolvation lines: 250 °C; heat block temperature: 400 °C. Multiple-reaction monitoring (MRM) was performed.

2.3. Chromatographic conditions

An LCMS-8030 system from Shimadzu Corp. (Kyoto, Japan) consisting of a binary pump and a temperature-controlled autosampler maintained at 4 °C was used. Formic acid, formic acid-ammonium formate buffer, acetic acid, and acetic acid-ammonium acetate buffer were each tested as eluent A. Methanol was employed as eluent B. Lab Solutions LCMS Version 5.72 was used for data analysis. An Ascentis express C18 column (100 mm × 2.1 mm, 2.7 μm) (Sigma-Aldrich) was used for the sample separation. The column oven was maintained at 50 °C.

2.4. Preparation of stock solutions

Stock solutions of cortisol and cortisone were prepared from the respective powders as 1 mg/mL solutions in methanol and stored at −20 °C.

2.5. Calibration curve

Standard solutions (STD) for calibration curve were freshly prepared on the day when urine samples were extracted. All dilutions were made with eluents A and B. The final concentration of eluent A was adjusted to 60% in eluent B (in accordance with the mobile phase at the start of the chromatographic measurements). The final concentration of the internal standard (IS, cortisol-d4) was adjusted to 300 ng/mL in all the STD mixtures.

In brief, cortisol-d4 (0.1 mg/mL) was diluted to 3.0 μg/mL. The stock solutions of cortisol and cortisone (1 mg/mL) were diluted to 40 μg/mL in the same solution (cortisol-cortisone mixture), and were further diluted to 400 ng/mL. Finally, STD samples of concentrations 50, 100, 200, and 300 ng/mL were prepared by dilution of the cortisol-cortisone mixture and cortisol-d4 in the same solution. To prepare STD samples of concentration 1, 5, 10, and 20 ng/mL, the 400 ng/mL solution (cortisol-cortisone mixture) was further diluted to 40 ng/mL before the final dilution.

2.6. HPLC analysis of creatinine

HPLC analyses were performed with binary gradient elution using an LC-20AD pump (Shimadzu, Kyoto, Japan) and an analytical column, Kinetix EVO C18 (100 mm × 2.1 mm I.D. 2.6 μm) (Shimadzu). The mobile phase consisted of 10 mM sodium phosphate buffer (pH 5.8) with 0.1 M $NaSO_4$ (C) and methanol (D). The flow rate was 0.3 mL/min. The spectrophotometric detector SPD-20A (Shimadzu) was set at 234 nm. Analyses were performed at 40 °C. The gradient program was as follows: 0–2.0 min, 0% D; 2.0–3.0 min, 0–80% D; 3.0–3.9 min, 80% D; and re-equilibration at 0% D for 11 min. Centrifuged urine samples were diluted 100-fold with distilled water. The injection volume was 1 μL.

2.7. Urine samples

Sampling of morning and daytime spot urine was performed. Aliquots of the urine samples were kept at −20 °C until analysis. The study design was approved by the Medical Ethics Committee of Saga Medical School, Saga, Japan.