Newly Identified Steroid Hormone in Urine of Patients with Cushing’s Syndrome: 3α,11β-Dihydroxy-4-Androsten-17-one

TAKASHI SUZUKI, MAKOTO UEKI*, HIDENARI SAKUTA**, MAKOTO FUJISAKI*, HIROKO YASUDA** AND TEIZO ITO**

Department of Research and Laboratory, Self Defense Forces Central Hospital, Setagaya-ku, Tokyo 154-0001, Japan
*Department of Endocrinology, Mitsubishi Kagaku Bio-clinical Laboratory, Itabashi-ku, Tokyo 174-8555, Japan
**Department of Internal Medicine, Self Defense Forces Central Hospital, Setagaya-ku, Tokyo 154-0001, Japan

Abstract. We have previously reported that the urine of patients with Cushing’s syndrome, including pituitary adenoma cases and adrenal adenoma cases, consistently show a conspicuous peak in the chromatographical analysis of 17-ketosteroid fraction but not in the urine of control subjects. The substance emerges just before 11β-hydroxy-androsterone (11β-OH-A) in capillary gas chromatography. In the present study, we have identified an “unknown peak substance” observed in the urine of Cushing’s syndrome patients using gas chromatography-mass spectrometry (GC/MS). Trimethylsilyl ether (TMS)-derivative of the substance was found to have a molecular weight (MW) of 448, which is similar to that of 11-OH-A (MW: 450). From these findings, we hypothesized that the substance had the structure of a C-19 steroid with two hydroxyl groups at positions C-3 and C-11, one keto-group at C-17 and a double bond between C-4 and C-5 of the A ring. We hypothesized that the unknown peak substance was 3α,11β-DH-A. To confirm this speculation we synthesized 3α,11β-DH-A and compared the elution pattern of it with that of the “unknown peak substance” using GC and GC/MS. We found that both substances were indistinguishable by GC and GC/MS analysis. These results suggest that the unknown substance observed in the urine of patients with Cushing’s syndrome is 3α,11β-DH-A.

Key words: Cushing’s syndrome, Steroid hormone, 17-Ketosteroid, Urine, Identification

IN spite of recent advances in medical technology of body imaging, it is still essential, however, to perform endocrinological tests to demonstrate the excessive and autonomic secretion of cortisol for the diagnosis of Cushing’s syndrome. For this purpose, urinary free cortisol and/or its metabolites such as 17-hydroxycorticosteroid (17-OHCS) and 17-ketosteroid (17-KS) are usually estimated [1, 2].

However, it has been shown that there is a substantial overlap in the basal value of urinary free cortisol between patients with Cushing’s syndrome and obese subjects [3]. This overlap can be discerned with a conventional overnight dexamethasone suppression test (DST). However, it would be of great benefit to the physician if a biological marker was available which could enable one to detect Cushing’s syndrome specifically without implementing DST.

Previously we have reported that the urine of patients with Cushing’s syndrome due to adrenal or pituitary adenoma consistently shows an unknown peak in the 17-KS fraction [4–6] using high performance liquid chromatography (HPLC) [7] and capillary gas chromatography (GC). This peak emerges just before the peak for 11β-hydroxy-androsterone (11β-OH-A) in GC and is relatively specific to Cushing’s syndrome [5].

In the present study, we attempted to identify this “unknown peak substance”. The results from this study suggest that the substance which contributes to the elevation of the 17-KS fraction in urine of patients with Cushing’s syndrome is likely to be 3α,
11β-dihydroxy-4-androsten-17-one (3α,11β-DH-A).

Methods

Urinary samples were obtained from patients with Cushing’s syndrome and healthy control subjects. Sample preparation and gas chromatography-mass spectrometry (GC/MS) analysis were carried out as described elsewhere [8]. To obtain trimethylsilyl ether derivatives (TMS) of steroids, we used a TMS derivatization kit according to the manufacturer’s instructions.

Fig. 1. Synthesis of 3α,11β-dihydroxy-4-androsten-17-one (1A) from hydrocortisone acetate (4). Abbreviation: Ac = acetyl, SGC = silica gel chromatography.
instructions. Using hexamethyldisilazane/trimethylchlorosilane (TMS-HT), two hydroxy groups of dihydroxy-steroids were replaced with TMS [8].

To synthesize 3α,11β-dihydroxy-4-androsten-17-one (3α,11β-DH-A) (1A) [9, 10, 11] and 3β,11β-dihydroxy-4-androsten-17-one (3β,11β-DH-A) (2A), hydrocortisone acetate (4) was selected as a starting material (Fig. 1). The synthesis of (1A) and (2A) was carried out using a method described by Fukushima et al. [12]. After reducing (4) with LiAlH₄, the crude obtained was oxidized with NaIO₄. Then, the keto-group at carbon-3 (C-3) and the side chain at C-17 was reduced to form substances (1A) and (2A). In this reaction, the keto-group at C-3 (3) was also produced as a by-product. These three substances were acetylated to produce a mixture of (1B), (2B) and (3), and applied to silica gel chromatography (SGC) to obtain two separate fractions, one containing (1B) and (2B), and the other containing (3). Then, the former fraction, including (1B) and (2B), was hydrolyzed with KOH and separated into (1A) and (2A) with SGC.

The ¹H-NMR and mp of (1A) and (2A) obtained through the above procedure were concordant with those previously reported in the literature [9] (data not shown), and both MS spectra of the synthesized chemicals showed a maximum mass/charge ratio (m/z)

![Graph](attachment:image.png)

**Fig. 2.** GC analysis of the 17-KS fraction of urine from a patient with Cushing’s syndrome. (a) GC elution patterns of synthesized 11β-OH-A and synthesized 11β-OH-E. 11β-OH-A appeared earlier than 11β-OH-E.; (b) GC elution pattern of urine from a patient with Cushing’s syndrome with high urinary 17-KS secretion. Note a small peak before 11β-OH-A peak (arrow); (c) GC elution pattern of the urine from healthy control subjects. Although there appeared to be a subtle peak prior to the appearance of the 11β-OH-A peak, it was unclear (arrowhead). Abbreviations: 11β-OH-A = 11β-hydroxy-androsterone, 11β-OH-E = 11β-hydroxy-ethiocholanolone. 17-KS = 17-ketosteroid.
of 304 (data not shown). Thus, the structural formulas of the synthesized chemicals were confirmed to be (1A) and (2A) with a molecular weight (MW) of 304.

Results

In order to identify the “unknown peak substance” which was observed in the urine of patients with Cushing’s syndrome, analysis of a urine sample collected from these patients was performed using GC and GC/MS. The “unknown peak substance” eluted at the retention time of 10.025 min, just before the peak of 11β-OH-A in GC analysis (Fig. 2).

The pooled urine collected from patients with Cushing’s syndrome was applied to GC/MS. The fractional pattern of the TMS derivative of the “unknown peak substance” exhibited the highest peak at 343 and other peaks at 358, 419 and 448 (Fig. 3a). The molecular weight (MW) of the TMS derivative of the “unknown peak substance” was estimated to be 448, which resembled the MW (450) of the TMS derivative for 11β-OH-A (Fig. 3b).

On the basis of the results of GC/MS spectra and the structure of the TMS derivative of the unknown substance and that of 11β-OH-A, we speculated that the “unknown peak substance” might have a structure similar to 11β-OH-A (Fig. 4c and d). We speculated that the “unknown peak substance” may have a structure similar to that of a C-19 steroid with two hydroxyl groups at C-3 and C-11 and one keto-group at C-17 like 11β-OH-A. Although the position of the double bond was obscure, we hypothesized it to be between C-4 and C-5 of the A-ring like cortisol, because the substance was likely to be a metabolite of cortisol which is excessively produced in patients with Cushing’s syndrome.

Thus, the unknown substance was tentatively speculated to be a cortisol-metabolite, 3α,11β-DH-A (1A). To confirm this hypothesis we synthesized 3α,11β-DH-A (1A) and 3β,11β-DH-A (2A) and compared the fractional patterns and relative retention time of this steroids with the “unknown peak substance” in GC and GC/MS. The fractional pattern and relative re-

---

Fig. 3. GC/MS analysis of the TMS derivative of the unknown peak fraction obtained from the 17-KS fraction of urine of a patient with Cushing’s syndrome (a) and TMS derivative of synthesized 11β-OH-A (b). Abbreviations: 11β-OH-A = 11β-hydroxyandrosterone, 17-KS = 17-ketosteroid, TMS = trimethylsilylether.
Fig. 4. Speculated structure of the “unknown peak substance” TMS derivatives of the substance possess MW of 448 (a), which was similar to the MW of the TMS derivative of 11β-OH-A (MW: 450, b). The “unknown peak substance” was speculated to have a structure similar to that of 11β-OH-A (MW: 306, c) but have a double bond between C-4 and C-5 in the A-ring (MW: 304, d). Abbreviations: TMS = trimethylsilyl ether derivatives.

Fig. 5. GC pattern of (1A), 11β-OH-A, 11β-OH-E and (2A), and GC/MS analysis of (1A)(TMS)₂. (a) (1A), 11β-OH-A, 11β-OH-E and (2A) elute in this order. (b) GC/MS analysis of (1A). Note that GC/MS pattern for (1A) (TMS)₂ resembles that of the unknown substance (TMS)₂ shown in figure 3 (lower). Abbreviations: 11β-OH-A = 11β-hydroxy-androsterone, 11β-OH-E = 11β-hydroxy-ethiocholanolone.
tention time of the “unknown peak substance” in GC coincided with the fractional patterns of synthesized 3α,11β-DH-A (1A) but not with that of 3β,11β-DH-A (2A) (Figs. 2B and 5a). In addition, the GC/MS pattern for the TMS derivative of the “unknown peak substance” resembled that of the TMS derivative for 3α,11β-DH-A (1A-(TMS)2) (Figs. 3b and 5b). Namely, the “unknown peak substance” and synthesized 3α,11β-DH-A (1A) were indistinguishable with GC or GC/MS.

**Discussion**

Previously, we reported that an “unknown peak substance” was consistently observed in the urine of patients with Cushing’s syndrome originating from either adrenal adenoma and/or pituitary adenoma but not in healthy control subjects [4, 5]. In the present study we have shown that the “unknown peak substance” is likely to be 3α,11β-DH-A (1A), a known steroid metabolite [9, 10, 11].

There are at least two pathways in humans which are associated with the synthesis of 3α,11β-DH-A (1A) in vivo. In one of these pathways, adrenosterone is metabolized to form 11β-hydroxy-4-androsten-3, 17-dione, and then to 3α,11β-DH-A (1A) [9, 13, 14].

In the other pathway, cortisol is metabolized to form 11β-hydroxy-4-androsten-3,17-dione and then to 3α, 11β-DH-A (1A) [9, 14, 15]. 3α,11β-DH-A observed in the urine of patients with Cushing’s syndrome appears to be derived from the latter pathway. Although we speculated that the substance might be a metabolite of cortisol, a hydrocortisone test should be carried out to confirm this hypothesis.

3α,11β-DH-A (1A) is not a novel steroid [13–15]. However, the clinical or pathophysiological significance of this C-19 steroid has not been reported to date. Therefore, to our knowledge, this is the first report which shows the clinical association of 3α,11β-DH-A (1A) and Cushing’s syndrome. But the clinical value of this steroid as a “marker” of Cushing’s syndrome has yet to be elucidated.

**Acknowledgements**

We would like to thank Drs. Masuo Morisaki and Noriko Kobayashi of the Department of Medical Chemistry, Kyoritsu College of Pharmacy, Tokyo, JAPAN for synthesizing 3α,11β-dihydroxy-4-androsten-17-one and 3β,11β-dihydroxy-4-androsten-17-one and for critical reading of this manuscript.

**References**


11. Fukushima DK, Dobriner S, Bradlow HL, Zumoff B,


