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Determination of urinary cortisol, cortisone and 6-sulfatoxymelatonin using dilute and shoot ultra-high pressure liquid chromatography–tandem mass spectrometry

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ABSTRACT

Human sleep is a natural part of every individual's life. Clear relationship between sleep and endocrine system has been already established. In particular, melatonin and cortisol are known to affect and regulate sleep/wake patterns. Here we report the development of an ultra-high pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for simultaneous measurement of 6-sulfatoxymelatonin (MT6s), cortisol and cortisone in urine. A separate method was developed for measurement of creatinine in urine. These levels were used to normalise the levels of analytes. First void morning urine samples were collected from 24 healthy volunteers. Samples were diluted 1:1 in water prior to injection onto reversed-phase C18 column and analysed using UHPLC–MS/MS method. Linear calibrations were obtained for all analytes with correlation coefficient in the range 0.998–0.999. The observed concentration was found to be in the range 92–105% for cortisol, 92–107% for cortisone and between 93 and 120% for MT6s of the reference levels. The total run time of 6 min with all peaks of interest eluting within 3 min was obtained. This demonstrates the feasibility of utilising the method for large multi-scale studies, where high throughput is required for studying the circadian rhythm of melatonin and cortisol secretion. These hormones play significant role in circadian rhythm and sleep/wake cycle; therefore it is important to monitor the levels of these endocrine markers in individuals suffering from sleep disorders. It is also beneficial with clinical applications to analyse melatonin and cortisol simultaneously in order to assess their interrelationships of these substances, such as their effect on diurnal rhythm and sleep.

1. Introduction

Human sleep is a natural part of every individual's life. It has been estimated that we spend about one third of our lives asleep [1]. Sleep is also known to have a modulatory effect on many components of the endocrine system, such as growth hormone and prolactin [2]. Cortisol and melatonin, in particular, affect sleep and display circadian and diurnal rhythmicity [3–6]. Their levels tend to run in opposition to each other, melatonin levels increases before night time sleep and reaches a maximum at approximately 3–4 am [7], whereas cortisol levels rise before the dawn, rapidly increase after awakening and decrease over the course of the day [8]. This pattern of secretion has been shown to be stable across days and weeks amongst a wide age range of individuals [8].

The primary function of cortisol is to increase the circulating level of energy providing compounds by way of inducing cellular gluconeogenesis, glycogenolysis, selective lipolysis, and heightened breakdown of proteins [9]. Cortisol also increases cardiac output by elevating the heart rate and blood pressure and increasing response to catecholamines [9]. Thus, it has been suggested, that the rise in levels of cortisol before dawn stimulates the brain and diverts energy to the muscles, which facilitates awakening [10]. The predominant metabolite of cortisol is inactive cortisone and both cortisol and cortisone are excreted in urine [11].

Melatonin is known to indirectly cause the nocturnal decreases in core body temperature and facilitates sleep [5]. As such, its secretion coincides with sleepiness and the greatest decline in

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the core body temperature over a 24 h period [7]. In humans, the main metabolite of melatonin is 6-sulfatoxymelatonin (MT6s), and urinary concentrations account for up to 90% of administered melatonin, which closely parallels the plasma melatonin profile [12,13].

Disturbances in the circadian patterns of both melatonin and cortisol may negatively affect sleep [14,15].

Methods for the determination of cortisol as well as melatonin and their metabolites in body fluids based on enzyme linked immunosorbent assay (ELISA) [16–18] and radioimmunoassay (RIA) [17,19,20] are used because they are relatively simple and can be automated. These are limited to determination of a single analyte, have a narrow dynamic range and show antibody cross-reactivity with other structurally related molecules. High performance-liquid chromatography (HPLC) with fluorescence detection [21] and gas chromatography hyphenated to mass spectrometry (GC-MS) [22] for analysis of melatonin requires long analysis time and derivatisation respectively, for GC-MS analysis this is approximately an hour [22].

The development of liquid chromatography with tandem mass spectrometry (LC–MS/MS) methods provide high sensitivity, specificity and reduced analysis time for measurement of multiple analytes with peaks of interest eluting within 3 min [23,24]. It has been reported that LC–MS/MS can offer better specificity and accuracy than immunoassay-based methods [25]. Several LC–MS/MS methods for analysis of melatonin and/or cortisol in plasma, saliva or urine have been previously reported, however, they require sample preparation steps such as liquid–liquid extraction [26,27], solid phase extraction (SPE) [28,29], on-line SPE [30] that significantly increases workload and total analysis time.

At present LC–MS/MS methods cannot be used for the simultaneous measurement of cortisol, cortisone and MT6s in urine or for assessing interrelationships of these analytes and their effect on diurnal rhythm and sleep. The simultaneous measurement of these sleep biomarkers in urine would be a valuable tool for the diagnosis of sleep disorders where blood samples are often difficult to obtain. Furthermore, there is increasing evidence which suggests that simultaneous measurements of more than one biomarker in a sample provides greater confidence for the acceptance or rejection of specific diagnoses [23,31,32]. In this paper a new method for the simultaneous measurement of cortisol, cortisone and MT6s in urine using an LC–MS/MS approach is presented.

2. Materials and methods

2.1. Participants

Twenty-four healthy children aged 4–11 years (12 male, 15 female) were recruited from a mainstream schools in South-East UK. The age range was chosen in order to avoid puberty. Participants were screened for any medication containing melatonin and cortisol or those that may affect the level of these analytes. Individuals taking such medications were not included in the study. Foods which could affect levels of melatonin and cortisol, including bananas and caffeinated drinks, were also avoided by study participants.

All participants and their parents were informed of the details of the study and gave their written consent. The study was approved by Middlesex Natural Sciences Ethics sub-Committee (approval letter number 524).

2.2. Sample collection

Fasting first void morning urine samples were collected into 200 mL storage urine pots. Samples were stored in the participants' household refrigerator (4 °C), aliquoted and frozen (−20 °C) within 24 h until analysis.

2.3. Standards and reagents

Cortisol and cortisone were obtained from Sigma–Aldrich (Pool, Dorset, UK). Due to the lack of commercially available standard of 6-sulfatoxymelatonin, for the development of LC–MS/MS method, standards for melatonin sulphate were obtained from an ELISA kit (IBL International, Hamburg, Germany). Creatinine was obtained from Acros Organics (Geel, Belgium). 6-α-Methylprednisolone, indole-3-acetamide and 1-(3-aminopropyl)imidazole used as internal standards for cortisol/cortisone, MT6s and creatinine, respectively, were obtained from Sigma–Aldrich. Acetonitrile of LiChrosolv LC–MS-grade was acquired from Merck Millipore (Feltham, UK).

2.4. Preparation of stock and standards solutions

Stock solutions of cortisol and cortisone in methanol were prepared at a concentration of 0.1 mg/mL. The primary stock solutions were used to prepare working standards in methanol at concentrations of 2.78, 5.56, 16.67, 50 and 100 ng/mL. Standard solutions from MT6s ELISA kit were used at concentrations of 3.11, 6.22, 18.67, 56 and 112 ng/mL. A solution of creatinine in water at concentration of 1 mg/mL was prepared. Calibration standards were prepared at concentration of 0.125, 0.25, 0.5 and 0.75 mg/mL. All the standard solutions for the calibration studies were spiked with the internal standards (see Section 2.5).

2.5. Sample preparation

Frozen urine samples were thawed and centrifuged for 5 min at 1500 g, and then diluted in water (1:1, v/v). For the analysis of cortisol, cortisone and MT6s, 500 μL of diluted samples were spiked with a small volume (2.5 μL) of each of the internal standards (10 μg/mL) and vortexed for 30 s. For the analysis of creatinine, 600 μL of samples diluted in water (1:1, v/v) were spiked with 30 μL of the internal standards (10 μg/mL) and vortexed.

2.6. LC–MS/MS analysis

An ultra-high pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) system comprising of a Shimadzu UHPLC Nexera system interfaced to a triple quadrupole mass spectrometer LCMS-8030 (Shimadzu Corporation, Japan) equipped with an electrospray ionisation (ESI) source was used. The chromatographic separations were performed using a Kinetex 2.1 × 50 mm, 2.6 μm, C18 column (Phenomenex, Macclesfiled, UK). The UHPLC system was composed of two LC-30AD pumps, a column oven (CTO-20AC) maintained at 40 °C and a model DGU-20A3 solvent degasser. A binary gradient elution profile composed of eluent 'A' – 0.1% formic acid in water and 'B' – 0.1% formic acid in acetonitrile delivered at a flow rate of 0.4 mL/min was used. For the separation of cortisol, cortisone, MT6s and their internal standards a linear gradient from 2 to 90% 'B' over 4 min followed by a hold at 90% 'B' until 4.5 min then a step within 0.1 min to 2% 'B' and re-equilibration from 4.6 to 6.1 min was used. For the separation of creatinine and its internal standard a linear gradient from 2 to 90% 'B' over 2 min followed by a hold at 90% 'B' for 0.5 min then step over 0.1 min to 2% 'B' and re-equilibration at 2% 'B' from 2.6 min to 4.1 min was used. The desolvation line temperature was maintained at 300 °C while the heat block temperature was set at 400 °C. An interface voltage of 4.5 kV was applied to the electrospray probe. Nitrogen was used as the nebulising gas at a flow rate of 2.8 L/min, additionally, the flow rate of drying gas was set at 16 L/min. Detection and quantification