



Serum cortisol concentration in patients with major depression after treatment with fluoxetine

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ABSTRACT

Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis and elevated cortisol levels is characteristic of the pathophysiology of major depressive disorder (MDD). The aim of this study was to determine whether increased plasma cortisol levels appear in patients with major depression and if effective antidepressant treatment by fluoxetine leads to regulation of cortisol level. This aim was realized by describing and validation of methods of determining fluoxetine and cortisol in serum and searching for correlation between their concentrations in patients with endogenous depression, the therapeutic effect as assessed in Hamilton Depression Rating Scale (HDRS), age and sex of patients. Plasma cortisol and fluoxetine levels were measured using high performance liquid chromatography (HPLC) methods with applying Shimadzu chromatograph with UV detection. Plasma cortisol and fluoxetine levels were measured at time zero (before therapy) and after 6 h, 24 h, 2, 4, 6 and 8 weeks of fluoxetine administration in patients with major depression qualified for therapeutic drug monitoring (TDM). The study included 21 patients (14 women, 7 men; mean age 29–75 years) and 24 healthy comparison subjects. The patients had a mean score on the 21-item HDRS. As the effect of fluoxetine administration the decrease of the level of cortisol was observed in patients who responded to the therapy (the reduction of points in HDRS scale in at least 50%). The validation parameters of HPLC method of fluoxetine and cortisol determination indicate the possibility of applying them for determination of both: the level of concentration of the drug in therapeutic drug monitoring and the level of cortisol in serum of patients with endogenous depression.

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1. Introduction

Deregulation of the hypothalamic-pituitary-adrenal (HPA) axis is thought to play a role in the etiology of major depressive disorder (MDD). A number of studies have shown, that in depression we deal with irregularity of the HPA axis, therefore effective antidepressant treatment should lead to normalization of the HPA axis activity (Pariente, 2003; Burke et al., 2005). In recent decades many researchers tried to investigate a relation between the HPA axis and depression (Bouquet et al., 1981; Abreu Feijo de Mello et al., 2003; Boyle et al., 2005). These observations were mostly focused on the role of cortisol—stress hormone—in the etiology of depression (Schüle, 2006).

Numerous studies suggest, that the above normal cortisol concentration is also influencing the development of cognitive abilities disorder, that is characteristic of depression as well (Boyle et al.,

2005; Holsboer and Ising, 2008). The increased secretion of glucocorticoids is responsible for appearance of psychotic symptoms (Holsboer and Ising, 2008).

In ill patients it was observed that the difference between the basic level of cortisol and the level of cortisol under stress stimulus is smaller than in healthy patients; the curve of 24-hour-secretion of cortisol was flatter in ill patients as well (Burke et al., 2005).

If the HPA axis activity disorder is the basis for the depression, it can be assumed, that the restoration of the proper activity in this area would improve the health condition of patients. The examination conducted by DeRijk et al. (2008) in vivo on rats, which were subjected to antidepressant therapy (including fluoxetine), showed the decrease in the basic and stress-stimulated concentrations of adreno corticotropin hormone (ACTH) and corticosterone.

The analysis by Schüle (2006) proves that the treatment of depression by applying fluoxetine decreases the level of corticosterone in the liquor cerebrospinal in the span of several weeks. Other authors point out, that the chronic treatment with antidepressants lowers the corticotropin-releasing hormone (CRH) mRNA and the concentration of CRH in hypothalamic of rats, as well as increases the concentration of corticosterone in liquor cerebrospinal in which

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increases the effectiveness of the HPA negative feedback (Weber et al., 2006).

The analysis by Huang and Herbert (2006), indicate that both the flattened curve of 24-hour-secretion of corticosterone and the too high concentration of this hormone may cause lowering the effectiveness of fluoxetine, and especially its influence on increasing proliferation of progenitor cells of hippocampus.

The aim of this work was to examine whether in the group of patients with affective disorder elevated plasma cortisol levels appear, as well as to find out if the antidepressant therapy using fluoxetine leads to the normalization of the cortisol level.

2. Materials and methods

2.1. Materials

Cortisol, fluoxetine, 6- α -methylprednisolone and protryptiline HCl (used as internal standards—IS) and tetrahydrofuran (99.9%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, dichloromethane, *n*-hexane, potassium dihydrogen phosphate, phosphoric acid (85%), sodium chloride and sodium hydroxide were purchased from J. T. Baker (Deventer, Holland) and used as analytical-grade reagents. Standard serum for validation method was received from Cormay Control Serum NH (Cormay, Poland).

2.2. Patients and treatment

The study included $N = 21$ patients (14 women and 7 men; mean age 29–75 years) and 24 healthy comparison subjects (16 women and 8 men; mean age 20–81 years). Patients were recruited from the Departments of Psychiatry at the Medical University of Warsaw and had clinical diagnoses of major depression. Before medications were administered, informed consent was obtained from all patients. The Local Bioethical Committee approved the trial. Patients treated with fluoxetine qualified for the therapeutic drug monitoring were subject of this treatment. The Hamilton Depression Rating Scale (HDRS) was used to assess patients' psychological status and efficacy of antidepressant therapy before treatment and 2, 4, 6 and 8 weeks after its commencement. The patients had a mean score HDRS of 31.14 (± 5.75). The initial HDRS in patients fluctuated between 22 and 44, which means a tough and a very tough depression on the 21-item Hamilton Depression Rating Scale. A response to treatment was defined as a 50% or more reduction in pre-treatment scores. Patients were dosed with 20 mg of fluoxetine per day⁻¹. The blood was collected under normal conditions, that is at 8 a.m. Plasma cortisol and fluoxetine levels were determined by high performance liquid chromatography (HPLC) with absorbance detection at the time zero (before therapy), and after 6, 24 h of administration fluoxetine as well as after 2, 4, 6, 8 weeks of treatment.

2.3. HPLC method

Many analytical methods have been developed for the determination of cortisol and fluoxetine in serum. The most of the described in the literature are the methods of liquid chromatography (LC) coupled with a spectrophotometer detector (HPLC/UV) (Wong et al., 1990; Nichols et al., 1994; Huang and Herbert, 2006), of liquid chromatography coupled with a mass-selective detector (LC/MS) (Frerichs and Tornatore, 2004; Kushnir et al., 2004) of gas liquid chromatography (Dixit et al., 1991), or of gas liquid chromatography coupled with a mass-selective detector (Thienpont et al., 1991, 1996). Comparable to these methods are methods with applying isotope dilution – the method of liquid chromatography coupled with a massspectrometry (ID-LC-MS) (Shibasaki et al., 1997; Furuta et al., 2000; Jung et al., 2004).

The above mentioned methods, when applying the proper equipment and the techniques of the newest generation, are expensive. The methods of determining and extraction of cortisol and fluoxetine by applying liquid chromatography coupled with a spectrophotometer detector (HPLC-UV), that we studied and optimized, are considerably cheaper, if considering the equipment used and the chemical reagents that are necessary.

The chromatographic system, from Shimadzu, consisted of a LC-10 AS pump, equipped with a 100 μ l injection loop, and a programmable SPD-10 Av UV-vis absorbance detector. Separation for cortisol was performed by Waters C18 precolumn and Waters Symmetry C18 (5 μ m, 150 mm $\times$ 4.6 mm) analytical column with a mobile phase consisting of methanol: water: tetrahydrofuran (100:110:2.5, v/v/v) set at a flow rate of 1.0 ml min⁻¹. The UV detection was set at 252 nm. The retention time of cortisol and internal standard (6- α -methylprednisolone) was found to be 8.1 and 13.8 min respectively.

Separation of fluoxetine was performed by Supelguard LC-8-DB precolumn and Supelcosil LC-8-DB (5 μ m, 150 mm $\times$ 4.6 mm) analytical column with a mobile phase consisting of acetonitrile : 100 mM KH₂PO₄, pH = 3 (40:60, v/v) set at a flow rate of 1.0 ml min⁻¹. The UV detection was set at 228 nm. The retention time of fluoxetine and internal standard (protryptiline) was found to be 8.3 and 6 min respectively.

2.4. Sample preparation

Samples for both cortisol and IS were prepared for HPLC analysis using the following extraction procedure: 500 μ l of plasma was mixed with 50 μ l of IS (6- α -methylprednisolone in methanol: water 1:1 v/v), 250 μ l sodium hydroxide (0.1 M) and 50 μ l of methanol. After adding 2 ml of dichloromethane the samples were shaken 10 min and frozen. The organic layer was subsequently transferred into a glass tube and evaporated under a stream of nitrogen at 45 °C. The residue was reconstituted in 500 μ l of methanol: water (1:1, v/v). Injection volume for HPLC analysis was 100 μ l.

Samples for both fluoxetine and IS were prepared for HPLC analysis using the following extraction procedure: 500 μ l of plasma was mixed with 50 μ l of IS (protryptiline in methanol), 50 μ l sodium hydroxide (1 M) and 3 g NaCl. The sample centrifuged (2000 $\times$ g, 5 min). To a liquid phase 6 ml of hexane: acetonitrile (98:2, v/v) was added, the samples were shaken for 10 min and centrifuged again. The organic layer was subsequently transferred into a glass tube and evaporated under a stream of nitrogen at 45 °C. The residue was reconstituted in 500 μ l of the mobile phase. Injection volume for HPLC analysis was 100 μ l.

Data recording was carried out using Chromax-2004 Software. The HPLC method of cortisol and fluoxetine determination was validated.

2.5. Method of validation

The precision and accuracy of the method were determined by replicate analyses of serum samples containing different concentrations (low, medium and high) of cortisol and fluoxetine. The repeatability (intermediate precision) was determined by performing analyses of the concentrations of these analyses during one day under the same conditions by one operator. The intermediate (day-to-day) precision was determined by analyzing low, medium and high concentrations of cortisol and fluoxetine on different days by two operators (each of them analyzed one sample of each concentration on 21 different days).

The first calibration curves were prepared from serum containing cortisol in concentrations of: 25, 50, 100, 200, 500, 1000 ng ml⁻¹. All samples contained 5 ng ml⁻¹ 6- α -methylprednisolone.

The calibration curves for fluoxetine were prepared from serum containing fluoxetine in concentrations of: 25, 50, 100, 200, 400 and 600 ng ml⁻¹. All samples contained 5 ng ml⁻¹ protryptiline.

2.6. Statistical analysis

Statistical analysis employed the SAS v.9 software. The area under the curve was calculated by the trapezoid method. The plasma concentrations of cortisol, fluoxetine and HDRS scores were analyzed using repeated measures of MANOVA (multivariate analysis of variance). To detect differences in initial cortisol and fluoxetine levels we compared using the Student *t* test.

3. Results and discussion

The HPLC method of cortisol and fluoxetine determination was validated. The response was linear within the studied range of 25–1000 ng ml⁻¹ for cortisol and 25–600 ng ml⁻¹ for fluoxetine. The calibration curve was established by applying the linear regression method. The linear regression parameters were calculated from peak areas/peak IS. The typical linear regression equations were: $y = 0.0071x + 0.1354$ ($R^2 = 0.9999$) for cortisol and $y = 0.4531x - 0.0035$ ($R^2 = 0.9995$) for fluoxetine. Repeatability of the HPLC method for quantification of cortisol and fluoxetine the following coefficients of variation (CV %) were obtained <15% and intermediate precision were obtained <10%. The recovery of the examined analytes was 102.83% for cortisol and 92.5% for fluoxetine. The limit of quantification (LOQ) was established at 12.75 ng ml⁻¹ for cortisol and 13.4 ng ml⁻¹ for fluoxetine. The limit of detection (LOD) was established at 7.01 ng ml⁻¹ for cortisol and 9.57 ng ml⁻¹ for fluoxetine. LOQ and LOD were calculated according to "HPLC Methods for Pharmaceutical analysis" by Lunn and Schmuff (1996).

The level of cortisol in the blood serum of healthy subjects (the control group, $N = 33$) ranges between 51.09 and 263.22 ng ml⁻¹ (mean cortisol levels 116 ± 75.3 ng ml⁻¹) and were in the physiological range by Burtis et al. (2006), which confirms that our methodology of determining cortisol is comparable to the methodology applied in other studies described in literature.

One of the tasks we focused on was the analysis of the changes of presence of cortisol in serum of patients with MDD during 8 weeks of fluoxetine treatment. Cortisol concentration in blood

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