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EVALUATION OF CORRELATION BETWEEN THE PHARMACOGENETIC PROFILES OF RISPERIDONE TREATED PSYCHIATRY PATIENTS WITH PLASMA AND URINE CONCENTRATION OF RISPERIDONE AND ITS ACTIVE MOIETY 9-OH RISPERIDONE DETERMINED WITH OPTIMIZED BIOANALYTICAL LC METHOD

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ABSTRACT

Atypical antipsychotic risperidone is widely used first-line monotherapy in schizophrenia and combined therapy in bipolar disorders. Therapeutic plasma concentrations of risperidone and its active moiety are directly influenced by genetic variations in metabolic CYP450 enzymes (CYP2D6 and CYP3A4/5) and transporter (ABCB1) protein and additional environmental factors. Since active metabolite 9-OH risperidone has a greater percentage of the pharmacologically active fraction and is equipotent to the parent drug risperidone, it is assumed that it contributes significantly to therapeutic and adverse effects.

Unpredictable dose/concentration ratio, narrow therapeutic index, number of interactions, along with serious adverse reactions (ADR), raises the need for individualization of risperidone treatment and establishing of good therapeutic regime using TDM.

A simple and reliable validated bioanalytical liquide-liquide extraction HPLC/UV method was applied for the simultaneous determination of risperidone and its active metabolite, 9-OH risperidone, in human plasma and urine of 52 hospitalized schizophrenia/bipolar disorder patients treated with risperidone as monotherapy and in polytherapy. All the patients were previously genotyped for CYP2D6 (EM=30, EM/IM=14, IM=4 IM/PM=1 and PM=3) and ABCB1 using Real-Time PCR methods with TaqMan SNP genoty¬ping suitable assays according to the guidelines of the manufacturer (Life Technologies, USA).

The influence of CYP2D6 phenotype on metabolic ratio MR (Ris/9-OHRis) in plasma ($p=0.012$) and in urine ($p=0.048$) was confirmed. Statistically significant correlation ($R2=55.53\%$, $Rho=0.844$, $p<0,0001$) for MR in both plasma and urine indicates that urine may be utilized as appropriate media for initial CYP2D6 phenotype identification and selection of patients on risperidone treatment with high risk for ADR.

Key words: risperidone, 9-OH risperidone, plasma, urine, liquide- liquide extraction, HPLC

INTRODUCTION

Atypical antipsychotic risperidone is the most widely used neuroleptic as first-line monotherapy in schizophrenia and combined therapy in bipolar disorders.

Risperidone is selective antagonistic for serotonin 5-HT2 and dopamine D2 receptors. Antipsychotic effect is linked to blockade of more than 65% D2 receptors. Occupancy of more than

72% of dopamine 2 receptors in tuberoinfundibular pathway is considered responsible for prolactin elevation, whereas occupancy of over 80% of these receptors cause extrapyramidal effects (EPS). [1] Risperidone plasma concentrations are influenced by many physiological factors such as age, renal and hepatic function. It is confirmed that concentration-dose ratio is increased approximately by 35% for every decade in patients that are over 40 years old. [2]

Therapeutic plasma concentrations of risperidone and its active moiety are directly influenced by genetic variations in metabolic CYP 450 enzymes (CYP2D6 and slightly CYP3A4/5) and transporter (ABCB1) enzymes, and are influenced by environment. Particularly drug interactions associated with intake of inducers or inhibitor may influence CYP or P-gp and alter the pharmacological profile of risperidone and its active metabolite, and their therapeutic effect [3, 4]. The metabolic transformation of risperidone is determined by the genetic variations in CYP2D6 enzyme, and depending on the number of active copies of the gene patients are classified as extensive metabolizers (EM), intermediate metabolizers (IM), ultrarapid metabolizers (UM) and poor metabolizers (PM). Bork et al, confirmed that the CYP2D6 poor metabolizers have three times higher risk for adverse reactions associated with risperidone treatment in comparison with EM and IM and six times higher risk for treatment failure in comparison with EM [5, 6]

Main metabolite 9-OH risperidone has almost the same pharmacological antipsychotic activity as risperidone [7, 8] and the clinical response is a result of concentration of active moiety-AM (sum of risperidone and 9-OH risperidone). Risperidone is usually administered at oral daily doses ranging from 1 to 8 mg, which result in concentration of 20-60ng/ml of active moiety for optimal clinical outcome [9]. Most of the patients that are on per-oral form of risperidone exhibit plasma levels of 9-OH risperidone between 5 and 10 times higher than their plasma levels of risperidone. It is supposed that 9-OH risperidone is responsible for risperidone induced prolactin secretion, probably because it crosses the blood brain barrier (BBB) to a lesser extent and dopamine receptors on tuberoinfundibular pathway are unprotected. Risperidone is highly bound (90%) to plasma proteins, while the active metabolite 9-OH risperidone binding is less strong (77% bound). Since active metabolite is equipotent to the parent drug and has a greater percentage in the pharmacologically active fraction, 9-OH risperidone is assumed that contributes significantly to both therapeutic and adverse effects. Maximum plasma concentrations of risperidone are reached after 1 hour in extensive metabolizers and after 3 hours in poor metabolizers. Risperidone has linear elimination kinetics and steady state plasma concentrations are reached in period of one day for risperidone and five days for its active metabolite 9-OH risperidone. Risperidone and its active metabolite are dominantly eliminated in urine and partly in feces. The half-life of risperidone and 9-OH risperidone are 3 and 21 hours, while in poor metabolizers are 20 and 30 hours, respectively.

Risperidone has a narrow therapeutic index, unpredictable plasma concentration because of differences in genetics, age, gender, absorption, autoinduction and disease state between individuals. Therefore, the routine analysis of risperidone along with 9-OH risperidone may provide optimal therapeutic monitoring of this antipsychotic treatment and identification of patients with higher risk for therapeutic failure and adverse reactions occurrence. [10]

Unpredictable dose/concentration ratio, narrow therapeutic index, date for number of clinically relevant interactions of risperidone with other drugs, as well as serious adverse reactions, raises the need for individualization of risperidone treatment and establishing of good therapeutic regime using therapeutic drug monitoring (Therapeutic drug monitoring, TDM).

The aim of this study was to evaluate the correlation between the pharmacogenetic profile of risperidone treated patients with simultaneously analyzed plasma and urine concentration of risperidone and its active moiety 9-OH risperidone determined with reliable optimized and validated liquid - liquid extraction method followed by RP-HPLC with UV detection.

MATERIAL AND METHODS

Human plasma sampling

Plasma/urine samples were obtained from 52 schizophrenia/bipolar disorder patients undergoing chronic risperidone therapy hospitalized at Clinic of Psychiatry, Faculty of Medicine, Uni-

versity “Ss. Cyril and Methodius”, Skopje. The participation of each subject was voluntary and could be cancelled by any individual at any time during this study (according to the Helsinki II Declaration). The Ethics Committee at the Faculty of Pharmacy and the Faculty of Medicine, Ss. Cyril and Methodius University – Skopje, approved the research protocol for this study and all volunteers signed the Study Informed Consent form.

Sampling time of risperidone in relation to dose ingestion was important for the interpretation of the drug concentration. According to literature results, blood samples for TDM of risperidone should be collected after eight weeks of treatment and after a minimum of two weeks of stable dose of the drug. Blood samples were drowned before the morning dose and after a minimum of 15 days. Thus, blood samples were collected at 08.00 h in the morning, just before the first daily drug administration, into EDTA tubes, and centrifuged at 3000 rpm for 10 min. The supernatant plasma was transferred into test tubes and frozen at - 20°C until analysis. At the same time urine sample was collected and stored at - 20°C until analysis.

DNA isolation

The genomic DNA was extracted from peripheral lymphocytes in the blood samples obtained in EDTA vacutainers, using Proteinase K digestion, phenol chloroform extraction and ethanol precipitation. DNA yields and purity were measured at 260 nm and 260/280nm respectively (NanoDrop 2000, Thermo Scientific) and DNA integrity was confirmed with electrophoresis on 1% agarose gels, stained with ethidium bromide.

Genotyping

The genotyping was performed with Real-Time PCR based on the allelic discrimination method (MxPRo 3005P, Staratgene, La Jolla, CA, USA) using TaqMan SNP genotyping assay for CYP2D6 (EM=30, EM/IM=14, IM=4, IM/PM=1 and PM=3) and ABCB1gene with Real-Time PCR methods using TaqMan SNP genoty¬ping suitable assays according to the guidelines of the manufacturer (Life Technologies, USA).

Preparation of solutions for application with liquid/liquid extraction

Plasma and urine samples were prepared by liquid/liquid extraction according to modified and adopted procedure, described by Jovanović et al., 2010. [11] Procedure of liquid/liquid extraction was performed for samples collected from risperidone treated patients. To the final volume of 1500 µl plasma/urine, clozapine (IS) was added to final concentration of 100 ng/ml plasma/urine and 0.5M NaOH (150 µl/plasma; 600 µl/urine). The mixture was continuously vortexed for 30 s and extraction solvent was added (hexane (Merck): ethyl acetate (Merck) (30:70) (v/v). Mixture of organic phase: (plasma/urine) ratio 1:1 (v/v) was centrifuged for 10 minutes at 12000 rpm. Organic phase was evaporated to dryness at thermo block at temperature of 37-40°C. The residue was dissolved in 50µl methanol and centrifuged for 2 minutes at 12000 rpm. 30 µl of the sample was injected into the HPLC system.

Previously validated method was applied for simultaneous analyzation of risperidone/9-OH risperidone in plasma/urine. The assay was carried out on Agilent 1100 HPLC system equipped with a vacuum degasser (G1322A Degasser), quaternary pump (G1311A QuatPump), autosampler (G1313A ALS), column compartment (G1316A COLCOM), diode array detector (G1315B DAD), and ChemStation for LC 3D software for data handling (Wilmington, DE).

The determination was performed with reversed-phase column LiChrospher 60 RP B selected (125 x 4 mm; 5 µm) (Merck) using acetonitrile and phosphate buffer (5 mM NaH-2PO4xH2O, pH 3,0) with gradient elution (from 20% (v) acetonitrile, rising to 80% (v) in period of 10 minutes for plasma; from 18% (v) acetonitrile, rising to 82% (v) in period of 10 minutes for urine) as a mobile phase at a flow rate of 1 ml/min. The temperature was 35°C, volume of injection 30 µl and UV detection was set at 280 nm. [12]

RESULTS AND DISCUSSION

The working concentration range for risperidone and 9-OH risperidone (10 – 150 ng/ml) was selected in agreement with published data for plasma concentration of risperidone and its active metabolite 9-OH risperidone. Higher

concentrations then concentration when the optimal therapeutic effect is achieved (active moiety AM, risperidone + 9-OH risperidone, ranges between 20-60ng/ml), are published for patients that are on concomitant therapy with CYP2D6 and ABCB1 inhibitors. [11, 13, 14] There was limited information on urine concentration of risperidone and 9-OH risperidone, the same concentration range as plasma samples (10 - 150 ng/ml) was used in the patients' population. In our optimized and validated method clozapine was selected as an internal standard.

No interference from endogenous plasma components was present and extraction procedure gave excellent results when was applied to plasma/urine samples of patients undergoing chronic treatment with risperidone, both in monotherapy and polytherapy. Using the chromatographic conditions satisfactory separation for risperidone, its active metabolite and internal standard is obtained and no interference from endogenous plasma/urine components was present (the peaks from blank plasma/urine were < 20% from LLOQ of the analytes and less then 5% of the LLOQ of internal standard). No interfering peaks due to the co-administered drugs except carbamazepine were eluted at the retention times of analytes. [12]

The optimized method was applied to the simultaneous determination of risperidone and its active metabolite, 9-OH risperidone, in 52 plasma samples and 46 urine samples taken from patients with diagnosed psychiatric disorder (schizophrenia or bipolar disorder) under chronic risperidone treatment (2 – 6 mg/day). 32 of the patients were on co-medication with biperiden (2-6 mg/day) and lorazepam (2.5-10 mg/day), and 2 of the patients were co-medicated with carbamazepine (400 mg/day). The obtained results for risperidone, 9-OH risperidone, metabolic ratio (MR, risperidone/9-OH risperidone) and active moiety (AM, risperidone+9-OH risperidone) from plasma/urine obtained from patient population are presented in Table 1.

Table 1. *Average plasma concentration and standard deviation of risperidone, 9-OH risperidone, metabolic ratio (MR, risperidone/9-OH risperidone) and active moiety (AM, risperidone+9-OH risperidone) from plasma/urine obtained from patient population.*

	Plasma (52 patients)				**Urine (46 patients)**			
	9-OH risperidone	**risperidone**	Metabolic Ratio (ris/9-OH-ris)	**Active Moiety (ris+9-OH- ris)**	**9-OH risperidone**	**risperidone**	Metabolic Ratio (ris/9-OH-ris)	**Active Moiety (ris+9-OH- ris)**
	ng/ml	**ng/ml**		**ng/ml**	**ng/ml**	**ng/ml**		**ng/ml**
average	71.46	14.01	0.44	85.47	969.68	150.03	0.32	1133.71
SD	47.87	10.57	0.26	51.91	1137.93	185.27	0.23	1236.24

All 52 patients treated with risperidone were classified in five CYP2D6 phenotypes. Three poor metabolizers and one IM/PM were identified as a subpopulation highly prompt on ADR when treated with risperidone and no ultrarapid metabolizer (UR) was identified. The obtained results were in line with the confirmed frequency of 5-10% of poor metabolizers whereas ultrarapid CYP2D6 phenotype was found in only 1-2% of Caucasian population. [15] The patients were also genotyped for the three most commonly analyzed ABCB1 gene polymorphisms C1236T, G2677T/A, and C3435T. No statistically significant influence of these three SNPs on plasma concentration of dose corrected concentration of risperidone and 9-OH risperidone was observed in plasma and urine was confirmed. ABCB1 gene polymorphisms didn't influence the metabolic ratio (MR, ris/9-OHris), nor active moiety (AM, ris+9-OHris) in both biological fluids.

Our study has established statistically significant influence of CYP2D6 phenotype on pharmacokinetic parameters of risperidone. The influence of CYP2D6 phenotype on metabolic ratio MR (Ris/9-OHRis) in plasma (p=0,012) and in urine (p=0,048) was confirmed (Table 2, a and b respectively).

Table 2. *Influence of CYP2D6 phenotype on metabolic ration (MR) Ris /9-OH-Ris in plasma (a) and urine (b)*

Influence of CYP2D6 phenotype on MR (ris/9-OH ris) in plasma				**Kruskal-Wallis**
Phenotype	patients	Average	SD	P-value
EM	30	0.086	0.120	**0.012**
IM	4	0.199	0.258	
IM/EM	14	0.073	0.157	
IM/PM	1	1.181		
PM	3	1.481	0.508	
Total	52	0,193	0.395	

Influence of CYP2D6 phenotype on MR (ris/9-OH ris) in urine				**Kruskal-Wallis**
Phenotype	patients	Average	SD	P-value
EM	18	0.199	0.405	**0.048**
IM	2	0.065	0.088	
IM/EM	12	0.230	0.469	
IM/PM	1	0.890		
PM	3	1.999	1.154	
Total	36	0.371	0.698	

The obtained chromatograms from the analyzed patients' plasma and urine for different CYP2D6 phenotypes are presented in figures below. In figures 1 and 2 are presented the chromatograms obtained after plasma/urine sample preparation from a patient on risperidone 2 mg/day treatment, without co-medication. The patient has phenotype of CY2D6 poor metabolizer with (*4/*4) genotype. The correlation between metabolic ratio (MO) of risperidone/9-OH risperidone in plasma and urine is observed.

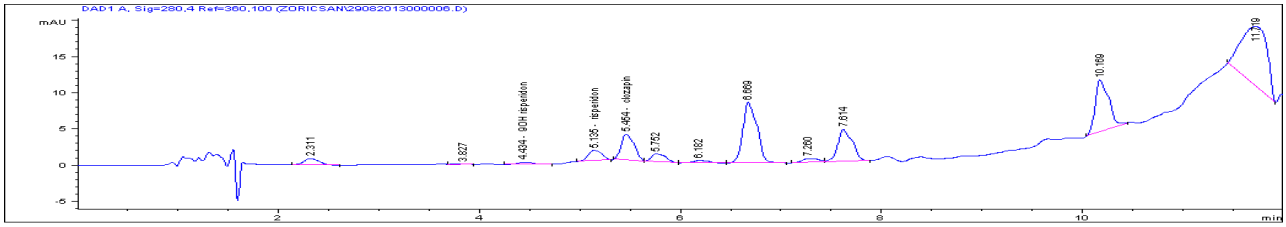

Figure 1. *Chromatograms of plasma sample from a patient that is poor metabolizer (PM)*

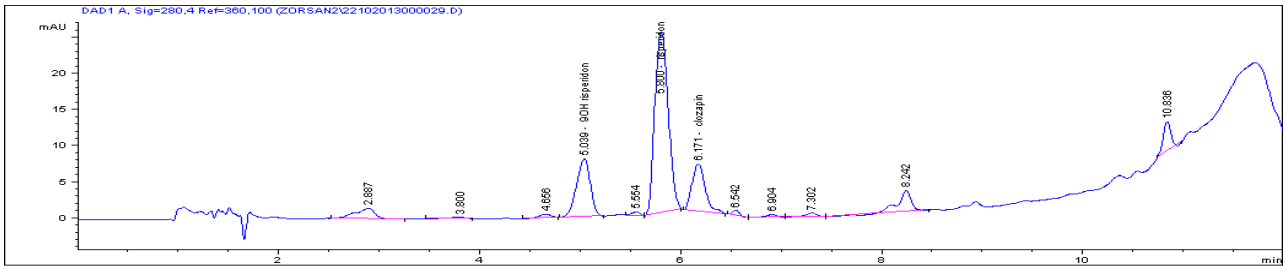

Figure 2. *Chromatograms of urine samples from a patient that is poor metabolizer (PM)*

In figures 3 and 4 are presented the chromatograms obtained after plasma/urine sample preparation from a patient on risperidone 2 mg/day treatment with concomitant use of biperiden (2 mg/day) and promazine (100 mg/day). The patient has IM/EM phenotype for CYP2D6 with (*1/*4) genotype. The correlation between metabolic ratio (MR) of risperidone/9-OH risperidone in plasma and urine is confirmed. No interference is observed between risperidone, its active metabolite and IS with the active substance used as a co-medication. Software evaluation of peak purity confirmed that there was no additional peak that co-elute with peaks of analytes and IS. That excludes the influence of biperiden and promazine in this optimized HPLC condition when they are used as co-medication with risperidone.

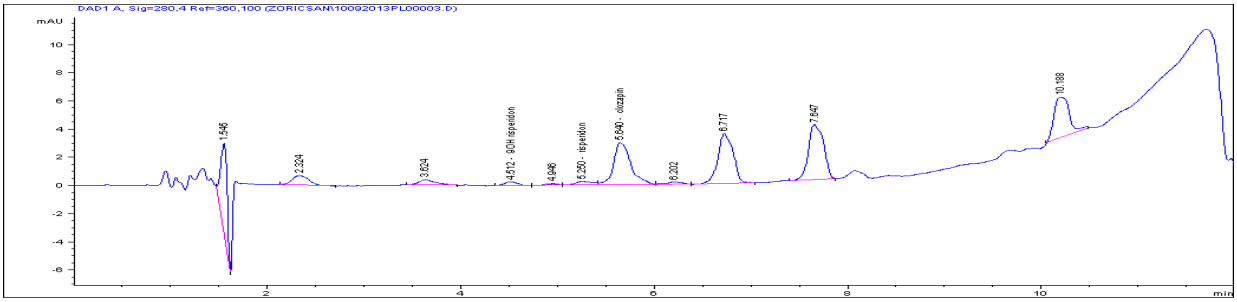

Figure 3. *Chromatograms of plasma samples from a patient that is intermediate/extensive metabolizer (IM/EM)*

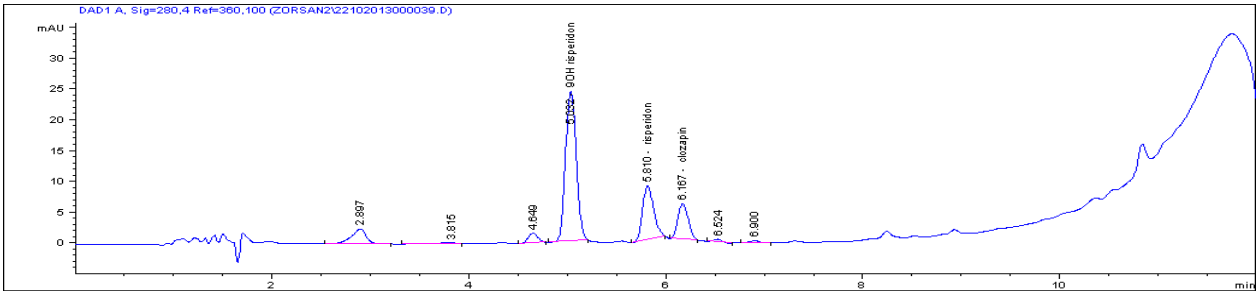

Figure 4. *Chromatograms of urine sample from a patient that is intermediate/extensive metabolizer (IM/EM)*

On figures 5 and 6 are presented the chromatograms obtained after plasma/urine sample preparation from a patient on risperidone (4 mg/day), treatment with concomitant use of biperiden (2 mg/day) and lorazepam (2.5 mg/day). The patient has EM phenotype for CYP2D6 with genotype (*2/*2). The correlation between metabolic ratio (MO) of risperidone/9-OH risperidone in plasma and urine is confirmed. No interference is observed between risperidone, its active metabolite and IS with the active substance used as a co-medication. Software evaluation of peak purity confirmed that there was no additional peak that co-elute with peaks of analytes and IS. That excludes the influence of biperiden and lorazepam in this optimized HPLC condition when they are used as co-medication with risperidone.

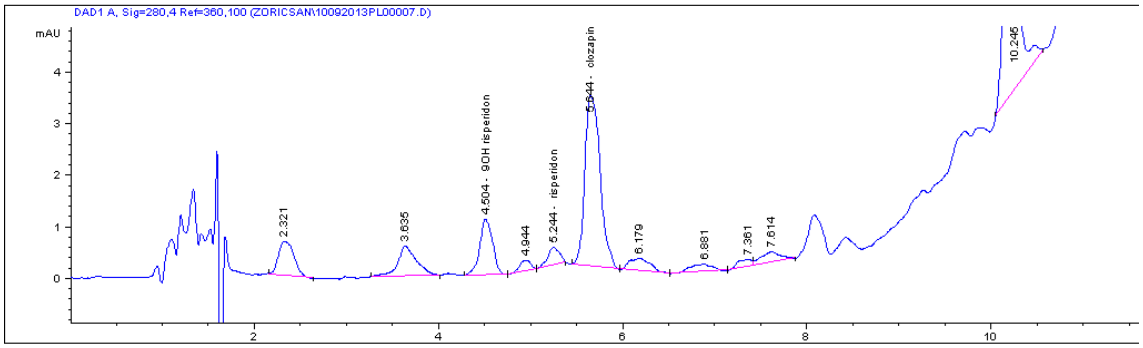

Figure 5. *Chromatograms of plasma sample from a patient that is extensive metabolizer (EM)*

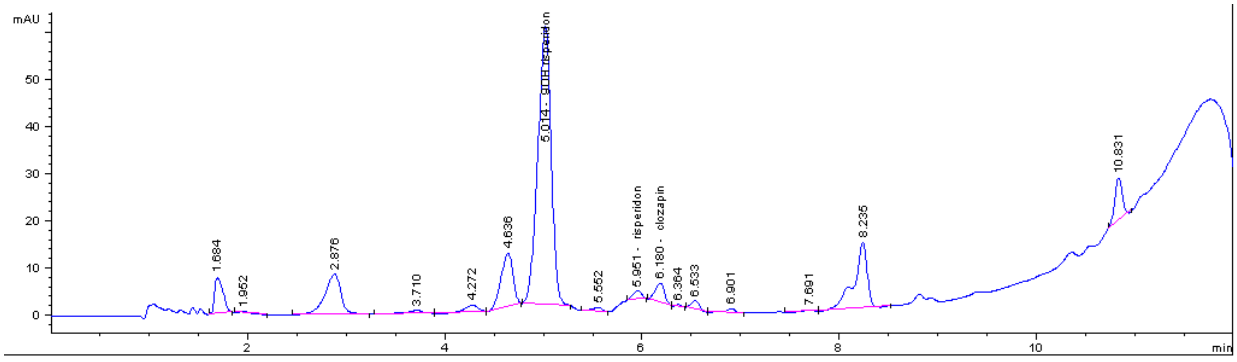

Figure 6. *Chromatograms of urine sample from a patient that is extensive metabolizer (EM)*

In figures 7 and 8 are presented the chromatograms obtained after plasma/urine sample preparation from patient on risperidone (4 mg/day), treatment with concomitant use of carbamazepine (400 mg/day). The patient has EM phenotype for CYP2D6 with genotype (*1/*2). Presented chromatograms show that carbamazepine co-elutes with the peaks from risperidone and clozapine, and has high concentration in plasma/urine. Optimized HPLC condition is not suitable for therapeutic drug monitoring in risperidone treated patients when they are in carbamazepine co-medication. Two patients which use this therapeutic regime were excluded from this study.

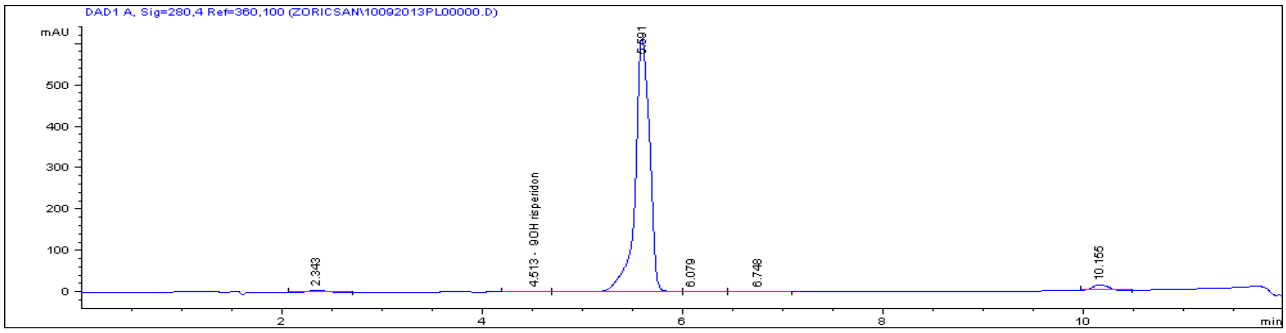

Figure 7. *Chromatograms of plasma sample from a patient that is co-medicated with carbamazepine*

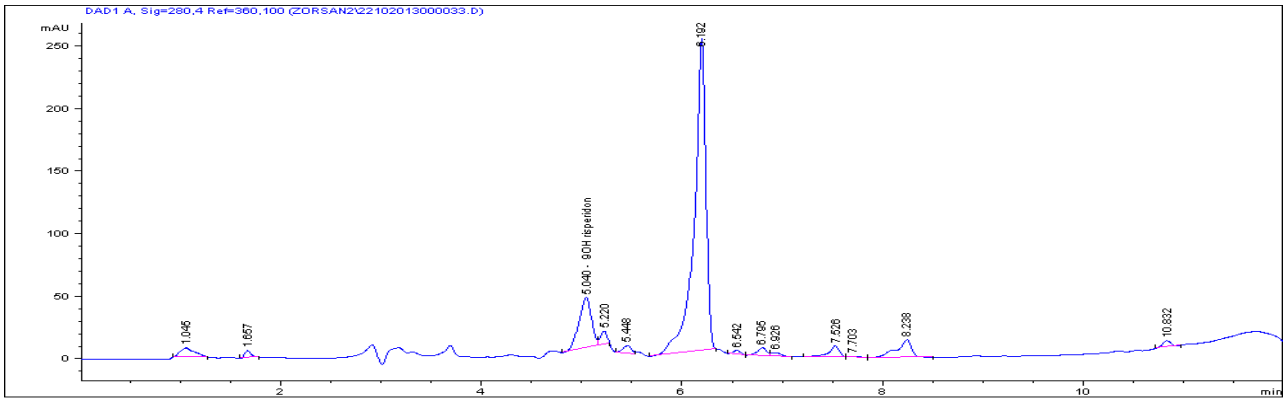

Figure 8. *Chromatograms of urine samples from a patient that is co-medicated with carbamazepine*

Statistically significant correlation was obtained for metabolic ratio (MR) risperidone/9-OH risperidone in plasma plotted to metabolic ratio (MR) in urine (R2=55.53%, Rho=0.744, p<0, 0001) defined with linear regression double square root model: Y = (a + b*sqrt(X))2, (p=0, 0001) (Figure 9). The model is determined by following equitation:

Plasma RATIO (Ris/9OHRis) = (0.0748346 + 0.638939*sqrt (Urine RATIO (Ris/9OH Ris)))2

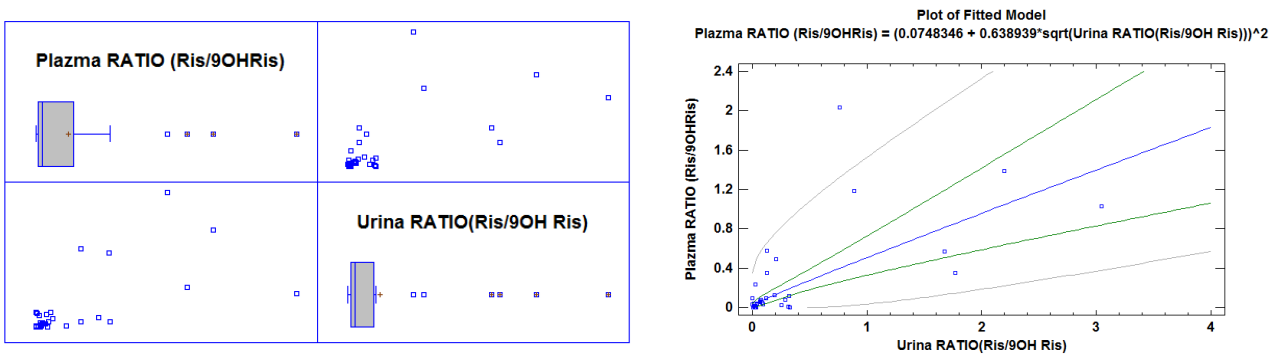

Figure 9. *Correlation between risperidone/9-OH risperidone metabolic ratio (MR) in plasma and urine*

This model explains 55.36% of the variability of MR (Ris/9OH-Ris) in the plasma after logarithmic transformation with relatively high coefficient of correlation of 0.744 between the variables. Statistically significant correlation (p=0.0001; SD=0.247; CI 95%) indicates that MR (Ris/9OHRis) obtained from the urine could be used for determination of MR in plasma for risperidone treated patients. Taking into consideration the fact that risperidone metabolic ratio MR is reflecting CYP2D6 phenotype of the patient and could be treated as the biomarker for CYP2D6 genotype in schizophrenic patients [16], the optimized bioanalytical LC method could be utilized for selection of patient population that is on high risk for adverse reaction occurrence (CYP2D6 PM phenotype) from risperidone treatment, and help in initial dosing for the patient.

CONCLUSION

A simple and reliable bioanalytical HPLC method was successfully applied to separate and determine the risperidone and 9-OH risperidone in plasma and urine obtained from patients treated with risperidone as monotherapy and in polytherapy. Proposed method is suitable for a reliable therapeutic drug monitoring of risperidone treated patients, and could be particularly advantageous when multiple blood sampling are needed, as in pharmacokinetic studies.

The influence of CYP2D6 phenotype on metabolic ratio MR (Ris/9-OHRis) in plasma (p=0,012) and in urine (p=0,048) was confirmed. Statistically significant correlation (R2=55,53%, Rho=0.844, p<0,0001) for MR in both plasma and urine indicates that urine may be utilized as appropriate media for initial CYP2D6 phenotype identification and selection of patients on antipsychotic risperidone treatment with high risk for adverse drug reactions.

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Резиме

ЕВАЛУАЦИЈА НА КОРЕЛАЦИЈАТА НА ФАРМАКОГЕНЕТСКИОТ ПРОФИЛ НА ПСИХИЈАТРИСКИ ПАЦИЕНТИ СО КОНЦЕНТРАЦИЈАТА НА РИСПЕРИДОН И НА НЕГОВИОТ АКТИВЕН МЕТАБОЛИТ 9-ОН РИСПЕРИДОНА ВО ПЛАЗМА И УРИНА ОПРЕДЕЛЕНА СО ОПТИМИЗИРАН БИОАНАЛИТИЧКИ LC МЕТОД

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Атипичниот антипсихотик рисперидон е широко применуван како прволиниска монотерапија кај шизофренијата и како дел од комбинираната терапија кај биполарните растројства. Терапевтските плазма-концентрации на рисперидон и неговиот активен метаболит се под директно влијание на генетските варијации на метаболните CYP450 ензими (CYP2D6 и CYP3A4/5) и на транспортниот (ABCB1) протеин, како и дополнителни фактори од надворешната средина. Бидејќи активниот метаболит 9-ОН рисперидон има поголем процент на фармаколошки активната фрекција и е еквипотентен со рисперидонот, се смета дека значително придонесува во терапевтскиот ефект и појавата на несакани реакции.

Непредвидливиот доза/концентрација/однос, тесниот терапевтски индекс, бројните интеракции и сериозните несакани реакции ја зголемуваат потребата од индивидуализацијата на третманот со рисперидон и воспоставување добар терапевтски режим со примена на терапевтски мониториг на лековите.

Едноставен, конзистентен и валидиран биоаналитички HPLC/UV метод по течно-течна екстракција е применет за симултано определување на рисперидон и неговиот активен метаболит 9-ОН рисперидон во плазма и во урина кај 52 хоспитализирани пациенти со дијагностицирана шизофренија/биполарни растројства, третирани со рисперидон како монотерапија или како дел од

политерапија. Сите пациенти се претходно генотипизирани за CYP2D6 (EM=30, EM/IM=14, IM=4 IM/PM=1 и PM=3) и ABCB1 со примена на Real-Time PCR методи со соодветни TaqMan SNP тестови за генотипизација, во согласност со инструкциите од производителот (Life Technologies, USA).

Потврдено е влијанието на CYP2D6 фенотипот на метаболниот однос MO (Ris/9-OHRis) во плазма (p=0,012) и во урина (p=0,048). Определената статистички значајна корелација (R2=55,53%, Rho=0.844, p<0,0001) за MO и во плазма и во урина индицира дека уринатa може да се користи како соодветен медиум за иницијална CYP2D6 фенотипска идентификација и селекција

Клучни зборови: рисперидон, 9-OH рисперидон, плазма, урина, трчно-трчна екстракција, HPLC, CYP2D6, ABCB1