Determination of Risperidone and its Active Metabolite 9-Hydroxyrisperidone in Plasma by Dispersive Liquid Liquid Microextraction – HPLC-UV

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Abstract

A reliable method has been proposed for the simultaneous determination of risperidone and its active metabolite 9-hydroxyrisperidone in plasma by dispersive liquid-liquid microextraction (DLLE) in combination with high-performance liquid chromatography-ultraviolet detector. All important variables influencing the extraction efficiency, such as pH, types of the extraction solvent and the disperser solvent and their volumes, ionic strength and centrifugation time were investigated and optimized. The method was validated over the concentration range 10-200 ng/ml. Mean recoveries were 96.8% for risperidone and 98.3% for 9-hydroxyrisperidone. Intra- and inter-day relative standard deviations were less than 11.5% for both compounds, while accuracy, expressed as percent error, ranged from 1.4 to 12%. The limit of quantitation was 3 ng/ml for both analytes. The method was successfully applied for the determination of risperidone and 9-hydroxyrisperidone in plasma.

Keywords: *Risperidone; 9-hydroxyrisperidone; dispersive liquid-liquid microextraction; high-performance liquid chromatography; plasma.*

Introduction

Risperidone (RSP), a benzisoxazole derivative, is one of the newer antipsychotic drugs used in the treatment of schizophrenia and other psychotic disorders[1,2]. Clinical studies indicate that daily doses of 4-6 mg are effective in most patients[3]. RSP is rapidly and completely absorbed from the gastrointestinal tract after oral administration and undergoes extensive metabolism by hydroxylation and N-dealkylation. The main metabolite of RSP, 9-hydroxyrisperidone (9-OH-RSP), has a similar activity as the parent compound, and the serum concentration of active moiety is thus the sum of the serum concentrations of RSP and 9-OH-RSP (Figure 1)[4,5].

The methods for the determination of antipsychotic drugs in biological samples are mainly based on the separation techniques of gas chromatography (GC) with flame

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photometric detection (FPD)[6] and flame ionization detection (FPD)[7], capillary electrophoresis (CE)[8] and high-performance liquid chromatography (HPLC)[9] coupled with UV detection[10], gas chromatography flame photometric detection (GC-FPD)[6], gas chromatography flame ionization detection (GC-FID)[6,7], coulometric detection[11], and high-performance liquid chromatography diode-array detection (HPLC-DAD)[12]. The use of some novel hyphenated techniques was also reported, such as LC-MS[13] and LC-MS-MS[14]. Nowadays, more and more scientists focus on exploring the application of MS because of its high selectivity and superior sensitivity. However, the high cost involved in the instrumental setup and the sequential instrumental operation/maintaining, makes it unaffordable in many studies. Among the aforementioned methods, HPLC-UV is most commonly used, mainly due to its low cost and easy accessibility.

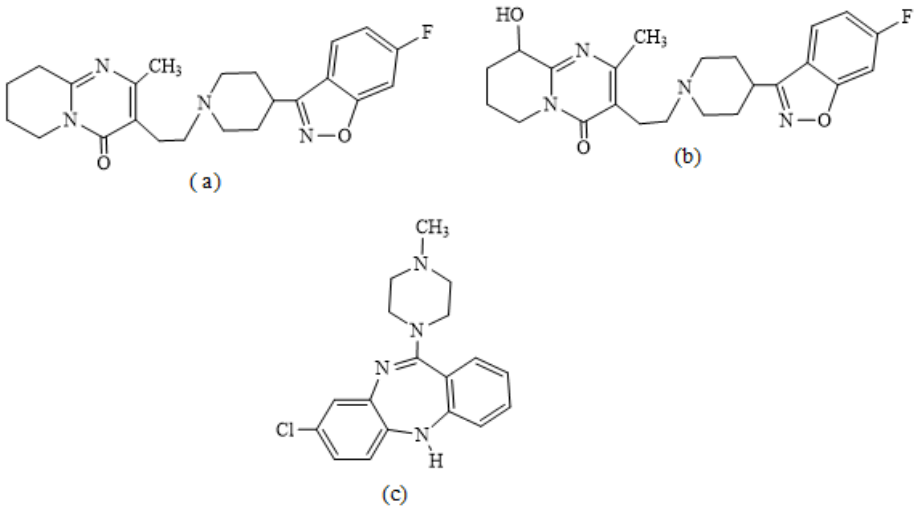

Figure 1: Chemical structures of (a) risperidone, (b) 9-hydroxyrisperidone and (c) clozapine (IS).

Owing to the low concentration of the target drugs and the complex matrices in blood samples, pretreatment of samples is usually required prior to instrumental analysis. Some separation/preconcentration procedures including liquid–liquid extraction (LLE)[15] and solid-phase extraction (SPE)[16,17] have been applied for the determination of antipsychotic drugs. However, LLE is tedious, time-consuming and normally requires large amounts of organic solvents that are potentially hazardous to human health while SPE requires a specific device loaded with certain adsorption material as well as a high-pressure delivery system that can be relatively expensive. It should be noted that some of microextraction methods such as hollow fiber-based liquid phase extraction[18] and fiber-in-tube solid-phase microextraction[19] have been successfully used for the analysis of some drugs in the biological fluids. Recently, a novel liquid phase microextraction technique named dispersive liquid–liquid micro-extraction (DLLME) has been proposed by Rezaee and co-workers, which is based on a ternary component solvent extraction system including extraction solvent, disperser

solvent and aqueous samples containing the analyte of interest[20]. It possesses many advantages: simple, rapid, low cost, high enrichment factor, high recovery and easy operation. DLLME has been widely applied for the assay of environmental water samples and it also shows good prospect in the analysis of analytes in complex matrices such as biological fluids[21-23]. Fisichella et al. developed a DLLME method for the determination of many different classes of drugs including main drugs of abuse (cocain and metabolites, amphetamines and analogues, LSD, ketamine, opiates, methadone and fentanyl and analogues), Z-compounds and 44 benzodiazepines and antipsychotic drugs in blood samples followed by analysis with liquid UHLC-MS/MS[24].

In the present study, DLLME was applied for the analysis of RSP and 9-OH-RSP in human blood using HPLC-UV. The factors influencing the extraction efficiency and analytical determination were evaluated in detail. The developed method was successfully applied to real blood samples analysis.

Materials and Methods

Chemicals

The reference standards of RSP, 9-OH-RSP and Clozapine (IS) were obtained from Sigma-Aldrich (Darmstadt, Germany). Dichloromethane (CH_2Cl_2), chlorobenzene (C_6H_5Cl), chloroform ($CHCl_3$) and carbontetrachloride (CCl_4), methanol, ethanol, acetone, acetonitrile, sodium chloride (NaCl), and sodium hydroxide (NaOH) were also purchased from Sigma-Aldrich (Darmstadt, Germany).

Instruments and chromatographic conditions

A liquid chromatographic system consisting of a 1200 series binary pump (Agilent, Waldbronn, Germany) equipped with a UV detector set at 280 nm wavelength was used. Chromatographic separation was carried out on a C_{18} BDS-Hypersil analytical column (3 µm, 100x4.6 mm I.D.). The mobil phase of KH_2PO_4 (0.1 M, pH 3.3 with 20% H_3PO_4) and acetonitrile (75:25,v/v) was delivered at a flow rate of 1mL/min. The run time of assay was 4 min and the retention times for 9-hydroxyrisperidone, risperidone and clozapine (I.S.) were 2.4, 2.7 and 3.3 min, respectively.

Drug solutions

Stock solutions of RSP, 9-OH-RSP and clozapine (I.S.) (1mg/ml of each) were prepared in methanol. Working solutions were prepared by diluting aliquots of the stock solutions with methanol. These solutions were stored at -20 ^{o}C until analysis.

Preparation of plasma sample

Drug-free blood from healthy donors was used for the validation studies. Calibrators were prepared by spiking known amounts of working standard solutions in 1 ml blank plasma to yield the final concentrations of 10-200 ng/ml for both analytes. The concentration of the internal standard in each calibrator was 50 ng/ml. Acetonitrile

was added to the samples in the ratio of 1:1 (v/v) and vortexed for 2 min to precipitate the plasma proteins. The resulting mixture was centrifuged for 10 min at 14000 rpm at 10 min then filtered to obtain a clear solution. 1 ml of the clear supernatant was transferred into a 10.0 ml glass test tube with a conical bottom and diluted with 5.0 ml of deionized water.

Dispersive liquid liquid microextraction (DLLME) procedure

The pH of a 5.0 ml of the prepared sample was adjusted to 10.0 with 1 M NaOH. Then, 5% (w/v) of NaCl (5 g/ 100ml) was added to the sample and the solution was placed in a 5 ml glass test tube with a conical bottom. 0.60 ml of methanol (as disperser solvent) containing 40.0 µl carbon tetrachloride (CCl_4) (as extraction solvent) was rapidly added to the sample solution by using a 1.0 ml syringe resulting immediately in a cloudy solution. Then the mixture was gently shaken. In order to separate the phases, the mixture was centrifugated for 1 min at 10000 rpm, the extraction solvent was sedimented in the bottom of the tube, sedimented phase was removed using a 1.0 ml syringe and evaporated to dryness at 40 ^{o}C under nitrogen. The residue was reconstituted in 200 µl of mobile phase and and 50 µl was injected into HPLC for analysis. The chromatogram of a blank sample, after DLLME, is shown in Figure 2A, while the chromatogram of the same plasma sample after DLLME, spiked with risperidone (100 ng/ml), 9-hydroxyrisperidone (100 ng/ml), and IS (50 ng/ml) is shown in Figure 2B.

Results and Discussion

Optimization of DLLME system

Selection of extraction solvent and its volume

A satisfactory extraction solvent should be selected on the basis of density higher than that of water, high extraction efficiency, low solubility in water, non-volatility during extraction and good HPLC behavior. Halogenated hydrocarbons solvents are denser than water and are the most widely used solvents in DLLME due to being easily removed from the bottom of the conical vial after centrifugation. Therefore, several organic solvents including chlorobenzene (C_6H_5Cl), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), and carbon tetrachloride (CCl_4) with density values of 1.11, 1.32, 1.47, and 1.59 g/ml, respectively, were investigated. The extraction capability was studied using a mixture of 0.60 ml of methanol and 40 µl of the above solvents to extract the standard solutions at pH 10.0. The results indicate that when CH_2Cl_2 or $CHCl_3$ were chosen as extraction solvents, no cloudy solutions were formed, and after centrifugation, no extract droplets were found in the bottom of the test tube. However, a cloudy solution was formed when CCl_4 or C_6H_5Cl were employed as extraction solvents and droplets of the extract were settled in the bottom of the test tube after centrifugation. The difference in the behaviour of extraction solvents is probably due to

the difference in their solubility in water. After chromatographic analysis of the obtained extracts, CCl_4 and C_6H_5Cl showed similar extraction capacities for RSP, 9-OH-RSP and IS. In this study, CCl_4 was chosen as the extraction solvent due to its highest relative density value.

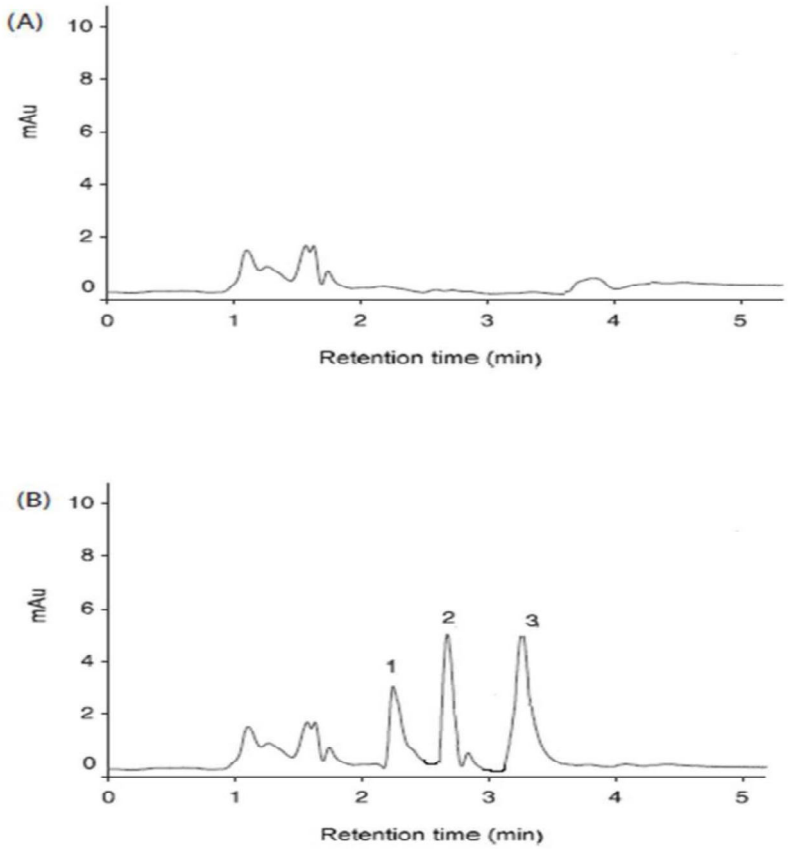

Figure 2: Chromatogram of A) a blank plasma sample after the DLLME procedure, and B) the same plasma sample spiked with 100 ng/ml of risperidone and 100 ng/ml of 9-hydroxyrisperidone as well as IS. Peaks:1= 9-hydroxyrisperidone, 2= risperidone, 3=IS.

To examine the effect of the volume of extraction solvent, the volume of CCl_4 was varied in the range of 10-50 µl, keeping other experimental conditions constant. Figure 3 shows that the extraction recovery was increased by increasing the volume of CCl_4 to 40 µl. However, a reduction in the extraction recovery for RSP and 9-OH-RSP is observed when the volume of CCl_4 exceeded 40 µl. Based on these observations, the volume of 40 µl was selected as optimal extraction volume of CCl_4.

Selection of dispersive solvent and its volume

The main criteria in the selection of the disperser solvent is its miscibility in the organic (extraction solvent) and aqueous (sample solution) phases. So, methanol, ethanol, acetone, and acetonitrile were chosen for this purpose. The effect of different kind of disperser solvents on the extraction capabilities of RSP, 9-OH-RSP and the IS are presented in Figure 4. It was found that using methanol as a disperser solvent attains the highest extraction efficiency and the best extraction precision for RSP and

its metabolite as compared with other solvents. Therefore, methanol was selected as a dispersive solvent in the present study.

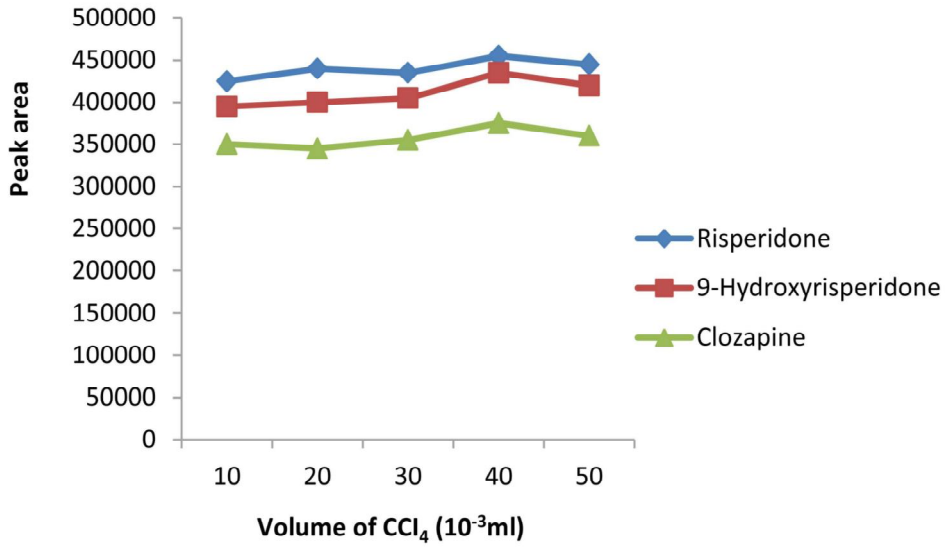

Figure 3: Effect of volume of extraction solvent (CCl_4) on extraction efficiency.

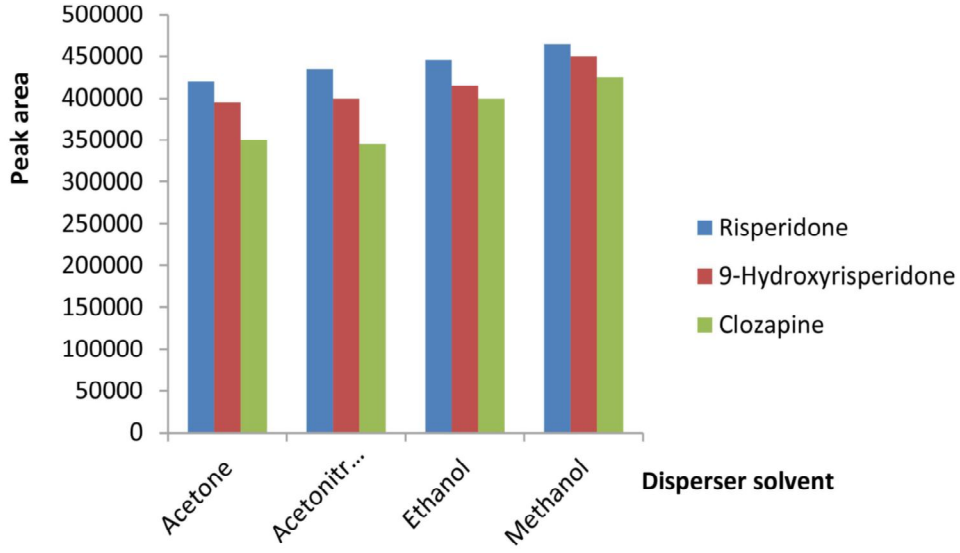

Figure 4: Effect of disperser solvent type on DLLME.

Dispersive volume has a key role in DLLME procedure. The influence of the volume of disperser solvent was investigated by using 0.2, 0.4, 0.6, 0.8 and 1 ml volumes. According to Figure 5, the extraction efficiency increases by increasing the volume of methanol to 0.6 ml and then decreases at volumes over 0.6 ml. Hence, in the present work, 0.6 ml of methanol was chosen as the optimal disperser volume.

Effect of pH

Obviously, pH was the key parameter for sample solution affecting both the extraction efficiency and DLLME selectivity. The effect of pH was studied in the pH range from 2 to 12; 1 M NaOH was used for pH adjustment. Figure 6 shows the effect

of pH on the peak area of the target analytes. The extraction efficiency continues getting improved by increasing the pH to 10, after which the area of target analytes remains constant. Hence, the optimal pH value for the above DLLME method is 10.

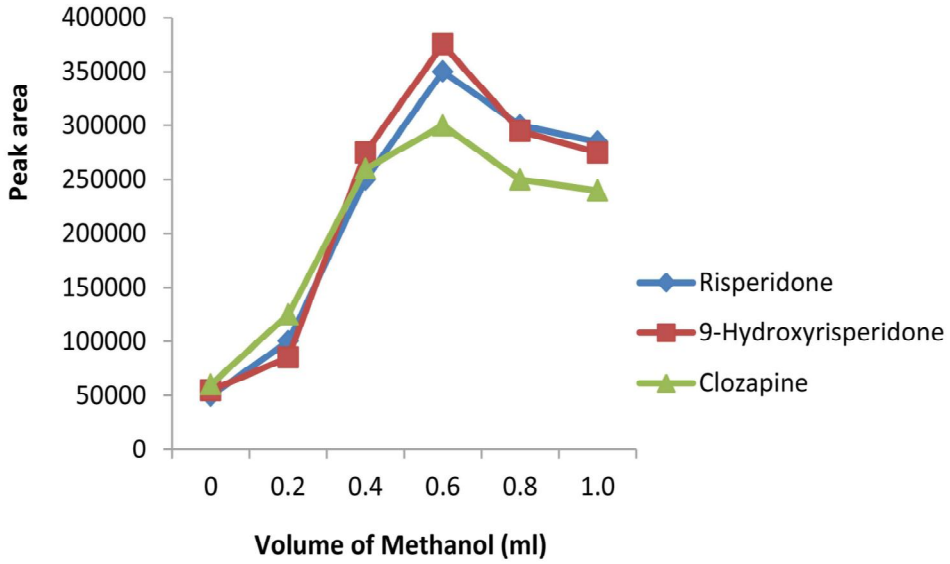

Figure 5: Effect of volume of disperser solvent (methanol) on DLLME.

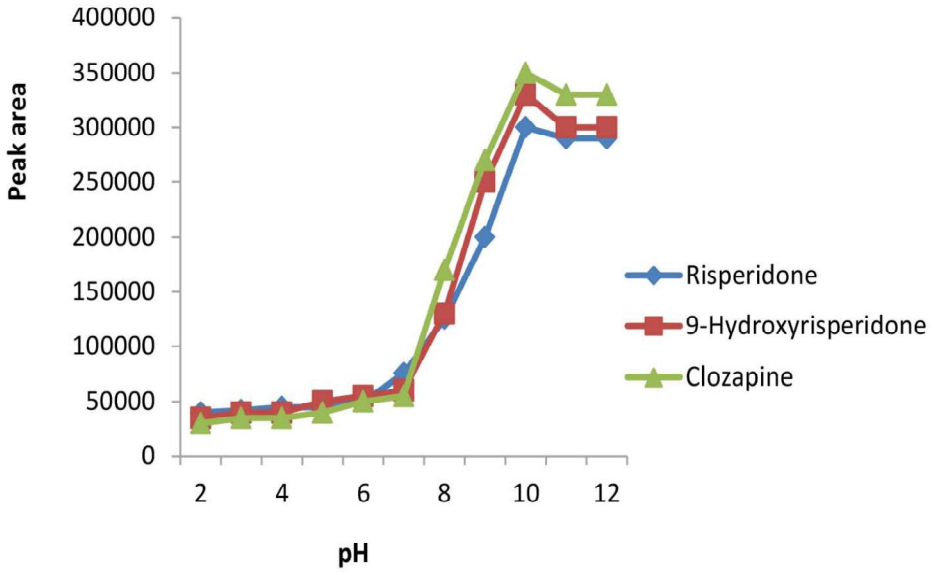

Figure 6: Effect of pH of aqueous solution on DLLME.

Effect of salt

To evaluate the possibility of salting out effect, the extraction efficiency was studied at different sodium chloride (NaCl) concentrations over the range of 1-15% (w/v). There was an incresae in the extraction efficiency with the increase in the salt concentration up to 5% after which a drop in efficiency was observed. Additionally, phases separation could not be accomplished at NaCl concentration above 10%. As a result, the optimum DLLME conditions were as follows: 40 µl CCl_4 as extraction solvent; 0.6 ml methanol as disperser solvent; pH=10 and 5% NaCl addition.

Effect of the extraction time, centrifugation time and rate

According to previous studies[25-28], the DLLME method is time-independent. To investigate the effect of extraction time on the extraction efficiency of target analytes, the extraction time was varied from 1 to 5 min. The results indicate that there is no obvious influence of the extraction time on extraction efficiency. This can be explained as follows: After injecting the mixture of disperser solvent (methanol) and extraction solvent (CCl_4), numerous small droplets of extract were instantaneously dispersed among the aqueous solution as cloudy phase, indicating the infinitely large interface between the extraction solvent and the aqueous phase. Therefore, quick equilibrium was achieved due to the fast transition of analytes from the aqueous phase to the extraction solvent. Consequently, 1 min of extraction time was chosen in the this work.

In DLLME, the most time consuming step is centrifugation. The effect of centrifugation rate and time were examined in the range of 4000-10000 rpm and 1-5 min, respectively. According to the obtained results, 10000 rpm and 1 min were selected as optimum centrifuge rate and time, respectively.

Method validation parameters

Linearity and recovery

The validation method followed the international analytical guidelines[29]. The standard calibration curves for RSP and 9-OH-RSP were linear over the range 10-200 ng/ml. The correlation coefficient (R^2) for RSP and 9-OH-RSP were 0.997 and 0.998, respectively. The mean recoveries for RSP and 9-OH-RSP calculated at three different concentrations (20, 80 and 140 ng/ml) are listed in Table 1.

Table 1: Recovery of the analytical method (n=10).

Added conc. (ng/ml)	Recovery(%) (mean±SD)		
	Risperidone	9-Hydroxyrisperdone	Clozapine(IS)
20	94.3±2.2	96.1±6.3	
80	96.8±5.5	97.6 ±1.2	
140	99.4±3.6	101.1 ±7.5	
50			91.2±1.3

Precision and accuracy

Intra- and inter-day precision and accuracy were evaluated by analyzing blank plasma spiked with five different concentrations of RSP and 9-OH-RSP. Intra- and inter-day precision were assessed by analyzing five samples at three concentrations (30,100 and 120 ng/ml) and 10 samples at two concentrations (10 and 50 ng/ml) for 5 days, respectively. Accuracy was expressed as percent error, [(measured concentration-spiked concentration)/spiked concentration] × 100%, while precision was quantitated by calculating intra- and inter-day RSD (Table 2).

Table 2: Accuracy and precision of the determination of risperidone and 9-hydroxyrisperidone in spiked plasma.

Added conc. (ng/ml)	Found conc. (ng/ml) (mean±SD)	Intra-day(n=5) (RSD,%)	Inter-day(n=10) (RSD,%)	Relative Error (%)
Risperidone				
10	9.5±0.4		4.2	-5.0
30	31.1±1.2	3.9		3.6
50	49.3±3.6		7.3	-1.4
100	102.2±5.6	5.5		2.2
120	116.0±4.1	3.5		-3.3
9-Hydroxyrisperidone				
10	11.2±0.2		1.7	12.0
30	28.3±3.2	11.3		-5.6
50	46.4±1.3		2.8	-7.2
100	93.7±7.5	8.0		-6.3
120	111.1±2.6	2.3		-7.4

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined as 3 and 10 times the baseline noise respectively. The values of LOD and LOQ were 1 ng/ml and 3 ng/ml, respectively, for both analytes in plasma.

Selectivity

None endogenous substances interfered with the detection of risperidone, 9-hydroxyrisperidone or I.S. Numerous anxiolytics, hypnotics, antidepressants and neuroleptic drugs did not interfere with the method (Table 3).

Table 3: Compounds tested for interference.

Drug	Retention Time(min)	Drug	Retention time(min)
Midazolam	2.0	Citalopram	6.7
Lorazepam	2.2	Haloperidol	7.3
Imipramine	2.9	Carbamazepine	8.9
Venlafaxine	3.6	Paroxetine	n.d.
Fluoxetine	3.9	Olanzapine	n.d.
Amitryptiline	4.2	Chlorpromazine	n.d.
Clomipramine	4.9	Phenobarbital	n.d.
Quetiapine	5.2		

Application

The present method was applied in the routine therapeutic drug monitoring of risperidone and 9- hydroxyrisperidone in psychiatric patients[9]. The method was also used to determine the plasma concentration of risperidone in the reported case of a patient treated daily with 6 mg. The plasma levels of the described patient were 48

ng/ml and 14 ng/ml for risperidone and 9-hydroxyrisperidone, respectively. The active moiety (sum of risperidone plus metabolite) was 62 ng/ml. The chromatogram is presented in Figure 7.

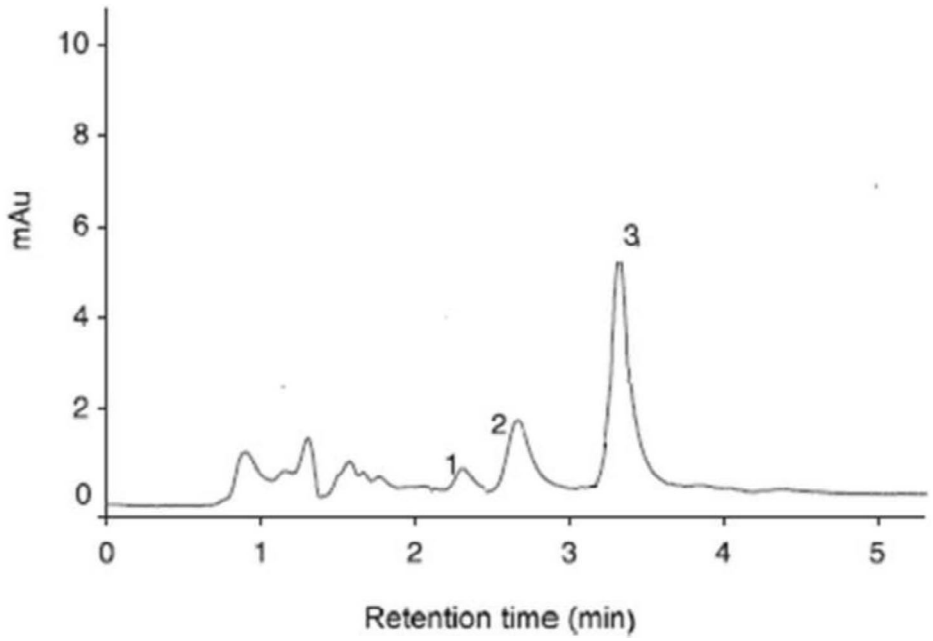

Figure 7: Chromatogram of a plasma sample from a patient who took 6 mg of risperidone per day. Peaks:1= 9-hydroxyrisperidone, 2= risperidone, 3=IS.

Conclusions

A new method of dispersive liquid-liquid microextraction combined with HPLC-UV has been described for the determination of risperidone and 9-hydroxyrisperidone in bood samples. The method has the advantages of easy operation, simplicity, economy, high enrichment factor, high recovery and lower detection limits. The present method is the only alternative method reported so far which can be applied to therapeutic drug monitoring in routine patient care and clinical studies investigating the influence of various factors on interindividual variability in pharmacokinetics of risperidone and 9-hydroxyrisperidone.

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