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Analysis of risperidone and 9-hydroxyrisperidone in human plasma, urine and saliva by MEPS-LC-UV

Roberto Mandrioli[a], Laura Mercolini[a], Domenico Lateana[a], Giancarlo Boncompagni[b], Maria Augusta Raggi[a,*]

[a] Laboratory of Pharmaco-Toxicological Analysis, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Alma Mater Studiorum - University of Bologna, Via Belmeloro 6, I-40126 Bologna, Italy
[b] Psychiatric Diagnosis and Care Services, "M. Malpighi" Hospital, Via Palagi 9, I-40139 Bologna, Italy

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ABSTRACT

Risperidone is currently one of the most frequently prescribed atypical antipsychotic drugs; its main active metabolite 9-hydroxyrisperidone contributes significantly to the therapeutic effects observed. An original analytical method is presented for the simultaneous analysis of risperidone and the metabolite in plasma, urine and saliva by high-performance liquid chromatography coupled to an original sample pre-treatment procedure based on micro-extraction by packed sorbent (MEPS). The assays were carried out using a C8 reversed-phase column and a mobile phase composed of 73% (v/v) acidic phosphate buffer (30 mM, pH 3.0) containing 0.23% triethylamine and 27% (v/v) acetonitrile. The UV detector was set at 238 nm and diphenhydramine was used as the internal standard. The sample pre-treatment by MEPS was carried out on a C8 sorbent. The extraction yields values were higher than 92% for risperidone and 90% for 9-hydroxyrisperidone, with RSD for precision always lower than 7.9% for both analytes. Limit of quantification values in the different matrices were 4 ng/mL or lower for risperidone and 6 ng/mL or lower for the metabolite. The method was successfully applied to plasma, urine and saliva samples from psychotic patients undergoing therapy with risperidone, with satisfactory accuracy results (recovery > 89%) and no interference from other drugs. Thus, the method seems to be suitable for the therapeutic drug monitoring of schizophrenic patients using the three different biological matrices plasma, urine and saliva.

1. Introduction

Risperidone (3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidine]ethyl]-6,7,8,9-tetrahydro-2-methylpyrido[1,2-a]pyrimidin-4-one, RISP, Fig. 1a) is currently one of the most frequently prescribed antipsychotic drugs worldwide. It is mainly used in the treatment of acute and chronic schizophrenic psychoses and has also been approved for the treatment of manic-depressive disorder [1]; it seems to be active against obsessive-compulsive disorder in association with selective serotonin reuptake inhibitors (SSRIs) [2–4]. In bipolar disorders, it is mainly used during manic episodes with psychotic symptoms [5]. RISP is commonly included in the atypical antipsychotic class, since it seems to act on a variety of central receptors (such as D_2, D_3, 5-HT_2 and α) [6] and has therapeutic and side effect profiles quite different for those of older neuroleptic drugs (such as phenothiazines and butyrophenones) [7]. In particular, RISP seems to cause significantly less extrapyramidal effects, except at very high dosages, while maintaining the same effectiveness against positive symptoms of the illness such as delirium and delusions [8]. It is active also on negative symptoms of the disorder and can reduce affective symptoms (such as depression, sense of guilt, anxiety) associated to schizophrenia [9]. The most frequent side effect of RISP therapy is hyperprolactinaemia [10], which is directly related to the dose.

RISP is usually administered orally at doses ranging from 4 to 16 mg/day, divided in two administrations. A modified-release microgranule formulation also exists, which allows the intramuscular injection of a single dose (25–50 mg) every 15 days [11]. It is mainly metabolised in the liver by cytochrome P450 (CYP) isoforms 2D6 and 3A4, producing the most important metabolite, 9-hydroxyrisperidone (3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidine]ethyl]-6,7,8,9-tetrahydro-9-hydroxy-2-methylpyrido[1,2-a]pyrimidin-4-one, 9OHR, Fig. 1b). This metabolite possesses pharmacological properties similar to those of RISP and usually reaches plasma levels equal to one to three times those of the parent drug. For this reason, the sum of the two compounds' levels is usually considered as the therapeutically relevant amount and is called the "active moiety" [12].

Since several years, many psychiatrist agree that therapeutic drug monitoring (TDM) can be a powerful tool to optimise the ther-

* Corresponding author. Tel.: +39 051 2099740; fax: +39 051 2099740.
E-mail address: mariaaugusta.raggi@unibo.it (M.A. Raggi).

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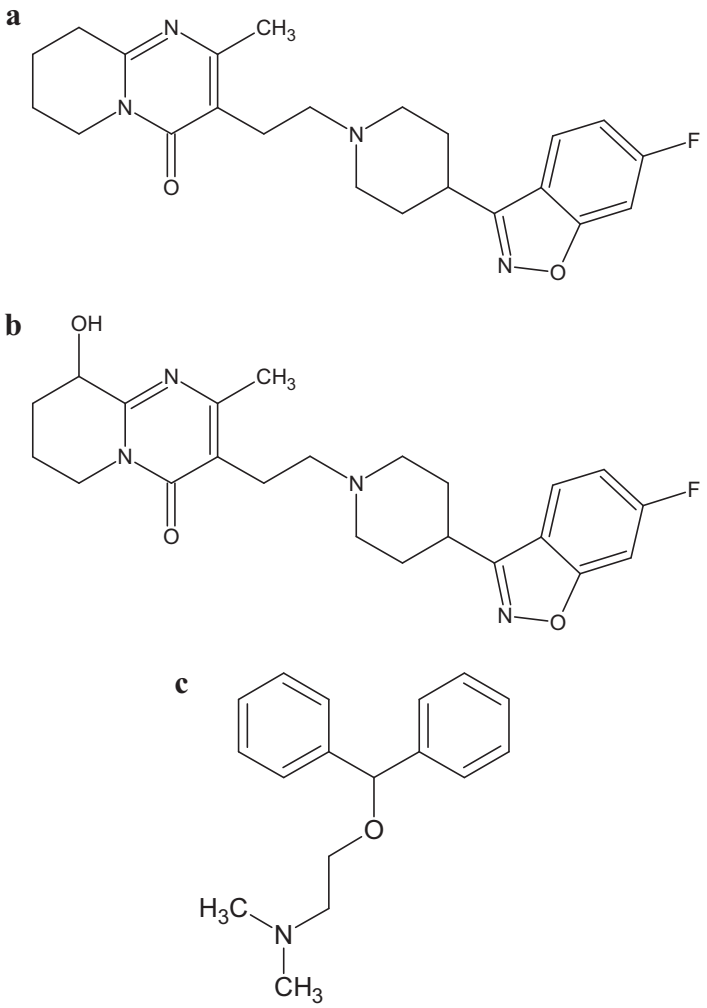

Fig. 1. Chemical structures of (a) risperidone (RISP), (b) 9-hydroxyrisperidone (9OHR) and (c) diphenhydramine (IS).

apy, personalising drug dosages and scheduling and thus obtaining net health and economic benefits [7]. In fact, therapy personalisation can reduce side and toxic effects, thus avoiding unnecessary and expensive hospitalisations and drug administrations. Regarding the intramuscular formulation, TDM can help in assessing the effective dose in comparison to the effective oral one. In order to carry out a reliable TDM, the plasma levels of RISP and 9OHR are usually determined; however, blood sampling is often complicated, especially in psychotic patients, who are typically very suspicious of any invasive procedure.

For this reason, alternative matrices would be welcome to avoid unnecessary stress and to obtain simpler procedures. For example, saliva levels could be useful in many situations, both to supplement the information provided by plasma assays and to partially substitute the latter during long-term, established therapy. Obviously, saliva sampling is not invasive and much more easily accepted than blood sampling, however saliva levels do not always strictly reflect blood concentrations. Urine testing is similarly not invasive, however it reflects compound elimination rates, not current levels available for therapeutic activity; in this sense, it provides complementary information to both plasma and saliva analysis. Regarding RISP specifically, no conclusive data is available on the possible relationship between plasma and saliva levels, although linearity has been found in a preliminary study with a limited dataset [13]. For these reasons, an original method has been developed for the analysis of RISP and 9OHR in human plasma, urine and saliva. The sample pre-treatment is based on micro-extraction by packed sorbent (MEPS), a recent technique that uses the basic principles of solid phase extraction (SPE) to obtain a simple, fast and reliable procedure with minimal consumption of solvents and sorbent.

Several methods can be found in the literature for the analysis of RISP and 9OHR in biological fluids; most of them deal with plasma [14–21] or serum [22] and some with urine [23,24] or saliva [25,26], but none carries out the analysis in all three matrices. Moreover, many of these methods are based on expensive or uncommon instrumentation and procedures, not always available or possible in clinical laboratories, such as mass spectrometric [16,20,24,25] or coulometric detectors [14,21,26], column switching [22] or luminol chemiluminescence [23].

The aim of this study was the development of a fast and feasible HPLC-UV method for the simultaneous analysis of RISP and 9OHR in different matrices and namely plasma, urine and saliva.

2. Experimental

2.1. Chemicals and solutions

RISP and 9OHR, reference pure compounds, were purchased from Janssen Pharmaceutical (Titusville, USA). Diphenhydramine (2-diphenylmethoxy-*N*,*N*-dimethyletaneamine, Fig. 1c), used as the Internal Standard (IS), HPLC-grade acetonitrile and methanol were purchased from Sigma-Aldrich (St. Louis, USA). Potassium dihydrogen phosphate, sodium hydroxide, 85% (w/w) phosphoric acid, and triethylamine, all pure for analysis, were purchased from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, USA).

Stock solutions of the analytes and the IS (1 mg/mL) were prepared by dissolving suitable amounts of each pure substance in methanol. Standard solutions were obtained by diluting stock solutions with the mobile phase and directly injected into the HPLC. Stock solutions were stable for at least two months when stored at −20 °C (as assessed by HPLC assays); standard solutions were prepared fresh every day.

2.2. Instrumentation and chromatographic conditions

The chromatographic system was composed of a Jasco (Tokyo, Japan) PU-980 chromatographic pump and a Jasco UV-975 spectrophotometric detector set at 238 nm.

Separations were obtained on a Varian (Walnut Creek, USA) Chromsep C8 reversed-phase column (150 × 4.6 mm I.D., 5 μm) coupled to a C8 cartridge precolumn (3 × 3 mm I.D., 5 μm) and kept at room temperature. The mobile phase was a mixture of acetonitrile (27%, v/v) and a pH 3.0, 30 mM phosphate buffer containing 0.23% (v/v) triethylamine (73%, v/v). The mobile phase was filtered through a Sartorius (Göttingen, Germany) membrane filter (47 mm diameter, 0.2 μm pore size, nylon) and degassed by an ultrasonic bath. A flow rate program was used as follows: 0.0–6.0 min, constant 1.0 mL min^{-1} flow rate; 6.1–7.0 min, linear gradient 1.0–2.0 mL min^{-1}; 7.1–12.0 min, constant 2.0 mL min^{-1} flow rate; 12.1–12.5 min, linear gradient 2.0–1.0 mL min^{-1}. The injections were carried out through a 50-μL loop. Data processing was handled by means of a Varian (Walnut Creek, USA) Star Chromatography 4.0 software.

The MEPS procedure was carried out by means of a SGE Analytical Science (Ringwood, Australia) apparatus, consisting of a 250-μL HPLC syringe with a removable needle; the syringe was fitted with a BIN (Barrel Insert and Needle) containing the C8 sorbent and was used to draw and discharge samples and solutions through the BIN.

2.3. Sample collection and preparation

The biological samples were collected from patients of the Mental Health Centre of the "M. Malpighi" Hospital (Bologna, Italy) subjected to therapy with oral RISP for at least 2 weeks at constant daily doses (or with intramuscular RISP for at least 30 days); the sampling was carried out in the morning, immediately before breakfast and 12 h after the last drug administration. Urine was collected in plastic containers during spontaneous urination; saliva was collected with a plastic Pasteur pipette directly from the patient's mouth, without any previous stimulation. Both urine and saliva were simply frozen at −20 °C until analysis, when they were thawed and centrifuged at 1400 × *g* for 10 min. Blood was collected by phlebotomy and drawn into glass tubes containing EDTA as the anticoagulant, then centrifuged (within 2 h from collection) at 1400 × *g* for 10 min; the supernatant (plasma) was transferred into polypropylene vials and stored at −20 °C until HPLC analysis. "Blank" samples were obtained in the same way from healthy volunteers not subjected to any pharmacological treatment.

The C8 MEPS cartridges were conditioned by passing 300 μL of methanol through the BIN (at a speed of about 10 μL/s) and then equilibrated by passing 300 μL of ultrapure water (at about 10 μL/s). The loading solution was a mixture of 50 μL of urine, 50 μL of pH 12, 15 mM phosphate buffer and 5 μL of IS solution (for urine), or a mixture of 100 μL of saliva or plasma, 100 μL of water and 5 μL of IS solution (for saliva and plasma); the loading mixture was drawn into the syringe and discharged back 10 times at about 5 μL/s.

The cartridge was then washed with 200 μL of water and then with 200 μL of a water/methanol mixture (70/30, v/v for urine or 90/10, v/v for saliva and plasma), at 10 μL/s.

Finally, the analytes were eluted by drawing and discharging 500 μL of methanol through the barrel at 5 μL/s.

The eluate was dried under vacuum (rotary evaporator), and redissolved with 100 μL of mobile phase. An aliquot of 50 μL of this solution was injected into the HPLC system.

2.4. Method validation

Validation was carried out on blank samples of plasma, urine and saliva collected from healthy volunteers as described in Section 2.3. Each sample, devoid of the analytes, came from a single donor.

2.4.1. Calibration curves

Aliquots of 5 μL of analyte standard solutions (prepared daily) at seven different concentrations containing the IS at a constant concentration were added to blank samples. The resulting mixture was subjected to the previously described MEPS procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analytes (expressed as ng/mL) and the calibration curves set up by means of the least-square method. The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated according to USP [27] and "Crystal City" [28] guidelines as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.4.2. Extraction yield (absolute recovery)

The procedure was the same as that described in Section 2.4.1, except the points were at 3 different concentrations, corresponding to the upper limit, lower limit and middle point of each calibration curve. The analyte/IS peak area ratios were compared to those obtained by injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

2.4.3. Precision

The assays described in Section 2.4.2 were repeated six times within the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision) [28], both expressed as RSD% values.

2.4.4. Selectivity

Blank biological samples from six different volunteers were subjected to the SPE procedure and injected into the HPLC; the resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was that no interference peak was to be higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different drugs active on the Central Nervous System were injected at concentrations higher than the respective therapeutic levels; if the resulting chromatograms contained any interference peak, the potentially interfering compounds were then subjected to the SPE and injected to ascertain if they could be extracted. The tested substances were antidepressants (citalopram, fluoxetine, paroxetine, reboxetine, sertraline and venlafaxine), antipsychotics (amisulpride, clozapine, haloperidol, levomepromazine, olanzapine and ziprasidone) and anxiolytics-hypnotics (clonazepam, delorazepam, diazepam, flurazepam, flunitrazepam and lorazepam).

2.4.5. Accuracy

Accuracy was evaluated by means of recovery assays. The assays described in Section 2.4.2 were carried out adding standard solutions of the analytes and the IS to real plasma, urine and saliva samples taken from psychotic patients subjected to therapy with RISP. The assays were repeated three times during the same day to obtain mean recovery and SD data.

3. Results and discussion

3.1. Choice of the experimental conditions

Our previous experience with the analysis of classical and atypical antipsychotics in human plasma [17] prompted us to study RISP starting from the same experimental conditions. Using this mobile phase (a 70/30, v/v mixture of acidic phosphate buffer and acetonitrile), a C8 column and a flow rate of 1 mL/min, RISP, and even more 9OHR, were eluted very close to endogenous components of the two matrices. For this reason, the acetonitrile percentage was decreased from 30 to 23%, increasing both the analyte retention times and the peak resolution. Regarding the IS, some compounds were tested, such as loxapine, diphenhydramine and amitriptyline; diphenhydramine has chemical–physical properties sufficiently similar to those of the analytes and is well resolved from both of them. For this reason, it was chosen as a prospective IS. However, since diphenhydramine is strongly retained by the column, a flow rate gradient from 1 to 2 mL/min was introduced, as reported in Section 2, in order to reduce analysis times. Diphenhydramine is an antihistaminic drug and a common ingredient of cold medicines. As such, analytical interference is possible if the patient takes such a medicine during RISP therapy; however, diphenhydramine therapy is typically acute, thus co-administration with RISP is easily avoided if the sampling time is correctly chosen.

Under these conditions the analytes' and IS peaks are neat, symmetric and well separated and run times are shorter than 10 min.

3.2. Analysis of standard solutions

Seven-point calibration curves were set up for RISP over the 2–200 ng/mL concentration range and for 9OHR over the 3–300 ng/mL range. Good linearity ($r^2 > 0.9996$) was obtained, with limits of quantitation (LOQ) of 2 ng/mL for RISP and 3 ng/mL for

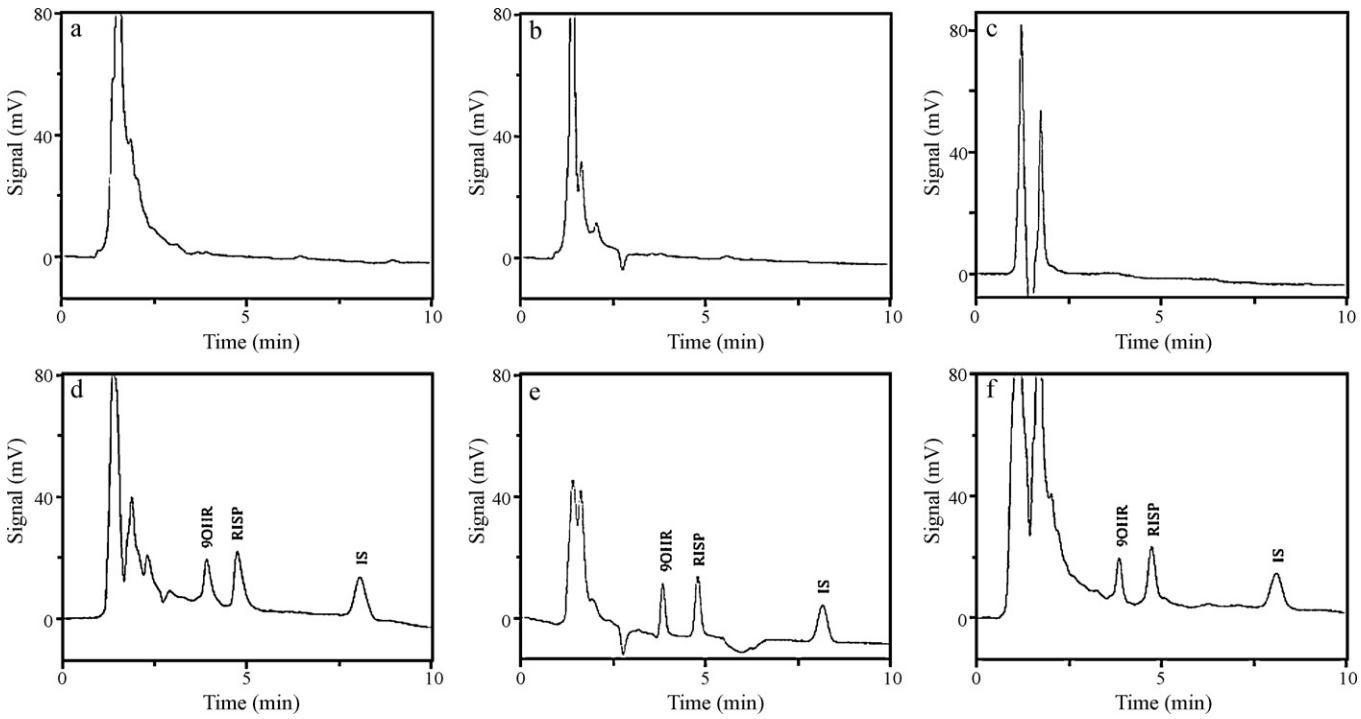

Fig. 2. Chromatograms of blank plasma (a), urine (b) and saliva (c) samples; the same blank plasma (d), urine (e) and saliva (f) samples spiked with 50 ng/mL of RISP and 9OHR and 200 ng/mL of the IS (concentrations in the injected solutions).

9OHR; the limits of detection (LOD) were 0.7 ng/mL for RISP and 1 ng/mL for 9OHR.

Precision was evaluated at three concentrations (3, 60 and 200 ng/mL), with the following results: RSD values were always lower than 3.7% for repeatability (intraday precision) and lower than 4.4% for intermediate precision (interday precision).

3.3. Development of the MEPS procedure

The sample pre-treatment procedure is a critical step of the analysis. For this purpose, MEPS was chosen: it is a relatively recent technique, based on the same general principle of solid-phase extraction (SPE), but with the sorbent contained within a very small barrel, which constitutes the needle assembly of an HPLC syringe. MEPS retains the high selectivity and good sample purification and extraction yields of SPE, while being fast, feasible and using small amounts of biological sample. Different kinds of sorbents were tried, such as cyanopropyl (CN), C8 and C18. The CN sorbent gave low extraction yields of the analytes; the C18 sorbent, while providing better extraction yields, gave unsatisfactory sample purification. The best results were obtained with the C8 sorbent, which was thus chosen for the MEPS procedure. All the main steps of the procedure (loading, washing, and elution) were optimised for the different matrices. Basic buffer addition was needed in the case of urine, since they are normally more acidic and their pH value is more variable than those of either plasma or saliva, which were just diluted with water. The analytes were sufficiently retained by the sorbent after 10 drawing/discharging cycles of the loading mixture, irrespective of the matrix (extraction yields: <40% with one cycle, <70% with 5 cycles); the main difference was in the washing steps. In fact, saliva and plasma needed less strong washing steps than urine: good purification was obtained with 200 μL of water followed by 200 μL of water/methanol 90/10 (v/v) mixture; urine needed 200 μL of the stronger water/methanol 70/30 (v/v) mixture (as well as the water step) to obtain comparable results. In all cases, an elution step consisting in two cycles with 250 μL of methanol proved sufficient for the complete elution of the analytes (extraction yields: <50% with 100 μL, <80% with 200 μL, not higher than 93% with volumes higher than 250 μL).

The eluate was then dried under vacuum and re-dissolved with 100 μL of mobile phase.

Using this MEPS procedure, good extraction yields of the analytes and the IS were obtained, while eliminating all endogenous interference. Fig. 2a, b and c reports the chromatograms of blank plasma, urine and saliva samples, respectively, after MEPS, while Fig. 2d, e and f reports the chromatograms of the same blank samples (plasma, urine and saliva, respectively) spiked with a known amount of RISP, OHR and the IS and subjected to the MEPS procedure. No interference can be detected in the blanks close to the retention times of the compounds of interest; peak shapes and resolution are good in the spiked samples, with asymmetry factors always lower than 1.3 and resolution values higher than 2.

3.4. Method validation

Satisfactory linearity ($r^2 > 0.9992$) was obtained over wide concentration ranges (100-fold) for both analytes on the three matrices. LOQ and LOD values were always lower than 7 ng/mL; the complete linearity, LOQ and LOD data are reported in Table 1.

Extraction yield (absolute recovery) and precision assays were carried out on blank matrices spiked with analyte concentrations corresponding to the lower limit, middle point and upper limit of the calibration curve. The results of these assays are reported in Table 2. As one can note, mean extraction yields were always higher than 90%. Precision results were also satisfactory: RSD values were always lower than 6.9% (4.5% for the IS) for repeatability and lower than 7.9% (5.9% for the IS) for intermediate precision.

Selectivity was evaluated by injecting into the HPLC standard solutions of several drugs, most of which are commonly co-administered during psychiatric therapy: other antipsychotics, antidepressants and anxiolytics-hypnotics. The complete list of the tested drugs and their retention times are reported in

Table 1
Linearity parameters.

Analyte	Matrix	Linearity range (ng/mL)	Linearity parameters, $y = ax + b$[a]		r^2	LOD (ng/mL)	LOQ (ng/mL)
			a	b			
RISP	Plasma	2–200	0.0193	0.0251	0.9995	0.7	2
	Urine	4–400	0.0209	0.0048	0.9993	1.5	4
	Saliva	2–200	0.0189	0.0282	0.9995	0.7	2
9OHR	Plasma	3–200	0.0148	0.0179	0.9995	1.0	3
	Urine	6–400	0.0138	0.0051	0.9994	2.0	6
	Saliva	3–200	0.0141	0.0189	0.9996	1.0	3

[a] y = analyte/IS peak area ratio; x = analyte concentration (ng/mL).

Table 2
Extraction yield and precision assays.

Analyte	Matrix	Concentration (ng/mL)	Mean extraction yield (%)[a]	Repeatability (RSD%)[a]	Intermediate precision (RSD%)[a]
RISP	Plasma	2	93	4.4	6.1
		100	93	3.6	5.1
		200	94	3.2	4.8
	Urine	4	93	6.8	7.8
		150	93	6.4	7.0
		400	94	6.0	6.7
	Saliva	2	93	4.5	6.0
		100	94	4.0	6.0
		200	95	3.5	5.8
9OHR	Plasma	3	91	4.1	5.7
		100	92	3.5	4.5
		200	93	3.3	4.0
	Urine	6	92	6.5	7.8
		150	93	6.0	6.9
		400	94	5.9	6.5
	Saliva	3	92	4.0	5.6
		100	92	4.0	5.4
		200	91	3.8	5.0
IS	Plasma	200	93	4.2	5.8
	Urine	200	93	4.1	5.7
	Saliva	200	91	4.4	5.8

[a] $n = 6$.

Table 3. As can be seen, none of them causes any interference in the analysis. Furthermore, six blank samples of each matrix were injected after MEPS and none of them produced peaks from endogenous compounds, which could interfere with the determination. Therefore, the method has demonstrated to be selective.

Table 3
Compounds tested for possible interference.

Therapeutic class	Compound	t_R (min)
(analytes and IS)	RISP	4.4
	9OHR	5.7
	IS	9.4
Antidepressants	Citalopram	8.7
	Fluoxetine	19.7
	Paroxetine	n.d.
	Reboxetine	n.d.
	Sertraline	13.0
	Venlafaxine	8.3
Antipsychotics	Amisulpride	2.6
	Clozapine	12.1
	Haloperidol	20.6
	Levomepromazine	13.7
	Olanzapine	11.4
	Ziprasidone	n.d.
Anxiolytics-hypnotics	Clonazepam	14.0
	Delorazepam	13.9
	Diazepam	13.0
	Flurazepam	3.3
	Flunitrazepam	n.d.
	Lorazepam	17.7

n.d. = not detected within a 30-min chromatographic run.

3.5. Analysis of patient samples

Having thus validated the method, it was applied to the analysis of plasma, urine and saliva samples from some psychotic patients of the Mental Health Centre of the "M. Malpighi" Hospital (Bologna, Italy) undergoing therapy with RISP. All biological fluids were collected early in the morning, immediately before any morning drug administration and 12 h after the previous one. Since the mean half-life of the active moiety is about 20 h, RISP is usually administered in two daily doses; thus, sampling after 12 h corresponds to the trough conditions. Fig. 3a, b and c shows samples (plasma, urine and saliva, respectively) from a patient taking 4 mg/day of oral RISP. The analyte concentrations found in these real samples were: RISP, 15 ng/mL in plasma, 17 ng/mL in urine and 18 ng/mL in saliva; 9OHR, 28 ng/mL in plasma, 95 ng/mL in urine and 30 ng/mL in saliva. In this sample and several others, plasma and saliva analyte levels were very similar under trough conditions. More extensive studies involving several patients are underway to determine whether a reliable correlation exists, as was also hypothesised in a previous study [13] (from limited data). In this case, saliva could be considered as a suitable, non-invasive substitute of plasma for TDM purposes.

Accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentration and of the IS at a constant concentration (500 ng/mL) were added to samples containing known amounts of RISP (i.e., samples which had already

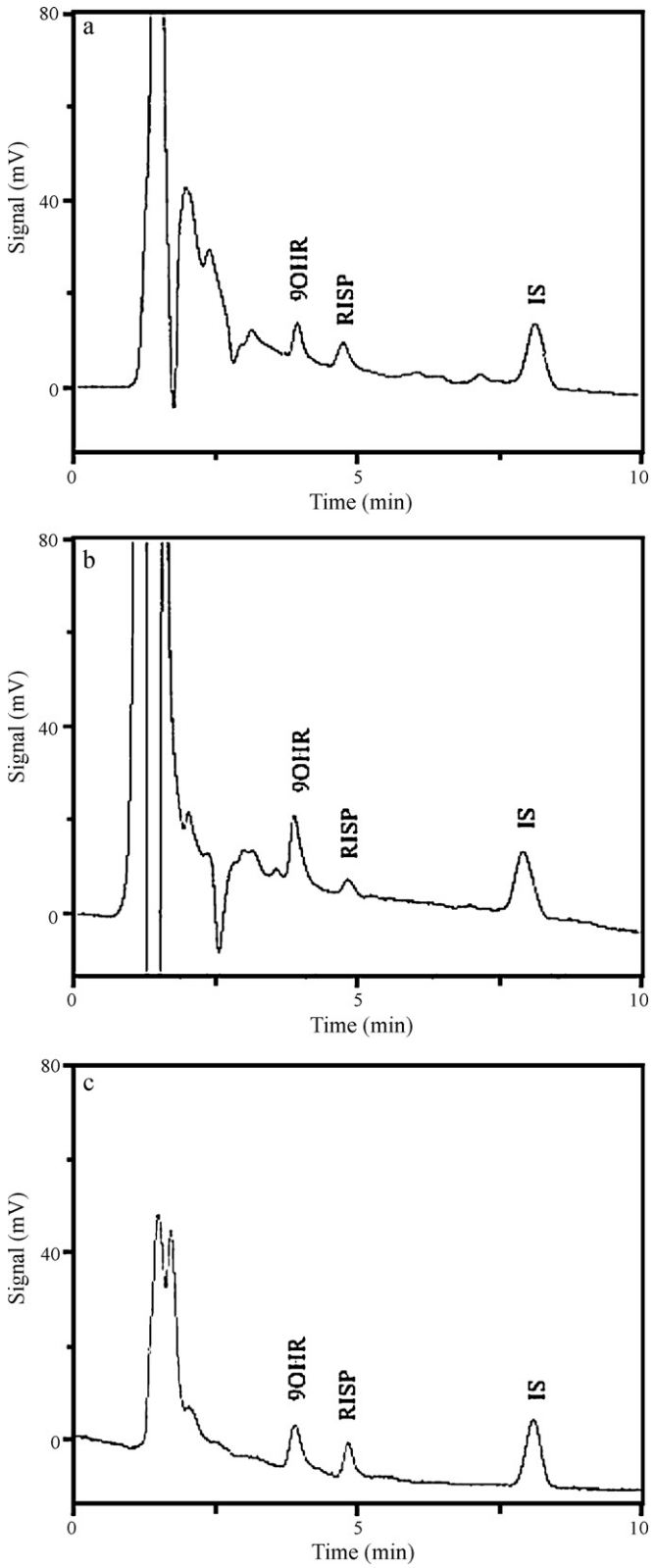

Fig. 3. Chromatograms of plasma (a), urine (b) and saliva (c) samples from a patient who was subjected to treatment with 4 mg/day of RISP.

Table 4
Accuracy assay results.

Analyte	Matrix	Concentration (ng/mL)	Mean recovery (%)[a]	SD[a]
RISP	Plasma	2	92	6.0
		50	91	4.1
		100	93	4.0
	Urine	4	91	5.5
		100	92	4.3
		200	94	4.1
	Saliva	2	94	5.6
		50	96	4.4
		100	99	4.3
9OHR	Plasma	3	91	6.1
		50	91	4.6
		100	90	4.2
	Urine	6	90	5.6
		100	91	4.4
		200	94	4.0
	Saliva	3	91	5.8
		50	95	4.6
		100	98	4.4

[a] $n = 3$.

been analysed). Then, the recovery of the added analytes was calculated, as well as the standard deviation of the assays. The results of the accuracy assays are reported in Table 4: mean recovery values were always higher than 89% (SD < 6.2). Thus, method accuracy is satisfactory.

4. Conclusion

The HPLC method presented here for the analysis of RISP is feasible, reliable and widely applicable. The MEPS procedure implemented for the sample pre-treatment, based on C8 cartridges, gives good extraction yields (>90%) and satisfactory precision (RSD% < 7.9%). The method is also selective: neither endogenous compounds nor any of the Central Nervous System drugs tested has produced any interference in the analysis of RISP and 9OHR in schizophrenic patients' plasma, urine and saliva. The use of MEPS has several advantages with respect to the liquid–liquid extraction procedures used elsewhere [15,20,21] for the analysis of RISP and 9OHR: in fact, the proposed MEPS procedure requires lower volumes of organic solvents (e.g., 0.5 mL of methanol vs. 8 mL of heptane–isoamyl alcohol [15] or 7 mL of pentane–methylene chloride [20]) and of sample (50–100 μL vs. 500–1000 μL) and provides better extraction yields (e.g., >90% vs. >84% [20]).

The proposed method is also advantageous for other reasons: first of all, because it has been validated for application to plasma, urine and saliva, while other methods are only applied to one or two of these matrices. Moreover, it uses feasible procedures and almost ubiquitous equipment and is thus applicable in a wide variety of clinical and research environments; previous methods often used very expensive or not easily available instrumentation and procedures, such as LC–MS [16,20,24,25], coulometric detection [14,21,26], column switching [22] or luminol chemiluminescence [23].

LC–MS methods possess superior selectivity and comparable precision and sensitivity, but as noted they are considerably more expensive in terms of instrumentation and reagent acquisition, of maintenance and of personnel training. Compared to the method recently published by some of the authors [26], the proposed method was independently developed; it has the main advantages of being validated for urine as well (instead of plasma and saliva alone). Its applicability to urine is advantageous, since this matrix gives useful insight into the metabolism and elimination of both the parent drug and its metabolite; the application to these three biological fluids within a large TDM program could lead

to establish significant chemical-clinical correlations. Moreover, the proposed method uses much more easily available and less expensive instrumentation (spectrophotometric instead of electrochemical coulometric detection).

The method seems thus to be suitable for the TDM of patients undergoing therapy with RISP and is a significant improvement with respect to currently available procedures.

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