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Determination of orphenadrine plasma levels using HPLC with diode array detection and a novel solid-phase extraction procedure in psychiatric patients

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ABSTRACT

Orphenadrine is an antimuscarinic agent mainly used for the treatment of parkinsonism and to alleviate the neuroleptic syndrome induced by antipsychotic drugs.

A new, rapid analytical method, based on liquid chromatography with diode array detection (DAD), has been developed and applied to the determination of orphenadrine in plasma of schizophrenic patients for therapeutic drug monitoring and toxicological purposes.

The chromatographic separation was performed on a pentafluorophenyl reversed phase column with a mobile phase composed of acetonitrile–phosphate buffer mixture. DAD detection was carried out at 220 nm. A careful and rapid solid-phase extraction procedure on cyanopropyl cartridges was chosen for plasma sample purification and pre-concentration obtaining good extraction yield values for the analyte (>96.0%). The assays showed a linear response for orphenadrine (30–1000 ng mL^{-1}). The method is also precise and selective.

Thus, the method developed seems to be suitable for routine analysis of orphenadrine in psychiatric patients. Moreover, it was applied to plasma samples from a psychotic patient who had tried to poison himself with 1000 mg of orphenadrine and was undergoing polypharmacy.

1. Introduction

Orphenadrine (*N,N*-dimethyl-2-[(2-methylphenyl)-phenyl-methoxy]-ethanamine, ORPH, Fig. 1a) is an anticholinergic drug of the ethanolamine antihistamine class with prominent central nervous system (CNS) and peripheral actions used to treat painful muscle spasm [1] and other symptoms and conditions as well as some aspects of parkinsonism [2]. It is also used to alleviate the extrapyramidal syndrome induced by antipsychotic drugs [2].

ORPH is administered in a dosage range between 150 and 300 mg day^{-1} but some patients may require a total of up 400 mg daily [2]. The ORPH therapeutic concentration in plasma usually ranges from 100 to 200 ng mL^{-1}; blood concentrations greater than about 500 ng mL^{-1} may cause toxic reactions, while those greater than about 5000 ng mL^{-1} can be lethal [3]. It is readily absorbed from the gastrointestinal tract and plasma peak levels are reached only after 4 h after ingestion of the drug. In case of large doses of ORPH, gastric emptying and the absorption are even slower due to its anticholinergic effect [4]. ORPH is completely metabolized to many metabolites; the most important metabolite is nororphenadrine. The mean half-life is in a range between 30.5 and 40.0 h after repeated oral administration [5].

Common side effects are dry mouth, dizziness, drowsiness, insomnia, constipation, orthostatic hypotension, and euphoria caused by the blockade of the central cholinergic receptors and by the inhibition of dopamine, serotonine and norepinephrine reuptake [2]. Toxic effects consist of neurotoxic and cardiotoxic effects such as convulsion, cyanosis, coma, arrhythmias, shock and cardiac arrest [6].

ORPH is known as an inducer and inhibitor of the microsomal cytochrome P450 (CYP) system in mammals [7]. Thus, pharmacological interactions could be evident in some patients in polypharmacy and determination of ORPH plasma levels may be so helpful in monitoring of patients on chronic therapy as well as assessing poisoning in patients.

To date, few analytical methods have been reported for the ORPH determination alone or together with other CNS drugs in plasma samples; they are based on HPLC-UV [4], or gas-chromatography (GC) [3,8] or LC with mass detection [9]. Extraction methods of ORPH from biological samples usually involved liquid–liquid

Abbreviations: CNS, central nervous system; CYP, cytochrome P450; TDM, therapeutic drug monitoring; ORPH, orphenadrine; IS, internal standard; DAD, diode array detection; SPE, solid-phase extraction; CN, cyanopropyl; RSD, relative standard deviation; LOQ, limit of quantification; LOD, limit of detection.

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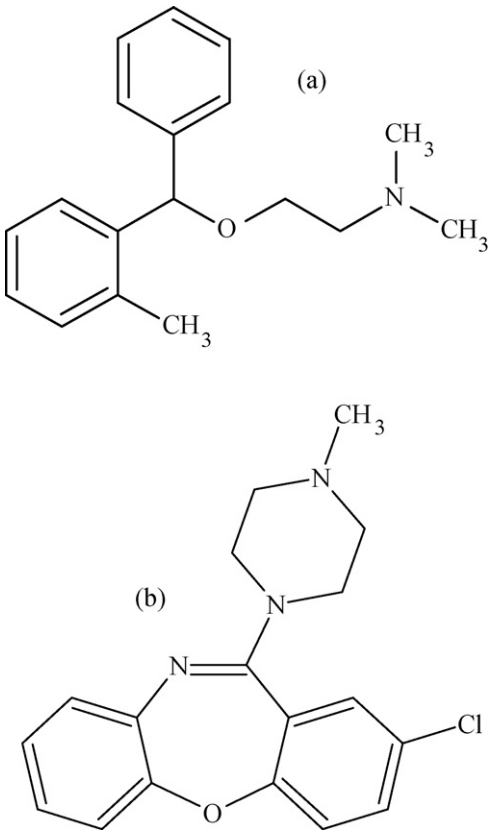

Fig. 1. Chemical structures of (a) orphenadrine (ORPH) and (b) loxapine, used as internal standard (IS).

extraction [3,4] or tip solid-phase extraction [8] or deproteinization [9] procedures. However, no paper dealing with the extraction of ORPH by means of a solid-phase extraction (SPE) was found in the literature.

The purpose of our study is to develop a rapid and reliable HPLC method with diode array detection (DAD) for determination of plasma ORPH coupled with an original SPE as sample preparation. This method will be applied to therapeutic drug monitoring (TDM) of psychiatric subjects treated with ORPH as well as to toxicological analysis.

2. Material and methods

2.1. Chemicals

Orphenadrine (*N*,*N*-dimethyl-2-[(2-methylphenyl)-phenylmethoxy]-ethanamine) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Clotiapine, chlorpromazine, phenytoin, ethosuximide, levodopa, trazodone, lorazepam, flurazepam, clonazepam, promethazine and triprolidine (substances tested for selectivity) were also purchased from Sigma–Aldrich (St. Louis, MO, USA). Aripiprazole (substance tested for selectivity) was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Loxapine (2-chloro-11-(4-methyl-1-piperazinyl)-dibenz[b,f] [1,4] oxazepine succinate, IS, Fig. 1b) used as the internal standard was kindly provided by Lederle Laboratories (Gosport, Hampshire, UK). Olanzapine, clozapine (and its metabolites, *N*-desmethylclozapine and clozapine *N*-oxide), quetiapine, valproic acid, biperidene, fluoxetine, sertraline and *E*,*Z*-fluvoxamine (substances tested for selectivity) were kindly provided by Eli Lilly (Indianapolis, IN, USA), Novartis Italia (Origgio, Italy), Astra Zeneca (Wilmington, New Zealand), Sanofi-Synthelabo (Paris, France), Abbott Italia S.p.A. (Campoverde, Italy), Eli Lilly Italia S.p.A. (Sesto Fiorentino, Italy), Pfizer Italia (Borgo S. Michele, Italy), Solvay Pharmaceuticals (Weesp,The Netherlands), respectively.

Acetonitrile and methanol for HPLC, 85.0% (w/w) phosphoric acid and potassium dihydrogen phosphate were produced by Carlo Erba (Milan, Italy). Triethylamine was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Ultrapure water (18.2 MΩ cm), obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA), was used for the preparation of all the solutions.

2.2. Preparation of standard stock and working solutions

Primary stock solutions of ORPH (1.0 mg mL^{-1}) were prepared by dissolving 10.0 mg of pure substance in 10.0 mL of methanol. The IS stock solutions (1.0 mg mL^{-1} of pure loxapine) were prepared by dissolving 14.6 mg of loxapine succinate in 10.0 mL of methanol (14.6 mg of loxapine succinate correspond to 10 mg of pure loxapine). Working standard solutions at different concentrations were obtained by diluting primary stock solutions with the mobile phase. Stock solutions were stable for at least 1 year when stored at −20 °C, as assessed by means of spectrophotometry and HPLC injections; working standard solutions were prepared freshly every day.

2.3. Sample collection

Blood samples were obtained from six psychiatric patients subjected to therapy with Disipal® tablets at daily doses between 150 and 300 mg from some psychiatric clinics of Bologna (Italy). One sample from each patient was included. Usually, blood samples were drawn 12 h after the last drug administration. The chromatographic assays were also carried out on blood samples from a schizophrenic patient of the "Ottonello" psychiatric clinic, Maggiore Hospital of Bologna (Italy) who took an overdose of 1000 mg of ORPH, all at once: one blood sample was drawn after 30 h and then after 60 h from the overdose.

All blood samples were stored in glass tubes containing EDTA as the anticoagulant, then centrifuged (within 2 h from collection) at 4000 rpm for 15 min at 5 °C; the supernatant (plasma) was then transferred to polypropylene tubes and stored at −20 °C until HPLC analysis, usually within three days. A Hettich (Tuttlingen, Germany) Universal 32 R centrifuge was used.

Blood samples from healthy volunteers (whose plasma was used as blank plasma) were treated in the same way.

2.4. Apparatus and chromatographic conditions

An Agilent 1100 Series chromatographic system (Palo Alto, CA, USA) equipped with a diode array detector (DAD) was used for ORPH chromatographic assays. The detector wavelength was set at 220 nm. The analyses were carried out on a Supelco (Bellefonte, PA, USA) Discovery reversed-phase pentafluorophenylpropyl column (HS F5, 150 mm × 4.6 mm I.D., 5 μm), kept at room temperature (25 ± 3 °C), using a mobile phase composed of phosphate buffer (pH 3.0; 104 mM)–triethylamine–acetonitrile (69.6:0.4:30.0, v/v/v). A Crison (Barcelona, Spain) MicropH 2000 pHmeter was used. The mobile phase was filtered through a Phenomenex (Torrance, CA, USA) membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic apparatus. The flow rate was 1.4 mL min^{-1}. The samples were injected into the HPLC system by means of a 50 μL loop.

2.5. Solid-phase extraction procedure

Solid-phase extraction (SPE) for the sample pre-treatment was carried out using IST (Hengoed, UK) Isolute cyanopropyl cartridges

(CN, 50 mg, 1 mL) by means of a Varian (Harbor City, CA, USA) VacElut apparatus.

The cartridges were activated with 3 × 1 mL of methanol and conditioned with 3 × 1 mL of water. Aliquots of 50 μL of loxapine (IS) standard solution (and analyte standard solution for blank plasma samples) were added to 500 μL of blank or patient plasma. The resulting mixture was diluted with 500 μL of water and loaded onto a previously conditioned CN cartridge. After loading, the cartridges were washed with 1 mL of water twice and then with 1 mL of a mixture of methanol–water (5:95, v/v). Elution was carried out with 1.5 mL of methanol. The eluate was brought to dryness in a rotary evaporator, re-dissolved with 125 μL of mobile phase, and then injected into the HPLC system.

2.6. Method validation

Method validation procedures were carried out according to USP XXVIII [10] and Crystal City [11] guidelines.

2.6.1. Extraction yield (absolute recovery) and precision

Aliquots of 50 μL of ORPH standard solutions at three different concentrations (in order to obtain analyte plasma concentrations of 30, 500 and 1000 ng mL^{-1}, respectively), containing the IS at a constant concentration (in order to obtain plasma concentration of 500 ng mL^{-1}), were added to 500 μL of blank plasma. After diluting with 500 μL of water the mixtures were subjected to the SPE procedure and injected into the HPLC. The analyte peak area was compared to those obtained injecting standard solutions at the same theoretical concentrations and the absolute recovery was calculated.

The assays described above 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*), both expressed as percentage relative standard deviation values (RSD %).

2.6.2. Calibration curves, limit of quantitation, limit of detection

Aliquots of 50 μL of ORPH standard solutions at six different concentrations (in order to obtain plasma concentrations of 30, 250, 500, 600, 800 and 1000 ng mL^{-1}, respectively), containing loxapine as the IS at a constant concentration (in order to obtain plasma concentration of 500 ng mL^{-1}), were added to 500 μL of blank plasma. The resulting mixtures were diluted with 500 μL of water, subjected to the previously described SPE procedure and injected into the HPLC system. This procedure was done in triplicate for each point. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}). The calibration curve was constructed by means of the least-square method. One stock solution was used for each replicate; different working solutions were prepared from the stock solutions and added to the blank plasma samples to obtain the different concentrations.

The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated according to official guidelines [10,11] as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.6.3. Selectivity

The method selectivity was evaluated by injecting into HPLC system standard solutions of several drugs usually co-administered in psychiatric practice such as antidepressants, antipsychotics, sedatives-hypnotics and antiepileptic agents. Moreover, blank plasma samples from six subjects (n = 1 for each subject) not receiving ORPH treatment were processed in the absence of the internal standard.

2.6.4. Stability

Stability of ORPH stock solutions (1 mg mL^{-1}) stored for 1 year at −20 °C was carried out by comparing analyte/IS peak area ratios with those of the fresh samples. When analysing the samples, primary stock solution was diluted with mobile phase to obtain analyte final concentration of 2000 ng/mL.

Stability of ORPH in control plasma samples was tested by making three consecutive injections of the same plasma sample, containing 500 and 1000 ng/mL of ORPH, after the SPE procedure, over 5 h at room temperature, three freeze–thaw cycles and 3 months stored at −20 °C.

2.6.5. Accuracy

Accuracy was evaluated by means of recovery assays. Aliquots of 50 μL, each one containing analyte standard solutions at three different concentrations (i.e. analyte plasma additions of 50, 150 and 500 ng mL^{-1}; n = 3 for each level) and the IS at a constant concentration, were added to 500 μL of plasma from psychotic patients treated with Disipal®; then the mixtures were subjected to the SPE procedure described above. Recovery values were calculated according to the following formula: 100 × ([after spiking] − [before spiking])/[added].

3. Results and discussion

3.1. Choice of chromatographic conditions

Preliminary experiments were carried out starting from a paper on the plasma analysis of the antipsychotic aripiprazole by means of HPLC with DAD detection [12], using a mixture of phosphate buffer (pH 3.0)–acetonitrile (65:35, v/v) as the mobile phase and a C8 (150 mm × 4.6 mm I.D., 5 μm) reversed phase column. Under these conditions, the ORPH peak showed low retention on C8 column because it was eluted at void peak. A concomitant analyte peak broadening occurred when the organic phase percentage was decreased from 35% to 30%. Thus, the C8 column was substituted with a pentafluorophenylpropyl (HS F5) bonded phase whose main property is the greater retention than C8 phase of the analytical compounds. The HS F5 column (kept at room temperature) provided a good separation of the analyte from the IS peak in an acceptable run time (<9.0 min), coupled with a good selectivity and peak shape.

Several compounds were tested as possible internal standards; the most suitable was found to be loxapine. In fact, loxapine has a relatively short retention time and chemical–physical properties similar to ORPH. A detection wavelength of 220 nm was chosen as a good compromise between the two absorbance maxima of ORPH bands. In fact, the maximum at 195 nm would confer higher sensitivity but selectivity would dramatically decrease; on the other hand, the maximum at 260 nm has a lower potential for interference but has a quite low absorbance value.

The chromatogram of a standard solution containing 2000 ng mL^{-1} of ORPH and IS is reported in Fig. 2a. As can be seen, the peaks are neat and well-resolved and the chromatographic run lasts 9.0 min. Retention times (t_R) are: ORPH, t_R = 8.3 min; IS, t_R =5.4 min.

3.2. Development of a solid-phase extraction procedure (SPE)

The sample pre-treatment is a crucial step in the pharmacoclinical analysis. Proteins and other biological interferences may result in precocious deterioration of the performance of separation columns and in increased column backpressure. Solid-phase extraction (SPE) is today the most commonly used sample pretreatment, that extract, pre-concentrate and clean-up compounds of interest from a biological matrix. Therefore, an original SPE

Table 1
Validation parameters of orphenadrine in human plasma.

Compound	Amount added (ng mL^{-1})	Extraction yield (%)[a]	Repeatability (RSD%)[a]	Interday precision (RSD%)[a]
ORPH	30	99.9	3.94	4.79
	500	97.2	3.11	4.01
	1000	96.1	2.94	3.89
IS	500	97.0	2.90	3.51

[a] Each value is the mean of six independent assays. The extraction yield was calculated from analyte peak area from spiked plasma samples compared to peak areas of the same analyte concentration in standard solutions.

procedure was developed for the ORPH determination instead of the liquid–liquid extraction (LLE) procedures [3,4], which are usually employed to clean up the biological matrix. In fact, SPE is more selective, feasible and rapid than LLE and it also requires lower amounts of polluting and potentially toxic organic solvents.

Different cartridges were tested: hydrophilic–lipophilic balance (HLB, 30 mg, 1 mL), C2 and C8 (50 mg, 1 mL) and cyanopropyl (50 mg, 1 mL) sorbents. The weakly lipophilic cartridges (C2) and the HLB sorbent gave extraction yields close to 80%, but they did not allow an adequate clean-up of the plasmatic matrix. On the other hand, more lipophilic sorbents, such as C8, gave rise to low extraction efficiency (60%) and was unsuitable for the separation of the analytes from endogenous interference. Only the cyanopropyl cartridges allowed good purification of the plasma matrix together with a high extraction yield values for ORPH. Therefore, the cyanopropyl cartridges were selected as the clean-up and the pre-concentration procedures. After loading with 500 μL of plasma sample and washing with water and a methanol/water mixture, the elution step was investigated. Acetonitrile proved to be almost ineffective as the eluent (analytes extraction yield < 50%); better results were obtained with methanol (1.5 mL). Furthermore, it was necessary to operate a 4-fold pre-concentration in the determination of ORPH plasma levels: the best loading/redissolution volume ratio was found to be 4:1, thus improving sensitivity of the analytical method. Using this SPE procedure, no interference from the matrix was present, as the chromatogram from a blank plasma sample shows (Fig. 2b), while Fig. 2c shows a blank plasma sample spiked with 500 ng mL^{-1} of ORPH and IS. The analytical peaks are neat and symmetric with good extraction yield values (>96.0%).

3.3. Method validation

Extraction yield and precision assays were carried out at three different concentration levels of ORPH, corresponding to the lowest level, highest level and middle point of the calibration curve. The results of these assays are reported in Table 1. As one can see, the results are satisfactory being the extraction yield values higher than 96.0%. The mean extraction yield of the IS was 97.0%. The precision was also satisfactory with RSD value always lower than 4.8%.

Calibration curve was set up for ORPH and good linearity (r_c = 0.9994) was found in the 30–1000 ng mL^{-1} concentration range. The limit of quantitation (LOQ) was 30 ng mL^{-1}, while the limit of detection (LOD) 10 ng mL^{-1}, in accordance with official requirements.

No obvious changes or degradation of ORPH were observed during stability tests on stock solutions or plasma samples.

3.4. Selectivity

The compounds tested for interference are reported in Table 2. As one can see, none of these were detected within 30 min and none interfered with the determination of ORPH. The analysis of blank plasma samples from six subjects was carried out; no unacceptable interferences at the retention time of ORPH and IS were observed, as one can see in the chromatogram of a blank sample in Fig. 2b.

3.5. Accuracy

Method accuracy was evaluated at three different concentration levels by adding known amounts of standard solutions of the analyte and the IS to real plasma samples taken from psychotic patients, whose drug (ORPH) content had been already determined (see Section 3.6). Results were satisfactory: recovery values were always higher than 89.0%.

3.6. Analysis of patient plasma samples for TDM purpose

The validated method was applied to the analysis of plasma samples from psychotic patients being treated with Disipal® also in combination with other CNS drugs. The chromatogram of a plasma sample from a patient taking 150 mg day^{-1} of ORPH together with clozapine, fluvoxamine and lorazepam is reported in Fig. 3a. A

Table 2
Compounds tested for interference.

Compound	Retention time (min)
Antipsychotics	
Olanzapine	n.d.
Clotiapine	10.0
Clozapine	3.0
N-desmethylclozapine	2.3
Clozapine *N*-oxide	3.8
Chlorpromazine	24.3
Aripiprazole	n.d.
Quetiapine	4.0
Antiepileptics	
Valproic acid	n.d.
Phenytoin	24.2
Ethosuximide	n.d.
Antiparkinsons	
Levodopa	n.d.
Biperidene	10.4
Antidepressants	
Sertraline	25.0
Fluoxetine	22.9
Trazodone	4.0
Z-Fluvoxamine	9.0
E-Fluvoxamine	15.0
Sedative-hypnotics	
Lorazepam	7.0
Flurazepam	6.5
Clonazepam	10.3
Others	
Promethazine	9.2
Triprolidine	4.2
Analytes	
Orphenadrine	8.3
Loxapine (IS)	5.4

n.d. = not detected within 20 min.

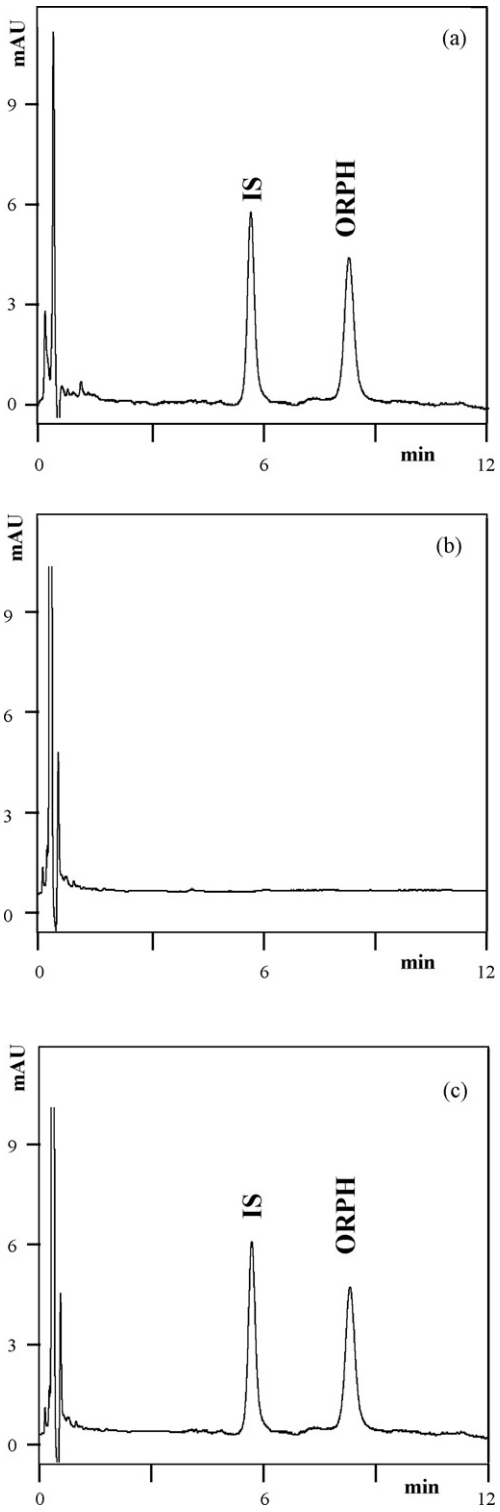

Fig. 2. Chromatograms of (a) a standard solution containing 2000 ng mL^{-1} of ORPH and IS; (b) a blank plasma sample; and (c) the same blank plasma sample spiked with 500 ng mL^{-1} of ORPH and IS.

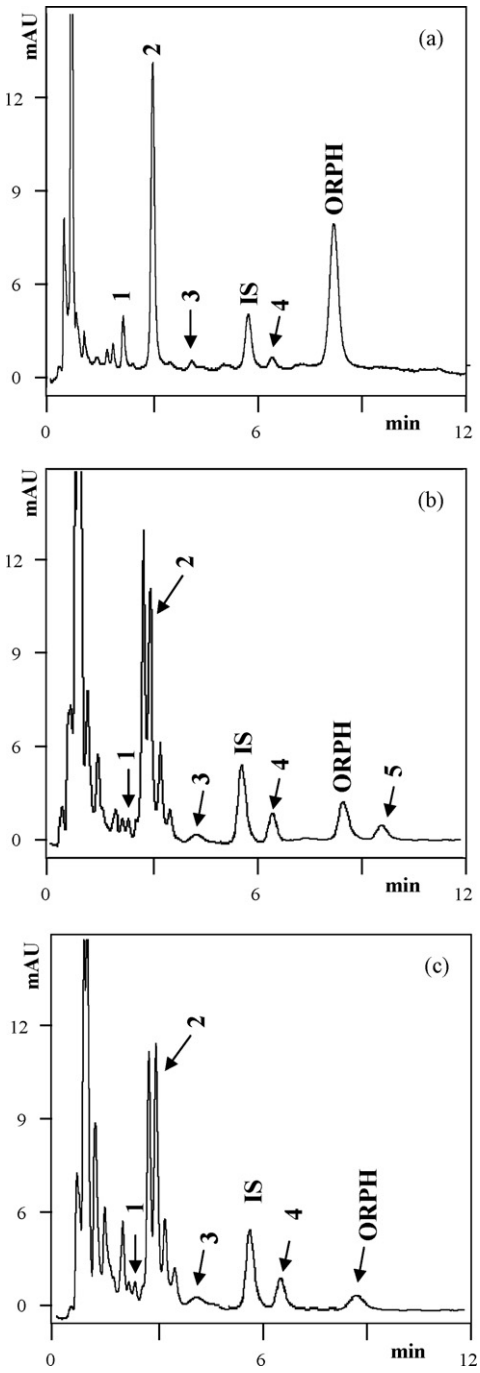

Fig. 3. Chromatograms of (a) a plasma sample from a schizophrenic patient taking 150 mg day^{-1} of ORPH and also clozapine, after 12 h from the last administration; (b) a plasma sample from a schizophrenic patient after 30 h from an overdose with 1000 mg of ORPH; and (c) a plasma sample from the intoxicated patient after 60 h from overdose. Chromatographic peaks: 1 = *N*-desmethylclozapine; 2 = clozapine; 3 = clozapine *N*-oxide; 4 = norорphenadrine; and 5 = promethazine.

blood sample was drawn 12 h after the last drug administration. As one can see, no interference from the matrix is apparent and analyte peak is neat and symmetric. Other peaks present in the chromatogram were due to clozapine and its metabolites, as results by comparison with injections of compound standard solutions (clozapine, t_R = 3.0 min; *N*-desmethylclozapine, t_R = 2.3 min; clozapine *N*-oxide, t_R = 3.8 min; see Table 2). The chromatographic peak at 6.2 min should be probably due to norорphenadrine, the main ORPH metabolite (as assessed by its DAD spectrum).

The ORPH concentration found in this plasma sample was very high; in fact, a level of 996 ng mL^{-1} was calculated, which is about 5 times the reported therapeutic plasma levels (range: 100–200 ng mL^{-1}) [3]. The high ORPH value is probably due to co-administration of fluvoxamine, which is a potent inhibitor of cytochrome P450.

3.7. Application to toxicological analyses

The analytical method was also applied to the toxicological analysis of ORPH in plasma of a schizophrenic patient who took an ORPH overdose equal to 20 tablets of Disipal® (i.e. 1000 mg of ORPH). ORPH levels found were 375 ng mL^{-1} (see Fig. 3b) and 254 ng mL^{-1} (see Fig. 3c) after 30 and 60 h by overdose, respectively. Moreover, the chromatographic peak at 6.2 min should be due to nororphenadrine, the ORPH metabolite. Although the high ingested dosage (1000 mg), the patient showed moderate symptoms of an acute intoxication, probably for the timely intervention by means of gastric lavage and charcoal administration. The patient presented confusion (Glasgow Come Scale value of 15), without any major clinical complications and recovered in few days. At the time of overdose, the patient was also undergoing treatment with other drugs, namely clozapine, clotiapine and promethazine. In fact, in Fig. 3b and c, some chromatographic peaks were found due to clozapine (t_R = 3.0 min) and its metabolites (*N*-desmethylclozapine, t_R = 2.3 min, and clozapine *N*-oxide, t_R = 3.8 min) and to promethazine (t_R = 9.2 min). The method thus demonstrated to be suitable also in poisoning cases of ORPH.

4. Conclusion

The analytical method presented herein for the analysis of ORPH in plasma samples was fast and feasible, as well as accurate and precise. Thanks to its selectivity, it can also be applied to the determination of the analyte in plasma when the patients are subjected to therapy with ORPH as well as in cases of poisoning.

With respect to other published methods, it has the advantage of better precision (RSD < 4.8%, while other methods report RSD values up to 9.9%) [8], better extraction yields [9] and lower costs than the reported LC or GC with a mass spectrometry detector [3,8,9], while maintaining short run times (<9 min). An original SPE procedure, using cyanopropyl cartridges, has been implemented to clean-up plasma samples and extract ORPH. The feasible SPE procedure implemented instead of liquid–liquid extraction [3,4] allows to obtain high extraction yields of the analyte with optimal purification of the biological samples without using several millilitres of polluting organic solvents.

The HPLC method coupled with SPE pre-treatment has been successfully applied to the analysis of plasma samples from schizophrenic patients. Therefore, it seems to be suitable for the therapeutic drug monitoring of ORPH in psychiatric patients and also in overdose case.

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