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Simultaneous determination of four antipsychotic drugs in plasma by high-performance liquid chromatography Application to management of acute intoxications

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Abstract

A specific reversed phase-high pressure liquid chromatography (RP-HPLC) method has been developed for the simultaneous determination of clozapine (CZP), loxapine (LXP), zuclopenthixol (ZPT) and flupenthixol (FPT) in plasma. These four antipsychotic drugs are frequently used for the treatment of schizophrenia and other neuropsychiatric diseases. Carpipramine, a dihydrodibenzazepine, was used as an internal standard (I.S.). A liquid–liquid procedure was used to extract the drugs from human plasma. The analysis was performed on a XTerra™ MS C_{18} column with UV detection. Calibration curves were linear in the range 50–1000 μg/l. The limit of quantification (LOQ) was 15 μg/l for clozapine and loxapine and 20 μg/l for zuclopenthixol and flupenthixol. The coefficient of variation (CV) for intra- and inter-day precision was 7.2% or less with accuracies within 10% for the three concentrations.

This isocratic and rapid method (run time < 10 min) is useful for the management of acute intoxication.
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Keywords: Clozapine; Loxapine; Zuclopenthixol; Flupenthixol

1. Introduction

Clozapine (CZP) and loxapine (LXP) are dibenzodiazepines whereas zuclopenthixol (ZPT) and flupenthixol (FPT) are thioxanthene derivatives (Fig. 1). These antipsychotics are widely used for the treatment of schizophrenia and other neuropsychiatric diseases. Consequently, they are frequently encountered in toxicology and in forensic chemistry [1–6].

Clozapine, loxapine and flupenthixol were each characterized using gas chromatography [7–9], gas liquid chromatography [10–12] or gas chromatography-mass spectrometry [13]. Clozapine or flupenthixol were resolved by liquid chromatography-mass spectrometry [14,15]. The four drugs were determined by HPLC and the detection was performed with an UV detector [16–25] or with a diode array detector [26,27]. Nevertheless only few paper described methods in which at least two of these neuroleptics were

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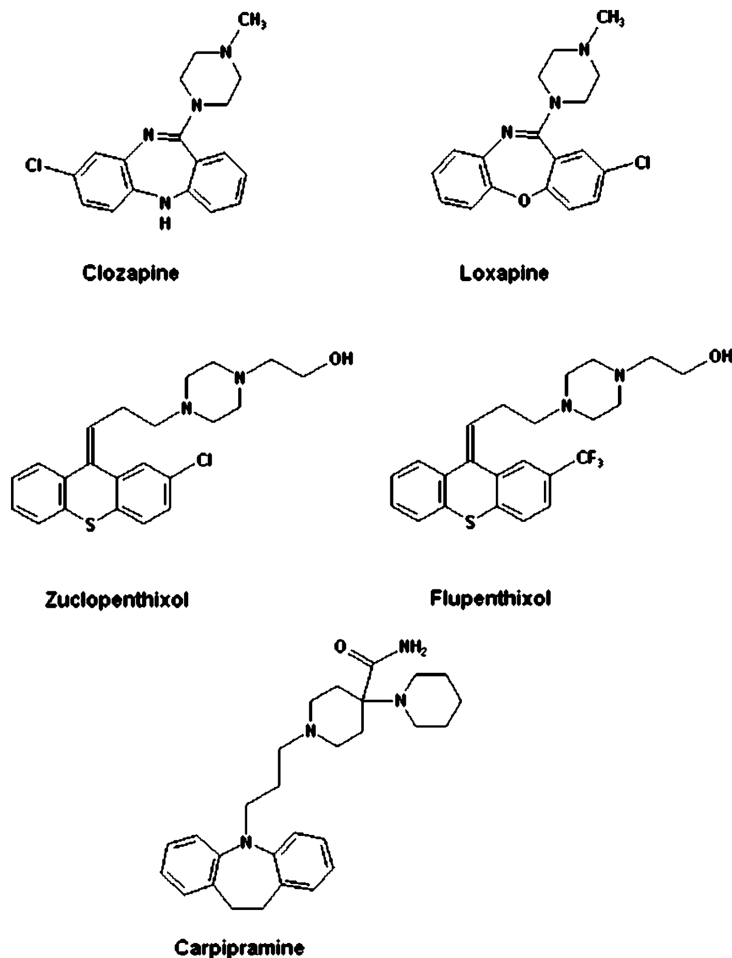

Fig. 1. Chemical structures of clozapine (CZP), loxapine (LXP), zuclopenthixol (ZPT), flupenthixol (FPT) and carpipramine (internal standard: I.S.).

separated. Clozapine and loxapine were resolved using capillary zone electrophoresis [28] or gas chromatography [29]. Clozapine and flupenthixol were both separated using HPLC [24]. Recently, a determination of clozapine, flupenthixol and zuclopenthixol with haloperidol, penfluridol and thioridazine was realized by liquid chomatography-tandem mass spectrometry with diode array detector using hair samples of psychiatric patients [30]. Most of the published techniques require a long-time analysis, or expensive equipment and therefore are not suitable for routine analysis of all four drugs.

The aim of the present study was to establish a simple, rapid and accurate reversed phase-high pressure liquid chromatography (RP-HPLC) method for the simultaneous measurement of CZP, LXP, ZPT and FPT in plasma after overdose. The assay requires a small sample volume, involves a single step liquid extraction with a specific internal standard (I.S. carpipramine) (Fig. 1) and a short chromatographic run.

2. Experimental

2.1. Chemicals and reagents

LXP was provided by Lederle (France), CZP and ZPT were kindly gifted by Lundbeck (Copenhagen,

Denmark), FPT was donated by Sanofi Synthelabo (Montpellier, France). Carpipramine (I.S.) was obtained from Specia (Paris, France). Acetonitrile was of HPLC grade and was obtained from Merck (Strasbourg, France). The reagent containing sulfonic pentane acid (Pic B5® Low UV) was a premixed product of Waters (Milford, MA, USA). Potassium dihydrogenophosphate (KH_2PO_4) and potassium phosphate dibasic (K_2HPO_4) were, respectively, obtained from SDS (Peypin, France) and Aldrich (Saint Quentin Fallavier, France). Water was deionized and glass-distilled prior to use. Drug-free human plasma was obtained from Aquitaine Establishment of Blood Transfusion (Bordeaux, France).

2.2. Apparatus

The chromatographic apparatus (Waters, Milford, USA) was equipped with a Model 501 constant flow pump, a Model 2487 ultraviolet detector and a 746 Data Module Integrator. In a preliminary study, two reversed phase columns were tested. The first one was a Waters Spherisorb® S5C8 (4.6 mm × 150 mm; 5 μm particle size) with a mobile phase consisting of acetonitrile-phosphate buffer (6.24×10^{-2} M) (40:60, v/v). To this mixture, 500 μl of diethylamine (DEA) and 10 ml of Pic B5® (pentane sulfonic acid) was added for 1 l. Finally the pH of this eluent was adjusted to 5.0 with orthophosphoric acid. The second method was carried out on a XTerra™ MS C_{18} analytical column (Waters) (3.9 mm × 150 mm; 5 μm particle size), with a mobile phase consisting of acetonitrile-phosphate buffer (6.24×10^{-2} M) (38:62, v/v). Finally, the pH was adjusted to 4.2 with orthophosphoric acid. These two mobile phases were filtered through a 0.5 μm filter and degassed prior to use. The flow rate was maintained at 1.0 ml/min in each case.

Compounds were detected at 220 nm. The unknown concentrations of CZP, LXP, ZPT and FPT were quantified using linear regression of response (drug/I.S. peak height ratio) versus CZP, LXP, ZPT and FPT concentrations.

2.3. Standard solutions

Stock solutions of all compounds included I.S. were prepared at a concentration of 1000 mg/l and stored at −20 °C until analysis. They were stable for at least 3 months.

The internal standard stock solution was diluted daily in bidistilled water to yield a 5 mg/l working solution. From the stock solution of CZP, LXP, ZPT and FPT, working solutions (2 mg/l) were made up daily in bidistilled water and the calibration standards were prepared freshly. The calibration standards were added into drug-free human plasma to yield concentrations of 50, 125, 250, 500 and 1000 μg/l for all the compounds. In the same manner, plasma quality controls (QC) spiked with 70, 280 and 700 μg/l were prepared to measure the accuracy and the precision of the method.

2.4. Extraction procedure

To 1 ml of calibration sample or patient plasma, 200 μl of I.S. (5 mg/l) and 200 μl of 1 M NaOH were added in a 5 ml tube. The mixture was extracted with 2 ml of diethyl ether. The sample was closed with a plastic cap. After vortexing for 1 min, the samples were centrifuged for 10 min at $1400 \times g$. Then, the organic supernatant was transferred in a clean 5 ml tube and evaporated to dryness under nitrogen. The residue was dissolved in 200 μl of mobile phase and 20 μl were injected onto the HPLC column.

2.5. Recovery

Extraction recoveries from human plasma were determined by comparison of HPLC responses from extracted samples, containing known amounts (50, 250 and 1000 μg/l) of each drugs, to those from unextracted and directly injected standards, spiked with the same amounts.

2.6. Analytical method validation

The method was validated by the QC samples prepared at three concentrations spanning the concentration range. Six samples of each quality control pool and calibration samples were analyzed on three different days. Precision and accuracy were determined. The precision was calculated as the coefficient of variation (CV) within a single run (intra-day) and between different assays (inter-day). Accuracy was expressed as the percentage (%) of bias [(found concentration − spiked concentration)/spiked concentration] × 100.

The limit of quantification (LOQ) was defined as the lowest clozapine, loxapine, zuclopenthixol and flupenthixol concentration that could be determined with a precision below 20% and with an accuracy between 80 and 120%, as determined in the inter-day analytical runs.

3. Results and discussion

3.1. Selection of HPLC conditions

The aim of this work was the development of a rapid HPLC assay with a total run time < 10 min, while maintaining suitable sensitivity and selectivity.

In a preliminary study, two HPLC columns (Spherisorb® S5C8 and XTerra™ MS C_{18}) were tested for the elution of the four compounds. On the Spherisorb® S5C8 column, the drugs were separated in the following order: CZP, LXP, ZPT, I.S. and FPT. The total run time was near 20 min. Changing the pH or the mobile phase composition to 45% acetonitrile led to a better run time but FPT and the I.S. were not resolved.

Using the XTerra™ MS C_{18} column provided a good and rapid separation of all the drugs: CZP (1.88 min), LXP (2.50 min), ZPT (4.29 min), FPT (6.23 min). As shown in Fig. 2, no other peak corresponding to these retention times were noted in the chromatogram of drug-free plasma, indicating that interfering endogenous substances were not present.

Table 1
Statistical data ($n = 8$) for linearity including standard deviation (S.D.)

	r^2	Slope (±S.D.)	Intercept (±S.D.)
CZP	0.9878 ± 0.0118	0.0053 ± 0.0002	0.0936 ± 0.0424
LXP	0.9961 ± 0.0046	0.0039 ± 0.003	0.0454 ± 0.0248
ZPT	0.9945 ± 0.0042	0.0012 ± 0.0002	0.0261 ± 0.0280
FPT	0.9905 ± 0.0137	0.0010 ± 0.0002	0.0192 ± 0.0058

3.2. Calibration curve

Calibration curves were linear in the range of 50–1000 μg/l between the drug concentrations and the peak height ratio of the drug to I.S. The coefficient of correlation, the regression equation were reported in Table 1 (weighting factor = 1).

3.3. Precision and accuracy

The results obtained for precision and accuracy are listed in Table 2 and expressed as CV (%) and % bias, respectively. CV for the repetability indicate that the method is precise for all the four drugs (intra-day precision less than 3.6% and inter-day precision less than 7.2%) and accurate (bias ranging from −9.1 to

Table 2
Precision and accuracy of the HPLC method for the analysis of CZP, LXP, ZPT and FPT in plasma

Compounds	Concentrations added (μg/l)	Intra-day studies ($n = 6$)			Inter-day studies ($n = 18$)		
		Concentrations found ± S.D. (μg/l)	% CV	% bias	Concentrations found±S.D. (μg/l)	% CV	% bias
CZP	70	63.6 ± 1.9	2.9	−9.1	64.9 ± 2.1	3.2	−7.3
	280	253.0 ± 4	1.5	−9.5	254.0 ± 2.0	0.9	−9.2
	700	686.0 ± 7.7	1.1	−2.0	678.0 ± 7.9	1.2	−3.2
LXP	70	67.5 ± 1.5	2.2	−3.5	65.6 ± 1.6	2.5	−6.2
	280	257.0 ± 5.6	2.2	−8.0	256.0 ± 1.3	0.5	−8.5
	700	656.0 ± 6.6	1.0	−6.2	650.0 ± 5.7	0.9	−7.2
ZPT	70	73.6 ± 2.7	3.6	5.1	73.8 ± 1.2	1.6	5.4
	280	292.0 ± 6.4	2.2	4.4	290.0 ± 7.3	2.5	3.7
	700	745.0 ± 8.5	1.1	6.4	757.0 ± 7.7	1.0	8.2
FPT	70	75.1 ± 2.5	3.4	7.3	69.4 ± 5.0	7.2	−0.8
	280	268.0 ± 5.6	2.1	−4.3	258.0 ± 8.0	3.1	−7.9
	700	709.0 ± 3.6	0.5	1.23	703.0 ± 9.7	1.4	0.5

Data were expressed as mean (±S.D.).

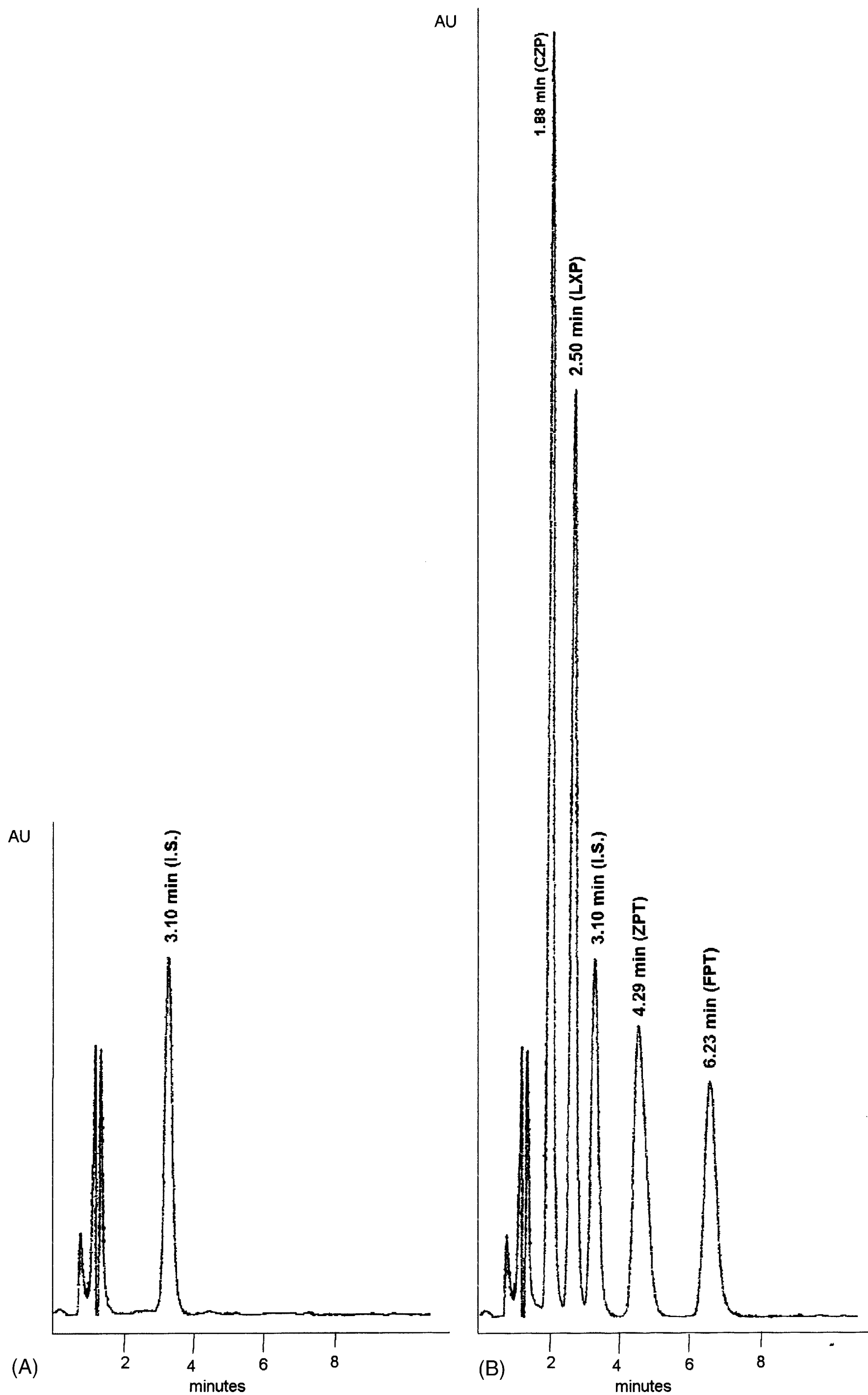

Fig. 2. Representative chromatograms of (A) blank plasma with internal standard (I.S.); (B) plasma spiked with 700 μg/l of clozapine (CZP), loxapine (LXP), zuclopenthixol (ZPT) and flupenthixol (FXP).

7.3% for the intra-day studies and from −9.2 to 8.2% for the inter-day studies).

The LOQ was found to be 15 μg/l ($n = 6$) for both CZP and LXP (with a precision of 8.6 and 7.4%, respectively, and a bias of −14.2 and −13.7%, respectively). The LOQ was determined at 20 μg/l ($n = 6$) for both ZPT and FPT (with a precision of 11.8 and 9.7%, respectively, and a bias of −16.9 and −18.4%, respectively).

The detection limit (LOD) (signal-to-noise ratio = 3) was 4 μg/l for CZP and LXP and 9 μg/l for ZPT and FPT.

3.4. Stability

To determine the influence of temperature and light on the stability of drugs, QC samples spiked with CZP, LXP, ZPT and FPT (70–280–700 μg/l, $n = 4$) were stored under different conditions at −20 °C for a month; >4 °C during 48 h; >20 °C for 24 h in daylight for one group of samples, and in the dark for another group.

No decomposition of CZP, LXP, ZPT and FPT was noted in the frozen samples after a month. Storage for

Table 3
Compounds studied for possible interferences

Compound	t_{r1}	t_{r2}	t_{r3}	t_{r4}	t_{r5}
Oxazepam	1.75	1.29	1.00[a]	0.73	0.50
Tiapride	0.55	0.41	0.32	0.23	0.15
Risperidone	0.80	0.60	0.46	0.33	0.22
OH-risperidone	0.72	0.53	0.41	0.30	0.20
Bromazepam	1.20	0.88	0.68	0.50	0.33
Lorazepam	1.93	1.43	1.11	0.81	0.54
Paroxetine	1.48	1.10	0.85	0.62	0.41
Diazepam	4.66	3.46	2.69	1.95	1.30
Moclamine	0.65	0.48	0.37	0.27	0.18
Venlafaxine	0.81	0.60	0.47	0.34	0.23
Nordazepam	2.73	2.02	1.57	1.14	0.76
Flunitrazepam	2.82	2.09	1.63	1.18	0.79
Clomipramine	2.91	2.16	1.68	1.22	0.81
Nortryptiline	2.01	1.49	1.16	0.84	0.56
Haloperidol	1.47	1.10	0.84	0.61	0.41
Zopiclone	NR	NR	NR	NR	NR
Milnacipram	NR	NR	NR	NR	NR
Amisulpride	NR	NR	NR	NR	NR

t_{r1}: relative retention time of CZP (1.88 min); t_{r2}: relative retention time of LXP (2.50 min); t_{r3}: relative retention time of I.S. (3.10 min); t_{r4}: relative retention time of ZPT (4.29 min); t_{r5}: relative retention time of FPT (6.23 min); NR: no response.

[a] Compound not extracted in our chromatographic conditions.

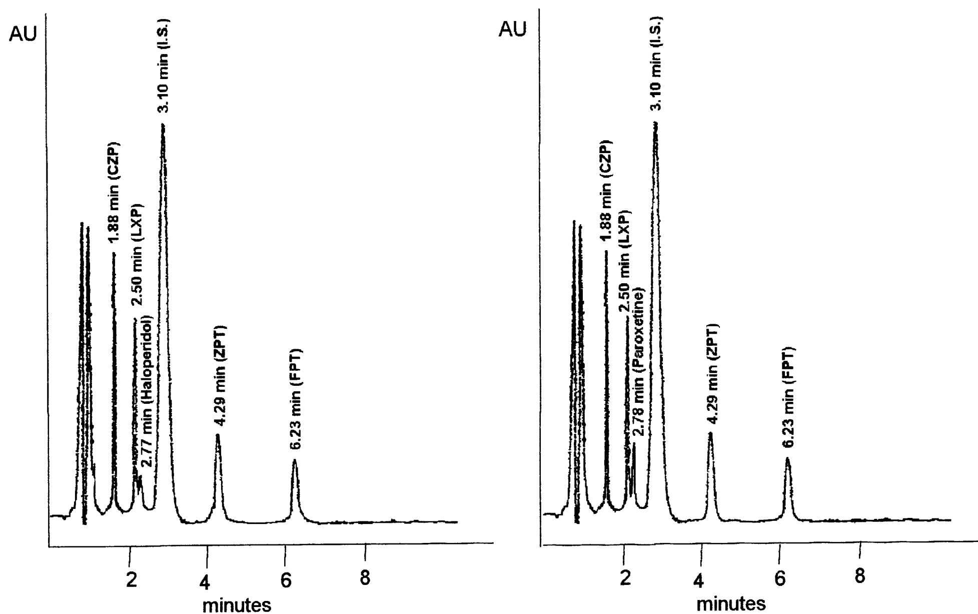

Fig. 3. Chromatograms for potential interferences: plasma spiked with 125 μg/l clozapine (CZP), loxapine (LXP), zuclopenthixol (ZPT), flupenthixol (FPT), haloperidol (10 μg/l) or paroxetine (50 μg/l) and carpipramine (internal standard: I.S.).

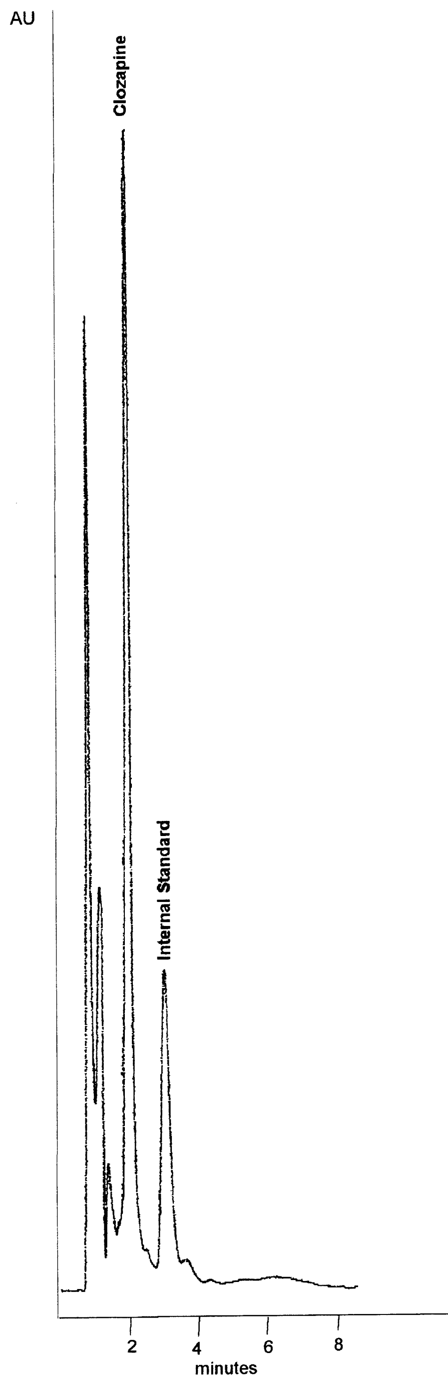

Fig. 4. Chromatogram of plasma obtained from a psychiatric patient sample (diluted 1:10, v/v, in blank plasma) following an overdose of clozapine at 220 nm.

48 h at >4 °C produced no significant decrease of the compounds concentrations. Storage for 24 h at >20 °C in daylight or in the dark showed good stability of all compounds with CV and bias values less than 10%.

3.5. Specificity

Potential interferences were investigated by assaying different blank plasma spiked with drugs commonly found in voluntary drug intoxications (Table 3). No significant chromatographic interferences were found with tested compounds (Fig. 3).

3.6. Clinical case

In voluntary drug intoxications, the ingested dose and the beginning of the intoxication are often unknown. Nevertheless, the expected concentrations are generally above the therapeutic concentration.

By using the described method, we were able to manage several cases of antipsychotic poisoning.

A typical patient chromatogram is shown in Fig. 4. Clozapine was identified and quantified in the plasma of a 37-year-old self-poisoned man. The determination

Fig. 5. Chromatogram of plasma obtained from a psychiatric patient sample following a non-fatal intoxication with zuclopenthixol at 97.5 μg/l.

of the clozapine concentration in this sample was carried out after dilution at 1:10 (v/v) in human plasma. The measured value is 6.98 mg/l, about 30 times higher than the mean therapeutic concentration [31].

We report also three cases of non-fatal intoxications with a mixture of drugs including zuclopenthixol at 31.4, 33.8 and 97.5 μg/l concentrations (Fig. 5). The therapeutic concentration for zuclopenthixol is reported under 100 μg/l [31].

4. Conclusion

The aim of this study was to provide a rapid and effective method employing the normal equipment found in a Clinical Toxicologic or Forensic Department for the simultaneous determination of four antipsychotics: clozapine, loxapine, zuclopenthixol and flupenthixol in human plasma.

The method described involves a simple liquid–liquid extraction procedure with a RP-HPLC analysis and UV detection providing a good separation of the four studied compounds. Moreover, this process allows the detection of the drugs with high selectivity from other psychotropic drugs which are widely co-prescribed, e.g. anxiolytics and antidepressants.

For loxapine, zuclopenthixol and flupenthixol, this method can only be used for plasma samples which contain a drug concentration above the therapeutic range. This method is well adapted to the management of antipsychotic overdoses or poisoning.

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