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High-performance liquid chromatographic assay of the X-ray contrast agent iopiperidol in plasma and urine of rats and humans

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Abstract

Iopiperidol is a non ionic iodinated compound currently under evaluation as a potential contrast medium with anticoagulant property for radiological examinations. An HPLC method for assaying iopiperidol in plasma and urine of rats and humans is described. The analysis is based on the reversed-phase chromatographic separation of iopiperidol and the internal standard (iopamidol) from the endogenous components of the biological fluids, and their detection by UV absorption at 244 nm. The selectivity of the method was satisfactory. The mean absolute recovery was greater than 80%. The precision and accuracy of the analytical methods were in the range 0.3 to 3.3 and −8.5 to +11%, respectively. The detection limits of iopiperidol in plasma (0.1 ml) and urine (0.25 ml) were 0.2 and 0.4 μg/ml, respectively.

Keywords: Iopiperidol

1. Introduction

Iopiperidol is *N,N′*-bis(2,3-dihydroxypropyl)-5-(3-hydroxy-2-oxo-1-piperidinyl)-2,4,6-triiodo-1,3-benzenedicarboxamide (Fig. 1). This heterocyclic non ionic iodinated compound is currently under evaluation as a potential X-ray contrast medium with anticoagulant properties for angiography and urography [1].

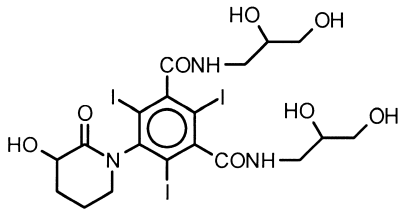

Fig. 1. Structural formula of iopiperidol relative molecular mass, 803.13.

Concentrations of iodinated diagnostic agents in biological samples can be determined by ultraviolet spectrophotometry [2], by assaying the total iodine content by radiochemical methods [3] including neutron activation [4–6] or by catalytic determination based on the Sandell Kolthoff reaction [7,8]. Other methods include inductively coupled plasma [9,10], X-ray fluorescence [11], laser spectroscopy [12] and direct potentiometry by sensitive iodine electrode [13]. Despite their reliability, these techniques are not molecular specific; they are unable to distinguish the contrast agent and the various chemical species of iodine present in the sample (parent compounds and potential metabolites).

The high-performance liquid chromatographic

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(HPLC) method described here enables the selective determination of iopiperidol in plasma and urine samples of rats and humans. Because the assays in plasma and urine required sample pretreatment, an internal standard method of calibration was employed using iopamidol as an internal standard (I.S.). The analysis is based on protein precipitation, reversed-phase chromatographic separation of iopiperidol and I.S. from the endogenous components of the biological fluids, and their detection during elution by ultraviolet light absorption at 244 nm.

2. Experimental

2.1. Apparatus

The assays were performed on a Merck–Hitachi (Tokyo, Japan) liquid chromatograph which consisted of a Model L-7100 pump and a Model L-7200 autosampler. The chromatographic system was connected to a Model L-4500A diode array UV–Vis detector (10 mm flow-cell path-length) linked to a Merck Hitachi work station. Analyses were performed on a LiChrospher RP-8 reversed-phase column (25 cm×4 mm I.D., particle size 5 μm) (E. Merck, Darmstadt, Germany) housed in a thermostated oven (45°C). A LiChrospher RP-8 precolumn (2.5 cm×4 mm I.D., particle size 5 μm) (Merck) was used to prevent contamination of the analytical column.

2.2. Materials

Iopiperidol (racemic form) and iopamidol, chemically (S)-*N,N′*-bis[2-hydroxy-1-(hydroxymethyl)-ethyl]-5-[(2-hydroxy-1-oxopropyl)amino]-2,4,6-triiodo-1,3-benzenedicarboxamide, used as an I.S., were synthesized by Bracco S.p.A. (Milan, Italy).

Purified water was obtained with a Millipore Milli-Q water purification system (Bedford, MA, USA). Analytical grade potassium dihydrogen phosphate, perchloric acid (35–36%, v/v), glacial acetic acid and HPLC grade acetonitrile were obtained from Merck. The mixed ion-exchange resin (anion and cation exchanger 5) was obtained from Merck. The ion-exchange resin was washed with water and excess water was eliminated by short suction filtration. Sodium heparin solution (5000 IU/ml) was obtained under the name of Liquemin® from Hoffman–La Roche (Grenzach–Wyhlen, Germany).

2.3. Biological samples

Plasma and urine samples of rats and humans were prepared. The plasma samples in both instances were obtained from whole blood which had been collected in test tubes containing sodium heparin solution (5000 IU/ml) at a ratio of about 1:50 (v/v) with blood and then centrifuged (10 min at 3500 *g*). Blank samples of plasma and urine were obtained from control rats. Test samples of rat plasma and urine were obtained from rats administered with iopiperidol solution [300 mg(iodine)/ml] at a dose of 300 mg(iodine)/kg. Samples of blank human plasma and urine were obtained from healthy subjects. No test sample was obtained from humans.

2.4. Stock standard solutions

Iopiperidol stock standard solutions were prepared by dissolving iopiperidol powder with purified water to give concentrations ranging from 0.0017 to 3.4 mg/ml for assays in plasma and from 0.070 to 10 mg/ml for assays in urine.

Aqueous solutions of iopamidol at concentrations of 2.0 and 5.0 mg/ml were used as I.S. for the assays. Stock standard solutions were stored in darkness at room temperature (20°C). Under these conditions, they were stable for at least 2 months.

2.5. Preparation of plasma samples

Each calibration standard solution was prepared by adding 30 μl of the desired stock standard solution and 30 μl of iopamidol (I.S.) at concentrations of 2.0 mg/ml to 100 μl of blank plasma from rats or humans. To this sample 30 μl of perchloric acid (35–36%) was added to precipitate the plasma proteins. After agitation and subsequent centrifugation (10 min at 1800 *g*), 10 μl of the clear supernatant was injected into the chromatograph. To determine the iopiperidol content in the plasma of rats treated with the iopiperidol [300 mg(iodine)/kg], 30 μl of purified water and 30 μl of the I.S. solution

were added to 100 μl of plasma test sample. The sample was then processed as described above.

2.6. Preparation of urine samples

Each calibration standard solution was prepared by adding 100 μl of the desired iopiperidol stock standard solution to 1 ml of rat or human urine previously diluted 1:4 with purified water and centrifuged (15 min at 4500 *g*). To this sample 100 μl of I.S. solution (iopamidol 5.0 mg/ml), 100 μl of glacial acetic acid and 1.9 g of ion mixed exchange resin were added. The suspensions were diluted to 5 ml with purified water and, after agitation for 30 min at room temperature, centrifuged (5 min at 3500 *g*). Ten μl of each supernatant were used for the chromatographic analysis. To determine the iopiperidol content in the urine of rats treated with the iopiperidol [300 mg(iodine)/kg], 100 μl of the I.S. solution and 100 μl of purified water were added to 1 ml of centrifuged urine test sample. This was then treated as described above.

2.7. Sample preparation for stability study

The stability of iopiperidol was tested in rat and human plasma and urine over the range of concentrations of pharmacokinetic relevance. The samples required for the stability study were prepared by dissolving directly iopiperidol powder with blank biological fluids of both species. These samples were stored in similar plastic test-tubes to those in which freshly collected samples are usually kept. The standard samples were prepared from a freshly made up stock solution using the same substance batch and the same kind of biological fluid as used in the preparation of test samples.

2.8. Chromatographic conditions

The chromatographic conditions were the same for both the plasma and urine samples. Elution was carried out isocratically with a 94:6 (v/v) mixture of 0.05 *M* KH_2PO_4 (pH 4.5) and acetonitrile at a flow-rate of 0.9 ml/min. The mobile phase was filtered through a 0.45 μm Millipore filter (Millex-HV) and degassed before use. The temperature of the thermostated oven containing the column was set at 45°C. The UV detection wavelength was 244 nm. The injection volume was 10 μl. The areas of the chromatographic peaks relative to iopiperidol and iopamidol (I.S.) were measured and used to calculate the analytical response ratio.

2.9. Calibrations and calculations

For the analyses of both biological fluids the analytical response ratio (y) of the iopiperidol peak area to the I.S. (iopamidol) peak area was calculated and plotted for each concentration (x) relative to each calibration standard solution. A least-squares linear regression was then performed [14]. Since the variances of peak areas at different concentrations showed strong heterogeneity, the weighting factors $1/\mathrm{var}(y|x)$ were introduced improving homogeneity of variances. This type of weight is suitable when, as in the present case, the variances of y values tend to be proportional to the corresponding x values [15]. A standard regression line was then fitted and punctual estimates of unknown concentrations were determined by inverse interpolation.

2.10. Method validation

2.10.1. Selectivity

The selectivity of the chromatographic method was evaluated by checking for interference from drug-free plasma and urine of rats and humans [16]. The selectivity was also checked in plasma and urine of rats for interferences due to potential metabolites of iopiperidol by comparing the chromatographic profiles of standard samples to those of test samples collected from rats after different period from their administration with iopiperidol solution. Furthermore selectivity was verified by analysis of the peak purity which was performed by comparison of three U.V. spectra recorded for samples taken at the beginning, apex and end of the iopiperidol and I.S. elutions.

2.10.2. Stability

The stability of iopiperidol either in plasma and urine of rats and humans stored at −19°C for 1 and 6 months in darkness or in the relative processed samples stored for 24 h in the autosampler at room temperature (20°C) in darkness was investigated. The stability study was performed on six replicates

Table 1
Chromatographic parameters for iopiperidol and iopamidol (I.S.) peaks in plasma and urine

Biological fluid	Analyte	Retention factor (t_0=1.5 min)	Plate number/m ($\times 10^3$)	Peak symmetry
Plasma	Iopiperidol	6.4	7.8	0.92
	I.S.	3.1	6.0	0.94
Urine	Iopiperidol	6.2	6.5	0.94
	I.S.	3.0	5.5	0.91

Table 2
Absolute recoveries for iopiperidol and iopamidol (I.S.) in plasma and urine

Biological fluid	Analyte	Concentration (μg/ml)	Mean absolute recovery (n=6), (%)
Rat plasma	Iopiperidol	91.2	92.1
		456	91.8
		912	94.1
	I.S.	615	92.5 (n=18)
Rat urine	Iopiperidol	90.5	80.2
		452	79.8
		905	82.4
	I.S.	503	77.9 (n=18)
Human plasma	Iopiperidol	21.0	90.1
		300	90.6
		903	88.6
	I.S.	629	90.1 (n=18)
Human urine	Iopiperidol	20.1	82.1
		502	81.8
		1.00×10^3	83.9
	I.S.	510	79.6 (n=18)

Table 3
Linearity of standard curves for iopiperidol in plasma and urine (62 observations for each curve); analysis days 1, 2 and 3 were performed after different time intervals[a]

Biological fluid	Day	$a\pm s_a$[b] ($\times 10^{-3}$)	$b\pm s_b$[c] ($\times 10^{-3}$)	r[d]
Rat plasma	1	−0.98±0.29	1.6000±0.0047	0.99975
	2	−0.593±0.084	1.5500±0.0029	0.99990
	3	0	1.5560±0.0029	0.99990
Rat urine	1	−1.09±0.13	1.9320±0.0027	0.99995
	2	−0.87±0.16	1.9010±0.0032	0.99990
	3	−1.68±0.38	1.9110±0.0091	0.99930
Human plasma	1	−0.554±0.048	1.4980±0.0039	0.99980
	2	−0.726±0.068	1.5190±0.0028	0.99985
	3	−0.74±0.12	1.5530±0.0041	0.99980
Human urine	1	0	1.9050±0.0040	0.99985
	2	0	1.9260±0.0029	0.99995
	3	−0.75±0.16	1.9210±0.0032	0.99990

[a] Equation from least-squares linear regression: $y=a+bx$.
[b] Intercept±standard deviation.
[c] Slope±standard deviation.
[d] Correlation coefficient.

using standard solutions at 100 and 500 μg (iopiperidol)/ml. The stability of the contrast agent was determined by comparing the analytical data obtained for the stored samples with those of freshly prepared standards. The application of a statistical procedure that takes into account the precision of the analytical method and the level of degradation that is pharmacokinetically relevant enabled the stability profile of iopiperidol to be defined. The stability was assessed by means of comparison in analytical response between stored and original (t_0) samples, using 10% as a maximum acceptable degradation [17].

2.10.3. Recovery

The recovery study was performed on six replicates using three standard solutions at concentrations ranging from 21.0 to 912 and from 20.1 to 1.00×10^3 μg/ml, respectively, for plasma and urine. Absolute recovery was measured as the analytical response

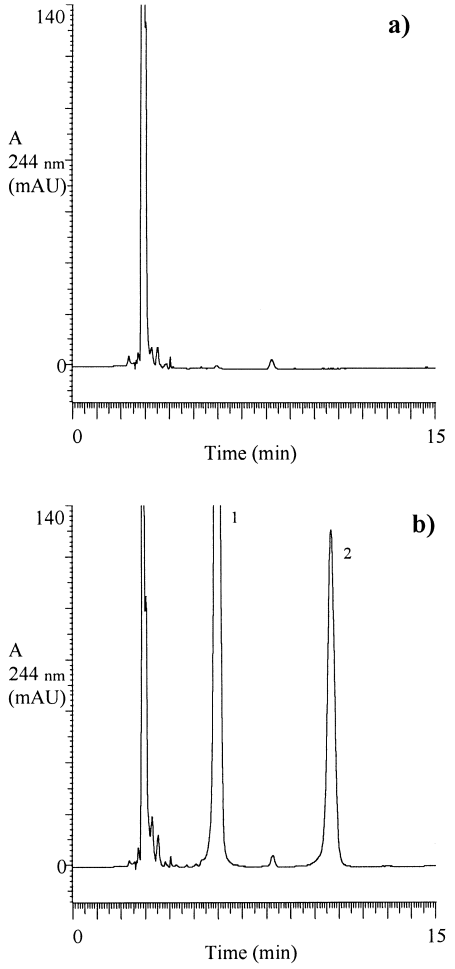

Fig. 2. High-performance liquid chromatograms of rat plasma extract monitored at 244 nm. Peak 1 is due to iopamidol (I.S.) and peak 2 is iopiperidol. (a) drug-free plasma extract from a rat maintained on a control diet; (b) extract of a plasma sample, spiked with iopamidol (I.S.), from a rat 45 min after intravenous contrast media administration (dose 300 mg (iodine)/kg body weight). Iopiperidol concentration (212 μg/ml) was estimated by interpolation on the standard curves.

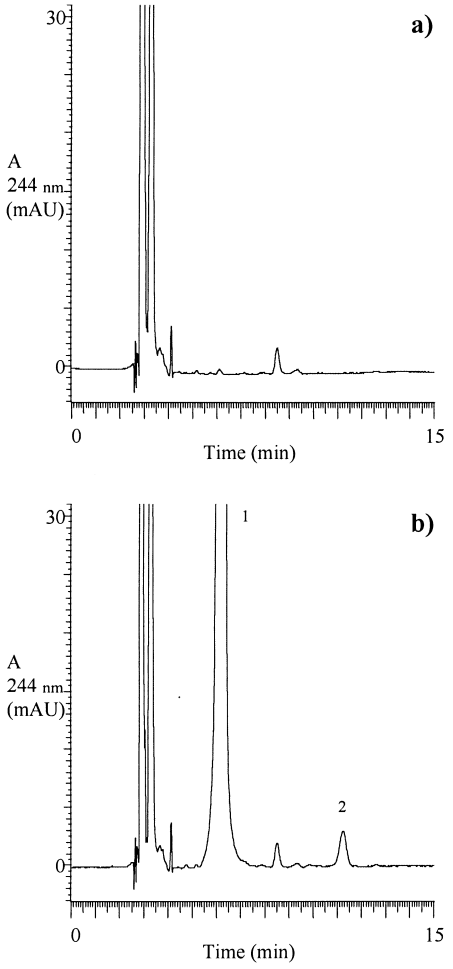

Fig. 3. High-performance liquid chromatograms of human plasma extract monitored at 244 nm. Peak 1 is due to iopamidol (I.S.) and peak 2 is iopiperidol. (a) drug-free plasma extract from a healthy subject; (b) human plasma extract spiked with iopamidol (I.S.) and iopiperidol (5.05 μg/ml).

(peak area) for a processed spiked matrix standard and expressed as a mean percentage of the response for pure standard which had not been subjected to sample treatment [16]. Absolute recovery of the I.S. was assessed for 18 replicates at concentrations ranging from 503 to 629 μg/ml for plasma and urine.

2.10.4. Linearity

Linearity was evaluated for nine concentrations ranging from 5.0 to 1.0×10^3 and from 7.0 to 1.0×10^3 μg/ml, respectively, for plasma and urine. Slope and intercept (when significantly different from zero) and goodness of fit (correlation coefficient) of the standard regression line were calculated [14].

2.10.5. Precision and accuracy

The evaluation of precision and accuracy of the analytical system was performed using nine standard solutions for each biological fluid at concentrations ranging from 5.0 to 1.0×10^3 μg/ml and 7.0 to 1.0×10^3 μg/ml, respectively, for plasma and urine. The assays were repeated six times (ten times for the extreme concentration levels of the range) for three different days separated by variable amounts of time ranging from 1 to 10 days. To evaluate the instrumental and sample preparation variability without introducing statistical error due to the calibration model, the analytical response instead of the interpolated concentration was adopted. Precision was expressed as the percentage standard deviation [s_r(%)] of the analytical responses (peak area ratio) [14]. Accuracy was evaluated by calculating the percentage difference between the estimated and the true concentrations of iopiperidol solutions [14]. For each day, the range and the mean of absolute values were determined.

2.10.6. Detection limit

The detection limits, expressed as concentration c_L, for iopiperidol in plasma and urine of rats and humans, were estimated as described by IUPAC [18,19], using the equation $c_L=3\times sb/S$ where sb is the blank standard deviation calculated experimentally by analyzing ten replicate blank samples, expressed as peak height and S is the sensitivity of the calibration model that corresponds to the slope (b) of the calibration line. In this case the regression line was performed by plotting the analytical response of the iopiperidol peak height (y) against concentration (x) of each calibration standard solution.

The software employed for statistical data processing was SYSTAT (Version 5 Edition; SYSTAT, Evanston, IL, USA, 1992) for personal computer.

3. Results and discussion

For each biological matrix, the chromatographic parameters of iopiperidol and I.S. peaks were calculated according to USP [21,22] and are reported in Table 1. Table 2 shows data for the absolute recovery of iopiperidol and I.S. The coefficient of correlation (r) and the regression parameters of the standard curves, which were fitted to data on three different days, are reported in Table 3. Detection limits of iopiperidol are shown in Table 8.

Table 4
Precision and accuracy for the assay of iopiperidol in rat plasma; analysis days 1, 2 and 3 were performed after different time intervals

Concentration (μg/ml)	Day	Precision (n=10) s_r (%)	Accuracy (n=10)		
			Range (%)		Mean of absolute values (%)
5.0	1	3.0	+1.3	+11	5.5
	2	1.5	−0.82	+3.6	1.7
	3	3.3	−7.9	+1.6	3.2
50.5	1	1.2 (n=6)	−8.4	−5.5	6.8
	2	1.6 (n=6)	−4.9	−1.0	3.0
	3	2.2 (n=6)	−6.2	−0.24	2.8
1.01×10^3	1	1.1	−5.1	−1.9	3.9
	2	1.2	−2.5	+1.5	0.91
	3	0.26	−1.2	−0.27	0.70

3.1. Assay of iopiperidol in plasma

Fig. 2 shows representative chromatograms of (a) a pre-dose sample taken from a rat, and (b) a plasma sample taken from a rat to which iopiperidol had been administered. Typical chromatograms of iopiperidol in human plasma samples are shown in Fig. 3. No interfering peaks at the retention times of iopiperidol and the I.S. were detected in the chromatograms of ten blank plasma samples of rat and human. The stability study indicated 3.7 and 4.8% as maximum degradation of iopiperidol, respectively, in rat and human plasma samples stored at −19°C for 6 months in darkness. No sign of degradation was appreciate for plasma processed samples stored 24 h at room temperature in darkness in the autosampler. The absolute recovery of iopiperidol, which was obtained for concentrations from 21.0 to 912 μg/ml, was in the range from 88.6 to 94.1%. The best

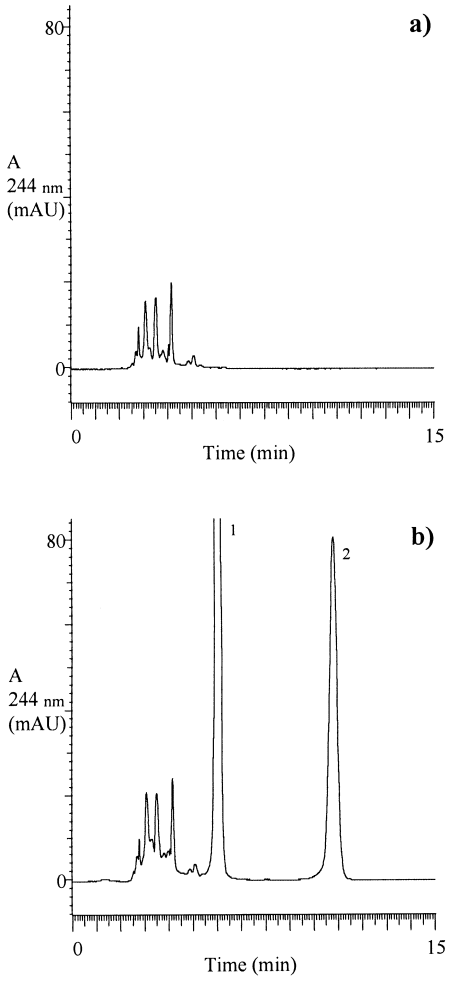

Fig. 4. High-performance liquid chromatograms of rat urine extract monitored at 244 nm. Peak 1 is due to iopamidol (I.S.) and peak 2 is iopiperidol. (a) drug-free urine extract from a rat maintained on a control diet; (b) extract of a urine sample, spiked with iopamidol (I.S.), from a rat 120–240 min (collection time) after intravenous contrast media administration (dose 300 mg (iodine)/kg body weight). Iopiperidol concentration (296 μg/ml; sample diluted ×100 with drug-free urine) was estimated by interpolation on the standard curves.

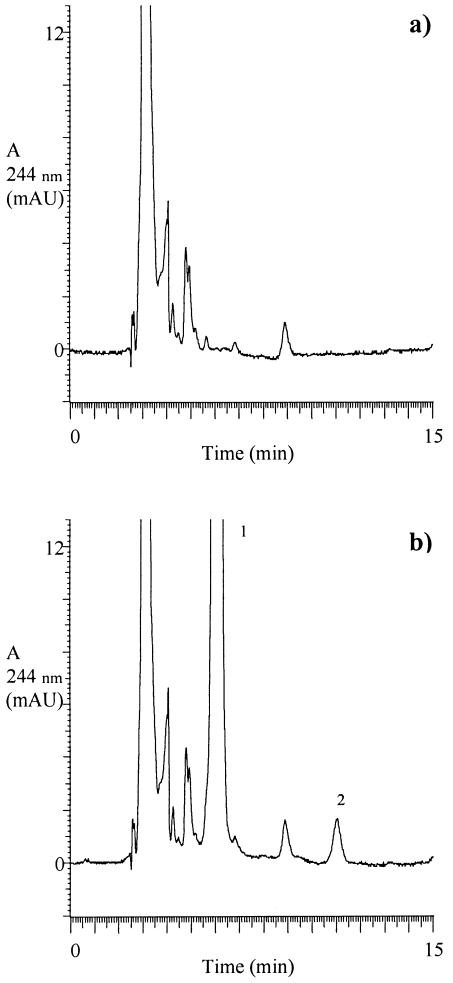

Fig. 5. High-performance liquid chromatograms of human urine extract monitored at 244 nm. Peak 1 is due to iopamidol (I.S.) and peak 2 is iopiperidol. (a) drug-free urine healthy subject; (b) human urine extract spiked with iopamidol (I.S.) and iopiperidol (7.01 μg/ml).

Table 5
Precision and accuracy for the assay of iopiperidol in rat urine, analysis days 1, 2 and 3 were performed after different time intervals

Concentration (μg/ml)	Day	Precision (n=10) s_r (%)	Accuracy (n=10)		
			Range (%)		Mean of absolute values (%)
7.1	1	2.0	−2.7	+2.6	1.5
	2	2.7	−1.6	+7.9	2.7
	3	2.9	+4.7	+12	7.6
51.0	1	2.9 (n=6)	−8.5	−0.77	4.5
	2	1.2 (n=6)	−3.0	−0.16	1.8
	3	1.1 (n=6)	−1.4	+0.88	0.94
1.02×10^3	1	1.0	−2.8	−0.21	1.5
	2	0.74	−2.1	−0.070	1.0
	3	0.87	−0.21	+2.3	0.87

correlation between the peak area ratios (iopiperidol to I.S.) and the concentration of iopiperidol was obtained over the range from 5.0 to 1.0×10^3 μg/ml. Data for the precision and accuracy of some estimated concentrations for analyses performed on three different days by using rat and human plasma samples are reported, respectively, in Tables 4 and 6. Detection limit was in the range from 0.15 to 0.27 μg/ml. No additive or coeluting peak due to potential metabolites of iopiperidol was detected in rat plasma by adopting the described chromatographic conditions.

3.2. Assay of iopiperidol in urine

Fig. 4 shows representative chromatograms of (a) a pre-dose sample taken from a rat, and (b) a urine sample taken from a rat to which iopiperidol had been administered. Typical chromatograms of iopiperidol in human urine samples are shown in Fig. 5. No interfering peaks at the retention times of iopiperidol and the I.S. were detected in the chromatograms of ten blank urine samples of rat and human. The stability study indicated 8.3 and 3.7% as maximum degradation of iopiperidol, respectively, in rat and human urine samples stored at −19°C for 6 months in darkness. No sign of degradation was appreciate for urine processed samples stored 24 h at room temperature in darkness in the autosampler. The absolute recovery of iopiperidol, which was obtained for concentrations from 20.1 to 1.0×10^3 μg/ml, was in the range from 79.8 to 83.9%. The best correlation between the peak area ratios (iopiperidol to I.S.) and the concentration of iopiperidol was obtained over the range from 7.0 to 1.0×10^3 μg/ml. Data for the precision and accuracy of some estimated concentrations for analyses performed on three different days by using rat and human urine are reported, respectively, in Tables 5 and 7. Detection limit was in the range 0.32 to 0.52

Table 6
Precision and accuracy for the assay of iopiperidol in human plasma; analysis days 1, 2 and 3 were performed after different time intervals

Concentration (μg/ml)	Day	Precision (n=10) s_r (%)	Accuracy (n=10)		
			Range (%)		Mean of absolute values (%)
3.4	1	1.4	−1.1	+3.1	1.1
	2	1.7	−0.3	+4.3	1.5
	3	2.7	+0.090	+8.1	1.9
50.1	1	1.3 (n=6)	−4.6	−1.1	3.0
	2	1.0 (n=6)	−4.7	−2.0	3.7
	3	0.92 (n=6)	−4.2	−1.9	3.1
2.01×10^3	1	2.2	−5.2	+1.9	1.6
	2	0.65	−0.98	+0.76	0.57
	3	0.89	−1.0	+2.0	0.90

Table 7
Precision and accuracy for the assay of iopiperidol in human urine; analysis days 1, 2 and 3 were performed after different time intervals

Concentration (μg/ml)	Day	Precision (n=10) s_r (%)	Accuracy (n=10)		
			Range (%)		Mean of absolute values (%)
3.4	1	3.3	−6.1	+3.9	2.8
	2	2.2	−3.5	+3.3	1.8
	3	3.3	−4.4	+5.1	2.8
50.1	1	0.59 (n=6)	−1.9	−0.34	1.1
	2	0.75 (n=6)	−0.020	+2.0	0.57
	3	2.0 (n=6)	−4.0	+0.90	1.5
2.01×10^3	1	0.74	−0.010	+2.1	0.95
	2	0.61	−1.5	+0.65	0.56
	3	0.44	−0.74	+0.77	0.33

μg/ml. No additive or coeluting peak due to potential metabolites of iopiperidol was detected in rat urine by adopting the described chromatographic conditions.

Iopiperidol has three stereochemical centers, enabling several diastereomeric forms and conformational isomers (atropisomers) which may be separated on a non-chiral stationary phase. Despite of the use of iopiperidol racemate the adopted chromatographic conditions with column temperature 45°C permitted a rapid interconversion of the iopiperidol atropisomers to give just one chromatographic band. This phenomena was well described by C. de Haën et al. for iomeprol, an iodinated compound having structural formula similar to that of iopiperidol [20]. Since the chromatographic peaks for iopiperidol and the I.S. are completely separated from any other peak in plasma and urine samples from rats and humans, the described assays can be said to have excellent selectivity for the investigated biological matrices. For the method validation, precision and accuracy were calculated by using the same calibration standard samples adopted to generate the calibration curve in order to use the maximum available information for studying the calibration model. On the basis of the results obtained, iopiperidol can be considered stable in the different biological matrices when stored under the above conditions. The stability of the processed samples and the absolute recovery, linearity, precision, accuracy and detection limits of the described methods are satisfactory for pharmacokinetic studies of iopiperidol in rats and humans. The methods could be also suitable, if appropriately validated, for other small animals or infants because the required amount of plasma and urine does not limit the number of data points obtainable from individual subjects. The fact that the proposed techniques are rapid and can be performed on readily available equipment makes them suitable for routine use.

Table 8
Detection limits (c_L) for iopiperidol in plasma (≥0.1 ml) and urine (≥0.25 ml)

Biological fluid	Day	c_L (μg/ml)
Rat plasma	1	0.15
Rat urine	1	0.52
Human plasma	1	0.27
Human urine	1	0.32

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