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Picogram determination of iloperidone in human plasma by solid-phase extraction and by high-performance liquid chromatography–selected-ion monitoring electrospray mass spectrometry

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Abstract

A very sensitive liquid chromatographic–mass spectrometric (LC–MS) method has been developed to quantitate iloperidone, **1**, and its principal metabolite, 4-[3-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]propoxy]-3-methoxy-α-methylbenzenemethanol, **2**, in human plasma. Iloperidone is currently used in clinical trials for the treatment of psychoses.

The analytes were extracted from human plasma using mixed-mode Bond-Elut Certify cartridges and quantitated using selected-ion monitoring electrospray ionization mass spectrometery (SIM-ES-MS). No interference was observed from endogenous compounds following the extraction of plasma samples from six different human subjects. The limit of quantitation for **1** and **2** was 250 pg/ml of plasma. The standard curves were linear over a working range of 250 pg to 20 ng/ml. Absolute recoveries from plasma ranged from 82 to 101% and 73 to 97% for **1** and **2**, respectively. Overall intra-day precision ranged from 0 to 9% and 0.9 to 12.5% for **1** and **2**, respectively.

The method was found to be rugged and very reliable due to the high specificity of SIM-ES-MS. The results obtained from this study confirm the application of solid-phase extraction combined with SIM-ES-MS in quantitating basic drugs in small quantities in biological extracts.

1. Introduction

Iloperidone, 1-[4-[3-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]propoxy]-3-methoxyphenyl]ethanone (**1**), is a dopamine-D_2/serotonin-5HT_2 antagonist that is currently undergoing clinical trials as a potential atypical antipsychotic. Its preclinical, biological profile suggested that iloperidone should be an efficacious antipsychotic with a low propensity to cause extrapyramidal side effects [1,2,3], as well as having a potential to be effective against the negative symptoms of schizophrenia [4].

Iloperidone is currently administered to subjects at doses of 1 mg to 16 mg daily. The levels of iloperidone and its major circulating metabolite, 4-[3-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]propoxy]-3-methoxy-α-methylben-

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zenemethanol (**2**), were found to be in the low nanogram or in picogram levels as determined by HPLC [5] after single oral doses of 1 to 5 mg. The HPLC method with ultraviolet detection for determination of **1** and **2** was developed initially to support human pharmacokinetic studies based upon a 4 to 8 mg dosage regimen. Clinical studies with oral doses of 1 to 2 mg demonstrated that plasma concentrations of **1** and **2** were in the low nanogram range. Though the HPLC–UV method had a limit of quantitation (LOQ) of 0.25 ng/ml of human plasma, a major problem encountered during the assay was the presence of other unidentified metabolites which would elute in the region of interest during the chromatography. This made it difficult to get reliable levels of analytes especially if they were present in low nanogram quantities. Furthermore, the extraction procedure, though efficient in recovery, was time-consuming. In order to monitor **1** and **2** at pg/ml concentrations in human plasma and to get more reliable plasma concentration time courses following oral administration of iloperidone at 1 to 3 mg doses, it was necessary to develop a more sensitive and specific assay method. An extraction procedure was developed to extract iloperidone and the major metabolite efficiently from the plasma sample. The extract was analyzed using selected-ion monitoring electrospray mass spectrometry (SIM-ES-MS). The application of electrospray mass spectrometry in quantitating compounds extracted from biological matrices has been described previously [6]. The method developed for the analyses of iloperidone had a LOQ of 250 pg/ml of human plasma with an analysis time of less than 11 min. Preliminary studies conducted in this laboratory showed that HPLC–APCI-MS–MS, which has been used recently to analyze a variety of compounds [7–13], was not as sensitive as SIM-ES-MS in quantitating iloperidone in human plasma extract. Since only a small fraction of the extracted sample was introduced to the mass spectrometer during a single analytical run, most of the sample was still available for reanalysis if needed. The application of this methodology for the quantitation of **1** and **2** in human plasma is described.

2. Experimental

2.1. Materials and reagents

Iloperidone, 1-[4-[3-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]propoxy-3-methoxyphenyl]ethanone (**1**), 4-[3-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]propoxy]-3-methoxy-methylbenzenemethanol (**2**) and 1-[4-[3-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]propoxy]-3-ethoxyphenyl]ethanone (**3**, internal standard) (Fig. 1) were obtained from the Department of Chemical Research, Hoechst-Roussel Pharmaceuticals (Somerville, NJ, USA). Acetonitrile, methanol, formic acid and ethyl acetate (all HPLC grade solvents, EM Science, Gibbstown, NJ, USA), ammonium hydroxide, glacial acetic acid, *o*-phosphoric acid, 85% and ammonium formate (Fisher Scientific, Fair Lawn, NJ, USA), drug free human plasma

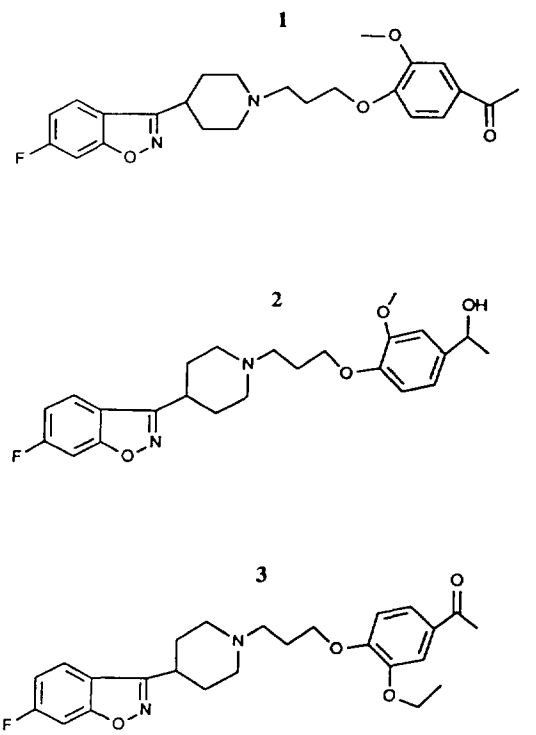

Fig. 1. Structures of **1**, **2** and **3**.

(Biological Specialities, Lansdale, PA, USA), Bond-Elut Certify 130 mg/10 ml cartridges (Varian, Harbor City, CA, USA) were purchased from their respective suppliers. Other reagents were of either HPLC or analytical grade and were used without further purification.

2.2. Instrumentation

A Spectra Physics SP 8800 ternary pump and a Waters autosampler (Model 717) were used for all LC–MS analyses. Chromatography was carried out using a base deactivated 100 × 4.6 mm, 5 μm, Hypersil C_{18} analytical column (Keystone Scientific, Bellefonte, PA, USA). No guard columns were used during the assays. Chromatography was carried out at ambient temperature. The mobile phase consisted of a 70:30 (v/v) mixture of acetonitrile and 2.5 m*M* ammonium formate, pH 3.5 solution. The pH of the aqueous phase was adjusted with formic acid prior to adding it to acetonitrile. The mobile phase was filtered through a 0.45-μm nylon filter (Rainin Instruments, Woburn, MA, USA) and degassed for 5 min under vacuum. The mobile phase was delivered at a flow-rate of 0.55 ml/min. A splitter was set up in the electrospray interface that led to approximately 20% of the HPLC effluent to be sprayed in the source of the mass spectrometer. Post-column plumbing consisted of capillary tubings obtained from Polymicro Technologies, Arizona. The internal diameters of these capillaries were 50 and 100 μm. The samples were introduced onto the HPLC column by the autoinjector set at a run time of 11 min.

Mass spectrometric detection was carried out using a PE-SCIEX API *III* triple-stage quadrupole instrument (PE-SCIEX, Thornhill, Toronto, Canada) using the electrospray interface. The electrospray process was modified (pneumatically assisted) by introduction of zero grade air at 40 p.s.i. (1 p.s.i. = $6.9 \cdot 10^3$ Pa) coaxial to the direction of liquid flow. The electrospray needle was maintained at 4500–5000 V. The curtain gas, nitrogen, was set at 0.9 l/min. The orifice potential and electron multiplier settings were + 55 V and −3.8 kV, respectively. The interface plate was heated to 65°C to prevent condensation of liquids. Dwell time of 266 ms was used during the analyses. To quantitate the analytes present in the human plasma extracts, the mass spectrometer was operated in the selected-ion monitoring mode (SIM). The eluent from HPLC was introduced into the source via the electrospray interface generating the positively charged pseudomolecular ions (MH^+). The MH^+ ions of **1**, **2** and **3** were at *m/z* 427, 429 and 441, respectively. The ions were monitored using the Q3 quadrupoles. The output signal from the mass spectrometer was interfaced to a Quadra 950 Macintosh computer operating RAD and MacQuan softwares (PE-SCIEX) for data collection, peak integration and analysis. Peak-area ratios of analytes (either *m/z* 427 or 429) to the internal standard (*m/z* 441) were used for the construction of calibration curves. MS–MS studies were carried out using argon as the collision gas at a thickness of $200 \cdot 10^{12}$ molecules cm^{-3}. The mass spectrometer was operated in such a way that the protonated parent ions of **1** (*m/z* 427) and **2** (*m/z* 429) were selected by the first quadrupole filter (Q1) to be fragmented to daughter ions in Q2 quadrupole. The resulting fragment ions were analyzed using Q3 quadrupole.

2.3. Standard solutions

Separate standard stock solutions of **1**, **2** and **3** (obtained as free bases) were prepared in 0.1% acetic acid solution using volumetric flasks to give concentrations of approximately 1 mg/ml of each. The stock solutions were diluted in series to prepare a number of solutions of known concentrations of **1**, **2** and **3**. The dilutions, carried out with distilled water, gave a series of working standards with concentrations of 100, 10, 1, 0.1 and 0.01 ng/μl. A working standard solution of 0.1 ng/μl of **3** (internal standard) was used during the entire assay. The stock solutions of **1**, **2** and **3** were kept refrigerated and discarded one month after their preparations. The working standards were prepared fresh on the day of assay.

2.4. Calibration standards

A set of seven calibration standards in duplicate were analyzed with each run. For the analyses of clinical samples, three sets of QC samples (low, medium and high concentrations) in duplicate were included in the assay. For quantitative purposes, the peak areas generated by selected ions of **1**, **2**, and **3** were obtained. The ratios of the analyte peak areas to the internal standard peak areas were obtained. A weighted ($1/x$) linear least-squares regression of the plasma concentrations and measured peak area ratios was used to construct the calibration curves.

2.5. Sample preparation and extraction procedure

The frozen plasma samples were thawed at room temperature and centrifuged to separate out the suspended proteins. After aliquoting 1-ml samples of control plasma, quality control samples and the unknowns into separate disposable culture tubes (100 × 13 mm), 25 μl of 0.1 ng/μl of **3** was added to each tube. After vortexing, the samples were extracted on Bond-Elut Certify cartridges as described below.

Ten extraction cartridges were placed in the holders of a Vac-Elut (Varian, Harbor City, CA, USA) manifold and conditioned by adding 2 ml of methanol and eluting under a full vacuum. Using a dispenser, 2 ml of 8.3 m*M* phosphoric acid was added immediately to the reservoir of the cartridges. The phosphoric acid was drawn through the cartridges at full vacuum. The vacuum was released as soon as the acid solution was almost depleted from the reservoir. The plasma samples, which were diluted with 2 ml of the same acid solution, were loaded onto these cartridges and drawn slowly through the columns at a rate of less than 1 ml/min. Each cartridge was then rinsed with 1.0 *M* acetic acid solution followed by a rinse with 2 ml of 8.3 m*M* phosphoric acid solution. The cartridges were dried for 5 min under full vacuum prior to a final rinse with 2 ml of methanol. The cartridges were dried again for an additional 2 min before the basic compounds were eluted with 2% ammonium hydroxide in ethyl acetate. The collected fractions were dried under a stream of nitrogen at 37°C. The dried residues were reconstituted in 200 μl of the HPLC mobile phase and 20 μl injected onto the HPLC column.

2.6. Recovery

The extraction recoveries of **1**, **2** and **3** from human plasma were evaluated by comparing the areas of the peaks of the extracted and non-extracted analytes. The recovery study was carried out by spiking control human plasma samples with **1** and **2** at these levels (in duplicate): 0.25, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/ml. These samples were extracted as described above. Another set of control human plasma samples was extracted without any prior addition of the analytes. Once the control extracts were dried, appropriate amounts of analytes were added (same levels as the extracted samples) and the final volume made up to 200 μl with the HPLC mobile phase. Aliquots (20 μl) of both the extracted and non-extracted samples were analyzed by LC–MS. The placement of the reconstituted extracts in the autosampler tray was such that the extracted and non-extracted samples containing the same amounts of analytes were analyzed immediately following each other. This was to minimize the influence of any change in the mass spectral response over a period of time. The areas of the analytes were obtained and the percent recovery at each concentration level calculated by dividing the areas of the extracted analytes by the areas of the non-extracted standards. The recovery of the internal standard was obtained only at one level (3 ng/ml) which was used during the assay.

2.7. Linearity, limit of quantitation and specificity

Linearity of the calibration curve was assessed over a concentration range of 0.25 to 20 ng/ml of plasma. Control plasma samples were spiked to give concentrations of 0.25, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/ml of both **1** and **2**. Internal

standard (**3**) was added to each sample prior to the extraction. The linearity of each standard curve was confirmed by plotting the ratio of the drug and internal standard peak areas versus drug concentrations. A standard curve was prepared and assayed with quality control and the unknown samples. The limit of quantitation was defined as the smallest quantity of the analyte that could be determined with acceptable precision and accuracy. At the LOQ, an acceptable precision was when a value of ≤20% R.S.D. was obtained for a set of measured concentrations of the analytes. Another criteria for the LOQ was that the mean value should be within ±20% of the theoretical value. Plasma samples were spiked with 250 and 400 pg of **1** and **2** ($n = 6$ at each level), extracted, quantitated from a standard curve, and the precision and accuracy at these two levels determined. The specificity of the assay was checked by analyzing control and subjects' predose plasma samples. A total of six different plasma samples were analyzed to check for interferences prior to validating the method.

2.8. Precision and accuracy of the method

The intra-day and inter-day precision and accuracy study was carried out by analyzing quality control samples prepared by an independent scientist not directly involved in the study. Quality control (QC) samples were prepared by spiking human plasma samples with standards of **1** and **2**. Three levels of QC were prepared (QC low, 0.5 ng/ml; QC medium, 5.0 ng/ml and QC high, 15.0 ng/ml) and stored in aliquots of 2.3 ml at −20°C. Intra-day precision and accuracy was carried out by analyzing a set of QC samples ($n = 6$) at each of the three levels. An intra-day precision and accuracy study was carried out by analyzing QC samples in triplicate at each level on six different days.

2.9. Stability studies

A number of studies were carried out to establish the stability of the analytes under different storage conditions. Bench-top stability of the analytes was tested by analyzing the QC samples which were thawed and placed at room temperature for 2 and 4 h. The low and medium QC samples in triplicate were left at room temperature for 2 and 4 h, extracted and analyzed using a standard curve prepared on the same day. The effects of the freeze–thaw cycles were investigated using the QC samples. The study involved analyses of QC samples taken through two freeze–thaw cycles. QC samples at all three levels (in triplicate) were used during this study. The stability of the extracted samples left in the autosampler was also assessed. The study involved analyses of the extracted QC samples (all three levels in triplicate) and calibration standards (seven calibration points in duplicate) left in the HPLC mobile phase over a 24-h time period. The following day, a new set of calibration samples were extracted and a new calibration curve prepared. Analyses were initiated after the extractions of the new calibration samples were completed. The levels of the analytes were determined using standard curves from both days. The aim of this study was to show that the samples, if left in the tray for 24 h, could be analyzed the following day. Furthermore, if needed, a new calibration curve prepared on the second day could be used to analyze samples extracted on the previous day. Long-term storage stability of the samples was checked by analyzing the QC samples after four months of storage at −80°C. The stability of the QC samples stored at −20°C was verified by the intra-day and inter-day precision and accuracy study.

3. Results and discussion

The solid-phase extraction procedure using Bond-Elut Certify cartridges described in this paper was found to be very reliable in that reproducible and high recoveries of the analytes **1**, **2** and **3** were obtained. Although the use of this particular cartridge type has been described in the literature [14], it has not been widely used for the analysis of pharmaceutical compounds which are basic in nature. Studies conducted in this laboratory have shown that a number of

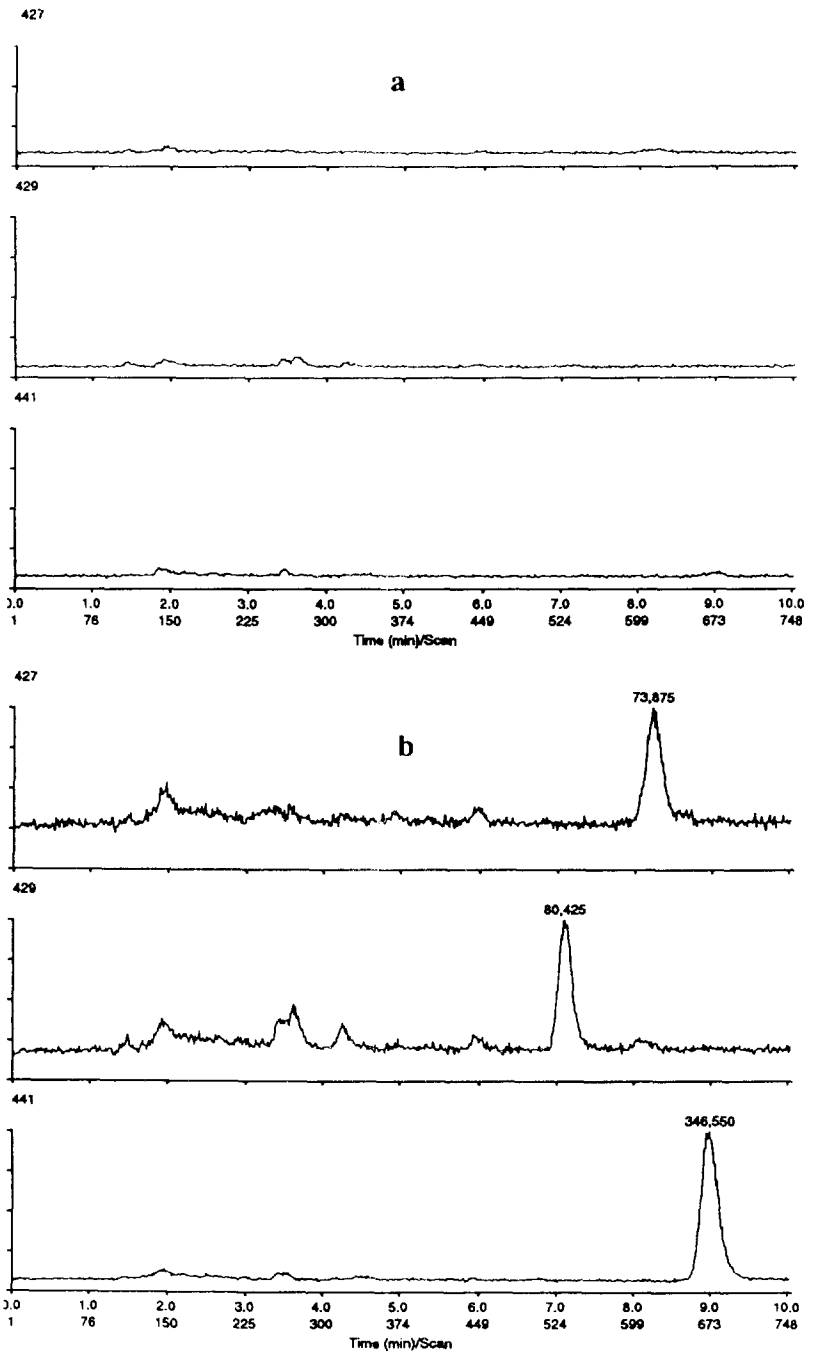

Fig. 2. SIM traces of ions at m/z 427, 429 and 441 present in (a) control human plasma extract, and (b) spiked (500 pg) human plasma extract.

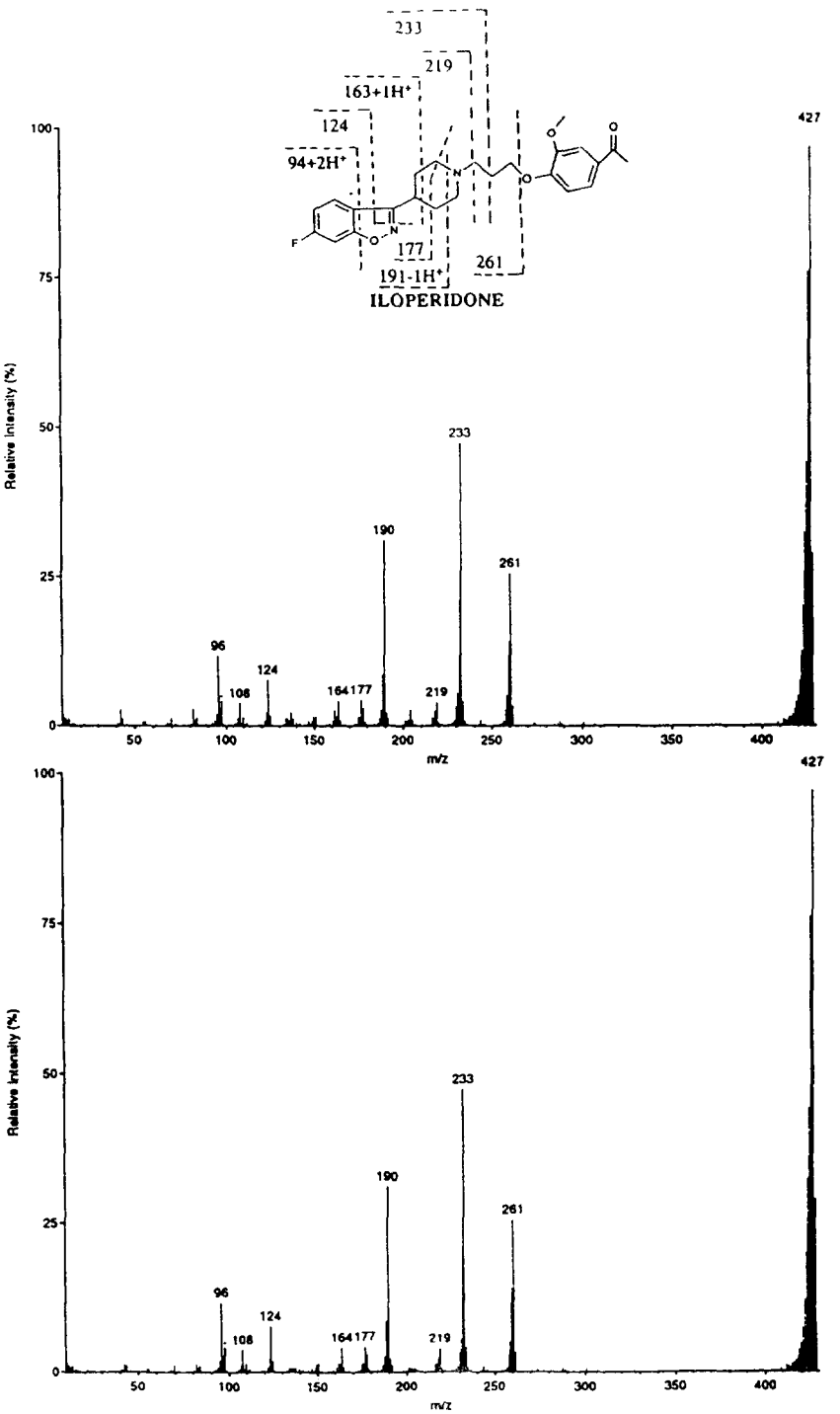

Fig. 3. Daughter ion spectrum of pseudomolecular ion of (upper panel) synthetic standard of **1** (MH^+ at m/z 427) and (lower panel) **1** present in plasma of a subject dosed with iloperidone.

basic compounds with different structures could be efficiently extracted from plasma, bile and urine using these cartridges. The extraction procedure led to isolation of only basic compounds from the biological matrices. The procedure usually provided a very clean extract that could be analyzed by HPLC with UV detection or by coupling GC or LC to a mass spectrometer.

The electrospray mass spectrometry of compounds **1**, **2** and **3**, prepared as solutions in the HPLC mobile phase, introduced to the mass spectrometer yielded only the protonated molecular ions at *m/z* 427, 429 and 441, respectively. These ions were chosen for the quantitation of **1** and **2** in human plasma. The fact that a predominant portion of the ion current was carried by the MH^+ ions provided an ideal quantitative situation and resulted in a good limit of detection. By monitoring these ions, approximately 1 pg of **1** and **2** injected onto the LC–MS system was detected with a signal-to-noise ratio of 3:1. This high sensitivity of detection allowed development of an assay for **1** and **2** with LOQ of 250 pg/ml of biological fluid. The LOQ was defined as the lowest point on the standard curve for which the precision and accuracy were within $\leq 20\%$. Since only a small fraction of the reconstituted samples was introduced to the mass spectrometer, further studies are being conducted in this laboratory to explore the possibility of extending the dynamic range of the assay below 250 pg/ml by reconstituting extracts in smaller volumes. The internal standard, 1-[4-[3-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]propoxy]-3-ethoxyphenyl]ethanone, **3**, was chosen as the appropriate internal standard due to its structural resemblance to **1**, its elution after the analytes of interest and the presence of no interfering peaks from plasma extract. Selected-ion monitoring at *m/z* 427, 429 and 441 of extracted control human plasma indicated that no interferences were present in any of the six different plasma samples analyzed. A sample chromatograph from one of the control plasma extracts is shown in Fig. 2. To confirm the identities of **1** and **2** in plasma from subjects dosed with iloperidone (**1**), MS–MS studies were carried out on the pseudomolecular ions at *m/z* 427 and 429 present in the extracts. The MS–MS fragmentation pattern and the LC–MS retention times of **1** and **2** were then compared with those produced by the selected ions in the plasma extract. The daughter ion spectrum of **1** present in the calibration standard (20 ng/ml) and that obtained from a subject dosed with **1** are shown in Fig. 3. The results obtained indicated that this method is specific for the analytes being quantitated in the human plasma. The linear range of the calibration curve was assessed over a concentration range of 0.25 ng to 20 ng per ml of plasma. Correlation was obtained between the ratio of peak areas of analytes to the internal standard and the drug concentrations. The correlation coefficients of curves obtained on five

Table 1
Calibration curve parameters obtained for **1** and **2** extracted from human plasma on five different occasions

Day of assay	Compound **1**			Compound **2**		
	Slope	Intercept	Correlation coefficient	Slope	Intercept	Correlation coefficient
1	0.37189	0.008187	0.999219	0.378299	0.00207	0.998443
2	0.33977	−0.002527	0.998645	0.330046	0.02175	0.997110
3	0.409777	0.01233	0.998811	0.371017	0.01753	0.998364
4	0.411436	0.006433	0.998383	0.376264	−0.02246	0.998735
5	0.39312	−0.005054	0.999018	0.361095	−0.00164	0.997152
Mean	0.38519			0.36334		
σ_{n-1}	0.0299			0.0198		
%R.S.D.	7.78			5.4		

Table 2
Intra-day precision and accuracy data for the analyses of 1 and 2 in human plasma

Nominal concentration (ng/ml)	Mean ($n = 6$) observed concentration (ng/ml)	R.S.D. (%)	Percentage of theory
Compound 1			
0.526	0.582	9.43	110.60
5.260	5.817	2.32	110.60
15.78	17.087	7.22	108.30
Compound 2			
0.503	0.500	7.80	99.32
5.030	5.279	4.70	104.95
15.09	15.856	10.34	105.08

different days for each analyte are shown in Table 1. The recovery of **1** was determined to be between 82 and 101% over the entire range of the calibration curve. The recovery for **2** was similar except that it showed a greater variability in recovery values over the entire range (73 to 97%).

The intra-day assay precision and accuracy results are shown in Table 2. The %R.S.D. values, which are a measure of the precision of the assay, were less than 10.5% for both **1** and **2** at all the QC concentrations studied. The percent theoretical concentrations, which represent the accuracy of the assay method, were all within ±11% for both **1** and **2** when all three levels of QC samples were analyzed. The inter-day precision and accuracy data are listed in Table 3. QC samples quantitated on six different days gave %R.S.D. values ranging from 0 to 9% for **1** and 0.9 to 12.5% for **2** at all three QC concentrations. The accuracy of the assay method as determined by the percent theoretical concentration ranged from 89 to 107% for **1** and 84 to 114 for **2** at all three levels.

A number of studies were carried out to assess the stability of the analytes in human plasma under a number of different storage conditions. The bench-top stability study showed that the plasma samples containing the analytes can be left on bench-top at room temperature over 4 h without any significant changes in their concentrations. The stability of the analytes in plasma subjected to a number of freeze–thaw (−20°C to ambient temperature) cycles showed that after three cycles, the concentrations of the analytes had not changed. The results from this study are shown in Table 4. The %R.S.D. and percentage of theoretical values obtained (Table 4) indicate that the plasma samples can be thawed and refrozen at least two times prior to analyses. Another study, which was carried out to evaluate the stability of the plasma extract left in the autosampler tray after reconstituting in the

Table 3
Inter-day variability for the assay of quality control samples spiked with **1** and **2** at three different levels

Nominal concentration (ng/ml)	Initial mean concentration[a] (ng/ml)	Mean concentration[b] (ng/ml)	Coefficient of variation[c]
Compound 1			
0.53	0.48	0.51	8.67
5.26	5.40	5.32	6.86
15.78	16.30	15.18	6.93
Compound 2			
0.50	0.55	0.51	5.84
5.03	5.40	5.22	10.10
15.09	16.81	15.87	6.36

[a] $n = 3$.
[b] Mean of six analyses performed over a period of four months.
[c] $n = 6$.

Table 4
Freeze–thaw stability of **1** and **2** QC samples (human plasma)

Compound	QC low (ng/ml)	QC medium (ng/ml)	QC high (ng/ml)
Cycle No. 2			
1	0.600	5.839	15.410
	0.493	5.853	16.165
	0.456	5.712	15.817
Mean	0.516	5.801	15.797
R.S.D. (%)	14.49	1.34	2.39
Percent of theoretical	98.17	110.29	100.11
2	0.429	5.549	16.715
	0.449	5.905	16.056
	0.439	6.117	16.044
Mean	0.439	5.857	16.272
R.S.D. (%)	2.28	4.90	2.36
Percent of theoretical	87.27	116.40	107.83
Cycle No. 3			
1	0.538	5.113	15.620
	0.462	5.233	15.970
	0.606	5.410	14.714
Mean	0.535	5.252	15.435
R.S.D. (%)	13.52	2.85	4.20
Percent of theoretical	101.77	99.85	97.81
2	0.535	4.980	16.429
	0.498	5.003	17.554
	0.530	5.826	15.127
Mean	0.521	5.2695	16.370
R.S.D. (%)	3.83	9.16	7.42
Percent of theoretical	103.55	104.76	108.48

HPLC mobile phase, showed that no degradation of analytes had taken place over the 24-h storage period. This was confirmed by reanalyzing the extracts with a freshly prepared standard curve on the day of the analyses. The results also showed that analyses could be performed on samples using a calibration curve prepared 24 h after the initial extraction. This is useful especially if the calibration samples are unsatisfactory from the first run. The long-term stability of the QC samples stored at −20°C was also evaluated. It was demonstrated that **1** and **2** were stable in human plasma stored at −20°C for at least six months.

The method has been applied successfully to the determination of **1** and **2** in plasma of subjects dosed with 2 mg of iloperidone and is currently used to support other clinical studies with this compound. The method has been found to be rugged and very reliable due to the high specificity of SIM-ES-MS. The results obtained from this study confirm the application of this technique in quantitating basic drugs present in small quantities in biological extracts.

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