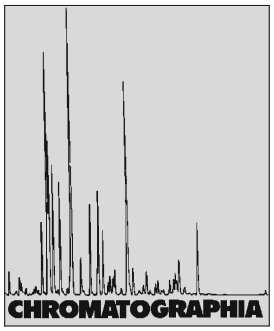
LC–ESI–MS Determination of Flupentixol in Human Plasma



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Abstract

Flupentixol and an internal standard, loperamide were extracted from human plasma by liquid–liquid extraction and analyzed on a Thermo Hypersil HyPURITY C18 column, with 10 mM ammonium acetate–acetonitrile–methanol (26:62:12, *v/v/v*) as mobile phase, coupled with electrospray ionization mass spectrometry (ESI–MS). The protonated analyte was quantified by selected-ion monitoring (SIM) with a quadrupole mass spectrometer in a positive-ion mode. The calibration curve was linear ($r = 0.9990$) over the concentration range: 0.039–2.5 ng mL^{-1}. Intra-day and inter-day precision (RSD%) were less than 13.05%. The established method was successfully applied for the determination of pharmacokinetics of flupentixol in human plasma.

Keywords

Column liquid chromatography
Electrospray ionization–mass spectrometry
Equivalence
Pharmacokinetics
Flupentixol in human plasma

Introduction

Flupentixol is a potent antipsychotic used for treatment of schizophrenia and other psychotic diseases, but frequently associated with extrapyramidal symptoms. It also has anxiolytic and antidepressant properties in low doses. Melitracen is a tricyclic antidepressant with activating properties in low dose. The combination of flupentixol and melitracen is used as an anxiolytic and antidepressant drug without any serious side effects due to lower drug dosage (0.5 mg of flupentixol and 10 mg of melitracen per tablet) [1]. Determination of melitracen in human plasma has been reported before [2, 3]. There are also several reports on the determination of flupentixol. Tanaka et al. [4] and Walter et al. [5] detected flupentixol in human serum with LC–UV which required relatively large sample volume (1 mL) and long analysis time (30 min). The lower limit of quantification (LLOQ) was 0.5 ng mL^{-1}. The LLOQ was 1 ng mL^{-1} for flupentixol in human serum using LC–ESI–MS [6]. Plasma samples were directly injected into LC–APCI–MS–MS [7]. The LLOQ was 0.523 ng mL^{-1}. The mass detection was in the multiple reaction monitoring (MRM) mode. Weinmann et al. [8] and Che et al. [9] detected the flupentixol in hair and human plasma by LC–MS–MS, the LLOQ for flupentixol in hair and in plasma was 0.05 ng mg^{-1} and 0.026 ng mL^{-1}, respectively. However, these methods were generally not suitable for the studies involving low oral dose (including the new formulation

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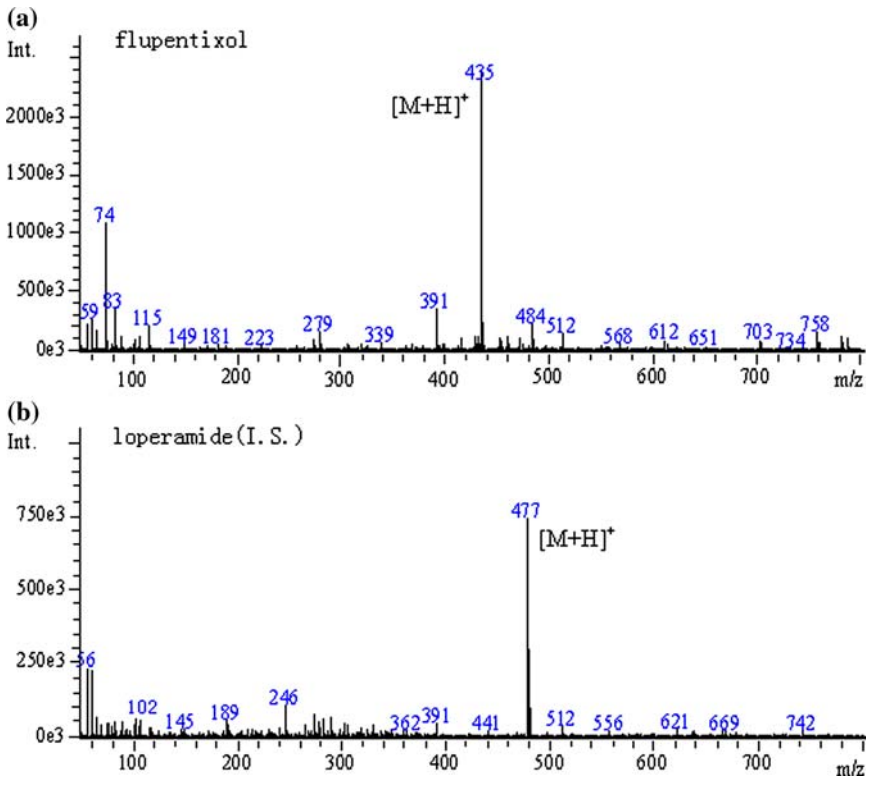

Fig. 1. ESI–MS positive ion scanning spectra of **a** flupentixol and **b** loperamide (IS)

used in the present study) and for high-throughput analysis due to the low sensitivity, long analysis time or the requirement for the expensive instrumentation. In the present investigation, we focused on LC-single quadruple mass because of its availability in most pharmacokinetics laboratories as well as its sufficient sensitivity, selectivity and effectiveness. The established method enables rapid, sensitive, precise, and accurate determination of flupentixol in human plasma samples.

Experimental

Chemicals and Reagents

Flupentixol standard (99.5%) was supplied by Bright Future Pharm. Fty, HK, China. Internal standard (IS), loperamide (98.9%) was purchased from Huanghe Pharmaceutical Limited (Jiangsu, China). Acetonitrile was supplied by Caledon Laboratories (Georgetown, Canada). LC grade methanol was obtained from Tedia (Fairfield, USA). Other reagents were of analytical grade, and water used was Milli-Q grade. Drug-free and drug-containing plasma samples were taken from the volunteers. Samples were stored at −20 °C until further use for analysis.

Instrumentation and LC–MS Condition

The LC system consisted of a Shimadzu LC-10Advp pump, an SCL-10Advp system controller, a CTO-10Avp column oven, an FCV-10ALvp low-pressure gradient unit, a DGU-14A degasser (Shimadzu, Kyoto, Japan). The mass spectrometer was an LCMS-2010 single quadrupole equipped with electrospray ionization interface (Shimadzu, Kyoto, Japan). The data were collected and processed using LCMS solution software.

Chromatographic separations for flupentixol were performed using a Thermo Hypersil HyPURITY C18 (150 mm × 2.1 mm, 5 μm) analytical column. The oven temperature was set at 40 °C,. The mobile phase containing 10 mM ammonium acetate (adjusted pH to 3.6 with acetic acid)–acetonitrile–methanol (26:62:12, *v*/*v*/*v*) was used at a flow rate of 0.22 mL min^{-1}.

An LC–MS-2010 quadrupole mass spectrometer was interfaced with electrospray ionization (ESI) probe. The temperatures were maintained at 250, 250 and 200 °C for the probe, CDL and block, respectively. The voltages were set at 4.5 kV, −30 V, 25 V, 150 V and 1.6 kV for the probe, CDL, Qarray 1, 2, 3 bias, Q-array radio frequency (RF) and detector, respectively. The flow rate of nebulizer gas and dried gas were set at 1.5 L min^{-1}. The ions of selection monitoring were decided by positive scanning from *m*/*z* 0–800. For the quantification of flupentixol, the positive protonated molecule ions at *m*/*z* 435 (flupentixol, $[M + H]^+$) and 477 (loperamide, IS, $[M + H]^+$) were monitored. Tuning of mass spectrometer was performed with the help of autotuning function of LC–MS solution software (Version 2.02) using tuning standard solution (polypropylene glycol). Optimization and calibration of mass spectrometer were achieved with autotuning.

Preparation of Stock Solutions, Calibration Curves and Quality Control Samples

Primary stock solutions of flupentixol and standard solutions of loperamide (IS) were prepared at 100.0 μg mL^{-1} in methanol, respectively. Working solutions were obtained by diluting the stock solutions with methanol. All standard stock solutions and samples were prepared once a month and stored at −20 °C. Seven point-calibration curves (0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 ng mL^{-1}) were freshly prepared by serial dilution of stock solution with drug-free plasma. Quality control (QC) samples were separately prepared by adding standard solution to drug-free plasma at concentrations of 0.078 (low), 0.312 (medium), and 1.583 ng mL^{-1}

(high) of flupentixol. Fifty microliters of IS (loperamide,15 ng mL^{-1}) were added to calibration curve samples and QC samples, respectively.

Sample Collection and Preparation

Eighteen healthy male volunteers received the investigation. The average age of the volunteers was 22.7 years within the range of 20–25. The mean of body weights was 62.0 kg (55–80 kg) and the mean of body heights was 171.0 cm (165–179 cm). All the subjects were non-drinkers and non-smokers. Subjects included were based on their medical history, clinical examination results and routine laboratory test results. All eligible subjects provided written informed consent for participation in the study. A 2 × 2, crossover, randomized, open-label design was used. Subjects were randomly assigned to receive the reference formulation followed by the test formulation with a 2-week washout period between doses. After a 12-h (overnight) fast, subjects received a single oral dose of three tablets (amounting to 1.5 mg of flupentixol) with 200 mL of water. Blood samples were collected in heparinized tubes pre-dose (0 h) and at 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 48, 72, and 96 h post-dose. Plasma was immediately separated by centrifugation at 14000 rpm and stored at −20 °C until analysis.

A plasma sample (600 μL) of flupentixol was placed in a 2 mL Eppendorf tube. After the addition of 50 μL of 15 ng mL^{-1} solution of IS (loperamide), the tube was briefly vortexed and 1 mL of ethyl acetate/n-hexane (5:95, *v*/*v*) was added into the tube. After vortexing for 3 min, the tube was centrifuged at 14,000 rpm for 3 min at room temperature and the organic phase was transferred to another clear 1.5 mL Eppendorf tube. The extract was evaporated to dryness under gentle nitrogen stream at 60 °C.The residue was redissolved in 50 μL of mobile phase. The tube was vortexed for 30 s and centrifuged at 14,000 rpm for 3 min, and 5 μL of supernatant was injected into the analytical column. Complete separation for analytes was achieved within 6.0 min.

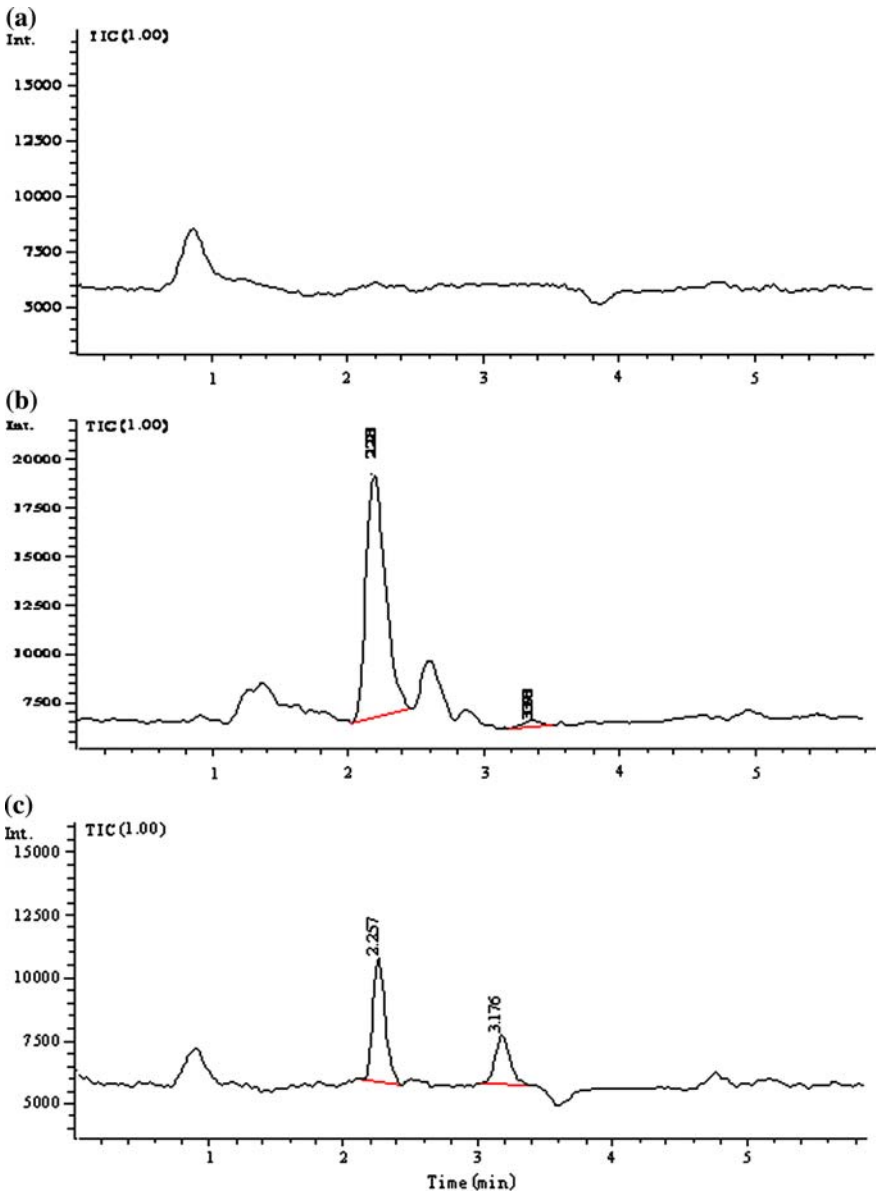

Fig. 2. Total ion chromatograms (TIC) of flupentixol and loperamide (IS). Positive ion monitored at *m*/*z* 435 (flupentixol), 477 (loperamide,IS), **a** blank plasma and human plasma sample spiked with **b** 0.039 ng mL^{-1} flupentixol and 50 ng mL^{-1} loperamide, and **c** human plasma sample 6 h after oral administration of 1.5 mg flupentixol and spiked with IS. The retention time of flupentixol and loperamide (IS) was 3.2 and 2.3 min, respectively

Stability Studies

The stability of flupentixol and IS in human plasma under different storage conditions was evaluated as follows: QC samples were subjected to short-term room temperature conditions, to four freeze-thaw cycles stability studies, to 40-day storage conditions (−20 °C) and to processed sample kept at room temperature. All the stability studies were conducted by QC samples with five determinations for each. For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time (around 24 h) during routine sample preparation. Samples were extracted and analyzed as described above.

Table 1. Intra-day and inter-day accuracy and precision of flupentixol in human plasma

Concentration (ng mL^{-1})	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
	Mean concentration founded	RSD (%)	Accuracy (%)	Mean concentration founded	RSD (%)	Accuracy (%)
0.039	0.036	11.10	92.31	0.037	13.05	94.87
0.078	0.073	10.88	93.72	0.075	12.28	96.15
0.312	0.300	7.24	96.30	0.301	7.53	96.47
1.583	1.553	5.07	98.09	1.550	5.76	97.92

Statistical Analysis

ANOVA was used to check the difference of the means of the pharmacokinetic parameters between the two preparations at a significant level of 0.05. Bioequivalence was determined by two one-sided t tests. After logarithmic conversion, $AUC_{0\text{-}96\ h}$ and C_{max} underwent the analysis of variance to obtain the standard deviation of different groups. Then, two one-sided t tests were carried out to determine the bioequivalence. If the 90% confidence limit of the trial preparation $AUC_{0\text{-}96\ h}$ and C_{max} falls within 80–125% of the reference preparation $AUC_{0\text{-}96\ h}$ and C_{max}, we may conclude that the trial preparation and the reference preparation are bioequivalent. T_{max} also underwent the analysis of variance.

Results and Discussion

Optimization of LC–MS Condition and Sample Preparation

The choice of ionization mode was guided by base peak with higher intensity in the LC–MS analysis. The mass spectra of flupentixol and loperamide (IS) obtained from scan mode were characterized by a protonated molecular ion $[M + H]^+$ as base peak. To confirm ionization mode, the mass spectra were measured in ESI and APCI positive and negative mode using injection flupentixol and IS. In both ionization modes, the base peak intensity of positive ion was higher than those of negative ion, and the efficiency of ionization in ESI was higher than APCI. Figure 1 shows the positive ion mass spectra of flupentixol and loperamide (IS) by ESI selective ion monitoring. Therefore, selective ion monitoring (SIM) mode ($[M + H]^+$ at m/z 435 and 477 was used for quantitative analysis of flupentixol and loperamide (IS), respectively.

The choice of the chromatographic conditions selected was based on symmetry of peak shape and reduction of chromatographic analysis time. The Thermo Hypersil HyPURITY C18 column was selected for all the analytes since it provided symmetrical peak shape and obtained the highest ion intensity to flupentixol. The separation and ionization of flupentixol and IS were affected by composition of mobile phase. The mobile phase pH affected not only the retention time, but also the ionization efficiency of flupentixol and IS. The retention time was prolonged by increasing the mobile phase pH. The acidity of mobile phase enhanced the ionization of flupentixol and IS. Thus the sensitivity was improved by increasing acidity of mobile phase because of raising the ionization efficiency. Acetic acid 1% was used to adjust the pH. Mobile phases with pH 3.6 and 3.9 were applied to increase the sensitivity of flupentixol analysis. Thus, mobile phase consisting of 10 mM ammonium acetate-acetonitrile-methanol (26:62:12, $v/v/v$) with pH 3.6 was used in flupentixol analysis.

Direct precipitation protein with acetonitrile, perchloric acid or trichloroacetic acid was used for processing plasma samples and for obtaining low recovery. Various liquid–liquid extraction methods were investigated for the extraction of flupentixol from plasma. By the comparison of extraction efficiency of different organic solvents including cyclohexane, n-hexane, isopropanol and ethyl acetate, the results showed that isopropanol-n-hexane (2:98, v/v) offered relatively high recovery among the above solvents, but interference was also added. However, ethyl acetate- n-hexane (5:95, v/v) gave the higher recovery and had less interference than other organic solvents.

Method Validation

Specificity was assessed by analyzing six different human blank plasma samples. Typical chromatograms are shown in Fig. 2. Matrix interference was evaluated by comparing the peak areas of analyte in extracted samples of blank plasma from six different drug-free volunteers spiked with known concentrations with the corresponding peak areas obtained by direct injection of standard solutions. No matrix effects were detected in the study.

The calibration curves were constructed by analyzing seven independent standard plasma samples. The peak areas of flupentixol were measured and plotted against the concentrations of flupentixol spiked with blank plasma. The regression equation was $y = 0.883x + 0.0232$ with a correlation coefficient r of 0.9990. It was found to be linear over the range of 0.039–2.5 ng mL^{-1}, with a lower LLOQ at 0.039 ng mL^{-1} ($S/N \geq 10$; RSD $< 15\%$).

The recoveries of flupentixol and IS were calculated by comparing the areas obtained from QC samples and IS spiked before extraction with those obtained from QC samples and IS spiked after extraction ($n = 5$). The mean extraction recoveries were 59.82 and 59.94% for flupentixol and loperamide (IS), respectively.

Precision and accuracy were assessed by investigating QC samples. The intra-day and inter-day repeatability of the

methods for plasma is summarized in Table 1 by analysis of replicates ($n = 5$) of LLOQ and QC samples containing 0.039, 0.078, 0.312 and 1.583 ng mL^{-1} of flupentixol. The precision of the methods was described as RSD among each assay. The intra-day RSDs were always below 11.10% and the inter-day RSDs were within 13.05%. The accuracy was evaluated by analysis of the QC samples spiked with standard solutions and expressed as a percentage of measured concentrations versus known concentrations. Precision and accuracy were calculated at each concentration. The results of the precision and accuracy of the proposed methods were acceptable for bioequivalency study.

The results of the stability study indicate reliable stability behavior under experimental conditions of the regular analytical procedure. Flupentixol is stable at room temperature for at least 24 h. The analyte is also stable in human plasma when stored at −20 °C for at least 40 days and at room temperature for at least 24 h. It is also stable under the influence of four freeze-thaw cycles.

Bioequivalency Study

Table 2 shows the pharmacokinetic parameters of the new flupentixol formulation and the reference flupentixol formulation. The bioequivalency of the new flupentixol formulation was determined with respect to C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$ and $T_{1/2}$. As can be seen from Table 2, the pharmacokinetic parameters of test formulation were very close to those of the reference formulation. In this study, a single-dose of the test formulation was found to be bioequivalent to the reference drug based on the rate and extent of absorption in 18 healthy Chinese male volunteers.

Table 2. Pharmacokinetic properties of two oral formulations of single-dose of flupentixol 1.5 mg

Property	Test formulation (T)	Reference formulation (R)	T/R
C_{max} (ng/mL)	0.495 (0.150)	0.491 (0.153)	1.053 (0.26)
T_{max} (h)	6.7 (2.6)	6.7 (2.9)	1.189 (0.32)
AUC_{0-t} [ng/(mL h)]	22.1 (7.4)	21.4 (7.0)	1.044 (0.14)
$AUC_{0-\infty}$ (ng/(mL h))	26.1 (9.0)	25.4 (8.3)	1.032 (0.14)
$T_{1/2}$ (h)	33.5 (8.4)	35.1 (7.4)	0.965 (0.21)

Values are mean (SD)

Conclusion

A sensitive, rapid and specific LC–MS method has been described for the determination of flupentixol in human plasma. The appropriate mobile phases were selected and charged ions $[M + H]^+$ were selectively monitored. The method involved one-step liquid–liquid extraction with adequate recovery. The established method was successfully applied for a bioequivalency study. The pharmacokinetic parameters of flupentixol (1.5 mg) in healthy volunteers were obtained. The *cis*-isomer of flupentixol is much more active than the *trans*-isomer. A further study will be required to separate the *cis*- and *trans*-isomers.

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