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Simultaneous determination of flupirtine and its major active metabolite in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A rapid, selective and sensitive HPLC–tandem mass spectrometry method was developed and validated for simultaneous determination of flupirtine and its active metabolite D-13223 in human plasma. The analytes and internal standard diphenhydramine were extracted from plasma samples by liquid–liquid extraction, and chromatographed on a C_{18} column. The mobile phase consisted of acetonitrile–water–formic acid (60:40:1, v/v/v), at a flow rate of 0.5 ml/min. Detection was performed on a triple quadrupole tandem mass spectrometer by selected reaction monitoring (SRM) mode via atmospheric pressure chemical ionization (APCI). The method has a limit of quantitation of 10 ng/ml for flupirtine and 2 ng/ml for D-13223, using 0.5-ml plasma sample. The linear calibration curves were obtained in the concentration range of 10.0–1500.0 ng/ml for flupirtine and 2.0–300.0 ng/ml for D-13223. The intra- and inter-run precision (RSD), calculated from quality control (QC) samples was less than 7.2% for flupirtine and D-13223. The accuracy as determined from QC samples was less than 5% for the analytes. The overall extraction recoveries of flupirtine and D-13223 were determined to be about 66% and 78% on average, respectively. The method was applied for the evaluation of the pharmacokinetics of flupirtine and active metabolite D-13223 in volunteers following peroral administration.

Keywords: Flupirtine; D-13223

1. Introduction

Flupirtine (Fig. 1) is a centrally acting analgesic with a long duration of action and apparently a low addiction and tolerance liability [1]. Its activity appears not to be affected significantly by naloxone and it may therefore lack opiate activity. Flupirtine undergoes hepatic biotransformation to two primary metabolites, which have been identified, an acetylated metabolite and *p*-fluorohippuric acid [2]. The acetylated metabolite (D-13223, Fig. 1) has been showed to possess almost 20–30% analgesic activity of the parent compound [1].

A variety of methods have been employed for the analysis of flupirtine in plasma and urine including measurement of radiolabelled drug [2,3], direct fluorescence spectrometry [2,3], thin layer chromatography [2,3] and HPLC with fluorescence detection [4,5]. The HPLC-fluorescence method has also been

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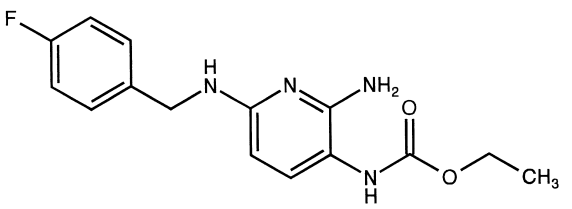
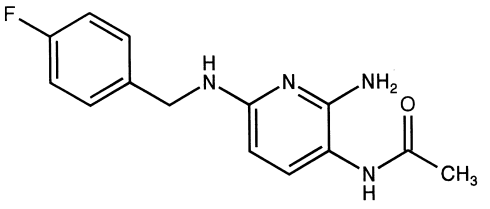
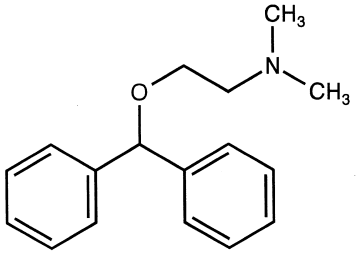


Flupirtine



D-13223



Diphenhydramine (IS)

Fig. 1. Chemical structures of flupirtine, its metabolite D-13223 and diphenhydramine (internal standard).

used for the analysis of the active metabolite D-13223 with a limit of quantitation (LOQ) of 500 ng/ml [4]. The LOQ achievable with the method is only adequate for determining the pharmacokinetic profiles of flupirtine (10 ng/ml), but not for D-13223 following peroral administration. To study the pharmacokinetics of flupirtine and D-13223, an analytical method with higher sensitivity is needed. In this paper we describe a rapid, selective and sensitive HPLC–tandem mass spectrometry method for simultaneous determination of flupirtine and its active metabolite D-13223 in human plasma. The method was demonstrated to be sufficiently sensitive for pharmacokinetic studies for flupirtine and its active metabolite D-13223, using 0.5-ml plasma samples.

2. Experimental

2.1. Reference compounds and chemicals

Flupirtine maleate and its acetylated metabolite D-13223 were obtained from ASTA Medica (Frankfurt, Germany). Diphenhydramine hydrochloride was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were of HPLC-grade, and other chemicals used were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrumentation

A Shimadzu LC-10AD pump (Kyoto, Japan) was used. Chromatography was performed on a Diamonsil C_{18} column (250×4.6 mm, 5 μm, Dikma, Beijing, China), using a mobile phase of acetonitrile–water–formic acid (60:40:1, v/v/v). The flow-rate was 0.5 ml/min. The column temperature was maintained at 25°C.

A Finnigan TSQ™ triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (San Jose, CA, USA) was used for mass analysis and detection. The mass spectrometer was operated in the positive ion detection mode with the corona discharge current set at 5 μA. The vaporizer temperature was 400°C. Nitrogen was used as the sheath gas (c.a. 0.6 MPa) and the auxiliary gas (3 L/min) to assist with nebulization. The interface capillary was heated to 250°C to provide optimum desolvation. The APCI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity at unit resolution. Quantitation was performed using selected reaction monitoring (SRM) of the transitions m/z 305→m/z 195.9 for flupirtine, m/z 275→m/z 165.9 for D-13223, and m/z 256→m/z 166.9 for the internal standard, respectively, with a scan time of 0.3 s per transition. Argon was used as the collision gas at the pressure of approximately 1.9 Pa. The collision energies of 30, 25 and 20 V were used for

flupirtine, D-13223 and the internal standard, respectively.

2.3. Preparation of standards

The stock standard solutions of flupirtine and D-13223 were prepared by dissolving the accurately weighted standard compounds in methanol to give final concentrations of 500 μg/ml for each analyte. The solutions were then successively diluted with a mixture of methanol–water–formic acid (60:40:1, v/v/v) to achieve standard working solutions at concentrations of 7500/1500, 6000/1200, 2500/500, 1000/200, 400/80, 125/25 and 50/10 ng/ml for flupirtine/D-13223. A 120 ng/ml internal standard working solution was prepared by diluting the 400 μg/ml stock standard solution of diphenhydramine with the mixture of methanol–water–formic acid.

The standard working solutions (100 μl) were used to spike blank plasma sample (0.5 ml) either for calibration curves for both compounds of interest (flupirtine and D-13223) or for quality control in prestudy validation and during the pharmacokinetic study.

All the solutions were stored at 4°C and were brought to room temperature before use.

2.4. Sample preparation

To a 0.5 ml aliquot of plasma were added 100 μl of the mixed solution of methanol–water–formic acid, 100 μl of the internal standard and 100 μl of 3 *M* phosphoric acid. The samples were vortexed for 10 s, and mixed for 3 min by ultrasonication. The mixture was adjusted to pH 8–9 with 0.3 ml of 2 *M* sodium hydroxide, followed by extraction with 3 ml of diethyl ether–dichloromethane (1.5:1, v/v). Following the extraction, the organic layer was removed and evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase, and vortex mixed. A 20-μl aliquot of the solution was injected into the LC–MS–MS system.

2.5. Data acquisition and analysis

Data acquisition was performed with Xcalibur 1.1 software (Finnigan). Peak integration and calibration were performed using Finnigan LCQuan software. Peak area ratios of analytes to the internal standard were utilized for the construction of calibration curves, using $1/x^2$ weighted linear least-squares regression of plasma concentrations and the measured peak area ratios. Concentrations of analytes in quality control (QC) or unknown samples were calculated by interpolation from the calibration curves.

2.6. Method validation

The calibration curves for the determination of flupirtine and D-13223 were prepared by analyzing spiked plasma samples. The spiked plasma samples at three concentration levels (see Table 1) were used as QC samples and analyzed by LC–MS–MS system.

During prestudy validation [6], the calibration curves were defined in three runs based on triplicate assays of the spiked plasma samples, and QC samples were determined in replicates ($n=6$) on the same run. Overall assay performance was assessed by calculating the accuracy and intra- and inter-run

Table 1
Summary of precision and accuracy from QC samples of human plasma extracts (in prestudy validation, $n=18$)

Analyte	Added C (ng/ml)	Found C (ng/ml)	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)
Flupirtine	10.0	10.3	7.0	3.5	3.3
	200.0	202.2	5.5	5.1	1.1
	1200.0	1214.8	7.0	4.3	1.2
D-13223	2.00	2.10	6.9	2.1	4.7
	40.0	40.4	3.8	7.1	1.0
	240.0	242.1	6.2	7.1	0.9

precision of QC samples analyzed. During routine analysis each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknowns.

2.7. Application of the analytical method

The LC–MS–MS procedure developed was used to investigate the plasma profile of flupirtine and its active metabolite D-13223 after a single oral dose of an immediately released formulation of 100 mg flupirtine maleate. A clinical study on eight healthy male volunteers was conducted.

3. Results and discussion

3.1. LC–MS–MS optimization

The LC–MS–MS method for the detection of flupirtine and D-13223 in human plasma was investigated. Each compound was at first directly introduced in mass spectrometer using APCI ionization and parameters such as corona discharge, orifice voltage, ring voltage, flow of sheath and auxiliary gas (N_2), were optimized in order to obtain the protonated molecular ions of flupirtine and D-13223. Fig. 2 shows the full-scan Q1 mass spectra of flupirtine and D-13223 where the abundant protonated molecular ions $[M+H]^+$ with small amounts of solvent clustered ions $[M+H+CH_3CN]^+$ were observed. The most suitable collision energy was determined by observing the maximum response obtained for the fragment ion peak m/z. The product ion mass spectra of the analytes and internal standard were presented in Fig. 3, where $[M+H]^+$ ion of each analyte was selected as the precursor ion. The most abundant fragment ions at m/z 195.9, 165.9 and 166.9 were chosen in the SRM acquisition for flupirtine, D-13223 and the internal standard, respectively.

During the early stage of method development, attempts were also made to use electrospray ionization (ESI) as an alternative ionization method for flupirtine and D-13223 analysis. In the ESI full-scan Q1 spectra of the analytes, the clustering ions $[2M+H]^+$ were observed besides the most intensive $[M+H]^+$ and less abundant $[M+H+CH_3CN]^+$. When the source collision-induced dissociation (CID) energy was set, the abundance of $[2M+H]^+$ decreased. The product ion spectra of $[M+H]^+$ and response in the ESI mode were similar to those in APCI mode. When standard working solutions of the two analytes were directly injected into LC–MS–MS system, it was observed that the responses of the analytes at higher concentrations (500–1500 ng/ml for flupirtine, 100–300 ng/ml for D-13223) were much lower than those expected by the linear extrapolation. The relative error in these cases was about −50% using the mobile phase described above and −30% using acetonitrile–water as mobile phase. The narrower linear range (10–200 and 2–40 ng/ml for flupirtine and D-13223, respectively) would not be adequate to the pharmacokinetic study. Therefore, APCI source was chosen in preference to ESI due to the wider linear range for the analytes, and also due to lower levels of chemical noise.

The chromatographic conditions were also opti-

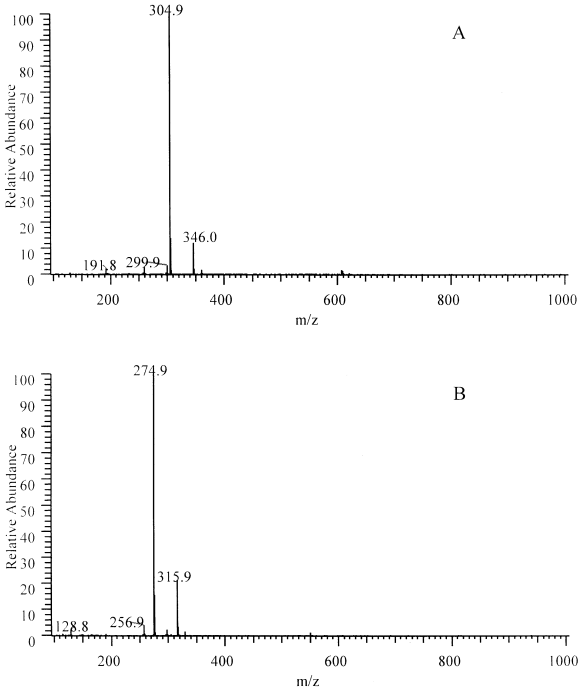


Fig. 2. Full-scan Q1 spectra of (A) flupirtine and (B) D-13223 dissolved in the mobile phase.

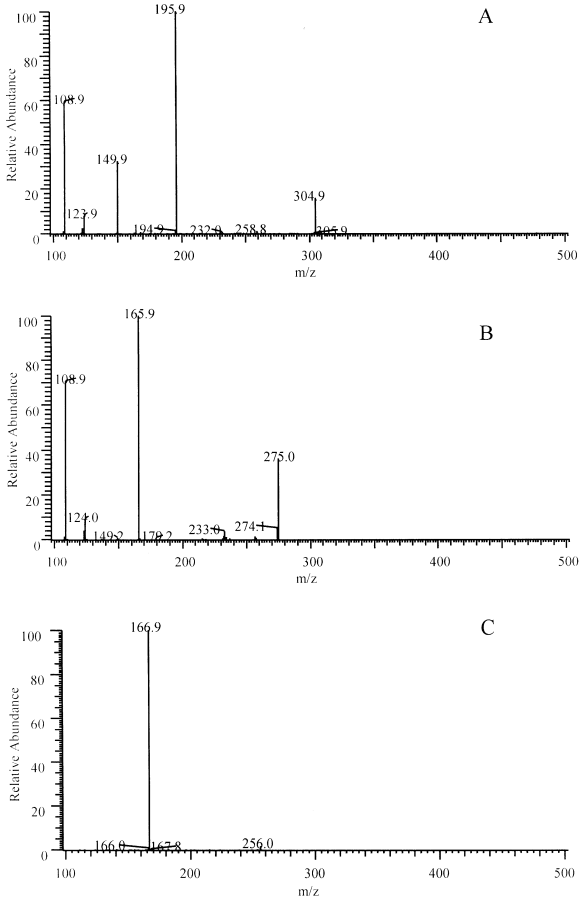


Fig. 3. Product ion spectra of $[M+H]^+$ of (A) flupirtine and (B) D-13223.

mized in the experiment. It was found that the acid modifier in mobile phase had no significant effect on the sensitivity of the analytes under APCI conditions. The solvent clustered ions could be observed using either methanol or acetonitrile as the mobile phase in APCI full-scan Q1 spectra. To achieve the symmetrical peak shapes and short chromatographic analysis time, the mobile phase consisting of acetonitrile–water–formic acid was used in the experiment. In addition, during sample preparation, it was proved to be necessary to reconstitute the residues with the mobile phase, otherwise the chromatographic behaviors of the analytes would be seriously deteriorated.

3.2. Method validation

3.2.1. Assay specificity

The specificity of the method was demonstrated by comparing chromatograms of six independent plasma samples from volunteers — each as a blank sample and a spiked sample. Fig. 4 demonstrates that no interferences from endogenous substances with the analytes and internal standard were detected. Typical

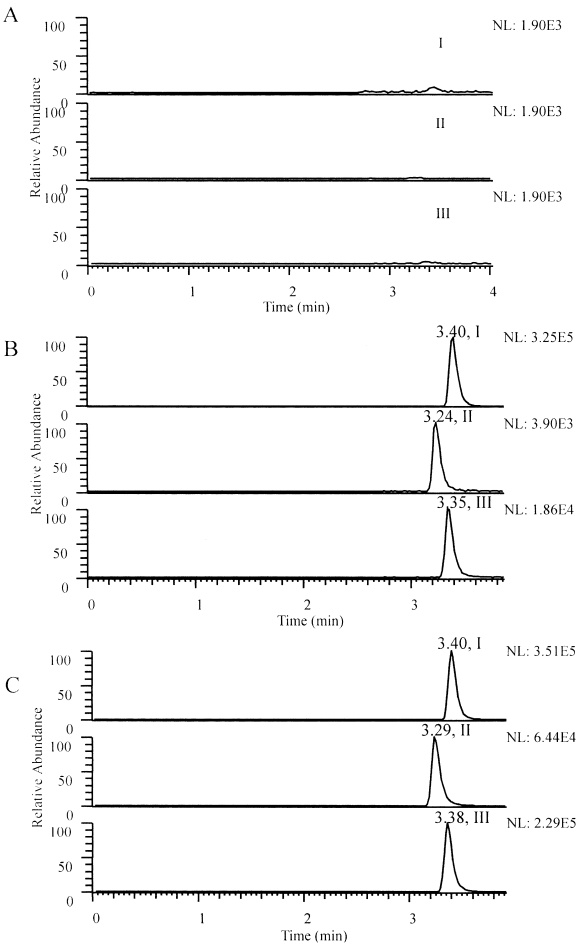


Fig. 4. Representative SRM chromatograms. (A) A blank plasma sample; (B) a blank plasma sample spiked with flupirtine and D-13223 at the limit of quantitation (10 and 2 ng/ml); (C) a volunteer sample 1.0 h after administration of 100 mg flupirtine maleate (flupirtine, 380.4 ng/ml and D-13223, 59.8 ng/ml). Peaks I, II, and III refer to the internal standard, D-13223 and flupirtine, respectively.

retention times for D-13223, flupirtine and the internal standard were 3.2, 3.3, and 3.4 min, respectively.

It has been reported that endogenous matrix components could interfere with the ionization of the target analytes in the LC–MS interface, therefore a suitable chromatographic separation of endogenous extracts to the analytes is necessary [7,8]. In the present paper, the matrix effects on analytes and the internal standard were evaluated by comparing the peak area of the compounds in the samples spiked after extraction with that of each compound in mobile phase injected into the system. The results indicated that no co-eluting ''unseen'' endogenous species interfered with the ionization of the analytes and internal standard.

3.2.2. Linearity of calibration curve and lower limit of quantitation

Linear calibration curves were obtained over the concentration range of 10–1500 ng/ml for flupirtine and 2–300 ng/ml for D-13223 in human plasma. Typical equations of calibration curves were as follows:

Flupirtine: $y = -4.622 \times 10^{-4} + 13.40 \times 10^{-4}x$
$r = 0.9979$

D-13223: $y = 1.572 \times 10^{-4} + 15.45 \times 10^{-4}x$
$r = 0.9984$

The lower limit of quantitation, defined as the lowest concentrations analyzed with acceptable accuracy and precision, was 10.0 ng/ml for flupirtine and 2.0 ng/ml for D-13223, respectively. These limits are already sufficient for pharmacokinetic studies, although much lower LOQ of flupirtine and D-13223 could be achieved, for example to 0.5 ng/ml for each analyte.

3.2.3. Assay precision and accuracy

Intra- and inter-run precision were assessed from the results with QC samples. The mean values and RSD for QC samples were calculated over three validation runs. Six replicates of each QC level were determined in each run. These data were then used to calculate the intra- and inter-run precision (RSD) by using a one-way analysis of variance (ANOVA).

The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed in the relative error (RE).

Table 1 shows the intra- and inter-run precision and accuracy for flupirtine and D-13223 from the QC samples. The intra-run precision, calculated from QC samples, was less than 7% for each QC level of flupirtine and D-13223. The inter-run precision, calculated from QC samples, was less than 5.2% for each QC level of flupirtine and less than 7.2% for each QC level of D-13223. The accuracy as determined from QC samples was within 4% for each QC level of flupirtine and 5% for each QC level of D-13223, respectively.

3.2.4. Extraction recovery

The extraction recoveries of flupirtine and D-13223 were determined by comparing peak areas obtained from plasma sample with those found by direct injection of standard solution at the same concentration. The results showed that the extraction recoveries of flupirtine were 62.2±6.1, 66.5±4.3, and 69.1±4.5% at concentrations of 25.0, 200.0, and 1200.0 ng/ml, respectively; and the extraction recoveries of metabolite D-13223 were 78.2±3.5, 78.1±2.8, and 78.1±6.0% at concentrations of 5.0, 40.0, and 240.0 ng/ml, respectively.

In the experiment, a 100-μl of 3 *M* phosphoric acid was added to the plasma samples before extraction, to destroy the binding of both analytes to plasma protein so that they could be easily extracted into the organic solvent. Adjusting the pH values of plasma samples before adding the organic solvent was also an important step, in order to obtain the maximum extraction recovery.

3.2.5. Analyte stability

The stability of flupirtine and D-13223 in human plasma was investigated under a variety of storage and process conditions. The analytes were found to be stable (>85%) in human plasma after three cycles of freeze (−20°C)–thaw (room temperature) and for at least 4 h at room temperature. The analytes were also shown to be stable for at least 18

Table 2
Stability of flupirtine and D-13223 in human plasma ($n=6$)

	Flupirtine (ng/ml)			D-13223 (ng/ml)		
	25.0	200.0	1200.0	5.00	40.0	240.0
Freeze–thaw stability (relative error,%)						
0 cycle	−1.1	–	5.1	4.9	–	0.6
3 cycles	0.0	–	−5.4	0.9	–	2.3
Storage stability (≤ −20°C, relative error,%)						
0 day	–	−0.4	–	–	0.7	–
18 days	–	−4.9	–	–	3.3	–
Processed plasma samples at room temperature (relative error,%)						
0 h	–	1.5	–	–	2.9	–
12 h	–	4.8	–	–	−7.7	–
24 h	–	−21.6	–	–	−5.9	–
Unprocessed plasma samples at room temperature (relative error, %)						
0 h	–	1.5	–	–	2.9	–
4 h	–	2.6	–	–	3.4	–

days under −20°C freezer conditions. Flupirtine was found to be unstable after 12 h of storage in reconstitution solutions at room temperature, whereas D-13223 was stable for at least 24 h under the same conditions. These data are summarized in Table 2.

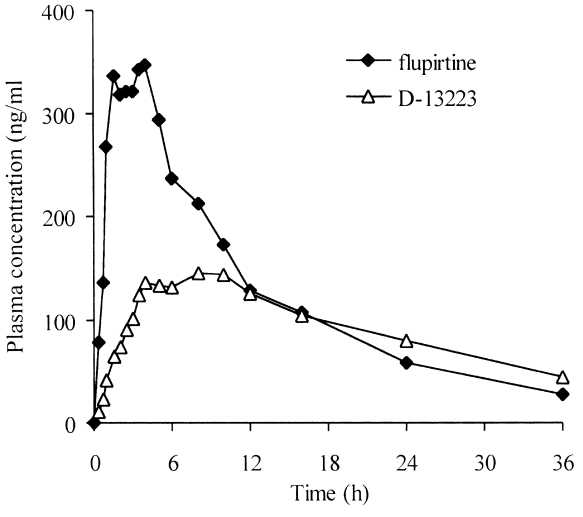


Fig. 5. Mean plasma concentration–time profiles of flupirtine and its active metabolite D-13223 after an oral administration of 100 mg flupirtine maleate to eight healthy volunteers.

3.3. Application of the analytical method in pharmacokinetic studies

After a single oral administration of 100 mg flupirtine maleate to eight healthy subjects, plasma concentrations of flupirtine and its active metabolite D-13223 were simultaneously determined by the described LC–MS–MS method. Fig. 5 shows mean plasma concentration–time curves of flupirtine and D-13223 after administration ($n=8$).

4. Conclusion

An APCI LC–MS–MS method has been developed and validated for the determination of flupirtine and its active metabolite D-13223 in human plasma samples collected following oral low doses. The method has a lower limit of quantitation of 10 and 2 ng/ml for flupirtine and D-13223,

respectively, using a 0.5-ml plasma sample and has been shown to be sensitive, reliable, selective and reproducible. The method has been shown to be successful in applications supporting clinical studies.

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