A selective ^{19}F nuclear magnetic resonance spectroscopic method for the assay of the neuroleptic drug *cis(Z)*-flupentixol in human serum

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Abstract

This investigation was carried out to evaluate ^{19}F nuclear magnetic resonance as an analytical tool for the measurement of the *cis(Z)* and *trans(E)* stereoisomers of the antipsychotic drug flupentixol in human serum. The method is based on the integration of appropriate signals of both analytes and an internal standard. The proposed method was applied to the analysis of real samples without any interference, manipulation of large samples, and lengthy instrument time. Experimental parameters were selected to optimize accuracy, precision, and analysis time. The calibration curves in human serum matrix were linear for *cis(Z)*- and *trans(E)*-flupentixol over the ranges 4.0–50.0 and 2.6–25.0 μg/mL, respectively, with respective minimum detectable limits (S/N = 3) of 1.67 and 1.72 μg/mL. The method was validated through spike and recovery for the two isomers of flupentixol from a human serum matrix.
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Flupentixol is the most important member of the thioxanthene class of drugs, which are effective in the systematic treatment of psychoses. They are most appropriately used in the therapy of schizophrenia, organic psychoses, and other idiopathic psychotic illnesses. Their occasional use may be indicated in severe depression with psychotic features and in the management of patients with organic psychotic disorders. However, these drugs have other clinically useful properties, including antiemetic, antinausea, and antihistamine effects and the ability to potentiate analgesics, sedatives, and general anesthetic actions [1].

The structure of flupentixol is based on the thioxanthene ring to which the side chain is linked by a C=C double bond. Because of the double bond linking of the side chain to the thioxanthene ring structure, flupentixol exists as two geometric isomers, namely *cis(Z)* and *trans(E)* isomers (Scheme 1). It is interesting to note

that, in pharmacological studies, the neuroleptic activity is associated with the *cis(Z)* isomer, while the *trans(E)* isomer is practically inactive [2]. The oral form of flupentixol, administered as tablets or drops, is an equimolar mixture of the active *cis(Z)* and the inactive *trans(E)* isomers, while the parenteral preparation for intramuscular injection is the decanoic acid ester of the isolated *cis(Z)* isomer.

There is considerable interest in sufficiently selective and sensitive methods for the aim of comparison of the oral and i.m. forms and for studies of different metabolite pathways of *cis(Z)*- and *trans(E)*-flupentixol in biological fluids [3]. In addition, neuroleptics are frequently associated with severe neurological side effects and a serum-concentration-guided drug dosage might help to reduce the number of patients suffering from such side effects due to overdosing.

Several methods have been designed for the analysis of thioxanthene neuroleptic drugs in pharmaceutical preparations including ultraviolet–visible spectroscopy [4,5], fluorimetry [6,7], and chromatographic methods [8–10]. However, only a few of the published methods

Cis(Z)-flupentixol

Trans(E)-flupentixol

Scheme 1.
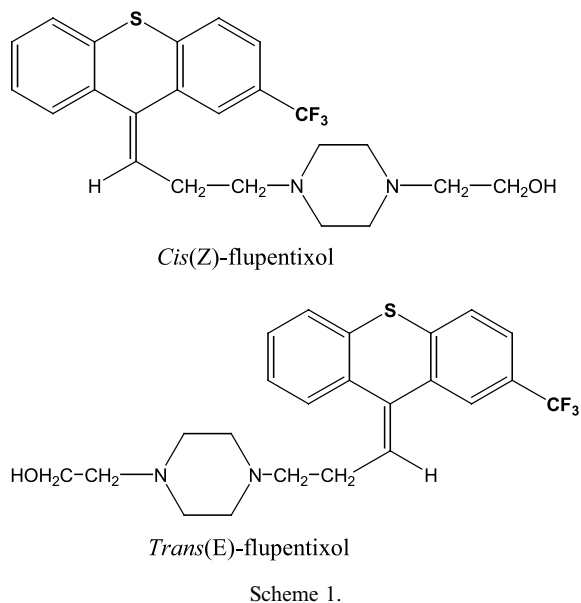
were designed to measure blood concentrations of the highly potent neuroleptic drug flupentixol with sufficient selectivity and sensitivity, because blood-level monitoring has completely different requirements with respect to sensitivity and selectivity to other compounds in the matrix [11–18]. In all of these methods, a one-step extraction is necessary for separation of sample from complex blood matrix and required relatively large sample amounts such as 2 mL of serum [19]. Withdrawal of large blood volumes might, however, cause problems in medical treatment, especially if many samples are required for frequent drug monitoring or pharmacokinetic analysis.

As a basis for quantitative determination of fluorinate species, flourine-19 nuclear magnetic resonance (^{19}F NMR) offers several potential advantages compared to chromatography. ^{19}F NMR spectroscopy has a large spectral window associated with it. Typically organofluorine nuclei lie within a window of 300 ppm, resulting in a small probability of peak overlap between molecules. Moreover, the spin 1/2 ^{19}F isotope is 100% abundant and possesses a sensitivity which is 81% of a ^{1}H nucleus. The technique can be used for spectral identification of an analyte and for quantification, when an internal standard of known concentration is included [20–24].

In this study, it was found that the presence of trifluoromethyl groups in the cis and trans isomers of flupentixol enables ^{19}F NMR for the analysis of drug content of serum samples. This feature provided an alternative method to study the metabolism of these compounds. Experimental parameters for ^{19}F NMR analysis of the two isomers of flupentixol are selected to optimize the NMR method with respect to accuracy, precision, and analysis time. The purpose of the method was to establish the minimum detectable level main-

taining a convenient sample size (500 µL) and a rapid instrumental time period (7 min).

Experimental

Materials

The cis(Z)- and trans(E)-flupentixol reference materials were obtained from Lundbeck (Denmark) and were used as received. Deuterated acetonitrile and HPLC-grade solvents (acetonitrile and methanol) were purchased from Aldrich. 4-Fluorotoluene was obtained from Merck and used as received. Drug-free human serum was obtained from the Central Blood Transfusion Service of Tarbiat Modarres University (Tehran, Iran).

Sample processing

Blood was immediately centrifuged and the serum supernatant was added to equivolume of acetonitrile (5 mL of each) in 12-mL glass tubes supplied with Teflon-covered screw-caps. The samples were deproteinized by vortex-mixing on a multitube vortexer for 30 s. After centrifugation at 3500g for 4 min at room temperature, the upper layer was transferred into a clean 10-mL glass tube and used to prepare calibration samples.

An acetonitrile/methanol (2:1 v/v) mixture was selected as a stock solvent to properly dissolve the two isomers of flupentixol. In all NMR measurements, 20% w/w of deuterated acetonitrile was also added to lock the field frequency of the instrument.

Standard stock solutions of 1.0 mg/mL cis(Z)- and 1.0 mg/mL trans(E)-flupentixol were prepared in stock solvent, protected from light, and stored at 4 °C. These standard solutions were used to spike drug-free human serum for the calibration and recovery purposes. The calibration solutions were prepared in human serum samples on the day of analysis by mixing 25-µL portions of appropriately diluted standard stock solutions of cis(Z)- and trans(E)-flupentixol in the stock solvent with 475 µL of human serum, to yield spiked solutions of 4.0 to 50.0 µg/mL of cis(Z)-flupentixol and 2.4 to 25.0 µg/mL of trans(E)-flupentixol. A range of 3–15 µg 4-fluorotoluene was added as internal standard to the calibration samples.

Apparatus

All ^{19}F NMR spectra were recorded on a Bruker DRX 500 Avance (11.7 T) spectrometer operating at 470.59 MHz with a dedicated 5-mm QNP probe and running XWIN-NMR 2.6 software using 500 µL of samples. The spectra were acquired using 90° pulses with 16–512 scans collected into 128 k data points over a spectral width of 61,162 Hz. The 90° pulse width was

measured to be 12.1 μs for human serum. The acquisition time was 1.07 s followed by a relaxation delay of 5 s to ensure full T_1 relaxation, resulting in a total pulse recycle time of 6.07 s (T_1 relaxation values were determined to be 1.2 and 1.1 s for *cis*(Z)- and *trans*(E)-flupentixol, respectively). The spectra were recorded at 300 K and chemical shifts are reported relative to trichlorofluoromethane (CFCl$_3$) at 0.00 ppm.

NMR processing for final solutions of all samples included phase correction (performed manually for each replicate) and baseline correction over the entire spectral range. In all instances, the baseline was additionally corrected over the integrated regions. Areas of the peaks were determined by electronic integration of expanded regions around diagnostic resonances, using an integral limit of ±20 Hz around the corresponding signals.

The T_1 measurements of the ^{19}F nuclei were carried out by the inversion-recovery method, using the standard sequence of 180°-F-90°-D$_1$ with a relaxation delay D$_1$ of 10 s and T_1cal Bruker program. It should be noted that, in the process of T_1 measurements, the minimal spectral windows required for each analyte were employed.

Method validation

The concentration of *cis*(Z)- and *trans*(E)-flupentixol were calculated from ^{19}F NMR signal integrals compared with that of a standard compound (4-fluorotoluene) spiked into the samples. Calibration curves of the two isomers of flupentixol were constructed by plotting the calculated ratios of *cis*(Z)- and *trans*(E)-flupentixol signal integrals and internal standard signal integral against the corresponding concentration flupentixol isomers. Moreover, the absolute quantity of these two isomers can be calculated by substituting this value in the formula

$$W_a = (W_{is}I_aMW_a)/(I_{is}MW_{is}),$$

where W_a is the measured weight of analyte (mg), W_{is} is the weight of internal standard (mg), MW_a is the molecular weight of analyte, MW_{is} is the molecular weight of internal standard, and I_a and I_{is} are the integrals of the selected resonances of analyte and internal standard, respectively.

Accuracy and precision

Accuracy was assessed by determining the concentration of spiked drug measured in human serum (8.6, 14.2, and 23.5 μg/mL for *cis*(Z)-flupentixol and 5.2, 13.5, and 18.1 μg/mL for *trans*(E)-flupentixol; $n = 3$ for each case) relative to the known concentration added. Precision was determined utilizing the relative standard deviation (%) of the within-day ($n = 3$) and between-day ($n = 5$) variations.

Results and discussion

The structures of *cis*(Z)- and *trans*(E)-flupentixol are presented in Scheme 1. The proton NMR pattern of aliphatic regions of the two isomers is the same in both chemical shift and multiplicity. The main difference in the two ^{1}H NMR spectra is observed in the chemical shift of triplet signals corresponding to the single proton of the double bond linked to the thioxanthene group that are presented in 5.98 and 5.95 ppm for *cis*(Z)- and *trans*(E)-flupentixol, respectively. On the other hand, the aromatic regions of the two isomers are also similar except that, in the aromatic region of *trans*(E)-flupentixol spectrum, a singlet signal is separated from the others and observed at 7.87 ppm, corresponding to the proton located between the CF$_3$ and the H—C= linked to the thioxanthene group. Thus, the simultaneous identification and determination of these isomers by the ^{1}H NMR spectroscopy method seems to be very difficult, if not impossible. In addition, a water suppression method is also necessary for the analysis of flupentixol isomers in biological fluid matrices, in conjunction with ^{1}H NMR spectra, to eliminate the intense water signal observed in these matrices [25].

Several previously published studies revealed that ^{19}F NMR spectroscopy can be employed as a powerful selective tool to analyze different fluorinated molecules [20–24]. Thus in this work, we were interested to apply ^{19}F NMR to the simultaneous identification and quantification of the two isomers of flupentixol in serum samples. Fig. 1A shows the ^{19}F NMR spectrum of *cis*(Z)-flupentixol, possessing a sharp singlet ^{19}F signal for the CF$_3$ group at −61.20 ppm. The CF$_3$ group signal of *trans*(E)-flupentixol located at −61.33 ppm (Fig. 1B) was well resolved from the corresponding signal of *cis*(Z)-flupentixol in the ^{19}F NMR spectrum of a mixture of *cis*(Z)- and *trans*(E)-flupentixol (Fig. 1C). The high sensitivity of ^{19}F nucleus (further enhanced by the availability of three equivalent fluorine nuclei in the CF$_3$ resonance) in conjunction with the wide range of fluorine chemical shifts provided a suitable and simple method for the identification and determination of *cis*(Z)- and *trans*(E)-flupentixol. On the other hand, since fluorine atoms are not naturally present in biological fluids, the ^{19}F NMR spectra of these matrices containing desirable F-substituted analyte and their metabolite are generally highly simplified, in comparison with the corresponding ^{1}H NMR spectra. The CF$_3$ signals corresponding to *cis*(Z)- and *trans*(E)-flupentixol in a human serum matrix are presented at −62.95 and −63.03 ppm, respectively (Fig. 2). The concomitant dispersion signal inherent to ^{19}F NMR spectroscopy enabled precise integration of the signal areas and allowed a simple identification and determination method for the analysis of two isomers of flupentixol in human serum samples.
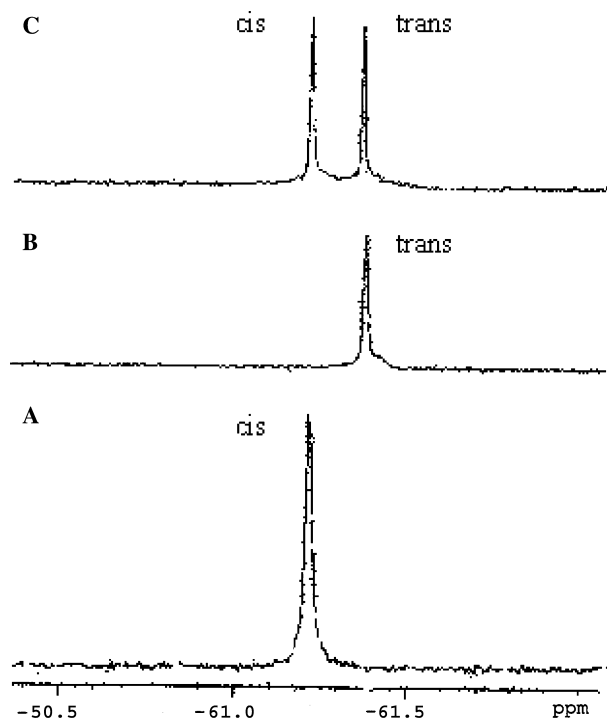
Fig. 1. ^{19}F NMR spectra of *cis(Z)*- (A), *trans(E)*-flupentixol (B), and their mixture (C) in acetonitrile/methanol (2:1 v/v) solvent.

Fig. 2. ^{19}F NMR spectra of blank human serum (A) and serum spiked with 4.0 and 2.6 (B), 7.0 and 3.8 (C), 10.0 and 5.2 (D), 12.0 and 6.2 (E); 15.0 and 7.0 μg/mL (F) *cis(Z)*- and *trans(E)*-flupentixol, respectively (each containing 4-fluorotoluene as internal standard).

To provide quantitative information needed for the two geometric isomers of flupentixol (identity assay) many NMR data collection parameters have been optimized. The magnitude and duration of the applied rf pulse are important parameters which affect both the signal-to-noise ratio and the quantitative accuracy of the signal integrals. Integration of the spectral peaks would then yield the required quantitative information [26]. In addition, the other parameter which is routinely open to the NMR spectroscopy for optimizing sensitivity is the recycling delay time (D_1), to return all magnetizations to equilibrium between pulses. This is obviously related to the spin-lattice relaxation time (T_1) of the nuclei. To carry out quantitative determinations, we first measured the T_1 values of *cis(Z)*- and *trans(E)*-flupentixol by the inversion recovery method [27], and the respective resulting values were found to be 1.2 and 1.1 s.

It has been suggested that a good approach for the quantitative analysis of complex mixtures containing nuclei with a short range of T_1 values is to use large pulse angles (70–90°) and a repetition time higher than the maximum T_1 by three- to fivefold. After performing optimization studies on the two drug isomers we selected a standard pulse angle of 90° and a relaxation delay of 5 s associated with an acquisition time of 1.07 s, which allowed accurate quantitation of *cis(Z)*- and *trans(E)*-flupentixol in human serum.

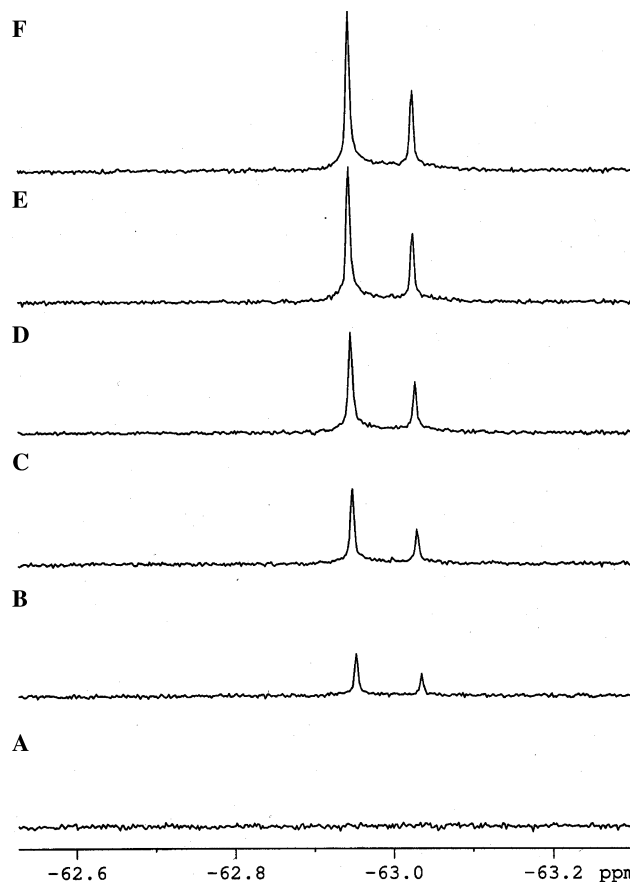Meanwhile, the quantitative procedure by NMR spectroscopy needs to take into consideration the ratio of signals belonging to different compounds with respect to those of an internal standard. Selection of the internal standard is dictated by high purity, chemical inertness, easy solubility, and good resolution from the analyte signals. In this work, 4-fluorotoluene was selected as a suitable internal standard, possessing a sharp singlet ^{19}F signal (located at −120.4 ppm) well separated from that of *cis(Z)*- and *trans(E)*-flupentixol. The quantitative procedure can be carried out on the basis of either peak integration (area) or peak intensity (height). It should be noted that the area measurements are in general easy to perform for the case of well-resolved component NMR signals.

Fig. 2A shows the ^{19}F NMR spectrum of a blank serum sample which contains no ^{19}F NMR signal in chemical shift windows employed for the quantitative studies of the *cis(Z)*- and *trans(E)*-flupentixol. The quantitative method was evaluated by spiking known concentrations of the two isomers of flupentixol into control serum samples. Figs. 2B–F show typical ^{19}F NMR spectra of spiked-serum samples over concentration ranges of 4.0–15.0 and 2.6–7.0 μg/mL for *cis(Z)*- and *trans(E)*-flupentixol, respectively. Calibration curves were constructed based on the calculated peak

area of each analyte relative to that of the internal standard in spiked samples against the corresponding concentrations of the two analytes. Linear regression analysis of the calibration plots resulted in correlation coefficients $R > 0.9969$ for cis(Z)- and trans(E)-flupentixol (Table 1). From these results and assuming a minimum usable sample volume of 500 μL and 64 scans, the minimum detectable amounts (S/N = 3) under the optimized experimental conditions were approximated as 1.67 and 1.72 μg/mL for cis(Z)- and trans(E)-flupentixol, respectively. It was also evident from this set of samples that the chemical shift values were independent of concentrations. Standard deviations for the three replicate measurements of each calibration were equal to ±0.003 ppm or less. In addition, the assay of the two isomers of flupentixol was accurate and precise as summarized in Table 2. The precision of the proposed method, as determined by the relative standard deviations of the within-day ($n = 3$) and between-day ($n = 5$) variations of samples of 8.6, 14.2, and 23.5 μg/mL for cis(Z)-flupentixol and 5.2 and 18.1 μg/mL for trans(E)-flupentixol were consistently within 1–7%. The accuracy, indicated as the concentration of drugs measured in the above-mentioned samples relative to the corresponding known concentrations, was found to be in the range 98–116%.

It should be noted that, in the presence of serum from various individuals, the ^{19}F NMR spectra showed some changes in chemical shifts and relaxations, due to some solvent effect and weak binding of the two isomers to proteins. However, in the presence of serum proteins, the trend of the ^{19}F NMR signals for both isomers was similar to that observed in the case of deproteinated serum samples and, consequently, showed no measurable difference in the amount of drugs present. It is our program in the next part of this research to determine the ability of the method for in vivo determinations using animal sera and extend it to human serum samples for patients.

It is noteworthy that the main metabolic reactions of flupentixol are sulfoxidation, side-chain N-dealkylation, and glucuronic acid conjugation. The main flupentixol metabolites are N-desalkylflupenthixol and flupenthixol sulfoxide, which can be found in plasma. However, the flupentixol metabolites (if present) will not interfere in ^{19}F NMR determinations of the drug. This is due to differences in the structures of the metabolites and the target drug so that their NMR signals will show the chemical shifts that are quite different from those of the flupentixol isomers.

The S/N ratio for a peak in the NMR is known to increase with scan number as the (S/N)$^{1/2}$. Therefore, the minimum quantifiable limit of analyte may be attained with ^{19}F NMR by increasing the scan numbers. This limit of quantitation (LOQ) would be obtained without any preconcentration step. If the sample is preconcentrated about 100 times via solid-phase extraction [27], the LOQ of this method is near that of to HPLC, although the analysis time of this method is three times lower than that of the HPLC method. The LOQ for the two analytes examined (at an S/N = 10) are summarized in Table 3. As is obvious,

Table 1
Linearities and detection limits for analysis of flupentixol isomers

Analyte	Linear range (μg/mL)	Regression equation[a]	Correlation coefficient ($n = 10$)	Estimated error	LOD[b] (μg/mL)
Cis(Z)-flupentixol	4.0–50.0	$I = 899.1C + 0.0006$	0.9969	0.0035	1.67
Trans(E)-flupentixol	2.4–25.0	$I = 837.9C + 0.0007$	0.9992	0.0012	1.72

[a] I, relative integral (arbitrary unit); C, concentration of analytes (μg/mL).
[b] Limit of detection (S/N = 3).

Table 2
Accuracy and precision of the assay of two isomers of flupentixol in serum samples

Concentration added (μg/mL)	Measured concentration (μg/mL)[a]		Accuracy (%)		Precision (RSD, %)	
	Within-day ($n = 3$)	Between-day ($n = 5$)	Within-day ($n = 3$)	Between-day ($n = 5$)	Within-day ($n = 3$)	Between-day ($n = 5$)
Cis(Z)-flupentixol						
8.6	8.6 ± 0.1	9.8 ± 0.3	100.6	113.6	1.2	2.8
14.2	13.5 ± 0.2	—	95.0	—	1.6	—
23.5	24.3 ± 0.5	27.7 ± 0.8	103.6	117.8	2.1	2.8
Trans(E)-flupentixol						
5.2	5.1 ± 0.1	6.05 ± 0.4	98.8	116.4	1.8	6.6
13.5	13.7 ± 0.5	—	101.6	—	3.4	—
18.1	18.2 ± 1.1	19.4 ± 1.5	100.6	107.5	6.0	7.8

[a] Mean ± SD.

Table 3
Limit of quantitation (LOQ) of *cis*(*Z*)-flupentixol as a function of scan number

Scan number	Scan time (min)	LOQ (µg/mL)
16	1.5	14.6
64	7	7.2
128	12	3.4
256	26	1.8
512	60	0.28

the limitation for reaching lower LOQs is the length of scan time.

To investigate the influence of temperature on the ^{19}F NMR assay of *cis*(*Z*)- and *trans*(*E*)-flupentixol, the NMR spectra of a mixture of the two isomers were recorded at the probe temperatures of 5 to 65 °C (10 °C interval). Since the probe temperature is controlled within ±0.05 °C during NMR measurements, the stray fluctuation in ambient temperature is not expected to affect the chemical shift values and precision of integration significantly. It was found that the signal separation of the two isomers was decreased from 39.2 to 31.7 Hz, by increasing probe temperature at the ranges indicated above.

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