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Quality assessment of fluoxetine and fluvoxamine pharmaceutical formulations purchased in different countries or via the Internet by ^{19}F and 2D DOSY ^{1}H NMR

Saleh Trefi, Véronique Gilard, Stéphane Balayssac, Myriam Malet-Martino*, Robert Martino

Groupe de RMN Biomédicale, Laboratoire SPCMIB (UMR CNRS 5068), Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse cedex 9, France

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Abstract

A simple and selective ^{19}F NMR method has been validated for the quantitation of fluoxetine (FLX) and fluvoxamine (FLV) in methanol solutions and in human plasma and urine. The regression equations for FLX and FLV showed a good linearity in the range of 1.4–620 μg mL^{-1} (3.3×10^{-6}–1.8×10^{-3} mol L^{-1}) with a limit of detection of ≈0.5 μg mL^{-1} (1.3×10^{-6} mol L^{-1}) and a limit of quantification of ≈2 μg mL^{-1} (4.6×10^{-6} mol L^{-1}). The precision of the assay depends on the concentrations and is comprised between 1.5 and 9.5% for a range of concentrations between 1.2×10^{-3} and 3.2×10^{-6} mol L^{-1}. The accuracy evaluated through recovery studies ranged from ≈96 to 103% in methanol solutions and in urine, and from ≈93 to 104% in plasma, with standard deviations <7.5%. The low sensitivity of the method precludes its use for the assay of these antidepressants in biofluids at least at therapeutic doses as the ranges of FLX and FLV plasma levels are 0.15–0.5 μg mL^{-1} and 0.15–0.25 μg mL^{-1}, respectively. The method was applied successfully to the determination of FLX and FLV contents in pharmaceutical samples (brand-named and generic) purchased in several countries or via the Internet. All the commercial formulations contain the active ingredient in the range 94–103% of stated concentration. A "signature" of the formulations (solid and liquid) was obtained with 2D Diffusion-Ordered SpectroscopY (DOSY) ^{1}H NMR which allowed the characterisation of the active ingredient and excipients present in the formulations studied. Finally, the DOSY separation of FLX and FLV whose molecular weights are very close was obtained by using β-cyclodextrin as host–guest complexing agent.

Keywords: ^{19}F NMR; 2D DOSY ^{1}H NMR; Fluoxetine; Fluvoxamine; β-Cyclodextrin

1. Introduction

The selective serotonin reuptake inhibitors (SSRIs) are antidepressant drugs that enhance serotoninergic neurotransmission through the selective inhibition of neuronal reuptake of serotonin. Fluoxetine (FLX) and fluvoxamine (FLV) (Fig. 1) are belonging to this class of drugs. FLX commercially known as Prozac® is one of the most widely used SSRIs in therapy and is often the drug of choice in the treatment of severe depressive disorder.

Various analytical methods for SSRIs determination in pharmaceutical or biological samples have been developed in recent years. Reported methods for the determination of FLX and FLV in pharmaceutical formulations or biofluids are mainly based on high-performance liquid chromatography (HPLC) [1–8]. To increase sensitivity and specificity, methods have been developed using gas chromatography (GC) with various types of detection, the most recent being mass spectrometry (MS) [9–11]. Capillary GC [12], capillary electrophoresis [13,14] and spectrophotometric methods [15] were also proposed.

Under appropriate recording and processing conditions [checking that excitation is uniform over the whole frequency range when a large spectral width is observed, avoidance of perturbation of the relative intensities of the resonance peaks due to their different longitudinal relaxation time (T_1) values and that of differential nuclear Overhauser enhancements when proton decoupling is applied, adequate digitization of the signals, optimization of data processing such as zero-filling, exponential multiplication to improve signal-to-noise (S/N) ratio, careful correction of phase and baseline distortions

* Corresponding author. Tel.: +33 5 61 55 68 90; fax: +33 5 61 55 76 25.
E-mail address: martino@chimie.ups-tlse.fr (M. Malet-Martino).

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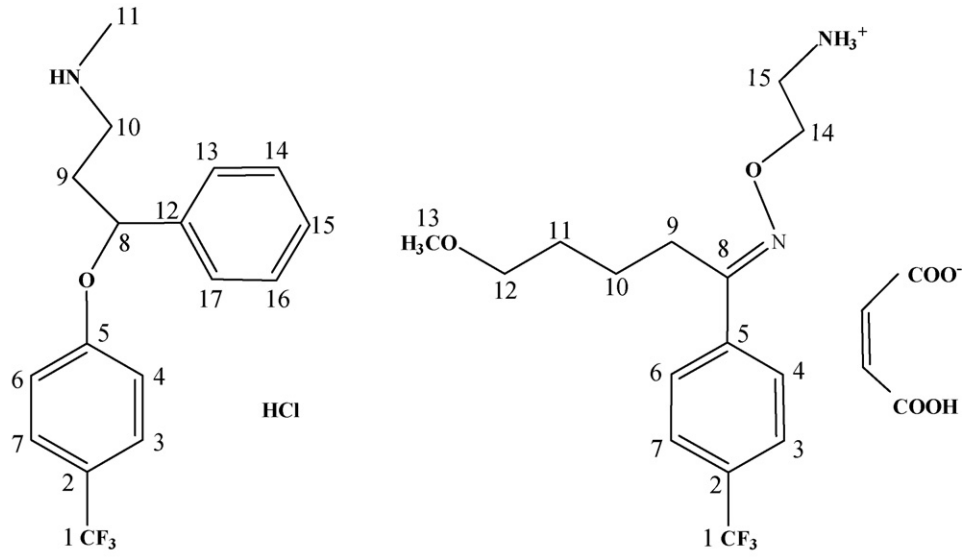

Fluoxetine hydrochloride

Fluvoxamine maleate
(E-isomer)

Fig. 1. Structures of fluoxetine and fluvoxamine.

of the spectrum, integration], the area of each NMR peak is directly proportional to the number of corresponding nuclei and thus NMR is a quantitative spectroscopic tool [16–18]. Surprisingly, even if both FLX and FLV are fluorinated drugs (Fig. 1), ^{19}F NMR has never been described for assaying these compounds in pharmaceutical preparations or biofluids. Nevertheless, a number of previously published results reveal that ^{19}F NMR is a powerful selective method for the quantitative analysis of various fluorinated drugs [17,19–25]. Indeed, the ^{19}F nucleus has favorable NMR characteristics: nuclear spin of 1/2, relatively narrow lines, 100% natural abundance, high sensitivity (83% that of proton), large chemical shift range (about 500 ppm), which minimizes signal overlap. Provided that the ^{19}F NMR spectrum is acquired under conditions of full T_1 relaxation, it is possible to quantify the absolute amounts of the components of the mixture by measuring integrals in the spectrum.

2D Diffusion-Ordered SpectroscopY (DOSY) ^{1}H NMR is a powerful technique based on measurement of diffusion coefficients often described as "in tube chromatography" as it leads to a virtual separation of species. It allows the fingerprinting of pharmaceutical formulations and can be used to determine the similarities or differences between samples. The variety of manufacturing procedures and the wide selection of excipients in the manufacture of various pharmaceutical products, even with the same active ingredient, means that 2D DOSY ^{1}H NMR spectra can differ for similar formulations manufactured by various producers.

The aim of this report is thus to provide a simple and selective ^{19}F NMR method to determine FLX and FLV contents in pharmaceutical formulations and biofluids. A validation procedure was thus carried out. Moreover, driven by healthcare insurance systems, generic drugs are substituted for more expensive brand-name drugs. Also, the Internet has revolutionized the way in which consumers purchase medications as many consumers believe that online pharmacies are more convenient than traditional pharmacies and offer cost savings. We thus used (i) ^{19}F NMR to compare the amounts of FLX and FLV in the brand-named prescription drugs Prozac® and Floxyfral® with those measured in several generic equivalents bought in several countries or via the Internet and (ii) 2D DOSY ^{1}H NMR to get qualitative information on the different pharmaceutical formulations, especially on their excipient composition. To the best of our knowledge, this study is the first report dealing with the analysis of FLX and FLV pharmaceutical formulations with ^{19}F NMR and 2D DOSY ^{1}H NMR.

2. Experimental

2.1. Materials

2.1.1. Chemicals

Pure FLX hydrochloride was purchased from European Pharmacopoeia (Strasbourg, France). FLV maleate (E-isomer), chromium(III) acetylacetonate ($Cr(acac)_3$) and β-cyclodextrin (β-CD) were obtained from Sigma–Aldrich (Sigma–Aldrich, Saint-Quentin Fallavier, France). Methanol and acetonitrile used for sample preparation were of analytical grade.

2.1.2. Commercial formulations of FLX and FLV (Table 1)

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2.1.2.1. Solid formulations. Ten FLX and four FLV commercial formulations were analysed. Two of them were the brand formulations from LILLY (Prozac®) and SOLVAY Pharma (Floxyfral®), the others were generic drugs from different countries. The declared amounts of the active ingredient in the various

Table 1
Commercial formulations of fluoxetin (1–15) and fluvoxamine (16–19) analysed in this study and amounts of active ingredient measured

	Formulation name	Pharmaceutical form and dosage	Batch number	Expiry date	Manufacturer name	Country of manufacturing	% of nominal concentration	*n*	S.D.
1	Prozac	Capsules 20 mg	5002A	03/2008	Lilly	France	99.3	4	2.6
2	Fluocim	Capsules 20 mg	0506B006	03/2008	Siegfried	Switzerland	100.3	3	0.3
3	Prouziac	Capsules 20 mg	019	09/2008	Massoud-Bahri & Co	Syria	94.2	4	0.7
4	Fluzac	Tablets 20 mg	38	01/2008	Balsam Pharma	Syria	103.3	4	7.5
5	Fluoxin	Capsules 20 mg	04002	12/2006	S.C. VIM Spectrum	Romania	99.0	3	1.1
6	pms-Fluoxetine	Capsules 20 mg	411657	01/2007	Pharmascience Inc.	Canada	102.8	3	3.5
7	Fluoxetina	Capsules 20 mg	X002	08/2008	Belmac	Spain	98.9	3	0.7
8	Proxetin	Capsules 20 mg	3667	05/2008	Mediphar Laboratories	Lebanon	94.6	4	2.8
9	Fludac	Capsules 20 mg	5017	03/2008	Cadila Pharmaceuticals	India	99.7	3	4.0
10	Salidep	Capsules 40 mg	007	06/2006	Mano Pharma	India	98.8	3	3.2
11	Prozac	Oral solution 20 mg/5 mL	6314B	09/2008	Lilly	France	98.3	4	1.0
12	Fluoxetine Arrow	Oral solution 20 mg/5 mL	2007	06/2008	Laboratoires Aerocid	France	99.0	4	0.9
13	Fluoxetine Biogaran	Oral solution 20 mg/5 mL	FB0206	09/2009	Laboratoires Aerocid	France	99.5	4	0.8
14	Fluoxetine Ratiopharm	Oral solution 20 mg/5 mL	G22808	07/2009	Merckle GmbH	Germany	98.3	4	1.1
15	Fluoxetine Teva	Oral solution 20 mg/5 mL	896005	08/2009	TEVA Santé	France	98.5	4	1.2
16	Floxyfral	Tablets 50 mg	0113	04/2009	Solvay Pharma	France	97.3	4	0.9
17	Fluvoxamine Merck	Tablets 50 mg	1002A	10/2007	Merck	Netherlands	95.1	4	0.6
18	Fluvoxamine Teva	Tablets 50 mg	C749	03/2008	TEVA	France	97.0	4	4.6
19	Fluvoxamine EG	Tablets 50 mg	06A02.26	11/2008	Eurogenerics	Netherlands	98.5	3	0.6

formulations were 20 mg (numbers 1–9) or 40 mg (number 10) for FLX and 50 mg for FLV.

2.1.2.2. Oral solutions. Five FLX commercial formulations were analysed, the brand formulation from LILLY (Prozac®) and four generic solutions. The declared amount of FLX was 20 mg in 5 mL of solution.

All samples, as received, were stored in the dark at ambient temperature and humidity. They were all analysed within expiry dates.

2.2. Preparation of the samples

2.2.1. Preparation of solutions for the validation of the method

Although a combination of FLX and FLV is not present in the same formulation but as both drugs can be found in biofluids from patients, the ^{19}F NMR analytical method was validated with a mixture of FLX and FLV. This is possible as both compounds have different ^{19}F chemical shifts (δ), 13.3 and 12.2 ppm for FLX and FLV, respectively (Fig. 2).

For preparing stock solutions, about 62 mg of FLX hydrochloride and 42 mg of FLV maleate exactly weighed were placed in a 100 mL calibrated flask. Methanol was then added and the mixture was shaken on a magnetic stirrer for 10 min. From these stock solutions, measured aliquots were taken and diluted with methanol to give different final concentrations of drugs in the ranges 0.0017–0.6228 mg mL^{-1} (4.95×10^{-6}–1.8×10^{-3} mol L^{-1}) for FLX and 0.0014–0.4235 mg mL^{-1} (3.3×10^{-6}–9.75×10^{-4} mol L^{-1}) for FLV.

2.2.2. Preparation of biofluid samples for the validation of the method

2.2.2.1. Plasma sample preparation. After addition of 5 mL of acetonitrile in 5 mL of human plasma for deproteinisation, the sample was vortexed for 1.5 min. After 5 min of centrifugation (3000 rpm), the supernatant was collected. Aliquots ($\leq$0.1 mL) of a stock aqueous solution containing FLX and FLV were then added. Final concentrations were respectively for FLX and FLV 0.090 and 0.032 mg mL^{-1} or 1.058×10^{-3} and 1.333×10^{-3} mg mL^{-1}. 2.5 mg of Cr(acac)$_3$ were added in all the samples (2.5 mL) before the ^{19}F NMR analysis.

2.2.2.2. Urine sample preparation. Solutions of FLX and FLV were prepared in human urine. Final concentrations were respectively for FLX and FLV 0.830 and 0.868 mg mL^{-1}, 0.103 and 0.138 mg mL^{-1} or 1.028×10^{-3} and 1.378×10^{-3} mg mL^{-1}. 2.5 mg of Cr(acac)$_3$ were added in all the samples (2.5 mL) before the ^{19}F NMR analysis.

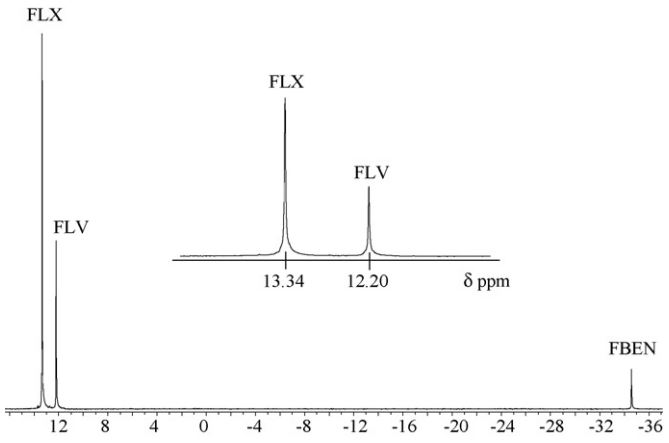

Fig. 2. ^{19}F NMR spectrum recorded at 282.4 MHz of a mixture of fluoxetine (FLX; 0.416 mg mL^{-1}) and fluvoxamine (FLV; 0.268 mg mL^{-1}) in methanol. The chemical shifts were reported relative to the resonance peak of CF_3COOH (5%, w/v aqueous solution) used as external chemical shift reference (δ = 0 ppm). FBEN is the reference for quantification.

Table 2
Validation parameters for the determination of fluoxetine and fluvoxamine by ^{19}F NMR

	Fluoxetine hydrochloride		Fluvoxamine maleate	
Linearity				
Intercept	$2.5 \times 10^{-3} \pm 2.7 \times 10^{-4}$		$6.6 \times 10^{-4} \pm 7.1 \times 10^{-4}$	
Slope	0.984 ± 0.013		0.991 ± 0.027	
R^2	0.9981 ± 0.0011		0.9983 ± 0.0013	
Range	0.0017–0.6228 mg mL^{-1}		0.0014–0.4235 mg mL^{-1}	
	4.95×10^{-6}–1.8×10^{-3} mol L^{-1} (9 concentrations)		3.3×10^{-6}–9.75×10^{-4} mol L^{-1} (8 concentrations)	
Precision	mol L^{-1}	mg mL^{-1}	mol L^{-1}	mg mL^{-1}
Theoretical concentration	1.20×10^{-3}	0.415	6.20×10^{-4}	0.269
Measured value (mean ± S.D.) ($n = 7$)	$1.17 \times 10^{-3} \pm 0.02 \times 10^{-3}$	0.406 ± 0.007	$6.08 \times 10^{-4} \pm 0.08 \times 10^{-4}$	0.264 ± 0.003
Precision (R.S.D.%)	1.6		1.3	
Theoretical concentration	9.89×10^{-5}	3.42×10^{-2}	6.47×10^{-5}	2.81×10^{-2}
Measured value (mean ± S.D.) ($n = 3$)	$9.87 \times 10^{-5} \pm 0.32 \times 10^{-5}$	$3.41 \times 10^{-2} \pm 0.11 \times 10^{-2}$	$6.28 \times 10^{-5} \pm 0.26 \times 10^{-5}$	$2.73 \times 10^{-2} \pm 0.14 \times 10^{-2}$
Precision (R.S.D.%)	3.2		4.2	
Theoretical concentration	9.89×10^{-6}	3.42×10^{-3}	6.47×10^{-6}	2.81×10^{-3}
Measured value (mean ± S.D.) ($n = 3$)	$10.08 \times 10^{-6} \pm 0.61 \times 10^{-6}$	$3.49 \times 10^{-3} \pm 0.21 \times 10^{-3}$	$6.05 \times 10^{-6} \pm 0.36 \times 10^{-6}$	$2.60 \times 10^{-3} \pm 0.16 \times 10^{-3}$
Precision (R.S.D.%)	6.1		6.0	
Theoretical concentration	4.95×10^{-6}	1.71×10^{-3}	3.23×10^{-6}	1.41×10^{-3}
Measured value (mean ± S.D.) ($n = 3$)	$5.12 \times 10^{-6} \pm 0.48 \times 10^{-6}$	$1.77 \times 10^{-3} \pm 0.17 \times 10^{-3}$	$3.31 \times 10^{-6} \pm 0.26 \times 10^{-6}$	$1.44 \times 10^{-3} \pm 0.11 \times 10^{-3}$
Precision (R.S.D.%)	9.4		7.8	
Accuracy				
Number of solutions analysed	13		11	
Range of concentrations (mol L^{-1})	5.10^{-6}–2.10^{-3}		3.10^{-6}–10^{-3}	
Mean recovery (%) ± S.D.	100.1 ± 3.4		98.8 ± 3.3	
LOD (24,000 scans)	1.3×10^{-6}	0.45×10^{-3}	1.3×10^{-6}	0.56×10^{-3}
LOQ (15,000 scans)	4.6×10^{-6}	0.16×10^{-2}	4.6×10^{-6}	0.20×10^{-2}

2.2.3. Preparation of formulation samples

A 50 mg FLV (or 20 mg FLX) tablet was powdered and transferred to a 200 mL (or 100 mL for FLX) volumetric flask. One FLX capsule (equivalent to 20 or 40 mg FLX) was emptied, and the content transferred to a 50 mL volumetric flask. The volumes were adjusted with methanol and the suspensions stirred for 30 min. After 15 min of settling, the supernatants were taken. For the determination of fluorinated impurities, an analogous procedure was used except that the final volumes were 3.5 mL for FLX and 5 mL for FLV, in order to enhance impurity concentrations in the solution analysed.

The oral solutions of FLX were 5-fold diluted in D_2O.

2.5 mg of $Cr(acac)_3$ were added in all the samples (2.5 mL) before the ^{19}F NMR analysis.

2.2.4. Preparation of samples for DOSY analysis

2.2.4.1. Analysis of formulations. For FLX solid formulations, the content of one capsule was stirred with 5 mL of a mixture of CD_3CN/D_2O (80/20) for 30 min. The suspension was then sonicated for 10 min and centrifuged (10 min, 3000 rpm). The supernatant was analysed. The same procedure was employed for FLV formulations except that the tablets were powdered before addition of the solvent mixture. For FLX oral solutions, a 1:1 dilution with D_2O was prepared before NMR analysis.

2.2.4.2. Complexation with β-cyclodextrin. Four solutions were prepared from three stock solutions of FLX, FLV and β-CD in D_2O. Solution A contained only FLX (0.25×10^{-3} mol L^{-1}) and FLV (0.25×10^{-3} mol L^{-1}); solution B contained FLX (0.25×10^{-3} mol L^{-1}) and β-CD (2.5×10^{-3} mol L^{-1}); solution C contained FLV (0.25×10^{-3} mol L^{-1}) and β-CD (2.5×10^{-3} mol L^{-1}). The fourth solution (solution D) was a mixture of FLX (0.25×10^{-3} mol L^{-1}), FLV (0.25×10^{-3} mol L^{-1}) and β-CD (2.5×10^{-3} mol L^{-1}).

2.3. Validation parameters

The linearity of the ^{19}F NMR calibration curves was determined for both FLX and FLV signals. The slope and other statistical parameters of the calibration curves were calculated with least-squares linear regression analysis.

The precision of the method is expressed in terms of standard deviation (S.D.) and relative standard deviation (R.S.D.) [26]. The ^{19}F NMR spectra of mixtures of FLX and FLV at various concentrations were recorded several times and S.D. and R.S.D. were determined.

The accuracy is the closeness of the measured value to the true one for the sample and is reported as percent recovery by the assay of known added amounts of both analytes in the sample [26]. To test the accuracy of the method, 13 solutions of FLX in the range 5.10^{-6}–2.10^{-3} mol L^{-1} and 11 solutions of FLV in the range 3.10^{-6}–10^{-3} mol L^{-1} were analysed.

The limit of detection (LOD) with the spectrometer employed after 24 h recording is 1.3×10^{-6} mol L^{-1} at an S/N ratio of 3, the S/N ratio being [2.5(peak height/noise height measured peak-to-peak)]. The limit of quantification (LOQ) after 15 h recording is 4.5×10^{-6} mol L^{-1} at an S/N ratio of 10.

2.4. NMR analysis

2.4.1. ^{19}F NMR analysis

^{19}F NMR spectra were recorded at 282.4 MHz with inverse-gated ^{1}H-decoupling on a Bruker WB-AM 300 spectrometer (Bruker SA, Wissembourg, France) using 10-mm diameter NMR tubes. The recording conditions were: probe temperature, 25 °C; sweep width 29,411 Hz; 32,768 data points zero-filled to 65,536; pulse width, 7 μs (flip angle ≈40°); pulse interval, 3.6 s; number of scans, 3000 to 24,000 for linearity, precision and accuracy studies and quantitation of FLX and FLV in spiked biofluids, depending on the concentration analysed, 5000 and 12,000 for the quantitation of FLX or FLV and the analysis of impurities, respectively, in pharmaceutical formulations; line broadening due to exponential multiplication, 1 Hz (5 Hz for the analysis of impurities in order to improve the S/N ratio). The chemical shifts were reported relative to the resonance peak of CF_3COOH (5% w/v aqueous solution) used as external chemical shift reference ($\delta = 0$ ppm).

The concentration of fluorinated compounds were measured by comparing the expanded areas (30 Hz cm^{-1}) of their respective NMR signals with that of the external standard for quantification placed in a coaxial capillary, namely a solution of sodium parafluorobenzoate (FBEN) in D_2O doped at saturation (≈3 mmol L^{-1}) with $Cr(acac)_3$, the paramagnetic agent used to shorten its T_1 relaxation time. The apparent concentration of the FBEN reference (2.32×10^{-4} mol L^{-1}) was previously measured against solutions of FLX and FLV of known concentrations under the recording conditions described above.

Phase and baseline correction over the entire spectral range were performed manually. The baseline was additionally corrected over the integrated regions. The areas were determined by manual integration using Bruker WinNMR software. An integral limit of around ±100 Hz around the signal of interest was applied. Each data is the mean of at least five integrations.

In order to check that the NMR conditions used allowed an accurate quantitation of FLX and FLV, their ^{19}F T_1 values were determined by the inversion-recovery pulse sequence method applied to (i) solutions of standard FLX (1.2×10^{-3} mol L^{-1}) and FLV (6.2×10^{-4} mol L^{-1}) in methanol containing 2.5 mg of $Cr(acac)_3$ and (ii) solutions obtained from two pharmaceutical formulations (number 3 for FLX and number 17 for FLV) prepared in methanol as described above and also containing $C(acac)_3$. The T_1 values were found to be 0.52 and 0.51 s for standard FLX and FLX in the pharmaceutical formulation 3, respectively, and 0.48 and 0.54 s for standard FLV and FLV in the pharmaceutical formulation 17, respectively. As the T_1 of FLX and FLV were not measured in plasma or urine, a ^{19}F NMR experiment with a longer interval between pulses (10.6 s) was carried out for FLX and FLV in each medium. The same concentration values were obtained, thus demonstrating that a pulse interval of 3.6 s for a flip angle of 40°

Table 3
Mean values ± S.D. ($n = 3$ unless otherwise specified) of the recoveries of fluoxetine and fluvoxamine in spiked human urine and plasma

	Drug added		Drug recoveries (%) ± S.D.	
	Fluoxetine HCl	Fluvoxamine maleate	Fluoxetine HCl	Fluvoxamine maleate
Urine	0.830 mg mL^{-1} (2.4×10^{-3} mol L^{-1})	0.868 mg mL^{-1} (2.0×10^{-3} mol L^{-1})	99.6 ± 2.5 ($n = 6$)	99.9 ± 2.6 ($n = 6$)
	0.103 mg mL^{-1} (3.0×10^{-4} mol L^{-1})	0.138 mg mL^{-1} (3.2×10^{-4} mol L^{-1})	97.2 ± 2.9	98.6 ± 3.5
	1.028×10^{-3} mg mL^{-1} (3.0×10^{-6} mol L^{-1})	1.378×10^{-3} mg mL^{-1} (3.2×10^{-6} mol L^{-1})	96.6 ± 7.1	98.0 ± 7.5
Plasma	0.090 mg mL^{-1} (2.6×10^{-4} mol L^{-1})	0.032 mg mL^{-1} (7.4×10^{-5} mol L^{-1})	104.2 ± 3.5	102.5 ± 2.9
	1.058×10^{-3} mg mL^{-1} (3.1×10^{-6} mol L^{-1})	1.333×10^{-3} mg mL^{-1} (3.1×10^{-6} mol L^{-1})	96.8 ± 5.7	93.3 ± 7.5

is sufficient to record the spectra under conditions of full T_1 relaxation.

2.4.2. ^1H and 2D DOSY ^1H NMR

^{1}H NMR experiments were performed on a Bruker AVANCE 500 spectrometer operating at 500.13 MHz equipped with a 5 mm proton cryoprobe at 298 K on 600 μL samples.

All chemical shifts in the ^{1}H NMR spectra were referred to an internal trimethylsilylpropane sulfonic acid (TMPS) reference. Typical acquisition parameters were as follows: probe temperature, 25 °C; sweep width 10,000 Hz; 32,768 data points zero-filled to 65,536; pulse width, 8 μs (flip angle 90°); pulse interval, 4.6 s; number of scans, 128. Spectra were acquired with a classical water suppression sequence using selective irradiation for eliminating residual water signal from HOD. The 2D NMR experiments (gCOSY, gHSQC, gHMBC) were acquired using standard Bruker sequences.

For 2D DOSY ^{1}H NMR, a stimulated echo bipolar gradient pulse sequence including spoiler gradient of −7.92 G cm^{-1} was used with a pulse field gradient length of 1 ms, a gradient recovery delay of 3 ms, and a diffusion time of 100 ms. A 3-9-19 pulse sequence with gradients of 9.25 G cm^{-1} was added for signal water suppression when recording the DOSY NMR spectra of FLX oral solutions. Sequence parameters were adapted in order to have the intensity of typical NMR signals of FLX ($H_{4,6}$) or FLV ($H_{3,4,6,7}$) strongly decreased (at least divided by 50) at 95% of the full gradient strength. Forty experiments were recorded with gradient intensity linearly sampled from 5 to 95%. The gradient system had been calibrated to 46.25 G cm^{-1} at maximum intensity. Spectra were recorded at 298 K, except for FLX oral solutions. In this case, temperature was 305 K in order to lower the viscosity of the formulation.

All data were processed using Gifa 5.2 software with the inverse Laplace Transform method using the Maximum Entropy algorithm (MaxEnt). The processing parameters were 2048 points along the Laplace spectrum diffusion axis and 20,000 MaxEnt iterations. The inverse Laplace Transform was computed only on the columns presenting a signal 32 times greater than the noise level of the experiment. DOSY spectra are presented with chemical shift on the horizontal axis and self-diffusion coefficients expressed in μm^2 s^{-1} on the vertical axis.

3. Results

3.1. Validation of the ^{19}F NMR analytical method

Fig. 2 depicts a typical ^{19}F NMR spectrum of a mixture of FLX (0.416 mg mL^{-1}) and FLV (0.268 mg mL^{-1}) in methanol that shows two signals at 13.34 ppm and 12.20 ppm attributed to FLX and FLV, respectively. Even if these chemical shifts are rather close, the resolution between the two signals is sufficient to ensure an accurate quantitation of each compound. Indeed, the half-height width ($\Delta\nu_{1/2}$) of each signal in our experimental conditions does not exceed 2.1 Hz whereas the frequency difference ($\Delta\nu$) between the two signals is about 320 Hz.

The ^{19}F NMR analytical method for determination of FLX and FLV was validated for linearity, precision, accuracy, LOD and LOQ. The results reported in Table 2 show that the ^{19}F NMR method is appropriate for the quantification of FLX and FLV.

3.2. Application to human biofluids spiked with FLX and FLV

Drug recoveries of both FLX and FLV in spiked human biofluids (urine and plasma) are reported in Table 3. A good recovery is obtained for both drugs even if the standard deviation is increased for the lowest concentrations (≈1 μg mL^{-1}).

3.3. Application to the quantitation of FLX and FLV in pharmaceutical formulations

FLX and FLV contents in pharmaceutical formulations are reported in Table 1. All samples contain the active ingredient between 94 and 103% of stated concentration. Fluorinated impurities were searched in five formulations, three containing FLX (formulations 1, 3 and 9) and the other two containing FLV (formulations 16 and 17). No fluorinated impurity could be detected in formulations 1 and 9 whereas 0.02% of an unknown fluorinated compound resonating at 12.2 ppm was observed in

Table 4
^{1}H NMR characteristics of fluoxetine hydrochloride and fluvoxamine maleate

	Chemical shift (δ ppm)	Multiplicity (J Hz)
Fluoxetine		
$H_{3,7}$ and $H_{4,6}$	7.41 and 6.92	AA'XX' (8.7)
H_{15}	7.21	m
$H_{13,14,16,17}$	7.25–7.33	m
H_8	5.45	dd (4.3, 8.4)
H_{10}	3.09 and 3.16	m and m
CH_3-N	2.59	s
H_9	2.19 and 2.27	m and m
Fluvoxamine		
$H_{3,4,6,7}$	7.65	A_2B_2 (9.6)
H_{14}	4.29	t (5.1)
H_{15}	3.25	t (5.1)
CH_3O	3.15	s
H_{12}	3.30	t (6.2)
H_9	2.77	t (7.3)
$H_{10,11}$	1.43	m
maleate	6.15	s

formulation 3. A signal at 36.1 ppm was found in both FLV formulations analysed accounting for 0.03% in formulation 16 and 0.005% in formulation 17. Fluoride ion (F^-) was not observed in FLX and FLV formulations.

3.4. 2D ^{1}H Diffusion-Ordered SpectroscopY (DOSY NMR)

3.4.1. ^{1}H NMR spectra of FLX and FLV

The ^{1}H NMR resonances of FLX and FLV were assigned by 2D ^{1}H NMR experiments (gCOSY, gHSQC, gHMBC) and comparison to other studies [27,28] (Table 4 and Fig. 1 for the numeration of protons).

3.4.2. 2D DOSY ^{1}H NMR of solutions of solid commercial formulations

A major advantage of DOSY NMR is that this technique provides global information on the composition of a formulation. Four solid formulations (brand and generic) of FLX and FLV were analysed with 2D DOSY ^{1}H NMR. The DOSY spectra with their corresponding 1D spectra are presented in Fig. 3. The peaks at 3.66 and 1.99 ppm correspond to the residual signals of water and acetonitrile, respectively, and have high diffusion coefficients. All the peaks of a same ingredient are lined up. The value of the self-diffusion coefficient was measured for each peak, and an average self-diffusion coefficient was determined. Several excipients could be observed depending on the formulation.

Differences can be seen between the brand-named and the generic formulation of FLX (Fig. 3A and B, respectively) whereas no difference was observed for FLV formulations. In spectrum 3A corresponding to the brand-named formulation Prozac®, the characteristic signals of FLX are detected and an average self-diffusion coefficient $D = 1276 \pm 9\ \mu m^2\ s^{-1}$ is measured. The sole excipient partially soluble in CD_3CN/D_2O (80/20) is dimethicone, a polydimethylsiloxane, which presents ^{1}H NMR signals typical of di- and tri-methysilyl moiety at ≈0 ppm [29] and a self-diffusion coefficient of $913\ \mu m^2\ s^{-1}$. The DOSY NMR spectrum of the same batch recorded in D_2O (data not shown) also showed the signals of corn starch (a broad singlet at 5.3 ppm, a narrow singlet at 5.19 ppm, and a broad multiplet between 3.5 and 4 ppm) with a very low diffusion coefficient ($D < 100\ \mu m^2\ s^{-1}$) typical of a macromolecule. From the DOSY spectrum of the Syrian generic capsule of FLX (Prouziac; spectrum 3B), it is obvious that the formulation differs from that of Prozac®. Indeed, the major excipient is lactose (instead of corn starch) that gives characteristic ^{1}H NMR resonances at 5.11 ppm (d, $J = 3.5$ Hz), 4.53 ppm (d, $J = 8.0$ Hz), 4.37 ppm (d, $J = 8.0$ Hz) and 3.45–3.86 (m). The self-diffusion coefficient of lactose was measured at $883 \pm 20\ \mu m^2\ s^{-1}$.

The DOSY NMR spectra of brand and generic formulations of FLV are identical (only the spectrum of the generic is presented in Fig. 3C). Beyond the key active compound FLV maleate, these two formulations contain mannitol as a filler that leads to multiplets located between 3.58 and 3.76 ppm. The tablet binder hypromellose (hydroxypropylmethyl cellulose) shows three aligned signals at 1.11, 3.37 and 3.54 ppm. Macrogol (polyethylene glycol), that enhances the effectiveness of the tablet binder, gives one signal at 3.61 ppm. The tablet lubricant sodium stearyl fumarate gives six signals at 6.81 (d), 6.51 (d), 4.14 (t), 1.65 (quin; not observed in the DOSY spectrum), 1.27 (broad s) and 0.88 ppm (t). The self-diffusion coefficients measured for each component of both formulations are reported in Table 5. Diffusion coefficients were lower in the brand-named formulation than those measured in the generic formulation, probably due to change in viscosity as a result of the variable amounts of some excipients in the pharmaceutical formulations.

3.4.3. 2D DOSY ^{1}H NMR of oral solutions

The five oral solutions of FLX analysed by 2D DOSY 1H NMR present two kinds of spectral pattern. The DOSY spectra of the two formulations Prozac® (Fig. 4A) and Fluoxetine Teva are very similar. In addition to the active pharmaceutical ingredient FLX, the major excipients detected were (i) glycerol (only the dedoubled AB system centered at 3.59 ppm is observed), (ii) saccharose (5.40 ppm, d; 4.20 ppm, d; 4.03 ppm, t; 3.81–3.89 ppm, m; 3.75 ppm, t; 3.66 ppm, s; 3.46 ppm, t), (iii) glucose [only the two signals at 5.22 ppm (d) and 4.63 ppm (d) are observed on the DOSY NMR spectrum; the other signals at 4.09 ppm (only a d of a dd system can be observed), 3.39 ppm (m) and 3.23 ppm (dd) do not give observable peaks on the

Table 5
Self-diffusion coefficients[a] ($\mu m^2\ s^{-1}$) measured in the formulations of fluvoxamine studied with 2D DOSY ^{1}H NMR

	Floxyfral®	Fluvoxamine Merck
Fluvoxamine	1205 ± 12	1501 ± 20
Macrogol	432[b]	639[b]
Stearyl fumarate	965 ± 20	1285 ± 53
Mannitol	1097 ± 8	1434 ± 10
Hypromellose	308 ± 29	491 ± 29
Maleate	1688[b]	1883[b]

[a] The value of the self-diffusion coefficient was measured for each peak, and an average self-diffusion coefficient was determined for each formulation.
[b] Only one peak was observed.

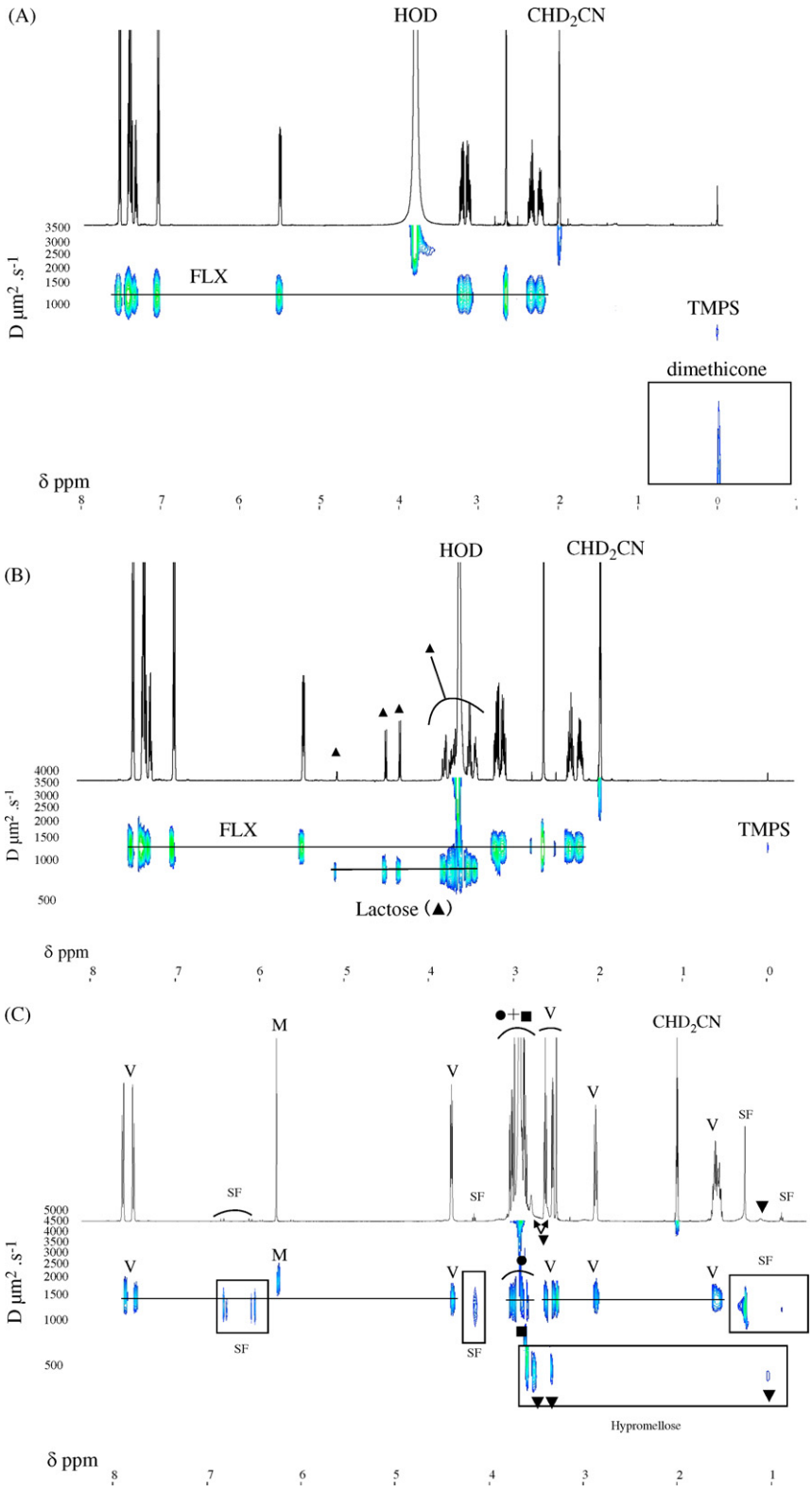

Fig. 3. 2D DOSY ^{1}H NMR spectra recorded at 500 MHz in CD_3CN/D_2O (80/20) of solid formulations of fluoxetine (A, B) and fluvoxamine (C). (A) Prozac from France; (B) Prouziac from Syria (generic); (C) Fluvoxamine Merck from Netherlands (generic). FLX, fluoxetine; TMPS, trimethylsilylpropane sulfonic acid; ▲, lactose; V, fluvoxamine; M, maleate; SF, sodium stearyl fumarate; ▼, hypromellose; ●, mannitol; ■, macrogol. A deeper section of some signals is shown in the boxes.

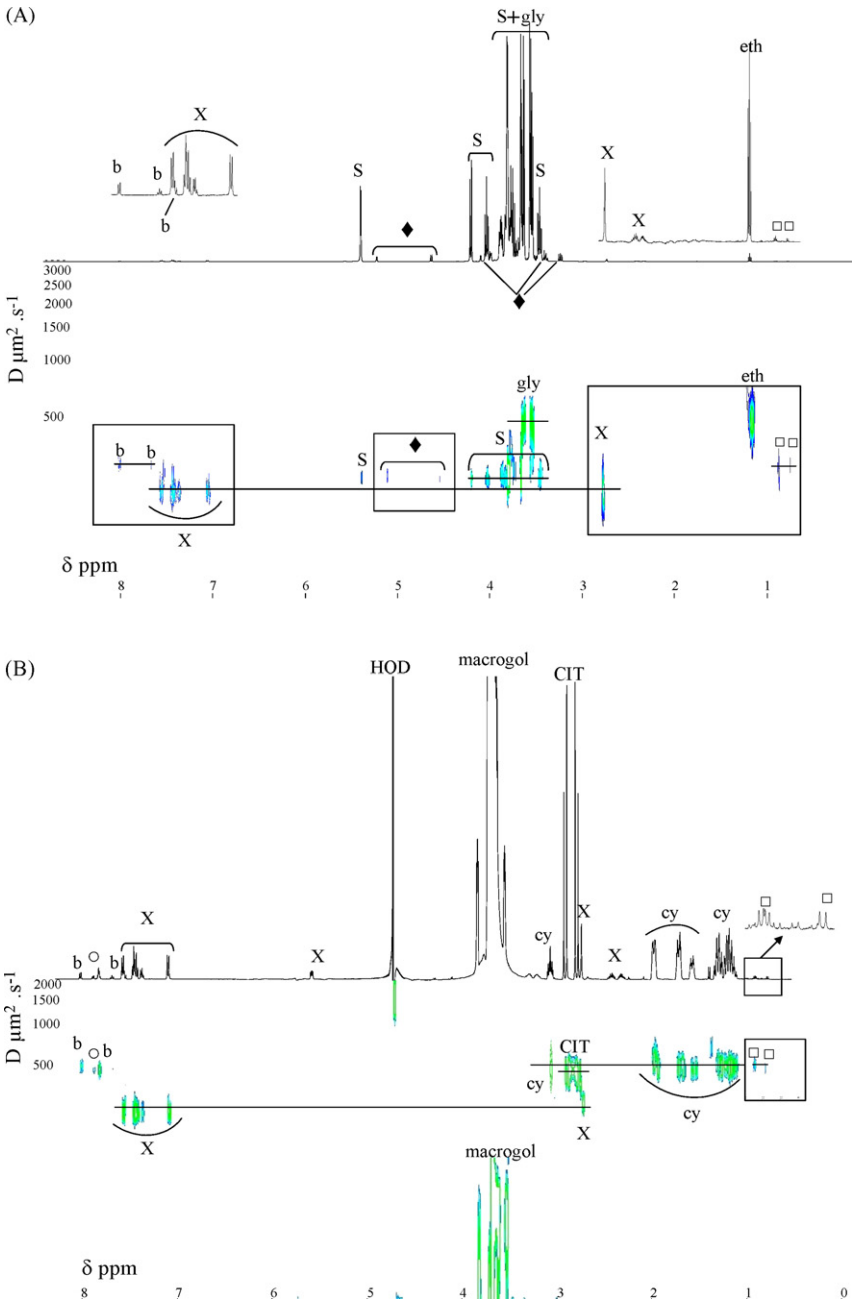

Fig. 4. 2D DOSY ^{1}H NMR spectra recorded at 500 MHz of liquid formulations of fluoxetine. (A) Prozac from France; (B) Fluoxetine Arrow from France (generic). X, fluoxetine; b, benzoic acid; s, saccharose; ♦, glucose; gly, glycerol; eth, ethanol; □, menthol; o, sodium saccharine; cy, sodium cyclamate; CIT, citric acid. A deeper section of some signals is shown in the boxes.

DOSY spectrum], (iv) ethanol from mint aroma (only the t at 1.18 ppm is detected), (v) benzoic acid [8.01 ppm, m; 7.67 ppm, tt; 7.54 ppm, m (not detected in the DOSY spectrum)] and (vi) menthol (only two signals at 0.90 ppm (dd) and 0.77 ppm (d) can be observed in the DOSY NMR spectrum). The other three formulations (from Ratiopharm, Biogaran and Arrow) have the same spectral signature; each contains FLX, macrogol (huge s at 3.69 ppm) to adjust the viscosity, two sweetening agents sodium cyclamate (3.06 ppm, m; 1.96 ppm, dd; 1.70 ppm, td; 1.55 ppm, td; 1.22 ppm, m) and sodium saccharine (7.88 ppm, m; 7.82 ppm, m), benzoic acid, menthol and citric acid (AB system centered at 2.83 ppm) (Fig. 4B). In all these DOSY spectra, the self-diffusion coefficients were quite low (<1000 $\mu m^2\ s^{-1}$) due to the high viscosity of the solutions. This is particularly noticeable for macrogol whose diffusion coefficients are very different between FLV tablets (Fig. 3C) and FLX oral solutions (Fig. 4B).

3.4.4. 2D DOSY ^{1}H NMR of a mixture of FLX and FLV

In DOSY NMR, the spectra of different molecules are separated according to their self-diffusion coefficient D, which generally decreases with increasing molecular weight (MW). The relationship between MW and D, for two molecular species A and B, is $MW_A/MW_B \approx (D_B/D_A)^3$. The difference between

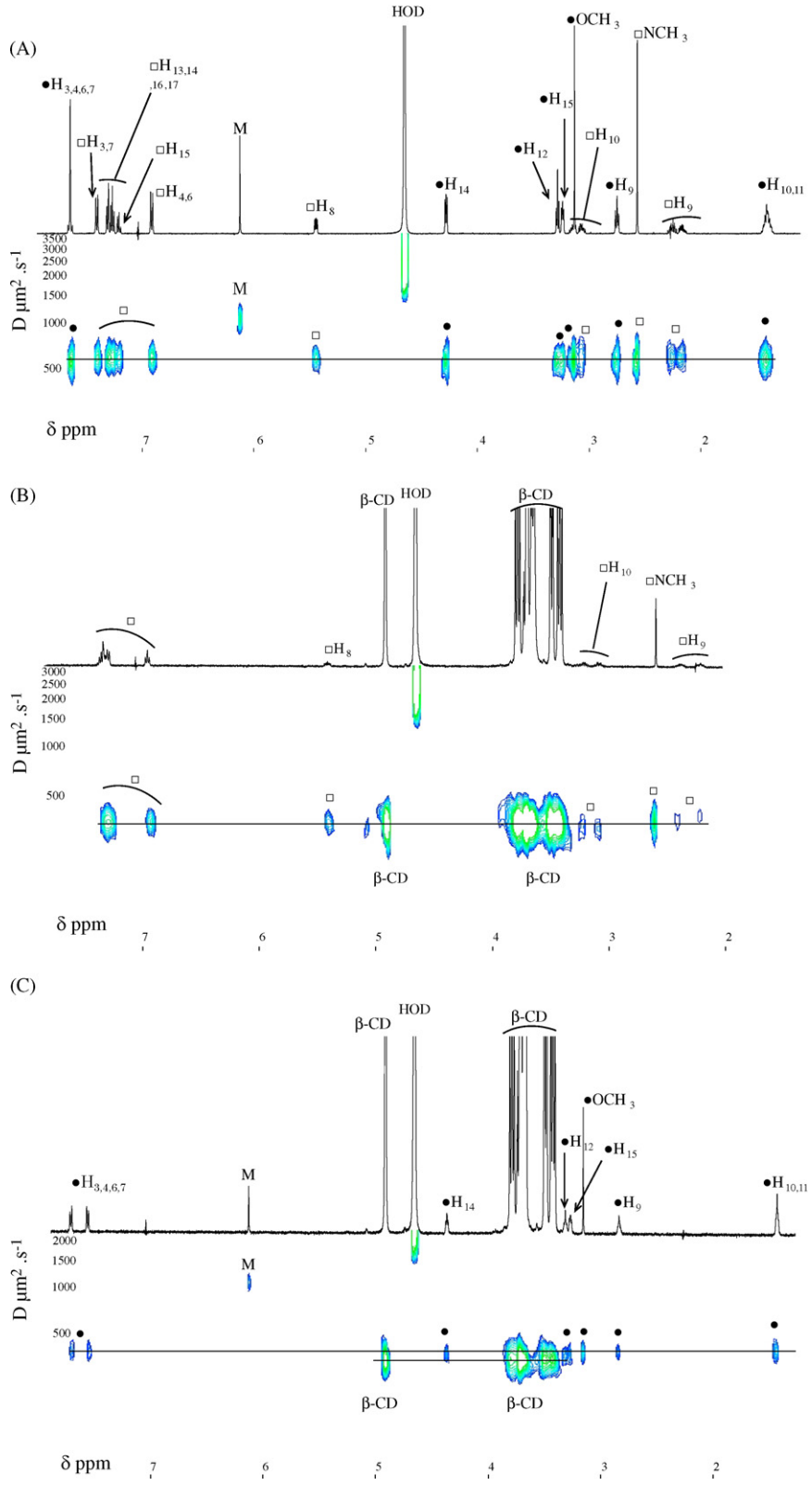

Fig. 5. 2D DOSY ^{1}H NMR spectra recorded at 500 MHz in D_2O of mixtures of fluoxetine (FLX; □) and fluvoxamine (FLV; ●) with β-cyclodextrin (β-CD). Solution A: FLX (0.25×10^{-3} mol L^{-1}) and FLV (0.25×10^{-3} mol L^{-1}). Solution B: FLX (0.25×10^{-3} mol L^{-1}) and β-CD (2.5×10^{-3} mol L^{-1}). Solution C: FLV (0.25×10^{-3} mol L^{-1}) and β-CD (2.5×10^{-3} mol L^{-1}). Solution D: FLX (0.25×10^{-3} mol L^{-1}), FLV (0.25×10^{-3} mol L^{-1}) and β-CD (2.5×10^{-3} mol L^{-1}). A deeper section of some signals is shown in the boxes.

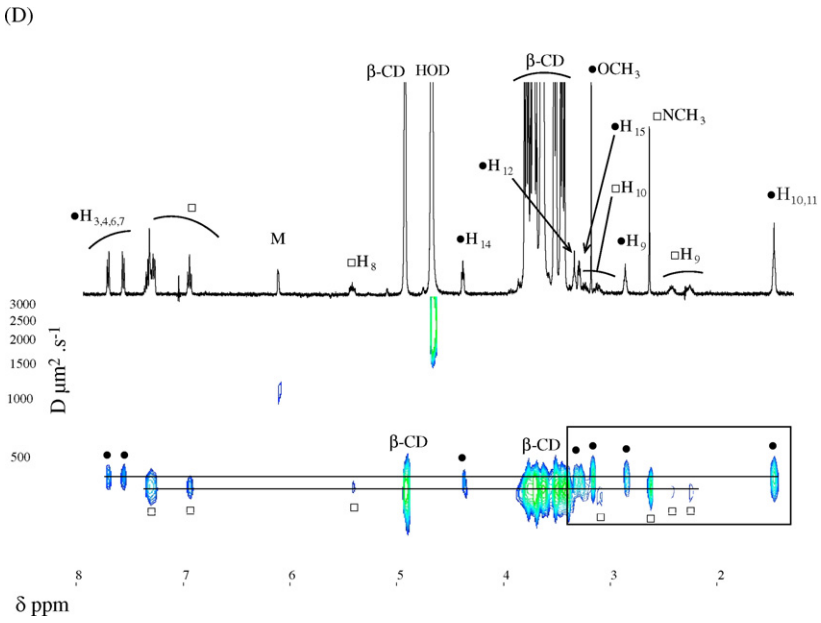

Fig. 5. (*Continued*).

the two MW must be sufficient to get different D and so a separation of the signals. One considers generally that the ratio Δm/mean MW ($\Delta m = MW_A - MW_B$) must not be inferior to 0.10–0.15 to observe separated signals along the diffusion axis. The MW of FLX and FLV are very close (309 and 318 for FLX and FLV bases, respectively). In the DOSY spectrum of a mixture of FLX and FLV (solution A), the signals of both compounds are all lined up (Fig. 5A). Cyclodextrins as host–guest complexing agents were thus used to modify the diffusion coefficients. Cyclodextrins are polymeric carbohydrates possessing hollow cavities which can accommodate a variety of guests in aqueous solution. So three solutions of β-CD with (i) FLX in a molar ratio 10:1 (solution B; Fig. 5B), (ii) FLV in a molar ratio 10:1 (solution C; Fig. 5C), and (iii) both FLX and FLV in a molar ratio 10:1:1 (solution D; Fig. 5D) were analysed.

Complexation of FLX and FLV with β-CD induced minor modifications in the ^{1}H NMR spectra of these drugs particularly in the region of aromatic protons. For instance, the splitting pattern of $H_{3,4,6,7}$ of FLV which is an A_2B_2 system (Fig. 5A) becomes an AA'XX' system (Fig. 5C). Slight modifications were also observed for the aromatic protons of FLX. The doublet corresponding to H_4 and H_6 of FLX (Fig. 5A) appears as a triplet (Fig. 5B) due to the resolution of the two FLX enantiomer signals. Indeed, the ^{19}F NMR spectrum of the same solution showed two signals of equal intensity for FLX with a $\Delta\nu$ of 11.5 Hz.

Very close values of diffusion coefficients were measured for FLX ($362 \pm 20\ \mu m^2\ s^{-1}$) and β-CD ($357 \pm 3\ \mu m^2\ s^{-1}$) in solution B, whereas two distinct values of diffusion coefficients were observed in solution C for FLV ($394 \pm 12\ \mu m^2\ s^{-1}$) and β-CD ($356 \pm 2\ \mu m^2\ s^{-1}$) (Table 6). The values of the diffusion coefficients are significantly lowered (from 588 to 394 $\mu m^2\ s^{-1}$ for FLV and from 612 to 362 $\mu m^2\ s^{-1}$ for FLX) in the presence of β-CD, which most probably indicates a partial inclusion of guests. In the case of FLX, the inclusion is greater as the same diffusion coefficient is observed for β-CD and FLX, whereas FLV diffusion coefficient is slightly higher than that of β-CD. This difference in inclusion capacity is confirmed in the DOSY spectrum of solution D which shows that β-CD is able to induce a virtual separation of FLX and FLV (Fig. 5D).

4. Discussion

A simple, precise and selective ^{19}F NMR spectroscopic method was developed for determining FLX and FLV in pharmaceutical preparations and human plasma and urine (Tables 2 and 3). Comparisons of the analytical methods for FLX and FLV determination in pharmaceutical formulations or biofluids (only the most recent publications have been considered for biofluids) are reported in Tables 7 and 8, respectively. Clearly, the main advantages of ^{19}F NMR are (i) its specificity as only fluorinated molecules (provided they are present at sufficient concentrations) are detected, and (ii) the fact that it avoids

Table 6
Self-diffusion coefficients[a] ($\mu m^2\ s^{-1}$) measured with 2D DOSY ^{1}H NMR in solutions containing fluoxetine and fluvoxamine with or without β-cyclodextrin (β-CD)

	Solution A	Solution B	Solution C	Solution D
Fluoxetine 0.25×10^{-3} mol L^{-1} (0.086 mg mL^{-1})	612 ± 3	362 ± 20	–	359 ± 27
Fluvoxamine 0.25×10^{-3} mol L^{-1} (0.109 mg mL^{-1})	588 ± 1	–	394 ± 12	406 ± 6
β-CD 2.5×10^{-3} mol L^{-1}	–	357 ± 3	356 ± 2	355 ± 2

[a] The value of the self-diffusion coefficient was measured for each peak, and an average self-diffusion coefficient was determined for each formulation.

Table 7
Comparison of analytical techniques of fluoxetine (FLX) and fluvoxamine (FLV) determination in pharmaceutical preparations (capsules (C), tablets (T), oral solutions (S))

Method [a]	Accuracy: % recovery (mean of x concentrations)		LOD ($\mu g\,L^{-1}$)		LOQ ($\mu g\,L^{-1}$)		Assay of pharmaceutical preparations % of nominal concentration		Ref.
	FLX	FLV	FLX	FLV	FLX	FLV	FLX	FLV	
							C 20 mg Prozac from Italy		[2]
Direct spectro	97.4						97.5%		
Derivative spectro	98.9						99.1%		
HPLC UV	99.1						100.9%		
HPLC fluorimetric	100.2						100.2%		
HPLC UV	99.7 ± 1.4 (x = 6)	100.5 ± 1.7 (x = 6)	1–10		3.3–33.3		- C 20 mg from Spain: 100.4% (Adofen), 102.1% (Reneuron), 100.8% (Prozac), 102.6% (Astrin) - T 20 mg from Spain: 99.3% (Adofen), 100.9% (Reneuron), 98.9% (Prozac) - S 20 mg from Spain: 100.9% (Adofen), 102.6% (Reneuron), 101.0% (Prozac), 102.4% (Astrin)	T from Spain 98.2% (Dumirox 50 mg) 99.5% (Dumirox 100 mg)	[1]
C-GC-FID	103.7 ± 5.5 (x = 7)	102.3 ± 3.9 (x = 7)	9.75	101.0	26.5	248.0	- C 20 mg from Spain: 98.5% (Adofen), 107.5% (Reneuron), 100.0% (Prozac)	T from Spain: 101.7% (Dumirox 100 mg)	[30]
C-GC-MSD			11.0	18	35.0	42.0	- T 20 mg from Spain: 98.8% (Adofen), 97.5% (Reneuron), 102.5% (Prozac) - S 20 mg from Spain: 100.3% (Reneuron), 97.5% (Prozac)		
C-GC-FID	100.3 ± 1.0 (x = 8)	100.3 ± 1.6 (x = 8)	19.4	30.1	64.9	100.3	- C 20 mg from Spain: 101.0% (Adofen), 100.7% (Reneuron), 100.0% (Prozac), 100.6% (Astrin), 100.5% (FLX-Bayvit), 101.6% (FLX-Normon), 98.5% (FLX-Alter), 98.8% (FLX- Ratiopharm) - T 20 mg from Spain: 100.9% (Adofen)	T from Spain: 101.8% (Dumirox 50 mg)	[31]
C-GC-FID	100.4 ± 1.5 (x = 5)	100.9 ± 1.1 (x = 5)	10.1	105.3	33.5	300	C 20 mg from Spain: 100.8% (Prozac)	T from Spain: 101.2% (Dumirox 100 mg)	[12]
C-GC-MS-SIM	99.8 ± 1.1 (x = 8)	100.6 ± 1.3 (x = 8)	12.4	10.5	41.5	35.1	- C 20 mg from Spain: 100.8% (Adofen), 102.4% (Reneuron), 102.3% (Prozac), 102.7% (Astrin), 100.7% (FLX-ICN), 101.1% (FLX-Bayvit), 100.3% (FLX-Normon), 99.2% (FLX-Alter), 98.1% (FLX-Ratiopharm) - T 20 mg from Spain: 101.9% (Adofen)	T from Spain: 98.4% (Dumirox 50 mg)	[9]
CZE	98.1 ± 1.1 (x = 5)	100.0 ± 1.0 (x = 5)	1000		2500		- C 20 mg from Spain: 100.5% (Adofen), 102.9% (Reneuron) - T 20 mg from Spain: 97.8% (Reneuron) - S 20 mg from Spain: 101.8% (Adofen), 102.7% (Reneuron), 103.0% (Prozac)		[32]
CZE	98.9 ± 1.0 (x = 7)	99.4 ± 1.0 (x = 7)	30–110		110–380		- C 20 mg from Spain: 95.7% (Adofen), 96.2% (Reneuron) - T 20 mg from Spain: 97.6% (Prozac) - S 20 mg from Spain: 98.3% (Astrin)	T from Spain 95.6% (Dumirox 50 mg) 95.9% (Dumirox 100 mg)	[13]
Spectrofluorimetry	99.0 ± 0.8 (x = 3)		70		200		C 20 mg Prozac from Italy 98.8%		[33]
CZE	101.0 ± 1.5 (x = 3)		100		250		100.9%		
Spectrophotometry							C 20 mg from India (MO/TB): 100.5/100.7% (Fludac), 101.5/101.3% (Prodep), 101.0/99.9% (Oxedap), 99.8/99.2% (Prodac), 95.0/95.1% (Nuzac), 100.6/98.9% (Loftil), 99.9/99.6% (Flunat), 101.4/98.9% (Trizac)		[34]
Methyl orange (MO) [b]	100.1 ± 0.4 (x = 3)								
Thymol blue (TB) [b]	99.9 ± 0.4 (x = 3)								
Spectrophotometry[c]			33	610	880	2040	T 20 mg from Poland: 98.9% (Prozac)	T 50 mg from Poland: 99.7% (Fevarin)	[35]
Spectrophotometry[d]							T 20 mg from Iran: 99.3%		[15]
Batch			1400		4800				
Flow injection			850		2840				
Capillary isotacophoresis					430	670	T 20 mg from Slovak Republic: 99.0% (Prozac), 98.0% (Deprex), 100.5% (Portal)	T from Slovak Republic: 99.8% (Fevarin 100 mg), 99.2% (Fevarin 50 mg)	[36]
Electroanalytical method		99.0 ± 0.5 (x = 3)		2.0		6.9		T from Portugal: 100.6% (Dumyrox 50 mg)	[37]
^{19}F NMR	100.1 ± 3.4 (x = 13)	98.8 ± 3.3 (x = 11)	450	560	1600	2000	See Table 1	See Table 1	This study

[a] Spectro: spectrophotometry; HPLC UV: high performance liquid chromatography with UV detection; C-GC-FID: capillary gas chromatography ionization detection; C-GC-MSD: capillary gas chromatography mass spectrometry detection; C-GC–MS–SIM: capillary gas chromatography–mass spectrometry–single ion monitoring; CZE: capillary zone electophoresis.

[b] Yellow ion-pair complex due to the action of MO and TB on FLX in acidic and basic medium, respectively.

[c] Reaction of FLX or FLV with pyrocatechol violet.

[d] Competitive complexation reaction of FLX with phenolphthalein-β-cyclodextrin inclusion complex.

Table 8
Comparison of analytical techniques of fluoxetine (FLX) and fluvoxamine (FLV) determination in human biofluids (only the articles published in 2005 and after are reported in this table)

Method[a]	Biofluid	Pretreatment	Extraction recovery		LOD ($\mu g\,L^{-1}$)		LOQ ($\mu g\,L^{-1}$)		Reference
			FLX	FLV	FLX	FLV	FLX	FLV	
HPLC fluorescence	Plasma	Extraction and derivatisation with NBD-Cl[b] and extraction	99.9%		0.5		1		[3]
HPLC-tandemMS	Serum		95%	100%			2.17	1.17	[6]
HPLC-MS SSI	Plasma	SPE		96.0%		130		200	[5]
HPLC UV	Plasma	SPE		95.6%		1.7		5.0	[4]
HPLC visible	Plasma Urine	Derivatisation with NQS[c] and extraction		Pl: 96.0 U: 96.5		Pl: 1.4 U: 1		Pl: 5 U: 2	[8]
SPME-HPLC UV	Plasma	SPME on line	1.9–6.8%		10		25		[7]
GC–MS	Urine	Derivatisation as acetyl compounds and SPME extraction	114%, 110%	116%,107%	0.25	0.38			[11]
C-GC–MS–SIM	Urine	SPE	95.2–106.1%		5.7				[10]
Nonaqueous CE	Urine	SPE	85–99%		10 for 6 mL urine 4 for 10 mL urine		32 for 6 mL urine 13 for 10 mL urine		[14]
Spectrophotometry	Urine	Competitive complexation reaction of FLX with phenolphthalein-β-cyclodextrin inclusion complex	96.5%						[15]
Batch Flow injection					1400 [d] 8500 [d]		4800 [d] 28,400 [d]		
Electroanalytical method	Serum	Extraction	75.8–88.5%			2.0 [d]		6.9 [d]	[37]
^{19}F NMR	Plasma Urine		Pl: 100.5% U: 97.8%	Pl: 97.9% U: 98.8%	450 [d]	560 [d]	1600 [d]	2000 [d]	This study

[a] HPLC-tandem MS: high performance liquid chromatography–tandem mass spectrometry; HPLC–MS SSI: high performance liquid chromatography–mass spectrometry with sonic spray ionization method; SPME: solid-phase microextraction; GC–MS: gas chromatography–mass spectrometry; C-GC–MS–SIM: capillary gas chromatography–mass spectrometry–single ion monitoring; CE: capillary electophoresis.
[b] NBD-Cl: 4-chloro-7-nitro-2,1,3-benzoxadiazole.
[c] NQS: 1,2-naphtoquinone-4-sulphonic acid sodium salt.
[d] Values determined in water.

Table 9
Comparison of validation parameters for the ^{19}F NMR quantitative determination of some fluorinated drugs

	5′-Deoxy-5-fluorouridine and α-fluoro-β-alanine[a] [40]	Fluoroquinolones[b,c] [21]	Flupentixol[d] [23]	Haloperidol [24]
Spectrometer ^{19}F resonance frequency	Cameca 250 FT 250 MHz	Bruker A200 188 MHz	Bruker DRX 500 470.59 MHz	Bruker DRX 500 470.59 MHz
Linearity Correlation coefficient (range of concentrations (mol L^{-1}))	5′dFUrd: 0.999 (1.5×10^{-5}–1.4×10^{-1}) FBAL: 0.999 (2.6×10^{-5}–1.0×10^{-1})	N: 0.998 P: 0.998 O: 0.988	Z isomer 0.9969 (9.2×10^{-6}–1.15×10^{-4}) E isomer 0.9992 (5.5×10^{-6}–5.75×10^{-5})	0.9968 (1.6×10^{-4}–1.6×10^{-3})
Precision (R.S.D.) (range of concentrations (mol L^{-1}))	3% for S/N ≥ 30 5% for S/N = 15	N: 0.7% P: 0.35% O: 0.6%	1–7% (1.2×10^{-5}–5.4×10^{-5})	3–7% ($\approx 8 \times 10^{-4}$)
Accuracy (%recovery ± S.D.)		N: 99.5 ± 0.7 P: 100.3 ± 0.35 O: 99.8 ± 0.59	98–116 (1.2×10^{-5}–5.4×10^{-5})	97–103% ($\approx 8 \times 10^{-4}$)
LOD (S/N = 3) mol L^{-1}	10^{-5} (78,000 scans)		3.9×10^{-6} (64 scans)	3.7×10^{-6}
LOQ (S/N = 10) mol L^{-1}			1.7×10^{-5} (64 scans) 6.4×10^{-7} (512 scans)	1.6×10^{-4} (64 scans) 5.3×10^{-5} (850 scans)

[a] 5′-Deoxy-5-fluorouridine: 5′dFUrd; α-fluoro-β-alanine: FBAL.
[b] norfloxacin: N; pefloxacin: P; ofloxacin: O.
[c] Concentrations used for the validation are not indicated.
[d] Flupentixol bears a $-CF_3$ group.

any pre-treatment of the sample (in some cases, the sample can be concentrated to increase the sensitivity). Indeed, the existing methods developed for the assay of FLX and FLV usually necessitate sample pre-concentration, derivatisation and/or extraction steps prior to analysis. Its main drawback is its low sensitivity, which is not a hindrance for the quantitation in pharmaceutical preparations but hampers the assay in biofluids. The ranges of FLX and FLV therapeutic plasma levels are 160–500 μg L^{-1} and 150–250 μg L^{-1}, respectively [38], preventing the use of ^{19}F NMR in routine assays. However, the method can be employed for toxicological studies as FLX and FLV toxic plasma levels are above 1000 μg L^{-1} and 650 μg L^{-1}, respectively [38].

The development of quantitative NMR has been accelerated since about 10 years by the increase of the sensitivity of high-field NMR spectrometers as well as by modern software packages that allow an accurate and precise data processing, and quantitative NMR is now well validated [16,18]. Nevertheless, all the relevant parameters for ^{19}F NMR recording and data acquisition and processing in order to determine absolute concentrations were taken into account 30 years ago [39,40]. Validation parameters for ^{19}F NMR quantitative determinations of several fluorinated drugs are shown in Table 9 and can be compared with those obtained in this study (Table 2). The linearity is correct in all the studies. The precision of the method evaluated with R.S.D. is comparable as well as the accuracy assessed by measuring the average recovery of samples spiked with analytes. If the LOD and LOQ are quite similar (except in the 1985 study), the number of scans to measure them are extremely different. This can be explained by the difference in the fields of the spectrometers employed and probably also by the fact that the apparatus used in the present study is much older. Since NMR signals are Lorentzian lines, the determination of LOD and LOQ based on the S/N ratio is not the best method. The approach based on the S.D. of the response and the slope of a calibration curve using solutions containing the analytes in the range of LOD [26] should have been more appropriate. However, the length of time necessary with the spectrometer used for recording ^{19}F NMR spectra of analyte solutions at very low concentrations makes the second method difficult to carry out. Nevertheless, the S/N ratio gives a good estimate of the LOD and is thus employed in several studies using quantitative ^{19}F NMR [23,24].

All the formulations tested in this study had FLX concentrations within the specification of the US Pharmacopeia, which recommends that FLX capsules should contain not less than 90% and not more than 110% of the labelled amount of the active ingredient (Table 1) [41]. Our data are in agreement with those reported by several authors [1,2,9,12,13,15,30–37] who,

Table 10
^{1}H NMR (500 MHz) chemical shift change (Δδ) values[a] for various protons of fluoxetine hydrochloride in the presence of β-CD in D_2O at 25 °C

	[β-CD]/[FLX]	$H_{3,7}$	$H_{4,6}$	H_8	H_9	$H_{9'}$	H_{10}	$H_{10'}$	H_{11}
[28]	1.4	−0.138	0.047	−0.021	0.131	0.093	0.005	0.040	0.050
This study	10	−0.138	0.018	−0.047	0.148	0.052	0.058	0.020	0.036

Negative values indicate upfield shift.
[a] Chemical shift differences were calculated by subtracting the values of the chemical shifts measured in presence of β-CD to those measured for fluoxetine alone.

for the validation of various analytical techniques or for quality assessment of pharmaceutical products, assayed FLX or FLV in pharmaceutical formulations (tablets, capsules, oral solutions) from different countries (Italy, Spain, India, Poland, Iran, Slovak Republic, Portugal) and found contents comprised between 95 and 108% or 96% and 102% of declared amount of FLX or FLV, respectively (Table 7). The intrabatch variability is correct as it is <5% for all the 19 formulations of FLX and FLV assayed except one (formulation 4), and even <2% for twelve pharmaceutical preparations.

The lack (or the paucity) of information on formulations purchased via the Internet and also on some generic drugs make difficult for control laboratories to determine the composition of pharmaceutical formulations (active ingredient and excipients). Moreover, the knowledge of pharmaceutical additive content (filler, binder, lubricant, disintegrant, flavor, colorant. . .) of a drug formulation is of great interest, particularly in the early development phase of a drug when formulation optimisation occurs [42]. Our study shows differences between the brand-named and the generic capsules of FLX (Fig. 3A and B, respectively) whereas no difference was observed for FLV tablets. The five oral solutions of FLX analysed by DOSY NMR present two kinds of spectral pattern. The DOSY spectra of the two formulations Prozac® and Fluoxetine Teva are very similar (Fig. 4A) but different from those of the other three formulations analysed that have the same spectral signature (Fig. 4B).

Although this part of our work does not correspond stricto sensu to quality assessment of FLX and FLV formulations as they are not marketed in CD-complexed form, a method that allows the separation of these two drugs in 2D DOSY ^{1}H NMR spectra is proposed. Indeed, the analysis by this technique of a mixture of drugs whose molecular weight difference is particularly low (Δm/mean MW ≈3% for free bases) is challenging. β-CD was thus used to induce changes in the diffusion coefficients of the species. The use of β-CD for complexation of FLX has already been reported showing a partial inclusion of the guest [28]. A comparison of the ^{1}H NMR chemical shift change ($\Delta\delta$) values for various protons of FLX hydrochloride in the presence of β-CD between our study and that of Ali et al. [28] is reported in Table 10. Measured Δδ are close between the two studies. Most noticeable is the very significant upfield shift for the $H_{3,7}$ signal in the presence of β-CD explained in terms of close proximity of these protons with fluorine in non-complexed state while in complexed state the fluorine atoms may form hydrogen bonds with the 6′-OH of β-CD, thus weakening the interaction between fluorine and $H_{3,7}$ [28]. As we used a great excess of host compared with guest ([H]/[G] = 10), the changes in chemical shifts for β-CD protons between spectra of mixtures of FLV or FLX with β-CD compared to pure β-CD are not very large. However, the signals for $H_{3'}$ and $H_{5'}$ of β-CD, situated inside the β-CD cavity [43], exhibited an upfield shift that, for the signal of $H_{3'}$, reaches 0.021 ppm for FLV complexed to β-CD and 0.025 ppm for the β-CD-FLX complex. The modifications in ^{1}H NMR chemical shifts of host and guest are in accordance with measurement of self-diffusion coefficients in DOSY spectra showing a partial inclusion of FLV and FLX in β-CD with FLX having a better capacity of inclusion.

5. Conclusion

A simple, accurate and reproducible ^{19}F NMR method has been developed for the determination of FLX and FLV contents in pharmaceutical samples. The results reported herein demonstrated that the quality of pharmaceutical formulations of FLX and FLV sold in several countries or via the Internet is correct. However, the method is not sufficiently sensitive for the assay of these antidepressants in biofluids at least at therapeutic doses. 2D DOSY ^{1}H NMR, which is now considerably easier to use, thanks to improvements in spectrometer hardware and DOSY software, is an interesting tool for the control of generic formulations as it shows the similarities and differences in formulation components. ^{19}F and ^{1}H DOSY NMR provide a "NMR toolbox" that could be additional analytical techniques particularly useful in the early stages of formulation development of new fluorinated drugs and also in the fight against counterfeit drugs.

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