Research Article

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Bioanalysis

Quantification of infliximab and adalimumab in human plasma by a liquid chromatography tandem mass spectrometry kit and comparison with two ELISA methods

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Background: This study compared the performance of plasma infliximab and adalimumab quantification using a commercially available kit (mAbXmise kit) and mass spectrometry readout to that of two ELISA methods in patients treated for inflammatory bowel disease. **Methods & results:** The mAbXmise method based on liquid chromatography tandem mass spectrometry (LC-MS/MS) was linear from 2 to 100 μg/ml. It was validated according to international guidelines. Regarding cross-validation for infliximab (n = 70), the mean bias with LC-MS/MS assay was approximately threefold higher with the commercial ELISA assay compared with the in-house ELISA (-6.1 vs -1.8 μg/ml, respectively). The mean bias between the LC-MS/MS assay and in-house ELISA was -1.2 μg/ml for adalimumab (n = 35). **Conclusion:** The LC-MS/MS method is a powerful alternative to immunoassays to monitor concentrations of infliximab and adalimumab.

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TNF-α plays a major role in the inflammatory process in inflammatory bowel disease (IBD). Inhibiting this cytokine through the use of therapeutic monoclonal antibodies (mAb) such as infliximab (IFX) and adalimumab (ADL) ensures a significant rate of clinical response in patients [1]. The pharmacokinetics of ADL and IFX is one factor contributing to interindividual drug response variability. Several studies have reported that therapeutic drug monitoring (TDM) for ADL and IFX provides clinical benefits in terms of both efficacy and safety [2,3]. Consequently, TDM could be useful to optimize patient management during the induction and maintenance phases of treatment with IFX or ADL [2,4].

Several methods to measure TNF-α inhibitors based on ELISA have been developed [5–11]. Unfortunately, different ELISA approaches may result in inconsistent results, and in different labs, the same ELISA can result in different readouts. For example, a recent comparison of three commercially available ELISA kits for IFX quantification revealed significant discrepancies between results obtained with different tests [12,13]. This can be improved (and likely standardized) by using a workflow that is based on liquid chromatography tandem mass spectrometry (LC-MS/MS) for quantification of large molecules in complex matrices such as plasma. In addition,

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immunoassay flaws such as the high-dose hook effect and the presence of antireagent antibodies can be improved by using an LC-MS strategy [14]. In an attempt to overcome some of these shortcomings, pharmacologists and bioanalysts have turned to separation methods, such as LC-MS/MS [15,16]. Indeed, compared with ELISA, LC-MS/MS provides optimal specificity; it is also compatible with simultaneous quantification of IFX and ADL. Moreover, the use of internal standards (IS) reinforces the reproducibility of these approaches by normalizing the results in case of variations in sample preparation or during the detection process [17–20]. Finally, the recent availability of commercial sample preparation kits can help deploy these novel methods more broadly in laboratories performing TDM.

The aim of the present study was to compare the performance of plasma IFX and ADL quantification using a commercially available kit (mAbXmise kit) and an MS readout to that of two ELISA methods and thus demonstrate the suitability and robustness of the LC-MS approach for standard practice.

This study was conducted in the context of a noncontractual collaboration between academic laboratories and a private company to assess the possibility of transfering LC-MS/MS TDM mAbs technology to our center.

Materials & methods

Reagents & lab ware

The multiplex IFX and ADL mAbXmise kit was obtained from Promise Proteomics (Grenoble, France). mAbXmise is a ready-to-use kit based on a patented workflow (patent nos. EP3165928, EP3371602, EP3165928, EP3165922) designed for the simultaneous quantification of IFX and ADL in plasma. (The exact composition of the kit cannot be disclosed due to patents.) It uses full-length isotopically labeled IFX and ADL as internal standards (SIL); its purity is >95% and isotopic incorporation is >98%. The mAbXmise kit includes reagents, calibration standards (n = 7 including zero) and three internal quality controls (IQC) prepared from reference solutions of Remicade and Humira, labware (mAbXmise plate, PuriXmise plate), and solutions (CutXmise enzyme and CutXStop) to prepare samples for injection into the LC-MS/MS system. Calibration standards and controls were prepared by spiking drug-free human plasma with commercial IFX and ADL (Remicade and Humira). LC-MS/MS-grade acetonitrile was purchased from Merck-Sigma (MO, USA), LC-MS-grade water and formic acid were from Fisher Chemicals (Illkirch, France). The commercial Lisa-Tracker kit was purchased from Theradiag (Marne la Vallée, France). Drug free human plasma and serum were obtained from Etablissement Français du Sang (Grenoble, France).

Preparation of calibration curves & IQCs

Pharmacokinetic data from pharmacokinetic/pharmacodynamic (PK/PD) studies guided to choice of calibration standards and IQC values [21]. Two independent stock solutions were prepared for each mAb: one was used to prepare calibration standards (CAL), and the other was used for the IQCs. All stock solutions were prepared from reference solutions of IFX and ADL. Briefly, 4-ml volumes of CAL solutions at 10, 25, 50, 100, 250 and 500 µg/ml and IQC solutions at 30, 125 and 375 µg/ml were prediluted in PBS 1x. These prediluted solutions (4 ml) were then diluted in 16 ml of blank plasma to produce the final concentrations: 2, 5, 10, 20, 50 and 100 µg/ml (CAL) and 2, 6, 25 and 75 µg/ml (IQC).

Sample preparation with mAbXmise kit

Samples were prepared according to the manufacturer's instructions. Briefly, as summarized in Figure 1, 20 µl of sample (calibration standard, IQC or test plasma) were loaded into wells on the mAbXmise plate and diluted with 80 µl of Buffer A from the kit. Plates were incubated for 1 h at room temperature with agitation. IFX and ADL along with their full-length isotopically labeled forms were extracted by immunocapture on the PuriXmise plate. Then samples were eluted before a drying step in a speed-vacuum (Martin Christ, Osterode am Harz, Germany). After resolubilization, samples were digested using CutXmise enzyme overnight at 37°C. Finally, CutXStop was used to stop digestion before the injection of 20 µl of the sample into the LC-MS/MS system.

LC-MS/MS conditions & instrumentation

The procedure used to select proteotypic peptides for IFX and ADL is described in supplementary material (Supplementary Table 1). The final list of selected MRM transitions is given in Supplementary Table 1. The chromatographic system used was an Exion system with binary pumps (Sciex, MA, USA), the autosampler temperature was set to 15°C, and the column oven was maintained at 40°C. Chromatographic separation of peptides was achieved on a BioZen 2.6-µm Peptide XB-C18 LC column measuring 100 × 2.1 mm (Phenomenex,

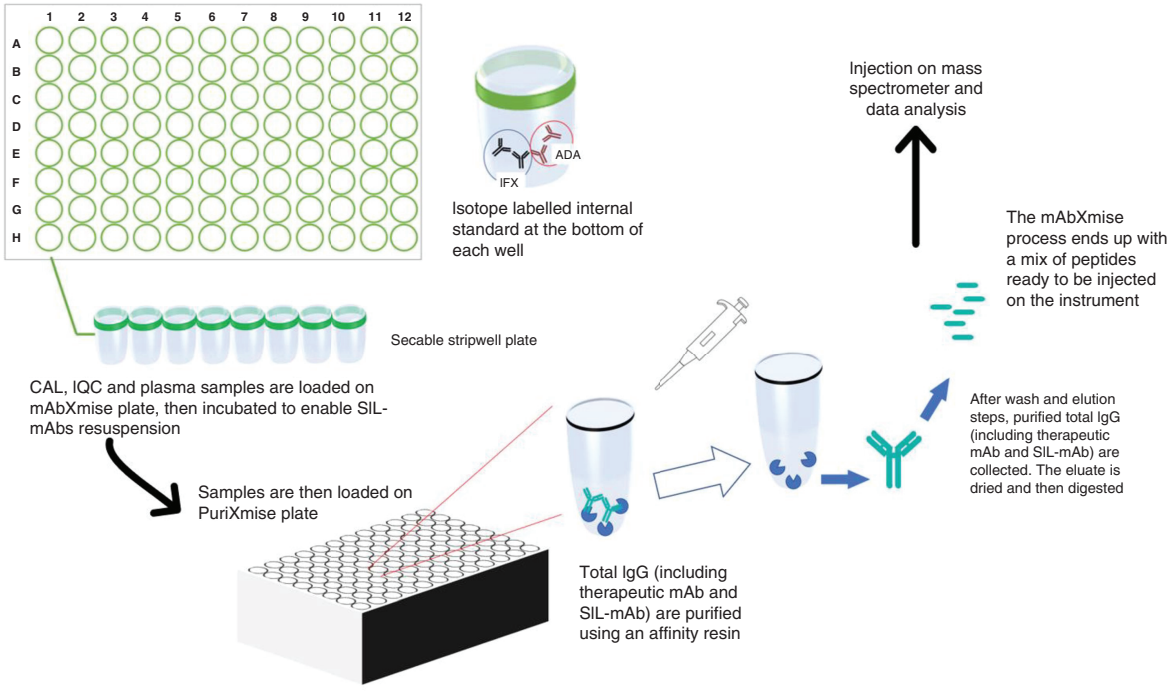

Figure 1. Summary of the mAbXmise process. Plasma samples were loaded onto a mAbXmise plate alongside calibrators and internal quality control samples provided in the kit. Full-length isotopically labeled IFX and ADL, coated on the plate, are solubilized in the plasma samples and will serve as internal quantification standards. Total IgG were purified, recovered and then digested. At the end of the process, the samples collected are ready for injection into the liquid chromatography tandem mass spectrometry system.
ADL: Adalimumab; CAL: Calibrators; IFX: infliximab; IQC: Internal quality control; mAb: Monoclonal antibody.

CA, USA). An elution gradient was applied to achieve chromatographic separation. The mobile phase was a mix of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The elution gradient included the following steps: 95% of phase A from 0 to 1 min, decrease to 80% of phase A from 1 to 2 min, decrease to 60% of phase A from 2 to 12 min, decrease to 10% of phase A from 12 to 12.1 min, keep 10% of A from 12.1 to 14.5 min, back to 95% of A from 14.5 to 14.6 min and keep 95% of A up to 20 min for system stabilization. The flow rate was 100 µl/min throughout the run. Mass spectrometer was a triple-quadrupole 6500 QTRAP (Sciex). Source parameters were curtain gas 30 psi, Ionspray voltage 5500 V and source temperature 550°C. Ion source gas 1 was applied at 40 psi, and ion source gas 2 was set at 45 psi. Declustering potential was set as variable, inlet potential and collision cell exit potential were set at 12 and and 19, respectively.

Method validation

The specifications targeted for the LC-MS method validation described here were determined using the international guidelines published when the experiments were performed (2018/US FDA and 2011/European Medicines Agency [EMA] 2011) [22,23]. Although these guidelines have not been specifically developed for biologics, the parameters described were consistent with those requested by French certification institution COFRAC for certifying analytical methods used for diagnostic in clinical labs in France. A gap analysis with CLSI C64, which has been published recently, will be conducted soon, and the analytical performances will be updated accordingly.

Linearity & lower limit of quantification

Calibration curves using a zero and six calibration standards (2, 5, 10, 20, 50 and 100 µg/ml) for both mAbs were built by plotting the ratio of the peak area of analyte of interest to its corresponding IS versus the nominal concentration of the analyte. Linearity of calibration curve was assessed over 5 days. For each linearity assessment, double blank, zero samples and CAL samples (between 2 and 100 µg/ml) were prepared on a single plate. A minimum of 75% of the standard calibration samples had to be within ±15% of the nominal concentration,

except ±20% for the lower limit of quantification (LLOQ) defined as the lowest amount that can be quantified with precision and accuracy within ±20%.

Accuracy & precision

Accuracy and precision were assessed using LLOQ (2 µg/ml) and three IQCs corresponding to low (6 µg/ml), medium (25 µg/ml) and high (75 µg/ml) concentrations. For intrarun tests, eight replicates of IQC (low, medium, high) and LLOQ samples were injected on the same day. Intraday accuracy and precision were determined using eight samples injected the same day (day 1). Interday accuracy and precision were determined by injecting IQC samples (n = 16) at low, medium and high concentrations, and LLOQ samples (2 µg/ml). Eight replicates were injected on day 1, four on day 2 and four on day 3. Precision was assessed by calculating the coefficient of variation (CV) of concentrations measured in the 16 replicates, injected on 3 days. Accuracy was reported as the relative difference between the concentration measured and the theoretical value. Accuracy and precision should be less than 15% for IQCs or 20% for LLOQ.

Biosimilars of IFX

Accuracy and precision were also assessed for two biosimilars of IFX, Flixabi and Inflectra. Six replicates of the three IQC levels (low, medium and high) were measured on the same day.

Selectivity, carryover & matrix effect

Selectivity was investigated using 'drug-free' plasma samples from six individual sources of plasma. The evaluation was made using six double blanks (processed matrix sample without analyte and without IS) and six zero samples (CAL0, processed matrix with iS). Evidence of interference was carefully sought by examination of the chromatograms for drug-free samples in the retention window where peaks of analytes and IS were expected. For each sample, signal in blank samples should be <20% of the LLOQ area for analytes and <5% for IS. Carryover was estimated by injecting the highest calibration standard (100 µg/ml for each mAb), immediately followed by an extracted blank sample. The peak area measured at the retention time for analytes in the blank extract should be <20% of that for the corresponding area measured in the LLOQ sample. Carryover should not exceed 5% at the retention time corresponding to the IS.

Matrix effects were investigated using six lots of blank matrix from individual donors. The matrix effect for analyte is investigated by resuspending a mix of digested IFX and ADL mAbs in solvent (mobile phase) or in digested plasma. Each condition was prepared and injected in triplicate. The same was done for SIL-IFX and SIL-ADL to determine matrix effect for IS. For each analyte and the IS, the matrix factor (MF) was determined by calculating the ratio of the peak area in the presence of plasma to the peak area in solvent. The IS normalized MF was calculated by dividing the MF of the analyte by the MF of the IS. Determination of MF was done at 6 and at 35 µg/ml.

Stability of incurred samples

Stability of incurred patient samples stored under frozen condition and that handled three freeze–thaw cycles was assessed according the EMA guidelines. Approximately 10% of the cohort (nine IFX samples and five ADL samples) was reanalyzed. Between the first analysis and the reanalysis, samples were stored for at least 2 months at -80°C. The percent difference between the initial concentration and the concentration measured during the repeat analysis should not be greater than 20% of their mean for at least 67% of the repeats.

Metrological traceability

The metrological traceability was determined using the WHO Standards for IFX and ADL. WHO adalimumab reference standard (ref. 17/236) and WHO infliximab reference standard (Re 16/170) were purchased from NIBSC (Ridge, UK). Datasheets provided specify that the content of each ampoule is ~50 µg. Ampoules of the WHO IFX and ADL were resuspended with 1 ml of purified water to obtain standard solutions at 50 µg/ml. Concentrations of each standard solutions (nondiluted) were then determined using the mAbXmise protocol in four replicates.

The metrological traceability was determined using Supelco Certified Reference Materials (CRM), recently made available for IFX (I-042–0.25 ml) and ADL (A-166–0.25 ml). The vials were produced by Cerilliant Corporation (TXD, USA) and purchased from Sigma-Aldrich (MO, USA); batch numbers were FN04212001 for ADL and FN04022002 for IFX. Data sheets provided for these batches specify the content of each ampoule is

9.98 ± 0.27 mg/ml for ADL and 9.56 ± 0.33 mg/ml for IFX. Solutions of both CRM IFX and ADL were diluted at 50 µg/ml in a solution of phosphate-buffered saline 1X, 1% bovine serum albumin and 0.05% Proclin150. Concentrations were then determined using the mAbXmise protocol in four replicates.

Clinical application & methods comparison

The cohort for the clinical comparison included 105 IBD patients, 70 treated with IFX and 35 treated with ADL. Whole blood was sampled as part of routine clinical care (therapeutic drug monitoring) at the gastroenterology department, Centre Hospitalier Universitaire de Rennes (Rennes, France). Blood was collected at steady state in heparin–lithium-containing tubes just before the next mAb infusion (trough concentration). After centrifugation (4°C, 10 min, 2000×*g*), plasma was aliquoted in polypropylene tubes, and stored at -20°C until analysis. The study was approved by the local ethics committee at the Centre Hospitalier Universitaire de Rennes (authorization no. 21.54). Patients were informed that their data, collected during their follow-up at the center, could be used for research purposes; they gave their consent for this use of their data.

Three laboratories were involved in the comparison of performance for IFX assay between the mAbXmise kit and the two ELISA methods. The mAbXmise kit was applied at Promise Proteomics (Grenoble, France), and the in-house ELISA method, adapted from Ternant *et al.* [24]. was applied at the laboratory of pharmacology, Centre Hospitalier Universitaire de Rennes. IFX concentrations were also assayed using the commercial Lisa-Tracker kit at the immunology laboratory, Centre Hospitalier Universitaire Cochin (Paris, France). All analyses were performed according to the manufacturers' instructions. Results obtained with the mAbXmise kit and an ELISA method for ADL assay were also compared; two laboratories were involved: Promise Proteomics for the mAbXmise kit and the laboratory of pharmacology at the Centre Hospitalier Universitaire de Rennes for the in-house ELISA method [24].

Statistical analysis

The MedCalc statistical package (version 19.2.6; MedCalc Software, Mariakerke, Belgium) was used for data analysis. Samples for which the concentrations measured were below the LLOQ for both mAbs (<2 µg/ml) were excluded from the statistical analysis. The nonparametric regression proposed by Passing-Bablok *et al.* [25] was used to determine whether there was a linear relationship between the different techniques when assaying plasma IFX or ADL concentrations. The regression equation was expressed along with the 95% CI to estimate slope and intercept. A Bland–Altman plot [26] was used to assess method agreement. Numerical results are reported as both mean bias and the Bland–Altman limits of agreement (LOA), along with their respective 95% CIs [95% LOA].

Results

First, the mAbXmise method was validated according to the criteria set out in the international guidelines [22,23].

Chromatograms

Supplementary Figure 1 displays typical chromatographic profiles (selected reaction monitoring mode) for a blank plasma sample (A & D), the LLOQ for each mAb (B & E), and plasma from IBD patients treated with either IFX (C) or ADL (F). As shown, the peptides for IFX and ADL were adequately separated from each other and from any potentially interfering peaks. Unlabeled peptides coeluted perfectly with their labeled analogs.

Limit of quantification & linearity

For each mAb, linearity of the method was determined over the calibration range based on linear regression. Calibration curves were linear between 2 and 100 µg/ml with $r^2 \geq 0.99$ for both ADL and IFX. The back-calculated concentrations of the calibrators were within ±15% of the nominal value for all standards and thus meet the acceptance criteria.

Slopes, intercepts and coefficients of determination obtained for mean values and standard deviations were as follows (n = 6): y = 0.10105X + 0.07985 and r^2 = 0.9964 for IFX_DILLTQSPAILSVSPGER, y = 0.09892X + 0.00656 and r^2 = 0.9946 for IFX_SINSATHYAESVK and y = 0.10697x + 0.14935 and r^2 = 0.9964 for ADL_APYTFGQGTK, where x is the concentration in µg/ml and y is the area ratio.

Accuracy & precision

Results for intraday and between-day precision and accuracy for ADL and IFX are reported in Table 1. They met

Table 1. Intra- and inter-assay accuracy and precision of adalimumab and infliximab measured in plasma with mAbXmise kit: results obtained with originator drugs Remicade® and Humira®.

Nominal concentration (μg/ml)	Intraday			Interday		
	Mean calculated concentration (μg/ml)	Accuracy (%, n = 8)	Precision (%, n = 8)	Mean calculated concentration (μg/ml)	Accuracy (%, n = 16)	Precision (%, n = 16)
ADL – APYTFGQGTK						
LLOQ	1.9	-4.4	5.3	2.2	9.6	11.6
6	6.0	0.2	2.6	6.1	2.4	2.1
25	26.4	5.7	1.8	25.7	2.8	3.1
75	76.9	2.5	1.9	73.7	-1.7	4.9
IFX – DILLTQSPAILSVSPGER						
LLOQ	2.2	9.3	10.1	2.2	10.8	1.3
6	6.2	3.6	4.5	6.1	2.3	1.3
25	26.0	4.1	4.7	25.3	1.4	3.2
75	75.6	0.8	2.1	72.1	-3.9	4.4
IFX – SINSATHAESVK						
LLOQ	2.2	10.4	7.3	2.2	12.2	5.7
6	6.0	-0.2	6.1	6.0	0.3	1.9
25	25.7	2.8	4.3	25.4	1.5	1.5
75	72.5	-3.4	4.5	70.8	-5.7	4.6

Precision was expressed as coefficient of variation and accuracy as bias compared with the nominal concentration.
ADL: Adalimumab; IFX: Infliximab; LLOQ: Lower limit of quantification (2 μg/ml).

the acceptance criteria because bias and CV were <20% for LLOQ and <15% for the three IQC concentrations for at least one peptide.

Biosimilars

For the two infliximab biosimilars (Inflectra and Flixabi), the bias on IQC were <15% of the nominal values, with a precision of <15% (Table 2).

Selectivity, carryover & matrix effect

For IFX, no peptidic interferences were detected with both peptides and for the unlabeled and labeled forms of each peptide on the six individual plasma samples analyzed. For ADL, however, an interfering signal was observed with the unlabeled form of peptide APYTFGQGTK because a peak was observed at the corresponding retention time of this peptide in some drug-free plasma samples. This artifact was observed for each transition monitored for peptide APYTFGQGTK. To further explore the extent of interference, 11 additional plasmas from untreated individuals were analyzed. The interference gave a signal higher than the LLOQ in 11.8% of drug-free human plasma. In these tests, the mean contribution of this interference in plasma carrying it on the ADL concentration was 30% of the signal of the LLOQ.

No carryover was observed when a blank sample was injected after the standard containing the highest calibration concentration. The ratio blank/LLOQ areas for IFX were <7% (unlabeled DILLTQSPAILSVSPGER_IFX) and <1% (labeled DILLTQSPAILSVSPGER_IFX) and for ADL were <3% (unlabeled APYTFGQGTK) and <2% (labeled APYTFGQGTK).

Results of matrix effect for proteotypic peptides of ADL and IFX are reported in Table 3. Despite an absolute matrix effect observed for each analyte, especially at low concentration, no concerning relative matrix effect was observed because coefficients of variation of IS-normalized MF were <15%.

Stability

Concentrations of IFX and ADL measured in incurred patient samples before and after long-term storage at -80°C were similar (mean bias between two measures of 4.1%) and fulfilled the acceptance criteria (Supplementary Table 2), meaning that freeze–thaw cycles and storage conditions applied to samples used for methods comparison did not led to any stability issue that could bias results.

Table 2. Accuracy and precision of infliximab measured in plasma with mAbXmise kit: results obtained with biosimilars of infliximab (Inflectra® and Flixabi®).

IFX – DILLTQSPAILSVSPGER	Nominal concentration (μg/ml)	Mean calculated concentration (μg/ml)	Precision (%) (n = 6)	Accuracy (%) (n = 6)
Flixabi				
	6	5.9	6.1	-1.7
	25	24.1	4.7	-3.6
	75	72.1	2.4	-3.9
Inflectra				
	6	5.6	4.9†	-6.7
	25	25.4	5.1	1.6
	75	71.9	2.3	-4.2
IFX – SINSATHYAESVK	**Nominal concentration (μg/ml)**	**Mean calculated concentration (μg/ml)**	**Precision (%) (n = 6)**	**Accuracy (%) (n = 6)**
Flixabi				
	6	5.7	2.3	-5
	25	23.1	7.0	-7.6
	75	68.1	5.5	-9.2
Inflectra				
	6	5.9	4.3†	-1.7
	25	25.5	3.3	2.0
	75	76.2	5.9	1.6

†n = 5.
Precision was expressed as coefficient of variation and accuracy as bias compared to the nominal concentration.
IFX: Infliximab.

Table 3. Matrix effect of adalimumab and infliximab.

Proteotypic peptide	Matrix effect (ratio peak area peptides in plasma/peak are peptides in water)							
	Concentration: 6 μg/ml				Concentration: 35 μg/ml			
	MF	Mfi	nMF	CV nMF (%)	MF	Mfi	nMF	CV nMF (%)
ADL – APYTFGQGTK	0.63	0.40	0.64	8.4	0.51	0.52	1.02	7.6
IFX – DILLTQSPAILSVSPGER	1.60	2.02	1.27	9.4	1.34	1.38	1.03	6.3
IFX – SINSATHAESVK	0.32	0.25	0.79	6.5	0.29	0.26	0.91	3.9

MF values are expressed as mean of 6 lots of matrix.
ADL: Adalimumab; CV: Coefficient of variation; IFX: Infliximab; MF: Matrix factor of the analyte; MFi: Matrix factor of the internal standard; nMF: IS-normalized matrix factor.

Metrological traceability

For the two WHO standards tested, the concentrations measured were <20% of the nominal value. The mean bias (n = 4) was 8.2% (CV = 9.6%), 2.8% (CV = 12.0%) and -6.3% (CV = 1.9%), respectively, for peptides IFX_SINSATHYAESVK, IFX_DILLTQSPAILSVSPGER and ADL_APYTFGQGTK, with CV% <15%.

For the two Supelco CRM standards tested, the concentrations measured were 10.79 ± 0.90 mg/ml for IFX (mean of peptides IFX_SINSATHYAESVK and IFX_DILLTQSPAILSVSPGER, n = 4 replicates, CV = 8.33%) and 10.11 ± 0.51 mg/ml for ADA (peptide ADL_APYTFGQGTK, n = 4 replicates, CV = 5.02%), respectively. Thus, the concentrations determined with mAbXmise kit were within ±15% of the nominal value.

Clinical application & methods comparison

Of the 105 IBD patients recruited, plasma IFX and ADL concentrations were measured at a median of 75 months (interquartile range [IQR], 42–112 months) and 18 months (IQR, 8–44 months) after starting treatment, respectively. Overall, 70 samples were assayed for IFX. One sample could not be assayed using the commercial ELISA kit because the volume of leftover plasma was sufficient. The concentrations measured in samples were all above the LLOQ (2 μg/ml). Figure 2 presents Passing-Bablock and Bland–Altman plots for each assay pair. Table 4 summarizes the method agreement between each assay pair. The Bland–Altman analysis revealed a mean bias of -1.8 [-8.8, 5.3] μg/ml between LC-MS/MS and in-house ELISA and -6.1 [18.2, 5.9] μg/ml between LC-MS/MS

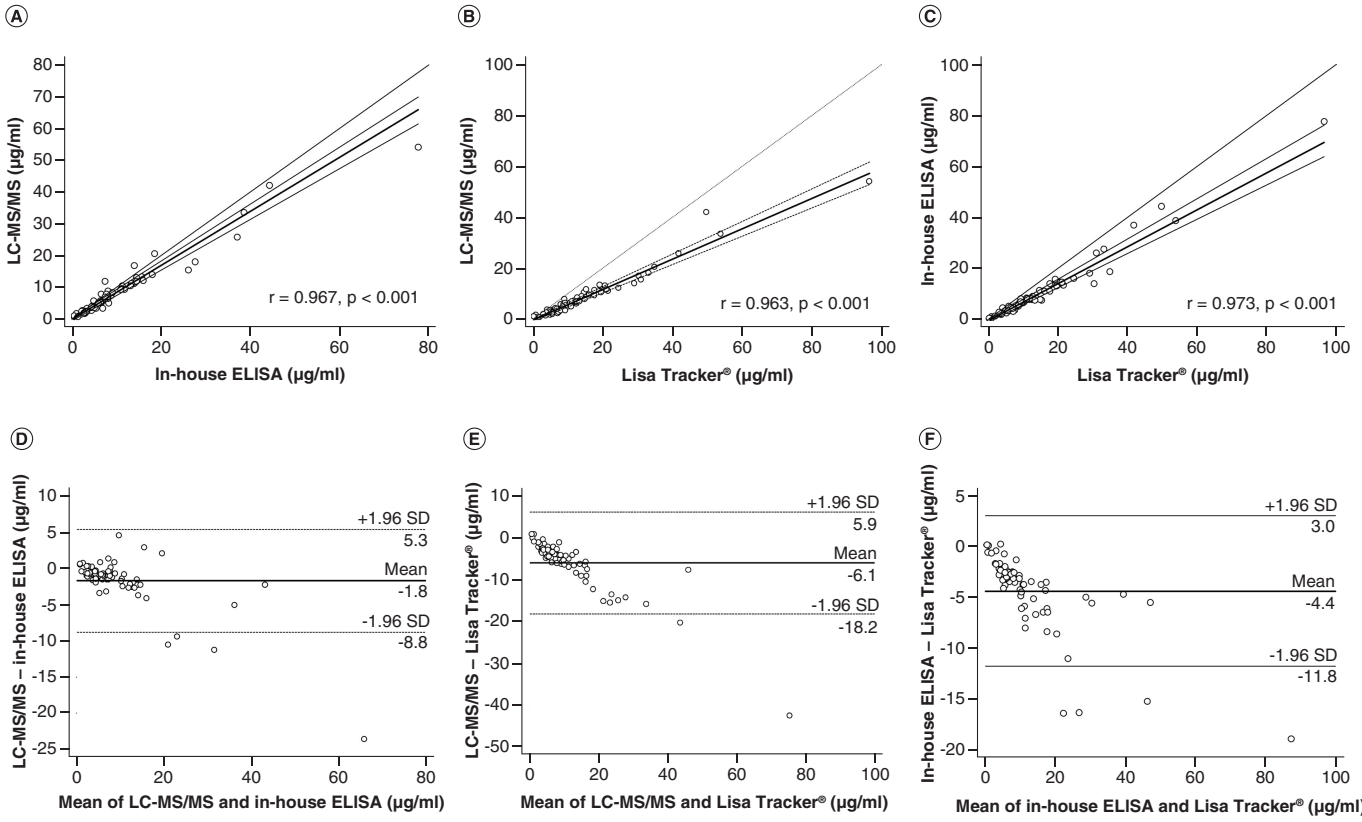

Figure 2. Methods comparisons for infliximab. Passing-Bablok regression plot of infliximab concentrations as measured by **(A)** LC-MS/MS and in-house ELISA, **(B)** LC-MS/MS and the commercial Lisa-Tracker® kit, **(C)** in-house ELISA and the commercial Lisa-Tracker® kit. Concentrations were measured in samples from patients with inflammatory bowel disease treated with IFX (n = 70). Bland-Altman analysis of the difference between **(D)** LC-MS/MS and in-house ELISA, **(E)** LC-MS/MS and the commercial Lisa-Tracker® kit, **(F)** in-house ELISA and the commercial Lisa-Tracker® kit. The mean ±2 standard deviation lines (95% limits of agreement) are plotted for reference. ELISA: Enzyme-linked immunosorbent assay; LC-MS/MS: Liquid chromatography tandem mass spectrometry.

Table 4. Methods comparisons.

Parameter	Passing–Bablok		Bland–Altman absolute differences
	Slope [95% CI]	Intercept [95% CI]	Bias [95% LOA]
Infliximab			
LC-MS/MS vs in-house ELISA (n = 70)	0.85 [0.80, 0.90]	-0.12 [-0.42, 0.31]	-1.8 [-8.8, 5.3]
LC-MS/MS vs kit Lisa-Tracker (n = 69)	0.60 [0.56, 0.64]	-0.31 [-0.77, 0.09]	-6.1 [-18.2, 5.9]
In-house ELISA vs kit Lisa-Tracker (n = 69)	0.72 [0.67, 0.79]	-0.46 [-1.07, 0.03]	-4.4 [-11.8, 3.0]
Adalimumab			
LC-MS/MS vs in-house ELISA (n = 34)	0.80 [0.71, 0.91]	1.14 [0.12, 2.11]	-1.2 [-5.6, 3.3]

LC-MS/MS: Liquid chromatography tandem mass spectrometry; LOA: Limits of agreement.

and the commercial kit Lisa-Tracker. The bias between LC-MS/MS and ELISA tended to be higher for high concentrations levels (Figure 2).

For ADL, 34 samples were included in the statistical analysis because the concentration measured for one sample (2.8%) was below the LLOQ for both methods. The Passing–Bablok regression equation is shown in Figure 3; Bland–Altman analysis is reported in Table 4. Using Bland–Altman analysis, the mean bias was -1.2 [-5.6, 3.3] µg/ml, again with a trend to higher bias for highest concentrations levels (Figure 3).

Table 5 presents the qualitative agreement between different assays based on distinct cutoffs for therapeutic IFX and ADL concentrations. The best qualitative agreement for IFX was observed between LC-MS/MS and in-house ELISA assays, with 33% and 17% of discordant samples for therapeutic (3–7 µg/ml) and supratherapeutic

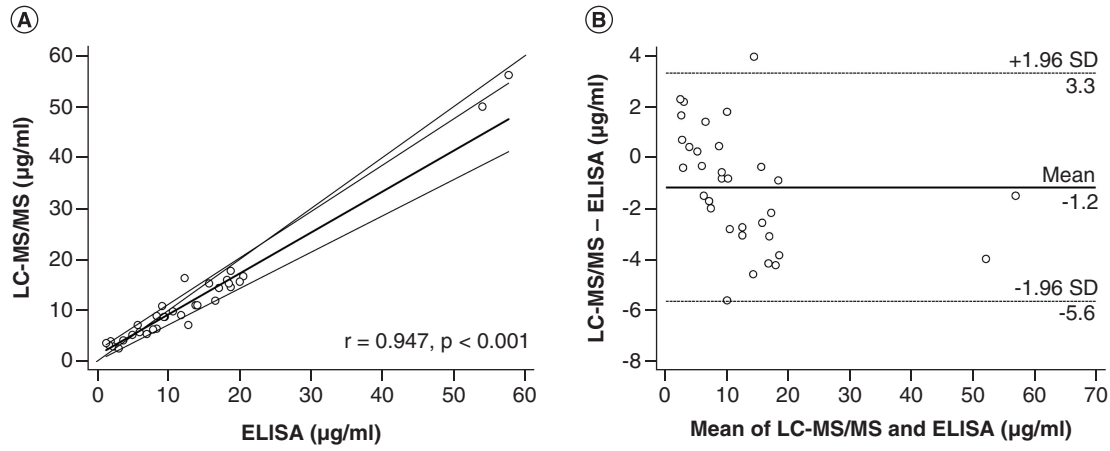

Figure 3. Methods comparisons for adalimumab. (A) Passing–Bablok regression plot of adalimumab concentrations measured by LC-MS/MS and in-house ELISA in samples from patients with inflammatory bowel diseases treated with Adalimumab (n = 34). **(B)** Bland–Altman analysis of the difference between LC-MS/MS and in-house ELISA. The mean ±2 standard deviation lines (95% limits of agreement) are plotted for reference.
LC-MS/MS: Liquid chromatography tandem mass spectrometry.

Table 5. Qualitative agreement in the infliximab and adalimumab concentrations between ELISA and liquid chromatography tandem mass spectrometry assays.

Infliximab		LC-MS/MS				Agreement (%)
		Subtherapeutic (n)	Therapeutic (n)	Supratherapeutic (n)	Total	
In-house ELISA	Subtherapeutic (n)	8	0	0	8	100
	Therapeutic (n)	8	18	1	27	67
	Supratherapeutic (n)	0	6	29	35	83
	Total	16	24	30	70	
Infliximab		**LC-MS/MS**				**Agreement (%)**
		Subtherapeutic (n)	**Therapeutic (n)**	**Supratherapeutic (n)**	**Total**	
Lisa-Tracker kit	Subtherapeutic (n)	4	0	0	4	100
	Therapeutic (n)	9	4	0	13	31
	Supratherapeutic (n)	2	21	29	52	56
	Total	15	25	29	69	
Infliximab		**In-house ELISA**				**Agreement (%)**
		Subtherapeutic (n)	**Therapeutic (n)**	**Supratherapeutic (n)**	**Total**	
Lisa-Tracker kit	Subtherapeutic (n)	4	0	0	4	100
	Therapeutic (n)	4	9	0	13	69
	Supratherapeutic (n)	0	18	34	52	65
	Total	8	27	34	69	
Adalimumab		**LC-MS/MS**				**Agreement (%)**
		Subtherapeutic (n)	**Therapeutic (n)**	**Supratherapeutic (n)**	**Total**	
In-house ELISA	Subtherapeutic (n)	5	0	0	5	100
	Therapeutic (n)	2	12	3	17	71
	Supratherapeutic (n)	0	0	13	13	100
	Total	7	12	16	35	

LC-MS/MS: Liquid chromatography tandem mass spectrometry; subtherapeutic: Infliximab concentration <3 µg/ml, adalimumab concentration <4 µg/ml; supratherapeutic: infliximab concentration >7 µg/ml, adalimumab concentration >12 µg/ml; therapeutic: infliximab concentration ∈3-7 µg/ml, adalimumab concentration ∈4-12 µg/ml. According to Gibson *et al.* [2].

(>7 µg/ml) concentrations, respectively. In contrast, the rates of discordance between LC-MS/MS and the commercial Lisa-Tracker kit were 69 and 44% for therapeutic and supratherapeutic concentrations, respectively.

Finally, the qualitative agreement between LC-MS/MS and in-house ELISA assays for ADL was performant, with 71% of concordant samples in the therapeutic range (4–12 µg/ml).

Discussion

ELISA is the most commonly used method for TDM of IFX and ADL [27]. However, a recent work showed substantial discrepancies of plasma IFX level assayed with three commercial ELISA kits [12], which may lead to inconsistent results between commercially available kits. Chromatographic methods coupled with mass spectrometry detection (LC-MS/MS) represent attractive alternatives to immunoassays [19,28–30]. For instance, they allow multiplexing analysis and also have higher specificity and reproducibility than ELISA methods [20]. Commercial kits for mAbs purification suitable for LC-MS/MS analysis such as mAbXmise, provide comprehensive solutions including a full set of standardized reagents, CAL, IQC and single-use labware, specifically designed for TDM of mAbs used in IBD. It may be attractive for labs aiming to simplify their analytical process.

In the present study, the LC-MS/MS method using the mAbXmise kit was successfully validated according to international guidelines [22,23]. Stabilities of mAbs stored in whole blood or serum/plasma or after extraction have been reported previously [20,24,30,31], and the present study confirmed long-term storage stability at -80°C of both mAbs. This point is critical to guarantee that no drug loss occurs in samples during storage in the context of analysis of the same samples by several laboratories. Whatever the mAb, analytical validation fulfilled acceptance criterion for all the targeted peptides used for quantification over the range measured, with intraday and between-day precision ranging from 1.3 to 11.6%. Compared with ELISA and to LC-MS methods using Fab-selective proteolysis such as nSMOL [29], a strength of the LC-MS/MS assay is the use of stable isotope-labelled full-length analogs as IS that could reduce the analytical variability, especially during the sample preparation process. Finally, the LC-MS/MS-based mAb quantification provides good specificity. Nevertheless, an unpredictable interference with ADL signal in some patient plasma samples was observed in this study. The detection of this interference was reported previously [32]. Because the interference was observed for all four transitions of the peptide APYTFGQGTK, it is likely that this peptide comes from an immunoglobulin present in the repertoire of some patients with a biochemical structure similar to ADL. From our experience, separation of this peak with specific chromatographic conditions is not successful; this interference has also been detected with high-resolution mass spectrometry instruments (data not shown), confirming this hypothesis. In most cases, the impact of the interference on ADL quantification is negligible (<20% of the LLOQ signal). Further investigations are ongoing to solve this issue.

Another advantage of the LC-MS/MS method presented is its fivefold higher upper limit of quantification compared with the two ELISA assays tested in this study (100 vs 20 µg/ml for both mAbs). Thus, in case of high plasma concentrations, the lack of requirement for further sample dilution should reduce inaccuracies related to this dilution step. Furthermore, by avoiding the need for reanalysis of samples after dilution, the higher upper limit should make the delivery of results faster. This time advantage is of particular interest for TDM during the induction phase, when IBD patients can have high circulating concentrations of ADL or IFX. The large linear dynamic range of the LC-MS/MS assay may also be required in maintenance therapy as more and more patients are treated with subcutaneous formulation of anti-TNF. In this context, mAbs concentrations are likely to be higher than those observed with intravenous administration. For instance, in IBD, median trough concentrations observed with the recently launched subcutaneous IFX are greater than 20 µg/ml (median: 21.8 [range: 0.1–54.4] µg/ml according to Schreiber *et al.* [33]). Finally, the fidelity of the LC-MS/MS method over a wide range of concentrations is an additional asset when seeking to characterize pharmacokinetic profiles (including peak concentration measurement) for IFX and ADL during PK/PD studies. A weakness of the method is the lower sensitivity of the LC-MS/MS assay described here compared with the ELISA assays tested; this is an area for future improvement. However, the LLOQ remains relevant for clinical decision-making, as indicated by the results of the qualitative agreement at subtherapeutic concentrations.

In recent years, the introduction of biosimilars for IFX and ADL has resulted in cost savings and led to wider availability of biological treatments for IBD. In this context, it is worth evaluating both innovator and biosimilar quantification when developing a TDM assay. To the best of our knowledge, no such data have been reported. Biosimilars differ from the innovator drug by glycosylation. However, no concerns regarding glycosylation are expected on proteotypic peptides after digestion because it is on the Fc fragment of the mAb and proteotypic peptides of IFX come from digestion of the complementarity-determining regions. In the present study, the satisfying results of within-day experiments for two biosimilars of IFX (Inflectra and Flixabi) for both peptides of IFX confirm that the digestion process did not significantly alter the relevance of proteotypic peptides used for

IFX quantification. In this context and according to EMA guidelines [22], a partial validation including within-day accuracy and precision was enough to attest to the robustness of the LC-MS/MS method for plasma quantification of these biosimilars. Taken together, these results support the suitability of the method presented for TDM of IFX in IBD patients treated with either Inflectra or Flixabi. It was impossible to evaluate the performance of the present method for biosimilars of ADL because these medicines were not available in our centers.

Several studies have reported wide interassay variability between LC-MS assays and other methods for IFX [18,19,34,35]. Most of these studies included an average of 20 patients. A major strength of this comparison study is the large number of patients included (n = 70), which strengthens confidence in the results. Thus, the cross-validation results between the LC-MS/MS assay and the in-house ELISA assay met all validation criteria for the Bland–Altman plot, with a mean bias of -1.8 μg/ml. Nevertheless, it is important to note that the bias between LC-MS/MS and ELISA methods is enhanced for high concentrations, probably because of the difference in linearity ranges between the two methods. The same observation during ADL cross-validation supports this hypothesis. Given that the therapeutic range for IFX is 3–7 μg/ml, these results suggest that the two analytical methods are clinically interchangeable. In addition, the results in terms of qualitative agreement, to aid clinical decision-making, were consistent. Conversely, significant discrepancies were observed in the comparison with the Lisa-Tracker kit. The overestimation observed with Lisa-Tracker kit could be partly explained by nonspecific binding. This result suggests that this commercially available kit cannot be used in place of the other two assays. These findings are consistent with previous results reported by Jourdil *et al.* [35].

For ADL, analytical and qualitative agreement were found to be excellent between the LC-MS/MS and in-house ELISA methods, supporting interchangeability of the two methods for TDM applications in IBD patients treated with this innovator mAb. In the future, similar evaluations will need to be applied to commercially available ELISA kits and other LC-MS/MS methods for both ADL and IFX.

Using an LC-MS/MS method, Nemoz *et al.* [36] recently reported that a threshold value of 6.2 μg/ml IFX was associated with biological remission in IBD patients. The results obtained here suggest that the recommended target concentrations could partly depend on the IFX quantitation method used. In this context, the PK–PD relationship should be determined using an LC-MS/MS method to quantify IFX or ADL to refine the therapeutic range.

Conclusion

The multiplex LC-MS/MS method presented here, based on the mAbXmise kit, was successfully validated for quantification of IFX and ADL in plasma, and then applied to samples obtained from IBD patients treated with innovator drugs. The results show that this method is also suitable for TDM in IBD patients treated with IFX biosimilars, such as Inflectra and Flixabi. The results of cross-validation for IFX show that the mean bias with the LC-MS/MS assay was about threefold higher with the commercial ELISA assay compared with in-house ELISA. These data emphasize the critical nature of the choice of analytical assay used for routine TDM of IFX when seeking to obtain comparable results between laboratories. Overall, the mAbXmise method based on LC-MS/MS is a powerful alternative to ELISA methods for routine TDM of IBD patients treated with IFX or ADL and to explore the PK/PD relationship of these mAbs.

Future perspective

Therapeutic drug monitoring of therapeutic monoclonal antibodies is likely to be more and more widely use is the routine follow-up of patient with inflammatory bowel diseases. The use of separation analytical methods that allow multiplex analysis should develop in parallel to meet this clinical need and to accommodate the instrumental resources of a large number of laboratories.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2022-0057

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Summary points

Background

- This study aimed to validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method using a ready-to-use kit (mAbXmise) to quantify infliximab and adalimumab in human plasma simultaneously; it also compared LC-MS/MS performance with that of two ELISA methods.

Experimental

- Validation of the LC-MS/MS method according to international guidelines for bioanalytical methods.
- One hundred and five plasma samples were analyzed to compare agreement of the concentrations measured with the LC-MS/MS assay and the two ELISA assays.

Results & discussion

- The LC-MS/MS assay analytical performances fulfilled the acceptance criteria.
- The mAbXmise assay was linear from 2 to 100 μg/ml.
- The assay allows accurate measure of infliximab from the originator drug and the biosimilar.
- Results from the LC-MS/MS assay were consistent with those obtained using an in-house ELISA assay for infliximab and adalimumab.

Conclusion

- LC-MS/MS is a promising alternative to ELISA for therapeutic drug monitoring of monoclonal antibodies drugs used in inflammatory diseases.

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- **This article points out the question of interpretation of results when different analytical assays are used.**