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Quantification of dimethyl-ifosfamide and its *N*-deschloropropylated metabolites in mouse plasma by liquid chromatography–tandem mass spectrometry

Alain Deroussent[a,b,*], Stéphanie Rodriguez[a], Sonia Martelli[a], Atmane Seck[a], Estelle Dubus-Daudigeos[a], Didier Desmaële[c], Gilles Vassal[a], Angelo Paci[a]

[a] *CNRS UMR 8203, Vectorologie et Thérapeutiques Anticancéreuses, Université Paris XI, Institut de Cancérologie Gustave Roussy, IRCIV, Villejuif, France*
[b] *Mass Spectrometry Facility, Institut de Cancérologie Gustave Roussy, IRCIV, Villejuif, France*
[c] *CNRS UMR 8076 BioCIS, Faculté de Pharmacie, Université Paris XI, Châtenay-Malabry, France*

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ABSTRACT

Among antitumor oxazaphosphorine drugs, the prodrug ifosfamide (IFO) and its analogs require metabolic activation by specific liver cytochrome P450 (CYP) enzymes to become therapeutically active. New 7,9-dimethyl-ifosfamide analogs have shown greater cytotoxic activity than IFO, whereas side-chain oxidation still occurred leading to monochloroacetone after *N*-dechloropropylation. A sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay was developed and validated for the simultaneous quantitation of the prodrug 7*S*,9*S*-dimethyl-ifosfamide (diMeIFO) and its two inactive metabolites, N^2- and N^3-deschloropropyl-dimethylifosfamide (N^2-DCP-diMeIFO and N^3-DCP-diMeIFO) in mouse plasma. After protein precipitation with methanol, the analytes were separated by isocratic reversed-phase chromatography with (methanol/ammonium formate pH 5.5, 60:40, v/v) and detected by tandem mass spectrometry using multiple reaction monitoring of transitions ions *m/z* 289 → 168 for diMeIFO, *m/z* 213 → 168 for N^2-DCP-diMeIFO, *m/z* 213 → 92 for N^3-DCP-diMeIFO and *m/z* 261 → 154 for IFO (internal standard). The calibration curves were linear over the concentration range of 20–10,000 ng/mL for the three analytes. Mean extraction recoveries from mouse plasma were 99, 96, 99 and 100% for diMeIFO, N^2-DCP-diMeIFO, N^3-DCP-diMeIFO and IFO, respectively. The lower limit of quantitation for diMeIFO and its metabolites was 20 ng/mL in 50 μL plasma. The method was accurate with calculated bias from −5.8 to 4.0% for diMeIFO, from −1.1 to 10.6% for N^2-DCP-diMeIFO and from −6.9 to 9.8% for N^3-DCP-diMeIFO, and precise with coefficients of variation lower than 6.8%, 7.8% and 14.3%, respectively. The assay was successfully applied to a preliminary pharmacokinetic study of diMeIFO and of its metabolites in mice.

1. Introduction

The oxazaphosphorines belong to the alkylating drug class with a wide spectrum of antineoplasic activity. This group includes cyclophosphamide (CPM), ifosfamide (IFO) and trofosfamide, which are important therapeutic agents due to their antitumor and immunomodulatory activities [1]. As prodrugs, they require metabolic activation by specific liver cytochrome P450 (CYP) enzymes to become therapeutically active. Thus, they are metabolised by CYP3A4, CYP2B6 and CYP2C9, leading to ring opening and subsequent production of the active nitrogen mustard, which displays cytotoxicity by DNA cross-linking. IFO, a bis-alkylating agent, was introduced into clinical trials in the 1970s, but its early use was limited by severe urotoxicity consisting in haemorragic cystitis. This side effect led to the development of sodium mercaptoethanesulfonate (mesna) as a safe and effective means of regional uroprotection [2]. Further studies have demonstrated IFO activity against a wide range of tumour types, from soft tissue sarcomas to lymphomas both in adult and paediatric patients [3]. Main adverse effects of IFO include urotoxicity, myelosuppression, nausea and vomiting, neurotoxicity and nephrotoxicity [4]. Chloroacetaldehyde (CAA) metabolite, a by-product of the *N*-dechloroethylation pathway, is known to be responsible for both nephrotoxicity and metabolic neurotoxicity which may be associated with IFO treatment [5–7].

New side-chain modified IFO analogs, 7,9-dimethyl-ifosfamide (diMeIFO), designed to reduce side-chain hydroxylation leading to metabolite monochloroacetone (MCA) through methylation of the C7- and C9-positions, have been synthesized and evaluated [8]. DiMeIFO has an oxazaphosphorine structure with a *N*-chloropropyl

* Corresponding author at: CNRS UMR 8203, Mass Spectrometry Facility, Institut de Cancérologie Gustave Roussy, 114, rue Edouard Vaillant, 94805 Villejuif, France. Tel.: +33 01 42 11 46 95; fax: +33 01 42 11 52 45.
E-mail address: alain.deroussent@igr.fr (A. Deroussent).

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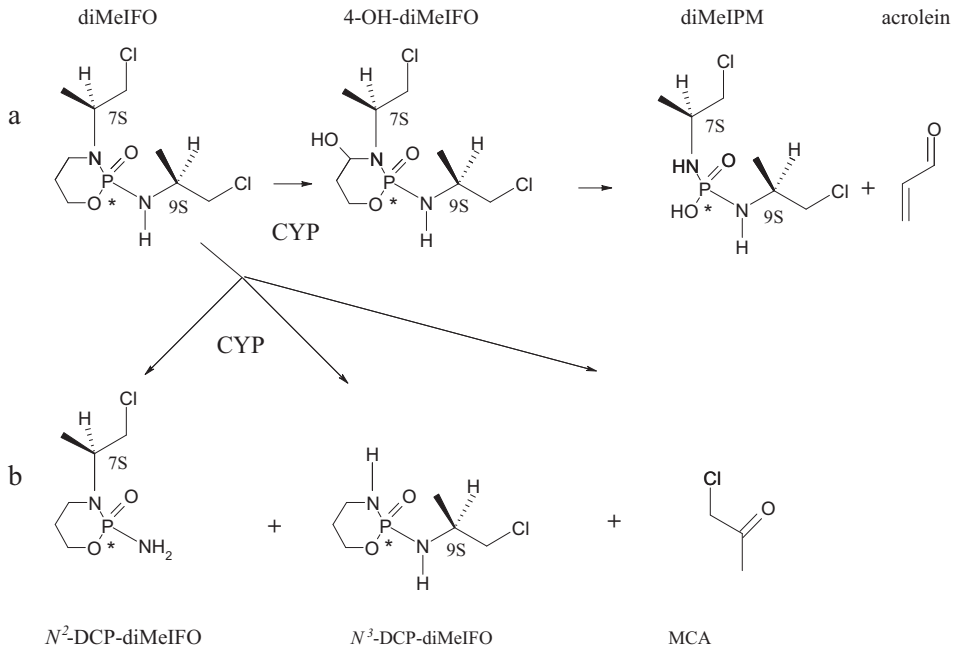

Fig. 1. Activation (a) and inactivation (b) pathways of 7*S*,9*S*-diMeIFO metabolism.

group linked to each nitrogen (Fig. 1). While steric hindrance should decrease enzymatic access to these analogs, the methyl group electron-donor effect reduces oxidability and avoids CAA formation. Experiments on co-cultured microsomal cell assays have demonstrated that the cytotoxic activities of two dimethylated analogs, specially the 7*S*,9*S*-diMeIFO, are greater than those of IFO and CPM, in agreement with alkylating activity assays and metabolic investigations [9].

IFO and its dimethylated analogs are prodrugs requiring biotransformation via cytochrome P450 (CYP) enzymes in order to exert their cytotoxic activity. The hepatic metabolism of diMeIFO has been studied *in vitro* with hepatic rat microsomes [10] and occurs in the liver by two major pathways (Fig. 1). The first pathway involves the 4-hydroxylation of diMeIFO and leads to the production of the cytotoxic dimethyl-isophosphoramide (diMeIPM) mustard and of the urotoxic acrolein by-product. As IFO, 4-hydroxylation of diMeIFO is catalyzed by CYP 3A4 and by CYP 2B6 as demonstrated by induction of rat liver microsomes [9]. The second pathway involves the oxidation of either the exocyclic-N^2- or endocylic-N^3-chloropropyl moieties producing the therapeutically inactive *N*-deschloropropyl-dimethylifosfamide (N^2-DCP-diMeIFO and N^3-DCP-diMeIFO). Similarly to CAA with IFO, an equimolar quantity of MCA should be probably formed during these *N*-dealkylation reactions. For IFO, the *N*-dechloroethylation pathway is regioselective. Furthermore, as it contains a chiral center at the phosphorus atom, enzymatic kinetic resolution is observed and, as a result, (*R*)-IFO and (*S*)-IFO have distinct efficacy and toxicity profiles [11]. Clinical studies and *in vitro* experiments have demonstrated that (*S*)-IFO is more extensively cleared by *N*-dechloroethylation than (*R*)-IFO. *In vitro* metabolism of enantioselectively synthesized of 7*S*,9*S*- and 7*S*,9*R*-diMeIFO analogs has been evaluated by Storme et al. [9]. Further *in vivo* animal studies of the most active 7*S*,9*S* analog need a specific and sensitive quantitative assay based on high performance liquid chromatography–electrospray mass spectrometry (HPLC-ESI/MS) [11].

The simultaneous determination of the oxazaphosphorines, such as IFO and CPM, and their metabolites has been developed using gas chromatography (GC) with sensitive and specific detectors. Thus, the GC–NPD (nitrogen–phosphorus detector) method allowed simultaneous determination of underivatized IFO, N^2-DCE-IFO and N^3-DCE-IFO in plasma after liquid–liquid extraction [12,13]. GC–NPD proved to be more sensitive than GC–MS with a lower limit of quantitation of 50 ng/mL in plasma [14]. Nevertheless, GC–MS assays were used for the determination of IFO in human plasma [15,16], but sample preparation needed derivatization before analysis. Recently, several quantitative LC–MS assays of oxazaphosphorines (CPM and glufosfamide) have been developed in plasma [17,18], but only a few were validated for IFO in human plasma [11] and performed separately with its deschloroethylated metabolites using high sensitive LC–MS/MS [19].

The goal of this work is to develop a specific and sensitive quantitative LC–MS/MS method for the simultaneous determination of diMeIFO and its metabolites N^2-DCP-diMeIFO and N^3-DCP-diMeIFO in mouse plasma in order to compare the pharmacokinetic profile of diMeIFO with IFO. The monitoring of *N*-dechloropropylation metabolism can indirectly quantify *in vivo* the production of MCA metabolite. In this paper, we report the validation of this assay and its use for a preliminary pharmacokinetic application.

2. Experimental

2.1. Chemicals and reagents

Ifosfamide (IFO, HOLOXAN®) (2-chloroethyl)-[3-(2-chloroethyl)-2-oxo-2λ^5-[1,3,2]oxazaphosphinan-2-yl]-amine was provided with 99% purity by Baxter SA (Maurepas, France). 7*S*,9*S*-dimethylifosfamide (diMeIFO) or (2-chloro-1-methyl-ethyl)-[3-(2-chloro-1-methyl-ethyl)-2-oxo-2λ^5-[1,3,2]oxazaphosphinan-2-yl]-amine was synthesized by Alpha chimica (Châtenay-Malabry, France) with 99% purity assessed by LC–MS/MS. N^2-DCP-diMeIFO or 3-(2-chloro-1-methyl-ethyl)-2-oxo-2λ^5-[1,3,2]oxazaphosphinan-2-yl)-amine and N^3-DCP-diMeIFO or (2-chloro-1-methyl-ethyl)-(2-oxo-2λ^5-[1,3,2]oxazaphosphinan-2-yl)-amine were synthesized by BioCIS (Châtenay-Malabry, France) with 99% purity. Their chemical structures were assessed by ^{31}P-, ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy and by electrospray tandem mass spectrometry. HPLC grade methanol was supplied by Carlo Erba (Rodano, Italy). Ammonium formate was provided by Sigma (St.

Quentin-en-Fallavier, France) and formic acid by Merck (VWR, Fontenay-sous-Bois, France). Deionized water was prepared using a Milli-Q (Millipore, St. Quentin-en-Yvelines, France). Drug-free heparinised mouse plasma was purchased to Euromedex (Souffelweyersheim, France).

2.2. Stock solutions

Stock solutions of diMeIFO, N^2-DCP-diMeIFO and N^3-DCP-diMeIFO were prepared separately at 1 mg/mL in methanol. Two solutions of the three mixed compounds were prepared at 200 μg/mL and 10 μg/mL in methanol. The stock solution of IFO as internal standard (IS) is prepared at 1 mg/mL in methanol. Then, the final IS working solution was diluted at 100 ng/mL in methanol. All solutions were then stored at −20 °C.

2.3. Pharmacokinetic profile in mice

Female Swiss athymic mice (average body weight of 25 g) were bred in the Animal Experimentation Unit of Institut Gustave Roussy (IGR). Experiments were carried out under the conditions established by the European Union (directive n^r 56/609/CEE) and validated by the IGR ethic committee. Food and water were given at libitum. Seven xenografted mice with rhabdomyosarcoma were treated with diMeIFO at a dose of 300 mg/kg by *intraperitoneal* injection (600 μL of a solution prepared at 10 mg/mL in (dimethylsulfoxide/chloride sodium, 10:90, v/v). Blood mouse samples (200 μL) were collected twice before injection and once at 1, 5, 15, 30 and 60 min after drug administration. The samples were drawn in heparinarised tubes and then centrifuged at 3000 × *g* for 15 min. Each plasma sample was frozen and stored at −20 °C prior to extraction and analysis in duplicate.

2.4. Plasma preparation and analyte extraction

Calibration standard and quality control (QC) plasma samples were prepared in 475 μL mouse plasma by adding a 25 μL volume of appropriate working solutions containing diMeIFO, N^2-DCP-diMeIFO and N^3-DCP-diMeIFO to provide eight standards at 20, 50, 100, 500, 1 000, 2500, 5000 and 10,000 ng/mL and five QCs at 20, 50, 200, 2000 and 8000 ng/mL. Standard, QC and unknown plasma samples (50 μL) were extracted by protein precipitation with 100 μL volume of methanol containing internal standard (100 ng/mL methanol). After mixing, the plasma samples were centrifuged at 4 °C at 10,000 × *g* for 10 min. Then, 75 μL of the supernatant were diluted in a glass microvolume vial with 75 μL ammonium formate 5 mM before LC–MS/MS analysis with a 10 μL sample injection.

2.5. LC–MS/MS conditions

Analyses were performed using a 1100 series HPLC system (Agilent Technologies, Massy, France) including an autosampler, a binary pump and a degasser fitted with an Uptisphere® C18 5 μm column, 2.1 mm i.d. × 100 mm length (Interchim, Montluçon, France). Isocratic elution was achieved with a flow rate of 0.25 mL/min using the mobile phase (methanol/5 mM ammonium formate, pH 5.5, 60:40, v/v). The run time is 8.0 min. Before each sample injection, the autosampler was washed twice with 20 μL of methanol/water (80:20, v/v). Detection was performed with a Quattro®-LCZ triple quadrupole mass spectrometer equipped with the orthogonal electrospray source (Micromass, Waters, Manchester, UK). Analytes were detected in the positive electrospray ion mode using tandem mass spectrometry with multiple reaction monitoring (MRM). The dwell time was set at 250 ms for all compounds. The capillary voltage and the cone voltage were set at 3500 V and 30 V, respectively. The source temperature and the nebulisation gas temperature were set at 90 °C and 250 °C, respectively. Nitrogen gas flow was set at 500 L/h. Collision gas (argon) pressure was set at 1.3×10^{-3} mbar. Product ion mass spectrum was achieved for each analyte by infusion at 10 μL/min with a Harvard syringe pump. Mass transitions monitored were m/z 213 → 168 for N^2-DCP-diMeIFO, m/z 213 → 92 for N^3-DCP-diMeIFO, m/z 289 → 168 for diMeIFO and m/z 261 → 154 for IFO. The collision energy was optimized at 20 eV for the three compounds and at 25 eV for IFO. Mass spectra were processed using Masslynx™ and analytes were quantified with Quanlynx™ software (Micromass, Waters, Manchester, UK).

3. Analytical assay validation

The LC–MS/MS assay was validated according to the international guidelines requirements (ICH Q2R) in terms of selectivity, linearity, sensitivity, accuracy and precision [20] and based on the recommendations published on-line by the Food and Drug Administration (FDA) [21]. Extraction yields and matrix effects evaluation was based on Matuzewski recommendations [22].

3.1. Selectivity

Several control samples prepared from different mice plasma were analyzed for testing interference using the described LC–MS/MS assay. Selectivity for the four analytes was performed as well with mouse plasma spiked at LLOQ.

3.2. Extraction recovery and matrix effects

Extraction recoveries in mice plasma for the four analytes (IFO, diMeIFO, N^2-DCP-diMeIFO and N^3-DCP-diMeIFO) were determined at three levels of QC samples: 200, 2000 and 8000 ng/mL. They were calculated as the ratio of analyte peak area from extracted QC plasma to mean peak area from extracted blank plasma spiked with the neat solutions.

The matrix effect variation was evaluated using extracted blank samples prepared from different mice plasma spiked with the analytes at three QCs concentrations. Matrix effects for the compounds (N^2-DCP-diMeIFO, N^3-DCP-diMeIFO, diMeIFO and IFO) were determined as the ratio of analyte peak area from extracted blank plasma spiked with the neat solutions to the mean peak area of the neat solutions at the same concentration prepared in solvent (methanol/ammonium formate 5 mM, 50:50, v/v).

3.3. Linearity and limits of quantification and of detection

Calibration curves were plotted between the peak area ratio of the analyte and of the internal standard against the nominal concentration of analyte. $1/x^2$ weighted least-squares linear regression was fitted over the 20–10,000 ng/mL concentration range using Quanlynx™ software. Mean regression equations were expressed as $y = a\,x + b$, where y corresponds to the analyte/IS peak area ratio and x the concentration of analyte. The lower limit of quantitation (LLOQ) of the assay was the lowest concentration of analyte with recommended precision and accuracy lower than 20% [20,21]. The lower limit of detection (LLOD) for the three analytes was calculated with signal to noise equal to 3, using the ratio of the standard deviation of the intercept (b) and of the mean slope (a) from three calibration curves.

3.4. Accuracy and precision

QCs were prepared for each analyte in mouse plasma at five concentrations (20, 50, 200, 2000, 8000 ng/mL) of three replicates

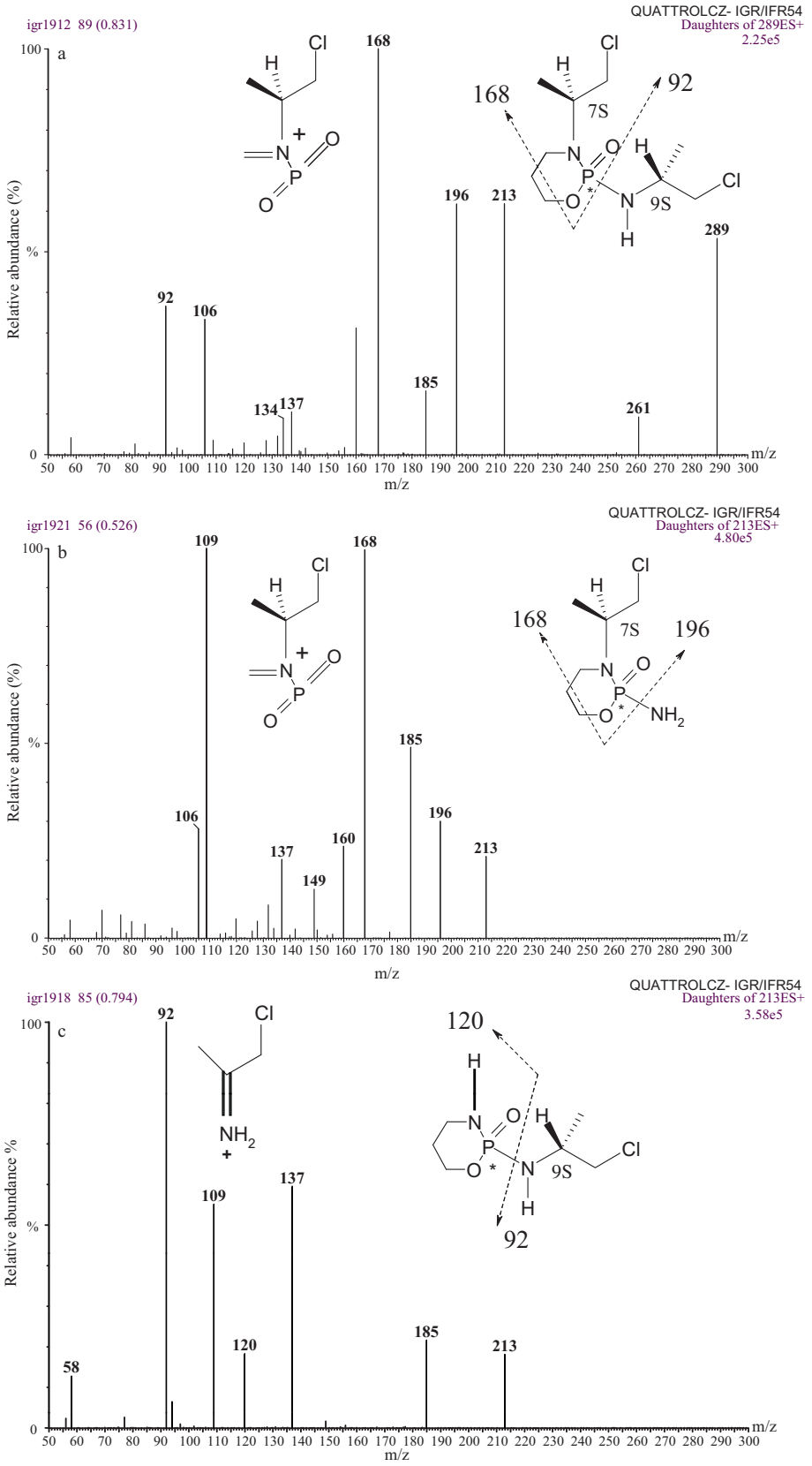

Fig. 2. Product ion mass spectrum and chemical structure of (a) diMeIFO, (b) N^2-DCP-diMeIFO and (c) N^3-DCP-diMeIFO.

for each run over three days. In addition, a twenty-fold dilution of QC plasma of 20,000 ng/mL was performed in order to evaluate the variation of preparation of unknown samples with higher concentrations than the upper limit. Accuracy was measured by the deviation or bias (%) of the mean found concentration from the actual concentration. Within-run precision or repeatability expressed as the coefficient of variation (CV_r), was investigated for the five levels of QCs with three replicates prepared the same day. Between-run

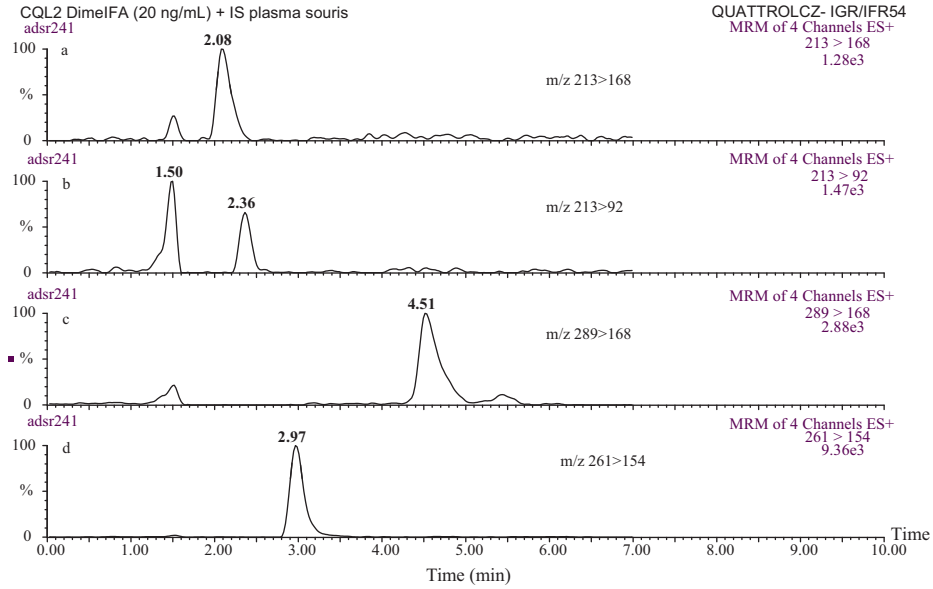

Fig. 3. MRM chromatograms of mouse plasma sample spiked at LLOQ of 20 ng/mL of (a) N^2-DCP-diMeIFO, with a retention time (t_R) of 2.1 min, (b) N^3-DCP-diMeIFO, with t_R of 2.4 min, (c) diMeIFO, with t_R of 4.5 min and (d) 200 ng/mL of IFO (IS), with t_R of 3.0 min.

or intermediate precision, expressed as the coefficient of variation (CV_i), was evaluated for each QC level with nine replicates.

3.5. Stability

Stability of the three extracted analytes from QCs plasma was evaluated in solvent (ammonium formate pH 5.5/methanol (50:50, v/v) on the autosampler at room temperature of 20 °C. Stability of the analytes in mouse plasma (three QC levels) was determined at room temperature and after three freezing at −20 °C and thawing cycles at room temperature. The analytes were considered stable when the accuracy bias was within ±5% of the nominal concentration.

4. Results and discussion

4.1. Mass spectra of diMeIFO and its N-deschloropropylated metabolites

The electrospray mass spectra of IFO, diMeIFO, N^2-DCP-diMeIFO, N^3-DCP-diMeIFO standards were performed to assay their molecular mass. For diMeIFO (M = 288.1), the pseudo-molecular ion [MH, $^{35}Cl^{35}Cl]^+$ was observed at m/z 289.1 with its chloride isotopic ion [MH, $^{35}Cl^{37}Cl]^+$ at m/z 291.1. For N^2-DCP-diMeIFO and N^3-DCP-diMeIFO (M = 212.1), the pseudo-molecular ion [MH, $^{35}Cl]^+$ was observed at m/z 213.1 with their chloride isotopic ion [MH, $^{37}Cl]^+$ at m/z 215.1.

Then, the product ion mass spectrum of the three compounds and of internal standard (IFO) was achieved under CID-MS/MS conditions. For diMeIFO, the selected precursor ion (m/z 289) provided the main product ion of m/z 168 according to the proposed cleavage (Fig. 2a), whereas the mass spectrum of IFO showed a precursor ion at m/z 261 and product ions at m/z 154 and 92, as shown by previously published results [10]. However, the same precursor ions (m/z 213) of N^2-DCP-diMeIFO and N^3-DCP-diMeIFO produced intense product ions at m/z 168 and m/z 92, respectively, according to their specific fragmentation pathways (Fig. 2b and c).

4.2. Optimisation of the LC–MS/MS conditions

Isocratic chromatographic elution used an optimised composition of methanol and ammonium formate buffer (5 mM, pH 5.5) to provide the selective separation and detection of the two hydrophilic *N*-deschloroalkylated metabolites from the parent drug, diMeIFO, within a short run time of 5 min. This analytical method allowed the separation based on reversed-phase chromatography and on the different MRM detections of the three compounds and of IFO (IS). The retention times were 2.1, 2.4, 3.0 and 4.5 min, for N^2-DCP-diMeIFO, N^3-DCP-diMeIFO, IFO and diMeIFO, respectively (as shown Fig. 3). An additional peak eluted at 5.3 min corresponding probably to a minor diastereoisomer of the racemic diMeIFO compound due to the chirality of phosphorus atom.

4.3. Performance results of the LC–MS/MS assay

Assay selectivity using the specific mass transitions was shown by analysis of control mouse plasma spiked with IS (IFO) at 200 ng/mL plasma (Fig. 4). MRM chromatograms of control mouse plasma showed at the retention time of each analyte no co-eluting peaks (area < 10%) of diMeIFO, N^2-DCP-diMeIFO and N^3-DCP-diMeIFO at the LLOQ level.

4.3.1. Extraction recovery and matrix effect

The extraction recovery for the three compounds was calculated in mouse plasma at three QC levels and for IS at 200 ng/mL using protein precipitation. Mean extraction recoveries of 99, 96, 99 and 100% were found for diMeIFO, N^2-DCP-diMeIFO, N^3-DCP-diMeIFO and IFO, respectively (Table 1). These good values agreed with those previously described with ethyl acetate extraction for IFO and its metabolites in plasma [15]. No significant matrix effect (ME) for diMeIFO drug and for IFO internal standard was observed in extracted mouse plasma spiked with three QC levels. ME ratios of 98 and 92% were obtained for diMeIFO and IFO, respectively (Table 1). But, although some ion suppression (<30%) occurred for N^2-DCP-diMeIFO and N^3-DCP-diMeIFO, the ME variability of several mouse plasma samples never exceeded 16%, which indeed demonstrated that the proposed extraction procedure was able to, if not eliminate, at least normalize these matrix effects.

4.3.2. Linearity and sensitivity

The calibration curve was linear over the concentration range of 20–10,000 ng/mL for each compound using $1/x^2$ weighted least-squares regression. The coefficient of determination (r^2) was

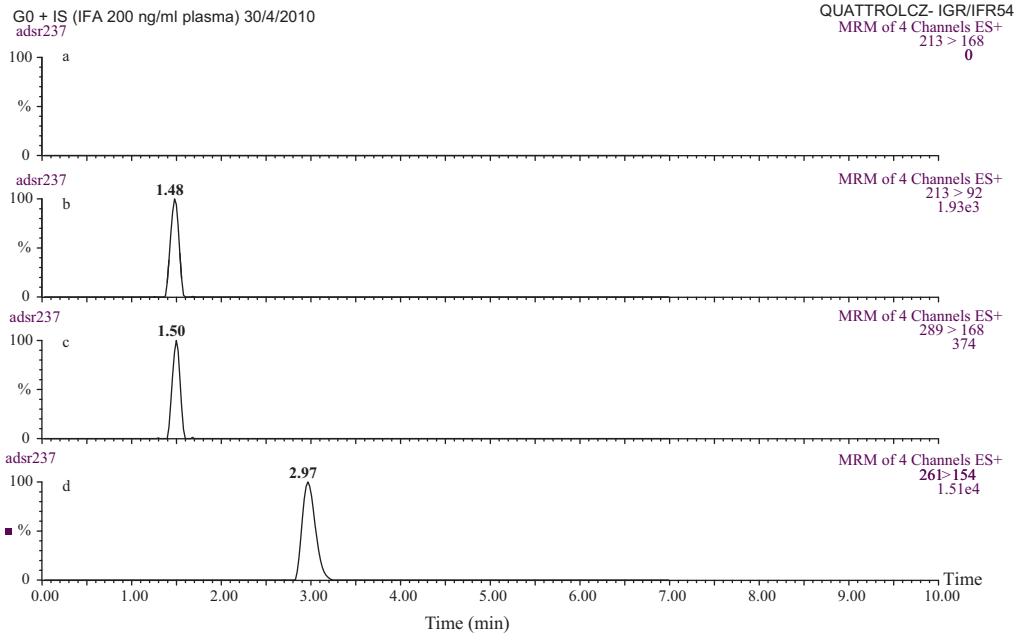

Fig. 4. MRM chromatograms of control mouse plasma spiked with IFO (200 ng/mL) monitoring transitions of (a) m/z 213 → 168, (b) m/z 213 → 92, (c) m/z 289 → 168 and (d) m/z 261 → 154.

Table 1
Extraction yield and matrix effect of the four compounds in mouse plasma.

Compound	diMeIFO	N^2-DCP-diMeIFO	N^3-DCP-diMeIFO	IFO
Extraction yield (%)	99 (17%)	96 (29%)	99 (23%)	100 (8%)
Matrix effect (%)	98 (16%)	76 (15%)	70 (16%)	92 (5%)

Mean of nine replicates with precision (CV %) were performed for the three QC levels.

better than 0.99 for diMeIFO and N^2-DCP-diMeIFO and 0.98 for N^3-DCP-diMeIFO. The regression equations were $y = 10.10 \times 10^{-3}$ $x + 0.24 \times 10^{-3}$ for diMeIFO, $y = 1.78 \times 10^{-3}$ $x + 10.59 \times 10^{-3}$ for N^2-DCP-diMeIFO and $y = 2.34 \times 10^{-3}$ $x + 34.26 \times 10^{-3}$ for N^3-DCP-diMeIFO. The slope of each regression line was reproducible with relative standard deviation less than 10%. Thus, the LLOD were determined at 10 ng/ml for the three analytes. The LLOQ for all compounds was set as 20 ng/mL in mouse plasma in agreement with accuracy bias lower than ±20%.

4.3.3. Accuracy and precision

The inter-assay accuracy, the intermediate precision (CV_i) and repeatability (CV_r) of the assay for QCs at five concentrations from 50 to 8000 ng/mL are summarized in Table 2. Within- and between-run QCs accuracy bias ranged from −5.8 to 4.0% for diMeIFO (CV < 7.1%), from −1.1 to 10.6% for N^2-DCP-diMeIFO (CV < 9.1%) and from −6.9 to 9.8% for N^3-DCP-diMeIFO (CV < 14.3%). For LLOQ at 20 ng/mL, accuracy bias was within 17% with CV of 15.3, 6.6 and 7.8% for diMeIFO and its two metabolites, respectively. In addition, as some early-collected mouse plasma samples needed to be diluted with drug-free mouse plasma before analysis within the calibration range, no effect of the dilution factor was observed in QCs (accuracy bias of 4% with CV of 11%). The accuracy values for the three analytes were within ±15% for all QCs and ±20% for LLOQ with acceptable precisions. Thus, the LC–MS/MS assay can be considered accurate and precise for the three analytes within the range 20–10,000 ng/mL and can be extended to higher concentrations.

4.3.4. Stability

Observed accuracies between freshly extracted analytes from mouse plasma and from samples kept for 4 h at room temperature showed variations from −2.1 to 6% for diMeIFO and from

Table 2
Between-run accuracy and precision and repeatability for the three analytes in QCs mouse plasma.

Compound	Concentration (ng/mL)	20	50	200	2000	8000
diMeIFO	Mean c (ng/mL)	20.5	50.4	200.3	1884	7945
	Bias (%)	2.3	0.9	0.1	−5.8	−0.7
	CV_i (%)	15.3	5.9	6.8	5.4	6.0
	CV_r (%)	5.5	3.5	2.2	7.1	5.1
N^2-DCP-diMeIFO	Mean c (ng/mL)	23.3	50.7	219.5	2069	7911
	Bias (%)	16.4	1.4	9.7	3.4	−1.1
	CV_i (%)	6.6	7.8	4.4	7.7	4.4
	CV_r (%)	2.3	5.7	4.3	9.1	6.1
N^3-DCP-diMeIFO	Mean c (ng/mL)	21.9	48.7	216.2	1987	7482
	Bias (%)	9.3	−2.6	8.1	−0.7	−6.9
	CV_i (%)	7.8	13.6	10.9	8.1	14.3
	CV_r (%)	11.4	12.2	4.5	9.2	9.1

Precision (CV_i %) and repeatability (CV_r %) were determined with nine and three replicates for each QC.

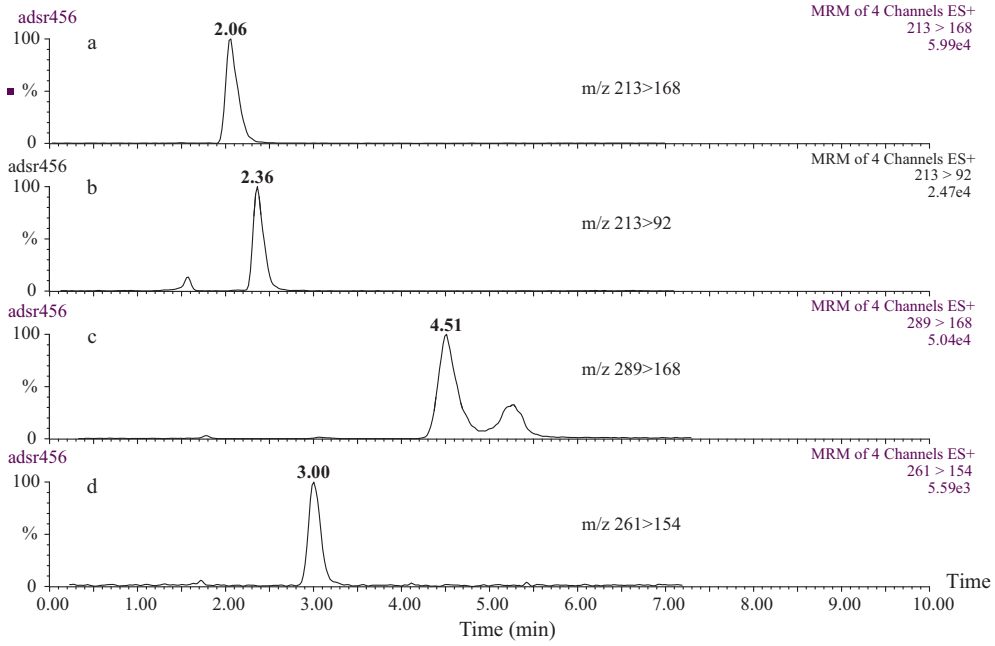

Fig. 5. MRM chromatograms of (a) N^2-DCP-diMeIFO at 719 ng/mL, (b) N^3-DCP-diMeIFO at 1344 ng/mL, (c) diMe-IFO at 4958 ng/mL and (d) 200 ng/mL of IFO (IS) in mouse plasma sample collected at 15 min after injection at 300 mg/kg.

3.2 to 13% for N^2-DCP- and N^3-DCP-diMeIFO. Accuracy deviation for plasma samples after three freeze/thaw cycles showed variations lower than −11% for diMeIFO, −3% for N^2-DCP- and 12% for N^3-DCP-diMeIFO. Thus, the three compounds can be considered stable in mouse plasma for 4 h preparation time and after freezing/thawing. Moreover, there was no significant variation (<10%) of extracted analytes in solvent (pH 5.5/methanol 50:50, v/v) for 24 h at room temperature, for one week at 4 °C and for 2 months storage at −20 °C in methanol/DMSO (90:10, v/v).

The LC–MS/MS assay showed good sensitivity and recovery in mouse plasma for the direct determination of diMeIFO and its two *N*-deschloropropylated metabolites. The LC–MS/MS sensitivity in mouse plasma was improved twice compared with that of the previous GC/NPD assay [14] using a 50 μL plasma sample. The method showed rapid preparation, compared to other liquid/liquid plasma extractions used with other GC assays. Thus, this LC–MS/MS assay could be used to quantify the prodrug diMeIFO and its metabolites in plasma for pharmacokinetic studies.

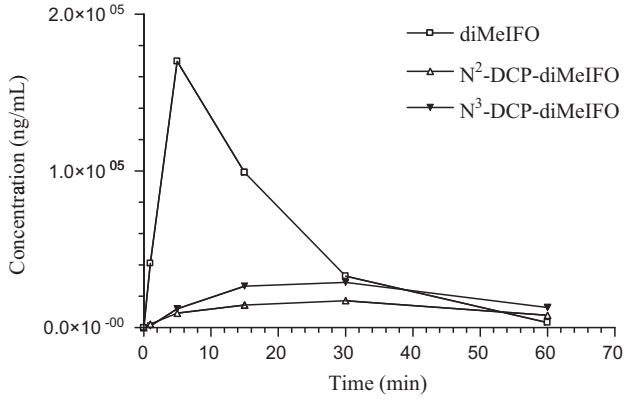

Fig. 6. Plasma concentration-time profiles of diMeIFO and of its metabolites N^2-DCP-diMeIFO and N^3-DCP-diMeIFO after 300 mg/kg injection of diMeIFO in mice.

4.4. Pharmacokinetic profile in mice

The validated LC–MS/MS assay of diMeIFO and its metabolites was applied to a preliminary pharmacokinetic study in mice treated with a dose of diMeIFO at 300 mg/kg. The LC–MS/MS analysis of diluted mouse plasma at 15 min was shown in Fig. 5. Then, the pharmacokinetic profiles of diMeIFO and its metabolites were displayed (Fig. 6) and allowed to calculate the pharmacokinetic parameters such as the half-time ($t_{1/2}$) and the maximal concentration (C_{max}). A short half-time ($t_{1/2}$) of 9.0 min in mouse plasma was observed close of that of IFO ($t_{1/2}$ 7.0 min) previously described [23,24]. At 30 min, maximal concentrations of 17,310 and 28,919 ng/mL were determined for the metabolites N^2-DCP-diMeIFO and N^3-DCP-diMeIFO, respectively. Thus, *in vivo* diMeIFO was rather metabolised into N^3-DCP- than N^2-DCP-diMeIFO, as previously demonstrated *in vitro* with the 7*S*,9*S* analog [10].

5. Conclusion

The validated LC–MS/MS assay is suitable to the quantification of diMeIFO and its N^2- and N^3-deschloropropylated metabolites in mouse plasma using IFO as internal standard. The method shows good specificity, linearity, accuracy and precision for the determination of all compounds in the range of 20–10,000 ng/mL using 50 μL of mouse plasma. This LC–MS/MS assay could be useful for further preclinical pharmacokinetic studies of diMeIFO prodrug. *In vivo* biotransformation of diMeIFO into N^2- and N^3-deschloropropylated metabolites will be evaluated and could provide an indirect determination of monochloroacetone, produced by the side-chain oxidation of diMeIFO metabolism.

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