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Journal of Chromatography B, 820 (2005) 251–259
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatography–mass spectrometry assay for quantitation of ifosfamide and its *N*-deschloroethylated metabolites in rat microsomal medium

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Received 18 November 2004; accepted 30 March 2005
Available online 25 April 2005

Abstract

A specific and sensitive quantitative assay has been developed using high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS) for the simultaneous quantitation of the antitumor drug ifosfamide (IFM) and its two metabolites, N^2-deschloroethylifosfamide (N^2-DCE-IFM) and N^3-deschloroethylifosfamide (N^3-DCE-IFM) in microsomal medium. The analytes and the internal standard (cyclophosphamide) were isolated by ethylacetate extraction from rat liver microsomes. They were analysed on a Nucleosil® C18 HD column (125 mm × 4 mm, 5 μm) using a step gradient with the mobile phase (2 mM ammonium formate and methanol). The HPLC–ESI-MS method used selected ion monitoring of ions *m*/*z* 199.1 Th and *m*/*z* 261.1 Th and was validated in the concentrations ranges of 100–5000 ng/mL for IFM and 50–2500 ng/mL for its *N*-deschloroethylated metabolites (DCE-IFM) with good accuracy and precision (CV less than 15%). The low limits of quantitation (LLOQ) were found at 50 ng/mL for *N*-deschloroethylated metabolites and at 100 ng/mL for the parent drug (IFM). The method was applied for the determination of ifosfamide and its *N*-deschloroethylated metabolites in rat microsomal incubations.

Keywords: Ifosfamide; Cyclophosphamide; Microsomal metabolism; HPLC–ESI-MS; Neurotoxicity

1. Introduction

The bisalkylating agent ifosfamide (IFM) 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 [1]. Further studies have demonstrated IFM activity against a wide range of tumour types, from soft tissue sarcomas to lymphomas both in adult and paediatric patients. Main adverse effects of IFM include urotoxicity, myelosuppression, nausea and vomiting, neurotoxicity and nephrotoxicity [2]. Le Cesne et al. have shown that high-dose regimen of IFM (HD-IFM) allowed the circumvention of resistance to standard-dose ifosfamide in advanced soft-tissue sarcomas, indicating that there is a dose–effect relationship [3]. Since the systematic use of adjuvant treatments such as mesna, granulocyte-macrophage colony-stimulating factor (GM-CSF) and setrons, and the increase of IFM dosages, neurotoxicity and nephrotoxicity are the limiting factors for IFM-based chemotherapy. Indeed, in some studies, up to 40% of the treated patients show neurological disorders (depending on the dose quantity and the

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doi:10.1016/j.jchromb.2005.03.023

administration mode) and 5% presents a Fanconi syndrome. These toxicities seem to be more frequent with children [4,5].

Ifosfamide is a prodrug: metabolism is needed to obtain its active form. The initial activation reaction in IFM metabolism is mediated by the cytochrome P450 enzyme CYP3A4 (Fig. 1). The hydroxylation on the Carbon-4 of the oxazaphosphorine ring leads to 4-hydroxy-ifosfamide (4-OH-IFM), which is in equilibrium with its tautomeric form, the aldo-ifosfamide. The latter form may then either be oxidized by an aldehyde dehydrogenase (ALDH1) to carboxy-ifosfamide, an inactive metabolite, or it can spontaneously be decomposed by a retro-Michaël reaction to form acrolein and the isophosphoramide mustard (IPAM) which is the active moiety. IPAM is a bisalkylating agent. Acrolein is held responsible for urotoxicity. Up to 50% of a dose of IFM undergoes a separated oxidative *N*-dealkylation reaction, resulting from the loss of chloroethyl side-chains and producing N^2-deschloroethylifosfamide (N^2-DCE-IFM), N^3-deschloroethylifosfamide (N^3-DCE-IFM) and N^2,N^3-dideschloroethylifosfamide (N^2,N^3-diDCE-IFM). An equimolar quantity of chloroacetaldehyde (CAA) is formed in each of these *N*-dealkylation reactions [6,7]. This metabolite is known to be responsible for both nephrotoxicity and metabolic neurotoxicity which may be associated with IFM treatment [8].

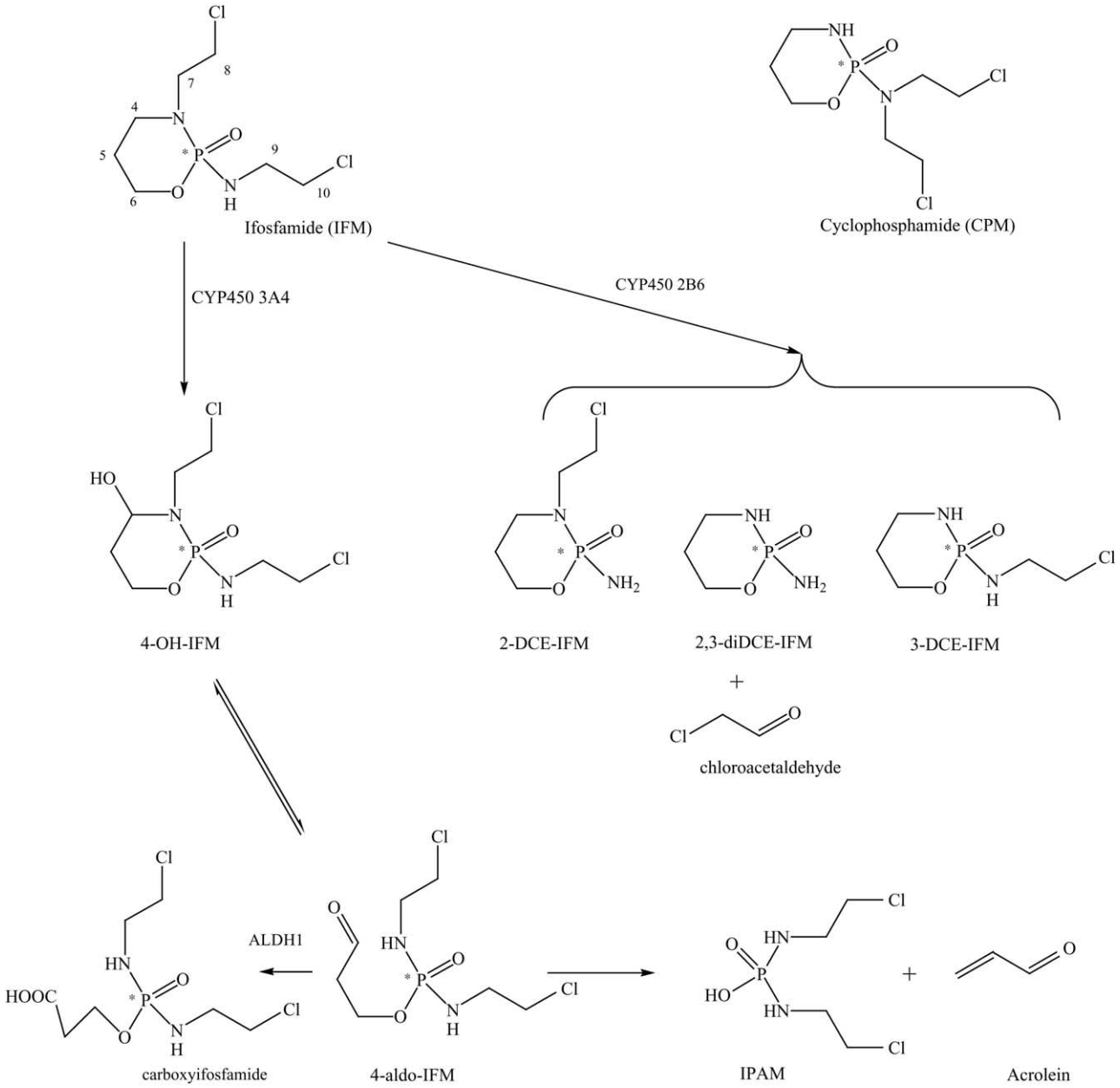

Fig. 1. Activation and inactivation pathways of ifosfamide metabolism.

The direct quantitative determination of the oxazaphosphorines, such as IFM and cyclophosphamide (CPM), and their metabolites is difficult, because of their high polarity and their chemical and thermal properties. Thus, several analytical methods have been developed using gas chromatography (GC) or high performance liquid chromatography (HPLC) combined with different detection techniques [9]. The UV detection of oxazaphosphorine compounds is also problematic due to their poor spectral properties. After a GC separation, the most appropriate detector seems to be a nitrogen phosphorus detector (NPD). Because NPD has high selectivity and sensitivity for oxazaphosphorine compounds, as well as a small solvent peak in comparison with flame ionization detector [10]. A GC–NPD technique allowed simultaneous determination of IFM, N^2-DCE-IFM and N^3-DCE-IFM in plasma after liquid–liquid extraction and without derivatization [11]. Kerbusch et al. compared GC–NPD with GC-positive ion electron-impact ion-trap tandem mass spectrometry [12]. GC–NPD proved to be superior to GC–MS^2 in terms of sensitivity (LOQs 50 ng/mL and 250–500 ng/mL, respectively), and detection range and as well for accuracy and precision. Moreover, mass spectrometry detection has been used successfully with GC or HPLC for the oxazaphosphorine compounds determination [10]. The described GC–MS methods were used successfully for the sensitive determination of IFM in human plasma or in urine [13], but sample preparation needed derivatization before analysis and was time-consuming.

The purpose of this work was to develop a simple, sensitive and effective quantitative HPLC–ESI-MS method to study the in vitro metabolism of IFM and other oxazaphosphorines analogues in rat microsomes. In precedent works [14], we have developed synthesis of methylated IFM analogues to reduce side-chain hydroxylation which leads to toxic metabolites such as chloroacetaldehyde (CAA). The monitoring of the N^2-DCE-IFM and N^3-DCE-IFM formation can indirectly quantify the production of toxic and labile CAA.

A fast and effective assay is necessary to determine the concentrations of deschloroethylated metabolites (DCE-IFM) and of IFM in microsomal medium that will be useful to confirm the enzymes involved in the side-chain oxidation of IFM and the synthesized analogues. Since there is no available assay for the concomitant direct detection of IFM and its metabolites without derivatization in rat liver microsomes, the present HPLC–ESI-MS method has been developed and validated for the simultaneous determination of N^2-DCE-IFM, N^3-DCE-IFM and IFM.

2. Experimental

2.1. Chemicals and reagents

Ifosfamide (HOLOXAN®) (2-(2-chloroethylamino)-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide) and cyclophosphamide (ENDOXAN®) (2-[bis-(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide) were obtained from Baxter SA (Maurepas, France). N^2-DCE-IFM ([3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphonan-2-yl]amine) and N^3-DCE-IFM (*N*-(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphinan 2-oxide) were synthesized according to previously developed techniques [14]. Their purity and chemical structures were assessed by ^{31}P, ^{13}C, ^{1}H NMR study, and mass spectrometry. Reagents and HPLC grade solvents (methanol, ethylacetate) and ammonium formate were obtained from Carlo Erba (Rodano, Italy). Deionized water was prepared using a UHQ II system (USF^{ELGA} SA, Trappes, France). Thermally inactivated rat liver microsomes were prepared for validation of the analytical method. Rat microsomes were prepared according to Abernathy et al. [15].

2.2. Liquid chromatographic and mass spectrometric conditions

Analyses were performed using a HPLC Surveyor® chromatographic system consisting in a quaternary pump and an autosampler (ThermoFinnigan, Courtaboeuf, France) controlled by the Xcalibur® software. Analytes were separated on a Nucleosil® C_{18} HD column 5 μm, 125 mm × 4 mm, (Macherey-Nagel, Hoerdt, France) and a LiChroCART® 4-4 precolumn RP-18e, 5 μm, 4 mm × 4 mm, (Merck KgaA, Darmstadt, Germany. A step gradient with a flow-rate of 0.5 mL/min was achieved with the mobile phase consisting of eluant A (2 mM ammonium formate aqueous, pH 5.7) and eluent B (methanol). The initial mixture of eluents A and B was set at (80:20, v/v) until 9 min, allowing the separation of N^2-DCE-IFM and N^3-DCE-IFM. Then, the gradient was increased up to (55/45, v/v) from 9 to 9.5 min and was kept for 10.5 min, then back again to the initial conditions for 4 min. The gradient conditions were set in order to reduce the analysis time and to allow good separation of IFM and CPM (IS). Thus the duty cycle of the assay was 25 min.

HPLC–ESI-MS analyses were performed with a LCQ_{DUO}® ion-trap mass spectrometer (ThermoFinnigan, Courtaboeuf, France), operating in electrospray positive-ion and in selected ion monitoring (SIM) mode for the detection of IFM (*m*/*z* 261.1 Th) and the internal standard CPM (*m*/*z* 261.1 Th), and for N^2-DCE-IFM (*m*/*z* 199.1 Th) and N^3-DCE-IFM (199.1 Th).

For the MS detection setup, two equimolar solutions of both metabolites and both drugs (IFM and CPM) were properly diluted in the initial mobile phase (A/B, 80:20, v/v). Each solution was infused at 0.5 mL/min and a set-up process optimized the cone tension, lenses and octapole parameters in order to obtain the best detection of the ions *m*/*z* 199.1 Th and 261.1 Th. The ESI probe temperature was set at 250 °C. The ion-spray voltage and the capillary voltage were set at 4500 and 8 V, respectively. Nitrogen was used as sheath gas and helium was used as auxiliary gas. The sheath gas and the auxiliary gas flow-rates were set to 80

and 10 arbitrary units, respectively. A divert valve directs the HPLC-flow in the first 2.5 min of the chromatographic run to waste.

2.3. ESI-MS/MS mass spectra of standards

The HPLC–ESI-MS/MS analyses of standards were performed using the same HPLC–MS system operating in electrospray positive-ion mode with collision induced dissociation. The electrospray product ion mass spectra (ESI-MS/MS) of IFM and CPM were obtained selecting the parent ion *m/z* 261.1 Th with relative collision energy of 23% (corresponding to 1.15 V peak-to-peak resonance excitation radio frequency (RF) voltage). The product ion mass spectra of N^2-DCE-IFM and N^3-DCE-IFM were obtained selecting the precursor ion *m/z* 199.1 Th with relative collision energy of 28%.

2.4. Stock solutions, calibration and quality control standards

The stock solutions of IFM (2 mg/mL, 7.76 mM), CPM (1 mg/mL, 3.83 mM), N^2-DCE-IFM and N^3-DCE-IFM (1 mg/mL, 5.04 mM) were prepared in methanol from weighing and stored at −20 °C. A working solution of CPM (internal standard) was prepared at 200 μg/mL in methanol/water (50:50, v/v).

Calibration standards and quality control samples were prepared by addition of pure analytes from separate stock solutions to boiled rat microsomal medium. Each standard and QC was prepared, extracted and diluted as described in the microsomal incubation and sample extraction. All the concentrations indicated below are the concentrations of the injected solutions.

Calibration curves were recorded using eight different known concentrations (i.e. 100, 200, 500, 1000, 2000, 3000, 4000, 5000 ng/mL for IFM and 50, 100, 250, 500, 1000, 1500, 2000, 2500 for its two metabolites). CPM was spiked in order to have a 1000 ng/mL CPM solution injected on column.

Three quality control samples, which were assigned as low, medium, and high QC values (QC_L, QC_M and QC_H) were prepared as follows: QC_L (150 ng/mL for IFM and 75 ng/mL for DCE-IFM), QC_M (1500 ng/mL for IFM and 750 ng/mL for DCE-IFM) and QC_H (4500 ng/mL for IFM and 2250 ng/mL for DCE-IFM).

2.5. Microsomal incubation and sample extraction

Each incubation sample contained 1 mg of rat liver microsomal proteins, 0.1 M potassium dihydrogeno/hydrogenophosphate buffer (pH 7.4), 5 mM magnesium chloride, 40 mM glucose-6-phosphate, 2 mM NADP, and 20 IU of glucose-6-phosphate dehydrogenase in a final volume of 0.5 mL. Samples were pre-incubated for 10 min at 37 °C. Afterward, 1 μmol of IFM (38 μL of a 10 mg/mL solution) was added in order to obtain a 2 mM concentration. Incubations were carried out for 30 min and stopped by adding 5 mL of ice-cold ethylacetate. In all samples, 20 μg of CPM (IS) was added to obtain a concentration of 40 μg/mL.

Each microsomal incubation mixture (pH 7.4) was extracted with 5 mL ethylacetate by shaking for 10 min. The organic phase was decanted following centrifugation ($6000 \times g$) for 10 min and evaporated to dryness using a AES1010 SpeedVac® system (Savant Instruments, Farmingdale, NY, USA). The residue was dissolved in 1 mL of methanol, and then an aliquot was diluted to a twentieth in the initial mobile phase before analysis. Twenty microliters of these diluted solutions were injected into the HPLC–ESI-MS system. Each microsomal incubation extract was run in duplicate.

Microsomes were boiled for 15 min before incubation in order to inactivate their enzymatic activities. These boiled microsomes were used for the blank samples (negative tests) and for the calibration standards and quality control samples.

2.6. Extraction recovery

Recovery values for the three analytes (IFM, N^2-DCE-IFM and N^3-DCE-IFM) were determined comparing the QCs values obtained during the validation to values obtained with blank samples spiked with the theoretical 100% recovery amount. Extraction recovery of IFM and its deschloroethylated metabolites from microsomal medium was determined by the ratio of the experimental concentration of QC samples by their theoretical concentration.

2.7. Evaluation of the matrix effect and ion suppression

Several HPLC–MS (SIM) experiments were performed in order to evaluate the matrix effect and the ion suppression. Several blank samples prepared from different microsomal preparations were injected in the described chromatographic conditions. The matrix effect was evaluated on the three QCs levels as recently recommended in the literature [16–18]. Matrix effects for the four oxazaphosphorine compounds (N^2-DCE-IFM, N^3-DCE-IFM, IFM and CPM) were determined comparing the three QCs values prepared by dilution in methanol to the values obtained with blank samples spiked with the corresponding amounts. Thus, the matrix effect of the studied microsomal medium on the oxazaphosphorine compounds was calculated by the ratio of response of spiked samples over the response of corresponding methanolic samples.

2.8. Validation procedure of the HPLC–MS assay

The quantitative HPLC–ESI-MS method was validated according to the international requirements (ICH topic Q2A and Q2B), in terms of selectivity, linearity, accuracy and precision [19,20].

2.8.1. Selectivity

Selective baseline separation was achieved between each chromatographic peak as translated by the mass spectrometry detection.

2.8.2. Linearity and limit of detection and quantitation

Calibration curves were calculated on the relationship between the ratio of the peak area of the analyte (IFM or DCE-IFM) to that of the internal standard and the theoretical concentration of analyte. Least-squares linear regression was fitted with a $1/x^2$ weighing over the previously defined range using the calibration procedure of the Xcalibur LCQuan® software (ThermoFinnigan, Courtaboeuf, France). The low limit of quantitation (LLOQ) is the lowest concentration of analyte, which can be determined with precision and accuracy (less than 15%). For calibration curves, the equation is $y = bx + a$ where b is the slope and a the intercept. The limit of detection (LOD) was obtained by use of the slope (b) and the standard deviation of the intercept (S.D.a) from five calibration curves, as defined by ICH topic Q2B [19].

2.8.3. Accuracy

The method accuracy gives information about the recovery of the analyte from the sample. The solutions were spiked with three different known concentrations of each analyte (QC_L, QC_M and QC_H). The analysis of each QC sample was repeated six times. Accuracy was measured by the deviation or bias (%) of the mean found concentration from the actual concentration.

2.8.4. Precision

In accordance with ICH guidelines, precision (repeatability and intermediate precision) of the method was evaluated.

Repeatability (intra-day precision), expressed as the coefficient of variation of repeatability (CV_r), was performed for each level of QC six times and intermediate precision (inter-day precision), expressed as the coefficient of variation of intermediate precision (CV_i), was evaluated for each level of QC over five days.

3. Results and discussion

3.1. Optimisation of the HPLC–MS method

In terms of chromatographic conditions, methanol was chosen to get narrow and symmetric peaks. Different ammonium formate solutions (2, 5 and 10 mM in water) were tested. The best HPLC–MS response was obtained with 2 mM (pH 5.7) Allowing good ionization and stability of the studied oxazaphosphorine compounds. No interference was observed as shown on Fig. 2a.

Once mobile phase was chosen, the gradient conditions were determined to meet the following objectives: the separation with baseline return of the four compounds, a short analysis time and the optimisation of retention times. The gradient HPLC conditions were optimised to get the best separation of IFM and CPM, N^2-DCE-IFM and N^3-DCE-IFM and in order to develop a quantitative method in a convenient analysis time (run time of 20 min, duty cycle time of 25 min). In order to separate the deschloroethylated metabolites in a short time, we decided to investigate the use of methanol with a step gradient elution. The initial proportion of the organic phase (methanol) was set at 20% (v/v) until 9 min, allowing the separation of N^2-DCE-IFM and N^3-DCE-IFM. Then, the methanol proportion was increased up to 45% (v/v) from 9 to 9.5 min. These proportions were kept for 10.5 min in order to reduce the analysis time, keeping good separation of IFM and CPM (IS).

This analytical method allows the complete separation with baseline return of the three compounds using CPM as an IS. The retention times for N^2-DCE-IFM, N^3-DCE-IFM, IFM and CPM were 5.6, 7.6, 16.3 and 17.7 min, respectively (Fig. 2b and c).

3.2. Mass spectrometry study

The HPLC–selected ion monitoring (SIM) of molecular ions (m/z 199.1 Th and m/z 261.1 Th) was used for selective and quantitative detection of deschloroethylated metabolites and of IFM as shown by chromatograms (Fig. 2). For both N^2-DCE-IFM and N^3-DCE-IFM (MW 198.56), the pseudo-molecular ion $[MH, {}^{35}Cl]^+$ was observed at m/z 199.1 Th, with their chloride isotopic counterpart, ion $[MH, {}^{37}Cl]^+$ at m/z 201.1 Th. For IFM and CPM (MW 261.09), the ion $[MH^+, {}^{35}Cl, {}^{35}Cl]^+$ was observed at m/z 261.1 Th, with their chloride isotopic counterparts, ion $[MH^+, {}^{37}Cl, {}^{35}Cl]^+$ at m/z 263.1 Th and ion $[MH^+, {}^{37}Cl, {}^{37}Cl]^+$ at m/z 265.1 Th.

The electrospray product ion mass spectra (Fig. 3) of the following standards N^2-DCE-IFM, N^3-DCE-IFM, IFM and CPM were obtained to characterise each HPLC–MS peak with their m/z ion and their retention time, respectively, as they are two pairs of isomers.

For this purpose, we studied their fragment ions in product ion scan mode (Fig. 3). Metabolites and drugs are regioisomers, but they have different mass spectra. As shown on Fig. 3a and b, N^2-DCE-IFM (A) gave a specific transition (m/z 199.1 Th to m/z 182.0 Th) and a single fragment (m/z 170.9 Th) while for N^3-DCE-IFM (B), only this single fragment (m/z 170.9 Th) was detected. For IFM and CPM (C and D), the chosen specific fragments for MS/MS identification were at m/z 182.0 Th and m/z 140.1 Th, respectively (Fig. 3c and d).

3.3. Validation study of the HPLC–ESI-MS method

The calibration function was determined by linear regression over 50–2500 ng/mL concentration range for IFM metabolites and 100–5000 ng/mL concentration range for ifosfamide.

The mean correlation coefficients of the five calibration curves were $r^2 = 0.994$, 0.998 and 0.991 for N^2-DCE-IFM,

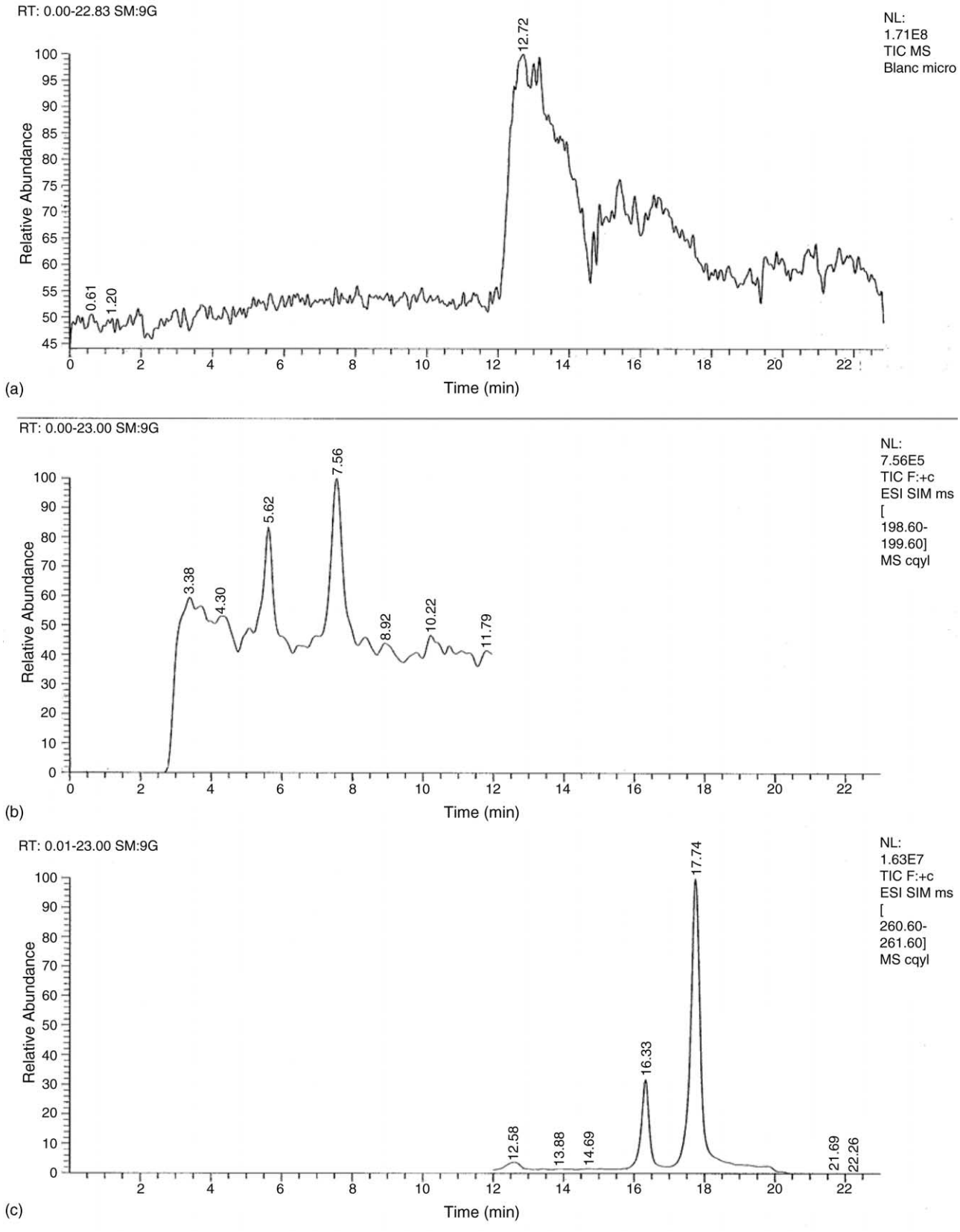

Fig. 2. Total ion-current chromatogram of blank microsome (a). HPLC/MS (SIM) chromatograms of LOQ rat microsomal QC_L sample spiked with 75 ng/mL for N^2-DCE-IFM and N^3-DCE-IFM, 150 ng/mL for IFM and 1000 ng/mL for CPM (IS): monitoring (b) *m/z* 199.1 Th (N^2-DCE-IFM, tr = 5.6 min and N^3-DCE-IFM, tr = 7.6 min); (c) monitoring *m/z* 261.1 Th (IFM, tr = 16.3 min and CPM, tr = 17.7 min).

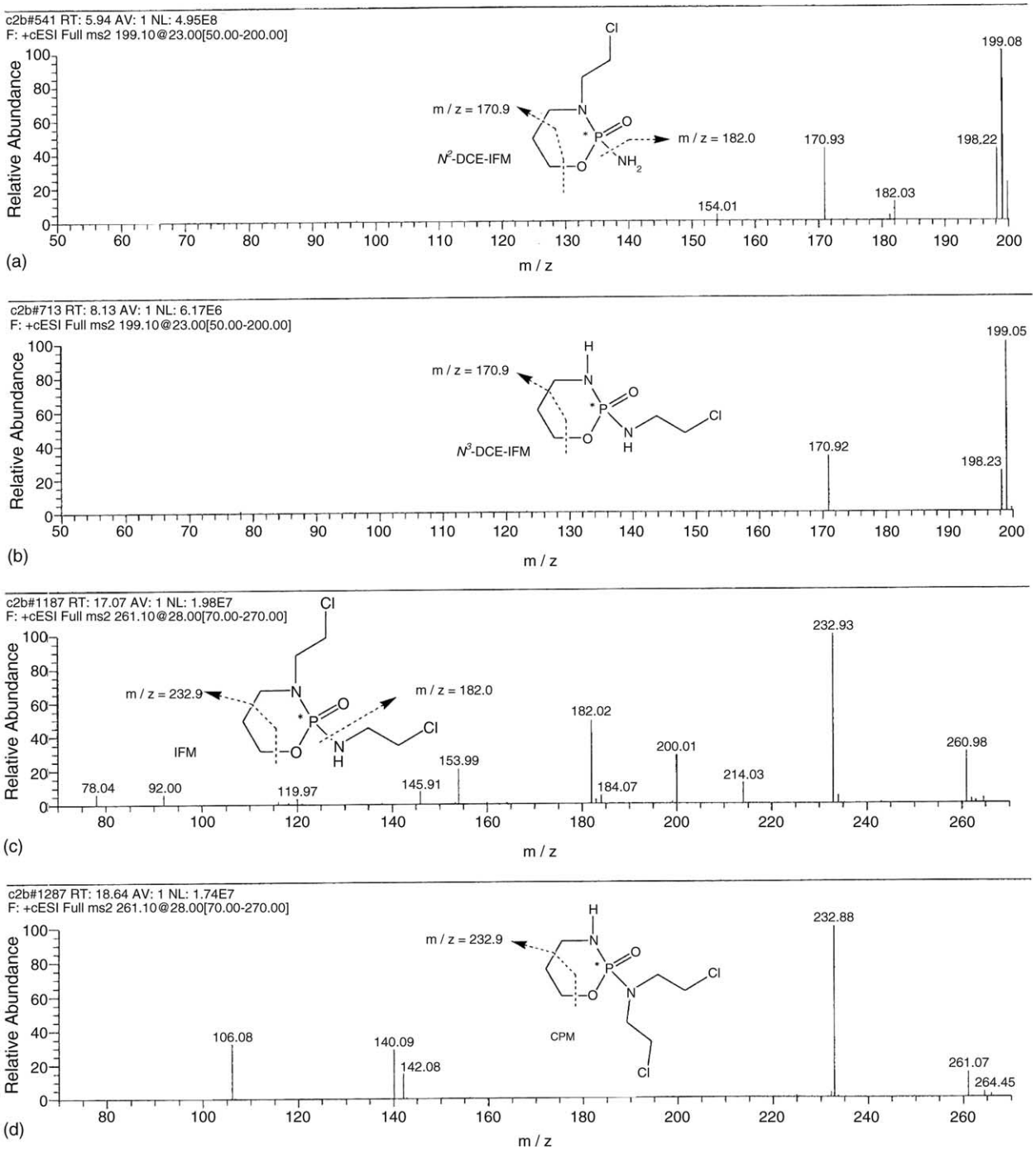

Fig. 3. Electrospray product ion mass spectra of N^2-DCE-IFM (a), N^3-DCE-IFM (b), IFM (c) and CPM (d).

N^3-DCE-IFM and IFM, with CV 0.4%, 0.1% and 0.9%, respectively. For N^2-DCE-IFM, the average equation was $y = 1.29 \times 10^{-3}x - 7.16 \times 10^{-3}$, where x corresponded to N^2-DCE-IFM/CPM (IS) peak areas ratio. For N^3-DCE-IFM, the average equation was $y = 0.96 \times 10^{-3}x + 18.35 \times 10^{-3}$, where x corresponded to N^3-DCE-IFM/CPM (IS) peak areas ratio. For IFM, the average equation was $y = 1.1 \times 10^{-3}x - 9.53 \times 10^{-3}$, where x corresponded to IFM/CPM (IS) peak areas ratio (see Table 1). The limits

Table 1
Linearity: calibration curves results

	N^2-DCE-IFM	N^3-DCE-IFM	IFM
a	-7.16×10^{-3} (150)	18.35×10^{-3} (57)	-9.53×10^{-3} (81)
b	1.29×10^{-3} (8)	0.96×10^{-3} (15)	1.10×10^{-3} (23)
r^2	0.994 (0.4)	0.998 (0.1)	0.991 (0.9)

Typical equation is: $y = bx + a$. Values are means of measurements performed on five curves fitted with least-square linear regression ($1/x^2$ weighing). Values in parentheses are R.S.D. (in %).

Table 2
Results of the accuracy study

	N^2-DCE-IFM	N^3-DCE-IFM	IFM
QC_L			
Mean	73.3 (75)	74.5 (75)	147.2 (150)
Bias (%)	−2.3	−0.7	−1.9
QC_M			
Mean	637 (750)	667 (750)	1360 (1500)
Bias (%)	−15.0	−11.0	−9.3
QC_H			
Mean	2531 (2250)	2436 (2250)	4521 (4500)
Bias (%)	12.5	8.3	0.5

The values were calculated on five different measurements. The actual values of the concentrations are in parentheses.

of detection as LOD = 3.3 × S.D.a/b' (where b' is the mean of the slope from five calibrations) were 29 ng/mL for N^2-DCE-IFM, 36 ng/mL for N^3-DCE-IFM, 23 ng/mL for IFM.

Moreover, the low limits of quantitation (LLOQ) were 50 ng/mL for N^2-DCE-IFM and for N^3-DCE-IFM, 100 ng/mL for IFM in agreement with accuracy and precision results lower than 15%.

The accuracy results of the three quality control samples (Table 2) showed the accuracy of the method according to the bias values calculated from the five analyses for each QC. Since the bias values were below 15.0% for N^2-DCE-IFM, below 11.0% for N^3-DCE-IFM and below 9.3% for IFM, the HPLC–ESI-MS method was accurate. The CV values for repeatability (CV_r) and for intermediate precision (CV_i) for each compound are summarised in Table 3. The method can be considered precise as CV_r are below 6.6% for N^2-DCE-IFM, below 8.0% for N^3-DCE-IFM and below 6.2% for IFM, and CV_i are below 8.2% for N^2-DCE-IFM, below 8.2% for N^3-DCE-IFM and below 8.8% for IFM.

According to these precision and accuracy studies values, analyzed samples should be diluted in order to assay injected concentrations between 50 and 2500 ng/mL for both metabolites and between 100 and 5000 ng/mL for IFM.

3.4. Evaluation of the matrix effect and the recovery

No significant matrix effect for the four analyzed compounds was observed in control microsome medium. Moreover, the experimental ratios characterising matrix effect (Table 4) showed less than 20% variation which is in good agreement with former recommendations [16,17].

Recovery values for the three analytes were determined comparing the QCs values obtained during the validation to values obtained with the spiked samples. According to Sottani et al. [21], the optimum pH for liquid–liquid extraction of cyclophosphamide metabolites is pH 7.0 (extraction yield over 90%). In our study, microsomal medium is buffered at pH 7.4, and remained stable during extraction. Because of the structural analogy between IFM/CPM and their metabolites, we chose to keep pH 7.4 for the extraction. Extraction yields were similar for methylene chloride and for ethylacetate. The latter was chosen as the extraction solvent for its ease of use (less dense and less toxic than methylene chloride).

For the assayed molecules (N^2-DCE-IFM, N^3-DCE-IFM, IFM), six determinations for all the QCs levels and six for 1000 ng/mL CPM were performed. Mean extraction recoveries were 60%, 55%, 95% and 101% for N^2-DCE-IFM, N^3-DCE-IFM, IFM and CPM, respectively (see Table 4). These values agreed with those previously described with ethylacetate extraction for the metabolites and IFM [9].

Table 3
Results of the repeatability (CV_r) and intermediate precision (CV_i) study

	N^2-DCE-IFM	N^3-DCE-IFM	IFM
QC_L			
CV_r (%)	5.3	8.0	5.9
CV_i (%)	8.2	7.0	7.5
QC_M			
CV_r (%)	2.0	4.8	2.5
CV_i (%)	2.5	5.2	3.6
QC_H			
CV_r (%)	6.6	6.8	6.2
CV_i (%)	5.3	8.2	8.8

Values were based on six different measurements for CV_r and five different measurements for CV_i.

3.5. Biological application: microsomal incubation of ifosfamide

To evaluate the neurotoxicity and nephrotoxicity of IFM, Kaijser et al. [22] determined the CAA amount in plasma, even if CAA is highly labile and reactive making it a bad marker of IFM *N*-deschloroethylation. Moreover, Kasel et al. described recently a LC–MS/MS technique for the quantitation of CPM and its metabolites in urine, limiting the quantitation to parent compound and its relatively stable metabolites [23]. For our purpose, to estimate the possible

Table 4
Matrix effect and extraction yield evaluation

	N^2-DCE-IFM	N^3-DCE-IFM	IFM	CPM
Matrix effect	1.01	1.11	1.17	1.07
Extraction yield (%)	60	55	95	101

Values are means of measurements performed on the three QCs levels.

N-deschloroethylation of IFM analogues, the CAA was not assayed as it could not be formed.

DCE-IFM are the best markers of IFM neurotoxicity. According to Weber and Waxman, rat liver cytochrome P450 2B are mainly responsible for IFM *N*-deschloroethylation [24]. Phenobarbital is a strong inducer of these P450 2B. We then performed metabolisation experiments using phenobarbital induced microsomes. Results presented are for rat microsomal incubations ($n = 3$) of 2 mM IFM. Mean metabolisation yield is 5% (95% IFM remained unchanged). Biotransformation of IFM into DCE-IFM is 0.8% (16% of the total metabolites), with a N^2-DCE-IFM/N^3-DCE-IFM ratio of 1.05. Using longer incubation time would enhance biotransformation yields and allow a better understanding of this metabolisation.

Our HPLC–ESI-MS method showed optimal separation and good sensitivity for the determination of *N*-deschloroethylated metabolites and IFM without derivatization. The LLOQ values were comparable with the described GC–NPD method and showed easy to use preparation, compared to the GC–MS. This method would be useful to confirm the enzymes involved in the side-chain oxidation of IFM.

4. Conclusion

The present work demonstrated the development of a HPLC–ESI-MS method using electrospray ion-trap mass spectrometry for the quantitation of IFM and their N^2- and N^3-deschloroethylated metabolites, which can be produced using rat microsomes. SIM mode detection allows the direct quantitation of both metabolites and CPM using CPM as internal standard. The HPLC–ESI-MS method was accurate and precise for the determination of *N*-deschlorethylated metabolites of IFM in the range of 50–2500 ng/mL and for the determination of IFM in the range of 100–5000 ng/mL in rat microsomal incubations. This method was found to be sensitive and selective with a low limit of quantitation of 50 ng/ml for N^2- and N^3-DCE-IFM and 100 ng/ml for IFM. The in vitro metabolic *N*-deschloroethylation of IFM was evaluated in rat microsomes and could be further studied in human microsomal medium to evaluate new structural IFM analogues with less neurotoxicity and nephrotoxicity.

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