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Analysis of ifosforamide mustard, the active metabolite of ifosfamide, in plasma

G.P. Kaijser[a], J.H. Beijnen[b], E. Rozendom[c], A. Bult[c], W.J.M. Underberg[c,*]

[a]*RL-CERM, Zône Industrielle "La Varenne", Rue Henri Goudier, 63203 Riom, France*

[b]*Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

[c]*Department of Pharmaceutical Analysis, Faculty of Pharmacy, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

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Abstract

Ifosforamide mustard is the active metabolite of ifosfamide, a cytostatic drug. In this study a sensitive and selective method for the analysis of ifosforamide mustard in plasma is described. The method consists of direct derivatisation of ifosforamide mustard in plasma with diethyldithiocarbamate and subsequent solid-phase extraction of the resulting derivative. The analysis of the derivatisation product was performed by high-performance liquid chromatography with UV detection. The calibration graph was linear in the concentration range 0.45–45 μM and the minimum detectable concentration was 0.45 μmol. The samples were stabilised by addition of semicarbazide and sodium chloride. A patient's plasma sample was analysed by means of the described method. The ifosforamide mustard concentration was 2.3 μM.

Keywords: Ifosforamide mustard; Ifosfamide

1. Introduction

Ifosforamide mustard (IFM) is the cytotoxic metabolite of the anticancer drug ifosfamide (IF). IF is a prodrug and has to be metabolised in order to exert its alkylating activities [1]. The cytochrome P450 system firstly hydroxylates IF to 4-hydroxyifosfamide. This compound is considered to be the transport form of IFM; IFM is too hydrophilic to enter the target cells whereas 4-hydroxyifosfamide (4-hydroxyIF) still possesses this capability. Inside the cells the 4-hydroxyIF is transformed into IFM and acrolein. Mehta et al. [2] have shown that phosphoramide mustard (PM), the mustard metabolite of cyclophosphamide (CP), reacts with guanosine and deoxyguanosine to form nucleoside–PM adducts, which can cause extensive damage during cell replication.

Since IFM is the ultimate alkylating metabolite of IF, it may be more appropriate to evaluate concentrations of IFM rather than IF in attempting to relate drug concentration to efficacy and toxicity of treatment, respectively.

The bio-analysis of IFM is hampered by its high reactivity, polarity and lack of a UV chromophore. Its high solubility makes it difficult to purify the analyte effectively from biofluids. Earlier studies report laborious liquid–liquid- (LLE) and solid-phase extractions (SPE) [3–5] or a combination of LLE and SPE [6] as sample pretreatment of IFM or

*Corresponding author.

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PM analysis. Thin-layer chromatography (TLC) [7], also in combination with LLE [8] or SPE [9,10], has been used to isolate the mustard compounds. After isolation, the analysis of IFM or PM may proceed by high-performance liquid chromatography (HPLC) [4], mass spectrometry (MS) [8] or photography-densitometry [9,10]. Some authors performed the analysis of IFM or PM after derivatisation and extraction of the derivative and subsequent fluorimetric [7,11] or gas chromatographic–mass spectrometrical (GC–MS) methods [3,5,6,12]. The derivatisation procedure with nitrobenzyl pyridine without prior separation is also still in use [12]; a major drawback of this assay is the lack of selectivity as all alkylating metabolites are measured. Due to the instability of 4-hydroxyIF, the precursor of IFM, IFM and acrolein can be formed during storage of patients' samples. This can be prevented by trapping the 4-hydroxy compound with semicarbazide [4,7,13].

In order to study the pharmacokinetics of IF, a selective method for IFM was needed which was suitable for routine analyses. A HPLC method for IFM after derivatisation with diethyldithiocarbamate (DDTC) has been developed. Furthermore, a stabilisation procedure for IFM in patients' plasma samples has been elaborated. However, since IFM is produced intracellularly, plasma levels of IFM may not have a high clinical relevance. Currently, work is in progress to adapt the assay to tissue analysis, so that it may be suitable to monitor the IFM levels intracellularly and a more appropriate correlation with efficacy and toxicity will be obtained.

2. Materials and methods

2.1. Chemicals

IF and IFM (cyclohexylamine salt) were kindly donated by Asta Medica AG (Frankfurt, Germany). The sodium diethyldithiocarbamate was purchased from Aldrich (Bornem, Belgium). All other chemicals were of the highest purity available and used as received. Throughout the study filtered demineralised water was used (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The HPLC system consisted of a Model 510 pump and U6K injection device (both from Waters, Milford, MA, USA). Separation was achieved on a LichroCART RP8 column, 125×40 mm, 5 μm particle size (Merck, Darmstadt, Germany) with a mobile phase of acetonitrile–water (32:68, v/v) containing 10 m*M* sodium phosphate buffer and 20 m*M* cyclohexylamine at a pH 7.0. The UV detection was performed with a type 785A variable-wavelength detector (Applied Biosystems, Maarssen, Netherlands) at 280 nm.

The capillary zone electrophoresis (CZE) was performed with a Prince CZE apparatus (Lauerlabs, Emmen, Netherlands) provided with a 70 cm fused-silica capillary of 75 μm internal diameter; 22.5 kV was applied. UV detection was performed on-line at 270 nm; 50 m*M* sodium phosphate buffers were used at pH values between 4 and 7.

The ^{1}H nuclear magnetic resonance (NMR) spectrum of the derivative was recorded at ambient temperature with a WP 200 instrument (Bruker Analytische Messtechniek GmbH, Karlsruhe, Germany) at a frequency of 200.13 MHz. Deuterium oxide was used as the solvent and the HOD-signal (at 4.76 ppm) as the internal standard.

The mass spectrometric (MS) measurements were performed with a Jeol SX102/102A fast atom bombardment (FAB) mass spectrometer (Tokyo, Japan) with xenon as the ionising agent. The energy was 6 keV and the measurements were made by positive ion monitoring.

2.3. Derivatisation procedure

To 500-μl aliquots of the plasma samples, stabilised with semicarbazide, 500 μl of a 0.5 *M* sodium phosphate buffer pH 8.0 (PPB) containing 1 *M* sodium chloride and 100 μl of a DDTC solution in water (0.1 g/ml) were added. The solutions were heated in a thermostatically controlled water bath at 70°C for 30 min. After cooling the samples at 0°C, a SPE procedure was performed with Seppack C_{18} cartridges (Waters). The cartridges were conditioned by eluting three times with 1 ml of methanol, followed by three times with 1 ml of the PPB

containing 25 μ*M* cyclohexylamine. Then the total sample volume (1.1 ml) was put onto the column. The cartridge was washed three times with the PPB containing 25 μ*M* cyclohexylamine, after which the IFM derivative was eluted with 1.0 ml of methanol. The methanol was evaporated under a stream of nitrogen at 30°C. The dry residue was dissolved in 100 μl of a 27.5% (v/v) acetonitrile solution. A 10-μl aliquot of this solution was injected into the HPLC system.

For the calibration graph plasma samples were prepared by adding 500 μl of a semicarbazide solution (2 *M* semicarbazide hydrochloride in PPB containing 1 *M* sodium chloride) to 5.0 ml drug-free plasma. Aliquots (500 μl) of the resulting solution were spiked with 0–500 μl of an IFM stock solution in PPB containing 1 *M* sodium chloride. The total volume of the samples was adjusted to 1.0 ml with PPB containing 1 *M* sodium chloride. The resulting solutions were then processed as described above.

2.4. Patients' samples

A plasma sample from a 30-year old patient receiving a continuous 24-h infusion of 3.5 g/m^2 was analysed. A blood sample was taken 20 h after the start of the infusion. A 5-ml aliquot of the blood was collected into a heparinised tube containing 500 μl of the semicarbazide solution described above. The blood cells were separated by centrifugation (10 min at 1600 *g*) and the plasma was stored immediately at −20°C. Aliquots of 500 μl plasma were used for the analysis as described above.

3. Results and discussion

3.1. Stability

4-Hydroxyoxazaphosphorines are unstable agents and degradation may lead to the formation of mustard compounds that interfere in the assay of free IFM. The addition of semicarbazide to a 4-hydroxyoxazaphosphorine, however, results in a semicarbazon compound [14] and thus prevents further breakdown.

PM stability has been shown to be dependent on pH, temperature and chloride ion concentration [15,16]. Extrapolating these results to IFM, stabilisation of plasma samples was investigated by adding sodium chloride. It appears that the 0.3 *M* plasma sodium chloride concentration resulting after the addition of the semicarbazide solution, decreases the degradation of IFM dramatically. An increase in the time at which 5% of the derivative has degraded from 24 h (without sodium chloride) to 4 days (with sodium chloride) was observed when the plasma was stored at −20°C. Therefore, analysis of the IFM containing samples has to take place within 5 days after withdrawal.

The derivatisation product appeared to be stable (less than 5% degradation) for at least 7 days when stored in methanol at 4°C and for at least 14 days when kept at −20°C.

3.2. Derivatisation

The derivatisation procedure is based on earlier reports concerning derivatisations of alkylating cytostatic agents like dianhydrogalactiol [17] and nitrogen mustard anticancer drugs [18] with DDTC. Most substitution or addition reactions employ electrophilic reagents, interacting with nucleophilic sites on target molecules. In clinical analysis this approach has the distinct disadvantage that extensive clean-up is often necessary prior to the derivatisation step because of the wide variety of endogenous nucleophiles present in biological fluids at high concentrations relative to the compound of interest. These endogenous compounds also react with the derivatising agent reducing the specificity of the method, potentially causing consumption of large amounts of reagent, and making separations more difficult. Also, such reagents (e.g. acylating agents) are often susceptible to degradation by water (the most abundant nucleophile in biological fluid). Nucleophilic reagents such as DDTC are much more specific because of the absence of electrophilic functions in endogenous compounds and are relatively stable in water [19,20]. These properties allow the derivatisation of IFM with DDTC to be carried out directly in plasma. The reaction product is less polar than IFM which increases the extractability of the analyte.

3.3. Structure identification

At first, the derivatisation reaction was performed in an aqueous solution at 50°C for 40 min. The residue resulting after SPE was subjected to various techniques: HPLC, CZE, FAB-MS and ^{1}H NMR. On theoretical grounds two derivatisation products of IFM with DDTC were expected: the mono-derivatised (MD) and the di-derivatised product (DD) (Fig. 1)

The HPLC chromatogram of an aqueous derivatisation solution, processed as described above, indeed shows two peaks that are absent in the blank chromatogram (Fig. 2). By reason of the higher polarity of MD relative to DD, the first peak (t_R=2 min) was attributed to MD and the second (t_R=4 min) to DD. The peak areas were about the same. Taking into account the higher molar absorptivity of DD with two DDTC groups, the ratio MD:DD was about 2:1.

The CZE electropherogram also shows two additional peaks that eluted after the time that neutral compounds elute, indicating a negative charge of the compounds (Fig. 3). Besides the charge, the velocity of the compounds in the capillary is also determined by their m/z ratio: the smaller this ratio, the faster the migration. Consequently, DD, with its higher mass, will elute before MD. The ratio DD:MD was 1:2. It was not possible to determine the pK_a value of the IFM derivatives as at pH values lower than 6.5 the derivatives degraded.

A FAB-MS spectrum of the derivatisation mixture after SPE was also registered (Fig. 4). The MD (m/z 334; MH^+) and DD (m/z 447; MH^+) were both found in the spectrum as well as their adducts with sodium (m/z 356 and 469, respectively) and with glycerol (m/z 378 and 491, respectively). From the spectra it is also seen that the species at m/z 334, 356 and 378 contain a single chlorine atom (m/z+2) while m/z 447, 469 and 491 lacking a chlorine atom in the molecule. Due to the presence of four sulphur atoms, the isotopes at m/z+1 of DD are more pronounced.

The ^{1}H NMR spectrum of a solution of the derivatisation mixture shows six signals: two quartets at 3.96 and 3.78 ppm (J=7.3 and 7.1 Hz, respectively; relative intensities 3.7 and 4.0), two triplets at 3.57 and 3.38 ppm (J=5.3 and 6.7 Hz, respectively, relative intensities 1.8 and 3.8, coupled with the multiplet at 3.05 ppm), a multiplet (overlapping triplets?) at 3.05 ppm (relative intensity 5.7), and two overlapping triplets at 1.20 ppm (relative intensity 10.8). The mass spectrum shows that the derivatisation reaction results in a mixture of two derivatives, of which one of the components still contains chlorine. Based on the assumptions that the quartets

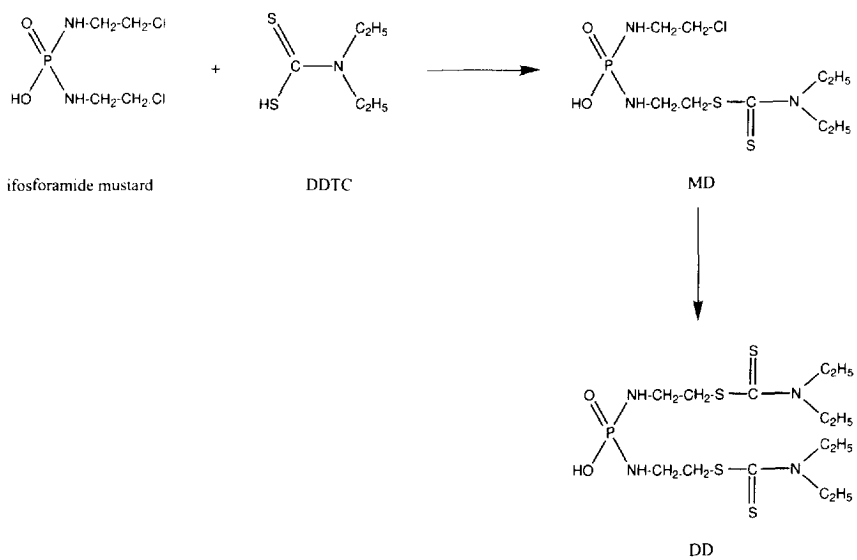

Fig. 1. Derivatisation reaction of IFM with diethyldithiocarbamate.

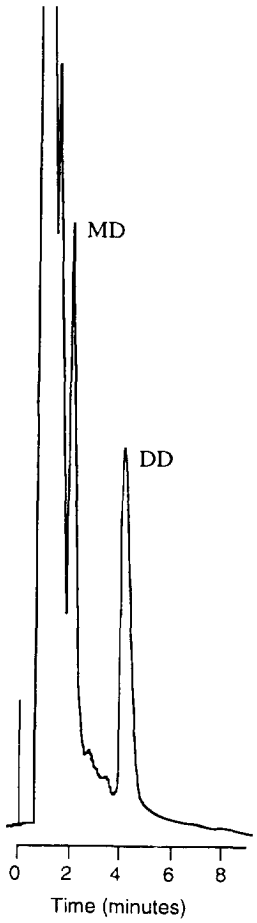

Fig. 2. HPLC chromatogram of a solution of IFM and DDTC after derivatisation at 50°C for 40 min. MD=mono-derivative of IFM, DD=di-derivative of IFM.

at 3.96 and 3.78 can be assigned to CH_2 parts of the C_2H_5 groups, the triplet at 3.57 ppm to a CH_2Cl, the triplet at 3.38 to CH_2SCS and the multiplet at 3.06 ppm to CH_2NP, the conclusion can be drawn that the mixture consists of 67% of MD and 33% of the DD. The same ratio DD:MD was found in HPLC, CZE and NMR analysis.

At this stage it appeared that the reaction conditions, yielding two products, were not optimal. The reaction conditions were then varied and the recovery of the two products was checked by means of HPLC. When the derivatisation was performed at 70°C for 30 min, a single peak resulted at 4 min (Fig. 5). After SPE of this reaction mixture, FAB-MS revealed that MD was absent with only DD

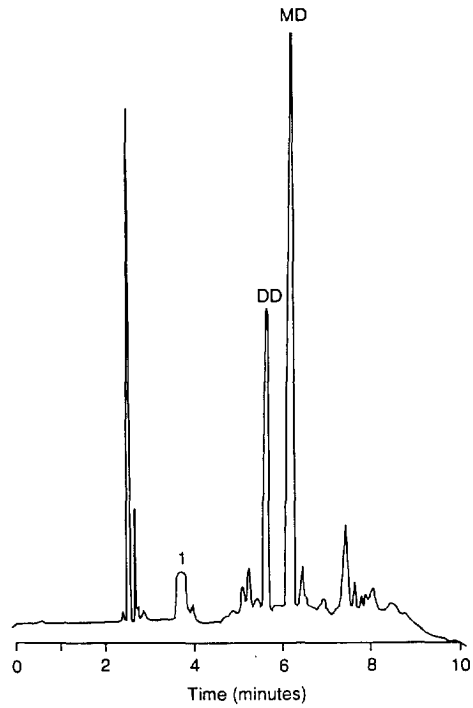

Fig. 3. CZE electropherogram of a solution of IFM and DDTC after derivatisation at 50°C for 40 min. MD=mono-derivative of IFM, DD=di-derivative of IFM, 1=elution time of neutral species.

formed. Consequently, these reaction conditions were selected for the derivatisation reaction of IFM with DDTC.

3.4. Chromatography and validation

The derivative decomposes at pH<6.5. Therefore, all solutions used in the derivatisation procedure were composed with a 0.5 *M* phosphate buffer solution of pH 8.0. The high semicarbazide hydrochloride concentration necessitated a solution with a high buffer capacity to maintain a pH>8.

Considering the fact that DD was retained by the C_{18} SPE columns, a reversed-phase HPLC system was attempted with a mobile phase consisting of a mixture of acetonitrile and a 10 m*M* sodium phosphate buffer pH 7.0. DD indeed showed some retention, but only with an acetonitrile content less than 5%. The bad peak shape, however, necessitated a different approach. Ion-pair chromatography with

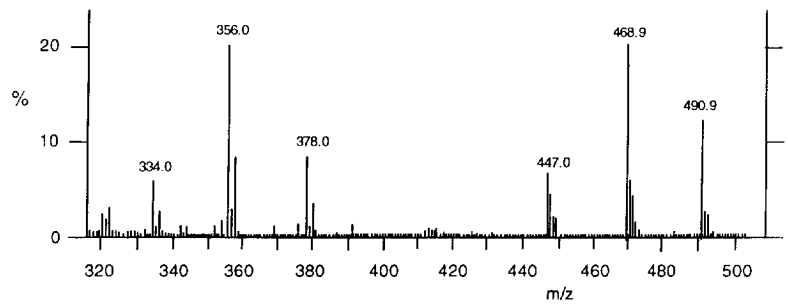

Fig. 4. FAB-MS spectrum of a solution of IFM and DDTC after derivatisation at 50°C for 40 min.

cyclohexylamine at a pH of 7.0 resulted in narrow, symmetrical peaks.

The on-line UV spectra of the peak shows a wavelength of maximum absorbance at 280 nm. This wavelength was chosen for the DD detection in HPLC. Detection at higher wavelengths circumvents the detection at 200 nm employed for underivatized IFM, which is interference prone.

The calibration graph of IFM in plasma was linear ($r>0.998$) in the concentration range 0.45–45 μ*M*.

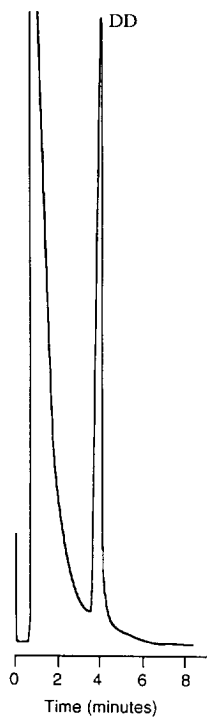

Fig. 5. HPLC chromatogram of a solution of IFM and DDTC after derivatisation at 70°C for 40 min. DD=di-derivative of IFM.

The equation of the graph was $y=0.09(\pm0.07)+2.30(\pm0.04)x$, in which y is the peak area of DD in the HPLC chromatogram and x is the IFM concentration in the sample. The limit of quantification is 0.45 μmol. The accuracy of the method was 98.3% with an intra-day variation of 5.1% and an inter-day variation of 5.7%.

3.5. Patients' samples

Fig. 6 shows the chromatogram of a patient's plasma sample. The IFM concentration was 2.3 μ*M*.

4. Conclusions

The presented method is suitable for the determination of IFM in plasma without elaborate extraction procedures. After derivatisation a product is obtained that is stable and can be detected at 280 nm. Patients' blood samples should be stabilised with semicarbazide to trap 4-hydroxyIF and with sodium chloride to stabilise IFM. The presented assay may be of great value in future studies of the correlation of IFM concentrations and toxicity and/or anti-tumor activity.

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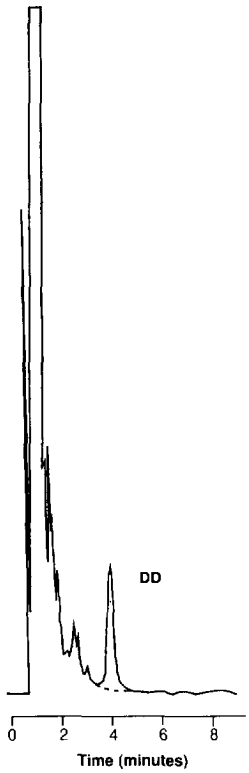

Fig. 6. HPLC chromatogram of a patient's plasma sample containing 2.3 μ*M* IFM. DD=di-derivative of IFM; (- - -)=blank plasma.

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