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Determination of ifosfamide by gas chromatography–mass spectrometry

Hilde Lambrechts, Eric O.O. Gheuens, Karel A. Van Cauwenberghe, Greet G.O. Pattyn, Allan T. Van Oosterom and Ernst A. De Bruijn *

Laboratory for Cancer Research and Clinical Oncology, Universiteitsplein 1 (S4), B-2610 Wilrijk (Belgium)

Piet A. Leclercq
Eindhoven University of Technology, Faculty of Chemical Engineering, Laboratory of Instrumental Analysis, P O Box 513, 5600 MB Eindhoven (The Netherlands)

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Abstract

Ifosfamide, an oxazaphosphorine, is thermally stable during elution in gas chromatography (GC) at temperatures above 200 °C, in contrast to its structural isomer cyclophosphamide. Both 2.65-μm and 0.32-μm OV-1 columns were efficient for GC of ifosfamide without derivatization. Mass spectrometry (MS), showed that intact ifosfamide was eluted without interference from naturally occurring metabolites in blood plasma. Ifosfamide can be monitored, by capillary GC–MS without derivatization, in blood plasma from cancer patients treated with the drug. Only a liquid–liquid extraction is required before injection of the sample. A single peak of ifosfamide is detected with molecular mass 260, fragmentation starts with loss of CH₂Cl ([M – CH₂Cl], m/z 211). The limit of determination for ifosfamide in human plasma was about 50 nM (10 ng ml⁻¹). Recovery, quality of calibration curves and reproducibility were suitable for the rapid determination of ifosfamide in the range 0.01–1000 μg ml⁻¹

Keywords. Gas chromatography, Mass spectrometry; Blood; Ifosfamide

Oxazaphosphorines are among the most frequently applied anticancer agents in clinical oncology. Cyclophosphamide has been an established anticancer agent for more than three decades whereas ifosfamide was first synthesized in 1965 in Germany. The agents require metabolic activation by hepatic metabolism which leads to a vast number of metabolites with high and low cytotoxic activity [1].

Data from early clinical investigations with ifosfamide, which started in 1967, demonstrated that the drug is active against a wide range of tumors. However, severe and dose-limiting urotoxicity and the occasional occurrence of renal failure hindered further investigations. The introduction of concomitant Mesna, in order to prevent development of hemorrhagic cystitis, led to renewed interest in ifosfamide [2]. Higher doses of the drug can presently be used in the treatment of different malignancies with myelosuppression being the dose-limiting factor [3].

The increasing application of ifosfamide has raised the demand for quantitative assays in order to establish the behaviour of the oxazaphosphorine in vitro and in vivo. Several methods have been introduced for the determination of ifosfamide, mostly based on gas chromatography (GC) and liquid chromatography (LC). Capillary GC was recently proven to be capable of eluting intact ifosfamide on 2.65-μm methylsilicone columns (Hewlett-Packard, HP-1; 5 m x 0.53 mm i.d.) without derivatization [4].
Mass spectrometry (MS) showed that intact ifosfamide \((m/z \ 260)\) was eluted in the temperature range \(150-300^\circ\text{C}\) whereas an isomer of ifosfamide, cyclophosphamide underwent partial degradation under similar conditions \([5,6]\). Data from these studies, however, indicated the possibility of using cyclophosphamide as an internal or external (chromatographic conditions) standard because the formation of the degradation product, intra-alkylated cyclophosphamide, was linear over a sufficient range and did not interfere with the determination of ifosfamide \([4-6]\). The present contribution describes the capability of capillary GC–MS in the determination of ifosfamide in blood plasma. Because only 10 \(\mu\text{l}\) of blood plasma is required, the method allows clinical drug monitoring with fingerprick blood below the \(\mu\text{g} \text{ml}^{-1}\) level.

**EXPERIMENTAL**

**Chromatographic systems**

Two GC systems were used: one GC–MS system and one system equipped with flame-ionization detection (FID), the latter for routine monitoring in clinical pharmacological sessions.

For capillary gas chromatography– mass spectrometry, a Hewlett-Packard 5790A (MSD) apparatus was interfaced to a Hewlett-Packard 9825B data system. Electron-ionization mass spectra at 70 eV were obtained at a rate of 2 s\(^{-1}\). Samples were injected splitless at 60°C during 30 s, and the oven was heated rapidly to 150°C (2 min), and then programmed from 150°C to 300°C at 10°C min\(^{-1}\). Purge off-time was 0.50 min; the head pressure was 20.0 psi and the interface temperature was 290°C. An OV-1 column (Hewlett-Packard, HP-UP, 25 m \( \times 0.20 \text{ mm i.d.}, 0.32 \mu\text{m}\) was used. The GC–MS system was used both to study the behaviour of oxazaphosphorine following GC \([4]\) and for random checking of patient plasma and urine samples determined by GC–FID.

The GC–FID system was a Carlo Erba HRGC 5160 Mega Series gas chromatograph (Carlo Erba, Milan, Italy) equipped with FID. A methylsilicone column (Hewlett-Packard, HP-1, 5 m \( \times 0.53 \text{ mm i.d.}, 2.65 \mu\text{m}\) was installed and helium was used as carrier gas. Samples were introduced by on-column injection at 85°C. The oven temperature was programmed to 100°C at 15°C min\(^{-1}\), then to 160°C at 50°C min\(^{-1}\), from 160°C to 180°C at 1°C min\(^{-1}\), and then to 220°C at 5°C min\(^{-1}\). Data were acquired with a Spectra Physics 4290 integrator.

**Sample pretreatment**

Ifosfamide can be extracted from biological fluids by a one-step procedure with ethyl acetate. Here, however, a two-step procedure was preferred in order to extend the lifetime of the columns used. To 10–250 \(\mu\text{l}\) of blood plasma, 0.1 M sodium hydroxide (30–800 \(\mu\text{l}\)) was added and mixed for 2 min, then 2.5 ml of ethyl acetate was added and vortexed for 5 min. After centrifugation (10 min, 3000 rpm), the upper layer was transferred to another tube and evaporated under a gentle stream of nitrogen at 35°C. Subsequently 1 ml of 90% methanol was added and the ifosfamide-containing methanol was washed twice with 2.0 ml of hexane. Finally, the methanol was evaporated and the residue was either stored or immediately used for chromatography. For introduction into the GC system, the residue was dissolved in 100 \(\mu\text{l}\) of ethyl acetate and 1 \(\mu\text{l}\) was introduced into the GC. Plasma for calibration curves and other analytical purposes was obtained from cancer patients before chemotherapy was started.

**RESULTS**

Chromatograms of ifosfamide introduced into the two GC systems used are presented in Fig. 1. Single peaks of ifosfamide (IF) were noted in both GC systems; no signs of degradation during injection or elution were noted.

Mass spectra demonstrated that intact ifosfamide entered the spectrometer; fragmentation started with loss of a \(\text{CH}_2\text{Cl}\) radical, and \([\text{M} - \text{CH}_2\text{Cl}]\) at \(m/z \ 211\), was found to be the base peak (Fig. 2). Further fragmentation yielded fragments with \(m/z \ 154\), \(m/z \ 134\) and \(m/z \ 92\) (among others), as depicted in Fig. 2.
Calibration curves for the concentration range which is relevant with respect to the concentrations occurring in patients, i.e., 1–100 µg ml⁻¹, were characterized by the equation \( y = 1.80(±0.28)x - 0.5(±0.5) \) \( (n = 6) \). Reproducibility was < 7% at the limit of determination (50 nM) and within-assay precision for the determination of ifosfamide in biological fluids obtained in clinical settings was 105.6% ± 5.9% at 1 µg ml⁻¹ and 98.3% ± 2.1% at 100 µg ml⁻¹.

A representative application of monitoring ifosfamide by GC–FID and GC–MS is given in Fig. 4. The concentrations in plasma reached the µg ml⁻¹ level rapidly after the start of a 4-h infusion with 2.5 g m⁻² ifosfamide. At the end of the first infusion, the concentration of ifosfamide was 64.7 µg ml⁻¹.

DISCUSSION

The oxazaphosphorines cyclophosphamide and ifosfamide are amongst the most frequently administered anticancer agents owing to their ther-
apeutic width, their therapeutic index and their characteristics in the pharmaceutical phase (oral and intramuscular administration is possible). Because both these drugs are prodrugs which are subject to intensive metabolism, methods capable of determining the parent drugs selectively in the presence of metabolites are preferable. It is not surprising that the methods available for cyclophosphamide can easily be adapted for ifosfamide, because the oxazaphosphorines are isomers. Furthermore, the formation of metabolites of ifosfamide follows a pattern similar to that of cyclophosphamide. The usual methods for the determination of ifosfamide in biological fluids are LC and GC [7].

The gas chromatography of oxazaphosphorines generally requires derivatization with trifluoroacetic or heptafluorobutyric anhydride, which is time-consuming and laborious [8-12]. In a series of papers, De Bruijn et al. [4-6,13] demonstrated that oxazaphosphorines can be handled reliably in GC without derivatization by exploiting the on-column intra-alkylation of cyclophosphamide. In contrast, unchanged ifosfamide was not subject to intra-alkylation during GC and it was demonstrated that this drug could be determined by GC in the presence of its naturally occurring metabolites without any derivatization [4]. Thus, GC offers a clear advantage over LC: derivatization is not essential, while the sensitive detection systems of GC can be exploited. The determination of underivatized ifosfamide by GC appeared to be possible with detection limits at the pg level when electron capture detection (ECD) was used [4]. The higher sensitivity obtained for ifosfamide than for cyclophosphamide by GC–ECD can in part be explained by the absence of intra-alkylation; approximately half the signal for cyclophosphamide is lost because two peaks are observed [4-6,13]. The concept was exploited in the present study. A simple two-step liquid-liquid extraction was used to isolate ifosfamide from biological fluids and MS proved that this drug was determined selec-
tively. Calibration curves made up in the concentration range to be expected in patients treated with the drug demonstrated sufficient linearity. Recoveries were approximately 70%.

When the GC system included a thick-film, short column and FID, the limit of determination was 13 ng ml⁻¹ (50 nM, signal-to-noise ratio 3 : 1). The limit of determination could be lowered to about 1 ng ml⁻¹ by using ECD or MS in the electron-ionization mode.

The GC–MS data demonstrated clear differences between the two isomers; when cyclophosphamide was introduced and eluted in GC, both unchanged and intra-alkylated cyclophosphamide entered the mass spectrometer [4] whereas only intact ifosfamide entered the spectrometer after GC. The latter was also found after injection of plasma extracts containing ifosfamide (Figs. 2 and 3). Furthermore, from the present data on plasma extracts, the absence of interference from naturally occurring metabolites with the signal for ifosfamide is evident. Therefore, it can be concluded that ifosfamide can be monitored by GC without derivatization and without the use of polar columns. Short thick film columns can be recommended because the elution times of ifosfamide can be reduced to a few minutes and because the lifetime of thick-film columns is longer than that of the OV-1 columns used in the present GC–MS studies. Moreover, the same system can be applied for the determination of cyclophosphamide (e.g., as internal standard) when the GC conditions are properly defined [4].

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REFERENCES