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# Enantioselective liquid chromatography–mass spectrometry assay for the determination of ifosfamide and identification of the *N*-dechloroethylated metabolites of ifosfamide in human plasma

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**Abstract**

A sensitive and specific liquid chromatography–mass spectrometry (LC–MS) method has been developed and validated for the enantioselective determination of ifosfamide [(*R*)-IF and (*S*)-IF] in human plasma and for the detection of the *N*-dechloroethylated metabolites of IF, 2-*N*-dechloroethylifosfamide [(*R*)-2-DCl-IF and (*S*)-2-DCl-IF] and 3-*N*-dechloroethylifosfamide [(*R*)-3-DCl-IF and (*S*)-3-DCl-IF]. IF, 2-DCl-IF and 3-DCl-IF were extracted from plasma using solid-phase extraction and resolved by liquid chromatography on a column containing a Chirabiotic T chiral stationary phase. The enantioselective separations were achieved using a mobile phase composed of 2-propanol:methanol (60:40, v/v) and a flow rate of 0.5 ml/min. The observed enantioselectivities ($\alpha$) for IF, 2-DCl-IF and 3-DCl-IF were 1.20, 1.17 and 1.20, respectively. The calibration curve was linear in the concentration range of 37.50–4800 ng/ml for each ifosfamide enantiomer ($r^2 > 0.997$). The lower limit of detection (LLOD) was 5.00 ng/ml. The inter- and intra-day precision ranged from 3.63 to 15.8% relative standard deviation (R.S.D.) and 10.1 to 14.3% R.S.D., respectively, and the accuracy ranged from 89.2 to 101.5% of the nominal values. The method was applied to the analysis of plasma samples obtained from a cancer patient who received 3.75 g/m$^2$/day dose of (*R*,*S*)-ifosfamide as a 96-h continuous infusion.
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## 1. Introduction

Ifosfamide (IF; Fig. 1) is an alkylating oxazaphosphorine anticancer agent routinely used in the treatment of a variety of pediatric and adult tumors including soft tissue sarcomas and lymphomas [1–5]. IF contains a chiral center at the phosphorus atom and is clinically administrated as a racemic (50:50) mixture of (*R*)-IF and (*S*)-IF.

IF is a prodrug and requires biotransformation via cytochrome P450 (CYP) enzymes in order to exert its cytotoxic activity. The hepatic metabolism of IF has been extensively studied and occurs in the liver by two major pathways, Fig. 1 [6–11]. One pathway involves the 4-hydroxylation of IF and eventually leads to the production of the cytotoxic isophosphoramide mustard [6]. The 4-hydroxylation of *R*-IF is catalyzed by CYP3A4 and the 4-hydroxylation of *S*-IF by CYP2B6 [10,11].

The second pathway involves the oxidation of either the exocyclic-N2- or endocylic-N3-chloroethyl moieties producing the therapeutically inactive 2-*N*-dechloroethyl-ifosfamide [(*R*)- and (*S*)-2-DCl-IF] and 3-*N*-dechloroethylifosfamide [(*R*)- and (*S*)-3-DCl-IF] [6,12], Fig. 1. (*S*)-IF is metabolized to (*R*)-3-DCl-IF by CYP3A4 and (*S*)-2-DCl-IF by CYP2B6 while the

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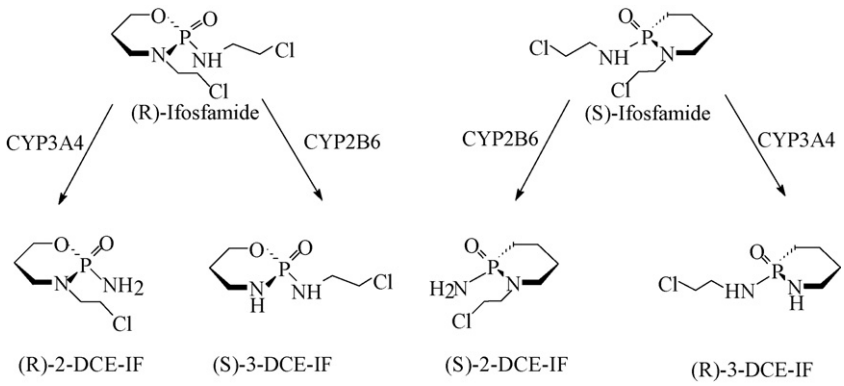


Fig. 1. The *N*-dechloroethylation of (*R*)- and (*S*)-ifosfamide by cytochrome P450 enzymes (CYP 3A4 and CYP 2B6) to yield the 2-*N*-dechloroethyl (2-DCE-IFF) and 3-*N*-dechloroethyl (3-DCE-IFF) metabolites. Reprinted with permission from [11].

(*R*)-IF enantiomer is metabolized to (*R*)-2-DCl-IF by CYP3A4 and (*S*)-3-DCl-IF by CYP2B6, where the apparent change in configuration at the chiral phosphorous moiety is a function of the Cahn–Ingold–Prelog nomenclature [11,13].

Clinically, the *N*-dechloroethylation pathway has been associated with treatment-ending central nervous system (CNS) toxicity [5,7–9]. The *N*-dechloroethylation pathway is regioselective and enantioselective [10,11] and, as a result, (*R*)-IF and (*S*)-IF have distinct efficacy and toxicity profiles. Clinical studies and in *in vitro* experiments have demonstrated that (*S*)-IF is more extensively cleared by *N*-dechloroethylation than (*R*)-IF [9,14–17]. In addition, clinical studies have indicated that treatment related CNS toxicities were associated with interpatient variations in the cumulative urinary excretion of the (*S*)-IF metabolite (*R*)-3-DCl-IF [9,16]. Thus, it is important that clinical studies include an enantioselective determination of the disposition of (*R*)- and (*S*)-IF as well as a measurement of the relative concentrations of their *N*-decholorethylated metabolites [16,17].

A number of enantioselective gas chromatography and HPLC methods have been developed for quantifying (*R*)- and (*S*)-IF and their respective *N*-dechloroethylated metabolites. Gas chromatography methods utilizing chiral stationary phases (CSPs) coated on glass capillaries have been extensively used for the enantioselective analysis of IF enantiomers in human plasma and urine [14,18–21]. These assays were also capable of the enantioselective resolution of 2-DCl-IF and 3-DCl-IF and, when coupled with mass spectrometric or nitrogen-selective detectors, able to quantify these compounds in biological matrices.

HPLC has also been used to resolve (*R*,*S*)-IF by applying different methods of detection including ultraviolet [22], fluorescence [10,15] or mass spectrometry detection [14,17,23,24]. Blaschke reported the initial separation using a polyacrylamide CSP [25] and subsequent enantioselective separations were achieved using the Chiracel OD [26] and AGP [27] CSPs. The latter assays were validated and used to assay plasma samples obtained during clinical studies. The Chiracel OD CSP was also used to simultaneously enantioselectively resolve (*R*,*S*)-IF, (*R*,*S*)-2-DCl-IF and (*R*,*S*)-3-DCl-IF in plasma and urine, but the liquid chromatography assay was not validated or applied to samples obtained during a clinical study [21].

However, a key problem in the application of enantioselective HPLC methods to routine clinical analysis is the fact that IF and its *N*-dechloroethylated metabolites lack a strong chromophore, see Fig. 1. When UV detection was used, the target compounds were monitored at $\lambda = 205–210$ nm, which made the analysis difficult due to solvent absorption at the same wavelength. A more sensitive achiral LC–MS method for the separation and analysis of IF and its *N*-dechloroethylated metabolites in rat microsomal medium has been recently reported [23], and our laboratory has reported preliminary results with an enantioselective LC–MS method [28]. In the latter approach, (*R*,*S*)-IF and (*R*,*S*)-2-DCl-IF were enantioselectively resolved using a CSP containing the macrocyclic antibiotic teicoplanin, the Chirabiotic T CSP, and detected by mass spectrometry using either an electrospray or atmospheric pressure chemical ionization interface. However, (*R*,*S*)-3-DCl-IF was not enantioselectively separated under the chromatographic conditions used in the study [28].

We now report the development and validation of an enantioselective LC–MS assay for the determination of (*R*)- and (*S*)-IF in human plasma. The assay utilizes the Chirabiotic T CSP chiral column and the chiral separations were achieved using a polar organic mode, where the mobile phase was composed of 2-propanol:methanol (60:40, v/v) solvents. The reported method is also able to enantioselectively resolve (*R*,*S*)-2-DCl-IF and (*R*,*S*)-3-DCl-IF and can be used to determine the relative concentrations of the compounds in human plasma. The assay has been applied to the study of plasma samples obtained from a clinical study employing (*R*,*S*)-IF.

## 2. Experimental

### 2.1. Materials

(*R*,*S*)-IF was obtained from Sequoia Research Products Ltd. (Oxford, UK). (*R*)-IF and (*S*)-IF were prepared by Advanced Separation Technologies Inc. (Whippany, NJ, USA) using an enantioselective HPLC separation of (*R*,*S*)-IF accomplished using a Chirabiotic T2 (10 μm) chiral column and a mobile phase composed of water:2-propanol (80:20, v/v) delivered at a flow rate of 30 ml/min. (*R*,*S*)-2-DCl-IF and (*R*,*S*)-3-DCl-IF were generously provided by the Central Analytical Services Department