Cancer Chemother Pharmacol (1997) 40: 57–59

ORIGINAL ARTICLE

R.F. Struck · D.M. McCain · S.W. Tendian
K.H. Tillery

Quantification of 4-hydroxyifosfamide in plasma of ifosfamide-treated mice

Received: 21 February 1996 / Accepted: 25 October 1996

Abstract *Purpose*: Ifosfamide is becoming an important clinical anticancer drug. Meaningful pharmacology studies require quantification of its activated and active metabolites, 4-hydroxyifosfamide (HOIfos) and isophosphoramide mustard (IPM), respectively. *Methods*: Current methodology for quantifying the unstable HOIfos in biological fluids consists of trapping acrolein as it is produced during the decomposition of this metabolite. However, unlike cyclophosphamide, ifosfamide is extensively metabolized to two dechloroethylated metabolites, which are susceptible to 4-hydroxylation and similarly are capable of yielding acrolein upon decomposition. Because the current method has the potential to yield higher than actual values for HOIfos, it was compared with an HOIfos-specific method that traps the first stage degradation product of HOIfos, aldoifosfamide, as its semicarbazone, and depends on the use of radiolabeled drug for quantification. Six experiments in mice were conducted with blood collection 15 or 30 min after drug treatment followed by determination of HOIfos in plasma by the two methods. *Results*: Comparison of plasma levels of HOIfos determined by the two methods indicated only minor differences between the two. *Conclusion*: These results suggest the possibility that the nonspecific method may be acceptable as a first estimation of levels of this metabolite in biological fluids until the development of a specific method that does not require radiolabeled drug, such as high-performance liquid chromatography or gas chromatography/mass spectrometry, has been developed.

Key words 4-Hydroxyifosfamide · Quantification · Mouse plasma

Supported by NIH, NCI Grants RO1 CA60844 and PO1 CA34200

R.F. Struck (✉) · D.M. McCain · S.W. Tendian · K.H. Tillery
Southern Research Institute, Birmingham, AL 35255-5305, USA

Introduction

Like its prototype cyclophosphamide (CPA), ifosfamide (Ifos) is considered to be an important clinical alkylating agent and similarly requires hepatic oxidative metabolism to generate an activated form, 4-hydroxyifosfamide (HOIfos), which subsequently yields an alkylating entity, isophosphoramide mustard (IPM). The metabolic pathway of Ifos is generally similar to that of CPA and is shown in Fig. 1. The metabolism of Ifos in vivo was first investigated by Norpoth et al. [9] and subsequently by others [10].

The major difference in the metabolism of Ifos and CPA is the former's greater susceptibility to dechloroethylation. Whereas dechloroethylCPA (DCECPA) is a minor metabolite of CPA in vivo, dechloroethylIfos (DCEIfos) and DCECPA, the two dechloroethylated metabolites of Ifos, are major metabolites of Ifos, as shown by Norpoth [8], who observed that, in 14 of 25 patients, urinary excretion of these two metabolites exceeded that of both Ifos and carboxyifosfamide, the metabolite analogous to carboxyphosphamide, which is typically the major urinary metabolite of CPA in experimental animals and patients [13]. Plasma concentrations are similarly high, in some patients equaling that of Ifos and achieving concentrations > 10 μg/ml [2, 3, 5, 7]. Consequently, a potential weakness exists in current methodology for quantifying HOIfos in biological fluids. In every case reported to date [1, 14], the method used has consisted of trapping acrolein liberated from the metabolite as it decomposes to the unstable aldoIfos and subsequently to IPM and acrolein. The potential weakness in the method is that any 4-hydroxylated derivatives of Ifos or its metabolites, namely 4-HODCEIfos and 4-HODCECPA, are similarly capable of liberating acrolein, and it is possible that these two metabolites may be produced in relatively large amounts because of the relative abundance of DCEIfos and DCECPA.

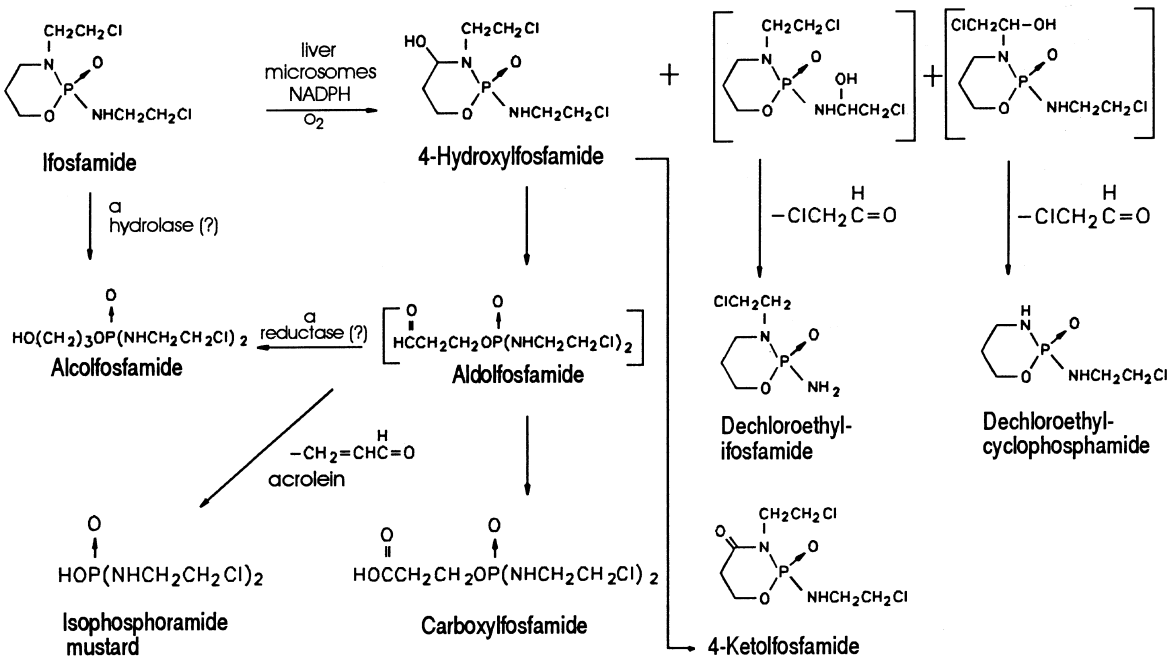

Fig. 1 Metabolism of ifosfamide

We report here the results of a comparison of the acrolein-trapping method with a specific method for quantifying 4-HOIfos in plasma of Ifos-treated mice which was based on trapping of this metabolite as its ring-opened semicarbazone, aldoifosfamide semicarbazone (AISC), and which utilized radiolabeled drug, thin-layer chromatography (TLC) and scintillation counting for quantification.

Materials and methods

Materials

[3-(2-Chloroethyl)-^{3}H]Ifos was purchased from Amersham, Arlington heights, Ill. Solvents and chemicals used were of reagent grade. TLC utilized Analtech silica gel G plates of 250 μm thickness. For use as a TLC standard, AISC was prepared by a previously reported method [11] from 4-hydroperoxyIfos [6] and characterized by fast atom bombardment mass spectrometry: $[M+1]^+$ 334 (2 Cl) and $[M+Na]^+$ 356 (2 Cl). 4-KetoIfos was prepared as reported [6], and DCEIfos was a gift from Dr. Marshall Goren, St. Jude Children's Research Hospital, Memphis, Tenn.

Drug treatment and plasma collection

CD2F1 mice (20 g, 6–8 weeks old, mixed male and female, (ten per group) were treated intraperitoneally (i.p.) with phenobarbital (80 mg/kg) for 4 days followed on day 5 with ^{3}H-Ifos (450 mg/kg, 10 μCi/mouse) in saline, and blood was collected on ice 15 and 30 min following carbon dioxide anesthetization. Plasma was collected by centrifugation at 4 °C. Half of each fraction was treated with 0.5 ml 0.4 *M* semicarbazide (pH 7). Both halves were stored at −20 °C pending analysis.

Isolation and quantification of plasma metabolites

Semicarbazide-treated plasma samples were allowed to melt and were extracted with methylene chloride (2 × 20 ml) to isolate Ifos and nonpolar metabolites. The total extract was dried over sodium sulfate, evaporated *in vacuo*, and the residue was dissolved in methanol and separated in triplicate by co-TLC with synthetic standards on silica gel in acetone/chloroform (1:3 v/v) for 15 cm followed by chloroform/methanol (9:1 v/v) for 10 cm to isolate in order (top to bottom of TLC plate) 4-ketoIfos, Ifos, DCEIfos and AISC. TLC spots were visualized with *p*-nitrobenzylpyridine (NBP) and collected for quantification by scintillation counting.

Using a previously reported procedure [14], plasma samples not treated with semicarbazide were separated into three equal fractions. Each fraction was treated with 0.6 ml of a solution containing 5 mg/ml 3-aminophenol and 6 mg/ml hydroxylamine hydrochloride. Samples were heated for 20 min at 95 °C, stored for 12 h at 5 °C, centrifuged, and their fluorescence determined at 350 nm excitation and 515 nm emission. Instrument calibration was accomplished with 4-hydroxyCPA in mouse plasma.

Extraction efficiency

Because of the unavailability of ^{3}H-HOIfos, extraction efficiency was determined for ^{3}H-aldophosphamide semicarbazone and was shown to be 82–90% in four determinations using ^{3}H-HOCPA, as previously reported [12].

Statistical analysis

Statistical tests were used to compare the HOIfos concentrations measured by the specific and acrolein methods. First, *F*-tests were used to compare the variances from the three determinations for each sample to determine whether the underlying variances were equal for the two methods. Then, for each of the six samples, the means determined with the two methods were compared using the appropriate two-sample Student's *t*-test, one for samples whose variances were significantly different (samples 1, 5, and 6). A paired-difference *t*-test was used for the comprehensive comparisons (all six samples, 15-min samples only, and 30-min samples only) to determine whether on average there was any difference in the results from the two methods.

Results and discussion

Six experiments were conducted using ten mice per group with blood collection for three groups 15 min after dosing and for three groups 30 min after dosing.

Utilizing the HOIfos-specific method, which traps HOIfos as AISC, co-TLC of each of the six samples in triplicate with synthetic standards in a solvent system of acetone/chloroform (1:3) for 15 cm followed by chloroform/methanol (9:1) for 10 cm, detection with NBP, collection of individual metabolite fractions, and quantification via scintillation counting gave the results shown in Table 1. Also included in Table 1 are the results of quantification of HOIfos in each of the six samples in triplicate employing the nonspecific, acrolein-trapping method in which acrolein is converted to the fluorescent compound 7-hydroxyquinoline [14].

Student's *T* tests of the data summarized in Table 1 showed that quantification by the two methods was not significantly different at the 5% level of significance. The data, therefore, do not support the hypothesis that significant metabolism of DCEIfos or of DCECPA to their corresponding 4-hydroxylated metabolites occurs in a mouse model to appreciably elevate the amount of acrolein trapped by the acrolein method.

The percentage of DCEIfos in the total yield of isolated drug and metabolites was relatively high (1–12% at 15 min for about 370 000 total cpm and 27–36% at 30 min for about 65 000 total cpm). It should also be noted that the specific method quantified only DCEIfos and not also DCECPA, typically the major dechloroethylated metabolite in plasma of Ifos-treated patients [2, 3, 5, 7], because the latter would not be radioactive, allowing for a possible underestimation of the levels of metabolites that could yield acrolein via the acrolein method following metabolic hydroxylation. There was, however, an insufficient amount of the hydroxylated, dechloroethylated metabolites in the mouse model to grossly elevate the apparent plasma levels of HOIfos quantified via the acrolein method. Although studies in patients indicate that relatively high levels of DCEIfos and DCECPA are present in human plasma, based on the study in mice, it is anticipated that significantly elevated apparent levels of HOIfos will not be observed in clinical studies using the acrolein method.

Consequently, the results of these investigations suggest the possibility that the acrolein method may serve as a first estimation for determination of plasma levels of HOIfos in patients until a specific method that does not require radiolabeled drug, such as high-performance liquid chromatography or gas chromatography/mass spectrometry as reported for 4-hydroxycyclophosphamide [4], is developed.

Table 1 Comparative plasma levels of HOIfos in mice following treatment with Ifos as determined by a specific method and a nonspecific acrolein trapping method

Sample	Time (min)	HOIfos (μg/ml)		Specific method
		Specific method	Acrolein method	Acrolein method
1	15	3.7 ± 0.3	5.3 ± 2.1	0.7
2	15	6.2 ± 0.5	5.6 ± 1.4	1.1
3	15	2.0 ± 0.6	4.3 ± 1.6	0.5
4	30	5.3 ± 0.6	3.3 ± 1.1	1.6
5	30	4.3 ± 0.2	4.2 ± 2.0	1.0
6	30	4.5 ± 0.3	4.9 ± 1.5	0.9

References

1. Arndt CAS, Balis FM, McCully CL, Colvin OM, Poplack DG (1988) Cerebrospinal fluid penetration of active metabolites of cyclophosphamide and ifosfamide in rhesus monkeys. Cancer Res 48: 2113
2. Boddy AV, Cole M, Pearson AD, Idle JR (1995) The kinetics of the auto-induction of ifosfamide metabolism during continuous infusion. Cancer Chemother Pharmacol 36: 53
3. Boddy AV, Proctor M, Simmonds D, Lind MJ, Idle JR (1995) Pharmacokinetics, metabolism and clinical effect of ifosfamide in breast cancer patients. Eur J Cancer 31A: 69
4. Chan KK, Hong PS, Tutsch K, Trump DL (1994) Clinical pharmacokinetics of cyclophosphamide and metabolites with and without SR-2508. Cancer Res 54: 6421
5. Granville CP, Gehrcke B, Konig WA, Wainer IW (1993) Determination of the enantiomers of ifosfamide and its 2- and 3-N-dechloroethylated metabolites in plasma and urine using enantioselective gas chromatography with mass spectrometric detection. J Chromatogr 622: 21
6. Hoborst HJ, Peter G, Struck RF (1976) Synthesis of 4-hydroperoxy derivatives of ifosfamide and trofosfamide by direct ozonation and preliminary antitumor evaluation in vivo. Cancer Res 36: 2278
7. Kaijser GP, Beijnen JH, Bult A, Wiese G, de Kraker J, Keizer HJ, Underberg WJ (1992) Gas chromatographic determination of 2- and 3-dechloroethylifosfamide in plasma and urine. J Chromatogr 583: 175
8. Norpoth K (1976) Studies on the metabolism of isophosphamide in man. Cancer Treat Rep 60: 437
9. Norpoth K, Wüst G, Witting U (1972) Quantitativ bestimmung des ifosfamids und eines ifosfamide-metaboliten in patient-enurin. Verh Dtsch Ges Inn Med 78: 1561
10. Sladek NE (1988) Metabolism of oxazaphosphorines. Pharmacol Ther 37: 301
11. Struck RF (1974) Isolation and identification of a stabilized derivative of aldophosphamide, a major metabolite of cyclophosphamide. Cancer Res 34: 2933
12. Struck RF, Alberts DS, Horne K, Phillips JG, Peng YM, Roe DJ (1987) Plasma pharmacokinetics of cyclophosphamide and its cytotoxic metabolites after intravenous versus oral administration in a randomized, crossover trial. Cancer Res 47: 2723
13. Struck RF, Kirk MC, Mellett LB, El Dareer S, Hill DL (1971) Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7: 519
14. Wagner T, Heydrich D, Jork T, Voelcker G, Hohorst HJ (1981) Comparative study on human pharmacokinetics of activated ifosfamide and cyclophosphamide by a modified fluorometric test. J Cancer Res Clin Oncol 100: 95