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Direct gas chromatographic determination of dechloroethylcyclophosphamide following microsomal incubation of cyclophosphamide

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Abstract

A method for the sensitive determination of dechloroethylcyclophosphamide (3-DCl) in microsomal incubation mixtures was developed. 3-DCl, a side-chain oxidation product of cyclophosphamide (CP), was isolated by extraction with acetic acid ethyl ester following solid-phase extraction on C_8 cartridges. Quantification of the metabolite was performed by direct capillary gas chromatography with a nitrogen–phosphorus detector without prior derivatization. The method showed good sensitivity and reproducibility with a detection limit of 1 ng/ml and a limit of quantification of 5 ng/ml. The suitability of the method is shown for the quantification of 3-DCl following incubation of CP with human liver microsomes.

1. Introduction

Cyclophosphamide (CP) is an antineoplastic drug which is widely used in cancer chemotherapy of various malignancies. It is used either alone or in combination with other cytostatics. The parent drug itself shows no cytotoxicity and therefore requires bioactivation via ring oxidation by the cytochrome P450 system in the human liver as shown in Fig. 1. Besides this metabolic activation there are some other pathways which lead to inactive metabolites. For example formation of 4-ketocyclophosphamide and carboxyphosphamide by further oxidation of the instable intermediates 4-hydroxycyclophosphamide and aldophosphamide is observed [1–3]. Inactivation of CP by means of side-chain oxidation (N-dealkylation) is also mediated by cytochrome P450 and leads to the formation of dechloroethylcyclophosphamide (3-DCl) and chloroacetaldehyde [4]. The latter seems to be responsible for central nervous system (CNS) toxicity of oxazaphosphorine therapy [5]. Fig. 1 shows a brief summary of these major pathways of CP metabolism and the role of the individual metabolites. Knowledge of the isoenzymes involved in activation and deactivation and the relative contribution of the different pathways would be useful for optimization of CP therapy.

Identification of the P450 isoenzyme responsible for side-chain oxidation in vitro (incubation

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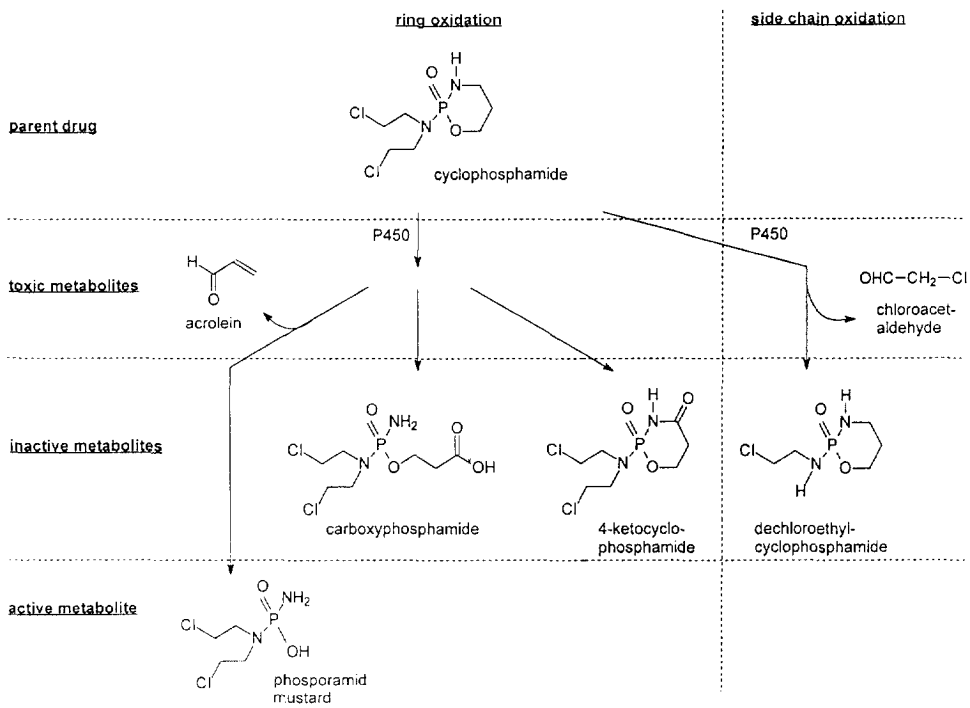

Fig. 1. Major pathways of cyclophosphamide metabolization.

of CP with human liver microsomes) requires a highly sensitive method. For measuring 3-DCl in plasma, urine or microsomal preparations various techniques have been described in the literature such as TLC [6,7], HPLC [8,9], GC [10,11] and ^{31}P NMR spectroscopy [12]. Besides these direct techniques for investigation of N-dealkylation via determination of 3-DCl, indirect methods were used (e.g. photometric determination of chloroacetaldehyde [13]). Most of these methods are not sensitive enough for the determination of 3-DCl following incubation with human liver microsomes (expected concentration range of only a few ng/ml). For example, with HPLC 3-DCl is only detectable because of its UV absorption at the low wavelength of 195 nm [8,9]. In this wavelength range most organic substances show strong absorption what causes loss of sensitivity. Similar to HPLC TLC densitometry [6,7] and NMR spectroscopy [12] have limits of detection in the lower μg/ml concentration range. The most sensitive technique is the GC determination of 3-DCl with a capillary column in combination with a nitrogen–phosphorus detection (NPD) system. The limit of quantification reached with this method was 0.2 nmol/ml in blood [10], 10 ng/ml in plasma and 25 ng/ml in urine [11]. Moreover, GC is readily combined with MS detection for verification of substance authenticity [9].

Since there is no assay available for the direct determination of 3-DCl formed in the presence of human liver microsomes we developed a GC method for the sensitive determination of 3-DCl. This method would be useful to identify the enzymes involved in the side-chain oxidation of CP.

2. Experimental

2.1. Chemicals

All solvents used were of HPLC quality; chemicals were of analytical grade. 2-DCl (2-dechloroethylifosfamide) and 3-DCl were a generous gift of Dr. J. Pohl (ASTA Medica, Frankfurt, Germany). CP monohydrate was ob-

tained from ICN (Meckenheim, Germany), and magnesium chloride hexahydrate was supplied by Merck (Darmstadt, Germany). NADPH, Trizma base [tris(hydroxymethyl)aminomethane) and Trizma–HCl [tris(hydroxymethyl)-aminomethane hydrochloride] were from Sigma (Deisenhofen, Germany).

2.2. Preparation of microsomes

Human and rat liver microsomes were prepared as described elsewhere [14]. Protein contents were determined according to the method of Lowry et al. [15].

2.3. Microsomal incubations

Incubations were carried out at 37°C for 1 h in the presence of increasing amounts of CP (20 μM to 18 mM). The protein content was 200 μg in a final volume of 250 μl. In addition, the incubation mixtures contained magnesium chloride (6 mM). The system was buffered with 0.05 M Tris buffer (pH 7.8). 2-DCl was added as internal standard at a final concentration of 100 ng/ml in the incubation mixture. The incubation was started by adding NADPH solution (end concentration 5 mM) and stopped by heating at 90°C for 10 min.

2.4. Extraction of 2-DCl and 3-DCl

For extraction of 2-DCl and 3-DCl and separation of CP, each of the microsomal incubation mixtures was transferred to a C_8 cartridge (3 ml, 200 mg, Varian, Harbor City, CA, USA) which was conditioned with 2 ml of methanol and 2 ml of water. The cartridges were then dried by vacuum on a SPE-24G column processor (Baker, Groß-Gerau, Germany). 2-DCl and 3-DCl were eluted with 2.5 ml of a methanol–water mixture (10:90, v/v) and then extracted with 5 ml of acetic acid ethyl ester by shaking for 15 min. The organic phase was evaporated to dryness in a stream of nitrogen and the residue dissolved in 50 μl of acetic acid ethyl ester. These extracts were analyzed by GC.

2.5. Standardization

Stock standard solutions of 2-DCl and 3-DCl were prepared in water at a concentration of 1 mg/ml, respectively. Working standard solutions were diluted from the stock solutions. All standard solutions were stored at −24°C until they were used.

Calibration samples were prepared by adding increasing amounts of 3-DCl to mixtures which contained denaturized rat liver microsomes instead of human liver microsomes. Calibration curves were obtained by plotting the peak-area ratios of 3-DCl and the internal standard 2-DCl against the substance concentration.

2.6. Instrumentation and GC conditions

2.6.1. GC determination of 3-DCl

A 5890 gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with a nitrogen–phosphorus detector was used. GC was performed in the splitless mode on a 15 m × 0.32 mm I.D. DB-17 capillary column (dimethylpolysiloxane with 50% phenyl groups), film thickness 0.25 μm (J&W Scientific, Fisons, Mainz, Germany). Helium was used as carrier gas at an inlet pressure of 85 kPa according to a linear carrier gas velocity of 30 cm/s (120°C). A 2-μl aliquot was injected at 200°C with an A200S autosampler (Fisons, Mainz, Germany). The detector temperature was 250°C, gas flow-rates were 3 ml/min (hydrogen), 100 ml/min (air) and 25 ml/min (helium, make-up gas), and the bead power was set at 30 pA. The initial oven temperature of 120°C was increased by 12°C/min to 200°C; this temperature was held for 9 min.

2.6.2. Identification of 3-DCl by GC–MS

A 5985 A mass spectrometer was coupled to a 5890 II gas chromatograph (Hewlett Packard). The GC conditions were the same as in the quantification experiments. MS was performed in the electron impact (EI) mode. MS conditions were: transfer-line temperature 250°C, source temperature 200°C, emission current 300 μA, electron energy 70 eV, multiplier voltage 2800 V. The spectrum obtained after incubation was

compared to that of the authentic reference compound under the same conditions.

2.7. Assay validation

To determine the accuracy and variability of the assay various amounts of 3-DCl were added to mixtures containing denaturized rat liver microsomes instead of human liver microsomes. These mixtures were analyzed alone (accuracy) or together with the samples in every series of experiments as quality controls (variability).

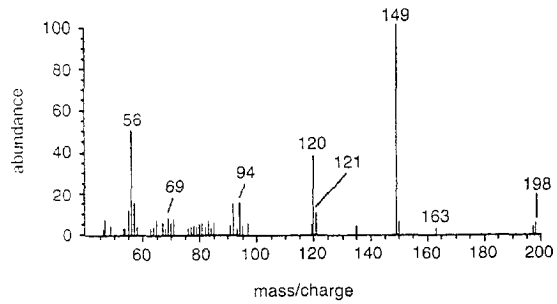

Fig. 2. Electron impact mass spectrum of dechloroethylcyclophosphamide.

3. Results and discussion

The method described allows the sensitive determination of 3-DCl in microsomal incubation mixtures with 2-DCl as internal standard in the presence of large amounts of CP. Sensitivity could be achieved by combination of high-resolution GC with highly selective NPD. Retention times were 6.6 and 7.3 min for 2-DCl and 3-DCl, respectively. CP produced two peaks at retention times of 9.1 and 10.3 min according to the original substance and an intramolecular cyclization product [16]. Although good separation of all these four compounds could be achieved, CP had to be removed by solid-phase extraction because of problems with overloading and contaminating the GC column by high amounts of CP which resulted in retention time shift and finally substance loss for 2-DCl and 3-DCl.

Authenticity of 3-DCl in microsomal mixtures was proven by GC–MS. Similar retention times and mass spectra were obtained for an authentic reference compound and the substance formed during the incubation. Fig. 2 shows the resulting EI mass spectrum of 3-DCl. Though 3-DCl was detectable with MS, NPD showed better sensitivity even when MS was carried out in the selected-ion monitoring mode. Therefore, the incubation mixtures were analyzed by high-resolution GC–NPD.

Standardization was carried out with denaturized rat liver microsomes instead of human liver microsomes in the presence of 2 m*M* CP. There was no formation of 3-DCl according to CP metabolization observed in these calibration samples. The internal standard 2-DCl was added before the incubation was started since no conversion of this compound by the microsomal enzymes was seen in control experiments. The method showed good linearity over the entire concentration range of 5–1000 ng/ml. Even at the lowest concentration the signal-to-noise ratio was better than 5. A typical standard curve for the determination of 3-DCl was $y = 0.088x + 0.2551$ ($r > 0.9996$). There was a good correlation ($y = 0.93x + 5.140$, $r = 0.9981$) between the concentration added and that measured by GC–NPD as the values for the accuracy show which are summarized in Table 1.

Reproducibility was tested by repeated analyses of mixtures of denaturized rat liver microsomes containing 2 m*M* CP spiked with known amounts of 3-DCl. Table 2 shows the intra-assay

Table 1
Accuracy of the determination of 3-DCl in microsomal incubation mixtures

Concentration added (ng/ml)	Concentration found (ng/ml)	Recovery (%)
5.0	4.8	96.0
10.0	9.6	96.0
20.0	20.2	101.0
50.0	46.0	92.0
100.0	90.8	90.8
200.0	205.5	102.8
500.0	499.6	99.9
1000.0	921.0	92.1

Table 2
Intra-assay and inter-assay precision for the determination of dechloroethylcyclophosphamide following microsomal incubation of cyclophosphamide

Concentration added (ng/ml)	n	Concentration found (ng/ml)	Bias (%)	C.V. (%)
Intra-assay variability				
5.0	5	4.77 ± 0.74	−4.6	15.5
100.0	5	102.5 ± 3.76	2.5	3.7
500.0	5	560.7 ± 20.1	12.1	3.6
Inter-assay variability				
5.0	6	5.07 ± 0.14	1.3	2.8
100.0	8	98.4 ± 5.1	−1.6	5.2
500.0	7	514.2 ± 32.4	2.8	6.3

and the inter-assay variabilities. Reproducibility was better than 5% in both cases except for the limit of quantitation (5 ng/ml) where it was still acceptable with 15%. At lower concentrations the variability increases to values of approximately 25%, the limit of detection is 1 ng/ml (signal-to-noise ratio = 3).

The suitability of the method described has been tested during an enzyme kinetic study. CP was incubated with human liver microsomes from different livers in the concentration range from 20 μM to 18 mM and the concentration of the 3-DCl formed was determined. Fig. 3 shows the resulting gas chromatogram of an extract after incubation of 1 mM CP compared to those of an incubation blank and a calibration sample. It is obvious that CP was effectively separated by solid-phase extraction since no corresponding peaks were detected in the retention time range of 9.0–10.5 min. Therefore, negative influences by the large surplus of CP in the chromatographic system could be eliminated. As it is depicted for one of the investigated livers in Fig. 4 the maximum rate of formation (V_{max}) and therefore the saturation of the enzyme could not be reached over the entire concentration range. The substrate-dependent formation of 3-DCl increased in a linear fashion over the entire range covered. As it is shown, the sensitivity of the method described is appropriate for the determination of 3-DCl in microsomal preparations.

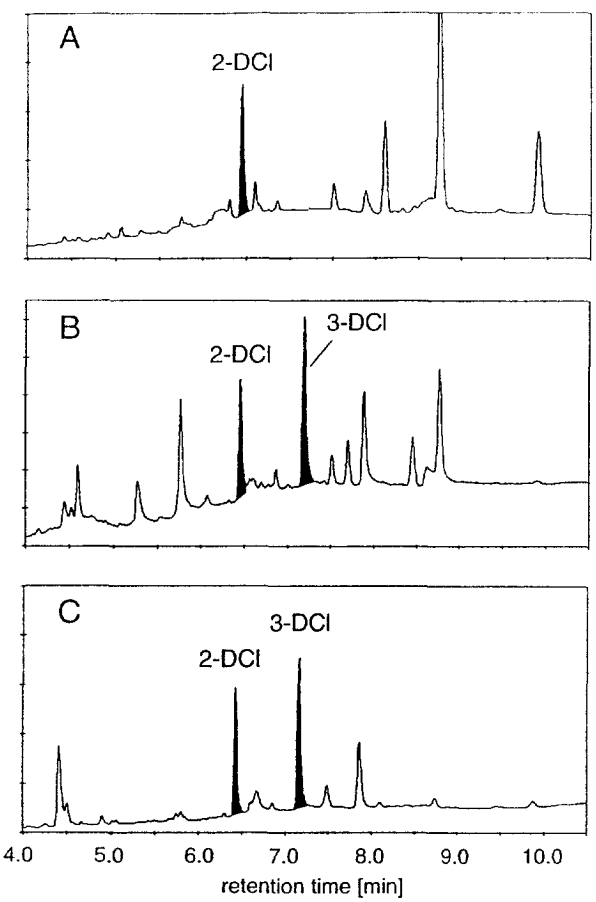

Fig. 3. Gas chromatograms of some incubation mixtures. (A) Incubation blank (human liver microsomes). (B) Extract after incubation of 1 mM cyclophosphamide with human liver microsomes. (C) Calibration sample containing 200 ng/ml 3-DCl (denaturized rat liver microsomes).

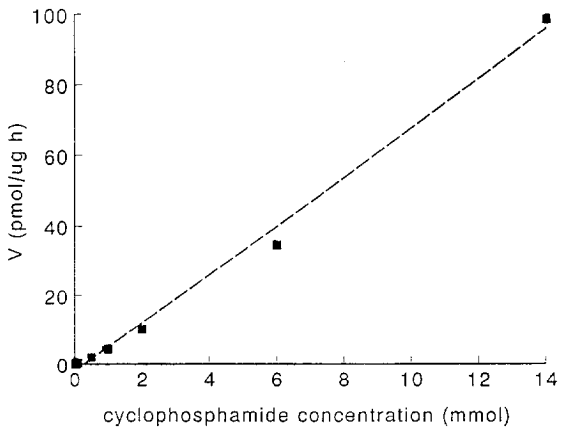

Fig. 4. Substrate-dependency of dechloroethylcyclophosphamide formation after incubation of cyclophosphamide with human liver microsomes.

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References

[1] T.A. Connors, P.J. Cox, P.B. Farmer, A.B. Foster and M. Jarman, Biochem. Pharmacol., 23 (1974) 115.
[2] C. Fenselau, M.-N.N. Kan, S.S. Rao, A. Myles, O.M. Friedman and M. Colvin, Cancer Res., 37 (1977) 2538.
[3] B.E. Domeyer and N.E. Sladek, Biochem. Pharmacol., 29 (1980) 2903.
[4] J.A. Ruzicka and P.C. Ruenitz, Drug Metab. Dispos., 20 (1992) 770.
[5] M.P. Goren, R.K. Wright, C.B. Pratt and F.E. Pell, Lancet, (1986) 1219.
[6] A.V. Boddy, Y. Furtun, S. Sardas, O. Sardas and J.R. Idle, J. Natl. Cancer Inst., 84 (1992) 1744.
[7] J.M. Hartley, L. Hansen, S.J. Hartland, P.W. Nicholson, F. Pasini and R.L. Souhami, Br. J. Cancer, 69 (1994) 931.
[8] J. Boos, M. Göring, F. Küpker, H. Jürgens and G. Blaschke, Pharm. Pharmacol. Lett., 3 (1993) 119.
[9] M.P. Goren, J. Chromatogr., 570 (1991) 351.
[10] V. Kurowski and T. Wagner, Cancer Chemother. Pharmacol., 33 (1993) 36.
[11] G.P. Kaijser, J.H. Beijnen, A. Bult, G. Wiese, J. de Kraker, H.J. Keizer and W.J.M. Underberg, J. Chromatogr., 583 (1992) 175.
[12] V. Gilard, M.C. Malet-Martino, M. de Forni, U. Niemeyer, J.C. Ader and R. Martino, Cancer Chemother. Pharmacol., 31 (1993) 387.
[13] D. Walker, J.-P. Flinois, S.C. Monkman, C. Beloc, A.V. Boddy, S. Cholerton, A.K. Daly, M.J. Lind, A.D.J. Pearson, P.H. Beaune and J.R. Idle, Biochem. Pharmacol., 47 (1994) 1157.
[14] P.J. Meier, H.K. Müller, B. Dick and U.A. Meyer, Gastroenterology, 85 (1983) 682.
[15] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[16] E.A. de Bruijn, A.T. van Oosterom, P.A. Leclercq, J.W. de Haan, L.J.M. van de Ven and U.R. Tjaden, Biomed. Environ. Mass Spectrom., 14 (1987) 643.