ELSEVIER

Journal of Chromatography B, 692 (1997) 163–168

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic determination of acrolein as a marker for cyclophosphamide bioactivation in human liver microsomes

Frank Bohnenstengel, Michel Eichelbaum, Ellen Golbs, Heyo K. Kroemer*

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Auerbachstrasse 112, 70376 Stuttgart, Germany

Received 10 July 1996; revised 24 September 1996; accepted 24 September 1996

Abstract

A high-performance liquid chromatographic method for the quantification of acrolein following incubation of cyclophosphamide (CP) with human liver microsomes was developed. Based on the formation of the fluorescent derivative 7-hydroxyquinoline by condensation of acrolein with 3-aminophenol quantitation was performed without prior extraction or other sample cleanup procedures. The method showed sufficient sensitivity with a limit of detection of 5 ng/ml and a limit of quantification of 10 ng/ml. The suitability of the method is shown for enzyme kinetic studies.

Keywords: Acrolein; Cyclophosphamide

1. Introduction

Cyclophosphamide (CP) is an alkylating antineoplastic substance which is used either alone or in combination with other drugs in many anticancer therapy regimes. The parent compound is a prodrug which is not cytotoxic and therefore requires bioactivation via ring oxidation. This reaction is mediated by the cytochrome P450 (CYP) system in human liver and leads to the instable intermediates 4-hydroxycyclophosphamide (4-OH-CP) and aldophosphamide (Aldo-CP) which are in tautomeric equilibrium [1,2]. The alkylating metabolite phosphoramide mustard (PM) is spontaneously formed from the latter via β-elimination of acrolein (2-propenal) which is responsible for urotoxic side effects of oxazaphosphorine therapy [3]. Moreover, acrolein has some teratogenic potency [4]. In parallel to this activation mechanism enzymatic inactivation is observed. Further oxidation of 4-hydroxycyclophosphamide (4-OH-CP) and aldophosphamide (Aldo-CP) leads to the formation of 4-ketocyclophosphamide and carboxyphosphamide, respectively [1]. Inactivation of CP is also achieved by cytochrome P450 3A4 mediated side-chain oxidation [5] leading to the formation of dechloroethylcyclophosphamide (DCl-CP) and chloroacetaldeyde [6] which seems to be responsible for CNS toxicity of oxazaphosphorines [7,8]. For optimization of CP therapy and consideration of drug–drug interactions knowledge of the P450 enzymes involved in activation and deactivation of CP and the relative contribution of the different pathways is pivotal. In the case of ifosfamide (IF) which is an isomer of CP Walker et al. [9] showed that CYP 3A4 is the major enzyme responsible for both ring and side-chain oxidation. A

*Corresponding author.

PII S0378-4347(96)00457-4

previous paper by Chang et al. [10] suggests that CYP 2B6 is responsible for bioactivation of CP. These findings appear to be questionable in view of the fact that 15% of the Caucasian and 70% of the Japanese patients do not express CYP 2B6 at all [11]. These patients would not bioactivate CP and therefore should be devoid of therapeutic efficacy, an effect which was not observed yet.

Identification of the enzyme involved in bioactivation of CP by means of in vitro methods (e.g., human liver microsomes or stable expressed enzymes) requires a highly specific and sensitive method. Since PM and above all 4-OH-CP/Aldo-CP are difficult to quantify due to their low stability measurement of CP bioactivation is usually achieved by determination of acrolein. For that purpose different methods have been described in the literature. Most of them include the formation of 7-hydroxyquinoline by condensation of acrolein with 3-aminophenol in presence of hydrochloric acid, a technique developed by Alarcon [12]. The easiest way for subsequent quantification is the direct fluorometric determination of the quinoline derivative. Though this method has the disadvantage of co-determination of other fluorescent substances it is still widely used for measurement of CP and IF bioactivation [9,10,13,14]. In order to avoid these problems some methods include separation of interfering substances by HPLC [15–17] without prior isolation procedures. Another HPLC method which includes the derivatization of acrolein with 2,4-dinitrophenylhydrazine [18] requires liquid–liquid extraction with chloroform. Since the in vitro characterization of cyclophosphamide bioactivation needs a highly sensitive and selective method we therefore developed a high-performance liquid chromatographic technique for determination of acrolein in presence of the microsomal fraction of human liver based on the derivatization with 3-aminophenol.

2. Experimental

2.1. Chemicals

All solvents used were of HPLC quality; chemicals were of analytical grade. Acrolein, 2-butenal, 3-aminophenol and hydroxylamine hydrochloride were obtained from Fluka (Neu-Ulm, Germany). CP monohydrate was from ICN (Meckenheim, Germany), magnesium chloride hexahydrate was supplied by Merck (Darmstadt, Germany). DCl-CP and PM were generous gifts of Dr. J. Pohl (ASTA Medica, Frankfurt, 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 elsewere [19]. Protein contents were determined according to the method described by Lowry et al. [20].

2.3. Microsomal incubations

Incubations were carried out at 37°C for 30 min in the presence of increasing amounts of CP (20 μ*M* to 32 m*M*). The system was buffered with 0.05 *M* tris buffer (pH 7.8). Protein content was 100 μg in a final incubation volume of 250 μl. In addition the mixtures contained magnesium chloride (6 m*M*). Incubation was started by adding NADPH solution (final concentration 5 m*M*).

2.4. Derivatization of acrolein samples with 3-aminophenol

Derivatization of the samples were carried out according to the method described by Alarcon [12]. The incubation was stopped by adding 250 μl of a reaction mixture consisting of 0.5% 3-aminophenol and 0.6% hydroxylamine hydrochloride in 3 *M* hydrochloric acid. A 10-μl volume of 2-butenal (25 μg/ml) was added as internal standard to yield a final concentration of 500 ng/ml in the incubation mixture and the samples were heated at 100°C for 10 min. After cooling the samples were centrifuged (5000 *g*, 5 min) for separation of microsomal proteins and 50 μl of the supernatant were injected onto the chromatographic system.

2.5. Standardization

Stock standard solutions of acrolein and 2-butenal were prepared in water in a concentration of 1 mg/ml, respectively. From these stock solutions working standard solutions were diluted. All standard solutions were stored at 4°C until they were used. Calibration curves showed that the standards were stable at least for 6 weeks. In order to get valid results they were newly prepared every month.

Calibration samples were prepared by adding increasing amounts of acrolein (final concentration 5, 10, 20, 40, 80, 100, 200, 400 or 600 ng/ml) to mixtures which contained denaturized rat liver microsomes instead of human liver microsomes. Calibration curves were obtained by plotting the peak-area ratios of acrolein and the internal standard 2-butenal against the substance concentration.

2.6. Instrumentation and HPLC conditions

For HPLC analysis a modular system from Shimadzu (Duisburg, Germany) was used. The system consisted of a LC-10AT pump unit, a RF-551 fluorescence detector, a SIL-9A auto injector with a 50 μl sample loop and a CR-R3A integrator. HPLC was performed on a 125×3 mm LiChrosorb 100 RP-8 (5 μm) column with a 20×4 mm RP-18 (5 μm) precolumn, both from Merck (Darmstadt, Germany). The mobile phase consisted of 0.5% phosphoric acid–acetonitrile (96:4 v/v), the flow-rate was 0.4 ml/min. The detector was set at 358 nm (excitation wavelength) and 505 nm (emission wavelength).

2.7. Assay validation

For determination of assay accuracy and variability various amounts of acrolein were added to samples containing denaturized rat liver microsomes instead of human liver microsomes. These mixtures were then analyzed alone (accuracy) or together with the human liver samples in every series of experiments as quality controls (variability). Protein binding of acrolein was investigated by comparison of acrolein concentrations in absence and in presence of denaturized rat liver microsomes.

3. Results and discussion

The method described allows the sensitive determination of acrolein following incubation of CP with human liver microsomes. Quantification was based on the formation of 7-hydroxyquinoline by condensation of acrolein (2-propenal) with 3-aminophenol in an acidic medium according to the method of Alarcon [12]. 2-Butenal was used as internal standard yielding 7-hydroxy-2-methylquinoline by the same derivatization reaction. The resulting derivatives could be easily and sensitively measured by fluorescence detection with an excitation wavelength of 358 nm and an emission wavelength of 505 nm. Except a centrifugation step for separation of the microsomal proteins no other sample cleanup procedures like liquid–liquid or solid-phase extraction are required. Although it has been described in the literature that the quinoline derivatives can be easily measured by fluorescence spectroscopy [9,10] they had to be separated by HPLC since we observed interfering substances in the microsomal incubation samples which could affect the results of quantitation. As shown in Fig. 1 these substances had their origin in the decomposition of NADPH. Derivatization of NADPH alone yielded two fluorescing peaks which had to be separated from the peaks of acrolein and 2-butenal by variation of column packing material and mobile phase. Best results were obtained with a C_8 column and a mobile phase consisting of 96% phosphoric acid (0.5%) and 4% acetonitrile. Under

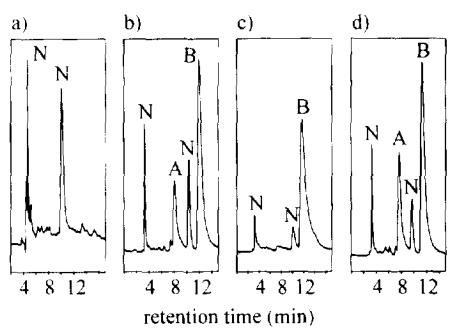

Fig. 1. HPLC chromatograms of some derivatized incubation mixtures (A: acrolein; B: 2-butenal; N: NADPH). (a) NADPH in water; (b) calibration sample containing 500 ng acrolein/ml (denaturized rat liver microsomes); (c) incubation blank (human liver microsomes); (d) incubation sample with 1 m*M* cyclophosphamide with human liver microsomes.

these conditions the NADPH peaks could effectively be separated from the peaks of acrolein and 2-butenal as it is also shown in Fig. 1. The retention times were 8.0 min (7-hydroxyquinoline) and 12.3 min (7-hydroxy-2-methylquinoline). Standardization was carried out with heat denaturized rat liver microsomes instead of human liver microsomes. The internal standard 2-butenal was added before the derivatization was started. There was no acrolein formation according to decomposition or metabolization of CP or the internal standard observed in calibration samples or incubation blanks.

Experiments in which acrolein was added to aqueous solutions or mixtures containing denaturized rat liver microsomes showed similar results for the measured acrolein concentrations. Therefore it can be concluded that protein binding of acrolein plays no important role in acrolein determination by the method presented here.

Additionally some experiments were carried out for investigation of acrolein formation due to decomposition of other CP metabolites which can be formed by microsomal oxidation (DCl-CP) or spontaneously (PM). Tests with high concentrations of these metabolites (6 m*M*) showed that DCl-CP and PM eliminated acrolein in 0.02 or 0.003% of the investigated metabolite concentration, respectively. These concentrations however are not achieved in microsomal incubations. Therefore acrolein formation by decomposition or metabolization of DCl-CP or PM could be neglected in our in vitro system.

Over the entire concentration range investigated (5–600 ng/ml) the method showed good linearity. A typical standard curve for acrolein determination was $y=0.0027x-0.0204$ ($r^2=0.9997$). The correlation between the acrolein concentration added and that measured by HPLC was excellent ($y=0.99995x+0.034$, $r^2=0.9993$) (values for the accuracy are summarized in Table 1). Reproducibility of the method was tested by repeated analyses of samples containing heat denaturized rat liver microsomes instead of human liver microsomes spiked with different amounts of acrolein. The CP concentration of these mixtures was 2 m*M*. The derivatization procedure was carried out as described for the incubation samples. Table 2 summarizes the values for the intra-assay and inter-assay variability. Reproducibility was better than 10% for all concentrations between 10 and 600 ng/ml. At lower concentrations the deviation from the nominal value increased to approximately 30% whereas the coefficient of variation was still acceptable with 14%. Therefore the limit of quantitation of the method was set at 10 ng/ml, the limit of detection is 5 ng/ml (signal-to-noise-ratio>3).

The method described has been used to determine the acrolein concentration following incubation of increasing amounts of CP (20 μ*M* to 32 m*M*) with human liver microsomes. The resulting HPLC chromatograms of a calibration sample, an incubation blank and an incubation sample containing 1 m*M* CP are depicted in Fig. 1. The results of the enzyme kinetic study are shown in Fig. 2. Contrary to DCl-CP formation after incubation of CP with human liver microsomes where a linear substrate dependency was observed [5] acrolein formation followed a Michaelis–Menten kinetic. As it is shown in Fig. 2 the maximum rate of formation (V_{max}) varied sub-

Table 1
Accuracy of the determination of acrolein as 7-hydroxyquinoline following incubation of cyclophosphamide with human liver microsomes ($n=5$)

Concentration added (ng/ml)	Concentration found (ng/ml)	Recovery (%)
10.0	11.2	112.0
20.0	20.0	100.0
40.0	36.6	91.4
80.0	74.8	93.4
100.0	97.8	97.8
200.0	208.9	104.4
400.0	407.7	101.9
600.0	593.2	98.9

Table 2
Intra-assay and inter-assay precision for the determination of acrolein as 7-hydroxyquinoline following microsomal incubation of cyclophosphamide

Concentration added (ng/ml)	n	Concentration found (ng/ml)	Deviation (%)	C.V. (%)
Intra-assay variability				
5.0	5	6.46±0.88	29.2	13.6
10.0	5	10.19±0.80	1.9	7.8
40.0	5	36.3±0.6	−9.3	1.6
100.0	5	91.8±0.5	−8.2	0.5
200.0	5	189.9±3.5	−5.1	1.8
600.0	5	597.4±6.6	−0.4	1.1
Inter-assay variability				
10.0	10	10.58±0.67	5.8	6.3
100.0	8	90.6±1.5	−9.4	1.3
200.0	5	184.2±4.8	−7.9	2.6
600.0	10	605.3±42.6	0.9	7.0

stantially in the 2 livers investigated. This example shows that selectivity and sensitivity of the method suffice for the determination of acrolein as its quinoline derivative in microsomal preparations. The method will be further used to characterize the P450 enzyme(s) involved in bioactivation of CP by incubation of CP with stable expressed enzymes and in presence of selective enzyme inhibitors.

Acknowledgments

This work was supported by the Dr. Mildred Scheel Foundation (Bonn, Germany), Grant No. 10-0952-Ei3 and the Robert Bosch Foundation (Stuttgart, Germany).

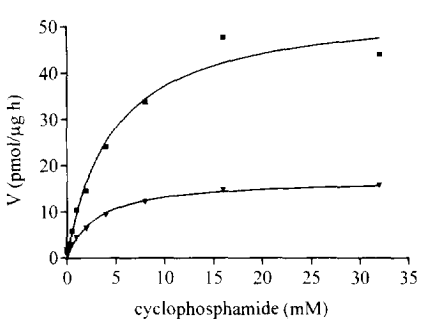

Fig. 2. Substrate-dependency of acrolein formation after incubation of cyclophosphamide with microsomes of 2 human livers.

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] N. Brock, J. Stekar, J. Pohl, U. Niemeyer and G. Scheffler, Arzneim. Forsch. Drug Res., 29 (1979) 659.
[4] U. Claussen, W. Hellmann and G. Pache, Arzneim. Forsch. Drug Res., 30 (1980) 2080.
[5] F. Bohnenstengel, U. Hofmann, M. Eichelbaum and H.K. Kroemer, Eur. J. Clin. Pharmacol., (1996) in press.
[6] J.A. Ruzicka and P.C. Ruenitz, Drug Metab. Dispos., 20 (1992) 770.
[7] M.P. Goren, R.K. Wright, C.B. Pratt and F.E. Pell, Lancet, (1986) 1219.
[8] I.W. Wainer, J. Ducharme, C.P. Granvil, M. Trudeau and B. Leyland-Jones, Lancet, 343 (1994) 982.
[9] D. Walker, J.P. Flinois, S.C. Monkman, C. Beloc, A.V. Boddy, S. Cholerton, A.K. Daly, M.J. Lind, D.J. Pearson, P.H. Beaune and J.R. Idle, Biochem. Pharmacol., 47 (1994) 1157.
[10] T.K.H. Chang, G.F. Weber, C.L. Crespi and D.J. Waxman, Cancer Res., 53 (1993) 5629.
[11] T. Shimada, H. Yamazaki, M. Mimura, Y. Inui and F.P. Guengerich, J. Pharmacol. Exp. Ther., 270 (1994) 414.
[12] R.A. Alarcon, Anal. Chem., 40 (1968) 1704.
[13] J.E. Low, R.F. Borch and N.E. Sladek, Cancer Res., 42 (1982) 830.
[14] I. Ikeuchi and T. Amano, Chem. Pharm. Bull., 33 (1985) 2416.

[15] J. Boos, M. Göring, F. Küpker, H. Jürgens and G. Blaschke, Pharm. Pharmacol. Lett., 3 (1993) 119.
[16] V. Kurowski and T. Wagner, Cancer Chemother. Pharmacol., 33 (1993) 36.
[17] S. Al-Rawithi, A. El-Yazigi and P.J. Nicholls, Pharm. Res., 10 (1993) 1587.
[18] P.J. Boor and G.A.S. Ansari, J. Chromatogr., 375 (1986), 159.
[19] P.J. Meier, H.K. Müller, B. Dick and U.A. Meyer, Gastroenterology, 85 (1983) 682.
[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.