Influence of Protein Binding on Acrolein Turnover In Vitro by Oxazaphosphorines and Liver Microsomes

Frank Baumann,* Renate Schmidt, Jens Teichert, and Rainer Preiss

Institute of Clinical Pharmacology, University Leipzig, Leipzig, Germany

For a correct determination of acrolein amounts generated in in vitro turnover experiments with oxazaphosphorines, it is necessary to characterize the interaction of acrolein with liver microsomal proteins. Acrolein, a highly reactive metabolite of oxazaphosphorines, readily forms covalent adducts with proteins by electrophilic attack on nucleophiles, such as the sulfhydryl group of cysteine, imidazole group of histidine, and amino group of lysine. The current investigations were mainly directed toward determination of the degree of acrolein-protein binding under conditions of in vitro experiments with liver microsome preparations. The acrolein concentration in protein dilution was determined by a fluorescence method. Moreover, the influence of sucrose and glycerine on the extent of acrolein-protein binding commonly used for

the stabilization of microsomal preparations during storage was investigated. The current investigations show evidence that the chemical reaction of acrolein with liver microsomal proteins strictly follows first order kinetics. The main part of the formed acrolein in the in vitro attempts is available as bound part. Results of these investigations indicate that the calibration should be carried out with mixtures from liver microsome preparations and known amounts of acrolein under the same conditions as the in vitro experiments to record the entirely formed acrolein part (free and bound) in oxazaphosphorine turnover experiments. Glycerine is recommended as a preservative to store liver microsomes instead of sucrose because the latter reacts with acrolein. *J. Clin. Lab. Anal.* 19:103–109, 2005. © 2005 Wiley-Liss, Inc.

Key words: Acrolein, liver microsome preparation, acrolein-protein binding

INTRODUCTION

Oxazaphosphorines such as cyclophosphamide, ifosfamide, and trofosfamide are widely used in the treatment of various cancer diseases. Oxazaphosphorines are prodrugs that require hydroxylation at C-4 by the microsomal liver cytochrome P450 system to exhibit antitumor activity. The 4-hydroxy-oxazaphosphorines decompose, forming mustards and acrolein. These mustards mediate the cytostatic effect of oxazaphosphorines, whereas acrolein seems to be responsible for the urotoxicity of oxazaphosphorines. Acrolein must be considered as a key component of the activation reaction of oxazaphosphorines. Furthermore, it is known that acrolein is a very active substance, which can readily reacts with sulfhydryl, imidazol, and amino groups of proteins (1–6). For a correct determination of acrolein amounts generated in in vitro turnover experiments of oxazaphosphorines, it is therefore necessary to characterize the interaction of acrolein

with liver microsomal proteins. In previously reported in vitro experiments with liver microsome preparations, only the nonbound fraction of acrolein has been evaluated (7–10). This problem seems to be undervalued. For the first time, methodical aspects of acrolein quantitation in microsomal protein preparations was discussed by our working group (11). The aim of the present study was to investigate various parameters that may affect the acrolein-protein binding in in vitro experiments with liver microsome preparations and oxazaphosphorines.

*Correspondence to: Frank Baumann, Universität Leipzig, Institut für Klinische Pharmakologie, Härtelstr. 16-18, 04107 Leipzig, Germany. E-mail: baumf@medizin.uni-leipzig.de

Received 14 January 2005; Accepted 11 March 2005

DOI 10.1002/jcla.20062

Published online in Wiley InterScience (www.interscience.wiley.com).

MATERIALS AND METHODS

Substances

ZnSO₄, Ba(OH)₂, sucrose, and hydroxylamine HCl were purchased from Sigma (Taufkirchen, Germany). Glycerine, concentrated HCl, and bidistilled water were obtained from Baker (Deventer, Netherlands) and acrolein from Fluka (Buchs, Switzerland). Potassium phosphate and ethylene diaminetetra acetic acid (EDTA) were from Merck (Darmstadt, Germany) and 3-aminophenol from Aldrich.

Preparation of Rat Liver Microsome Preparations

Immediately after excision, liver tissue samples were rapidly frozen in liquid nitrogen and stored at -70°C . Frozen tissue was homogenized in phosphate buffer (pH 7.4, 1/15 M potassium phosphate, 1 mM EDTA) and the homogenate was centrifuged at 11,000g and 2°C for 60 min. The microsomal pellet was resuspended in phosphate buffer containing 20% glycerine and stored at -20°C until use. Total protein concentrations were measured according to the method of Bradford (12). All samples were pooled and apportioned into aliquots.

Acrolein-Protein Binding Experiments

In a polypropylene tube, a solution of different acrolein content was added to a sample of microsome dilution and incubated in an Eppendorf Thermomixer (Hamburg, Germany). The binding rate was determined by taking samples at 6 time points over the course of 2 hr and the nonbound fraction of acrolein was analyzed by fluorescence measurement. For all experiments a constant rotation speed of 300 rpm (Eppendorf Thermomixer) was used. Concentrations of protein and sucrose, sampling time, pH value, and temperature were varied in the experiments (after 20 min). After a defined time, 100 μL 0.015 N HCl, 200 μL of a ZnSO₄ solution (5%), and 200 μL of a Ba(OH)₂ solution (2.5%) were added to 500 μL of samples or aqueous solutions with known acrolein amounts (for calibration) at room temperature. The precipitated BaSO₄ was removed by centrifugation (11,000g, 2 min). Nonbound respective free acrolein concentrations were detected in the supernatants using a fluorimetric assay. Three experiments were performed with exactly the same conditions and the concentrations of free acrolein were determined by repeated fluorimetric measurement ($n=2$). Concentrations of free or nonbound acrolein calculated from calibration curves obtained from analysis of aqueous solutions were correlated with the total amount of acrolein added to the mixture.

Fluorescence Assay for Measurement of Acrolein

After protein precipitation, 500 μL derivation reagent and 300 μL bidistilled water were added to 300 μL of the supernatant. 100 mL derivation reagent contained 500 mg 3-aminophenol, 600 mg hydroxylamine hydrochloride, and 25 mL concentrated HCl. Acrolein reacts with 3-aminophenol to form 7-hydroxyquinoline. Hydroxylamine stabilizes the solution of 3-aminophenol for a long time (13). The reaction was carried out in 1.5-mL polypropylene vials wrapped in aluminum foil at 90°C for 20 min. Subsequently, the reaction mixture was cooled to room temperature and transferred into cuvettes. The fluorescence measurements were carried out at 25°C with an excitation wavelength of 337 nm. The emission was recorded in the range from 300 to 600 nm (sensitivity: low; scan speed: fast; split width: 10 nm) with a maximum between 490 and 510 nm depending on the matrix (Fig. 1) using a Shimadzu RF-1502 spectrofluorometer (Duisburg, Germany).

Calibration curves were constructed by sampling known amounts of acrolein in aqueous solution with exactly the same conditions as used in the series of experiments with mixtures containing proteins after subtraction of the blank value. Because the fluorescence signal of blank values strongly depends on the matrix composition, there is strong need for a blank value subtraction.

The detection limit was 0.02 $\mu\text{g/mL}$ (threefold noise) and the coefficient of correlation for the calibration curve was always greater than 0.999. The within-run and between-run precision for 11.3, 1.89, and 0.44 $\mu\text{g/mL}$ acrolein were 1.5%, 2.2%, and 7.1%, as well as 4.5%, 10.3%, and 12.2%, respectively ($n=5$). Because within-run precisions were smaller than between-run precisions, a single calibration for each sample set was used.

RESULTS

Table 1 shows the time dependence of the reaction between acrolein and liver microsomal proteins of a pooled preparation. The reaction conditions were 37°C , pH 7.4, and 1 mg/mL protein.

Most relevant factors that affect acrolein-protein binding such as temperature, pH, and protein content are shown in Table 2. Generally, incubation times for the in vitro experiments are in the range 10–30 min, as it has been described in the literature. Hence, a reaction time of 20 min was chosen for all experiments. The results show that the degree of acrolein-protein binding clearly rises with increased pH values. Total protein contents were in range 0.25 to 2 mg/mL in these in vitro experiments (7–8, 11, 14–16). The degree of acrolein-protein binding, normalized on 1 mg protein/mL, decreases in a nonlinear manner with increased protein

Fig. 1. Fluorescence spectra of 7-hydroxyquinoline at excitation wavelengths from 337 nm.

TABLE 1. Time dependence on the reaction between acrolein and liver microsomes (pooled fraction) at different acrolein concentrations

1 µg/ml added acrolein concentration (t = 0 min)		2 µg/ml added acrolein concentration (t = 0 min)		5 µg/ml added acrolein concentration (t = 0 min)	
Time (min)	Measured acrolein concentration (µg/ml)	Time (min)	Measured acrolein concentration (µg/ml)	Time (min)	Measured acrolein concentration (µg/ml)
5	0.383 ± 0.129				
10	0.230 ± 0.066	10	0.815 ± 0.156	10	3.014 ± 0.359
20	0.125 ± 0.059	20	0.458 ± 0.233	20	2.240 ± 0.225
40	0.106 ± 0.028	40	0.277 ± 0.172	40	1.701 ± 0.222
60	0.091 ± 0.005	60	0.190 ± 0.114	60	1.458 ± 0.398
120	0.065 ± 0.006	120	0.130 ± 0.080	120	0.786 ± 0.146

N = 3 (3 experiments at each concentration); M = 2 (repeated measurements of each sample).

content. The temperature dependence was examined in the range 32–42°C.

After a heat denaturation at 90°C for 20 min the cooled liver microsome preparations were spiked with known acrolein amounts and the free acrolein concentration was measured after a reaction phase of 20 min at 37°C. The results were compared with values by an application of nondenatured proteins in Table 3. Acrolein reacts in a stronger extent with the heat-denatured proteins.

Glycerin and sucrose were used as preservative to store liver microsomes for a long time. It could be shown that glycerin does not bind acrolein (Table 4). Sucrose has been used in many studies for the preservation of liver microsomes. Therefore, in Table 5 the free acrolein part in different sucrose solutions is presented with and without microsome preparations. The results show that the measured free acrolein amounts decrease with increasing sucrose concentrations and this effect was enhanced in the presence of liver microsomes.

DISCUSSION

Acrolein is a very reactive representative of the α , β -unsaturated carbonyl compounds, which contain two electrophilic reaction centers. The following reaction schemes have been described in the literature for acrolein (1–6):

Acrolein reacts readily with primary amines to azomethines (Schiff bases) and with sulfhydryl to Michael addition reaction products, leading to acrolein-protein adducts. Representative amino acids containing free sulfhydryl or amino groups are cysteine, lysine, glutamine, asparagine, and arginine. Besides these reactions, a nucleophilic attack of acrolein at a nitrogen atom of the imidazole moiety contained in the amino acid histidine has been described. Moreover, acrolein shows facile reactivity with the ϵ -amino group of lysine to form FDP-lysine as the major product (1).

One has to take into account the fact that acrolein is highly protein-bound even in *in vitro* oxazaphosphorine turnover experiments with liver microsomal preparations. If disregarded, the concentrations of generated acrolein will be grossly underestimated due to the above mentioned acrolein-protein adducts.

The measured acrolein concentrations vs. time were fitted to an logarithmic function (Eq. [1]; c =acrolein concentration, t =time) to yield first-order rate constants, k_{1st} .

$$c = a_0 + a_1 * \ln t \quad (1)$$

We determined the order of the reaction n from the differential rate law after Eqs. [2] and [3].

$$r = -d(c)/dt = -a_1/t \quad (2)$$

$$\lg r = \lg k + n \lg (c) \quad (3)$$

The reaction rate r can be calculated at every time ($t > 0$ min) from the first derivative of the fitted function. The rate constant is k .

In Fig. 2, Eq. [3] is presented for all three examined acrolein concentrations. This complex reaction can be approximately described by first order kinetics. However, incubation time is the most important parameter,

which strongly affects the determination of total acrolein concentration. For the chosen concentration range, in *in vitro* experiments the protein-bound acrolein moiety overwhelms the nonbound acrolein part at all times.

The results show strong evidence that the extent of acrolein-protein binding increases with higher pH values. Provided that the above mentioned reactions of acrolein are the major reactions of acrolein turnover, the higher degree of acrolein-protein binding in the basic milieu is in good accordance with the base-catalyzed nature of these reactions.

The degree of acrolein-protein binding decreased in a nonlinear manner with increased protein content attributable to an increase in steric hindrance effects. The total amounts of added acrolein bound to components of liver microsome preparations within 20 min was between 25 and 95% under the experimental conditions employed. Besides incubation time, protein content is the most important parameter that must be held constant in the *in vitro* experiments. More than 99% of the proteins are removed from the solution by precipitation, as indicated by the total protein measurement of the supernatant. Compared to an aqueous solution, no different acrolein concentrations were determined fluorimetrically in the supernatant of a microsome preparation after barium sulfate precipitation of the microsomal proteins. Therefore, it can be assumed that acrolein is bound mainly to the proteins that have been precipitated by barium sulphate.

TABLE 2. Variation of different parameters on the reaction between acrolein and liver microsomes (pooled fraction) at various acrolein concentrations

Temperature dependence (20 min, pH 7.4, 1 mg/ml protein)			
Added acrolein concentration (μg/ml)	Measured acrolein concentration (μg/ml)		
	32°C	37°C	42°C
1	0.120 ± 0.052	0.078 ± 0.042	0.053 ± 0.007
2	0.526 ± 0.018	0.372 ± 0.122	0.208 ± 0.008
3.5	1.488 ± 0.045	1.230 ± 0.147	0.781 ± 0.122
5	2.557 ± 0.031	2.197 ± 0.151	1.627 ± 0.100

Influence of the pH-value (20 min, 1 mg/ml protein, 37°C)			
Added acrolein concentration (μg/ml)	Measured acrolein concentration (μg/ml)		
	pH 6.8	pH 7.4	pH 8.0
1	0.258 ± 0.052	0.119 ± 0.041	0.053 ± 0.010
2	0.725 ± 0.066	0.462 ± 0.060	0.158 ± 0.016
3.5	1.708 ± 0.164	1.279 ± 0.093	0.666 ± 0.056
5	2.735 ± 0.183	2.178 ± 0.186	1.358 ± 0.069

Variation of protein amount (20 min, pH 7.4, 37°C)			
Added acrolein concentration (μg/ml)	Measured acrolein concentration (μg/ml)		
	0.25 mg/ml	1 mg/ml	2 mg/ml
1	0.495 ± 0.124	0.113 ± 0.038	0.049 ± 0.015
2	1.268 ± 0.126	0.448 ± 0.060	0.119 ± 0.026
3.5	2.535 ± 0.089	1.274 ± 0.149	0.358 ± 0.051
5	3.766 ± 0.126	2.304 ± 0.077	0.882 ± 0.079

N=3 (3 experiments at each concentration); M=2 (repeated measurements of each sample).

TABLE 3. Amount of acrolein binding on heat-denatured (90°C, 20 min) and nondenatured liver microsomes at various acrolein concentrations*

Added acrolein concentration (μg/ml)	Measured acrolein concentration (μg/ml)	
	Nondenatured microsomes	Heat-denatured microsomes
1	0.109 ± 0.021	0.061 ± 0.009
2	0.435 ± 0.042	0.150 ± 0.012
3.5	1.261 ± 0.027	0.586 ± 0.092
5	2.230 ± 0.056	1.644 ± 0.124

*Conditions for reaction between acrolein and microsomes: 20 min, 37°C, pH 7.4, and 1 mg/ml protein.

In addition, we studied the binding of acrolein to liver microsome preparations after protein heat denaturation at 90°C for 20 min. After cooling, we subsequently

TABLE 4. Acrolein binding experiments with glycerine*

Added acrolein concentration (μg/ml)	Measured acrolein concentration (μg/ml)
12.480	13.148 ± 0.732
6.240	6.184 ± 0.414
2.496	2.576 ± 0.088

*Reaction conditions: 20 min, pH 7.4, 37°C, and 20% glycerine. N=3 (3 experiments at each concentration); M=2 (repeated measurements of each sample).

added known amounts of acrolein and measured the free acrolein content after incubation at 37°C for 20 min. The results indicate that the extent of protein-bound acrolein was significantly increased after heat denaturation (Table 3). It is known that protein denaturation unfolds the native structure of a protein and can significantly change their capacity to bind other molecules—typically decreasing specific affinity and increasing nonspecific affinity. The latter is attributable to the increased protein's surface and an increased number of nucleophilic reaction centers such as amino, sulfhydryl, or imidazole groups. It further suggests that heat denatured proteins of liver microsome preparations are unsuited for quantitative determination of acrolein generated in vitro by liver microsomes. This finding is in contradiction to the recommendations of Bohnenstengel et al. (8).

Further investigation of the acrolein affinity to glycerine and sucrose shows no binding of acrolein to glycerine (Table 4). However, measurable amounts of acrolein bound to different sucrose concentrations were detected in mixtures with and without liver microsomes. The results presented in Table 5 indicate that the measured free acrolein amounts decrease with increasing sucrose concentrations and this effect was enhanced in presence of liver microsomes. As a result of these investigations we recommend glycerine over sucrose as the preservative for liver microsome preparations. On the other hand, glycerine should not be used for the determination of acrolein by HPLC/UV-detection (11) because no sufficient chromatographic separation of the hydrazone derivatives formed by derivatization of acrolein and glycerine can be achieved. According to the method used for determination, a suitable preservative should be chosen.

For calibration of the total acrolein concentration generated in vitro by liver microsomes, calibration standards should be prepared by spiking the microsomal samples under identical reaction conditions as they are used for turnover experiments. In such experiments acrolein is consecutively formed from oxazaphosphorines and a constant rate of acrolein formation can be

TABLE 5. Acrolein binding of sucrose in presence and absence of liver microsomes

Different amounts of sucrose without liver microsomes (20 min, 37°C, pH 7.4)				
Added acrolein concentration (µg/ml)	Measured acrolein concentration (µg/ml) Different sucrose concentration			
	5 mg/ml	10 mg/ml	20 mg/ml	
1	0.770 ± 0.049	0.634 ± 0.035	0.358 ± 0.058	
2	1.437 ± 0.089	1.228 ± 0.112	0.696 ± 0.154	
3.5	2.607 ± 0.167	2.240 ± 0.110	1.374 ± 0.148	
5	3.864 ± 0.176	3.249 ± 0.125	1.969 ± 0.360	

Different amounts of sucrose and 1 mg/ml liver microsomal protein (20 min, 37°C, pH 7.4)				
Added acrolein concentration (µg/ml)	Measured acrolein concentration (µg/ml) Different sucrose concentrations			
	0 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml
1	0.109 ± 0.021	0.113 ± 0.031	0.081 ± 0.023	0.050 ± 0.013
2	0.435 ± 0.042	0.356 ± 0.060	0.259 ± 0.026	0.145 ± 0.055
3.5	1.261 ± 0.027	0.951 ± 0.052	0.699 ± 0.082	0.505 ± 0.040
5	2.230 ± 0.056	1.682 ± 0.060	1.221 ± 0.018	0.928 ± 0.036

N = 3 (3 experiments at each concentration); M = 2 (repeated measurements of each sample).

Fig. 2. Function $\lg r = \lg k + n \lg c$ for the calculation order of the reaction n at various acrolein concentrations; r , k , and c are the reaction rate of acrolein-protein binding, rate constant, and acrolein concentration, respectively.

assumed. A time equal to one-half of the incubation time should be sufficient to ensure protein-binding of generated acrolein. Therefore, we propose that a point of acrolein measurement after half of the incubation time should be adequate to characterize real circumstances in liver microsomal preparations. The total acrolein part should be redetermined following the scheme as described above or it must clearly be noted that only the free acrolein part was determined.

Summarizing the results obtained from oxazaphosphorine turnover experiments, we conclude that in liver microsome preparations the amounts of generated acrolein have been underestimated until now. To quantitate the total acrolein amount generated in oxazaphosphorine turnover experiments from microsomal protein preparations it is necessary to determine the proportion of protein-bound acrolein under identical conditions prior to measurement. In the present report we briefly describe a procedure for approximation of total acrolein amount in liver microsome preparations. Glycerine should be preferred to sucrose as preservative for liver microsome preparations in the case of acrolein determination by any type of fluorometric detection.

REFERENCES

1. Uchida K, Kanematsu M, Sakai K, et al. 1998. Protein-bound acrolein: potential markers for oxidative stress. *Proc Natl Acad Sci USA* 95:4882–4887.
2. Marinello AJ, Bansal SK, Paul B, et al. 1984. Metabolism and binding of cyclophosphamide and its metabolite acrolein to rat hepatic microsomal cytochrome P-450. *Cancer Res* 44:4615–4621.
3. Kanekal S, Kehrer JP. 1993. Evidence for peroxidase-mediated metabolism of cyclophosphamide. *Drug Metab Dispos* 21:37–42.
4. Gurtoo HL, Marinello AJ, Struck RF, Paul B, Dahms RP. 1981. Studies on the mechanism of denaturation of cytochrome P-450 by cyclophosphamide and its metabolites. *J Biol Chem* 256:11691–11701.
5. Calingasan NY, Uchida K, Gibson GE. 1999. Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease. *J Neurochem* 72:751–756.

6. Furuhashi A, Nakamura M, Osawa T, Uchida K. 2002. Thiolation of protein-bound carcinogenic aldehyde. An electrophilic acrolein-lysine adduct that covalently binds to thiols. *J Biol Chem* 277:27919–27926.
7. Walker D, Flinois J-P, Monkman SC, et al. 1994. Identification of the major human hepatic cytochrome P450 involved in activation and N-dechloroethylation of ifosfamide. *Biochem Pharmacol* 47:1157–1163.
8. Bohnenstengel F, Eichelbaum M, Golbs E, Kroemer HK. 1997. High-performance liquid chromatographic determination of acrolein as a marker for cyclophosphamide bioactivation in human liver microsomes. *J Chromatogr B* 692:163–168.
9. LeBlanc GA, Sundseth SS, Weber GF, Waxman DJ. 1992. Platinum anticancer drugs modulate P-450 mRNA levels and differentially alter hepatic drug and steroid hormone metabolism in male and female rats. *Cancer Res* 52:540–547.
10. Murray M, Butler AM, Stupans I. 1994. Competitive inhibition of human liver microsomal cytochrome P450 3A-dependent steroid 6 β -hydroxylation activity by cyclophosphamide and ifosfamide in vitro. *J Pharmacol Exp Ther* 270:645–649.
11. Preiss R, Schmidt R, Baumann F, et al. 2002. Measurement of 4-hydroxylation of ifosfamide in human liver microsomes using the estimation of free and protein-bound acrolein and codetermination of keto- and carboxy-ifosfamide. *J Cancer Res Clin Oncol* 128:385–392.
12. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle protein–dye binding. *Anal Biochem* 72:248–254.
13. Alarcon RA. 1968. Fluorimetric determination of acrolein and related compounds with m-aminophenol. *Anal Chem* 40:1704–1708.
14. Bohnenstengel F, Johnson S, Hofmann U, Eichelbaum M, Kroemer HK. 1995. Direct gas chromatographic determination of dechloroethylcyclophosphamide following microsomal incubation of cyclophosphamide. *J Chromatogr B* 672:271–276.
15. Bohnenstengel F, Hofmann U, Eichelbaum M, Kroemer HK. 1996. Characterization of the cytochrome P450 involved in side-chain oxidation of cyclophosphamide in humans. *Eur J Clin Pharmacol* 51:297–301.
16. Weber GF, Waxman DJ. 1993. Denitrosation of the anti-cancer drug 1,3-bis(2-chloroethyl)-1-nitrosourea catalyzed by microsomal glutathione S-transferase and cytochrome P450 monooxygenases. *Arch Biochem Biophys* 307:369–378.