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# Determination of cyclophosphamide metabolites by gas chromatography and thermionic specific detection Interindividual differences in hepatic biotransformation of cyclophosphamide in man in vitro

P.J.M. Sessink, W.H.J. Vaes, P.H.H. van den Broek, J. Noordhoek, R.P. Bos\*

*Department of Toxicology, Faculty of Medical Sciences, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, Netherlands*

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## Abstract

Sensitive methods for the determination of the cyclophosphamide metabolites nornitrogen mustard, 4-ketocyclophosphamide and carboxyphosphamide are presented. After liquid–liquid extraction and derivatization, the metabolites are determined by gas chromatography and thermionic specific detection. The methods were used to study the in vitro biotransformation of cyclophosphamide with S-9 liver fractions of human donors. The results show large interindividual differences in the formation of nornitrogen mustard and carboxyphosphamide. 4-Ketocyclophosphamide was not detected.

## 1. Introduction

Cyclophosphamide (CP) is an alkylating anti-neoplastic agent with activity against a variety of human tumours [1]. CP is also used in the treatment of autoimmune diseases and during renal and bone marrow transplantations [1]. The activity of CP is caused by its metabolites [1]. During bioactivation by hepatic cytochrome P-450, 4-hydroxycyclophosphamide is formed which is in equilibrium with its ring-opened tautomer aldophosphamide (Fig. 1). Both metabolites are detoxified by isoenzymes of the NAD-linked aldehyde dehydrogenase and aldehyde oxidase resulting in the formation of carboxy-

phosphamide (CAR) and 4-ketocyclophosphamide (KCP), respectively.  $\beta$ -Elimination of acrolein from aldophosphamide yields phosphoramidate mustard, a directly active alkylating species which reacts with DNA. Cleavage of phosphoramidate mustard results in the formation of nornitrogen mustard (NNM). Acrolein binds covalently to proteins. Most CP metabolites are ultimately eliminated by renal excretion.

In recently published studies, we have determined non-metabolised CP in urine samples of occupationally exposed hospital workers handling antineoplastic agents [2–5]. For the determination of low concentrations in urine a sensitive gas chromatographic–mass spectrometric method was developed [6]. In urine samples of many hospital workers CP was found. However, little is

\* Corresponding author.

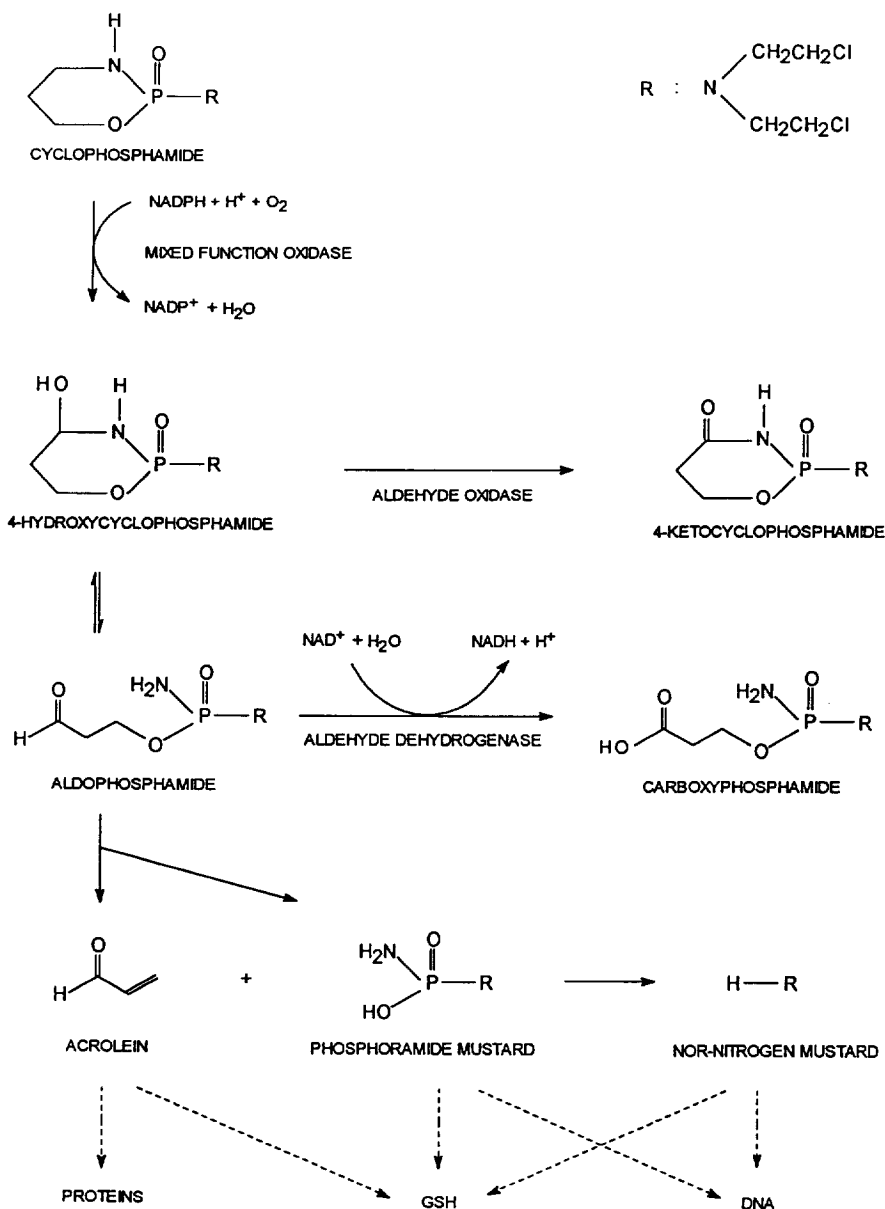


Fig. 1. Schematic presentation of the biotransformation of cyclophosphamide showing the most important reactions and metabolites (after Sladek [1]).

known about interindividual differences in biotransformation of this agent. Therefore, the *in vitro* biotransformation of CP was studied using (non)induced S-9 liver fractions. The rate of formation of the toxic metabolite NNM and the non-toxic metabolites KCP and CAR were measured. We were especially interested in the bal-

ance between toxicification (NNM formation) and detoxification (KCP + CAR formation) which is expressed by the toxic/non-toxic metabolites ratio  $\text{NNM}/(\text{KCP} + \text{CAR})$ .

The number of chromatographic methods available for the monitoring of CP metabolites is limited [7]. Therefore, new methods for the

determination of KCP, CAR and NNM in incubation mixtures are developed. After liquid–liquid extraction and derivatization, the metabolites are determined with gas chromatography and thermionic specific detection. NNM was determined after derivatization with benzenesulphonyl chloride. KCP and CAR were determined after derivatization with N,N-dimethylformamide dimethyl acetal.

## 2. Experimental

### 2.1. Chemicals

CP, 4-hydroxyperoxycyclophosphamide, throphosphamide (TRO), CAR and KCP were gifts from ASTA-Medica (Frankfurt am Main, Germany). Glucose-6-phosphate, sucrose and aniline were purchased from Sigma (St. Louis, MO, USA). 7-Ethoxy- and 7-pentoxoresorufin were from Boehringer (Mannheim, Germany). Nornitrogen mustard · HCl (NNM) and resorufin were from Aldrich (Milwaukee, WI, USA) and benzenesulphonyl chloride, EDTA and 4-morpholinepropanesulphonic acid (MOPS), were obtained from Janssen Chimica (Beerse, Belgium). N,N-dimethylformamide dimethyl acetal was purchased from Chrompack (Middelburg, Netherlands). NADP was from USB (Cleveland,

OH, USA). All other chemicals were of the highest purity obtainable.

### 2.2. Human livers

Pieces of human donor livers were used. They were kindly obtained from Dr. W.H.M. Peters, Division of Gastrointestinal and Liver Diseases, University of Nijmegen, Netherlands. Donor livers were made available anonymously. Consequently, limited information on the age and sex and no information about smoking and drinking habits was available. Their characteristics are presented in Table 1.

### 2.3. Preparation of the S-9 liver fractions

Liver homogenates were prepared (20%, w/v) in SETH-buffer (0.25 M sucrose, 2 mM EDTA and 10 mM Tris-HCl, pH = 7.4) with a Teflon–glass homogenizer. After centrifugation at 9000 g for 20 min, the floating fat layer was removed. The S-9 liver fraction was divided into a number of samples, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use.

### 2.4. In vitro incubations

The frozen S-9 liver fractions were thawed quickly at  $37^{\circ}\text{C}$  immediately prior to use and kept on ice. The incubation mixture contained 44

Table 1  
Total P-450 content, EROD and AH activities in human S-9 liver fractions

Liver	Sex	Age	Total P-450 (nmol $\text{mg}^{-1}$ protein)	EROD <sup>a</sup> (pmol $\text{min}^{-1}$ $\text{mg}^{-1}$ protein)	AH <sup>b</sup> (pmol $\text{min}^{-1}$ $\text{mg}^{-1}$ protein)
1	? <sup>c</sup>	?	0.09	4.7	0.17
2	male	16	0.11	3.6	0.18
3	?	?	0.09	3.7	0.13
4	?	20	0.22	4.0	0.32
5	male	47	0.09	8.0	0.15
6	?	?	0.12	12.9	0.19
7	?	?	0.08	2.7	0.06
Mean $\pm$ S.D.			0.11 $\pm$ 0.05	5.6 $\pm$ 3.6	0.17 $\pm$ 0.08

<sup>a</sup> 7-Ethoxyresorufin-O-deethylase.

<sup>b</sup> 4-Aniline-hydroxylase.

<sup>c</sup> Sex and age are not known.

mM MOPS buffer pH = 7.4, 2 mM NADP, 5 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 2 mM glucose-6-phosphate, 1 mM EDTA, and an amount of S-9 liver fraction based on 3.5 mg protein. After a preincubation of 5 min at 37°C, the incubation was started by adding 1.5  $\mu\text{mol}$  CP. The final incubation volume was 1 ml. The mixture was mildly shaken during the (pre)incubation at 150 rpm. The incubation was stopped after 30 min by the addition of 75  $\mu\text{l}$  of a 6 M HCl solution. The reaction mixture was immediately cooled on ice-water. All incubations were carried out double in triplicate. One series in triplicate was used for the determination of CAR and KCP. The other was used for the determination of NNM.

### 2.5. Enzyme measurements

Total cytochrome P-450 was determined from the dithionite-difference spectrum according to Rutten et al. [8]. Protein concentrations were measured with the method of Bradford using crystalline bovine serum albumin as standard [9]. 7-Ethoxyresorufin-O-deethylase (EROD) activity was determined according to Burke et al. [10] and 4-aniline-hydroxylase (AH) was measured according to Ishidate et al. [11]. Enzyme and total cytochrome P-450 measurements were performed in duplicate and protein concentrations were measured in triplicate.

### 2.6. Sample preparation and gas chromatographic analysis of the cyclophosphamide metabolites

#### CAR and KCP

After the incubation was stopped, 45  $\mu\text{l}$  10 M NaOH and 100  $\mu\text{l}$  1 M MOPS buffer pH = 8.0 were added. Excess of CP was removed by 4 extractions with 7 ml of diethyl ether by shaking for 5 min to avoid interference during the analysis. The ether layers were discarded and 200  $\mu\text{l}$  6 M HCl was added. The mixture was extracted twice with 4.5 ml ethyl acetate by shaking during 5 min. The ethyl acetate layers were combined in conical tubes with screw caps and dried under nitrogen at 40°C. After addition of 700  $\mu\text{l}$  toluene, 25  $\mu\text{l}$  N,N-dimethylformamide dimethyl

acetal and subsequently mixing, the tubes were closed for derivatization for 35 min at 115°C. The samples were cooled to room temperature and dried again under nitrogen at 50°C. Thereafter 200  $\mu\text{l}$  toluene, containing 1  $\mu\text{g}$  TRO (internal standard for controlling the sensitivity of the detector) was added, followed by sonification for 5 min. A 1- $\mu\text{l}$  sample was injected.

Gas chromatography with thermionic specific detection was performed on a Varian 3400 GC that was controlled by a Varian GC Star Workstation on a Grid 386 personal computer. Samples were injected splitless. A 15-m DB-5 column (J&W, Folsom, CA, USA) was used with 0.32 mm internal diameter and 0.25  $\mu\text{m}$  film thickness. The injector temperature was 320°C. The initial oven temperature was 85°C. After 1 min, the temperature was increased to 280°C following 4 segments (85–170°C, 20°C/min; 170–190°C, 5°C/min; 190–210°C, 10°C/min; 210–280°C, 20°C/min) where it remained constant for 6 min. Helium was used as carrier gas [column inlet pressure 12 p.s.i. (ca.  $8.2 \cdot 10^5$  Pa)]. The thermionic specific detector was set at 300°C. For quantification the peak-area ratios CAR/TRO and KCP/TRO were calculated. Quantification of the CAR and KCP derivatives was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference samples containing CAR (0, 5, 10, 20, and 25 nmol) and KCP (0, 13, 26, 38, and 51 nmol) dissolved in a blank incubation mixture. The reference samples containing CAR and KCP underwent the whole sample preparation procedure.

#### NNM

The incubation was stopped after 15 s, and 45  $\mu\text{l}$  10 M NaOH and 100  $\mu\text{l}$  1 M MOPS buffer pH = 8.0 were added. After 22 h at 37°C, 4.5 ml diethyl ether and 5  $\mu\text{l}$  benzenesulphonylchloride were added successively and the mixture was shaken during 90 min, and centrifuged during 5 min at 2500 rpm (1500 g). The ether layers were removed and dried under nitrogen at 30°C. After addition of 1 ml water, the solution was extracted twice with 2 ml *n*-hexane. The hexane layers were removed and dried under nitrogen at 40°C.

Finally, the extract was dissolved in 200  $\mu$ l toluene containing 1  $\mu$ g TRO (internal standard for controlling the sensitivity of the detector). A 1- $\mu$ l sample was injected.

For the analysis of NNM the same equipment and conditions were used as for the analysis of CAR and KCP, except for the oven programme. The initial oven temperature was 85°C. After 1 min, the temperature was increased by 15°C/min to 280°C, where it remained constant for 6 min. For quantification the peak-area ratio NNM/TRO was calculated. Quantification of the derivatized NNM and TRO was carried out by reference to calibration curves constructed from the analysis of freshly prepared reference samples containing 4-hydroxyperoxycyclophosphamide (0, 25, 50, 100, and 200 nmol) dissolved in blank incubation mixtures. For the determination of NNM we were not able to use calibration curves constructed from the analysis of freshly prepared reference samples containing NNM itself. It appeared that NNM was not formed within the incubation period but in a later stage out of metabolites generated during the incubation (Fig. 1). Therefore, the incubation mixture was put at 37°C during 22 h and 4-hydroxyperoxycyclophosphamide, which was completely converted to NNM, was preferred for the construction of the calibration curve instead of NNM itself.

#### Statistical analysis

Statistical analysis was carried out on a personal computer using InStat 1.1 as software package. Correlations were quantified with the Pearson correlation coefficient  $r$ .  $P$ -values (two-tailed) below 0.05 were considered to be of statistical significance.

### 3. Results and discussion

We have developed methods for the determination of the CP metabolites KCP, CAR and NNM in incubation mixtures. Prior to analysis, KCP and CAR were extracted from the incubation mixture with ethyl acetate and methylated with *N,N*-dimethylformamide dimethyl acetal.

Fig. 2 shows typical chromatograms of derivatized extracts of a blank incubation mixture spiked with KCP, CAR and TRO (internal standard) (A); a blank incubation mixture spiked with TRO (B); and an incubation mixture containing a S-9 fraction of a human donor liver (C). The retention times of KCP, CP, CAR, and TRO were 6.8, 6.9, 7.2 and 8.9 min, respectively. The CV. of the retention times was below 0.7%. In order to quantify the amount of KCP exactly, it was necessary to remove excess of CP. This was done by four extractions with diethyl ether prior to the extraction of the acidified incubation mixture with ethyl acetate. Finally, KCP and CP were completely separated and no interference with other compounds was observed. NNM was derivatized with benzenesulphonylchloride in diethyl ether according to the so-called Hinsberg-test resulting in the formation of a sulphonamide [12]. Fig. 3 shows typical chromatograms of derivatized extracts of a blank incubation mixture spiked with NNM (in situ formed out of 4-hydroxyperoxycyclophosphamide) and TRO (internal standard) (A); a blank incubation mixture spiked with TRO (B); and an incubation mixture containing a S-9 fraction of a human donor liver (C). The retention times of NNM, CP, and TRO were 7.6, 7.8 and 9.0 min, respectively. The CV. of the retention times was below 0.2%). No interference of NNM, CP and TRO with other compounds was observed. All calibration curves were linear, with a coefficient of correlation of a least 0.99. The limits of detection for CAR, KCP and NNM were 0.5, 0.5 and 0.1 nmol/ml incubation mixture (2.5, 2.5 and 0.5 pmol on column). The intra-assay precision (mean relative standard deviation of all triplicate incubations) was about 9% for NNM and 16% for CAR. The recovery of the extraction procedures is almost 100%.

Large differences in total P-450 content and EROD and AH activities were observed among the liver fractions of the donors (Table 1). Total P-450 content ranged from 0.08 to 0.22 nmol/mg protein. EROD and AH activity ranged from 2.7 to 12.9 and from 0.06 to 0.32 pmol/min per mg protein, respectively. Large differences were also found in NNM, CAR and total metabolite for-

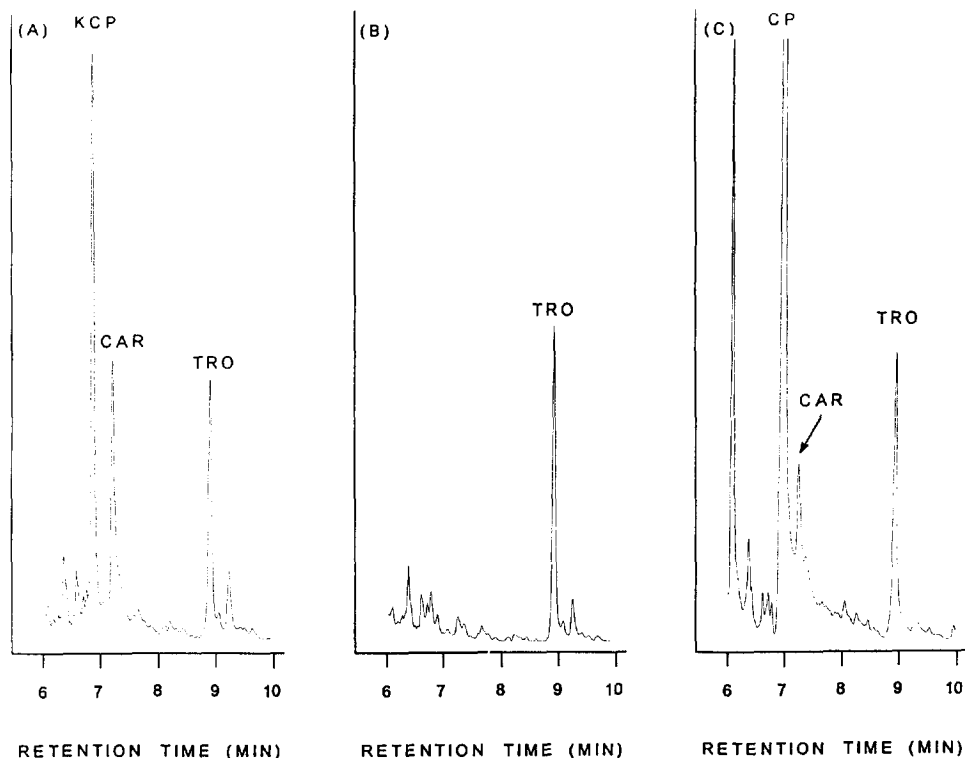


Fig. 2. Chromatograms of ethyl acetate extracts of incubation mixtures after derivatization with *N,N*-dimethylformamide dimethyl acetal. Peaks (retention time): KCP = 4-ketocyclophosphamide (6.8 min); CP = cyclophosphamide (6.9 min); CAR = carboxyphosphamide (7.2 min); TRO = trophosphamide (internal standard) (8.9 min). (A) Blank incubation mixture spiked with KCP (26 nmol/ml incubation mixture), CAR (10 nmol/ml incubation mixture) and TRO (15 nmol/ml toluene). (B) Blank incubation mixture spiked with TRO (15 nmol/ml toluene). (C) Incubation mixture containing S-9 liver fractions of liver 4.

mation and NNM/CAR ratio after incubation of CP with human S-9 liver fractions (Table 2). In none of the incubation mixtures KCP was detected. The highest amount of NNM was formed by liver 1. The NNM formation rate was eight times higher when compared to liver 7, having the lowest NNM formation rate. Liver 4 showed the highest CAR formation rate. This was fifteen times higher when compared to liver 6, having the lowest rate. Liver 4 also showed the highest NNM + CAR formation rate. This was eight times higher when compared to liver 6 with the lowest NNM + CAR formation. Liver 1 showed the highest NNM/CAR ratio. This was four times higher when compared to liver 4, the lowest one. It should be mentioned that the livers 1 and 4 with the highest NNM + CAR formation differed substantially in NNM/CAR

ratio. Comparable results were found for the livers 6 and 7 with the lowest NNM + CAR formation.

The results show large differences in the biotransformation of CP. Comparable results were found by Chang et al. although microsomes of human donor livers were used instead of S-9 liver fractions as was done in our study [13]. Besides, they studied CP 4-hydroxylase activity fluorometrically while we have determined CP metabolites with gas chromatography. They found that P-4501A1, 1A2 and 2E1 did not contribute to the hydroxylation of CP. In our study, we did not find a correlation between EROD (P-4501A1) activity and NNM, CAR, total metabolite formation and the NNM/CAR ratio. On the contrary, we found a correlation between CAR and total metabolite formation and AH (P-4502E1) activi-

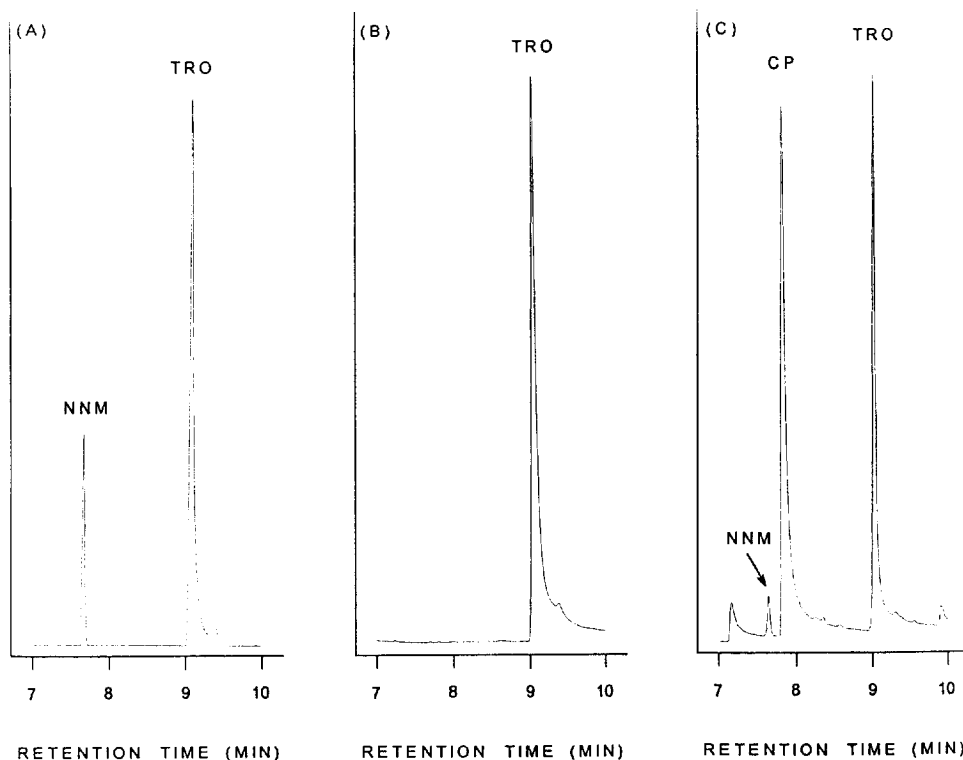


Fig. 3. Chromatograms of diethyl ether extracts of incubation mixtures after derivatization with benzenesulphonylchloride. Peaks (retention time): NNM = nornitrogen mustard (7.6 min); CP = cyclophosphamide (7.8 min); TRO = trophosphamide (internal standard) (9.0 min). (A) Blank incubation mixture spiked with NNM (100 nmol/ml incubation mixture) and TRO (15 nmol/ml toluene). (B) Blank incubation mixture spiked with TRO (15 nmol/ml toluene). (C) Incubation mixture containing S-9 liver fractions of liver 7.

ty ( $r = 0.8373$ ,  $P = 0.0187$  and  $r = 0.7954$ ,  $P = 0.0325$ , respectively). NNM formation and the NNM/CAR ratio were not correlated. Unfor-

tunately, it is not possible to establish the causal factor of the large differences in EROD and AH activity between the individual human S-9 liver

Table 2  
Formation of NNM and CAR after incubation of cyclophosphamide with human S-9 liver fractions<sup>a</sup>

Liver	NNM	CAR	NNM + CAR	NNM/CAR ratio
1	89	39	128	2.3
2	40	21	61	1.9
3	18	14	32	1.3
4	69	117	186	0.6
5	22	23 ( $n = 2$ )	45	1.0
6	15	8 ( $n = 2$ )	23	1.9
7	11	14	25	0.8
Mean $\pm$ S.D.	38 $\pm$ 31	34 $\pm$ 38	71 $\pm$ 61	1.4 $\pm$ 0.7

<sup>a</sup> Values of NNM and CAR formation are means  $\pm$  S.D. of duplicate ( $n = 2$ ) or triplicate incubations ( $\mu\text{mol}/\text{min}$  per mg protein).

preparations since no more data of the donors was available. Enhanced activity of EROD and AH do not per se reflect the induction of specific enzymes caused, for instance, by the life style factors smoking or alcohol. Genetic factors may also be of importance with respect to P-450 isoenzyme activities involved in the biotransformation of CP.

The large interindividual differences in especially the formation of NNM and CAR and the NNM/CAR ratio between the liver S-9 samples suggest large interindividual differences in the toxicity of CP due to differences in biotransformation. A high NNM/CAR ratio may be predictive for a relatively high level of DNA-alkylating metabolites in vivo. Otherwise a low NNM/CAR ratio may be predictive for the formation of detoxified metabolites.

We may assume that comparable differences in biotransformation will occur in man in vivo, for instance in treated patients and in occupationally exposed workers. This means that it is not possible to estimate the exact individual dose of CP in occupationally exposed workers on basis of their individual urinary excretion of CP. Therefore, we have applied the developed methods for the determination of KCP, CAR and NNM in urine of hospital workers. Unfortunately, the methods were not appropriate because we were not able to extract the metabolites and to derivatize them. Hence, only the method for the determination of CP in urine of hospital workers can be used to establish occupational exposure to CP and to estimate a group-based exposure level.

## Acknowledgement

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