Characterization and Quantitative Estimation of Activated Cyclophosphamide in Blood and Urine

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SUMMARY

An assay for the characterization and quantitative determination of activated cyclophosphamide has been described. The method is based on high reactivity of the krypta-ldehyde group in 4-hydroxycyclophosphamide with mercapto compounds to yield 4-(S-R)mercaptopyclophosphamide derivatives. The activated cyclophosphamide can be converted quantitatively to 4-(S-benzyl)mercaptopyclophosphamide with benzyl mercaptan, and the mercapto derivative can be separated by thin-layer chromatography on silica gel with ethyl acetate:methyl ethyl ketone as solvent. Using this assay, significant levels of activated cyclophosphamide in the blood of mice and rats, as well as of humans, were found to be present after cyclophosphamide application. The characterization of 4-(S-benzyl)mercaptopyclophosphamide formed was confirmed by comparing the thin-layer chromatographic and mass spectrometric data on this derivative obtained from an authentic sample of 4-hydroxycyclophosphamide.

INTRODUCTION

Studies on the metabolism of cyclophosphamide have shown that the drug requires activation by liver microsomes to gain its alkylating and cytotoxic properties (2, 3). Metabolism proceeds by "mixed function" hydroxylation at carbon 4 of the oxazaphosphorine ring of CP (11, 15) to yield the activated metabolite 4-OH-CP and its acyclic isomer aldophosphamide (24). As demonstrated previously (1, 4, 12), the relatively high oncostatic selectivity of CP as measured on Yoshida sarcoma-bearing rats is based exclusively on 4-OH-CP (17). Obviously, with regard to the oncostatic selectivity of cyclophosphamide, any hypothesis based on special reactions of 4-OH-CP depends on the demonstration that these metabolites do exist in significant concentrations in body fluids and tissues after CP application; however, because of the known instability of 4-OH-CP this proof has been found to be rather difficult, and controversial findings have been reported (9, 19, 21, 24).

In this paper, we describe a new method for the quantitative determination of activated CP in blood and urine. The method is based on the high reactivity of activated oxazaphosphorine ring with benzyl mercaptan and quantitative TLC of the product of this reaction. By means of this technique, significant levels of 4-OH-CP in the blood of mice, rats, and humans after cyclophosphamide injection were detected.

MATERIALS AND METHODS

1H-side-chain-labeled cyclophosphamide (specific activity, approximately 1.8 mCi/mmol) was a gift of Prof. Schaumloffel, Klinik und Poliklinik für Nuklearmedizin, 3550 Marburg, West Germany. It was purified by TLC on 1,3-propanediol-impregnated cellulose plates, as described previously (23). Ketocyclophosphamide and 4-hydroperoxycyclophosphamide were obtained by direct ozonization of CP, as described recently (17). 4-OH-CP was prepared by treating 4-hydroperoxycyclophosphamide with triphenylphosphine in CH2Cl2, according to the method of Takamizawa et al. (22). 4-[3H]OH-CP, having a 98% radiochemical purity, was obtained by a 2-fold recrystallization. Aldophosphamide (23) and the diastereomer of 4-OH-CP were obtained by incubating 4-OH-CP in 0.07 M phosphate buffer, pH 5.0, at 37° for 1 hr. Under these conditions the 2 4-OH-CP diastereomers and aldophosphamide were found to equilibrate at a ratio of about 1:1:1. The mixture was then separated by TLC on 1,3-propanediol-impregnated cellulose plates as reported (19, 23). After scraping off the appropriate zones and subsequent elution with CH2Cl2, the eluates were used for the benzyl mercaptan assay.

The benzyl mercaptan assay was performed at 0° in CH2Cl2 in the presence of TCA (1% w/v). Unless indicated, the concentration of benzyl mercaptan used in the experiments was 0.1 M.

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4 The abbreviations used are: CP, cyclophosphamide [2-bis(2-chlor-ethyl)aminetetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide]; 4-OH-CP, 4-hydroxycyclophosphamide; TLC, thin-layer chromatography; TCA, trichloroacetic acid.

5 Since under physiological conditions 4-OH-CP and aldophosphamide may be in equilibrium (24), the terms activated CP or 4-OH-CP always designate both partners of the isomeric pair if not indicated.

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TLC. 4-(S-Benzyl)mercaptocyclophosphamide and 4-ke
tocyclophosphamide were separated by TLC on precoated
silica gel plates (Merck 5721, Firma Merck, 6100 Darmstadt,
West Germany). The chromatogram was developed at 4° for
70 min in a sandich chamber (Firma Camag, Muttenz,
Switzerland) with ethyl acetate:methyl ethyl ketone (60:20,
v/v) as solvent. The alky1ating material on TLC plates was
identified by means of 4-(p-nitrobenzyl)pyridine spray (10,
19), and sulfur-containing compounds were identified by
means of iodine:azide spray (5).

For quantitative determination of labeled CP and metabo-
lites or derivatives, the appropriate zones containing radio-
activity were scraped off from TLC plates (4-mm bands) and
transferred directly to liquid scintillation vials.

Measurement of Radioactivity. Radioactivity was deter-
mined in a Philips scintillation spectrometer (PW 45/0/01)
or a Packard 3380/544 liquid scintillation counter. The scin-
tillation fluid contained 2 g butyl-2-(4'-t-butylphenyl)-5-(4"
TLC.

In Vivo Experiments. Rats (SPF, Sprague-Dawley, weighing
200 to 300 g) and mice (NMRI, weighing 20 to 30 g)
received i.p. injections of [3H]cyclophosphamide (25 mg/
kg) (specific activity, 0.3 mCi/mg). Blood samples were
obtained after cardiopunclume. In human studies
[3H]cyclophosphamide (0.5 to 1 μCi/mg) was given i.v. A
total of 6 patients who received cyclophosphamide for ad-
avanced cancers were subjected to this treatment. The hepa-
rinized blood samples (1.5 ml) were extracted twice with 70
ml CH2Cl2. After adding benzyl mercaptan (final concentra-
tion, 100 mm) and TCA (final concentration, 1% w/v), the
CH2Cl2 extract was concentrated in a vacuum under ice
cooling to a final volume of about 100 μl and then separated
by TLC as described above.

RESULTS

4-OH-CP reacts with sulphydryl compounds to give 4-(S-
R)mercaptocyclophosphamide (cf. Chart 1), a new class of
cyclophosphamide derivatives, which has been described
by us recently (8, 17, 18). Since the mercaptam derivatives
are relatively stable compounds when compared to 4-OH-CP,
the reaction of 4-OH-CP with various sulphydryl compounds
was studied to estimate 4-OH-CP in body fluids of humans
and laboratory animals. The reaction with benzyl mercap-
tan in organic solvents was chosen because of its good yield
and convenient separation of the products by means of TLC.

Chart 2 (dashed line) shows a thin-layer radiochromato-
gram of 4-OH-CP and 4-(S-benzyl)mercaptocyclophos-
phamide obtained after the equilibration of 4-[3H] OH-CP
with benzyl mercaptan in acetone for 24 hr at 4°.

The

Chart 2. Radiochromatogram of 4-OH-CP and its benzyl mercapto deriva-
tives. 3H side-chain-labeled 4-OH-CP (10 mm) was treated with benzyl mer-
captan (100 mm) in acetone at 4° for 24 hr. The reaction mixture was
separated on TLC, and the radioactivity of the silica gel zones was deter-
mimed as described. 1, phosphoramide mustard and decomposition prod-
ucts; 2, 4-OH-CP; 3a and 3b, 4-(S-benzyl)mercaptocyclophosphamide dia-
teomers; ———, + TCA; ———, without TCA.

mercapto derivative was found to be separated into 2
diastereomers exhibiting Rv values of 0.4 ± 0.03 and 0.78 ±
0.02 and for which the identity was proven by means of IR
and field desorption mass spectrometry (17, 19). Further-
more, small amounts of phosphoramide mustard and other
breakdown products were also visible near the starting
point. The yield of the fast-moving 4-(S-benzyl)mercap-
tocyclophosphamide diastereomer under these conditions
was found to be in the range of 40 to 60% depending on
the benzyl mercaptan concentration. If, however, the
reaction of 4-OH-CP with benzyl mercaptan was accom-
plished in the presence of TCA (1 to 2%, w/v), only the fast-moving
diastereomer (Rv 0.78) was formed with a yield of 98% with
respect to 4-OH-CP concentrations originally present (cf.
Chart 2). Under the acidic conditions the reaction is com-
pleted within 20 min at 0° as shown in Chart 3, thus pre-
venting any significant decomposition of the 4-OH-CP to be
assayed and rendering the suitability of the benzyl mer-
captan reaction for the quantitative determination of activ-
ated CP. Since the equilibrium of the reaction

\[
\frac{4(SR)\cdot H_2O}{4\cdot OH-CP\cdot RSH} = K_{eq}
\]

was found to be only slightly in favor of the products (in 6
experiments with different molar ratios of the reactants a
mean value of 14.3 ± 0.9 was found for K_{eq} at 0° in acetone), an excess of benzyl mercaptan must be present for quanti-
tative yield. The 4-(S-benzyl)mercaptocyclophosphamide
formed was found to be stable in CH2Cl2 for several days
when stored at −20°. After some weeks its partial conver-
sion into the slow-running diastereomer and its slight de-
composition could be observed. Regarding the mechanism
of this reaction it is not clear hitherto whether the nucleo-
philic sulphydryl group involved reacts directly with 4-OH-
CP or with its acyclic isomer aldothophosphamide; the dif-
ferent reaction mechanisms in question have been discussed.

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by us elsewhere (17). However, under acid catalysis, the 2 diastereomers of 4-OH-CP and also aldophosphamide (23) (for preparation see "Materials and Methods") are converted quantitatively by benzyl mercaptan into 1 common derivative that is chromatographically identical to the fast-running 4-(S-benzyl)mercaptocyclophosphamide diastereomer, shown in Chart 2 (17). No reaction of benzyl mercaptan with CP, ketocyclophosphamide, and carboxyphosphamide, or with other decomposition products of CP was observed under the conditions of this assay, thus rendering the test specific for activated CP.

For the determination of activated CP in blood or in urine, aliquot samples have to be extracted by 50- to 100-fold volumes of CH$_2$Cl$_2$ to remove 4-OH-CP and aldophosphamide quantitatively from the body fluids.

In Chart 4 a radiochromatogram of a CH$_2$Cl$_2$ extract from mouse blood drawn 30 min after an i.p. injection of [3H]cyclophosphamide is shown. In the absence of benzyl mercaptan (Chart 4, top) most of the radioactivity, representing CP and its main metabolites, except ketocyclophosphamide (R$_f$ 0.43), are found near the starting point, whereas only minor amounts of activated CP can be detected at R$_f$ 0.18, which is identical to 1 of the 4-OH-CP diastereomers (19). Apparently, even under these cautious conditions of the TLC system used, most (80%) of the activated cyclophosphamide must have been transformed or decomposed to products that remain near the starting point. This becomes evident by comparison with Chart 4, bottom, where the benzyl mercaptan procedure was applied on the same blood sample. In this case, a large peak of 4-(S-benzyl)mercaptocyclophosphamide appears at R$_f$ 0.78, representing approximately a 5-fold amount of activated cyclophosphamide when compared to the top curve of Chart 4. Furthermore, Chart 4 shows that the radioactivity found in the 4-(S-benzyl)mercaptocyclophosphamide peak (23% of the total radioactivity on the TLC plate) agrees very well with the sum of radioactivity in the 4-OH-CP diastereomer peak at R$_f$ 0.18 (5%) and the difference between radioactivities (16.5%) found at the starting points (Chart 4, top and bottom). Since further separation by TLC on 1,3-propanediol-impregnated cellulose (23) of the starting-point radioactivity (Chart 4, bottom) has shown this fraction to consist only of nonmetabolized CP, carboxyphosphamide, and small amounts of phosphoramid mustard, it may be concluded that the benzyl mercaptan procedure prevents activated cyclophosphamide either from breakdown to phosphoramide mustard or from transformation to an isomer or diastereomer not separable from the starting point under the TLC conditions used. Thus, in the absence of benzyl mercaptan only 20% of added 4-OH-CP could be recovered from mice blood as compared to almost 100% when the benzyl mercaptan procedure was applied.

Injection of [3H]cyclophosphamide to rats led to similar results, as demonstrated in Chart 4 for the mouse. However, in the rat the 4-(S-benzyl)mercaptocyclophosphamide peak was found to be smaller, representing a level of 5 to 10% of activated CP when referred to the sum of cyclophosphamide and metabolites extractable from the blood by CH$_2$Cl$_2$. This is in agreement with our previous findings with TLC on 1,3-propanediol-impregnated cellulose plates (24).

In humans only very low levels of activated CP were detected in blood. However, the benzyl mercaptan procedure allowed a quantitative estimation of such low levels due to the sharp TLC separation of the 4-(S-benzyl)mercaptocyclophosphamide peak from other CP metabolites and from nonmetabolized CP and due to the low radioactive background, as shown in Chart 4, bottom.

Thus, 2 hr after i. v. injection of [3H]cyclophosphamide (10 mg/kg) to patients, we could separate 1 to 3% radioactive 4-(S-benzyl)mercaptocyclophosphamide from the bulk of radioactive material present in the CH$_2$Cl$_2$ extract, which corresponds to blood levels of 0.5 to 1.5 nmoles/ml free (non-protein bound) activated cyclophosphamide.

To confirm the identity of the 4-(S-benzyl)mercaptocyclophosphamide isolated by our TLC system, the urine of a patient treated with 1 g cyclophosphamide was collected in 6 portions over 12 hr, extracted with CH$_2$Cl$_2$, and treated with benzyl mercaptan under TCA catalysis, as described above.

Within this time 27 µmoles (approximately 10 mg) were
Assay for Activated Cyclophosphamide in Body Fluids

Chart 5. Field desorption mass spectrum of 4-(S-benzyl)mercaptopcyclophosphamide isolated from urine of a patient treated with [3H]CP after derivatization with benzyl mercaptan.

found to be excreted as activated cyclophosphamide. Interference by other urinary substances of the TLC separation has not been observed.

After TLC on silica gel, the 4-(S-benzyl)mercaptopcyclophosphamide spot at Rf 0.78 was scraped off, eluted with CH₂Cl₂, and after evaporation subjected directly to field desorption mass spectrometry. Both the mass of the molecular ion [MH]⁺ = 383 and the fragmentation pattern were found to be identical with the benzyl mercaptan product of 4-OH-CP obtained by synthesis (Refs. 18 and 19; cf. Chart 5).

DISCUSSION

By comparing the therapeutic index on Yoshida sarcoma-bearing rats of cyclophosphamide with the corresponding data of its metabolites, it has recently been demonstrated (1, 4, 12) that only the primary metabolites 4-OH-CP and aldophosphamide, which are summarized here as activated cyclophosphamide, are the mediators of the relatively high oncostatic selectivity of CP in vivo. Other known CP metabolites such as phosphoramid mustard or nonnitrogen mustard, despite their high alkylating activity, as well as deactivated CP metabolites such as carboxycyclophosphamide and 4-ketocyclophosphamide, have been shown to exhibit neither oncostatic selectivity in vivo nor cytotoxic specificity in vitro (4, 12) against Yoshida sarcoma tumor cells.

However, it is still uncertain whether the primary metabolite of CP does really exist in significant amounts in the blood of laboratory animals or in humans after CP application, and some controversial findings have been published. Thus, Fenselau et al. (9) and Jardine et al. (16) could detect only phosphoramid mustard and nonnitrogen mustard in human blood as circulating metabolites after CP application, using a coupled gas chromatography-mass spectrometry technique. Colvin et al. (6) and Connors et al. (7) assumed phosphoramid mustard to be the most likely candidate for the "active" principle of cyclophosphamide because of its high cytotoxicity and alkylating activity. Struck et al. (21) have made extensive studies on this problem using combined TLC and mass spectrometric techniques. They were unable to detect measurable amounts of 4-OH-CP or aldophosphamide in mouse blood after injection of ¹⁴C ring- or side-chain-labeled cyclophosphamide, but found relatively high levels of phosphoramid mustard and nonnitrogen mustard. On the other hand, we could demonstrate in an early study (14) that, 30 min after cyclophosphamide injection to rats, about 85% of the total alkylating material in the blood corresponds to activated cyclophosphamide, being identical with the primary product of microsomal hydroxylation of CP. Since the total alkylating material in the blood, which corresponds approximately to the sum of CP metabolites, was determined to about 10% of the blood level of nonmetabolized cyclophosphamide (14), a relative level of about 8.5% "activated" cyclophosphamide may be calculated from these data, which is in very good agreement with the values obtained from the present study (cf. "Results").

Sladek (20) concluded from his 2-dimensional TLC data that equal amounts of aldophosphamide and carboxycyphosphamide exist in rat blood 1 hr after CP injection. More recently, we could demonstrate by means of TLC on 1,3-propanediol-impregnated cellulose plates (24) relatively high levels of both 4-OH-CP and aldophosphamide in the mouse (15 min posttreatment) and rat serum (120 min post-treatment) after injection of [³H]CP. In addition, the metabolic pattern in the urine of a rat has been evaluated at different times after CP injection, showing high levels of activated CP during the 1st 60 min. However, when the same technique was applied to human serum, no measurable amounts of activated cyclophosphamide were found.

Using the benzyl mercaptan technique, as a new independent method, we could confirm our previous findings on the existence of significant levels of activated CP in mouse serum after CP injection as shown in this paper. Since benzyl mercaptan, like other thiol compounds, has been shown to react very fast and specifically by a nucleophilic attack at carbon 4 with the activated oxazaphosphorine ring of the primary cyclophosphamide metabolites (17), and because the 4-(S-benzyl)mercaptopcyclophosphamide formed is much more stable than activated cyclophosphamide itself (8, 17), the benzyl mercaptan technique was shown to be well suited for the detection of low levels of activated cyclo-

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phosphamide, e.g., as in human blood.

Moreover, for the same reasons, mass spectrometric identification of the 4-(S-mercapto) derivatives of activated cyclophosphamide is much easier when compared to hydroxycyclophosphamide itself, as will be shown elsewhere (18).

The demonstration of measurable levels of activated cyclophosphamide in human serum after CP injection has encouraged us to undertake further pharmacokinetic studies with regard to the possible influence of the time course of activated cyclophosphamide levels in the blood on the toxic and therapeutic effects in vivo.

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